The molecular and cell biological characterization of cell-cell junctions in mesenchymally derived cells and tissues

Inaugural Dissertation

(Cumulative Thesis)

for the degree of

doctor rerum naturalium (Dr. rer. nat.)

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

presented by

Steffen Rickelt

born 20.09.1980 in Wolfen, Germany

May 2010

This work has been carried out in the laboratory of Prof. emer. Dr. Werner W. Franke in the Helmholtz Group for Cell Biology at the German Cancer Research Center (DKFZ), Heidelberg, Germany.

Referees: Prof. emer. Dr. Werner W. Franke
Prof. emer. Dr. Dr. h.c. Volker Storch

Oral examination: 17.06.2010

Do not go where the path may lead
go instead where there is no path and leave a trail.

Ralph Waldo Emerson (1803 - 1882)

Dedicated to my parents

1. Table of contents

2. Abbreviations and definitions

AJs	-	adhering junctions: collective term including all cadherin-based cell-cell junctions and half junctions such as desmosomes, adherens junctions (*puncta, fascia, zonula*) as well as the composite junctions
ARVC	-	arrhythmogenic right ventricular cardiomyopathy
ARVCF	-	armadillo repeat gene deleted in velo-cardio-facial (syndrome)
Cad	-	cadherin
cDNA	-	complementary deoxyribonucleic acid
DP	-	desmoplakin
Dsc	-	desmocollin
Dsg	-	desmoglein
EpCAM	-	epithelial cell adhesion molecule
JAM	-	junctional adhesion molecule
IF	-	intermediate-sized filament
IP	-	immunoprecipitation
mabs	-	monoclonal antibodies
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PG	-	plakoglobin
Pkp	-	plakophilin
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcriptase - polymerase chain reaction
SDS	-	sodium dodecylsulfate
siRNA	-	small interference RNA

3. Publications of Steffen Rickelt with the percentages of his specific contributions

This thesis is based on the following publications. The contribution to the specific manuscript of Steffen Rickelt is indicated in percent.

1. Schmitt, C. J., Franke, W. W., Goerdt, S., Falkowska-Hansen, B., **Rickelt, S.**, and Peitsch, W. K. (2007) Homo- and heterotypic cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein. J Invest Dermatol. *127*, 2191-2206.

   Contribution: 20%

1. **Rickelt, S.**, Franke, W. W., Dörflinger, Y., Goerdt, S., Brandner, J. M., and Peitsch, W. K. (2008). Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. Cell Tissue Res *334*, 401-422.

   Contribution: 80%

2. **Rickelt, S.**, Winter-Simanowski, S., Noffz, E., Kuhn, C., and Franke, W. W. (2009). Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer *125*, 2036-2048.

   Contribution: 90%

3. Franke, W. W., **Rickelt, S.**, Barth, M., and Pieperhoff, S. (2009). The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind. Cell Tissue Res *338*, 1-17.

   Contribution: 25%

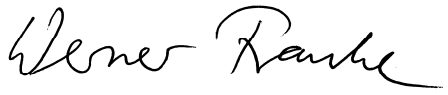
4. **Rickelt, S.**, Rizzo, S., Dörflinger, Y., Zentgraf, H. W., Basso, C., Gerosa, G., Thiene, G., Moll, R., and Franke, W. W. (2010). A novel kind of tumor type-characteristic junction: Plakophilin-2 as a major protein of adherens junctions in cardiac myxomata. Submitted.

   Contribution: 60%

5. Franke, W. W., and **Rickelt, S.** (2010). Spontaneous and Cumulative Syntheses of Epithelial Proteins and Glycoproteins and Their Assemblies to Novel Cell-Cell Junctions in Malignantly Transformed Cells: I. Carcinomatoid Dysplasia Forms of Human Hematopoietic K562 Cells. Submitted.

   Contribution: 50%

Heidelberg, 04.05.2010



Prof. Werner W. Franke

4. Summary

According to the prevailing textbook dogma there are two – and only two – distinct types of cell-cell connecting, calcium-dependent adhering junctions (AJs) characterized by dense cytoplasmic plaques which frequently anchor cytoskeletal filaments and represents architecturally and functionally important structures of epithelia and epithelia-derived tumor cells, including all carcinomas. These are the intermediate-sized filament-anchoring desmosomes (*maculae adhaerentes*) and the microfilament-anchoring adherens junctions which can appear in various sizes and morphotypes (*puncta, fasciae, zonulae*). The molecular composition of both kinds of AJs is by and large known and the molecule-specific diagnostic antibodies are used in cell and developmental biology as well as in diagnostic pathology. By comparison, very little is known as to the molecular composition of the AJs that connect non-epithelial cells, notably those of mesenchymal tissues and mesenchymally derived tumors.

Therefore and because of the general need for an improved immunocytochemically based armamentarium of diagnostic reagents and therapeutic methods, it has been the aim of this thesis to provide a first cell and molecular biological basis for the diagnostic identification and characterization of such AJs from mesenchymal-derived tumors. As I had initially noted marked ultrastructural and compositional differences between various AJ subtypes connecting non-epithelial cells I have selected for my research five specific novel kinds of such junctions which are distinguished by their molecular ensembles and organizations.

A first and widely occurring type is presented by AJs that have acquired, in addition to a "mesenchymal type" basis ensemble of N-cadherin and/or cadherin-11 as trans-membrane glycoproteins and a plaque assembled by some *armadillo*-type proteins including β-catenin, plakoglobin, proteins p120, p0071 and/or ARVCF together with actin microfilament-binding proteins such as α-catenin and α-actinin, the major plaque protein plakophilin-2 (Pkp2), not infrequently together with Pkp3. These molecules have hitherto only been known as important organizational building stones of desmosomes and certain desmosome-related junction forms such as the *areae compositae* of the heart muscle connecting cardiomyocytes. I have demonstrated the occurrence of AJs comprising Pkp2 – with or without Pkp3 – (*coniunctiones adhaerentes*) in several mammalian cell lines as well as in some tumors *in situ*, including tumor types for which this protein appears to be a general and diagnostically useful molecular marker such as cardiac myxomata. Based on a series of first experiments with siRNA-mediated gene product reduction experiments and on recent insights of a general role of Pkp2 as a cell-cell-attachment stabilizing protein as in the mammalian heart, I discuss the acquisition of Pkp to AJs of mesenchymally-derived

cells as a possible enhancer of structural stability and as obviously advantageous in cell proliferation. Therefore, I propose generally to distinguish the two major categories of mesenchymal AJs, those with and those without Pkp molecules.

As a third and functionally different, novel cadherin arrangement form I have discovered, in certain subtypes of human melanomas and melanocytes growing in cell culture, widely spread, surface membrane-integrated desmoglein Dsg2 glycoproteins which apparently occur as frequent but solitary molecules over rather extended areas of cell-cell contacts, i.e. without any junction-like local clustering. Detailed characterization of these Dsg2 molecules by biochemical as well as immunofluorescence and immunoelectron microscopy methods has revealed that they obviously exist out of any desmosomal complex and context. A special contribution of these Dsg2 molecules to cell-cell adhesion and cell recognition processes seem plausible and may contribute to special heterotypic cell associations and metastatic processes.

Finally, and this is certainly the most unexpected and though-provoking observation that I have followed in my thesis, I have characterized again by biochemical as well as immunofluorescence and electron microscopy methods and even in cell cloning series, the spontaneous, uninduced, often cumulative syntheses of cell junction molecules in long-established human hematopoietic cell culture lines (e.g., K562, RPMI 8226) and their assemblies to variously-sized and -structured cell-cell junctions. I have identified diverse types of cell-cell connection structures of the AJ category the vast majority of which are based on plasma membrane clusters of Dsg2 which rarely occurred in complexes with desmocollin Dsc2 and/or the major desmosomal plaque protein, desmoplakin, but more frequently were found inserted in plaque-like assemblies of Pkp2, with or without Pkp3, plakoglobin or rather rarely other *armadillo*-type junction proteins. Remarkably, even in the electron microscope some of these Dsg2-based AJs cannot be readily distinguished from true desmosomes. However, these punctate AJ structures based on desmosomal cadherins are not the only cell-cell contact structure contributions to the formation of tissue-like cell layers or even three-dimensional structures: Relative large amounts of the cell adhesion glycoprotein, epithelial cell adhesion molecule (EpCAM), are often also synthesized in these cells and accumulate on the plasma membrane, where they are anchored in extended subplasmalemmal plaque-assemblies rich in afadin, vinculin and α-actinin. It is obvious that such accumulations of carcinoma-characteristic molecules and structures now present a disturbing dilemma in tumor diagnosis and for therapeutic treatments. This spontaneous change of cell differentiation properties as well as of cell character and behavior is discussed, also in relation to corresponding observations in the literature.

The finding of such very different AJ structures connecting non-epithelial tumor cells have indicated that the textbook chapter on cell-cell junctions in cell and tumor biology should be reopened and filled with facts of ultrastructural and molecular analysis.

5. Zusammenfassung

Nach dem herrschenden Lehrbuch Dogma exestieren zwei – und nur zwei – verschiedene Typen von zell-zell-verbindenden, Calcium-abhängigen Zellkontakten ("Adhering Junctions", AJs), die durch dicht-gepackte zytoplasmatische Plaques gekennzeichnet sind und so regelmäßig Filamente des Zytoskeletts verankern. AJs sind somit architektonisch und funktionell wichtige Strukturen, die bisher insbesondere in Epithelien und davon abgeleiteten Tumoren, vor allem den Karzinomen, untersucht worden sind. Hier unterscheided man vor allem zwischen den Intermediärfilamente verankernden Desmosomen (*Maculae adhaerentes*) und den Mikrofilament-Bündel verankernden Zellkontakten der Adherenz-Kategorie, die in verschiedenen Größen und Morphotypen vorkommen (*Puncta, Fasziae, Zonulae*). Die molekulare Zusammensetzung dieser beiden AJ-Arten ist weitgehend bekannt, und spezifische molekular-diagnostische Antikörper werden routinemäßig in der Zell- und Entwicklungsbiologie sowie der diagnostischen Pathologie eingesetzt. Im Vergleich dazu ist sehr wenig über die molekulare Zusammensetzung der AJs, die nicht-epitheliale Zellen verbinden, bekannt, insbesondere die der mesenchymalen Gewebe und der mesenchymal-abgeleiteten Tumore.

Aus diesem Grund und wegen des allgemeinen Bedarfs an verbesserten immunzytochemisch-diagnostischen Reagenzien und molekular-therapeutischen Ansätzen war es das Ziel dieser Dissertation, eine zellbiologische und molekular-analytische Grundlage für die diagnostische Identifizierung und Charakterisierung solcher AJs von mesenchymal-abgeleiteten Tumoren zu liefern. Bereits zu Beginn meiner Untersuchungen hatte ich markante Unterschiede der Ultrastruktur und der molekularen Zusammensetzung bei verschiedenen AJ-Formen mesenchymaler Zellen festgestellt. Daher habe ich für meine weiteren Untersuchungen fünf neue und besonders auffällige Arten solcher AJs ausgewählt.

Der erste neuartige und weitverbreitete AJ-Typ ist einer, der ein grundlegendes "mesenchymales" Ensemble von N-Cadherin und/oder Cadherin-11 als transmembrane Glykoproteine aufweist, die in einem subplasmalemmalen Plaque verankert sind, der von typischen *armadillo* Proteinen wie β-Catenin, Plakoglobin, den Proteinen p120, p0071 und/oder ARVCF sowie den Aktin-Mikrofilamente-bindenden Proteinen α-Catenin und α-Actinin gebildet wird, hier aber zusätzlich das bedeutende Plaque-Protein Plakophilin-2 (Pkp2), oft zusammen mit Pkp3, enthält. Dieses Protein ist bisher nur als wichtiger organisatorischer Baustein von Desmosomen und bestimmten desmosomen-ähnlichen AJ-Formen wie der *Areae compositae* des Herzmuskels bekannt. Ich habe das Vorkommen solcher Pkp2-haltigen AJs – mit oder ohne Pkp3 – (*Coniunctiones adhaerentes*) in verschiedenen Säugetier-Zellkulturlinien gezeigt. Des Weiteren wurden diese AJs in einigen

Tumoren *in situ* nachgewiesen, einschließlich bestimmter Tumore, bei denen dieses Protein durchweg ein diagnostisch relevanter molekularer Marker zu sein scheint, wie zum Beispiel den kardialen Myxomen. Basierend auf ersten Experimenten mit einer siRNA-vermittelten Genprodukt-Reduktion und ausgehend von jüngsten Erkenntnissen über eine allgemeine Rolle von Pkp2 als stabilisierendes Zell-Zell-Verbindungs-Protein wie z.B. im Säugetier-Herz, diskutiere ich die Möglichkeit dass eine Integration von Pkp-Molekülen in AJs von mesenchymal-abgeleiteten Zellen wesentlich zur strukturellen Stabilisierung und Festigkeit beiträgt und vor allem einen Vorteil beim Zusammenhalten proliferierender Zellen darstellt. Ich schlage deshalb auch vor, in Zukunft bei solchen Tumoren zwischen Pkp-positiven und Pkp-negativen Tumoren zu unterscheiden.

Als dritte und strukturell völlig verschiedene Cadherin-Anordnung habe ich in einigen Subtypen von in Zellkultur-gewachsenen menschlichen Melanomen und Melanozyten das weit verbreitete in die Plasmamembran integrierte Glykoprotein Desmoglein Dsg2 entdeckt, dass dort sich offenbar regelmäßig in einzelnen Molekülen über große Areale der Zelloberfläche hin erstreckt und in Spiegelbild-symmetrischer Anordnung Zellen verbindet. Die Charakterisierung dieser Dsg2-Moleküle durch biochemische sowie immunfluoreszenz- und immunelektronenmikroskopische Methoden hat gezeigt, dass sie offensichtlich außerhalb jedes AJ-Zusammenhangs vorkommen und nicht mit anderen Cadherinen oder mit Plaque-Proteinen zu cis-Komplexen verbunden sind. Ein besonderer Beitrag dieser Dsg-Moleküle zu Zell-Zell-Adhäsions- und Zell-Erkennungprozessen scheint aber durchaus plausibel und kann daher unter Umständen eine Rolle in Zell-Zell-Verbänden und bei Metastasierungs-Prozessen spielen.

Sicherlich die am meisten unerwartete und auch herausfordernde Beobachtung, die ich in meiner Dissertation verfolgt habe und ebenfalls durch biochemische sowie immunfluoreszenz- und elektronenmikroskopische Methoden aber auch in Zellklonierungs-Serien belegt habe, ist die spontane, nicht induzierte, oft kumulative Synthese von Zellverbindungsmolekülen in bereits seit langem etablierten hämatopoetischen Zellkulturlinien (K562, RPMI 8226) und die Ansammlung solcher Moleküle zu einem Spektrum von Zell-Zell AJ-Strukturen. Ich habe verschiedene Arten von unterschiedlich großen, meist punktförmigen Zell-Zell-Verbindungs-Strukturen der AJ-Kategorie identifiziert, von denen die große Mehrheit auf distinkte Dsg2-Plasmamembran-Anhäufungen basiert, die selten auch in Verbindung mit Desmocollin Dsc2 bzw. dem wichtigen desmosomalen Plaque-Protein, Desmoplakin, auftreten. Am häufigsten habe ich AJ-Plaque-ähnliche Zusammensetzungen mit Pkp2 – mit oder ohne Pkp3 – mit Plakoglobin bzw. eher selten mit anderen *armadillo* Proteinen nachgewiesen. Diese punktförmigen AJ-Strukturen, welche auf desmosomalen Cadherinen basieren, können selbst in elektronenmikroskopischen Aufnahmen nicht von echten Desmosomen unterschieden werden. Sie sind aber nicht die

einzigen Zell-Zell-Kontaktstrukturen, die zur Bildung von epithel-ähnlichen Zellschichten oder sogar drei-dimensionalen Strukturen beitragen. Zusätzlich können solche hämatopoetischen Zellen auch relativ große Mengen des Zelladhäsions-Glykoproteins "Epithelial Cell Adhesion Molecule" (EpCAM) synthetisieren welches sich an der Plasmamembran anreichert – zunächst in punktförmiger, dann in kontinuierlicher plaque-artigen Struktur – wo es in erweiterten subplasmalemmalen Plaques verankert wird die reich an Afadin, Vinculin und α-Actinin sind.

Es ist offensichtlich, dass solch unerwartete Analyseergebnisse, vor allem etwa die zuletzt geschilderte Anhäufung von Karzinom-charakteristischen Molekülen und Strukturen in mesenchymalen Tumoren erst recht in unizellulären Bluttumorzellen, in der Tumordiagnostik und für die therapeutische Behandlung, Beunruhigungen hervorrufen. Diese spontanen Veränderungen der Zell-Differenzierungs-Eigenschaften sowie des Zell-Charakters und -Verhaltens werden in Bezug auf entsprechende vereinzelte Angaben in der Literatur diskutiert. In jedem Fall hat die Feststellung solcher sehr unterschiedlicher AJ-Strukturen, in nicht-epithelialen Tumorzellen gezeigt, dass die Lehrbuchkapitel über Zell-Zell-Kontakte in der Zell- und Tumorbiologie wiedereröffnet und mit Fakten der ultrastrukturellen und molekularen Analyse erneut und verbessert dargestellt werden sollten.

6. Introduction

During the past decades the cell and molecular biological discoveries of cell type-specific cytoskeletal and junctional molecules and their applications as immunocytochemical "markers" have been of increasing importance in clinical tumor diagnosis. One of the major improvements in the armamentarium of such diagnostically useful marker proteins was the definition of diverse types of the intermediate-sized filaments (IFs) forming major cell type-characteristic cytoplasmic structures in normal and malignantly transformed cells (e.g., Franke et al., 1978a, 1979b; Osborn and Weber, 1983; Banks-Schlegel, 1989; Fuchs and Weber, 1994). In particular the various keratins ("cytokeratins") are synthesized in cell type-specific patterns characteristic of certain epithelial and hair follicle cell types and also in tumors derived therefrom (Franke et al., 1978b, 1979a, b., 1981; Sun et al., 1979; Bannasch et al., 1980, 1981; Gabbiani et al., 1981; Moll et al., 1982; Moll and Franke, 1986; Moll, 1993; for recent reviews see Chu and Weiss, 2002; Moll et al., 2008).

Similarly, the molecular analysis of the intercellular junctions that often also serve as intracellular anchorage structures of the IF bundles, the desmosomes (*maculae adhaerentes*), has revealed cell type-specificity of their molecular composition. And this again has provided another set of valuable markers in tumor diagnostics, especially for the detection and characterization of carcinomas, including markers for stratified squamous epithelium-derived carcinomas (e.g., Franke et al., 1981, 1982, 1983b; Cowin and Garrod, 1983; Müller and Franke, 1983; Moll et al., 1986; Parrish et al., 1986; for recent reviews see Godsel et al., 2004; Holthöfer et al., 2007; Garrod and Chidgey, 2008; Schmidt and Koch, 2008; Franke, 2009).

The identification of the specific cell type under question and its level of differentiation is an essential part of tumor diagnosis, not only with respect to the histogenic origin but also as a basis for a prognosis as well as therapeutic decisions. In contrast, however, if it comes to questions of cell typing in the field of non-epithelial tissues and their tumors, the analytical knowledge and the availability of diagnostic marker proteins is still relatively poor. Therefore, in the study for my thesis I have systematically examined the molecular composition of potential marker structures of diverse cell types and tumors of non-epithelial origin, in particular the molecular components of the junctions connecting mesenchymal tumor cells.

6.1. Soft tissue tumors

The cytoskeletal and junctional molecules of tumors not derived from epithelia and neural cell types but from cells commonly subsumed under the collective term "mesenchymal" or "mesenchymal-derived" cells and the corresponding tumors, also known as “soft tissue tumors”, have not yet been systematically examined. Obviously these tumors are a very heterogeneous group, including tumors of connective tissue cells, diverse muscle types, bone or cartilage cells as well as tumors of vascular and blood-forming cells or lymphatic tissues (for recent reviews see e.g., Miettinen, 2003; Weiss and Goldblum, 2007). As some of these tumors still present great diagnostic and therapeutic problems, there have been several attempts over the past few decades to develop a useful diagnostic methodology and a comprehensive classification of the various soft tissue tumors (e.g., Lattes, 1983; Altmannsberger and Osborn, 1987; Weiss and Goldblum, 2001; Fletcher et al., 2002).

Soft tissue tumors are a highly multifarious group of tumors, comprising more than 50 histological subtypes. The aetiology of most benign and malignant soft tissue tumors is still unknown, although recent results suggest that most of them directly form from bone marrow-derived or soft tissue-resident mesenchymal cells (for references and discussions see Miettinen, 2003; Iwasaki et al., 2009). For example, the benign forms arising in more than 90 % dermally or subcutaneously often have a limited capacity for autonomous growth. Nearly one-third of benign soft tissue tumors are lipomas, one-third "fibrohistiocytic" and fibrous tumors, 10 % vascular and 5 % nerve sheath tumors (Fletcher et al., 2002; Miettinen, 2003; see there for references). Furthermore, benign soft tissue tumors outnumber malignant ones by at least two orders of magnitude.

Despite the fact that these tumors develop from mesenchymal elements, which represent almost two-third of the human body mass, malignant soft tissue tumors are rather uncommon tumors (Fletcher et al., 2002). Compared with carcinomas, they are relatively rare and constitute less than 1 % of all malignant cancers (Parker et al., 1996; see there for further references). On the other hand, the malignant tumors, generally known as sarcomas, are often locally aggressive and capable of both invasive and destructive growth and distant metastasis. The large majority of sarcomas seem to arise "spontaneously", i.e., without any obvious causative factors, whereas chemical carcinogens or radiation are rarely involved and certain viral infections have been reported to play a role in the formation of some sarcomas (cf. Eriksson et al., 1981, 1990; McClain et al., 1995; Karlsson et al., 1998; Weiss et al., 1998; Weiss and Goldblum, 2007). They can occur practically anywhere in the body but are preferentially located in the extremities. Sarcomas can be developed at any age and are more common in males, but

gender and age-related incidences vary amongst the specific histologic tumor types (see e.g., Weiss and Goldblum, 2001). While embryonal rhabdomyosarcoma and neuroblastoma develop almost exclusively in children, synovial sarcoma "Ewing-type tumors" and the alveolar rhabdomyosarcomas occur mostly in juvenile persons and young adults. Furthermore, malignant fibrous histiocytomas, liposarcomas or special fibrosarcomas as well as leiomyosarcomas are tumors mostly found in elderly persons.

Since histochemistry, including hematoxylin-eosin-staining and immunohisto-chemistry, are the most effective and cost-efficient ways to obtain correct diagnoses, these methods are widely used for soft tissue tumor diagnosis (for reviews see Folpe and Gown, 2001; Fletcher et al., 2002; Miettinen et al., 2003; Weiss and Goldblum, 2007). Nevertheless, electron microscopy, protein gel electrophoresis followed by immunoblot ("Western blot"), and polymerase chain reaction (PCR) analysis are also widely used for the detection, typing and staging of these tumors (e.g., Weiss and Goldblum, 2001; Miettinen et al., 2003).

While it is believed in a remarkably large part of the medical community that most soft tissue tumors can be diagnosed precisely and easily with conventional routine stainings of aldehyde-fixed, paraffin-embedded tissue sections, recent trials have revealed that the percentage of incorrect diagnoses not only of soft tissue tumors but also of carcinomas can be unexpectedly high in some forms, in particular if no appropriate immunohistochemistry has been applied (e.g. Rüdiger et al., 2002; Wetherington et al., 2002; for discussions of general methodological consequences and problems see also Wick et al., 1999; Taylor, 2000; Seidal et al., 2001; Wick and Mills, 2001; Rüdiger et al., 2003). In some specific soft tissue tumors, for instance the dermatofibrosarcoma protuberans, the accuracy was as low as 21 % (Rüdiger et al., 2003). Furthermore, in a critical review, 159 sarcomas diagnosed as pleomorphic malignant fibrous histiocytomas have been re-assessed using morphological as well as immunohistochemical and ultrastructural methods (cf. Fletcher, 1992). Unexpectedly, however, of these malignant fibrous histiocytomas, 97 cases (63 %) proved to be specific sarcomas, 20 turned out as non-mesenchymal neoplasms, and 42 cases were unclassifiable, however, only 13 % were eligible for consideration as malignant fibrous histiocytomas (Fletcher, 1992). These examples show the frequency of false diagnoses of non-epithelium-derived tumors and consequently point to the general and urgent need for an improvement and broadening of the spectrum of available immunohistodiagnostic markers, especially as most of these tumors prominently appear in younger patients. Thus, one has to conclude that at present the “catalog” of diagnostic markers for soft tissue tumors is still rather small and insufficient (see, e.g., Folpe and Gown, 2001; Fletcher et al., 2002; Miettinen, 2003).

6.2. The cell-cell adhering junctions connecting the cells of mesenchymally-derived tumors

As the general immunohistodiagnostic experience has shown, the architectonic proteins of frequent and relatively stable, e.g., cold stable and extraction-resistant structures are the most promising candidates for immunohistochemistry (see the developments of carcinoma-specific reagents mentioned at the beginning of this chapter), it was obvious that next to the IF proteins the components of cell-cell junctions would be of special diagnostic interest, as they often are also critically involved in tumor cell invasion, segregation and metastasis. Moreover, they are also target structures in several recent therapeutic approaches such as in the application of reagents interfering with N-cadherin (Li et a., 2007; Shintani et al., 2008; Perotti et al., 2009; for recent review see Blaschuk and Devemy, 2009) or epithelial cell adhesion molecule (EpCAM)-based cell-cell connections (Naundorf et al., 2002; Punt et al., 2002; Hartung et al., 2005; Brischwein et al., 2006; for recent reviews see Baeuerle and Gires, 2009; Deonarain et al., 2009).

This holds in particular as several pathologists have demonstrated that the ultrastructural morphology of the cell-cell contact structures of diverse kinds of non-epithelium-derived tumors appear to be different from the cell-cell junctions characterized so far. These authors have often used carefully chosen special names to refer to these apparently "novel looking" junctional structures as "desmosome-like", "rudimentary", "primitive", "diminutive", "poorly developed" or "intermediate" (e.g., Ghadially, 1980; Erlandson, 1981; Henderson et al., 1986; Dickersin, 2000). Some examples of light- and electron-microscopic descriptions of such novel junction types in anthologies of prominent pathologists are collected in Tables 1A - F. Moreover, as these novel cell-cell contact structures do not fulfil the molecular criteria of any of the junctions hitherto known, their presence or absence in non-epithelial tumors has been studied in remarkable detail and numbers (e.g., more than 1260 cases in the article by Quinonez and Simon, 1988). At the end, however, the laborious repetitive studies have not resulted in definitions of clear structure types primarily as objective, non-morphological criteria could not be applied. Therefore, it has been the aim of my thesis to characterize the various cell-cell junctions of soft tissue tumors by molecular biological criteria.

Tables 1A - F. Examples of diagnostically mentioned electron-microscopically described junctions of non-epithelial tumors which have not yet been molecularly characterized and classified but only referred to with misleading names.

A. Ghadially, 1980 ***Diagnostic Electron Microscopy of Tumours***

Fibroadenoma of breast: "desmosome-like junctions" (Fig. 37)
Atrial myxoma: "desmosome-like structures" (Fig. 41)
Cardiac myxoma: "unclear junctions" (Fig. 97)
Schwannomas: "desmosome-like structures" (Figs. 42 and 120)
Myofibrosarcoma: "desmosome-like junctions" (Fig. 114)
Phaechromocytoma: "desmosome-like structures" (Fig. 44)

B. Erlandson, 1981 ***Diagnostic Transmission Electron Microscopy of Human Tumors. The Interpretation of Submicroscopic Structures in Human Neoplastic Cells***

Pleomorphic angiosarcoma: "cell junctions" (Fig. 3.89)
Metastatic hemangiopericytoma: "primitive cell junctions" (Fig. 3.92)
Anaplastic germ cell tumor: "primitive cell junctions" (Fig. 4.34B)
Sertoli cell tumor: "long intermediate junctions" (Fig. 4.37)
Metastatic primitive neuroectodermal tumor: "primitive cell junctions" (Fig. 4.39)
Synovial sarcoma: "abortive junctional complexes" (Fig. 4.40)
Ewing's sarcoma: "primitive cell junctions" (Fig. 4.41)

C. Henderson, Papadimitriou and Coleman, 1986

Ultrastructural Appearances of Tumours. Diagnosis and Classification of Human Neoplasia by Electron Microscopy

Stromal myofibroblasts (in carcinomas): "intermediate junctions" (Fig. 3.16)
Cerebellar medulloblastoma: "small intermediate junctions" (Fig. 17.4)
Cerebral ependymoma: "junctional complexes" (Fig. 17.10)
Perineurioma: "small intermediate junctions" (Fig. 18.9)
Malignant fibrous histiocytoma: "junctions lacking ... morphology of desmosomes" (Fig. 20.7)
Gastric smooth muscle tumor: "intermediate junctions" (Fig. 21.4)
Leiomyosarcoma (poorly differentiated): "small intermediate junctions" (Fig. 21.8)
Angiosarcoma: "poorly developed junctions" (Figs. 23.2 and 23.3.)
(Malignant) haemangiopericytoma: "intermediate junctions" (Fig. 23.8)
Haemangiopericytoma: "... small ... plaques" (Fig. 23.9)
Epithelioid sarcoma: "small intermediate junctions" (Fig. 24.5)
Clear cell sarcoma: "[unspecified] intercellular junctions" (Fig. 24.6)
"poorly developed intercellular junctions" (Fig. 24.7)
Small cell tumor resembling atypical Ewing's sarcoma:
"[unspecified] intercellular junctions" (Fig. 25.9)
Follicular centre cell lymphoma: "desmosomal junctions" Fig. 27.12)

C. - *continued.*

Hairy cell leukemia: "zipper-like intercellular junctions" (Fig. 28.15)
Testicular seminoma: "intermediate junctions" (Fig. 30.3)
Endodermal sinus tumor: "intercellular junctions" (Figs. 30.6 and 30.7)
Blastemal Wilms' tumor (monomorphic): "intercellular junctions" (Figs. 30.8 - 30.11)

D. Quinonez and Simon, 1988 ***Cellular Junctions in a Spectrum of Human Malignant Tumors***

(observations reported in one of the earlier articles A-C are not included here)

Sarcoma botryoides: "desmosomes", "tight junctions" (Figs. 1 and 5)
Granulous cell tumor: "desmosomes" (Fig. 2)
Liposarcoma: "long desmosomes" (Fig. 3)
Ewing´s sarcoma: "tight junctions", "paired subplasmalemmal densities" (Figs. 4 and 6)

E. Dickersin, 2000 ***Diagnostic Electron Microscopy. A Text/Atlas***

Dendritic cell sarcoma: "desmosomes" (Fig. 3.100)
Neuroblastoma: "intercellular junctions" (Fig. 4.11)
Primitive neuroectodermal tumor: "diminuitive junctions" (Fig. 4.19)
Nephroblastoma: "small intercellular junctions" (Fig. 4.40)
Nephroblastoma (Wilms' tumor): "foci of junctional complexes" (Figs. 4.43 and 4.4.)
Desmoplastic small round tumor: "multiple intercellular junctions" (Fig. 4.56)
Fibrosarcoma (in dermatofibrosarcoma): "prominent junctions" (Fig. 6.10)
Malignant fibrous histiocytoma: "intercellular junctions" (Figs. 6.28 and 6.29)
Synovial sarcoma: "small intercellular junctions" (Fig. 6.55)
Hemangioma: "prominent junctional complexes" (Figs. 6.92 and 6.93)
Hemangioendothelioma: "tight junctions", "lateral junctions" (Fig. 6.95)
Hemangiopericytoma: "intermediate junctions" (Fig. 6.107)
Sarcomatoid carcinoma: "myofibroblastic features of desmosomes" (Figs. 6.125 and 6.126)
Signet-ring stromal tumor: "prominent intercellular junctions" (Fig. 7.51)
Sertoli-stromal cell tumors (androblastomas): "junctional complexes" (Figs. 7.52 - 7.54)
Hepatoblastoma (embryonal): "intercellular junctions" (Fig. 9.56)

F. A short exemplary collection of terms showing that vague pseudomorphological names are also given to junctional structures of non-epithelium-derived tumors in recent years

Antonescu and Erlandson, 2001 - Fibrosarcoma: "rudimentary cell junctions" (Figs. 8 and 9)

Barrett et al., 2002 - Soft tissue perineurioma: "desmosome-like junctions" (Fig. 4)

Zamecnik et al., 2002 - Dermatofibrosarcoma protuberans: "desmosome-like junctions" (Fig. 10)

Rampisela and Donner, 2004 - Pleomorphic sarcoma: "desmosome-like junctions" (Fig. 2)

Suster and Moran, 2005 - Synovial sarcoma: "desmosome-type cell junctions" (Fig. 12)

Eyden et al., 2009 - Epitheloid sarcoma: "primitive junctions (not desmosomes)" (Fig. 3)

6.3. Cell-cell adhering junctions: A brief review

In current textbooks of cell biology, four major categories of cell-cell junctions are generally distinguished (for schematic overview see Figure 1; see also Bloom and Fawcett, 1975; Darnell et al., 1986; Drenckhahn and Zencker, 1994; Alberts et al., 2002; Kühnel, 2002; Pollard and Earnshaw, 2002; for a historic review see Franke, 2009): the gap junctions (*nexus*), the tight junctions (*zonulae occludentes*), the desmosomes (*maculae adhaerentes*) and the various forms of adherens junction structures (*puncta, zonulae or fasciae adhaerentes*).

Since the latter two, the desmosomes and the adherens junctions, are both characterized by a dense cytoplasmic plaque, are able to anchor cytoskeletal filament bundles and are based on clusters of transmembrane glycoproteins of the larger cadherin family, they have been collectively subsumed under the term "adhering junctions" (AJs; for reviews on ultrastructural organizations and specificities see, e.g., Farquhar and Palade, 1963; Staehelin, 1974; Drochmans et al., 1978; Geiger et al., 1985a, b). The molecular compositions of these AJs and several related structures have so far revealed members of the cadherin superfamily, comprising not only “classical” type I and type II cadherins but also some atypical cadherins (e.g., T- and Li-cadherin), protocadherins and cadherin-related proteins (for references see: Takeichi, 1990; Angst et al., 2001; Patel et al., 2003; Troyanovsky, 2005; for evolutionary aspects see also Hulpiau and van Roy, 2009).

The desmosomes are junctions that tether bundles of IFs to the plasma membrane and are present in all epithelial cells and tumors derived therefrom as well as in heart structures, meningothelia and meningioma tumors and in certain dendritic reticulum structures of lymph nodes (for references see Franke et al., 1982; Kartenbeck et al., 1984; Schmidt et al., 1994; Wacker, 1994; Akat et al., 2003; Moll et al., 2009). They are formed by cell type-specific combinations of desmogleins (Dsg1-4) and desmocollins (Dsc1-3) which are anchored to the plaque forming proteins plakophilins (Pkp1-3), desmoplakin and plakoglobin, a protein known as a common constituent of both adherens and desmosomal plaques (Cowin et al., 1986; Franke et al., 1987a, b; for recent reviews see Godsel et al., 2004; Holthöfer et al. 2007; Garrod and Chidgey, 2008; Schmidt and Koch, 2008; Waschke, 2008; Delva et al., 2009; Franke, 2009).

By contrast the adherens junctions are present in a variety of cell types and characterized by cell type-specific assemblies of type I cadherins, including E-cadherin, which is regarded as typical of epithelial cells, N-cadherin, characteristically occurring on mesenchymal and neuronal cells as well as P-cadherin, first identified in the placenta.

Additionally, type II cadherins, for example the mesenchymal cadherin-11 or VE-cadherin specific for vascular endothelial cell junctions, can also be found. These transmembrane glycoproteins are anchored in cytoplasmic plaque structures of varying sizes and thicknesses containing *armadillo*-type proteins such as plakoglobin, β-catenin as well as proteins p120, p0071 and/or ARVCF, together with the actin microfilament-binding proteins α-catenin, α-actinin, vinculin and afadin (see e.g., anthology of Behrens and Nelson, 2004; for a recent review see Meng and Takeichi, 2009).

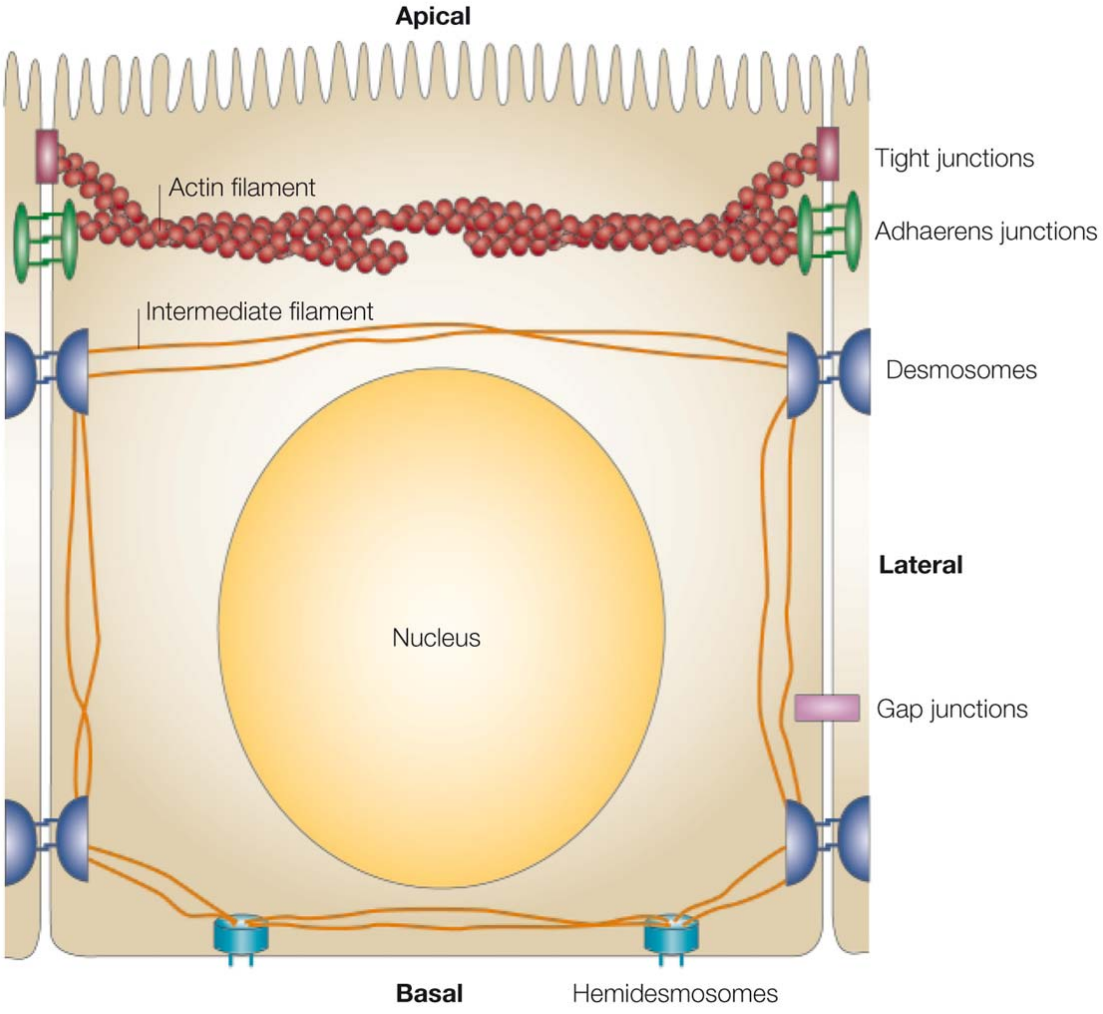


Figure 1. Schematic overview of a polarized epithelial cell showing the four major categories of cell-cell junctions as well as the hemidesmosomes, a specific asymmetric cell-matrix attachment structure. The tight junctions and the adherens junctions anchor actin microfilaments, whereas the desmosomes as well as the hemidesmosomes are usually associated with the intermediate filaments (modified from Matter and Balda, 2003).

6.4. The molecular composition of adhering junctions connecting cells of mesenchymal tissues and tumors: Novel categories

In addition to the "classical" AJs mentioned afore a number of further cell type-specific AJ forms have been identified in recent years that cannot be readily subsumed under the two major categories, i.e. desmosomes and adherens junctions. The diverse novel junctions connecting non-epithelial cells, including mesenchymal and mesenchymally-derived cells, are listed in Table 2 (see also Franke et al., 2009).

The hitherto most exclusively studied special AJ type is the cardiomyocyte-connecting composite junction (*area composita*) of the mature mammalian myocardium (Borrmann, 2000; Franke et al., 2006; Goossens et al., 2007), which represents an amalgamated form comprising components of both, desmosomal and *fascia adhaerens*-type proteins and glycoproteins, specifically Dsg2 and Dsc2 as well as N-cadherin and cadherin-11, anchored in plaques containing the major proteins of desmosomes such as desmoplakin, plakoglobin and plakophilin-2 (Pkp2) as well as proteins of the adherens junction category such as α- and β-catenin, p120, p0071 and ARVCF (Borrmann et al., 2006; Pieperhoff and Franke, 2007).

The second type of a morphologically exceptional junction is the *complexus adhaerens* of the endothelial and virgultar cells of the lymph node sinus (Schmelz et al., 1990, 1994; Schmelz and Franke, 1993; for the most recent review see Moll et al., 2009). In addition to VE-cadherin, often in colocalization with N-cadherin as transmembrane glycoproteins, these junctions are associated with a plaque containing desmoplakin and plakoglobin but also α- and β-catenin, protein p120 and the actin filament-anchoring proteins ZO-1 and afadin as well as the tight junction-typical transmembrane proteins claudin-1, claudin-5 and JAM-A (see, e.g., Valiron et al., 1996; Dejana, 2004; Hämmerling et al., 2006; Baluk et al., 2007; Pfeiffer et al., 2008; Moll et al., 2009).

Another extreme junctional structure is found in the anucleate prismoid fiber cells of the eye lens, which are surrounded and connected by extended plasma membrane contacts and a near-continuous cytoplasmic coat forming a giant cell-cell adhesive complex, the *cortex adhaerens* (Straub et al., 2003). These giant cortical plaque-supported structures, however, show marked regional differences. In some regions, in particular at the short polar sides, the membranes are tightly connected by clusters of N-cadherin in combination with cadherin-11, anchored in a cytoplasmic plaque composed of α- and β-catenin, protein p120 and plakoglobin in which, however, some other adherens junction plaque components such as proteins p0071 and ARVCF, afadin and all desmosomal proteins have not yet been detected. On the other hand, various other proteins can be identified in this cortical plaque such as ezrin, periplakin and periaxin.

By contrast, in primary mesenchymal cells of mouse embryos as well as in cultures of human bone marrow-, placenta- or adipose tissue-derived mesenchymal stem cells, rather small AJs, described as *puncta adhaerentia minima*, have been shown (Franke et al., 1983a; Wuchter et al., 2007),. They are characterized by N-cadherin and partly also cadherin-11, in combination with the typical adherens junction plaque proteins α- and β-catenin, p120, p0071 and ARVCF as well as plakoglobin and the actin micro-filament-binding proteins afadin, often together with ezrin and α-actinin (Wuchter et al., 2007). In addition to these "punctate" AJs, bone marrow-derived mesenchymal stem cells growing in culture also often form variably extended, often very long (up to 50 µm) invaginations forming near-continuous, sometimes gigantic AJs, the *manubria adhaerentia* (Wuchter et al., 2007).

Cell adhesion type	Occurrence	Associated filaments	Transmembrane proteins	Specific plaque proteins
Adherens junctions *Zonula adhaerens* *Fascia adhaerens* *Punctum adhaerens*	Epithelia Endothelia Myocardium Diverse mesenchymal derived cells	Microfilaments (Actin)	Classical cadherins (E-, N-, VE-, P- or R-cadherin) Nectin	Plakoglobin α-Catenin β-Catenin Protein p120 Protein p0071 Protein ARVCF α-Actinin Afadin Vinculin
Areae compositae Borrmann, 2000 Franke et al., 2006 Goossens et al., 2007	Cardiomyocytes of maturing and adult heart	Microfilaments (Actin) Intermediate-sized filaments	N-cadherin Cadherin-11 Desmoglein-2 Desmocollin-2	Plakoglobin α-Catenin β-Catenin Protein p120 Protein p0071 Protein ARVCF Desmoplakin Plakophilin-2 Proteins ZO-1–3 Vinculin
Cortex adhaerens Straub et al., 2003	Eye lens interior	nd*	Cadherin-11 N-Cadherin	Plakoglobin α-Catenin β-Catenin Protein p120 Vinculin Ezrin Desmoyokin Periaxin Periplakin
Complexus adhaerens Schmelz and Franke, 1993 Schmelz et al., 1994 Hämmerling et al., 2006 Moll et al., 2009	Endothelial and virgultar cells of lymph node sinus	nd	N-cadherin VE-cadherin Claudin-1 Claudin-5 JAM-A	Plakoglobin α-Catenin β-Catenin Protein p120 Desmoplakin Protein ZO-1 Afadin
Puncta adhaerentia minima ***Manubria adhaerentia*** Wuchter et al., 2007	Mesenchymal cells Myofibroblasts Mesenchymal cells growing in culture	Microfilaments (Actin)	N-cadherin Cadherin-11	Plakoglobin α-Catenin β-Catenin Protein p120 Protein p0071 Protein ARVCF α-Actinin Afadin Vinculin Ezrin

Table 2. The molecular components of junctions connecting non-epithelium-derived cells. * Actin microfilaments are seen near the junctions but their specific associations are not clear. nd, not decided yet.

7. Material and methods (additional information)

The details of the materials and the methods used in the different parts of the thesis have been described in the specific publications (see also “3. Publications of Steffen Rickelt...”). This chapter briefly summarizes some additional methods used in the course of this study.

7.1. Small interference RNA transfection experiments

Small interference RNAs (siRNAs) specific for human Pkp2 and N-cadherin as well as control siRNAs, including ON-TARGETplus SMARTpool, non-targeting control siRNA and lamin A/C siRNA, were purchased from Dharmacon Inc. (Chicago, IL, USA). All siRNA transfection experiments were essentially performed according to the suppliers instructions. The cells were seeded onto six-well plates which contained up to 4 poly-L-lysine-coated glass coverslips per well. Transfections were started 24 h after starting culturing, using lipid-based transfection reagents (DharmaFECT no. 1 and 4). Improved down-regulation results for cell cultures treated with siRNA specific for N-cadherin could be revealed upon double transfection (24 h and 48 h after starting culturing). The cells were then incubated for further 48 h with the transfection mixture, before the cell culture medium was replaced by normal growth medium to prevent cytotoxicity (Rickelt et al., 2009). Alternatively, cells were fixed after certain time points (in most cases 48, 72, 96 h) and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as well as immunofluorescence microscopy (for further details see the experiments with rat cardiomyocytes of Pieperhoff et al., 2008).

7.2. cDNA transfection experiments

Different normal human fibroblastoidal cell lines, including lung fibroblasts of lines WI38 and LL24 as well as skin fibroblasts of lines Hs295.SK and HG261 (cf. Rickelt et al., 2009), were used for transfection experiments. As transfection of fibroblastoidal cell lines is known to be difficult, different transfection protocols have been compared. For this reason, application protocols of different suppliers, including Mirus transfection reagents *Trans*IT-LT1 and *Trans*IT-293 (Mirus Bio LLC, Madison, WI, USA); Polyplus transfection reagent jetPEI (Polyplus-transfection Inc., Erlangen, Germany) and FuGENE 6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) have been used. Additionally, different reagents from Invitrogen (Karlsruhe, Germany) were tested (including Lipofectamine;

Lipofectamine 2000; LTX-PLUS; Superfect). All protocols for transfection of specific human Pkp2-cDNA, previously cloned into the eukaryotic expression vector pcDNA3.1 (cf. Mertens et al., 1996), were applied according to the individual manufacturer's instructions and subsequently analyzed using immunobiological and immunofluorescence microscopical techniques (see, e.g., Köser et al., 2003).

7.3. Reverse transcriptase-PCR

The total RNA from various cell culture lines was isolated with the "TriPure Isolation Reagent" (Roche Diagnostics) according to the manufacturer's instructions. Specific primers were applied in reverse transcriptase PCR (RT-PCR) experiments to obtain the different sequence intercepts (see Table 3). The PCR protocols were performed according to standard protocols (for details see Rickelt et al., 2009). The resulting PCR fragments were analyzed by electrophoresis on 2 % agarose gels.

	primers	primer sequences	hypothetical fragment length
αE-catenin	forward reverse	5'-TTTCTCAAGGAGGAGCTTGTG-3' 3'-TGCCTGGGATGCAGTATAGA-5'	429 bp
αN-catenin	forward reverse	5'-ATGACTTCGGCAACTTCACC-3' 3'-CTCATGACATCTGCCATGTC-5'	444 bp
αT-catenin	forward reverse	5'-GAAAAGATTGCTGAGCAAGT-3' 3'-GACATTTTCACTGTTTGCACTA-5'	464 bp
Plakophilin-2	forward reverse	5'-GCAAACCAGAGACTTGGAGAC-3' 3'-AGGAGAGGTTATGAAGAATGC-5'	373 bp (for Pkp2a) 505 bp (for Pkp2b)
GAPDH	forward reverse	5'-CCATCACCATCTTCCAGGAG-3' 3'-ATCCACAGTCTTCTGGGTGG-5'	349 bp

Table 3. PCR-primers used to investigate the occurrence of different α-catenins as well as plakophilin-2 and GAPDH in various mesenchymal derived cells.

8. Results

This thesis reports on some first attempts to elucidate the heterogeneous molecular compositions of the specific cell-cell adhering junctions (AJs) connecting normal as well as tumor cells of the mesenchymally-derived category. The first part deals with the discovery of an unexpected protein ensemble in a series of AJ types connecting a series of transformed mesenchymal cell types growing in culture and in some soft tissue tumors. The second part of the thesis focuses on the discovery of specific cell-cell contacts and on the molecular composition of AJs connecting melanocytes or melanoma cells in cell culture and *in situ*. Finally, I have studied in great detail the unexpected phenomenon of spontaneous cumulative syntheses of junctional proteins and glycoproteins as well as their assemblies to diverse AJ structures connecting cells of human hematopoietic tumor lines growing in culture.

8.1. Adhering junctions of mesenchymally-derived cells: I. The detection and characterization of adherens junctions containing plakophilin-2

The plaque protein plakophilin-2 (Pkp2) is a prominent component of simple epithelia, basal layers cells with proliferative potential in stratified epithelia, meningothelia and the dendritic reticulum cells of lymphatic tissues as well as in the composite junctions of the adult mammalian myocardium. Pkp2 is also an important component of the desmosomal ensemble of the junctions connecting tumor cells derived from the tissues mentioned, including all carcinoma types (see, e.g., Mertens et al., 1996, 1999; Borrmann et al., 2000; Akat et al., 2003, 2008; Grossmann et al., 2004; Hämmerling et al., 2006; for recent review see Bass-Zubek et al., 2009). Thus it was a great surprise to note that this protein – sometimes together with Pkp3 – can also occur out of any desmosomal context.

8.1.1. Molecular composition of adhering junctions of certain proliferative mesenchymally derived normal and malignantly transformed cells growing in cultures and in tumors

Using biochemical and immunolocalization analyses I could show in a number of mesenchymal or mesenchymally-derived normal and malignantly transformed cells growing in culture the early acquisition and then the general occurrence of junction complexes containing one or both transmembrane glycoproteins, N-cadherin and cadherin-11, which were anchored in a submembranous plaque comprising α-catenin and the *armadillo* proteins β-catenin, plakoglobin, p120, p0071 and/or protein ARVCF, which in turn are associated with a series of actin microfilament-binding proteins, including vinculin and α-actinin. Electron and immunoelectron microscopy then allowed the demonstration of the ultrastructural details of these AJs, which mostly display a rather thin electron-dense plaque of the *puncta adhaerentia*-type, also known from a wide range of cells including bone marrow-derived mesenchymal stem cells (Wuchter et al., 2007; see there for further references).

Surprisingly, however, I noted that cells of certain human and animal culture lines are connected by AJs which in addition to their typical molecular ensemble contained Pkp2 as a major and stable plaque component (for an example see Figure 2). Pkp2, which so far had been known only for its junction-organizing role in desmosomes and the composite junctions of cardiomyocytes (for recent reviews see Franke, 2009; Franke et al., 2009) was either exclusively present or in combination with Pkp3 whereas other desmosomal proteins, including desmosomal cadherins and desmoplakin, were absent. Particularly interesting in this context was the finding that SV40-transformed cell lines such as the “SV80 fibroblasts” and WI38VA13 embryonic cells were intensely Pkp2-positive while the corresponding non-transformed lines were not (see, e.g., Figure 1 in Rickelt et al., 2009). Therefore, I dedicated specific effectors to elucidate the structural role of Pkp2 and the mode of the frequent and spontaneous acquisition of Pkp2 to the plaques of certain rather actively proliferating mesenchymal cells. Specifically, I also tried to identify possible Pkp2-containing protein complexes by immunoprecipitation (IP) and sucrose gradient centrifugation and I found significant tight complexes of Pkp2 with N-cadherin as well as α- and β-catenin (Rickelt et al., 2009). As this new Pkp2-rich AJ type has been found in a diversity of highly proliferative cell culture lines, including samples derived form soft tissue tumors, I had to conclude that Pkp2 is a regular, frequent and characteristic plaque component typical of a special AJ subtype of mesenchymal and soft tissue tumor cells (for discussions see Franke et al., 2009; Rickelt el al., 2009).

When I had noticed in many transformed and tumor-derived cell culture lines the broad occurrence of relatively large amounts of Pkp2 I examined soft tissue tumors *in situ*.

As the existing antibodies were not readily applicable for sensitive detections of Pkp2 in sections through formaldehyde-fixed, paraffin-embedded tissue samples, I decided to generate improved, i.e. highly sensitive and stable monoclonal antibodies (mabs) specific for Pkp2, which were screened for immunostaining reactivity on formaldehyde-fixed cell cultures and tissue materials. In addition, improved buffers and application protocols for heat-induced antigen-retrieval had to be established (cf. Rickelt et al., 2010).

In a first extended series applying these novel and improved Pkp2-specific mabs to a variety of cell cultures and tissues I was able to demonstrate this plaque protein in the junctional structures of diverse human and animal mesenchymal cell lines (see, e.g., Table 1 in Rickelt et al., 2009) as well as on sections through some human tumors, notably some regions of rhabdomyosarcomas and an extended series of cardiac myxomas (for details see Rickelt et al., 2009, 2010). It is now clear that with the use of these improved antibodies, Pkp2 can serve as a novel cell type – and cell state – diagnostic marker in studies of cell cultures (for an example see Figure 2) as well as of tissues.

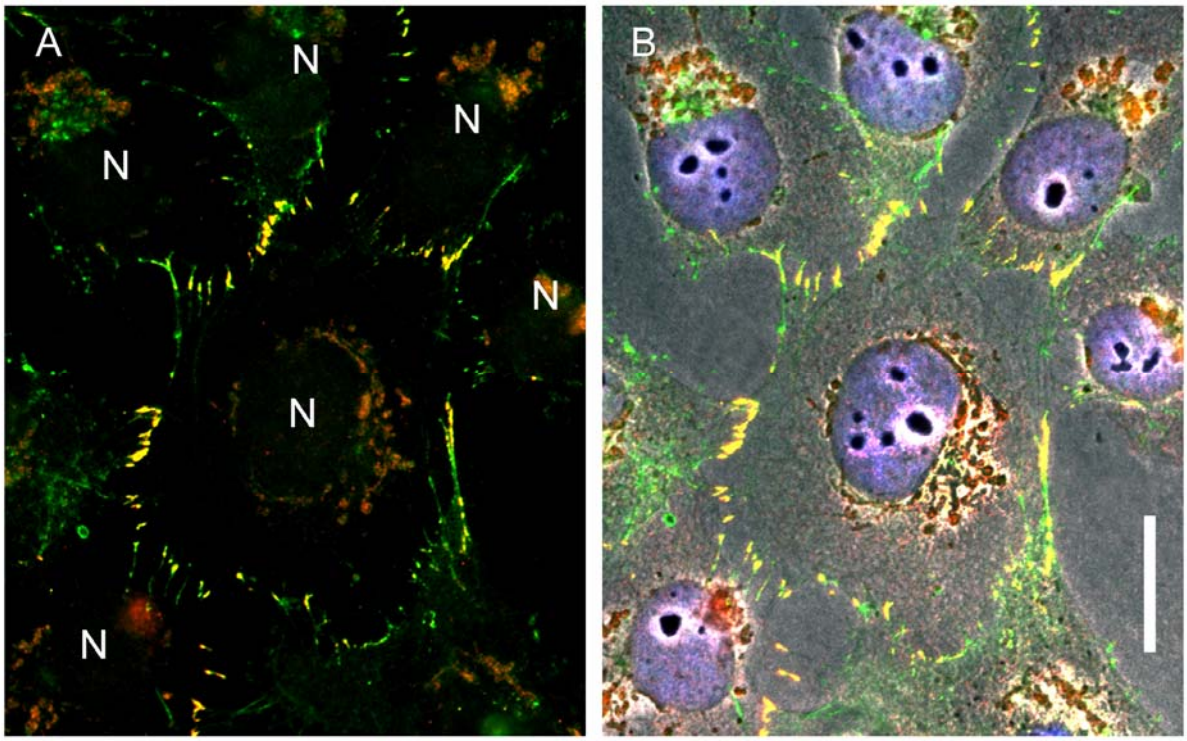


Figure 2. (**A**) Double-label immunofluorescence microscopy images of SV40-transformed human fibroblasts (line SV80) after reaction with antibodies to desmosomal protein Pkp2 (red) in comparison with the adherens junction protein β-catenin (green). In all cells shown, Pkp2 and β-catenin clearly colocalized in the AJs (**A**; yellow merge color). Note the frequency of such Pkp2-positive AJs in the confluent colonies of near-isodiametric cells. (**B**) The merged picture with a phase contrast background is shown. DAPI stain (blue) has been used to visualize nuclei. N, nucleus. Scale bar: 20 µm. This micrograph was taken and modified from the Int J Cancer 2009, 125:9; cover figure.

8.1.2. Small interference RNA-mediated plakophilin-2 reduction in cultured transformed mesenchymal cells

As small interference RNA (siRNA)-mediated Pkp2-gene product knock-down experiments in cell cultures of rat cardiomyocytes have revealed the important role of Pkp2 in the organization and stabilization of the composite junctions and even the demonstration of complete cardiomyocyte separations (Pieperhoff et al., 2008; for corresponding siRNA experiments see also Oxford et al., 2007; Fidler et al., 2008), I decided to also examine the role of Pkp2 in transformed mesenchymal cells in detail.

Therefore, I determined the influence of siRNA-mediated reduction of Pkp2-mRNA in human SV80 fibroblasts cultures using specific human lamin A/C-siRNA as a control for the specificity of mRNA knock-down (for details and references see Pieperhoff et al., 2008). Using SDS-PAGE immunoblot analysis I could show a specific down-regulation of Pkp2 in SV80 fibroblasts already after 48 h of PKP2-siRNA treatment whereas N-cadherin, used for comparison, remained rather stably synthesized even 96 h after transfection (Figure 3).

These findings could be supported by immunofluorescence microscopy. For example, Figure 4 shows SV80 fibroblasts treated with Pkp2-siRNA and stained with antibodies against α-catenin or Pkp2 in combination with N-cadherin antibodies. While the typical AJ proteins of these cells are still constantly produced and AJ-assembled, the Pkp2 staining is drastically reduced. By contrast, when I performed siRNA-mediated knock-down of N-cadherin in comparison with non-targeting control treatments, a specific and continuing reduction of N-cadherin was noted at different time points (48, 72 and 96 h; see also the upper right of Figure 3). These drastic reductions of N-cadherin were accompanied by noticeable, but not complete reductions of the cell-cell contact structures (data not shown). Interestingly, however, the total level of Pkp2 proteins in these cells remained rather unchanged (Figure 3). In further immunofluorescence microscopy studies of N-cadherin siRNA-treated cells, I was not able do detect any Pkp2 particles at the intercellular junctions. These results were not readily explainable but might be due to conformational changes of Pkp2, resulting in antibody inaccessibility, or to a change of localization of Pkp2 either into the nucleus (cf. Mertens et al., 1996) or as diffusely dispersed protein over the cytoplasm or along the entire plasma membrane as recently shown for desmoglein Dsg2 in melanocytes and melanoma cells grown in culture (Schmitt et al., 2007; Rickelt et al., 2008; see also 8.2.2).

Clearly, the most important finding of the siRNA-mediated knock-down experiments in Pkp2-containing transformed mesenchymal cells, was that a reduction of Pkp2 for an extended period of time, did not result in a sufficient dissociation or loosening of cell-cell

contacts and did not lead to the separation of cells as reported for rat cardiomyocytes (Pieperhoff et al., 2008 and references cited therein).

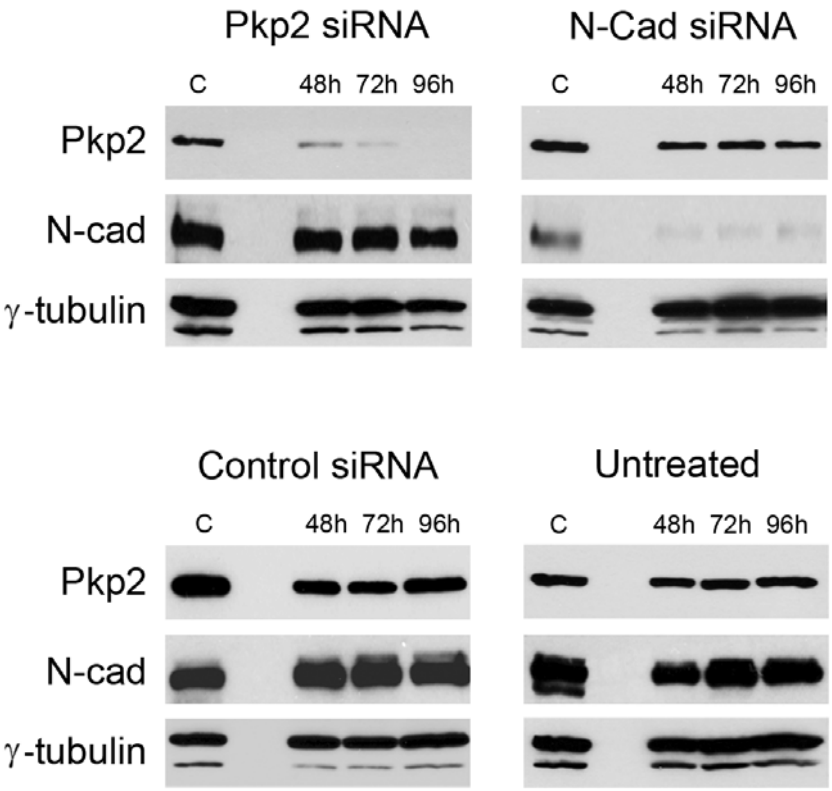


Figure 3. Selective reduction of plakophilin-2 (Pkp2) and N-cadherin (N-cad) upon siRNA-mediated interference in transformed human culture cells of line SV80, as seen in cell lysates analyzed by SDS-PAGE and immunoblotting with antibodies specific for Pkp2 and N-cadherin or for γ-tubulin as loading control. Cells at 48, 72 and 96 h after transfection have been used and compared with the initial cells (C). Pkp2 has been significantly down-regulated already 48 h after incubation with Pkp2-siRNA and this decrease continues for up to 96 h of siRNA exposure whereas N-cadherin remains stably expressed. By contrast, when N-cadherin has effectively been down-regulated a Pkp2 change has not been noticed. Cells treated with non-targeting siRNA as well as untreated cells have been used as controls.

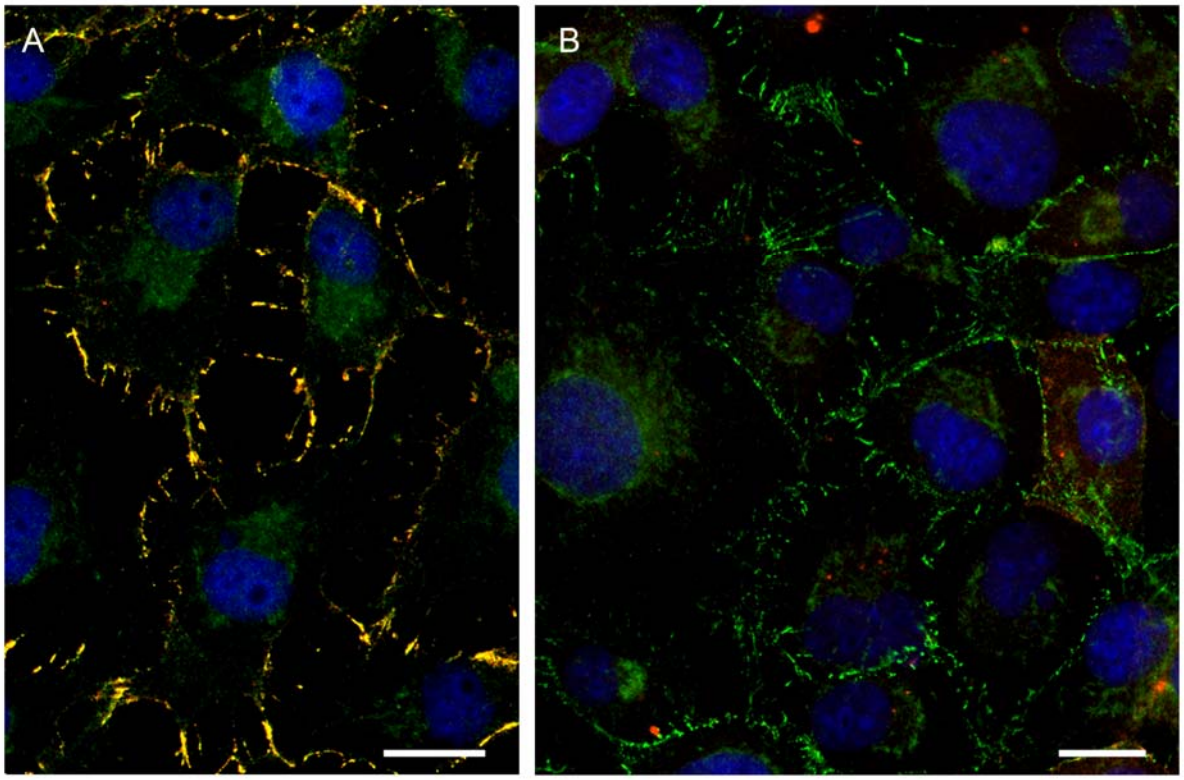


Figure 4. Double-label immunofluorescence microscopy of human SV80 fibroblasts after 96 h treatment with Pkp2-siRNA followed by immunostaining with antibodies against α-catenin (**A**; red) and Pkp2 (**B**; red), together with N-cadherin (green). Note that the genes encoding adherens junction proteins remain stably expressed (**A**, yellow merge color) while Pkp2 is drastically reduced after siRNA treatment (**B**). DAPI stain (blue) has been performed to show the nuclei. Scale bars: 20 µm.

8.1.3. Synthesis and stable integration of plakophilin-2 in adhering junctions of normal mesenchymal cells upon cDNA transfection

Since Pkp2 is present in AJs of various transformed fibroblastoidal cells, I performed human Pkp2 cDNA-transfection experiments in order to determine whether Pkp2 also associates with and integrates into the AJ ensembles of normal mesenchymal derived cells. Therefore, I used different normal fibroblastoidal cell lines that produce only "normal" *puncta adhaerentia*-type AJs without any Pkp2 (cf. Rickelt et al., 2009). To this end, different transfection protocols of various suppliers have been examined (see also 7.2.), using SDS-PAGE-separated polypeptides and immunoblotting for product identification (Figure 5). In addition, immunofluorescence microscopy with antibodies directed against Pkp2, N-cadherin or β-catenin has been performed (Figure 6). Preliminary data have shown an enrichment of transgenically expressed Pkp2 and a rather homogeneous Pkp2 immunostaining pattern in cell-cell contact regions (Figure 6), an observation previously also shown in our laboratory for similarly transfected HT-1080 fibrosarcoma cells (Köser et al., 2003). Thus, future and more detailed experiments will have to address the questions whether the "normal", mostly nucleoplasmic low concentration "background" Pkp2 or both associate with the AJ plaques of these non-epithelial cells and whether this AJ association correlates with cell proliferation as recently indicated by the rapid appearance of Pkp2 in very early steps of cell culture growth of valvular interstitial cells (cf. Barth et al., 2009).

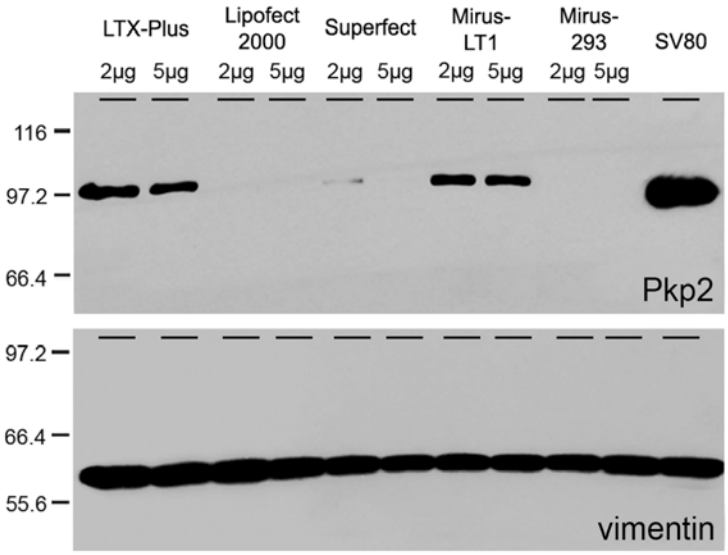


Figure 5. Characterization of human mesenchymally-derived skin fibroblasts of line HG261 transfected with either 2µg or 5µg cDNA encoding human Pkp2. The comparison of different transfection procedures, including LTX-Plus, Lipofectamine 2000; Superfect as well as Mirus *Trans*IT-LT1 and *Trans*IT-293, was performed, before immunoblots of near equal amounts of SDS-PAGE-separated proteins were allowed to react with antibodies specific for Pkp2 or vimentin. Note that in cells transfected with LTX-Plus or Mirus *Trans*IT-LT1 reagents, Pkp2 can be demonstrated in relatively moderate amounts whereas all the other reagents have not worked. Interestingly, the transfection rate has not been dependent on the amount of cDNA used. Immunoblots reacted with vimentin antibodies indicate not only the mesenchymal origin of the cells but also show that equal protein amounts had been loaded. Whole lysates of SV80 fibroblasts (*right lane*) were used as a positive control. Molecular weight reference bands (kDa) are shown on the left margin.

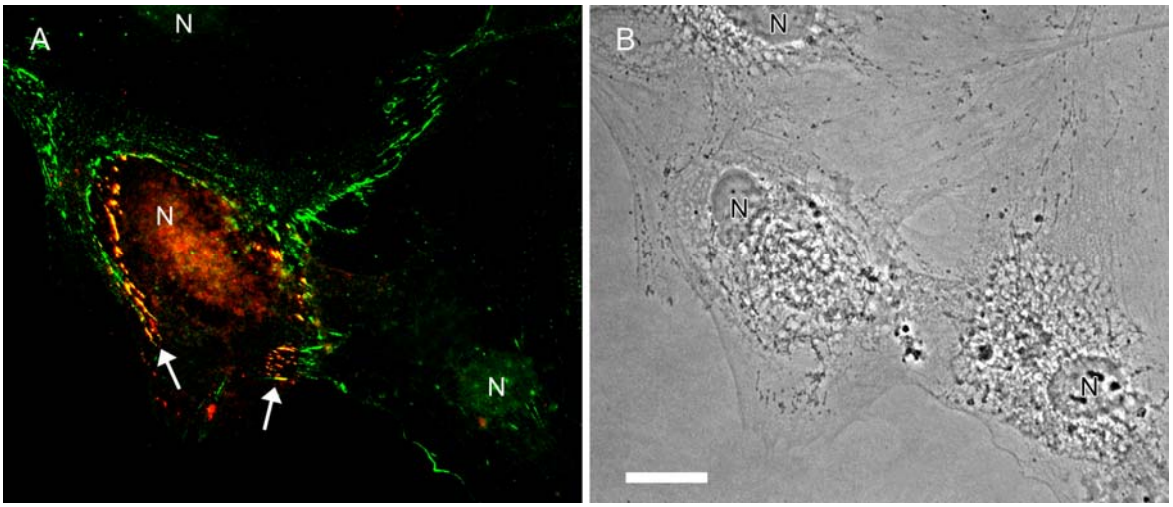


Figure 6. (**A**) Double-label immunofluorescence microscopy of human mesenchymally-derived skin fibroblasts of line HG261 transfected with cDNA encoding human Pkp2 which shows the protein Pkp2 (red) with the endogenous *puncta adhaerentia* protein β-catenin (green). Small arrows point to structures at which colocalization of both *armadillo* proteins (yellow merge color) is partially seen at adherens junctions whereas in non-transfected cells only rather weak and homogeneous β-catenin staining along the entire cell-cell contact region is seen. (**B**) The phase contrast image of (**A**). N, nucleus. Scale bar: 20 µm.

8.1.4. Exclusion of a possible role of αT-catenin in the acquisition of plakophilin-2 to the adhering junction plaques of mesenchymal cells

Previous investigations have revealed three different highly similar α-catenins: the ubiquitously expressed αE-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), the neural αN-catenin (Hirano et al., 1992), and the αT-catenin originally discovered in peritubular myoid cells of the testis (Janssens et al., 2001). The latter protein, αT-catenin, also occurs in some other tissues, however, the highest level has been found in the heart, where it occurs together with αE-catenin at the composite junctions of the intercalated disks (Janssens et al., 2001). In addition, myocardial αT-catenin is demonstrably involved in the binding to Pkp2 and the translocation to – and integration in – the plaque of composite junctions (Goossens et al., 2007).

Since α-catenins play a fundamental role in the AJ association of actin micro-filament bundles to the classical cadherin mediated cell-cell adhesion complex, I have examined the presence of the three catenins in several cell lines using the RT-PCR technique (for protocols and primer sequences see 7.3 and Table 3). Figure 7 presents the results obtained in 11 different "mesenchymal cell lines" and shows that αE-catenin occurs in all lines, while αN-catenin is detected only in bovine fibroblasts of line B1. By contrast αT-catenin is not found in any of these cell lines, an observation also confirmed by immunofluorescence microscopy (data not shown). In comparison, αT-catenin is well seen in heart cells (Figure 7) and Pkp2-mRNA is detectable in all cell lines.

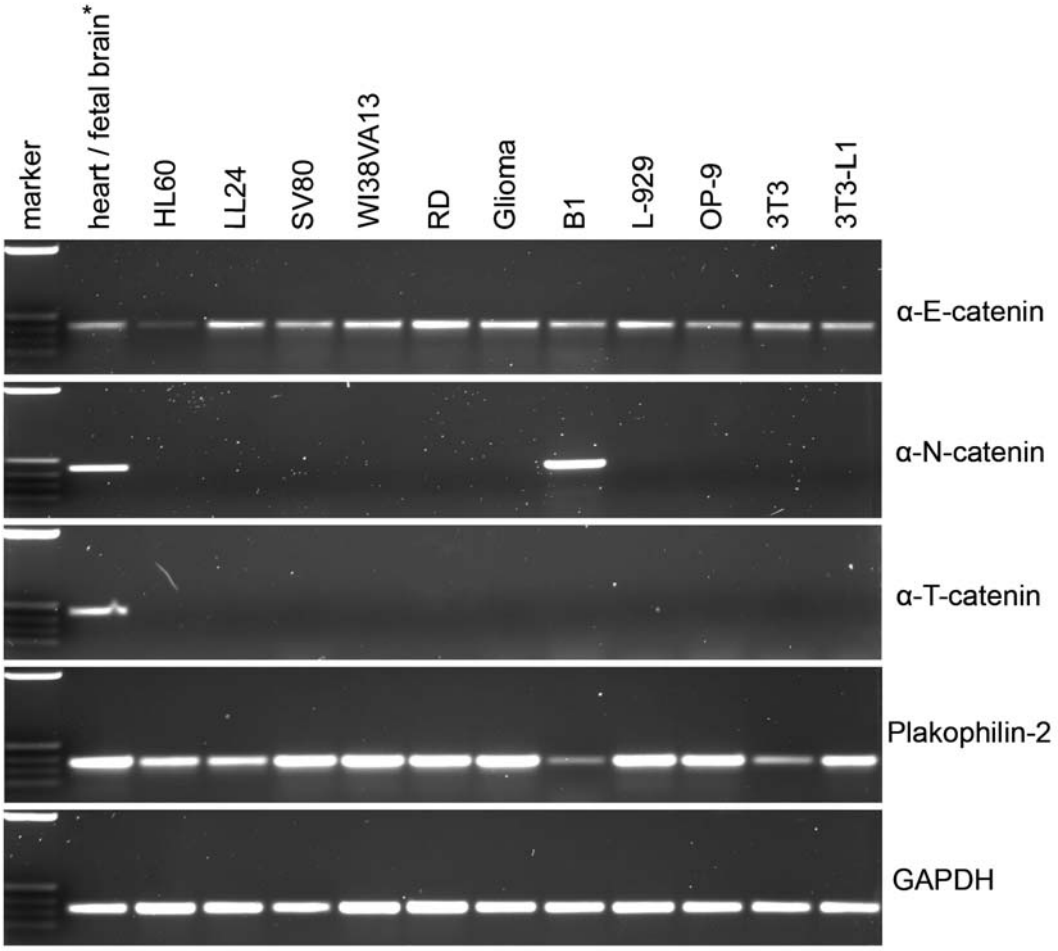


Figure 7. RT-PCR analyses of cells of different human cell lines such as multipotential leukemia cells of line HL60, lung fibroblasts of line LL24, SV40-transformed fibroblasts of lines SV80 and WI38VA13, rhabdomyosarcoma-derived cells of line RD and astrocytoma cells of line U333 (glioma), in comparison with bovine fibroblast of line B1 and mouse fibroblastoidal cells of lines L929, OP-9, 3T3 and 3T3L1 (for detailed characterization of the cells used, see cf. Rickelt et al., 2009), using primers specific for the different α-catenin subtypes or Pkp2 or for GAPDH as loading control. As positive controls, cDNA of human heart and/or fetal brain tissue were used. Note that αE-catenin-mRNA is detected in all cell lines examined, whereas αN-catenin appears only in bovine fibroblasts of line B1. In contrast, αT-catenin is absent in all cell lines examined. Pkp2-mRNA and GAPDH-mRNA are detected in all cells. Size markers used are indicated on the left margin and represent from top to bottom: 1400, 517, 397, 356 and 247 bp. * In some experiments, for the demonstration of αN-catenin RNA from fetal brain was loaded as a positive control instead of heart tissue derived RNA.

8.2. Adhering junctions of mesenchymally derived cells: II. Special forms of molecular organizations in normal and malignantly transformed melanocytic cells

8.2.1. Molecular composition of homotypic adhering junctions connecting melanocytes or melanoma cells grown in culture

Because of their special importance for the understanding of the carcinogenic process, of dedifferentiation processes, e.g., amelanotic melanomas and of melanoma cell metastasis, I have begun early in my thesis work to examine the molecular and cellular composition of AJs of different melanomas and melanoma cell culture lines, including lines derived from primary as well as lines from metastatic tumors (see, e.g., Table 3 in Schmitt et al., 2007). To characterize the molecular composition of homotypic cell-cell adhesion structures, SDS-PAGE-immunoblot analyses of total cell lysates from melanoma cells as well as immunofluorescence, electron and immunoelectron microscopy were used. The small plaque bearing structures of the *puncta adhaerentia*-type of melanoma cells revealed the presence of E-, P- and N-cadherin mostly in a mutually exclusive pattern, occasionally within the same cell showing overlapping localization or even coexistence of all three cadherins (Schmitt et al., 2007). These cadherins are anchored in typical but relatively thin cytoplasmic plaques assembled by α-catenin and the *armadillo* proteins β-catenin, plakoglobin, p120, p0071 and/or ARVCF as well as proteins ZO-1–3 and the actin microfilament-binding proteins vinculin and α-actinin. All cadherins could be co-immunoprecipitated with β-catenin, but, only P-cadherin was enriched in E-cadherin IPs indicative of the existence of heterotypic E- and P-cadherin complexes whereas N-cadherin seemed to occur in different complexes. Surprisingly, cadherin-11, a type II cadherin, was found only in one of the melanoma cell lines analyzed (Schmitt et al., 2007).

Similar cell-cell junctions have subsequently also been found in human epidermal melanocytes (Rickelt et al., 2008). But these cells showed generally an enrichment of E- and P-cadherin, whereas N-cadherin, cadherin-11 and VE-cadherin appeared to be absent. The latter had previously been reported to be present in a subset of highly aggressive melanoma cell lines (cf. Hendrix et al., 2001; Hess et al., 2006). In addition, melanocytes also contained the typical AJ plaque proteins detected in melanoma cell lines. The molecular AJ components of the examined melanocytic cell culture lines are summarized in Table 4.

8.2.2. Detection and characterization of a non-junction-bound form of the transmembrane glycoprotein desmoglein Dsg2 in melanocytes and melanoma cells

During molecular biological analyses to the AJ proteins of various melanoma cells, I have made the surprising observation that the transmembrane glycoprotein, desmoglein Dsg2 occurs in these cells which apparently do not possess any desmosomes (Schmitt et al., 2007). Dsg2, a protein normally synthesized in desmosomes of proliferative epithelial cells (Schäfer et al., 1994) and in the composite junctions connecting cardiomyocytes (Borrmann et al., 2006; Franke et al., 2006; for a recent review see Franke et al., 2009) has been regularly detected in MeWo and C32 melanoma cells not only by PCR analysis but also performing Western blot experiments. By contrast all other desmosomal cadherins are obviously absent, in contrast to recent findings reported for Dsg1 in different melanoma cell lines (Li et al., 2001b). However, Dsg1 has not been noted in my analyses, neither at the protein nor at the mRNA level (Schmitt et al., 2007). In addition I have also found Pkps1-3 in WM-115 melanoma cells as well as small amounts of Pkp3 in cells of line MeWo, whereas rather small amounts of plakoglobin has been detected in all melanoma lines analyzed (Table 4). Using double-laser confocal scanning microscopy localization an unexpected and highly specific Dsg2 distribution has been identified as shown in Figure 8. Here Dsg2 appears not to be enriched in any junction structure but seems distributed rather equally over the entire plasma membrane. Therefore, I have again performed immunoelectron microscopy to clarify the cellular localization at the adequate subcellular level. And indeed Dsg2 staining can be observed almost along the entire plasma membrane, with occasional weak reactions at small AJ plaques of the *puncta adhaerentia*-type, but in contrast to β-catenin without a specific enrichment at such sites (Schmitt et al., 2007). Again, IPs of MeWo cell lysates with antibodies to Dsg2, Pkp3, and β-catenin have not shown any co-precipitation, indicating that here Dsg2 appears in a different form or in different protein complexes, indicative of an existence as a primarily solitary cell surface component.

Encouraged by the surprising distribution pattern of Dsg2 in these melanoma cells, I then studied the presence of this desmosomal glycoprotein in melanocytes. As I have not found Dsg2 in human epidermal melanocytes *in situ*, I examined melanocytes grown in cell culture. Surprisingly, both cultured NHEM-f as well as NHEM-a melanocytes presented Dsg2 as a frequent, solitary cell surface protein (Rickelt et al., 2008). This finding is compatible with the fact that in NHEM-f melanocyte IP experiments, followed by MALDI-TOF analysis, neither α- and β-catenin nor E- and P-cadherin were enriched in the Dsg2 immunoprecipitates. In summary, these findings show for the first time the presence of

simple Dsg2 molecules out of a desmosomal context, in a novel, homogeneously dispersed kind of cell-cell adhesion system, which is extended over large areas of the plasma membrane and characteristic for a subset of melanocytic cells.

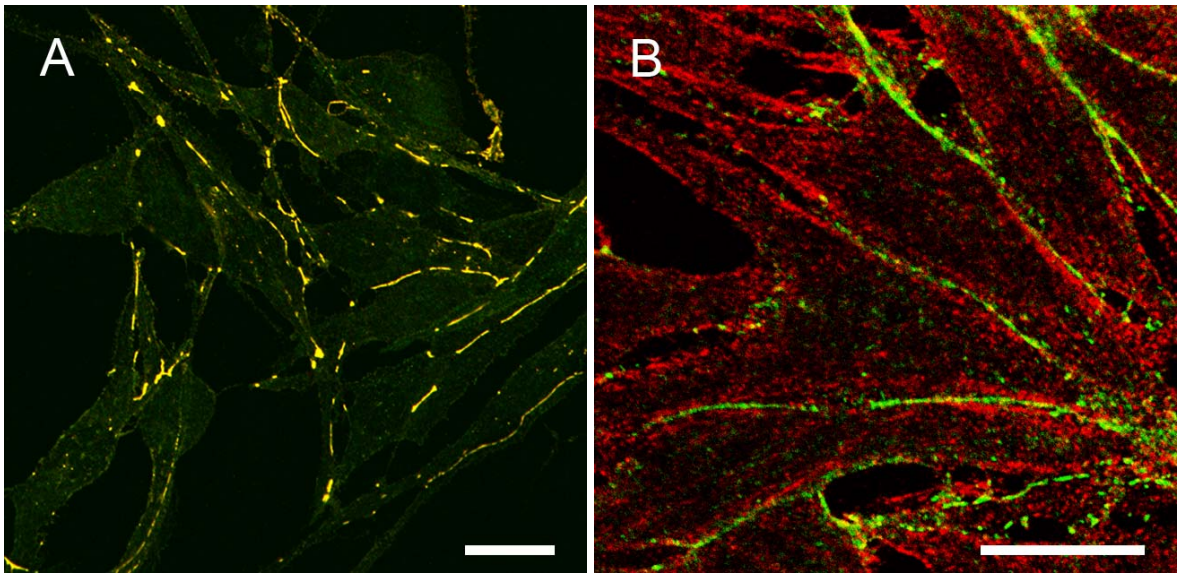


Figure 8. Double-label confocal immunofluorescence microscopy of human melanoma culture cells of line MeWo, immunostained with antibodies against the transmembrane glycoproteins N-cadherin (**A**; red) or desmoglein Dsg2 (**B**; red), in combination with the AJ plaque protein β-catenin (green). Note that, β-catenin and N-cadherin show colocalization at intercellular junctions (**A**; yellow merge color) whereas the localizations of β-catenin and Dsg2 are completely different (**B**). Scale bars: 20 µm. Micrographs modified from Schmitt et al., 2007.

Antigen	Melanocytes	Melanoma cells
Transmembrane Proteins		
E-Cadherin	+	+/-*
N-Cadherin	-	+/-*
P-Cadherin	+	+/-*
VE-Cadherin	-	-
Cadherin 6	-	-
Cadherin 11	-	+/-*
Desmoglein 1	-	-
Desmoglein 2	+	+/-**
Desmoglein 3	-	-
Desmoglein 4	-	-
Desmocollin 1	-	-
Desmocollin 2	-	-
Desmocollin 3	-	-
Occludin	-	-
Claudin 1	-	-
Claudin 4	-	-
Plaque Proteins		
α-Catenin	+	+
β-Catenin	+	+
Plakoglobin	(+)	+
Protein p120	+	+
Protein p0071	+	+
Plakophilin-1	-	+/-***
Plakophilin-2	-	+/-***
Plakophilin-3	-	+/-***
Desmoplakin	-	-
Intermediate Filament Proteins		
Vimentin	+	+
Keratins 8 and 18	-	-
Actin - Microfilament and Associated Proteins		
Afadin	+	+
α-Actinin	+	+
Vinculin	+	+
Drebrin	+	+
Protein ZO-1	+	+
Protein ZO-2	+	+

Table 4. AJ molecules in cultured human melanocytes as well as combined results from different melanoma cell lines, as determined by SDS-PAGE and immunoblotting (see also Schmitt et al., 2007; Rickelt et al., 2008). Symbol +/- indicates that positive as well as negative sublines have been noted by immunolocalization. * Five of the seven melanoma cell lines tested contain N-cadherin, two possess E-cadherin, four show P-cadherin and cadherin-11 has only been found in one line. ** Interestingly, two melanoma lines, MeWo and C32, synthesize the desmosomal cadherin Dsg2, in the absence of all other desmosomal cadherins and desmoplakin. *** Pkps1-3 have been found in WM-115 cells and Pkp3 occurs as the single Pkp in MeWo cells. Brackets indicate cell lines in which the specific protein either occurs only weakly or is detected only in some groups of cells.

8.3. Spontaneous and cumulative syntheses of desmosomal and epithelial cell adhesion molecule-associated proteins and their assemblies into cell-cell junction structures of certain cultured human hematopoietic tumor cells

Surprisingly, diverse rather stable junction-like cell-cell contact structures have been noted in colonies of certain cell culture lines generally assumed to be unicellular and never to form intercellular junctions of any kind. As these lines included some of the worldwide most studied hematopoietic cells, I have decided to extend my studies to the hematological tumor cell lines K562 and RPMI 8226.

The myelogenous leukemia-derived cell line K562 is generally used as a cell culture research model of hematopoiesis in modern biology, combining *inter alia* myelogenous and erythroleukemic properties (e.g., Lozzio and Lozzio, 1975; Andersson et al., 1979; Lozzio and Lozzio, 1979; Rowley et al., 1981, 1985; reviews: Köffler and Golde, 1980; Dimery et al., 1983; Drexler, 1994; Tsiftsoglou et al., 2003). Using biochemical as well as light- and electron-microscopic immunolocalization methods in combination with techniques of selecting and cloning such cells (for methods see, e.g., Knapp and Franke, 1989; Knapp et al., 1989) diverse types of non-induced, spontaneous and randomly occurring formations of novel kinds of AJs and half-AJ structures in clonal colonies and stable sublines of substratum-adherent monolayers or suspended spheroidal aggregates of K562 tumor cells have been characterized (Franke and Rickelt, 2010; see also Schäfer, 1995). These AJ structures vary greatly in size and molecular architecture but are mostly based on cis- as well as trans-connected clusters of the desmosomal transmembrane glycoprotein desmoglein, Dsg2. As already reported by Järvinen et al. (1990) we have noted masses of bundles of keratin IFs in these cells (see also Zauli et al., 1986) but in contrast to Järvinen et al. (1990) we have repeatedly found desmosomal protein-positive structures at the plasma membranes of such cells. Detailed analyses then have revealed such proteins assembled into small AJ-type structures in most of which Dsg2 is anchored in a submembranous plaque often containing plakoglobin and plakophilins Pkp2 and/or Pkp3 (for details see Figures 3 and 5 of Franke and Rickelt, 2010), sometimes mixed with other *armadillo* proteins such as proteins p120, p0071 and/or small amounts of β-catenin (cf. Table 5). Extensive immunocytochemical examinations for other cadherins, including desmogleins Dsg1, 3 and 4 as well as desmocollins Dsc1 and 3, and for α-catenin, neurojungin and protein ARVCF, have consistently yielded negative results in all K562 sublines characterized. Surprisingly, in two of the fifteen clonal sublines established I have detected both desmosomal cadherins, i.e., Dsg2 and desmocollin, Dsc2, which in some clones have shown the expected colocalization but in specific others have shown complete differential localization (for details see also Figures S1 of Franke and Rickelt, 2010;

see also Table 5). Additionally, I have also selected two sublines in which cells are connected by AJ-type structures containing the desmosome-typical protein desmoplakin (cf. Table 5). In some rare situations individual cells with small punctate or whisker-like reaction sites positive for N-cadherin. Other classical cadherins examined such as E-, P- or VE-cadherin or cadherin-11 have been absent.

In very densely-grown, substratum-adherent cell monolayer colonies of K562 cells a different kind of cell-cell contact structure has also been noted. These contact regions which extend over most of the cell periphery are characterized by a non-cadherin transmembrane glycoprotein, the epithelial cell adhesion molecule (EpCAM) anchored in a cortical cytoplasmic plaque intensely positive for afadin, α-actinin as well as vinculin and protein ZO-1 but essentially negative for the diverse *armadillo* proteins aforementioned (for details see Figures 8 and S2 of Franke and Rickelt, 2010; see also Table 6).

The discovery of an extended EpCAM-based cell-cell junctional system in hematopoietic tumor cells is another major surprise as the name EpCAM already indicates this type of plasma membrane-bound cis- and trans-homophilic, calcium-independent glycoprotein has so far been reported to be specific for certain, unusually highly proliferative epithelial and hair follicle cells (e.g., Tsubura et al., 1992; Litvinov et al., 1994a, b, 1996, 1997; Litvinov, 1995; Balzar et al., 1998; Winter et al., 2003). The close and massive association of EpCAM with a cortical plaque-like mass rich in α-actinin is, of course, in full agreement with the report of Balzar et al. (1998), that the rather short cytoplasmic carboxyterminal extension (26 amino acids long) is bound to α-actinin and thus connects the intracellular actin microfilament-based cytoskeleton to the extracellular adhesive functions of EpCAM.

The appearance of such cell-cell junctional as well as cell surface-exposed half-junctional structures is obviously not restricted to K562 cell, but also demonstrable in certain other hematological cell culture lines. Interestingly, in human RPMI 8226 myeloma-type hematopoietic cells even more complex changes of the cell-cell junction systems can be seen. In these cells I have regularly noted in addition to the Dsg2-based (Figure 9A-D) and the EpCAM-based plaque systems, typical *puncta adhaerentia*-type AJs. These are based on the transmembrane glycoprotein N-cadherin, anchored in a cytoplasmic plaque containing α-catenin and the *armadillo* proteins β-catenin (cf. Figure 9E-G), plakoglobin as well as proteins p120 and p0071, similar to the small AJs previously reported from bone marrow-derived mesenchymal stem cells (Wuchter et al., 2007).

Finally, I have noted further unexpected protein appearances in all sublines examined. Especially the rather heterogeneous IF assemblies of vimentin and keratins notably keratins 8, 18 and 19 have to be mentioned. These impressive IF bundle formations seem to exceed the relative amounts previously described in hematopoietic cell cultures (see, e.g., Zauli et al., 1986; Järvinen et al., 1990). Even more surprising was the

observation of microfilament bundles of the smooth muscle α-actin type in addition to β- and γ-non-muscle actins, an indication of a transformation to cells such as in myofibroblasts (Hinz et al., 2004, 2007). These additional extensive filament systems (for details see Figure 1 of Franke and Rickelt, 2010) are also clonally stable. Consequently, the cytoskeletal character of cultured hematopoietic cells cannot only differ from one line to another but can also differ between colonies of the same line and not infrequently accumulate major hallmark proteins of epithelial differentiation. Usually the original hematopoietic origin and differentiation can still be specifically challenged with a diversity of promoting or inhibitory drugs, including biological growth factors (e.g., Fukuda, 1981; Alitalo, 1990; Alitalo et al., 1990; Järvinen et al., 1990; Hickstein et al., 1993; Burger et al., 1994; LaMontagne et al., 1998; Shelly et al., 1998; Stopka et al., 1998; Bianchi et al., 1999; Kang et al., 1999; Woessmann and Mivechi, 2001; Huang et al., 2002; Takagaki et al., 2005).

Further experiments will have to show the possible mechanisms and functions of this non-genetic variability of cell-cell junctional structures and proteins. Moreover, the possible roles of these diverse AJ-type cadherin- or EpCAM-based structures in tumor spread and metastasis will have to be elucidated and diagnostic and therapeutic consequences will have to be considered.

	Incomplete AJ Subtypes						
Transmembrane AJ Cadherins	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
E-Cadherin	-	-	-	-	-	-	-
N-Cadherin	-	-	-	-	-	-	-
Desmoglein 1	-	-	-	-	-	-	-
Desmoglein 2	++	++	++	++	-	+	-
Desmoglein 3	-	-	-	-	-	-	-
Desmocollin 1	-	-	-	-	-	-	-
Desmocollin 2	-	-	-	+	-	+	-
Desmocollin 3	-	-	-	-	-	-	-
AJ Plaque Proteins							
α-Catenin	-	-	-	-	-	-	-
β-Catenin	-	-	+	-	+	+*	-
Plakoglobin	++	++	++	++	++	+	-
Protein p120	-	-	+	-	+	+*	-
Protein p0071	-	-	+	-	+	+*	-
Plakophilin 1	-	-	-	-	-	-	-
Plakophilin 2	+	++	++	++	++	+*	-
Plakophilin 3	+	+	+	+	+	+*	-
Desmoplakin	-	++	-	+	-	-	-

Table 5. Molecular characterizations of novel types of adhering junctions (AJs) with or without desmosomal proteins in clonal sublines of human hematopoietic K562 tumor cells, as determined by SDS-PAGE and immunoblotting as well as by immunocytochemistry. Symbols: +, moderate intensity of the reaction for the molecules tested; ++ high intensity of reaction; - not detected. Seven major different subtypes have so far been distinguished with respect to AJ formations: **(I)** AJs comprising desmosomal molecules, with the exception of any desmocollin and desmoplakin; **(II)** AJs with all desmosomal molecules, including desmoplakin, but without any desmocollin; **(III)** AJs comprising the desmosome-type molecules as in type (I) plus the *armadillo* plaque proteins β-catenin, p120 and p0071; **(IV)** AJs with a full desmosomal molecule complement, including desmoplakin and desmocollin Dsc2; **(V)** AJ-type assemblies similar to that presented in type (III) but without detectable desmocollins and desmogleins; **(VI)** punctate AJ-type assemblies positive for desmosomal cadherins, Dsg2 and Dsc2, and the plaque proteins as specified; * denote reactions of low and sometimes variable intensity; **(VII)** no demonstrable junction assemblies of AJ-type proteins.

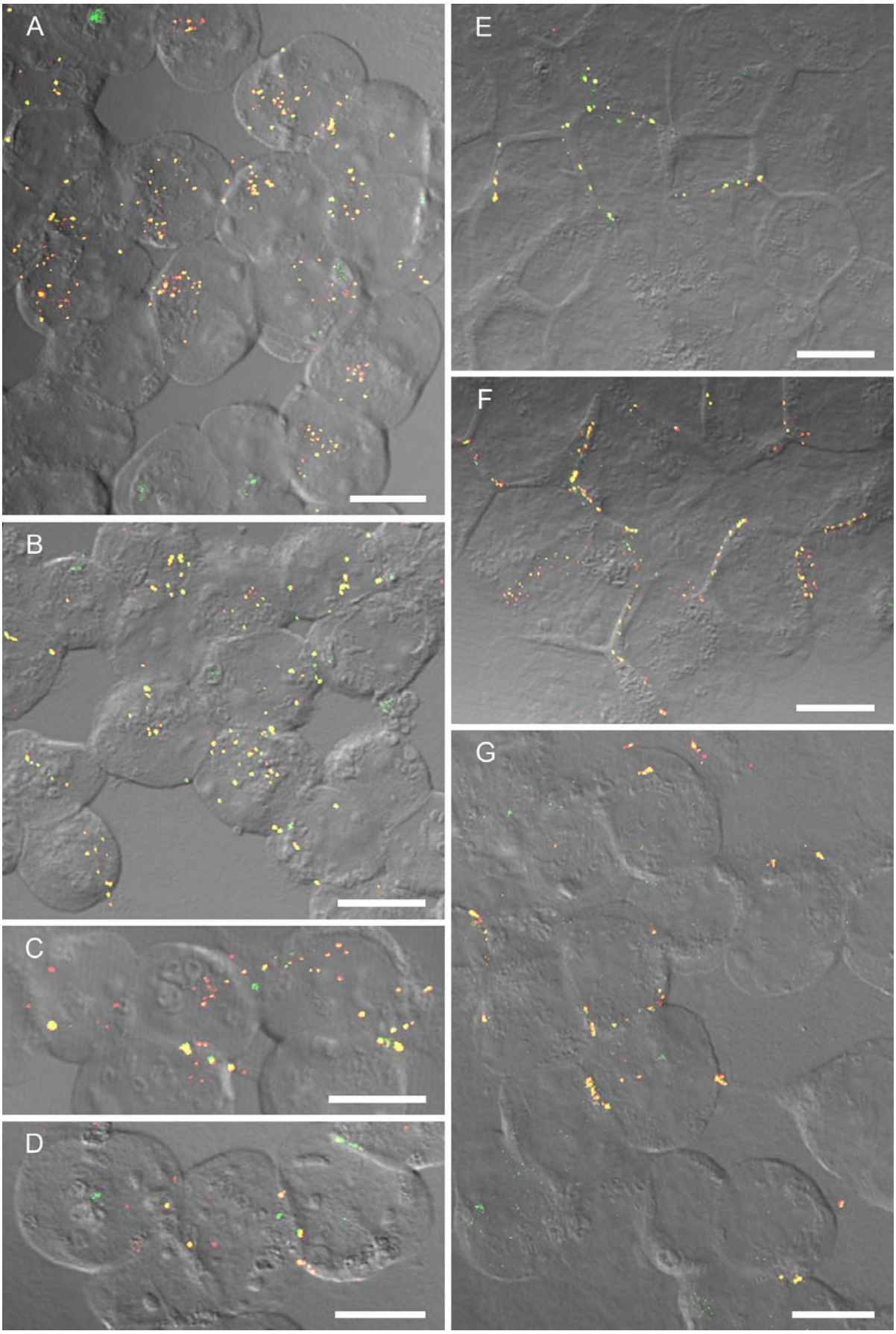

A
B
C
D
E
F
G

Figure 9. (**A**) Confocal laser-scanning immunofluorescence microscopy of substratum-adherent cultured cells of the hematopoietic line RPMI 8226, showing the results of double-label experiments, comparing the immunolocalizations of two different antibodies specific for the desmosomal glycoprotein, desmoglein Dsg2 (mab Dsg2 - clone 10G11 in red, in combination with the polyclonal antibodies Dsg2 - rb8 in green). Note the intense and near-complete colocalization in punctate structures (yellow) of both antibodies. (**B-D**) Double-label micrographs showing the colocalizations of different desmosomal proteins such as plakophilin Pkp2 (**B**; red) in combination with desmoglein Dsg2 (**B**; green) or Dsg2 (**C**; red) in comparison with desmoplakin (**C**; green) or Pkp2 (**D**; red) compared with desmoplakin (**D**; green). Note that in most, but not all of the punctate structures the colocalization of the specific desmosomal proteins is seen (yellow merge color). (**E-G**) Comparison of immunolocalizations of adherens junction proteins α-catenin (**E** and **F**; red) or N-cadherin (**G**; red) in combination with β-catenin (**E-G**; green) clearly shows their localization to *puncta adhaerentia*-type junctions. Note also that most, but not all N-cadherin colocalizes with the plaque protein β-catenin. All images are shown on a phase contrast background. Scale bars: 10 µm.

Transmembrane Glycoproteins
EpCAM
Subplasmamembraneous Cortex
Afadin
α-Actinin
Vinculin
Protein ZO-1

Table 6. Major molecular components of a special form of cell-cell junctions in human hematopoietic K562 tumor cells, colocalizing in cortical *puncta* or in extended zones.

9. General discussion

The results reported in this thesis have clearly demonstrated that in the field of mesenchymal cells, i.e. outside of the epithelial and neural cell systems, in addition to the special situations of the "fused" adhering junctions (AJs) of the composite junctions of the adult mammalian myocardium (e.g., Borrmann et al., 2006; Franke et al., 2006 for a recent review see Pieperhoff et al., 2010) and the heterogeneous adherens cortex of the anucleate cell structures of the eye lens (Straub et al., 2003), obviously a series of yet unknown cell-cell junction systems exists. The fact that I could identify not less than five major cell-cell junction types of different mesenchymal cells (Pkp2 positive- and negative-forms of *coniunctiones adhaerentes*, the diverse forms of "incomplete" AJs in certain hematopoietic cell lines, the dispersed Dsg2-based system of melanoma cells and melanocytes, and the occurrence of an EpCAM-based junction type in non-epithelial cell arrays) may be explained by the still existing lack of systematic AJ studies in mesenchymal cells. Consequently, this thesis should be regarded as a begin of studies elucidating further junctional structures and systems connecting mesenchymal cells.

Such a systematic study of the junctions connecting non-epithelial cells will certainly be valuable for our biological understanding of the molecular architecture and the functions of such cells as it will also provide novel molecule-specific reagents to improve the pathological recognition of certain soft tissue tumors (for reviews see Introduction; for examples indicative of the potential value of such reagents see, e.g., Table 1 A - E; note also cases of non-epithelial tissues in which a positive reaction for a single desmosomal antigen or for EpCAM has been noted, including potential perineurial tumors see, e.g., Fisher and Miettinen, 1997; Barrett et al., 2002; Zamecnik et al., 2002; Zamecnik and Michal, 2002; Suster and Moran, 2005; Eyden et al., 2009). Thus besides a better understanding for cell formation processes in developmental biology, the practical value of such research results for pathology might be enormous.

In view of the findings of a systemic synthesis of Pkp2 – with or without Pkp3 – and its integration into the AJ plaques of a major subtype of *coniunctiones adhaerentes* for a schematic presentation see Figure 10) one is inclined to speculate that this acquisition enhances the mechanical stability and biochemical resistance in both certain proliferative normal and transformed cells as it is also suggested by the occurrence of this protein in the AJ connections of very long processes of, e.g., myxoma tumors (cf. Rickelt et al., 2010) or of interstitial cardiac cells growing in primary cell culture (Barth et al., 2009). How widespread the occurrence of these Pkp-containing AJs is will be seen in future studies. That Pkp2 indeed contributes to the special stability to AJ-type

cell-cell junctions is probably most impressively shown by the sensitivity of the myocardial composite junctions with respect to Pkp2-gene mutations – even very small ones – in adult mammals, humans included (see, e.g., the role of AJ protein mutations in cardiomyopathies as recently reported in the references evaluated in Table 7). It may well be that studies of Pkp2-defects in transgenic animal experiments will help identifying and mechanistically understanding the functional contributions of special domains in the Pkp2 molecule to junctional stability of cell junctions.

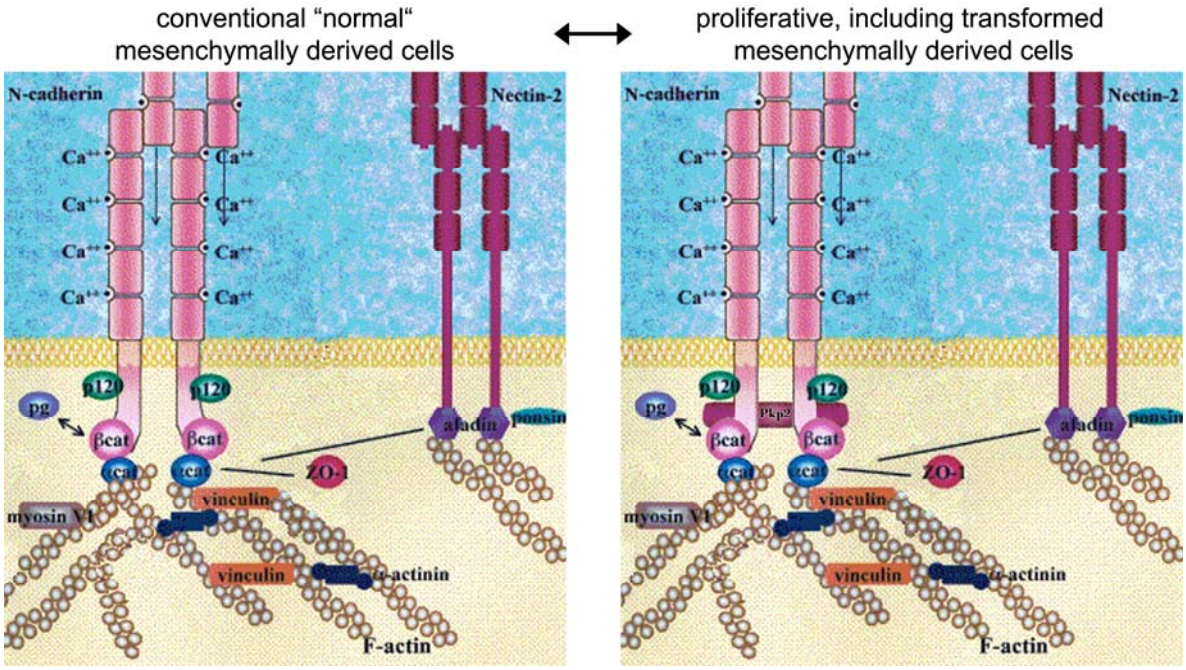


Figure 10. Schematic presentation of the hypothetical organization of adhering junction (AJ) proteins in the AJs "normal" mesenchymally derived cells in comparison with transformed ones. Shown is the cadherin-catenin-complex as well as the nectin-afadin-ponsin-system, which both provide anchorage to actin microfilament bundles (only one face of the symmetric AJ structure is demonstrated). Note that in the AJ subtype (*coniunctio adhaerens*) of transformed cells plakophilin-2 (Pkp2) occurs in a complex with typical *puncta adhaerentia* proteins. In certain kinds of cells, e.g., in cardiac myxoma cells, N-cadherin can be partly or totally replaced by cadherin-11. For the sake of clarity the *armadillo* proteins p0071 and ARVCF are not shown (for details see Hofmann et al., 2009). Ca^{++}, calcium ions; pg, plakoglobin; p120, protein p120; α-cat, α-catenin; β-cat, β-catenin; ZO-1, protein ZO-1 (modified from Perez-Moreno et al., 2003, to integrate the results of this thesis).

Protein	Reference	
Plakophilin-2	Gerull et al., 2004 Antoniades et al., 2006 Calkins, 2006 Nagaoka et al., 2006 Kannankeril et al., 2006 Dalal et al., 2006 Syrris et al., 2006a Tsatsopoulou et al., 2006 van Tintelen et al., 2006 Awad et al., 2006 Lahtinen et al., 2008 Otterspoor et al., 2007	Fidler et al., 2008 Joshi-Mukherje et al., 2008 Ram and Wagoner, 2008 Tandri et al., 2008 Wu et al., 2009 Qiu et al., 2009 (5 cases) Hall et al., 2009 Bhuiyan et al., 2009 (23 cases) Den Haan et al., 2009 (21 cases) Xu et al., 2010 (38 cases) Bauce et al., 2010 (7 cases) Cox et al., 2010 (58 cases)
Desmoplakin	Norgett et al., 2000 Rampazzo et al., 2002 Alcalai et al., 2003 Bauce et al., 2005 Norman et al., 2005 Sen-Chowdhry et al., 2005 Norgett et al., 2006 Uzumcu et al., 2006 Sen-Chowdhry et al., 2007	Tsatsopoulou et al., 2006 Yang et al., 2006 Den Haan et al., 2009 (1 case) Mahoney et al., 2010 Xu et al., 2010 (10 cases) Bauce et al., 2010 (5 cases) Cox et al., 2010 (1 case) Bolling et al., 2010
Desmoglein-2	Pilichou et al., 2006 Tsatsopoulou et al., 2006 Awad et al., 2006 Syrris et al., 2007 Yu et al., 2008	Bhuiyan et al., 2009 (4 cases) Den Haan et al., 2009 (8 cases) Xu et al., 2010 (10 cases) Bauce et al., 2010 (4 cases) Cox et al., 2010 (3 cases)
Desmocollin-2	Heuser et al., 2006 Syrris et al., 2006b Beffagna et al., 2007 Bhuiyan et al., 2009 (2 cases)	Simpson et al., 2009 Xu et al., 2010 (4 cases) Bauce et al., 2010 (2 cases) Cox et al., 2010 (3 cases)
Plakoglobin	McKoy et al., 2000 Protonotarios et al., 2001, 2002 Kaplan et al., 2004 Garcia-Gras et al., 2006	Asimaki et al., 2007 Asimaki et al., 2009 Den Haan et al., 2009 (1 case) Xu et al., 2010 (2 cases)

Table 7. Mutations in desmosomal proteins and glycoproteins contributing to ARVC cardiomyopathies and "sudden death" events (taken from Pieperhoff S., Barth M., Rickelt S., and Franke W.W., 2010; see therein for the complete reference list). Note the importance of the Pkp2-gene.

There is presently little to discuss as to the occurrence of a relatively large desmosomal cadherin, Dsg2, that is not integrated into any form of AJ but occurs as dispersed individual plasma membrane glycoproteins over large regions of a cell type, i.e. in the cases shown in this thesis melanoma cells and certain melanocytes growing in cell culture. Apparently, here the relatively long carboxyterminal "tail" portion (for details of these domains see Koch et al., 1990, 1991; for recent reviews see Koch and Franke, 1994; Garrod and Chidgey, 2008) is not anchored in a cytoplasmic plaque, at least it is not associated stably enough with any cytoplasmic protein that can be identified as co-immunoprecipitate. Nevertheless, the homogenous Dsg2-coating of large regions of the plasma membrane, which corresponds with a similarly Dsg2-coated surface of a closely adjacent membrane of the neighbouring cell, would be compatible with the concept that these dispersed cadherins are also engaged in calcium-mediated cell-cell attachment (for a detailed discussion of dermatological aspects see Schmitt et al., 2007; Rickelt et al., 2008). It is obvious that only cDNA-transfection or transgenic animal experiments expressing the Dsg2-gene in cells totally devoid of desmosomal proteins (for problems of correct target cells see, e.g., Chitaev and Troyanovsky, 1997; Köser et al., 2003) will be able to elucidate possible functions of such Dsg2 forms.

Even more provocative was the finding that blood tumor cells known to occur *in situ* as in cell cultures only as unicellular cell suspensions, are also able to synthesize AJs as well as EpCAM-based junctions and assemble with each other to form monolayers of densely-packed cells or even three-dimensional arrays. In the course of my thesis I have concentrated on two such hematopoietic cell lines: K562, a multipotential myelogenous line with demonstrable erythroleukemic properties, and RPMI 8226, a line with lymphoproliferative potential as demonstrated by the synthesis of immunoglobulin (a hypothetical presentation of their positioning in a survey scheme of blood cell formation is given in Figure 11). Since more than three decades these cell lines are among the worldwide most studied ones, serving as prime reference cells in haematology (for review see, e.g., Tsiftsoglou et al., 2003). Surprisingly, spontaneous synthesis and accumulation of several major epithelial molecules and structures could be noticed in individual cells (Franke and Rickelt, 2010; see also Schäfer et al., 1994; Schäfer, 1995), including in K562 cells the presence of IF bundles of the keratin type (for latter see Zauli et al., 1986; Järvinen et al., 1990; Schäfer, 1995).

In cultures of unicellular K562 cells, which are assumed not to contain any junctional proteins, the frequent appearance of diverse novel, clonally rather stable AJ structures have been seen and in molecular terms all the different AJ-types analyzed were based on the transmembrane glycoprotein, desmoglein Dsg2, in rare sublines accompanied by desmocollin Dsc2. This form of tightly clustered Dsg2 molecules is clearly

distinguishable from the Dsg2 molecules occurring as dispersed, non-junction-integrated proteins as described afore in certain subtypes of melanocytes and melanoma cells. These K562 AJ-resembling structures of variable sizes frequently also contain the desmosomal plaque proteins plakoglobin as well as plakophilins Pkp2 and Pkp3. Surprisingly, the additional occurrence of the main desmosomal plaque protein, desmoplakin, was only found in some rare subtypes. This seems puzzling in view of the important structural role that is generally ascribed to this protein (for reviews see Godsel et al., 2004; Delva et al., 2009). Even more interesting was the observation of the predominant absence of the glycoprotein desmocollin Dsc2, which in normal desmosomes is the isostoichiometric “twin” of Dsg2 (for reviews see Chitaev and Troyanovsky, 1997; Troyanovsky, 2005). Obviously, this absence of Dsc2 supports earlier conclusions from cDNA transfections into desmosome-lacking cultured fibrosarcoma-derived cells that heterophilic pairing of Dsg with a desmocollin is not an absolute requirement for AJ formation (Köser et al., 2003).

In addition, we have also repeatedly noted the acquisition of some *armadillo* proteins such as β-catenin and proteins p120 and p0071 to such AJ type plasma membrane structures which often connect adjacent cells into tissue-like higher order arrays not readily distinguishable from true epithelial structures.

The appearance of such Dsg2-based AJs is not even the only kind of junctional structures seen in such cultured hematopoietic cells. Small as well as larger EpCAM-based junctions anchored in a cortical actin-binding protein plaque system are also regularly seen. This is remarkable as EpCAM has hitherto been reported to be present only on plasma membranes of epithelia or epithelium-derived cells where they form special junctions (e.g., Momburg et al., 1987; Berendsen et al., 1988; Litvinov et al., 1994b; Balzar et al., 1999; Winter et al., 2003; for recent reviews see Trzpis et al., 2007; Munz et al., 2009). These two separate biochemically totally different junction systems are obviously not restricted to K562 cell, but have also been demonstrable in culture cells of the human RPMI 8226 myeloma line which in addition often also presents "normal" N-cadherin-based *puncta adhaerentia*.

Apparently, this new ability of hematological tumor cells to form AJ-type connections and thus to form tissue-like structures would certainly contribute to the stability of tumor cell aggregates as well as to potential metastatic interactions (for detailed discussions see Brock et al., 2009).

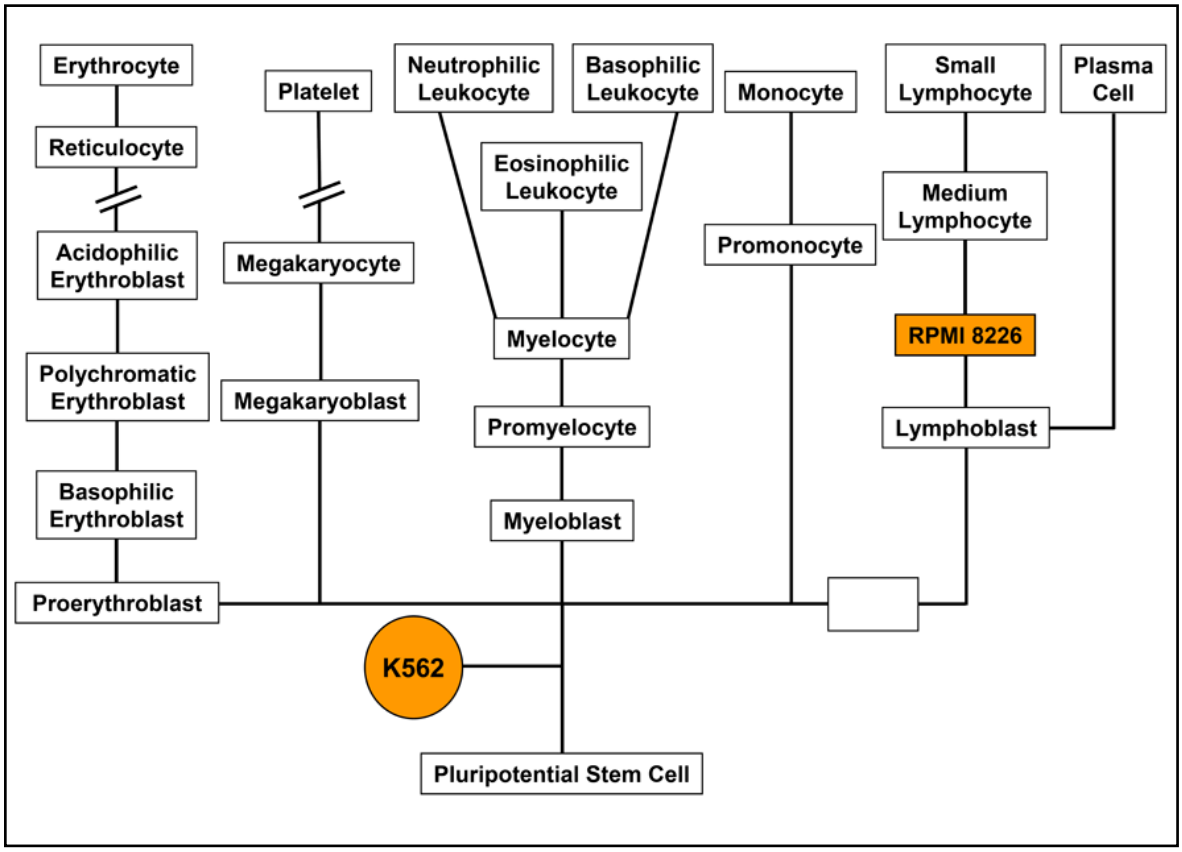


Figure 11. Schematic survey of differentiation pathways and the differentiation levels of the hematopoietic human tumor cell culture lines K562 and RPMI 8226.

10. References

Akat, K., Mennel, H. D., Kremer, P., Gassler, N., Bleck, C. K., and Kartenbeck, J. (2003). Molecular characterization of desmosomes in meningiomas and arachnoidal tissue. Acta Neuropathol *106*, 337-347.

Akat, K., Bleck, C. K., Lee, Y. M., Haselmann-Weiss, U., and Kartenbeck, J. (2008). Characterization of a novel type of adherens junction in meningiomas and the derived cell line HBL-52. Cell Tissue Res *331*, 401-412.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell., 4th edn (New York: Gerlach Science), pp. 1-1463.

Alitalo, R. (1990). Induced differentiation of K562 leukemia cells: a model for studies of gene expression in early megakaryoblasts. Leuk Res *14*, 501-514.

Alitalo, R., Partanen, J., Pertovaara, L., Holtta, E., Sistonen, L., Andersson, L., and Alitalo, K. (1990). Increased erythroid potentiating activity/tissue inhibitor of metalloproteinases and jun/fos transcription factor complex characterize tumor promoter-induced megakaryoblastic differentiation of K562 leukemia cells. Blood *75*, 1974-1982.

Altmannsberger, M., and Osborn, M. (1987). Mesenchymal tumor markers: intermediate filaments. Curr Top Pathol *77*, 155-178.

Andersson, L. C., Nilsson, K., and Gahmberg, C. G. (1979). K562: a human erythroleukemic cell line. Int J Cancer *23*, 143-147.

Angst, B. D., Marcozzi, C., and Magee, A. I. (2001). The cadherin superfamily. J Cell Sci *114*, 625-626.

Antonescu, C. R., and Erlandson, R. A. (2001). Fibrosarcoma mimicking plasmacytoma or carcinoma: an ultrastructural study of 4 cases. Ultrastruct Pathol *25*, 31-37.

Baeuerle, P. A., and Gires, O. (2007). EpCAM (CD326) finding its role in cancer. Br J Cancer *96*, 417-423.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E., and McDonald, D. M. (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med *204*, 2349-2362.

Balzar, M., Bakker, H. A., Briaire-de-Bruijn, I. H., Fleuren, G. J., Warnaar, S. O., and Litvinov, S. V. (1998). Cytoplasmic tail regulates the intercellular adhesion function of the epithelial cell adhesion molecule. Mol Cell Biol *18*, 4833-4843.

Balzar, M., Prins, F. A., Bakker, H. A., Fleuren, G. J., Warnaar, S. O., and Litvinov, S. V. (1999). The structural analysis of adhesions mediated by Ep-CAM. Exp Cell Res *246*, 108-121.

Banks-Schlegel, S. P. (1989). Keratin proteins: diagnostic aids in neoplasia. In: Cytoskeletal Proteins in Tumor Diagnosis., M. Osborn, and K. Weber, eds. (New York: Cold Spring Harbor Laboratory), pp. 127-137.

Bannasch, P., Zerban, H., Schmid, E., and Franke, W. W. (1980). Liver tumors distinguished by immunofluorescence microscopy with antibodies to proteins of intermediate-sized filaments. Proc Natl Acad Sci U S A *77*, 4948-4952.

Bannasch, P., Zerban, H., Schmid, E., and Franke, W. W. (1981). Characterization of cytoskeletal components in epithelial and mesenchymal liver tumors by electron and immunofluorescence microscopy. Virchows Arch B Cell Pathol Incl Mol Pathol *36*, 139-158.

Barrett, A. W., Hopper, C., and Landon, G. (2002). Intra-osseous soft tissue perineurioma of the inferior alveolar nerve. Oral Oncol *38*, 793-796.

Barth, M., Schumacher, H., Kuhn, C., Akhyari, P., Lichtenberg, A., and Franke, W. W. (2009). Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. Cell Tissue Res *337*, 63-77.

Bass-Zubek, A. E., Godsel, L. M., Delmar, M., and Green, K. J. (2009). Plakophilins: multifunctional scaffolds for adhesion and signaling. Curr Opin Cell Biol *21*, 708-716.

Behrens, J., and Nelson, W. J. (2004). Cell Adhesion., (Berlin, Heidelberg, New York: Springer Verlag), pp. 1-481.

Berendsen, H. H., de Leij, L., Postmus, P. E., Ter Haar, J. G., Poppema, S., and The, T. H. (1988). Detection of small cell lung cancer metastases in bone marrow aspirates using monoclonal antibody directed against neuroendocrine differentiation antigen. J Clin Pathol *41*, 273-276.

Bianchi, N., Osti, F., Rutigliano, C., Corradini, F. G., Borsetti, E., Tomassetti, M., Mischiati, C., Feriotto, G., and Gambari, R. (1999). The DNA-binding drugs mithramycin and chromomycin are powerful inducers of erythroid differentiation of human K562 cells. Br J Haematol *104*, 258-265.

Blaschuk, O. W., and Devemy, E. (2009). Cadherins as novel targets for anti-cancer therapy. Eur J Pharmacol *625*, 195-198.

Bloom, W., and Fawcett, D. W. (1975). A Textbook of Histology., (Philadelphia: W.B. Saunders Company), pp. 1-1033.

Borrmann, C. M. (2000) Molekulare Charakterisierung der Adhärens-Zellverbindungen des Herzens: Identifizierung einer neuen Art, der Area Composita., Ph. D. Thesis, Faculty of Biology, University of Heidelberg, Heidelberg, Germany, pp.1-119.

Borrmann, C. M., Mertens, C., Schmidt, A., Langbein, L., Kuhn, C., and Franke, W. W. (2000). Molecular diversity of plaques of epithelial-adhering junctions. Ann N Y Acad Sci *915*, 144-150.

Borrmann, C. M., Grund, C., Kuhn, C., Hofmann, I., Pieperhoff, S., and Franke, W. W. (2006). The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk. Eur J Cell Biol *85*, 469-485.

Brischwein, K., Schlereth, B., Guller, B., Steiger, C., Wolf, A., Lutterbuese, R., Offner, S., Locher, M., Urbig, T., Raum, T., Kleindienst, P., Wimberger, P., Kimmig, R., Fichtner, I., Kufer, P., Hofmeister, R., da Silva, A. J., and Baeuerle, P. A. (2006). MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. Mol Immunol *43*, 1129-1143.

Brock, A., Chang, H., and Huang, S. (2009). Non-genetic heterogeneity: a mutation-independent driving force for the somatic evolution of tumours. Nat Rev Genet *10*, 336-342.

Burger, P. E., Dowdle, E. B., Lukey, P. T., and Wilson, E. L. (1994). Basic fibroblast growth factor antagonizes transforming growth factor beta-mediated erythroid differentiation in K562 cells. Blood *83*, 1808-1812.

Chitaev, N. A., and Troyanovsky, S. M. (1997). Direct Ca2+-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. J Cell Biol *138*, 193-201.

Chu, P. G., and Weiss, L. M. (2002). Keratin expression in human tissues and neoplasms. Histopathology *40*, 403-439.

Cowin, P., and Garrod, D. R. (1983). Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. Nature *302*, 148-150.

Cowin, P., Kapprell, H. P., Franke, W. W., Tamkun, J., and Hynes, R. O. (1986). Plakoglobin: a protein common to different kinds of intercellular adhering junctions. Cell *46*, 1063-1073.

Darnell, J., Lodish, H., and Baltimore, D. (1986). Molecular Cell Biology., (New York: Scientific American Books), pp. 1-1187.

Dejana, E. (2004). Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol *5*, 261-270.

Delva, E., Tucker, D. K., and Kowalczyk, A. P. (2009). The desmosome. In: Cold Spring Harbor Perspectives in Biology., J. Nelson, and E. Fuchs, eds. (New York: Cold Spring Harbor Laboratory Press), 1:a002543.

Deonarain, M. P., Kousparou, C. A., and Epenetos, A. A. (2009). Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? MAbs *1*, 12-25.

Dickersin, G. R. (2000). Diagnostic Electron Microscopy. A Text/Atlas., 2nd edn (New York, Berlin, Heidelberg: Springer Verlag), pp. 1-1005.

Dimery, I. W., Ross, D. D., Testa, J. R., Gupta, S. K., Felsted, R. L., Pollak, A., and Bachur, N. R. (1983). Variation amongst K562 cell cultures. Exp Hematol *11*, 601-610.

Drenckhahn, D., and Zenker, W. (1994). Benninghoff Anatomie, Bd. 1 & 2., 15th edn (München: Urban & Schwarzenberg), pp. 1-985 & 981-866.

Drexler, H. G. (1994). Leukemia cell lines: in vitro models for the study of chronic myeloid leukemia. Leuk Res *18*, 919-927.

Drochmans, P., Freudenstein, C., Wanson, J. C., Laurent, L., Keenan, T. W., Stadler, J., Leloup, R., and Franke, W. W. (1978). Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis. J Cell Biol *79*, 427-443.

Eriksson, M., Hardell, L., Berg, N. O., Moller, T., and Axelson, O. (1981). Soft-tissue sarcomas and exposure to chemical substances: a case-referent study. Br J Ind Med *38*, 27-33.

Eriksson, M., Hardell, L., and Adami, H. O. (1990). Exposure to dioxins as a risk factor for soft tissue sarcoma: a population-based case-control study. J Natl Cancer Inst *82*, 486-490.

Erlandson, R. A. (1981). Diagnostic Transmission Electron Microscopy of Human Tumors., (New York, Paris, Barcelona: Masson Publishing USA, Inc.), pp. 1-193.

Eyden, B., Wang, G., and Yao, L. (2009). Epithelioid sarcoma: a case report with ultrastructural confirmation of myofibroblastic differentiation based on fibronexus junctions. Ultrastruct Pathol *33*, 61-66.

Farquhar, M. G., and Palade, G. E. (1963). Junctional complexes in various epithelia. J Cell Biol *17*, 375-412.

Fidler, L. M., Wilson, G. J., Liu, F., Cui, X., Scherer, S. W., Taylor, G. P., and Hamilton, R. M. (2008). Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations. J Cell Mol Med *13*, 4219 - 4228.

Fisher, C., and Miettinen, M. (1997). Parachordoma: a clinicopathologic and immunohistochemical study of four cases of an unusual soft tissue neoplasm. Ann Diagn Pathol *1*, 3-10.

Fletcher, C. D. (1992). Pleomorphic malignant fibrous histiocytoma: fact or fiction? A critical reappraisal based on 159 tumors diagnosed as pleomorphic sarcoma. Am J Surg Pathol *16*, 213-228.

Fletcher, D. M., Unni, K. K., and Mertens, M. (2002). World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Soft Tissue and Bone., (Lyon: IARCPress), pp. 1-427.

Folpe, A. L., and Gown, A. (2001). Immunohistochemistry for analysis of soft tissue tumors. In: Soft Tissue Tumors., S. Weiss, and J. Goldblum, eds. (St. Louis: Mosby), pp. 199-245.

Franke, W. W., Schmid, E., Osborn, M., and Weber, K. (1978a). Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci U S A *75*, 5034-5038.

Franke, W. W., Weber, K., Osborn, M., Schmid, E., and Freudenstein, C. (1978b). Antibody to prekeratin. Decoration of tonofilament like arrays in various cells of epithelial character. Exp Cell Res *116*, 429-445.

Franke, W. W., Schmid, E., Weber, K., and Osborn, M. (1979a). HeLa cells contain intermediate-sized filaments of the prekeratin type. Exp Cell Res *118*, 95-109.

Franke, W. W., Schmid, E., Winter, S., Osborn, M., and Weber, K. (1979b). Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp Cell Res *123*, 25-46.

Franke, W. W., Schiller, D. L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepier, R., and Platzer, B. (1981). Diversity of cytokeratins. Differentiation-specific expression of cytokeratin polypeptides in epithelial cells and tissues. J Mol Biol, 933-959.

Franke, W. W., Moll, R., Schiller, D. L., Schmid, E., Kartenbeck, J., and Müller, H. (1982). Desmoplakins of epithelial and myocardial desmosomes are immunologically and biochemically related. Differentiation *23*, 115-127.

Franke, W. W., Grund, C., Jackson, B. W., and Illmensee, K. (1983a). Formation of cytoskeletal elements during mouse embryogenesis. IV. Ultrastructure of primary mesenchymal cells and their cell-cell interactions. Differentiation *25*, 121-141.

Franke, W. W., Moll, R., Müller, H., Schmid, E., Kuhn, C., Krepler, R., Artlieb, U., and Denk, H. (1983b). Immunocytochemical identification of epithelium-derived human tumors with antibodies to desmosomal plaque proteins. Proc Natl Acad Sci U S A *80*, 543-547.

Franke, W. W., Kapprell, H. P., and Cowin, P. (1987a). Plakoglobin is a component of the filamentous subplasmalemmal coat of lens cells. Eur J Cell Biol *43*, 301-315.

Franke, W. W., Kapprell, H. P., and Cowin, P. (1987b). Immunolocalization of plakoglobin in endothelial junctions: identification as a special type of Zonulae adhaerentes. Biol Cell *59*, 205-218.

Franke, W. W., Borrmann, C. M., Grund, C., and Pieperhoff, S. (2006). The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. Eur J Cell Biol *85*, 69-82.

Franke, W. W. (2009). Discovering the molecular components of intercellular junctions – a historical view. In: Cold Spring Harbor Perspectives in Biology., J. Nelson, and E. Fuchs, eds. (New York: Cold Spring Harbor Laboratory Press), 1:a003061.

Franke, W. W., Rickelt, S., Barth, M., and Pieperhoff, S. (2009). The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind. Cell Tissue Res *338*, 1-17.

Franke, W. W., and Rickelt, S. (2010). Spontaneous and Cumulative Syntheses of Epithelial Proteins and Glycoproteins and Their Assemblies to Novel Cell-Cell Junctions in Malignantly Transformed Cells: I. Carcinomatoid Dysplasia Forms of Human Hematopoietic K562 Cells. *Submitted*.

Fuchs, E., and Weber, K. (1994). Intermediate filaments: Structure, dynamics, function and disease. Annu Rev Biochem *63*, 345-382.

Fukuda, M. (1981). Tumor-promoting phorbol diester-induced specific changes in cell surface glycoprotein profile of K562 human leukemic cells. Cancer Res *41*, 4621-4628.

Gabbiani, G., Kapanci, Y., Barazzone, P., and Franke, W. W. (1981). Immunochemical identification of intermediate-sized filaments in human neoplastic cells. A diagnostic aid for the surgical pathologist. Am J Pathol *104*, 206-216.

Garrod, D., and Chidgey, M. (2008). Desmosome structure, composition and function. Biochim Biophys Acta *1778*, 572-587.

Geiger, B., Avnur, Z., Volkberg, T., and Volk, T. (1985a). Molecular domains of adherens junctions. In: The Cell in Contact., G.M. Edelman, and J.P. Thiery, eds. (New York, Chichester, Brisbane, Toronto, Singapore: Neurosciences Research Foundation, Inc.; Published by John Wiley & Sons, Inc.), pp. 461-489.

Geiger, B., Volk, T., and Volberg, T. (1985b). Molecular heterogeneity of adherens junctions. J Cell Biol *101*, 1523-1531.

Ghadially, F. N. (1980). Diagnostic Electron Microscopy of Tumors., (London, Boston: Butterworths & Co Ltd.), pp. 1-251.

Godsel, L. M., Getsios, S., Huen, A. C., and Green, K. J. (2004). The molecular composition and function of desmosomes. In: Cell Adhesion., J. Behrens, and W.J. Nelson, eds. (Berlin, Heidelberg, New York: Springer Verlag), pp. 137-193.

Goossens, S., Janssens, B., Bonne, S., De Rycke, R., Braet, F., van Hengel, J., and van Roy, F. (2007). A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs. J Cell Sci *120*, 2126-2136.

Grossmann, K. S., Grund, C., Hülsken, J., Behrend, M., Erdmann, B., Franke, W. W., and Birchmeier, W. (2004). Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. J Cell Biol *167*, 149-160.

Hämmerling, B., Grund, C., Boda-Heggemann, J., Moll, R., and Franke, W. W. (2006). The complexus adhaerens of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought. Cell Tissue Res *324*, 55-67.

Hartung, G., Hofheinz, R. D., Dencausse, Y., Sturm, J., Kopp-Schneider, A., Dietrich, G., Fackler-Schwalbe, I., Bornbusch, D., Gonnermann, M., Wojatschek, C., Lindemann, W., Eschenburg, H., Jost, K., Edler, L., Hochhaus, A., and Queisser, W. (2005). Adjuvant therapy with edrecolomab versus observation in stage II colon cancer: a multicenter randomized phase III study. Onkologie *28*, 347-350.

Henderson, D. W., Papadimitriou, J. M., and Coleman, M. (1986). Ultrastructural Appearances of Tumours. Diagnosis and Classification of Human Neoplasia by Electron Microscopy., 2nd edn (Edinburgh: Churchill Livingstone), pp. 1-434.

Hendrix, M. J., Seftor, E. A., Meltzer, P. S., Gardner, L. M., Hess, A. R., Kirschmann, D. A., Schatteman, G. C., and Seftor, R. E. (2001). Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. Proc Natl Acad Sci U S A *98*, 8018-8023.

Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M., and Kemler, R. (1991). The uvomorulin-anchorage protein alpha catenin is a vinculin homologue. Proc Natl Acad Sci U S A *88*, 9156-9160.

Hess, A. R., Seftor, E. A., Gruman, L. M., Kinch, M. S., Seftor, R. E., and Hendrix, M. J. (2006). VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry. Cancer Biol Ther *5*, 228-233.

Hickstein, D. D., Grunvald, E., Shumaker, G., Baker, D. M., Back, A. L., Embree, L. J., Yee, E., and Gollahon, K. A. (1993). Transfected leukocyte integrin CD11b/CD18 (Mac-1) mediates phorbol ester-activated, homotypic cell:cell adherence in the K562 cell line. Blood *82*, 2537-2545.

Hinz, B., Pittet, P., Smith-Clerc, J., Chaponnier, C., and Meister, J. J. (2004). Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell *15*, 4310-4320.

Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., and Gabbiani, G. (2007). The myofibroblast: one function, multiple origins. Am J Pathol *170*, 1807-1816.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. (1992). Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. Cell *70*, 293-301.

Hofmann, I., Schlechter, T., Kuhn, C., Hergt, M., and Franke, W. W. (2009). Protein p0071 - an armadillo plaque protein that characterizes a specific subtype of adherens junctions. J Cell Sci *122*, 21-24.

Holthöfer, B., Windoffer, R., Troyanovsky, S., and Leube, R. E. (2007). Structure and function of desmosomes. Int Rev Cytol *264*, 65-163.

Huang, M., Wang, Y., Collins, M., Gu, J. J., Mitchell, B. S., and Graves, L. M. (2002). Inhibition of nucleoside transport by p38 MAPK inhibitors. J Biol Chem *277*, 28364-28367.

Hulpiau, P., and van Roy, F. (2009). Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol *41*, 349-369.

Iwasaki, H., Nabeshima, K., Nishio, J., Jimi, S., Aoki, M., Koga, K., Hamasaki, M., Hayashi, H., and Mogi, A. (2009). Pathology of soft-tissue tumors: daily diagnosis, molecular cytogenetics and experimental approach. Pathol Int *59*, 501-521.

Janssens, B., Goossens, S., Staes, K., Gilbert, B., van Hengel, J., Colpaert, C., Bruyneel, E., Mareel, M., and van Roy, F. (2001). alphaT-catenin: a novel tissue-specific beta-catenin-binding protein mediating strong cell-cell adhesion. J Cell Sci *114*, 3177-3188.

Järvinen, M., Andersson, L. C., and Virtanen, I. (1990). K562 erythroleukemia cells express cytokeratins 8, 18, and 19 and epithelial membrane antigen that disappear after induced differentiation. J Cell Physiol *143*, 310-320.

Kang, C. D., Do, I. R., Kim, K. W., Ahn, B. K., Kim, S. H., Chung, B. S., Jhun, B. H., and Yoo, M. A. (1999). Role of Ras/ERK-dependent pathway in the erythroid differentiation of K562 cells. Exp Mol Med *31*, 76-82.

Karlsson, P., Holmberg, E., Samuelsson, A., Johansson, K. A., and Wallgren, A. (1998). Soft tissue sarcoma after treatment for breast cancer: a Swedish population-based study. Eur J Cancer *34*, 2068-2075.

Kartenbeck, J., Schwechheimer, K., Moll, R., and Franke, W. W. (1984). Attachment of vimentin filaments to desmosomal plaques in human meningiomal cells and arachnoidal tissue. J Cell Biol *98*, 1072-1081.

Knapp, A. C., Bosch, F. X., Hergt, M., Kuhn, C., Winter-Simanowski, S., Schmid, E., Regauer, S., Bartek, J., and Franke, W. W. (1989). Cytokeratins and cytokeratin filaments in subpopulations of cultured human and rodent cells of non-epithelial origin: modes and patterns of formation. Differentiation *42*, 81-102.

Knapp, A. C., and Franke, W. W. (1989). Spontanous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occuring at different levels of regulation. Cell *59*, 67-79.

Koch, P. J., Walsh, M. J., Schmelz, M., Goldschmidt, M. D., Zimbelmann, R., and Franke, W. W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. Eur J Cell Biol *53*, 1-12.

Koch, P. J., Goldschmidt, M. D., Walsh, M. J., Zimbelmann, R., and Franke, W. W. (1991). Complete amino acid sequence of the epidermal desmoglein precursor polypeptide and identification of a second type of desmoglein gene. Eur J Cell Biol *55*, 200-208.

Koch, P. J., and Franke, W. W. (1994). Desmosomal cadherins: another growing multigene family of adhesion molecules. Curr Opin Cell Biol *6*, 682-687.

Köffler, H. P., and Golde, D. W. (1980). Human myeloid leukemia cell lines: a review. Blood *56*, 344-350.

Köser, J., Troyanovsky, S. M., Grund, C., and Franke, W. W. (2003). De novo formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. Exp Cell Res *285*, 114-130.

Kühnel, W. (2002). Taschenatlas der Zytologie, Histologie und mikroskopischen Anatomie., 11th edn (Stuttgart: Thieme), pp. 1-535.

LaMontagne, K. R., Jr., Hannon, G., and Tonks, N. K. (1998). Protein tyrosine phosphatase PTP1B suppresses p210 bcr-abl-induced transformation of rat-1 fibroblasts and promotes differentiation of K562 cells. Proc Natl Acad Sci U S A *95*, 14094-14099.

Lattes, R. (1983). Tumors of the soft tissue. In: Atlas of Tumor Pathology., (Washington, DC: Armed Forces Institute of Pathology), pp. 1-264.

Li, G., Schaider, H., Satyamoorthy, K., Hanakawa, Y., Hashimoto, K., and Herlyn, M. (2001). Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene *20*, 8125-8135.

Li, H., Price, D. K., and Figg, W. D. (2007). ADH1, an N-cadherin inhibitor, evaluated in preclinical models of angiogenesis and androgen-independent prostate cancer. Anticancer Drugs *18*, 563-568.

Litvinov, S. V., Bakker, H. A., Gourevitch, M. M., Velders, M. P., and Warnaar, S. O. (1994a). Evidence for a role of the epithelial glycoprotein 40 (Ep-CAM) in epithelial cell-cell adhesion. Cell Adhes Commun *2*, 417-428.

Litvinov, S. V., Velders, M. P., Bakker, H. A., Fleuren, G. J., and Warnaar, S. O. (1994b). Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. J Cell Biol *125*, 437-446.

Litvinov, S. V. (1995). Ep-CAM: a homophilic cell-cell adhesion molecule with EGF-like domains. Trends Glycosci Glycotechnol *7*, 375-384.

Litvinov, S. V., van Driel, W., van Rhijn, C. M., Bakker, H. A., van Krieken, H., Fleuren, G. J., and Warnaar, S. O. (1996). Expression of Ep-CAM in cervical squamous epithelia correlates with an increased proliferation and the disappearance of markers for terminal differentiation. Am J Pathol *148*, 865-875.

Litvinov, S. V., Balzar, M., Winter, M. J., Bakker, H. A., Briaire-de Bruijn, I. H., Prins, F., Fleuren, G. J., and Warnaar, S. O. (1997). Epithelial cell adhesion molecule (Ep-CAM) modulates cell-cell interactions mediated by classic cadherins. J Cell Biol *139*, 1337-1348.

Lozzio, B. B., and Lozzio, C. B. (1979). Properties and usefulness of the original K-562 human myelogenous leukemia cell line. Leuk Res *3*, 363-370.

Lozzio, C. B., and Lozzio, B. B. (1975). Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood *45*, 321-334.

Matter, K., and Balda, M. S. (2003). Signalling to and from tight junctions. Nat Rev Mol Cell Biol *4*, 225-236.

McClain, K. L., Leach, C. T., Jenson, H. B., Joshi, V. V., Pollock, B. H., Parmley, R. T., DiCarlo, F. J., Chadwick, E. G., and Murphy, S. B. (1995). Association of Epstein-Barr virus with leiomyosarcomas in children with AIDS. N Engl J Med *332*, 12-18.

Meng, W., and Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. In: Cold Spring Harbor Perspectives in Biology., J. Nelson, and E. Fuchs, eds. (New York: Cold Spring Harbor Laboratory Press), 1:a002899.

Mertens, C., Kuhn, C., and Franke, W. W. (1996). Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J Cell Biol *135*, 1009-1025.

Mertens, C., Kuhn, C., Moll, R., Schwetlick, I., and Franke, W. W. (1999). Desmosomal plakophilin 2 as a differentiation marker in normal and malignant tissues. Differentiation *64*, 277-290.

Miettinen, M. (2003). Diagnostic Soft Tissue Pathology., 1st edn (New York, Edinburgh, London, Philadelphia: Churchill Livingstone), pp. 1-593.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell *31*, 11-24.

Moll, R., Cowin, P., Kapprell, H. P., and Franke, W. W. (1986). Desmosomal proteins: new markers for identification and classification of tumors. Lab Invest *54*, 4-25.

Moll, R., and Franke, W. W. (1986). Cytochemical cell typing of metastatic tumors according to their cytoskeletal proteins. In: Biochemistry and Molecular Genetics of Cancer Metastasis., K. Lapis, L.A. Liotta, and A.S. Rabson, eds. (Boston: Nijhoff Publishing), pp. 101-114.

Moll, R. (1993). Cytokeratine als Differenzierungsmarker: Expressionsprofile von Epithelien une epithelialen Tumoren., (Stuttgart, Jena, New York: Gustav Fischer Verlag), pp. 1-197.

Moll, R., Divo, M., and Langbein, L. (2008). The human keratins: biology and pathology. Histochem Cell Biol *129*, 705-733.

Moll, R., Sievers, E., Hämmerling, B., Schmidt, A., Barth, M., Kuhn, C., Grund, C., Hofmann, I., and Franke, W. W. (2009). Endothelial and virgultar cell formations in the mammalian lymph node sinus: endothelial differentiation morphotypes characterized by a special kind of junction (complexus adhaerens). Cell Tissue Res *335*, 109-141.

Momburg, F., Moldenhauer, G., Hämmerling, G. J., and Möller, P. (1987). Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res *47*, 2883-2891.

Müller, H., and Franke, W. W. (1983). Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. J Mol Biol *163*, 647-671.

Munz, M., Baeuerle, P. A., and Gires, O. (2009). The emerging role of EpCAM in cancer and stem cell signaling. Cancer Res *69*, 5627-5629.

Nagafuchi, A., Takeichi, M., and Tsukita, S. (1991). The 102 kd cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. Cell *65*, 849-857.

Naundorf, S., Preithner, S., Mayer, P., Lippold, S., Wolf, A., Hanakam, F., Fichtner, I., Kufer, P., Raum, T., Riethmüller, G., Baeuerle, P. A., and Dreier, T. (2002). In vitro and in vivo activity of MT201, a fully human monoclonal antibody for pancarcinoma treatment. Int J Cancer *100*, 101-110.

Osborn, M., and Weber, K. (1983). Tumor diagnosis by intermediate filament typing: a novel tool for surgical pathology. Lab Invest *48*, 372-394.

Oxford, E. M., Musa, H., Maass, K., Coombs, W., Taffet, S. M., and Delmar, M. (2007). Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. Circ Res *101*, 703-711.

Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. (1996). Cancer statistics, 1996. CA Cancer J Clin *46*, 5-27.

Parrish, E. P., Garrod, D. R., Mattey, D. L., Hand, L., Steart, P. V., and Weller, R. O. (1986). Mouse antisera specific for desmosomal adhesion molecules of suprabasal skin cells, meninges, and meningioma. Proc Natl Acad Sci U S A *83*, 2657-2661.

Patel, S. D., Chen, C. P., Bahna, F., Honig, B., and Shapiro, L. (2003). Cadherin-mediated cell-cell adhesion: sticking together as a family. Curr Opin Struct Biol *13*, 690-698.

Perez-Moreno, M., Jamora, C., and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. Cell *112*, 535-548.

Perotti, A., Sessa, C., Mancuso, A., Noberasco, C., Cresta, S., Locatelli, A., Carcangiu, M. L., Passera, K., Braghetti, A., Scaramuzza, D. Zanaboni, F., Fasolo, A., Capri, G., Miani, M., Peters, W. P., and Gianni, L. (2009). Clinical and pharmacological phase I evaluation of Exherin (ADH-1), a selective anti-N-cadherin peptide in patients with N-cadherin-expressing solid tumours. Ann Oncol *20*, 741-745.

Pfeiffer, F., Kumar, V., Butz, S., Vestweber, D., Imhof, B. A., Stein, J. V., and Engelhardt, B. (2008). Distinct molecular composition of blood and lymphatic vascular endothelial cell junctions establishes specific functional barriers within the peripheral lymph node. Eur J Immunol *38*, 2142-2155.

Pieperhoff, S., and Franke, W. W. (2008). The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species. Eur J Cell Biol *87*, 413-430.

Pieperhoff, S., Schumacher, H., and Franke, W. W. (2008). The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes. Eur J Cell Biol *87*, 399-411.

Pieperhoff, S., Barth, M., Rickelt, S., and Franke, W. W. (2010). Desmosomal molecules in and out of adhering junctions: normal and diseased states of epidermal, cardiac and mesenchymally derived cells., Dermatology Research and Practice. *In press*.

Pollard, T. D., and Earnshaw, W. C. (2002). Cell Biology., (Philadelphia, London, New York, St. Louis, Sydney, Toronto: Saunders), pp. 1-805.

Punt, C. J., Nagy, A., Douillard, J. Y., Figer, A., Skovsgaard, T., Monson, J., Barone, C., Fountzilas, G., Riess, H., Moylan, E., Jones, D., Dethling, J., Colman, J., Coward, L., and MacGregor, S. (2002). Edrecolomab alone or in combination with fluorouracil and folinic acid in the adjuvant treatment of stage III colon cancer: a randomised study. Lancet *360*, 671-677.

Quinonez, G., and Simon, G. T. (1988). Cellular junctions in a spectrum of human malignant tumors. Ultrastruct Pathol *12*, 389-405.

Rampisela, D., and Donner, L. R. (2004). Pleomorphic sarcoma as a mimicker of sarcomatous carcinoma. Ultrastruct Pathol *28*, 209-212.

Rickelt, S., Franke, W. W., Dörflinger, Y., Goerdt, S., Brandner, J. M., and Peitsch, W. K. (2008). Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. Cell Tissue Res *334*, 401-422.

Rickelt, S., Winter-Simanowski, S., Noffz, E., Kuhn, C., and Franke, W. W. (2009). Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer *125*, 2036-2048.

Rickelt, S., Rizzo, S., Dörflinger, Y., Zentgraf, H. W., Basso, C., Gerosa, G., Thiene, G., Moll, R., and Franke, W. W. (2010). A novel kind of tumor type-characteristic junction: Plakophilin-2 as a major protein of adherens junctions in cardiac myxomata. *Submitted*.

Rowley, P. T., Ohlsson-Wilhelm, B. M., Farley, B. A., and LaBella, S. (1981). Inducers of erythroid differentiation in K562 human leukemia cells. Exp Hematol *9*, 32-37.

Rowley, P. T., Ohlsson-Wilhelm, B. M., and Farley, B. A. (1985). K562 human erythroleukemia cells demonstrate commitment. Blood *65*, 862-868.

Rüdiger, T., Höfler, H., Kreipe, H. H., Nizze, H., Pfeifer, U., Stein, H., Dallenbach, F. E., Fischer, H. P., Mengel, M., von Wasielewski, R., and Müller-Hermelink, H. K. (2002). Quality assurance in immunohistochemistry: results of an interlaboratory trial involving 172 pathologists. Am J Surg Pathol *26*, 873-882.

Rüdiger, T., Höfler, H., Kreipe, H. H., Nizze, H., Pfeifer, U., Stein, H., Dallenbach, E., Fischer, H. P., Mengel, M., Von Wasielewski, R., and Müller-Hermelink, K. (2003). [Interlaboratory trial 2000 " Immunohistochemistry" of the German Society for Pathology and the Professional Association of German Pathologists]. Pathologe *24*, 70-78.

Schäfer, S., Koch, P. J., and Franke, W. W. (1994). Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. Exp Cell Res *211*, 391-399.

Schäfer, S. (1995) Molekularbiologische Charakterisierung des desmosomalen Cadherins Desmoglein-2., Ph.D. Thesis, Faculty of Biology, University of Heidelberg, Heidelberg, Germany, pp.1-150.

Schmelz, M., Moll, R., and Franke, W. W. (1990). A new type of intercellular junction: desmosomal proteins in the extended junctions of certain endothelial cells of the lymphatic systems. In: Cell Biology International Reports, Abstracts Supplement., (London, San Diego, New York: Academic Press), p. 54.

Schmelz, M., and Franke, W. W. (1993). Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: the syndesmos connecting retothelial cells of lymph nodes. Eur J Cell Biol *61*, 274-289.

Schmelz, M., Moll, R., Kuhn, C., and Franke, W. W. (1994). Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: II. Different types of lymphatic vessels. Differentiation *57*, 97-117.

Schmidt, A., Heid, H. W., Schäfer, S., Nuber, U. A., Zimbelmann, R., and Franke, W. W. (1994). Desmosomes and cytoskeletal architecture in epithelial differentiation: cell type-specific plaque components and intermediate filament anchorage. Eur J Cell Biol *65*, 229-245.

Schmidt, A., and Koch, P. J. (2008). Desmosomes in development and disease. In: Cell Junctions, S.E. La Flamme, and A.P. Kowalczyk, eds. (Weinheim: Wiley-VCH), pp. 235-249.

Schmitt, C. J., Franke, W. W., Goerdt, S., Falkowska-Hansen, B., Rickelt, S., and Peitsch, W. K. (2007). Homo- and heterotypic cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein. J Invest Dermatol *127*, 2191-2206.

Seidal, T., Balaton, A. J., and Battifora, H. (2001). Interpretation and quantification of immunostains. Am J Surg Pathol *25*, 1204-1207.

Shelly, C., Petruzzelli, L., and Herrera, R. (1998). PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events. Leukemia *12*, 1951-1961.

Shintani, Y., Fukumoto, Y., Chaika, N., Grandgenett, P. M., Hollingsworth, M. A., Wheelock, M. J., and Johnson, K. R. (2008). ADH-1 suppresses N-cadherin-dependent pancreatic cancer progression. Int J Cancer *122*, 71-77.

Staehelin, L. A. (1974). Structure and function of intercellular junctions. Int Rev Cytol *39*, 191-283.

Stopka, T., Zivny, J. H., Stopkova, P., Prchal, J. F., and Prchal, J. T. (1998). Human hematopoietic progenitors express erythropoietin. Blood *91*, 3766-3772.

Straub, B. K., Boda, J., Kuhn, C., Schnölzer, M., Korf, U., Kempf, T., Spring, H., Hatzfeld, M., and Franke, W. W. (2003). A novel cell-cell junction system: the cortex adhaerens mosaic of lens fiber cells. J Cell Sci *116*, 4985-4995.

Sun, T. T., Shih, C., and Green, H. (1979). Keratin cytoskeletons in epithelial cells of internal organs. Proc Natl Acad Sci U S A *76*, 2813-2817.

Suster, S., and Moran, C. A. (2005). Primary synovial sarcomas of the mediastinum: a clinicopathologic, immunohistochemical, and ultrastructural study of 15 cases. Am J Surg Pathol *29*, 569-578.

Takagaki, K., Katsuma, S., Kaminishi, Y., Horio, T., Tanaka, T., Ohgi, T., and Yano, J. (2005). Role of Chk1 and Chk2 in Ara-C-induced differentiation of human leukemia K562 cells. Genes Cells *10*, 97-106.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem *59*, 237-252.

Taylor, C. R. (2000). The total test approach to standardization of immunohistochemistry. Arch Pathol Lab Med *124*, 945-951.

Troyanovsky, S. (2005). Cadherin dimers in cell-cell adhesion. Eur J Cell Biol *84*, 225-233.

Trzpis, M., McLaughlin, P. M., de Leij, L. M., and Harmsen, M. C. (2007). Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol *171*, 386-395.

Tsiftsoglou, A. S., Pappas, I. S., and Vizirianakis, I. S. (2003). Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther *100*, 257-290.

Tsubura, A., Senzaki, H., Sasaki, M., Hilgers, J., and Morii, S. (1992). Immunohistochemical demonstration of breast-derived and/or carcinoma-associated glycoproteins in normal skin appendages and their tumors. J Cutan Pathol *19*, 73-79.

Valiron, O., Chevrier, V., Usson, Y., Breviario, F., Job, D., and Dejana, E. (1996). Desmoplakin expression and organization at human umbilical vein endothelial cell-to-cell junctions. J Cell Sci *109 (Pt 8)*, 2141-2149.

Wacker, H. H. (1994). Sinuswandzellen: Immunakzessorische Zellen des Lymph-knotensinus., (Stuttgart, Jena, New York: Gustav Fischer Verlag), pp. 1-217.

Waschke, J. (2008). The desmosome and pemphigus. Histochem Cell Biol *130*, 21-54.

Weiss, R. A., Whitby, D., Talbot, S., Kellam, P., and Boshoff, C. (1998). Human herpesvirus type 8 and Kaposi's sarcoma. J Natl Cancer Inst Monogr, 51-54.

Weiss, S. W., and Goldblum, J. R. (2001). Enzinger and Weiss's Soft Tissue Tumors., 4th edn (St. Louis: Mosby), pp. 1-1622.

Weiss, S. W., and Goldblum, J. R. (2007). Enzinger and Weiss's Soft Tissue Tumors., 5th edn (Philadelphia: Mosby), pp. 1-1268.

Wetherington, R. W., Cooper, H. S., Al-Saleem, T., Ackerman, D. S., Adams-McDonnell, R., Davis, W., Ehya, H., Patchefsky, A. S., Suder, J., and Young, N. A. (2002). Clinical significance of performing immunohistochemistry on cases with a previous diagnosis of cancer coming to a national comprehensive cancer center for treatment or second opinion. Am J Surg Pathol *26*, 1222-1230.

Wick, M. R., Ritter, J. H., and Swanson, P. E. (1999). The impact of diagnostic immunohistochemistry on patient outcomes. Clin Lab Med *19*, 797-814.

Wick, M. R., and Mills, S. E. (2001). Consensual interpretive guidelines for diagnostic immunohistochemistry. Am J Surg Pathol *25*, 1208-1210.

Winter, M. J., Nagelkerken, B., Mertens, A. E., Rees-Bakker, H. A., Briaire-de Bruijn, I. H., and Litvinov, S. V. (2003). Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak. Exp Cell Res *285*, 50-58.

Woessmann, W., and Mivechi, N. F. (2001). Role of ERK activation in growth and erythroid differentiation of K562 cells. Exp Cell Res *264*, 193-200.

Wuchter, P., Boda-Heggemann, J., Straub, B. K., Grund, C., Kuhn, C., Krause, U., Seckinger, A., Peitsch, W. K., Spring, H., Ho, A. D., and Franke, W. W. (2007). Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res *328*, 499-514.

Zamecnik, M., and Michal, M. (2002). EMA+ cells in dermatofibrosarcoma protuberans. A study of 11 tumors suggesting perineurial cell differentiation. Cesk Patol *38*, 55-62.

Zamecnik, M., Michal, M., Baumruk, L., and Mukensnabl, P. (2002). Dermatofibrosarcoma protuberans with EMa+ cells. Report of a case suggesting perineurial cell differentiation. Pathol Res Pract *198*, 767-774; discussion 775.

Zauli, D., Gobbi, M., Crespi, C., Tazzari, P. L., Miserocchi, F., Magnani, M., and Testoni, N. (1986). Vimentin and keratin intermediate filaments expression by K562 leukemic cell line. Leuk Res *10*, 29-33.

11. Acknowledgements and thanks

First of all I would like to thank my supervisor and mentor Prof. emer. Dr. Werner W. Franke, for his guidance, support and enthusiasm during the whole period of my thesis. He always had time and an open mind for fruitful discussions, the review of abstracts, posters, presentations and manuscripts as well as personal concerns. His encouragement and support to attend both national and international scientific courses and meetings introduced me to a fascinating field of research.

I also thank the other members of my thesis committee, i.e. Prof. emer. Dr. Dr. h.c. Volker Storch (Department of Morphology and Ecology, Institute of Zoology, University of Heidelberg, Heidelberg, Germany); Prof. Dr. Thomas W. Holstein (Department of Molecular Evolution and Genomics, Institute of Zoology, University of Heidelberg) and Prof. Dr. Jürgen A. Kleinschmidt (Division of Tumor Virology, German Cancer Research Center).

I would not have managed to perform the experiments and analysis without the help and guidance of my colleagues in the Helmholtz Group for Cell Biology at the German Cancer Research Center. In particular, I would like to thank Eva Gundel, Dr. Hans Heid, Cäcilia Kuhn, Stefanie Winter and Ralf Zimbelmann for the excellent advice and support. Similarly, I want to acknowledge the cell culture team, i.e. Michaela Hergt, Edeltraud Noffz and Heiderose Schumacher as well as Christine Grund, Silke Prätzel and Herbert Spring for their kind and patient introductions in various microscopical techniques.

Special thanks go to my colleagues and friends Mareike Barth, Yvette Dörflinger and Dr. Sebastian Pieperhoff. Without your support and discussions I would not have managed to get this far.

During this work I have efficiently collaborated with PD Dr. Wiebke K. Ludwig-Peitsch and Dr. Christian Schmitt (Department of Dermatology, Medical Center Mannheim, University of Heidelberg, Mannheim, Germany) and I would like to thank for their kind support.

Furthermore, I thank the former members of the Division of Cell Biology (German Cancer Research Center); especially I appreciate the helpful discussions with PD Dr. Ilse Hofmann, Dr. Lutz Langbein, PD Dr. Marion Schmidt-Zachmann and Dr. Beate Straub.

I'm also very grateful to Prof. Dr. Cristina Basso, Dr. Stefania Rizzo and Prof. Dr. Gaetano Thiene (from the Department of Medical-Diagnostic Sciences, University of Padua Medical School, Padua, Italy) as well as Prof. Dr. Roland Moll (Institute of Pathology, Philipps University Marburg, Marburg, Germany) and Prof. Dr. Ingrid Moll (University Medical Center Hamburg-Eppendorf, Department and Clinic of Dermatology and Venereology, Hamburg, Germany) for providing numerous tumor samples and good collaboration as well as to Prof. Dr. Hanswalter Zentgraf and his co-workers (Monoclonal Antibody Facility, German Cancer Research Center) for helping me to generate the monoclonal Pkp2-antibodies.

There are many grateful people inside and outside the institute, especially the members of the Beer&Talk sessions, including Dr. Karsten Böhnke, Sonja Breuninger, Ulrike Krebs, Christine Leufke, Jutta Leykauf, Tanja Schlechter and Britta Walter as well as my best friends Monique, Romy, Stefan and Ulrike for their endless patience during the whole time of my studies.

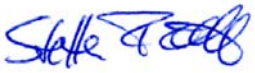
My special gratitude goes to my parents, who supported me during the whole course of my study of biology and my doctoral thesis research.

Last but not least my thanks go to Christina. With her encouragement and love when it was most required, she gave me the extra strength and motivation to get this thesis completed.

12. Declaration

Herewith I, Steffen Rickelt, declare that I have completed this thesis single-handedly without the unauthorized help of a second party. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged.

Heidelberg, 04.05.2010



Steffen Rickelt

13. Appendix: Publications of Steffen Rickelt

1. Schmitt, C. J., Franke, W. W., Goerdt, S., Falkowska-Hansen, B., **Rickelt, S.**, and Peitsch, W. K. (2007) Homo- and heterotypic cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein. J Invest Dermatol. *127*, 2191-2206.

2. **Rickelt, S.**, Franke, W. W., Dörflinger, Y., Goerdt, S., Brandner, J. M., and Peitsch, W. K. (2008). Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. Cell Tissue Res *334*, 401-422.

3. **Rickelt, S.**, Winter-Simanowski, S., Noffz, E., Kuhn, C., and Franke, W. W. (2009). Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer *125*, 2036-2048.

4. Franke, W. W., **Rickelt, S.**, Barth, M., and Pieperhoff, S. (2009). The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind. Cell Tissue Res *338*, 1-17.

5. **Rickelt, S.**, Rizzo, S., Dörflinger, Y., Zentgraf, H. W., Basso, C., Gerosa, G., Thiene, G., Moll, R., and Franke, W. W. (2010). A novel kind of tumor type-characteristic junction: Plakophilin-2 as a major protein of adherens junctions in cardiac myxomata. Submitted.

6. Franke, W. W., and **Rickelt, S.** (2010). Spontaneous and Cumulative Syntheses of Epithelial Proteins and Glycoproteins and Their Assemblies to Novel Cell-Cell Junctions in Malignantly Transformed Cells: I. Carcinomatoid Dysplasia Forms of Human Hematopoietic K562 Cells. Submitted.

Homo- and Heterotypic Cell Contacts in Malignant Melanoma Cells and Desmoglein 2 as a Novel Solitary Surface Glycoprotein

Christian J. Schmitt[1,2], Werner W. Franke[2], Sergij Goerdt[1], Berit Falkowska-Hansen[3], Steffen Rickelt[2] and Wiebke K. Peitsch[1,2]

During progression of melanomas, a crucial role has been attributed to alterations of cell–cell adhesions, specifically, to a "cadherin switch" from E- to N-cadherin (cad). We have examined the adhesion of melanoma cells to each other and to keratinocytes. When different human melanoma cell lines were studied by protein analysis and immunofluorescence microscopy, six of eight lines contained N-cad, three E-cad, and five P-cad, and some lines had more than one cad. Surprisingly, two N-cad-positive lines, MeWo and C32, also contained desmoglein 2 (Dsg2), a desmosomal cad previously not reported for melanomas, whereas other desmosome-specific proteins were absent. This finding was confirmed by reverse transcriptase–PCR, immunoprecipitation, and matrix-assisted laser desorption ionization–time of flight analyses. Double-label confocal and immunoelectron microscopy showed N-cad, α- and β-catenin in plaque-bearing puncta adhaerentia, whereas Dsg2 was distributed rather diffusely over the cell surface. In cocultures with HaCaT keratinocytes Dsg2 was found in heterotypic cell contact regions. Correspondingly, immunohistochemistry revealed Dsg2 in five of 10 melanoma metastases. Together, we show that melanoma cell adhesions are more heterogeneous than expected and that certain cells devoid of desmosomes contain Dsg2 in a non-junction-restricted form. Future studies will have to clarify the diagnostic and prognostic significance of these different adhesion protein subtypes.

Journal of Investigative Dermatology (2007) **127,** 2191–2206; doi:10.1038/sj.jid.5700849; published online 10 May 2007

INTRODUCTION

Under physiological conditions, melanocytes and keratinocytes form the ''epidermal melanin unit'' of the epidermis. Melanocytes are located in the basal layer, in a life-long stable ratio of 1:5 with basal keratinocytes. Dysregulation of this homeostasis may lead to uncontrolled proliferation of the melanocytes and, ultimately, to the development of malignant melanoma (MM). The exact molecular mechanism of this dysregulation is unknown, but an important role has been attributed to alterations in cell–cell communication and adhesion. For example, it has been reported that, compared with normal melanocytes, melanoma cells produce increased amounts of cell adhesion receptors of the immunoglobulin superfamily, correlated with enhanced tumorigenicity and invasiveness (reviewed by Haass *et al.*, 2005).

A group of special importance involved in development and progression of tumors are the cadherins (cads), calcium-dependent transmembrane glycoproteins mediating intercellular adhesion, mostly by homophilic interactions (see the reviews Duguay *et al.*, 2003; Wheelock and Johnson, 2003). So far, more than 80 members of the larger cad superfamily have been identified, including the classical type I and type II cads, which are components of adhering junctions, the desmosomal cads (desmogleins (Dsgs) 1–4 and desmocollins (Dscs) 1–3; for a recent review, see Getsios *et al.*, 2004), protocadherins, fats, seven-pass transmembrane cads, and Ret tyrosine kinases. The type I cads comprise E-cad, typically synthesized by epithelial cells, N-cad on neuronal and mesenchymal cells, and P-cad, first identified in the placenta, and differ from type II cads by the adhesive HAV domain (for review see Nollet *et al.*, 2000). Cads can mediate both homotypic cell–cell interactions, that is, between cells of the same type and heterotypic adhesions between two different cell types.

In normal epidermis, heterotypic melanocyte–keratinocyte adhesions are formed by E-cad (Tang *et al.*, 1994). However, during development of MM, E-cad often appears to be downregulated and replaced by N-cad (Hsu *et al.*, 1996). This phenomenon, also known as ''cadherin switch'', seems

[1]*Department of Dermatology, Medical Center Mannheim, University of Heidelberg, Mannheim, Germany;* [2]*Division of Cell Biology, German Cancer Research Center, Heidelberg, Germany and* [3]*Genetics of Skin Carcinogenesis, German Cancer Research Center, Heidelberg, Germany*

Correspondence: Dr Wiebke K. Peitsch, Department of Dermatology, Medical Center Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68135 Mannheim, Germany.
E-mail: wiebke.peitsch@haut.ma.uni-heidelberg.de

Abbreviations: cad, cadherin; cat, catenin; Dsc, desmocollin; Dsg, desmoglein; IP, immunoprecipitation; MM, malignant melanoma; PKP, plakophilin

Received 4 December 2006; revised 9 February 2007; accepted 19 February 2007; published online 10 May 2007

important not only for the pathogenesis of MM, but also for some tumrs of epithelial origin (see the reviews Christofori, 2003; Cavallaro and Christofori, 2004; Haass *et al.*, 2004). According to some authors (Li *et al.*, 2001a), N-cad can promote survival and migration of melanoma cells. For melanoma cells, it has also been reported that re-expression of E-cad might restore keratinocyte-mediated growth control and reverse malignancy (Hsu *et al.*, 2000; Li *et al.*, 2004).

In developmental biology, the differentiation and topogenesis of melanocytes provides remarkable examples of cell migration over long distances as well as of drastic changes of cell character, including pigmentation and cell–cell adhesion (for a ''classic'' review, see Le Douarin, 1984). Thus, in typical adhering junctions the originally dominant N-cad can be accompanied and taken over by E-cad and this may then be reversed during malignant growth (see, e.g., Tepass *et al.*, 2000; Niessen and Gumbiner, 2002; Duguay *et al.*, 2003; Foty and Steinberg, 2005).

According to a predominant working hypothesis in cancer research, the switch from E- to N-cad has several functional implications. First, it provides the melanoma cells with a new adhesive repertoire to interact with new, mostly mesenchymal neighbors such as fibroblasts (Li *et al.*, 2001a), blood vessels, and lymphatic tissues. Moreover, N-cad is also thought to be responsible for the transendothelial migration of melanoma cells (Sandig *et al.*, 1997; Li *et al.*, 2001a; Qi *et al.*, 2005). Second, the ''cadherin switch'' may provide proliferative and pro-migratory signals (Kuphal *et al.*, 2004; Qi *et al.*, 2005, 2006; Kuphal and Bosserhoff, 2006). As to other mechanisms regulating E- and N-cad expression in melanoma, several pathways and cell–cell cross-talk interactions have recently been discussed (Poser *et al.*, 2001; Qian *et al.*, 2004; Robert *et al.*, 2006; Liu *et al.*, 2006; see the review Huber *et al.*, 2005).

Functional implications of the ''cadherin switch'' on melanoma development and progression have been described by the Herlyn group and others, who mostly used *in vitro* cell culture systems, including two- and three-dimensional organotypic cultures as well as animal models (Hsu *et al.*, 2000; Li *et al.*, 2001a; 2004; Liu *et al.*, 2006). Immunohistochemical examinations on MMs and their metastases, however, have indicated that this concept in its purest form cannot be conferred 100% to the situation *in situ*. For example, a proportion of melanoma cells in metastases are still positive for E-cad and negative for N-cad (Danen *et al.*, 1996; Hsu *et al.*, 1996; Silye *et al.*, 1998; Sanders *et al.*, 1999). This has led us to search for further cell adhesion molecules involved, both in melanoma cell cultures, in two- and three-dimensional melanoma–keratinocyte cocultures, and in cryostat sections of melanoma metastases. We have found that the cad repertoire of melanoma cells – even within one cell – can be much more heterogeneous than expected. Moreover, most surprisingly, we made the observation that despite the absence of desmosomes and other desmosomal constituents, a number of melanoma cell lines in culture as well as a proportion of melanoma metastatic cells contain, in addition to classical cads, noticeable amounts of the desmosomal cad, Dsg2, as a widely spread, non-junction-bound transmembrane cell–cell adhesion protein.

RESULTS

Coexistence of classical cads in certain melanoma cells

To elucidate the molecular composition of plaque-bearing junctions in MM cells, we first performed immunocytochemistry on several cultured human melanoma cell lines, using antibodies to classical cads. In general, cells were grown to rather high density, as the confluency of cell cultures is known to affect the expression pattern of cads. In WM35 cells, for example (Figure 1), we found more than 90% of the cells to be intensely positive for N-cad, as expected (Figure 1b, c, e, and f). In addition, ~30% of the cells contained E-cad (Figure 1a, c, h, and i), thus confirming Smalley *et al.* (2005) and ~20% P-cad (Figure 1d, f, g, and i), both enriched at intercellular contacts, like N-cad. As seen by double-label confocal microscopy, these three classical cads often appeared in mutually exclusive patterns, but occasionally also within the same cell, showing at least partly overlapping localization (Figure 1c, f, and i, asterisks). In some cells, we could notice co-occurrence of all three cads. To examine our results with a biochemical identification method, immunoblot analyses were performed, showing specific bands for N-, E-, and P-cad in WM35 cells (Figure 1j, lane 5) as well as coexistence of N- and P-cad in another melanoma cell line, C32 (lane 4). In coimmunoprecipitation (IP) experiments, conducted again with WM35 cell lysates, all three cads were found to coimmunoprecipitate with the adhering junction plaque protein β-catenin (cat) (Figure 1k, lanes 2–4). N- and E-cad as well as N- and P-cad did not coprecipitate (lane 2). Remarkably, however, P-cad was specifically enriched in E-cad immunoprecipitates (lane 3), an observation made with two different solubilization buffers (1% Triton X-100 buffer, Figure 1k, and RIPA buffer; data not shown), suggestive of the existence of heterotypic E- and P-cad complexes.

Detection and biochemical characterization of Dsg2 in specific melanoma cell lines

Our findings on WM35 cells led us to a more detailed analysis of melanoma cell junctions. To this end, immunoblot analyses with diverse antibodies against proteins of adhering junctions, desmosomes, and tight junctions were performed on eight different human melanoma cell lines (Figure 2 and Table 1). Six of these eight lines contained N-cad, three WM35, SK-MEL-2, and Malme-3M, were positive for E-cad. In addition, P-cad was detected in five of eight lines, among those both N-cad- (WM-115, C32, and WM35) and E-cad-positive ones (SK-MEL-2, Malme-3M, and WM35). Line WM-115 also contained, in addition to N- and P-cad, cad 11, a cad typical for mesenchymal stem cells (Simonneau *et al.*, 1995; Wuchter *et al.*, 2007). Together, our analyses show that cad complements of melanoma cells are more heterogeneous than hitherto thought.

When the different melanoma cell lysates were immunoblotted with antibodies against desmosomal proteins (Figure 2; for a survey see Table 1), we detected Dsg2, a desmosomal cad normally synthesized in keratinocytes in the

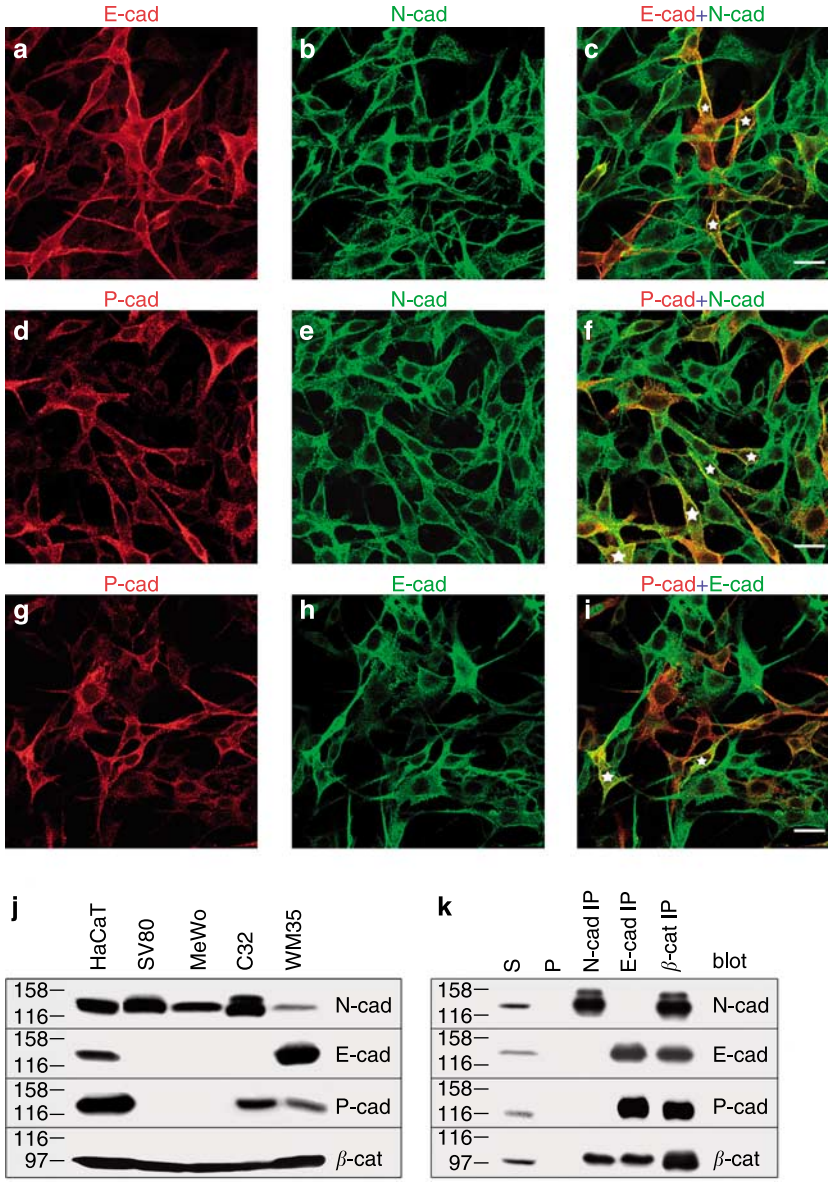


Figure 1. Patterns of coexistence of different classical cads in cultured melanoma cells. (**a**–**i**) Double-label immunofluorescence confocal microscopy of human WM35 melanoma cells using antibodies against N-cad (green in **b**, **c**, **e** and **f**), E-cad (red in **a** and **c**, green in **h** and **i**), and P-cad (red in **d**, **f**, **g**, and **i**), showing that the vast majority of the cells contain N-cad, which appears enriched at intercellular contacts and along cell borders, but also that a minor proportion of the cells is positive for E- and/or P-cad. The different classical cads can sometimes be found in the same cell (asterisks), but occasionally also in mutually exclusive cell patterns (**c**, **f**, and **i**). Bars = 20 μm. (**j**) Immunoblot analysis of the SDS-PAGE-separated polypeptides of total cell lysates from human HaCaT keratinocytes, SV80 fibroblasts, and different human melanoma lines (MeWo, C32 and WM35), confirming the coexistence of more than one classical cad in a given cell line, that is, N- and P-cad in C32 cells and N-, E-, and P-cad in WM35 cells. Equal amounts of proteins have been loaded. (**k**) Immunoblots of the SDS-PAGE-separated proteins of IPs from WM35 cell lysates, using N-, E-, and P-cad as well as β-cat antibodies. Note that N-cad coprecipitates neither with E- nor P-cad, whereas P-cad is pulled down in E-cad immunoprecipitates. S, supernatant before IP; P, material after preclearing. MW markers are indicated on the left margins.

basal epidermal layer, in two of eight melanoma lines, MeWo and C32, an unexpected finding confirmed with three different Dsg2 mAbs (clones DG3.10, 6D8, and 10G11; Figure 2). By contrast, all other desmosomal cads examined, that is, Dsg1 and 3 and Dscs 1–3, were absent. With regard to desmosomal plaque proteins, rather small amounts of plakoglobin, known as a common constituent of both desmosomal and adhering junction plaques (Cowin *et al.*, 1986), were detected in a number of melanoma cell lines, including the Dsg2-positive line C32. Moreover, the other Dsg2-positive line, MeWo, contained plakophilin (PKP) 3 and another line, WM-115, was positive for PKP1, 2, and 3, but negative for desmosomal cads. Interestingly, MeWo cells also synthesized another plaque protein, neurojungin, an arm-repeat protein described previously as a constituent of heterotypic adhering junctions specific for the outer limiting zone of the retina (Paffenholz *et al.*, 1999).

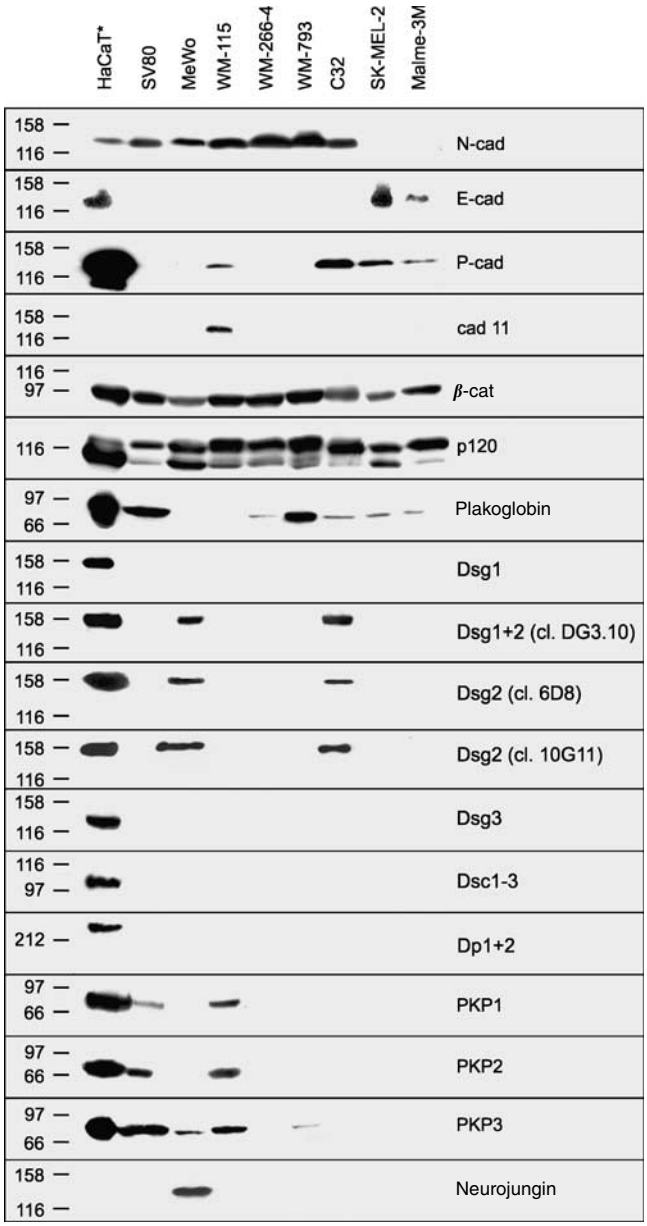


Figure 2. Immunoblot detection of adhering junction-associated and desmosomal proteins in different cultured human melanoma cell lines. Equal amounts of total proteins from seven human melanoma lines (MeWo, WM-115, WM-266–4, WM-793, C32, Sk-Mel-2, and Malme-3M) as well as from HaCaT keratinocytes and SV80 fibroblasts, loaded for comparison, were probed with antibodies against classical cads (N-, E-, and P-cad and cad 11), arm-type plaque proteins of adhering junctions (β-cat, protein p120^{ctn}, plakoglobin, and neurojungin), desmosomal cads (Dsg1–3 and Dsc1–3), and desmosomal plaque proteins (desmoplakin and PKP1–3). Five of the seven melanoma cell lines contain N-cad, two possess E-cad, four show P-cad and cad 11 is only found in one line. Interestingly, two melanoma lines, MeWo and C32, synthesize the desmosomal cad Dsg2, in the absence of all other desmosomal cads, a finding confirmed by immunoblotting with three different Dsg2 mAbs (clones DG3.10, 6D8, and 10G11). PKP1–3 are found in WM-115 cells and PKP3 occurs as the single PKP in MeWo cells, which, surprisingly, also contain the neuronal-type component of special kinds of adhering junctions (cf. Paffenholz *et al.*, 1999). *For immunoblot detection of Dsg1, whole cell lysates of human epidermis were loaded as positive control instead of HaCaT cell lysates.

Analyses of tight junction proteins revealed proteins ZO-1 and -2 in all melanoma cell lines examined (Table 1; see also Smalley *et al.*, 2005). However, proteins ZO-1 and -2 can occur both at adhering and at tight junctions (Itoh *et al.*, 1993, 1999) and indeed, it has been shown that in melanoma cells, ZO-1 is associated with adhering junctions (Smalley *et al.*, 2005). All other tight junction proteins probed, including the transmembrane constituents occludin and claudins 1 and 4, were not detected.

Clearly, the most unexpected result was the detection of Dsg2 in cells devoid of almost all other desmosomal constituents. To substantiate further this finding, PCR analysis was performed and the expected Dsg2 fragments were seen in both MeWo and C32 cells (Figure 3a). As another group had reported the detection of Dsg1 in different melanoma cell lines (Li *et al.*, 2001b), a finding contrasting with our own immunoblot results, we also conducted PCR experiments with Dsg1-specific primers. However, no amplification was seen in the melanoma lines (Figure 3a).

To identify candidates for interaction with Dsg2, lysates from lines C32 (Figure 3b) or MeWo (data not shown) were subjected to IP, followed by SDS-PAGE and matrix-assisted laser desorption ionization–time-of-flight analysis. In the Dsg2 immunoprecipitates, a ~160-kDa band appeared, which was identified as Dsg2 (Figure 3b, lane 3), but no other protein was specifically enriched. By contrast, in control IPs with β-cat antibodies, a 130-kDa band, corresponding to N-cad, and a 90 -kDa band representing α-cat were coimmunopreciptated with β-cat (Figure 3b, lane 4), that is, known functional protein complexes of adhering junctions. Essentially the same IP results were obtained in MeWo cells.

Subcellular localization of Dsg2 in melanoma lines

When we determined the subcellular distribution of Dsg2 by confocal microscopy, Dsg2 enrichment was observed at sites of cell–cell contacts, but also along free plasma membrane boundaries (Figure 4d and f). The classic plaque protein, β-cat, examined for comparison and control, was also accumulated at intercellular junctions, but appeared weaker at free cell borders (Figure 4b, c, e, and f) and did not codistribute with Dsg2 (Figure 4f). On the contrary, double labelling for β-cat and N-cad gave near-complete colocalization (Figure 4a–c) and the same applied for the comparison of α-cat and protein p120^{ctn} (data not shown). When MeWo cells were labelled for PKP3 and neurojungin and C32 cells for plakoglobin, all three proteins were diffusely distributed, without any specific enrichment at a distinct structure, and this was observed with different fixation and staining protocols (data not shown; see Materials and Methods). Co-IPs in MeWo cells did not reveal interactions of Dsg2 with PKP3 or β-cat (Figure 4g). When C32 cell lysates were immunoprecipitated with Dsg2 antibodies, minor amounts of plakoglobin were pulled down and a weak plakoglobin band also appeared in β-cat immunoprecipitates (data not shown). Yet, similar as in MeWo cells, Dsg2 and β-cat did not coimmunoprecipitate. Moreover, like in WM35 cells, no co-IP of N- with P-cad was observed (data not shown).

Table 1. Detection of proteins associated with adhering junctions, desmosomes, and tight junctions in different melanoma cell culture lines

	MeWo	WM-115	WM-226-4	WM-793	C32	SK-Mel-2	Malme-3M	WM35
Adhering junction proteins								
N-cadherin	+	+	+	+	+	–	–	+
E-cadherin	–	–	–	–	–	+	+	+
P-cadherin	–	+	–	–	+	+	+	+
VE-cadherin	–	–	–	–	–	–	–	–
Cadherin 11	–	+	–	–	–	–	–	–
Cadherin 6	–	–	–	–	–	–	–	–
α-catenin	+	+	+	+	+	+	+	+
β-catenin	+	+	+	+	+	+	+	+
$p120^{ctn}$	+	+	+	+	+	+	+	+
Vinculin	+	+	+	+	+	+	+	+
α-Actinin	+	+	+	+	+	+	+	+
Plakoglobin[1]	(+)	(+)	+	+	+	+	+	+
ZO-1[2]	+	+	+	+	+	+	+	+
ZO-2[2]	+	+	+	+	+	+	+	+
Drebrin[3]	+	+	+	+	+	+	+	+
Neurojungin	+	–	–	–	–	–	–	–
Desmosomal proteins								
Desmoglein 1	–	–	–	–	–	–	–	–
Desmoglein 2	+	–	–	–	+	–	–	–
Desmoglein 3	–	–	–	–	–	–	–	–
Desmocollin 1	–	–	–	–	–	–	–	–
Desmocollin 2	–	–	–	–	–	–	–	–
Desmocollin 3	–	–	–	–	–	–	–	–
Plakophilin 1	–	+	–	–	–	–	–	–
Plakophilin 2	–	+	–	–	–	–	–	–
Plakophilin 3	+	+	–	+	–	–	–	–
Desmoplakin 1	–	–	–	–	–	–	–	–
Desmoplakin 2	–	–	–	–	–	–	–	–
Plakoglobin[1]	(+)	(+)	+	+	+	+	+	+
Tight junction proteins								
ZO-1[2]	+	+	+	+	+	+	+	+
ZO-2[2]	+	+	+	+	+	+	+	+
Occludin	–	–	–	–	–	–	–	–
Claudin 1	–	–	–	–	–	–	–	–
Claudin 4	–	–	–	–	–	–	–	–

[1]Plaque component of both adhering junctions and desmosomes.
[2]Proteins occurring both at adhering and at tight junctions.
[3]For references with respect to adhering junction localization, see Peitsch *et al.* (1999, 2005).
(+) Trace amounts detectable by immunoblot analysis.

To clarify the molecular complement of melanoma cell junctions and, in particular, the localization of Dsg2, we performed electron and immunoelectron microscopy of MeWo cells (Figure 5). Ultrathin sections revealed small plaque-bearing adhering junctions of the puncta adhaerentia category (Figure 5a–c; arrows) the plaques of which showed associations with actin microfilaments as well as lateral neighborhood to intermediate-sized filaments (Figure 5b and c). In molecular terms, these puncta junctions were identified by their cat and $p120^{ctn}$ components, and immunoelectron microscopy in general showed marked and specific enrichment in their plaques (Figure 5d–f; arrowheads). In addition, minor β-cat reactions could also be detected in junction-free plasma membrane regions (Figure 5d and e; arrows). Dsg2

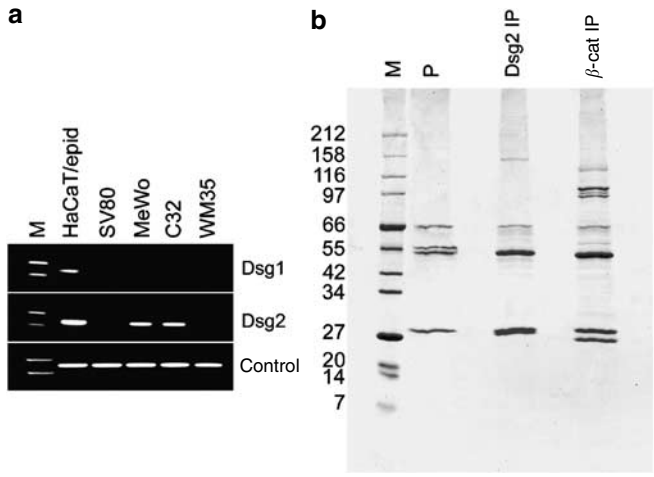


Figure 3. Specific detection of Dsg2 in MeWo and C32 cells at the mRNA level and by matrix-assisted laser desorption ionization–time of flight analysis. (**a**) PCR analysis of MeWo, C32, and WM35 melanoma cells using primers specific for the Dsg1 and 2 as well as for the actin-binding protein drebrin. Dsg2 is detected in MeWo and C32 melanoma cells, whereas none of the melanoma lines analyzed contains Dsg1. Human split skin has been employed as positive control for the Dsg1 PCR, HaCaT keratinocytes for the Dsg2 PCR, and SV80 cells as negative control. Size markers: 396 and 356 bp (Dsg1 and Dsg2 PCR) or 356 and 247 bp (drebrin PCR). (**b**) Coomassie blue-stained 4–20% acrylamide gel, showing proteins immunopreciptated from C32 cell lysates with antibodies to Dsg2 or β-cat. In the Dsg2 immunoprecipitate, a solitary band of ~160 kDa is seen, which has been identified as Dsg2 by matrix-assisted laser desorption ionization–time of flight analysis. In the β-cat immunoprecipitate, a band at ~130 kDa represents N-cad, and bands at 90–100 kDa contain α- and β-cat. Further bands at 66, 55, and ~30 kDa correspond to bovine serum albumin and the heavy and light Ig chains of the antibodies. P, material of the preclearing step; M, molecular weight marker, as indicated on the left margin.

was seen along the entire cell surface, but without any specific enhancements at plaques (Figure 5g).

Distribution of adhering junction proteins and Dsg2 in coculture systems

To assess the possible role of Dsg2 and the adhering junction proteins in heterotypic cell–cell adhesions of melanoma cells, coculture systems confronting melanoma cells with fibroblasts or keratinocytes of line HaCaT were established. When two-dimensional MeWo cell-fibroblast cocultures were reacted with antibodies to adhering junction proteins, including N-cad, α- and β-cat, and protein p120^{ctn}, they all labelled homotypic fibroblast and homotypic melanoma junctions as well as heterotypic melanoma–fibroblast junctions. As an example, the reaction of N-cad is shown in Figure 6a. Dsg2 staining of such cocultures revealed absence of this protein from fibroblasts, as expected, but demonstrated enrichment at the homotypic cell borders of MeWo cells (Figure 6c). In cocultures of C32 (Figure 6e–j) or MeWo cells with HaCaT keratinocytes, E-cad and desmosomal cads, except Dsg2, appeared exclusively in the keratinocytes: E-cad in linear arrays representing the series of adhering junctions (Figure 6e and f), the desmosomal proteins in their typical punctate arrays (data not shown). In contrast, reaction sites of β-cat (Figure 6g and h) as well as of α-cat, and protein p120^{ctn} (data not shown) were accumulated both along homotypic adhering junctions of melanoma and HaCaT cells and in regions indicative of heterotypic contacts. Correspondingly, Dsg2 was seen at the heterotypic interaction borders between melanoma and HaCaT cells (Figure 6i and j).

As a cell culture model of the situation *in vivo* organotypic cultures of MeWo or C32 cells with HaCaT keratinocytes were prepared. Keratinocytes and melanoma cells were mixed in a 5:1 ratio, corresponding to the physiological ratio in the basal epidermal layer and these cultures were exposed to an air–liquid interface for two weeks to allow differentiation of the keratinocytes and formation of a stratified epidermal equivalent (Figure 7). In MeWo–HaCaT organotypic cocultures, MeWo cells, a line originally generated from a melanoma lypmph node metastasis (cf. Table 3), formed cell clusters in subepidermal nests (Figure 7a–f). Immunostainings for β-cat (Figure 7a and b) and other adhering junction plaque proteins (data not shown) were positive not only at homotypic junctions between the keratinocytes on the one hand and MeWo cells on the other, but also at contact sites between the basal keratinocytes and the subepidermally located melanoma cells (Figure 7b). Dsg2 was detected in remarkable intensity at the MeWo cell contacts as well as in the basal layer of the epidermal equivalent, corresponding to the situation *in vivo* and at the boundaries between MeWo cells and basal keratinocytes (Figure 7e and f). By contrast, the reactions for Dsg 1 were rather inverse, showing strongly positive reactions in the upper epidermal layers. Clearly, this protein was absent from the melanoma cells (Figure 7c and d; cf. Figure 7i and j). Different from the MeWo cells, cells of the line C32 originated from a primary amelanotic melanoma, did not invade the epidermal equivalent but formed a compact multilayer tumor on top of it (Figure 7g–l). Otherwise, the distribution pattern of the adhering junction-associated and desmosomal proteins was similar to that noted in the MeWo–HaCaT organotypic cocultures.

Cad patterns in melanoma metastases *in situ*

To verify whether our results with cell culture systems might also be relevant for genuine tumors *in situ*, small samples of melanoma metastases from 10 different patients, originating from skin, lymph node, or lung tissue, all positive for melan-A, were analyzed by immunofluorescence microscopy (Figure 8 and Table 2). Eight of 10 metastases contained N-cad, enriched along cell boundaries (Figure 8a, c, d, f, j, and l) and in seven of the 10 metastases, at least a subpopulation of the tumor cells was positive for E-cad (cf. Figure 8e, f, n, and o and Table 2). Coexistence of both classical cads was noted in six metastases and double-label confocal microscopy revealed that they could occur both separately in different cell clusters and together within the same cell (Figure 8f). P- and VE-cad were detected each in two of 10 metastases, but only in small cell groups, representing less than 20% of the tumor cells (Table 2). Interestingly, two metastases contained some cells positive

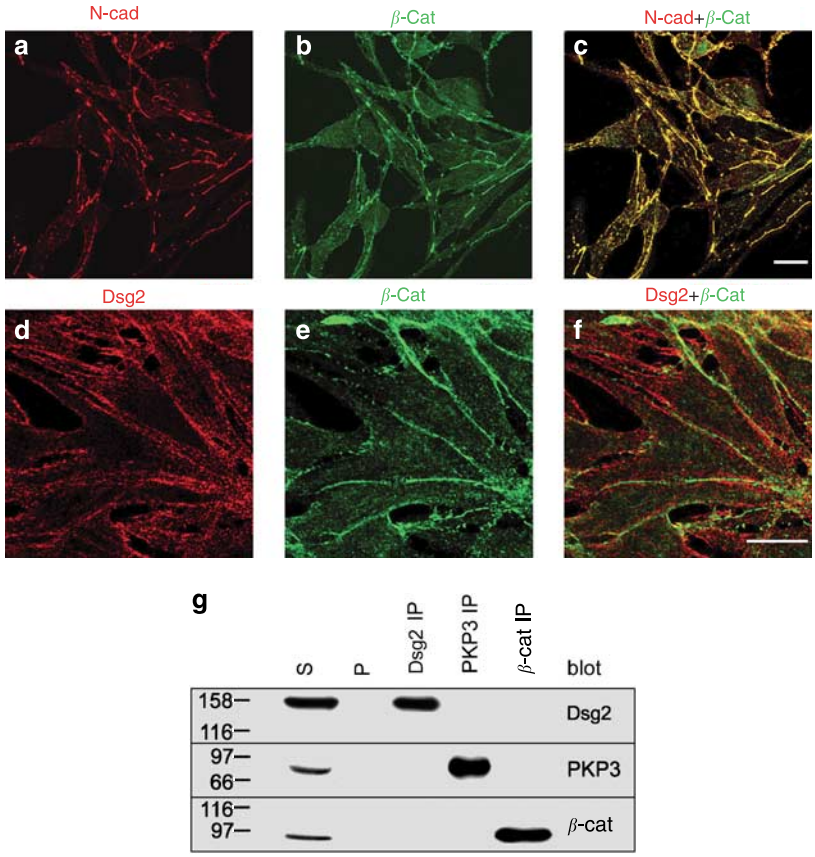


Figure 4. Differential distribution of Dsg2, compared with adhering junction proteins, in cultured melanoma cells. (**a**–**f**) Confocal microscopy of MeWo melanoma cells, stained for β-cat (green in **b**, **c**, **e**, and **f**) in combination with N-cad (red in **a** and **c**) or with Dsg2 (red in **d** and **f**). All three proteins are enriched at intercellular junctions and along free cell boundaries. Although β-cat and N-cad show (**c**) far-reaching colocalization , (**f**) localizations of β-cat and Dsg2 are completely distinct. Bar = 20 μm. (**g**) IP and immunoblot analysis of MeWo cell lysates with antibodies to Dsg2, PKP3, and β-cat, showing no co-IP, indicating that in the specific lysate they occur in different protein complexes.

for Dsg1, which at least, in one of these, appeared enhanced at cell boundaries (Table 2). Moreover, and most remarkably, Dsg2 was found in five of the 10 metastases and in four of them, even more than 50% of the tumor cells were positive (Table 2 and Figure 8g–o). As to subcellular distribution, significant cell border enrichment was noted in three of the five Dsg2-positive metastases (cf. Figure 8g, i, j, m, l, o), whereas the other two showed diffuse cytoplasmic staining (data not shown). When the Dsg2-positive metastases were double-labelled with antibodies to N- (Figure 8j–l) or E-cad (Figure 8m–o), it was evident that Dsg2 could occur both in N- and E-cad-containing cells (Figure 8l and o), apparently independent of the two classical cad pattern.

Surprisingly, in one metastasis, no. 864, which in its histology did not markedly differ from the other metastases, neither N-, E-, VE-, nor P-cad and cad 11 immunostaining was significant along cell–cell boundaries. However, most cell borders were labelled by antibodies against α- and β-cat and protein $p120^{ctn}$. Moreover, a variable number of tumor cells were positive for Dsg1, 2, and 3, all appearing accumulated along cell boundaries, whereas desmosome-specific plaque proteins were not detected by immunofluorescence microscopy. Therefore, we decided to extend our research on this specific subtype to a greater number of tumors.

Together, our observations *in situ* indicated that the cad patterns and combinations were more variable than anticipated and that metastases could comprise extremely heterogeneous subcompartments, reflecting our observations *in vitro*.

DISCUSSION

Our study has given three important results: first, the cad pattern of melanomas can be quite heterogeneous and certain melanoma cell lines as well as metastases can synthesize several – up to three – classical cads within the same cell. Second, a proportion of cultured melanoma cells and melanoma metastases contain, in addition, the desmosomal cad Dsg2, which, in the absence of desmosomes, is dispersed over the cell surface. Third, this complex cad complement contributes to the remarkable diversity of options of melanoma cells to form heterotypic cell–cell interactions. Thus, the facultative cad repertoire of melanoma cells might be much larger than previously thought, probably depending not only on the degree of malignancy but also on the specific growth conditions and the microenvironment.

Heterogeneous cad profiles and their implications

It is well known that under certain circumstances, melanoma cells can synthesize other cads than N- or E-cad. For example, P-cad has been identified in melanoma cells (Hsu *et al.*, 1996; Bauer *et al.*, 2005, 2006) and has been reported, similar to E-cad, to promote cell–cell adhesion and counteract invasion (Van Marck *et al.*, 2005). Moreover, the amount of P-cad appears to have a prognostic relevance, as a loss of P-cad is commonly seen in advanced melanomas and melanoma metastases (Bauer *et al.*, 2006). This seems in accordance with our findings on melanoma metastases in which only a very small subpopulation of cells was P-cad-positive. In addition to P-cad, melanoma cells may facultatively also synthesize VE-cad, a cad characteristic for endothelial cells, that has been related to highly aggressive melanoma cell lines undergoing ''vasculogenic mimicry'', a process facilitating hematogeneous metastasis (Hendrix *et al.*, 2001; Hess *et al.*, 2006). Our melanoma lines were all VE-cad-negative, but two of 10 metastases comprised small subgroups of VE-cad-positive cells.

Surprisingly, one of eight melanoma cell culture lines examined in our study contained cad 11, a type II classical cad originally identified in osteoblasts but later also detected in diverse other cells of mesenchymal origin which had so far not been described in melanoma cells. In an earlier study, six novel, yet not further characterized cad fragments, termed ME1–ME6, have been identified in melanoma cell lines by PCR (Matsuyoshi *et al.*, 1997).

Heterogeneity of subpopulations of melanoma cells might also have important implications for the growth and spreading of these tumors *in situ*. Although E-cad is known to inhibit and N-cad to enhance proliferation, migration, and metastasis, different immunohistochemical investigations have shown that a proportion of melanoma metastases still contains significant E-cad amounts (Danen *et al.*, 1996; Silye *et al.*, 1998; Sanders *et al.*, 1999; Andersen *et al.*, 2004). In our study, one of 10 metastases contained E-cad as the only classical cad and six of 10 synthesized both E- and N-cad, occasionally in different subcompartments of the tumor but sometimes even within the same cell. Therefore, loss of E-cad is apparently not a universal or an inevitable feature of tumor metastasis. Alternatively, it could also be possible that in some melanomas, E-cad is only temporarily downregulated during tumor development, but re-expressed later in metastases. Remarkably, and in contrast to other melanoma subtypes, it has been reported for uveal melanoma that E-cad-containing tumors show an increased risk for metastasis (Onken *et al.*, 2006).

Our biochemical analyses in a melanoma cell line containing E-, N-, and P-cad together, have shown that all three cads form complexes with the same set of plaque proteins of adhering junctions, that is, α- and β-cat, plakoglobin, and protein $p120^{ctn}$. Yet, although E- and N- as well as P- and N-cad occur in different complexes, E- and P-cad have been found to coimmunoprecipitate. Traditionally, the cell–cell binding specificities of cads have been considered as primarily homotypic. However, more recent studies have shown that their interactions can also be more promiscuous (e.g., Shimoyama *et al.*, 2000; Niessen and Gumbiner, 2002; see the review Patel *et al.*, 2003). Results of studies in cocultures of lens and liver cells (Volk *et al.*, 1987) or of epithelial cells and fibroblasts (Omelchenko *et al.*, 2001) have been taken as indications of heterotypic

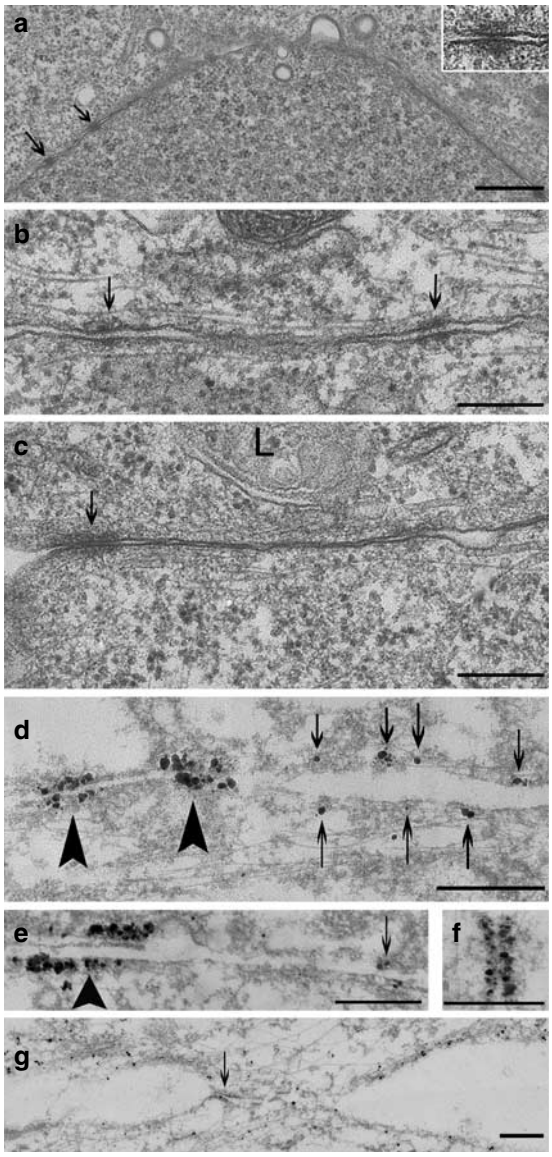


Figure 5. Electron and immunoelectron microscopy presenting cell–cell contacts of MeWo cells. (**a**–**c**) Conventional electron microscopy. In (**a**) the survey as well as in (**b** and **c**) more detailed, high-resolution micrographs, several small, plaque-bearing puncta adhaerentia junctions are seen (arrows in **a**–**c**; inset in **a**: higher magnification of such a junction). Note that at some sites devoid of plaques, the plasma membranes of neighboring cells are extremely close spaced, indicative of another form of junction (**c**). L, lysosome. Bar = 700 nm. (**d**–**g**) Immunoelectron microscopy, using (**d**–**f**) mAbs to β-cat and (**g**) to Dsg2 in combination with gold-coupled secondary antibodies and signal enhancement by the silver technique. β-cat is densely accumulated in the plaques of the puncta adhaerentia (arrowheads in **d** and **e**; higher magnification in **f**). Moreover, plaque protein reactions are sometimes also detected at plaque material of free plasma membranes (arrows in **d** and **e**). Dsg2 staining is observed diffusely along the plasma membrane, both at intercellular contact regions and along free cell membranes, with occasional weak reactions at plaques (arrow) but, in contrast to β-cat, does not show specific enrichment at these sites (**g**). Bar = 700 nm.

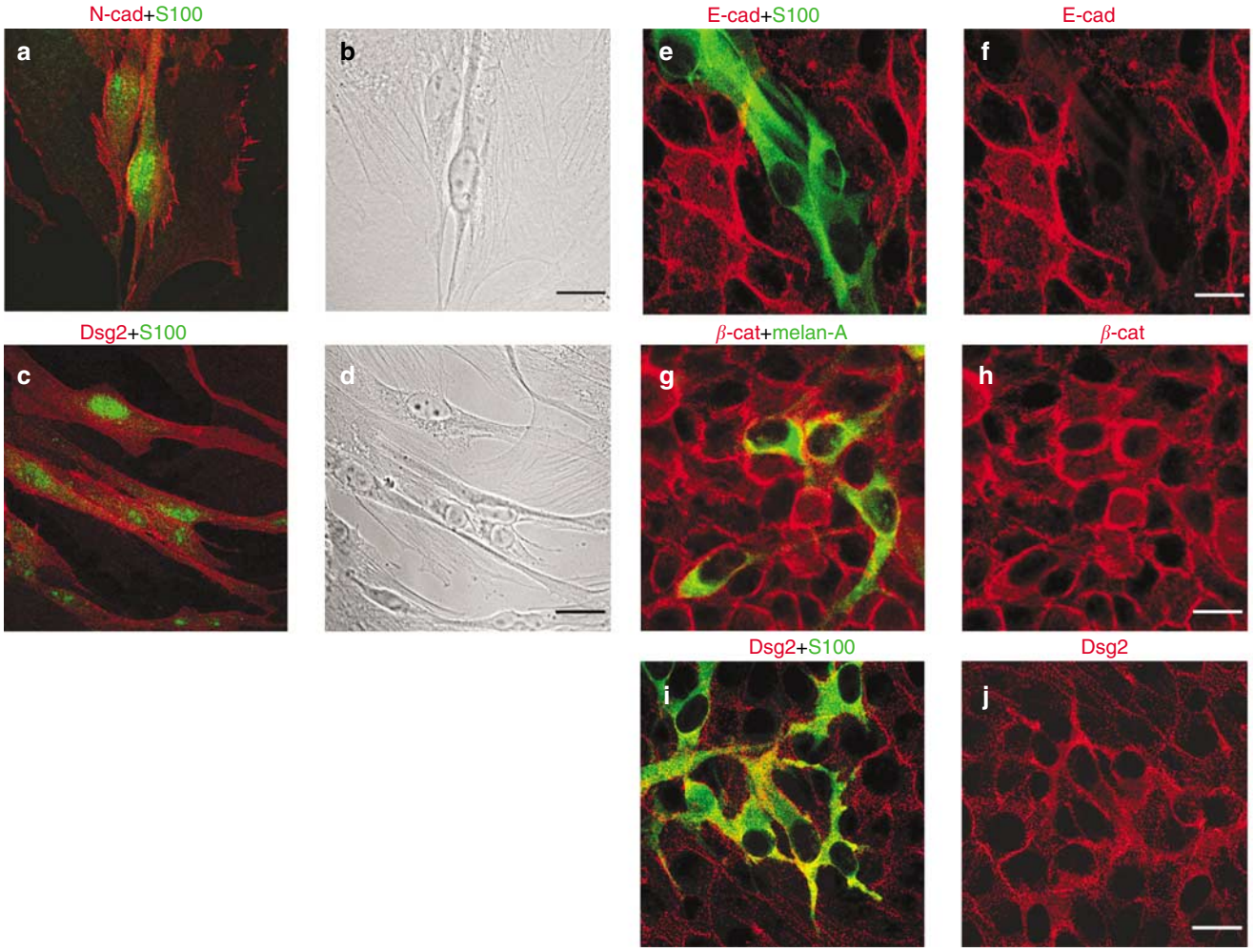


Figure 6. Localization of adhering junction proteins and Dsg2 in adherent cell cocultures. (**a**–**d**) Confocal microscopy of cocultures of primary human fibroblasts and MeWo melanoma cells, labelled for N-cad (red in **a**) or Dsg2 (red in **c**) in combination with S100 protein as melanoma marker (**a** and **c**; green). (**a**) N-cad is detected both in homotypic melanoma–melanoma and fibroblast–fibroblast junctions and in regions of heterotypic contacts between the two cell types, whereas Dsg2 staining is only seen on the surfaces of the S100-positive melanoma cells, here at intercellular contacts as well as along free cell membranes (**b** and **d**, phase contrast images). Bar = 20 μm. (**e**–**j**) Cocultures of HaCaT keratinocytes and C32 melanoma cells, immunostained for (**e** and **f**; red) E-cad, (**g** and **h**; red) β-cat, (**i** and **j**; red) Dsg2, and the melanoma cell markers, (**e** and **i**; green) S100 and (green in **g**) melan-A. (**e** and **f**) Here, E-cad is exclusively found along keratinocyte cell–cell borders containing adhering junctions. (**g** and **h**) By contrast, homotypic melanoma, homotypic keratinocyte, and heterotypic melanoma–keratinocyte contact sites are strongly positive for β-cat. (**i** and **j**) The same holds for Dsg2, which in HaCaT cells appears in the punctate pattern typical of demosomes. Bar = 20 μm.

adhesions formed by N- and E-cad. Moreover, L-cells transfected with E- or P-cad are capable of forming ''E–P heterocadherin'' adhesions between adjacent cells (Duguay *et al.*, 2003; Foty and Steinberg, 2005). On the other hand, it is now clear that certain cads may form lateral *cis*-heterodimers (e.g., Shan *et al.*, 2000) and specifically E- and P-cad *cis*-heterodimers have been identified in A431 carcinoma cells (Klingelhöfer *et al.*, 2000). As to the heterotypic E-P-cad complexes found in our study, their *cis*- or *trans*-nature remains to be determined.

Dsg2 as a novel solitary cell surface component characteristic of a subset of melanoma cells

A totally unexpected result of our study is that certain kinds of melanoma cells synthesize the desmosomal cad Dsg2, known as a widespread transmembrane glycoprotein of desmosomes of all proliferative epithelial cells, keratinocytes included (Schäfer *et al.*, 1994). Via its intracellular domain, Dsg2 interacts with plakoglobin which, in turn, can bind other desmosomal plaque proteins, the PKPs and desmoplakin, the latter constituting a linker to intermediate filaments (Troyanovsky *et al.*, 1993; Chitaev *et al.*, 1996; see the review Getsios *et al.*, 2004). In addition, Dsg2 is also an important constituent of the *area composita* complex connecting cardiomyocytes (Borrmann *et al.*, 2006; Franke *et al.*, 2006).

In our two melanoma cell lines, the Dsg2 distribution was radically different from that known from all other cell types. Here, the adhesive glycoprotein was spread over the cell surface, with occasional clusters at otherwise inconspicuous cell–cell contact sites. Biochemically, it was not found in

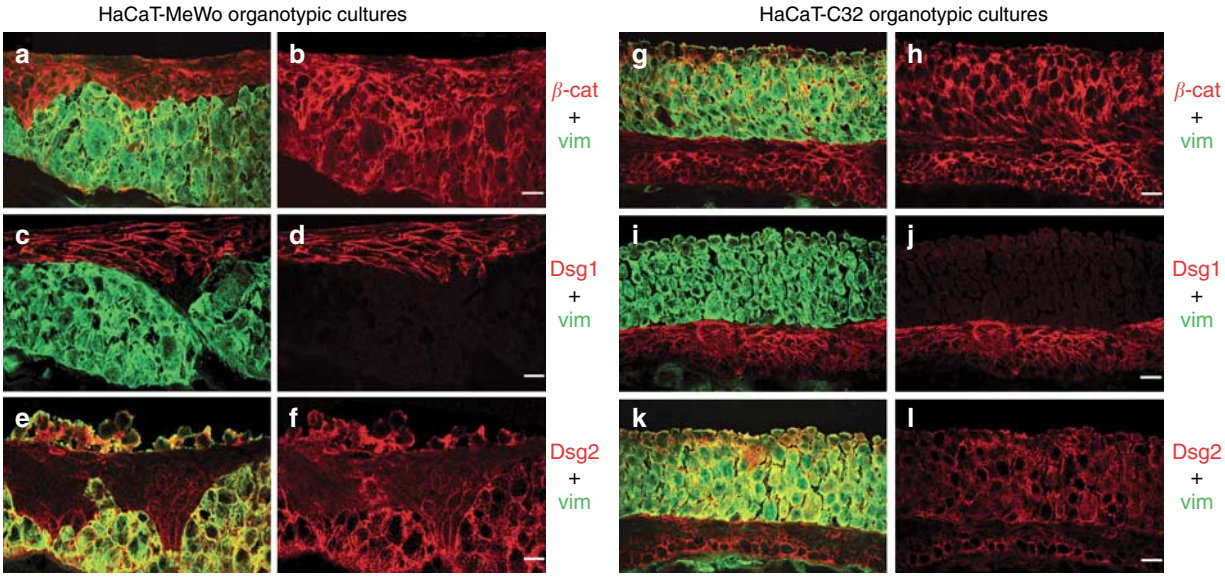


Figure 7. Immunolocalizations of junctional proteins in organotypic keratinocyte-melanoma cell cocultures. Vimentin (green in **a–k**) has been generally used as marker protein for the identification of melanoma cells. (**a–f**) In organotypic cultures of HaCaT keratinocytes with melanoma cells of line MeWo, the keratinocytes form a stratified and differentiated epidermis with a stratum corneum equivalent layer on top, whereas cells of line MeWo, derived from a lymph node metastasis, are densely aggregated in subepidermal cell clusters. As seen by double-label confocal microscopy, β-cat antibodies (**a** and **b**; red) mark homotypic junctions, both between keratinocytes and between melanoma cells as well as regions of heterotypic contacts between the HaCaT and the MeWo cells. Dsg1-positive reactions (**c** and **d**; red) are exclusively observed in the epidermis and here mostly in the upper layers. Clearly, the MeWo cells appear Dsg1-negative. By contrast, Dsg2 (**e** and **f**; red) is enriched in the basal epidermal layer. Moreover, strong Dsg2 immunoreactions are noted at junctions between the subepidermal MeWo cells as well as in areas of heterotypic MeWo cell–keratinocyte contacts. Bar = 20 μm. (**g–l**) In organotypic cocultures confronting HaCaT keratinocytes with C32 cells, originated from a primary melanoma, the melanoma cells do not invade the epidermal equivalent but form a dense multilayer conglomerate on top of it. Double-label confocal microscopy, using (**g** and **h**; red) antibodies to β-cat, (**i** and **j**; red) Dsg1, and (**k** and **l**; red) Dsg2, in combination with (**g–k**; green) vimentin as a melanoma cell marker, reveals distribution patterns for β-cat and the desmosomal cads Dsg1 and 2 that are similar to those in the organotypic HaCaT-MeWo cell cocultures. Bar = 20 μm.

complexes with any other junctional proteins. A similar cell surface distribution of Dsg2 was observed in the human fibrosarcoma cell line HT-1080, the only cell line reported so far to contain endogenous Dsg2 but no other desmosomal constituents (Chitaev and Troyanovsky, 1997). When this cell line was stepwise transfected with other desmosomal cads, junction protein complexes were formed. It was even possible to induce the formation of desmosomes by cotransfection of the other desmosomal proteins, of which the plaque protein, PKP2, played an especially important anchoring role (Koeser *et al.*, 2003).

When we systematically examined our Dsg2-positive melanoma cell lines for other desmosomal proteins, we only found plakoglobin, a constituent of both desmosomes and adhering junctions, which was synthesized in C32 cells in minor amounts and only in traces in MeWo cells. In addition, in MeWo but not in C32 cells, PKP3 was detected, another member of the $p120^{ctn}$ family of armadillo-related proteins (see the review Schmidt and Jäger, 2005). However, as seen by immunolocalization, both plakoglobin and PKP3 occurred predominantly in the cytoplasm and only small amounts of plakoglobin coimmunoprecipitated with Dsg2 in C32 cells. Thus, so far, Dsg2 appears as a primarily solitary cell surface component. This raises the question whether it can in fact as a single molecule type exert significant adhesive strength. When L-cells, a mouse fibroblast line, were transfected with cDNAs encoding single desmosomal cads or related chimeric proteins in the absence of other desmosomal components, the adhesive properties of the transfectants were only very weak (Amagai *et al.*, 1994; Chidgey *et al.*, 1996; Kowalczyk *et al.*, 1996). However, in contrast to our melanoma cell lines, these transfected L-cells were said to be devoid of any functional adhering junctions and thus may lack initiating components for desmosome formation (see the review Getsios *et al.*, 2004).

Another group has reported the synthesis of Dsg1 in several melanoma cell lines and its downregulation by autocrine hepatocyte growth factor, in parallel with down-regulation of E-cad (Li *et al.*, 2001b). In all the melanoma cell lines we analyzed, including line WM35 used by Li *et al.* (2001b), we did not detect Dsg1, neither at protein nor at the mRNA level. On the other hand, our observation that the Dsg1 antibody also used by Li *et al.* (clone 62, from BD Biosciences Pharmingen; Heidelberg, Germany), not only reacted with a 160-kDa band present in all melanoma cell lines but also in fibroblasts used as negative control as well as our matrix-assisted laser desorption ionization results that the 160-kDa component immunoprecipicated with this antibody was identified as epidermal growth factor receptor (data not shown) led us to the conclusion that this represents, most probably, a cross-reaction of this antibody between the two proteins.

Table 2. Classical and desmosomal cadherins in melanoma metastases in the lung, LN, or skin

Case no.	MM 770	MM 761	MM 782	MM 841	MM 864	MM 906	MM 941	MM 944	MM 948	MM 962
Primary melanoma	MUP	SSM, back, 1.03 mm, Clark level III	NM, shoulder, 1.5 mm, Clark level IV	NM, back, 4.5 mm	NM, chest, 1.0 mm, Clark level IV	SSM, chest, 1.9 mm, Clark level IV	Type unknown, shoulder, 3 mm	NM, knee, 4.0 mm, Clark level IV	NM, upper arm, 4.0 mm, Clark level III	Pedunculated, ulcerated melanoma shoulder, 2.6 mm
Metastatic site	Lung	LN, supraclavicular	LN, groin	LN, axilla	Lung	Skin, axilla	LN, groin	LN, groin	Skin, shoulder	Skin, abdomen
Cadherin pattern in metastases										
Classical cads										
N-cad	+	+++	+++	++	–	+++	–	+++	+++	+++
E-cad	+++	–	++	+	–	–	++	+++	+++	+++
P-cad	–	–	–	–	–	–	+	–	–	+
VE-cad	+	–	+	–	–	–	–	–	–	–
Desmosomal cads										
Dsg1	+	–	–	–	+++	–	–	–	–	–
Dsg2	+	+++	++	–	++	–	–	–	–	+++

LN, lymph node; MUP, melanoma of unknown primary; NM, nodular melanoma; SSM, superficial spreading melanoma.
Metastases with ⩾10% but <50% immunoreactive tumor cells were classified as ''+'', those with 50–75% reactive cells as ''++'', and those with ⩾75% as ''+++''.

We have identified Dsg2 not only in monocultures of melanoma cells but also in two- or three-dimensional cocultures and in regions of both homotypic melanoma cell and heterotypic melanoma–keratinocyte contacts. This implies the possibility that Dsg2 can indeed act as a heterotypic cell–cell adhesion molecule between keratinocytes and melanoma cells. It will therefore be mandatory to investigate the ultrastructural basis of such heterotypic contacts, especially as keratinocytes contain desmosomes and melanoma cells do not. Moreover, it will be important to characterize the subgroup of Dsg2-positive melanoma cells in greater detail and its impact on melanoma progression and metastasis. In this context, it may be remarkable that in our organotypic cocultures, one of the Dsg2-positive lines, C32, originated from a primary amelanotic melanoma, did not invade into the artificial epidermis, whereas the other line, MeWo, derived from a lymph node metastasis, formed subepidermal melanoma cell nests as well as Dsg2-positive contact sites with the basal keratinocytes.

Furthermore, it will be interesting to investigate normal melanocytes for Dsg2. Indeed, our studies on normal human epidermal melanocytes, both from newborn foreskin (NHEM-f_1, PromoCell, Heidelberg, Germany) and from adult skin (NHEM-a_1, PromoCell), cultured in serum-free medium, have shown some amounts of Dsg2, both on RNA and on protein level (unpublished results).

Together, our data indicate that a non-desmosome-integrated, ''free'' cell surface glycoprotein Dsg2 might represent a novel, primitive cell–cell adhesion system characteristic of a certain subset of melanomas. We propose to differentiate this subtype of melanoma cells in pathological diagnosis and to characterize it in detail with respect to interaction partners and regulatory proteins involved as well as with respect to its adhesive strength and pathophysiological implications, notably the tumor behavior of the Dsg2-positive melanomas.

MATERIALS AND METHODS

Antibodies

Murine mAbs against N-, E-, and P-cad (clone 56), Dsg1 (clone 62), α- and β-cat, and protein $p120^{ctn}$ were purchased from BD Biosciences Pharmingen, whereas mAbs against vinculin (clone 11–5) and α-actinin (clone BM-75.2) as well as rabbit antisera to α- or β-cat were obtained from Sigma (Deisenhofen, Germany). A rabbit antiserum to N-cad was from QED Bioscience Inc. (San Diego, CA) and a rabbit mAb to E-cad from Epitomics (obtained through Biomol GmbH, Hamburg, Germany). Other mAbs, directed against cad 11, P-cad (clone NCC-CAD-299), Dsg1 and 2 (clones 27B2 and 6D8), and claudin 4 were obtained from Invitrogen (Karlsruhe, Germany), as were polyclonal rabbit antibodies against proteins ZO-1 and -2, occludin, and claudin 1. A cad 6 mAb was from USBiological (obtained through Acris, Hiddenhausen, Germany) and a pan-Dsc rabbit antiserum (clone AHP 322) from Serotec (Düsseldorf, Germany). A mAb directed against cad 5 (VE-cad; clone BV9) was kindly provided by Professor Elisabetta Dejana (Department of Biomolecular and Biotechnological Sciences, School of Sciences, University of Milan, Italy; cf. Lampugnani *et al.*, 1992). The neurojungin mAb was from Dr Rainer Paffenholz (Ingenium

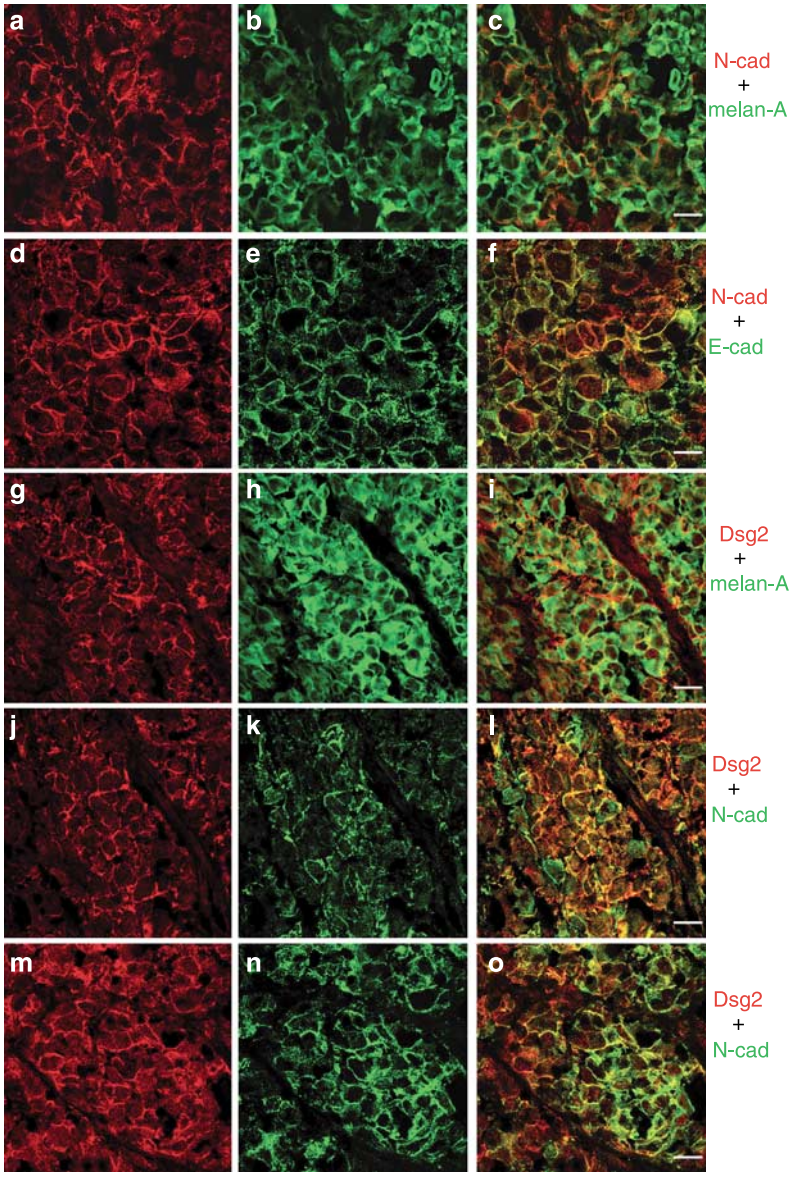


Figure 8. Double-label confocal microscopy presenting heterogeneous cad patterns in melanoma metastases. Cryostat sections of metastasis no. 962 (exemplatory shown) have been labelled with antibodies to N-cad (Red in **a**, **c**, **d**, and **f**; green in **k** and **l**), E-cad (**e**, **f**, **n**, and **o**; green), and Dsg2 (red in **d**–**m** and **h**–**o**) as well as with the melanoma cell marker melan-A (**b**, **c**, **h**, and **i**; green). In the metastasis presented, the vast majority of the melanoma cells exhibit strongly positive N-cad staining at cell–cell junctions (**a** and **c**; red). A major subset of the tumor cells also contains (**e** and **f**, green) E-cad, often appearing in the same cells as (**f**) N-cad. Moreover, large cell clusters also synthesize the desmosomal cad (**g**, **j**, **m**; **i**, **l**, **o**, red) Dsg2, which is markedly enriched along the cell borders. This protein can occur in melanoma cells containing (**l**) N-cad as well as in those positive (**o**) for E-cad, thus appearing independent of the classical cad profile. Bar = 20 μm.

Pharmaceuticals AG, Martinsried, Germany; cf. Paffenholz *et al.*, 1999) and for the demonstration of plakoglobin, a mAb (clone 11E4) from Professor Margaret J. Wheelock (University of Nebraska Medical Center, Omaha, NE) was used. Rabbit antibodies against Dsg2 (clone rb5) were a gift from Dr Lutz Langbein, guinea-pig antibodies against vimentin (clone GP1) from PD Dr. Ilse Hofmann (both Division of Cell Biology, German Cancer Research Center, Heidelberg, Germany). The following antibodies against desmosomal and other cytoskeletal proteins, most of which were generated in the Division of Cell Biology of the German Cancer Research Center, were purchased from Progen Biotechnik (Heidelberg, Germany): mAbs against desmoplakins 1 and 2 (clones DP-2.15, -2.17, and -2.20; Moll *et al.*, 1986), plakoglobin (clone PG 5.1; Cowin *et al.*, 1986), PKP1, 2, and 3 (clones PP1–2D6, PP2/86, and PKP3–270.6.2, respectively; Heid *et al.*, 1994; Mertens *et al.*, 1996; Schmidt *et al.*, 1999), Dsc1 and 3 (clones Dsc1-U100 and Dcs3-U114; Nuber *et al.*, 1996); Dsg1 (clone Dsg1-P23; Kurzen *et al.*, 1998), Dsg1 and 2 (clone DG 3.10; Schmelz *et al.*, 1986), Dsg2 (clone 10G11; Schäfer *et al.*, 1994), Dsg3 (clone Dsg-G194; Kurzen *et al.*, 1998), vimentin (clone VIM 3B4), and drebrin (clone MX823; Peitsch *et al.*, 2005). As melanoma markers, a mAb against melan-A and a rabbit antiserum against S100 protein (both from Progen Biotechnik) were used.

Table 3. Origins of the different melanoma cell culture lines used in this study

Melanoma cell line	Origin
MeWo	Lymph node metastasis
WM-115	Primary melanoma, skin
WM-266-4	Skin metastasis
WM-793	Primary melanoma, skin (SSM, vertical growth phase)
C32	Primary melanoma, skin (amelanotic melanoma)
SK-Mel-2	Skin metastasis
Malme-3M	Lung metastasis
WM35	Primary melanoma, skin (SSM, vertical and radial growth phase)

SSM, superficial spreading melanoma.
All lines were obtained from American Type Culture Collection.

For immunofluorescence microscopy, primary antibody complexes were visualized with secondary antibodies coupled to Cy3 (Dianova, Hamburg, Germany) or Alexa 488 (MoBiTec, Göttingen, Germany). For immunoblot analysis, horseradish peroxidase-conjugated secondary antibodies were applied in combination with the enhanced chemiluminescence system (NEN, Köln, Germany).

Cell culture

Human melanoma cells of lines MeWo, WM-115, WM-266–4, WM-793, C32, SK-Mel-2, Malme-3M, and WM35 as well as Simian virus (SV40)-transformed human SV80 fibroblasts and human U333 glioma cells were provided by American Type Culture Collection (Manassas, VA; for origins of the different melanoma lines, see Table 3). HaCaT keratinocytes were obtained from Professor Petra Boukamp (Genetics of Skin Carcinogenesis, German Cancer Research Center; Boukamp *et al.*, 1988). All cell lines, except WM35, were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) and 2 mM glutamine. WM35 cells were propagated in a 4:1 mixture of MCDB 153 medium (with 1.5 g/l sodium bicarbonate) and Leibovitz's L-15 medium (both from Biochrom), supplemented with 2 mM L-glutamine, 0.005 mg/ml bovine insulin, 1.68 mM $CaCl_2$, and 2% FCS. For isolation of primary human fibroblasts, human dermis was washed with phosphate-buffered saline (PBS) and cut into small pieces, which then were placed on glass coverslips and transferred into culture flasks containing DMEM plus 10% FCS, 2 mM glutamine, and 100 U/ml penicillin/streptomycin (Invitrogen). Cells were subcultured for the first time after ~2 weeks and were used for experiments in passage 4. Preparation and cell culture of human umbilical vein endothelial cells, used as controls for VE-cad immunoblots, was as reported previously (Peitsch *et al.*, 1999). All cell lines were maintained at 37°C with 5% CO_2 and subdivided twice a week.

For two-dimensional melanoma–fibroblast or melanoma–keratinocyte cocultures, cells were trypsinized, counted in a Neubauer chamber, and seeded on glass coverslips in a 5:1 ratio (fibroblasts: melanoma cells or HaCaT:melanoma cells, respectively). Cocultures were maintained for 3–4 days in DMEM plus 10% FCS before procession for immunofluorescence analysis.

Organotypic cocultures

Organotypic cocultures were prepared essentially as described by Stark *et al.* (1999). Dermal equivalents were generated with native type I collagen extracted from rat-tail tendons. The lyophilized collagen was redissolved with 0.1% acetic acid (final concentration: 4 mg/ml) and eight volumes of ice-cold collagen solution were mixed with one volume of 10 × Hank's buffered saline, followed by neutralization with NaOH up to a pH value of 7.0. Fibroblasts were trypsinized, counted, and resuspended in 100% FCS. One volume of fibroblast/FCS solution was added, resulting in a final concentration of 3.2 mg/ml collagen and 2×10^5 cells/ml. Of this mixture, 2.5 ml each were poured into PET membrane filter inserts (Falcon no. 3090, BD Biosciences), placed into special deep six-well trays (Biocoat 355464, BD Biosciences) and allowed to harden for 1 hour at 37°C. Glass rings (24 mm outer, 20 mm inner diameter) were put on the gels to compress them and to provide a central flat area for keratinocyte seeding. The gels were equilibrated over night with DMEM supplemented with 10% FCS and with 50 μg/ml ascorbic acid (Sigma). In total, 1×10^6 cells were seeded. The next day, HaCaT keratinocytes (passage 40), mixed with MeWo or C32 melanoma cells in a 5:1 ratio, were seeded on the top of the collagen matrix using DMEM with 10% FCS and 50 μg/ml ascorbic acid as culture medium, supplemented, for the first week, with 2 ng/ml epidermal growth factor (PromoCell) and 2 ng/ml transforming growth factor-α (R&D Systems, Minneapolis, MN). After submerse incubation over night, the cultures were raised to the air medium interface by lowering the medium level. The medium was changed every second day. Cultures were harvested after 14 days, embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen.

Tissues

Samples of human skin, split skin, and human MM metastases were obtained in the course of routine pathological diagnoses from the Departments of Dermatology and Pathology of the Medical Center Mannheim. Samples were snap-frozen in isopentane, which had been precooled to −80°C in liquid nitrogen and stored at −80°C. Procedures were performed with approval of the medical ethical committee of the Medical Center Mannheim of the University of Heidelberg, with patients' informed consent and according to the Declaration of Helsinki Principles. For analyses of the cad profiles in melanoma metastases, immunolabelled cryostat sections were evaluated by two independent investigators with respect to the proportion of immunoreactive tumor cells. Metastases with 10–50% immunoreactive tumor cells were classified as "+", those with 50–75% reactive cells as "++", and those with ⩾75% as "+++" (Table 2).

Immunoblotting, IP, and matrix-assisted laser desorption ionization analyses

Immunoblotting was performed as described by Peitsch *et al.* (1999), using total protein lysates of cultured cells or of human skin. For IP, cells grown to confluency were lysed either in a Triton X-100 IP buffer, containing 1% Triton X-100, 150 mM NaCl, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) or in a RIPA buffer composed of 1% Triton X-100, 0.1% SDS, 0.5% sodium desoxycholate, 1 mM dithiothreitol, 0.5 mM $CaCl_2$, 150 mM NaCl, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), both supplemented with a protease-inhibitor cocktail (Complete

Mini Inhibitor Tabs, EDTA-free; Roche Diagnostics, Mannheim, Germany), for 1 hour on ice. The supernatants obtained after centrifugation at 14.000 r.p.m. for 10 minutes (4°C) were subjected to IP, using pan mouse IgG or sheep anti-rabbit IgG Dynabeads (Dynal, Hamburg, Germany; cf. Peitsch *et al.*, 1999, 2005). Trypsin digestion and matrix-assisted laser desorption ionization–time of flight analyses were conducted by Dr Martina Schnölzer and Dr Tore Kempf (Protein Analysis Facility, German Cancer Research Center), as previously reported (Peitsch *et al.*, 1999).

RNA isolation, cDNA synthesis, and PCR

Isolation of total RNA from cultured cells and human split skin was performed with TriPure Isolation Reagent (Roche Diagnostics), according to the manufacturer's instructions. Cultured cells were vigorously vortexed and pipetted up and down for homogenization; split skin was pulverized in a micro-dismembrator (Braun Biotech International, Melsungen, Germany). The final concentrations of RNA were determined in an Ultrospec 2100 pro spectrometer (GE Healthcare Life Sciences, formerly Amersham Biosciences, Buckinghamshire, England), and quality was checked on formaldehyde-containing 1% agarose gels. For long-term storage at −80°C, RNA was precipitated with 2.5 volumes ethanol and 0.1 volume 3 M sodium acetate (pH 5.2).

Synthesis of cDNA was conducted with 10 μg total RNA as template, using 0.6 mM dNTP , 0,75 μg random primer dT7, 40 U protector RNAse inhibitor, and 10 U AMV reverse transcriptase in 50 μl 1 × AMV reverse transcriptase buffer (Roche Diagnostics), at 41°C for 1 hour, 51°C for 30 minutes, and 92°C for 3 minutes. The cDNA samples were replenished to a volume of 1 ml with TE buffer. For PCR, aliquots of 5 μl cDNA were utilized together with 0.2 mM deoxyribonucleotide triphosphate, 100 ng of each oligonucleotide primer and 1 U TAQ DNA polymerase in 50 μl $MgCl_2$-containing PCR buffer (Roche Diagnostics). The reaction profile was as follows: 3 minutes 94°C (initial denaturation), 34 cycles at 94°C (20 seconds), 54°C (30 seconds), and 72°C (1 minute), followed by a final elongation step at 72°C for 10 minutes. PCRs were performed for human Dsg1 (forward primer: 5′-GCACTGGTACAATTAATATTAACA-3′; reverse primer: 5′-TCCC TGGGTTCAGGCTGTGGTCCT-3′), human Dsg2 (forward primer: 5′-GCCAAGAAAGTACCAGTGTGCTGC-3′; reverse primer: 5′-CTTT CATCGTGGCTTCCTTGGCCA-3′) and, for control, for the actin-binding protein drebrin (forward primer: 5′-TTTAGATCTGCCGGCGT CAGCTTCAGCGGC-3′; reverse primer: 5′-CGCACTTGCGGGCAT CAGGCACAT-3′). PCR fragments were analyzed on 2% agarose gels.

Immunofluorescence and confocal laser scanning microscopy

For immunostainings, cultured cells grown on glass coverslips were fixed in 2% formaldehyde for 5–7 minutes, treated with NH_4Cl for blocking of free aldehyde groups (5 minutes), washed in PBS (5 minutes), and permeabilized with 0.1% Triton-X (2–3 minutes), followed by two washes in PBS. Alternatively, fixation was performed for 5 minutes at −20°C in methanol and permeabilization for 20 seconds in acetone (−20°C). For staining of tissues and organotypic HaCaT–melanoma cell cultures, frozen samples were sectioned at 5 μm thickness, using a Jung CM3000 cryomicrotome (Leica Microsystems, Wetzlar, Germany), air-dried for a minimum of 1 hour and either fixed in 2% formaldehyde as described above or in acetone for 10 minutes at −20°C. Before incubation with the first antibody, sections were blocked with 5% goat serum for 20 minutes. Both on cultured cells and on cryostat sections, primary antibodies were applied for 1 hour at room temperature, followed by three washes in PBS (5 minutes each), incubation with the secondary antibody (30 minutes, room temperature), washing with PBS (3 × 5 minutes), a short rinse in distilled water, and dehydration in 100% ethanol (1 minute). After air-drying, specimens were mounted with Fluoromount-G (Southern Biotech, obtained through Biozol Diagnostica, Eching, Germany).

PKPs are dual localization proteins, facultatively accumulating not only in intercellular contacts but also in the nucleus (Mertens *et al.*, 1996). The latter localization can best be visualized with a special short staining method guaranteeing minimal loss of soluble proteins, which was applied for PKP staining in addition to the above-mentioned protocols (Schmidt *et al.*, 1999). After methanol/acetone fixation, cells were incubated with primary antibodies only for 15 minutes, washed only twice for 2 minutes, covered with the secondary antibodies for 15 minutes, and washed twice for 2 minutes before mounting.

Immunofluorescence microscopical images were recorded with an Axiophot II photomicroscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam HR (Carl Zeiss), for confocal laser scanning microscopy a Zeiss LSM 519UV microscope was used.

Electron and immunoelectron microscopy

Electron and immunoelectron microscopy was accomplished essentially as described (Langbein *et al.*, 2002). Briefly, for conventional electron microscopy, cells grown on coverslips were fixed in 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2) for 30 minutes and then washed thrice in the same buffer. Postfixation was performed with 2% OsO_4 (cacodylate buffer) for 2 hours, followed first by several washes in distilled water and then by heavy metal staining (0.5% uranylacetate) overnight at 4°C. After three washes in distilled water, samples were dehydrated through an ethanol series and in propylenoxide, followed by embedding in Epon. Ultrathin sections for electron microscopy (EM) were made with a Reichert-Jung microtome (Utracut, Leica, Bensheim, Germany). For contrast enhancement, the sections were stained with 2% uranylacetate in methanol for 15 minutes and with lead citrate for 5 minutes.

For immunoelectron microscopy, cells were fixed in 2% formaldehyde and permeabilized with 0.1% Triton X-100 as mentioned above. Primary antibodies were applied for 2 hours. Antibodies conjugated with 1.4-nm gold particles (Nanogold, Biotrend, Cologne, Germany) were used as secondary reagent and incubated for 4 hours. Postfixation and silver enhancement were performed as described (Langbein *et al.*, 2002). Electron micrographs were taken at 80 kV, using an EM 910 (Carl Zeiss).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Martina Schnölzer and Dr Tore Kempf (Protein Analysis Facility, German Cancer Research Center, Heidelberg) for performing the MALDI–TOF analyses. PD Dr Selma Ugurel, Dr Jörg Faulhaber, and Dr Wolfgang Koenen (Department of Dermatology, Medical Center Mannheim, University of Heidelberg) are acknowledged for providing frozen tissue samples of melanoma metastases and human split skin. We would also like to thank Jutta Bulkescher, Yvette Dörflinger, and Christel Herbst (Department of Dermatology, Medical Center Mannheim) for excellent technical assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to W. K. Peitsch (project PE 896/1).

REFERENCES

Amagai M, Karpati S, Klaus-Kovtun V, Udey MC, Stanley JR (1994) Extracellular domain of pemphigus vulgaris antigen (desmoglein 3) mediates weak homophilic adhesion. *J Invest Dermatol* 103:609–15

Andersen K, Nesland JM, Holm R, Florenes VA, Fodstad O, Maelandsmo GM (2004) Expression of S100A4 combined with reduced E-cadherin expression predicts patient outcome in malignant melanoma. *Mod Pathol* 17:990–7

Bauer R, Hein R, Bosserhoff AK (2005) A secreted form of P-cadherin is expressed in malignant melanoma. *Exp Cell Res* 305:418–26

Bauer R, Wild PJ, Meyer S, Bataille F, Pauer A, Klinkhammer-Schalke M *et al.* (2006) Prognostic relevance of P-cadherin expression in melanocytic skin tumours analysed by high-throughput tissue microarrays. *J Clin Pathol* 59:699–705

Borrmann CM, Grund C, Kuhn C, Hofmann I, Pieperhoff S, Franke WW (2006) The *area composita* of adhering junctions connecting heart muscle cells of vertebrates.II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk. *Eur J Cell Biol* 85:469–85

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–71

Cavallaro U, Christofori G (2004) Multitasking in tumor progression: signaling functions of cell adhesion molecules. *Ann N Y Acad Sci* 1014:58–66

Chidgey MA, Clarke JP, Garrod DR (1996) Expression of full-length desmosomal glycoproteins (desmocollins) is not sufficient to confer strong adhesion on transfected L929 cells. *J Invest Dermatol* 106:689–95

Chitaev NA, Leube RE, Troyanovsky RB, Eshkind LG, Franke WW, Troyanovsky SM (1996) The binding of plakoglobin to desmosomal cadherins: patterns of binding sites and topogenic potential. *J Cell Biol* 133:359–69

Chitaev NA, Troyanovsky SM (1997) Direct Ca2+-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. *J Cell Biol* 138:193–201

Christofori G (2003) Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. *EMBO J* 22:2318–23

Cowin P, Kapprell HP, Franke WW, Tamkun J, Hynes RO (1986) Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell* 46:1063–73

Danen EH, de Vries TJ, Morandini R, Ghanem GG, Ruiter DJ, van Muijen GN (1996) E-cadherin expression in human melanoma. *Melanoma Res* 6:127–31

Duguay D, Foty RA, Steinberg MS (2003) Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. *Dev Biol* 253:309–23

Foty RA, Steinberg MS (2005) The differential adhesion hypothesis: a direct evaluation. *Dev Biol* 278:255–63

Franke WW, Borrmann CM, Grund C, Pieperhoff S (2006) The *area composita* of adhering junctions connecting heart muscle cells of vertebrates.I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. *Eur J Cell Biol* 85:69–82

Getsios S, Huen AC, Green KJ (2004) Working out the strength and flexibility of desmosomes. *Nat Rev Mol Cell Biol* 5:271–81

Haass NK, Smalley KS, Herlyn M (2004) The role of altered cell–cell communication in melanoma progression. *J Mol Histol* 35:309–18

Haass NK, Smalley KS, Li L, Herlyn M (2005) Adhesion, migration and communication in melanocytes and melanoma. *Pigment Cell Res* 18:150–9

Heid HW, Schmidt A, Zimbelmann R, Schäfer S, Winter-Simanowski S, Stumpp S *et al.* (1994) Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). *Differentiation* 58:113–31

Hendrix MJ, Seftor EA, Meltzer PS, Gardner LM, Hess AR, Kirschmann DA *et al.* (2001) Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. *Proc Natl Acad Sci USA* 98:8018–23

Hess AR, Seftor EA, Gruman LM, Kinch MS, Seftor RE, Hendrix MJ (2006) VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry. *Cancer Biol Ther* 5:228–33

Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE *et al.* (2000) E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am J Pathol* 156:1515–25

Hsu MY, Wheelock MJ, Johnson KR, Herlyn M (1996) Shifts in cadherin profiles between human normal melanocytes and melanomas. *J Investig Dermatol Symp Proc* 1:188–94

Huber MA, Kraut N, Beug H (2005) Molecular requirements for epithelial mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 17:548–58

Itoh M, Morita K, Tsukita S (1999) Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J Biol Chem* 274:5981–6

Itoh M, Nagafuchi A, Yonemura S, Kitani-Yasuda T, Tsukita S, Tsukita S (1993) The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J Cell Biol* 121:491–502

Klingelhöfer J, Troyanovsky RB, Laur OY, Troyanovsky S (2000) Amino-terminal domain of classic cadherins determines the specificity of the adhesive interactions. *J Cell Sci* 113:2829–36

Koeser J, Troyanovsky SM, Grund C, Franke WW (2003) *De novo* formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. *Exp Cell Res* 285:114–30

Kowalczyk AP, Borgwardt JE, Green KJ (1996) Analysis of desmosomal cadherin-adhesive function and stoichiometry of desmosomal cadherin-plakoglobin complexes. *J Invest Dermatol* 107:293–300

Kuphal S, Bosserhoff AK (2006) Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene* 25:248–59

Kuphal S, Poser I, Jobin C, Hellerbrand C, Bosserhoff AK (2004) Loss of E-cadherin leads to upregulation of NFkappaB activity in malignant melanoma. *Oncogene* 23:8509–19

Kurzen H, Moll I, Moll R, Schäfer S, Simics E, Amagai M *et al.* (1998) Compositionally different desmosomes in the various compartments of the human hair follicle. *Differentiation* 63:295–304

Lampugnani MG, Resnati M, Raiteri M, Pigott R, Pisacane A, Houen G *et al.* (1992) A novel endothelial-specific membrane protein is a marker of cell–cell contacts. *J Cell Biol* 118:1511–22

Langbein L, Grund C, Kuhn C, Praetzel S, Kartenbeck J, Brandner JM *et al.* (2002) Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. *Eur J Cell Biol* 81:419–35

Le Douarin N (1984) Pigment cells. In: *The neural crest.* (Le Douarin N ed.) Cambridge, London, New York, New Rochelle, Melbourne, Sydney: Cambridge University Press, 108–33

Li G, Fukunaga M, Herlyn M (2004) Reversal of melanocytic malignancy by keratinocytes is an E-cadherin-mediated process overriding beta-catenin signaling. *Exp Cell Res* 297:142–51

Li G, Satyamoorthy K, Herlyn M (2001a) N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res* 61:3819–25

Li G, Schaider H, Satyamoorthy K, Hanakawa Y, Hashimoto K, Herlyn M (2001b) Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. *Oncogene* 20:8125–35

Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R *et al.* (2006) Notch1 signaling promotes primary melanoma progression by activating mito-

gen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res* 66:4182–90

Matsuyoshi N, Tanaka T, Toda K, Imamura S (1997) Identification of novel cadherins expressed in human melanoma cells. *J Invest Dermatol* 108:908–13

Mertens C, Kuhn C, Franke WW (1996) Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *J Cell Biol* 135:1009–25

Moll R, Cowin P, Kapprell HP, Franke WW (1986) Desmosomal proteins: new markers for identification and classification of tumors. *Lab Invest* 54:4–25

Niessen CM, Gumbiner BM (2002) Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J Cell Biol* 156:389–99

Nollet F, Kools P, van Roy F (2000) Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol* 299:551–72

Nuber UA, Schäfer S, Stehr S, Rackwitz HR, Franke WW (1996) Patterns of desmocollin synthesis in human epithelia: immunolocalization of desmocollins 1 and 3 in special epithelia and in cultured cells. *Eur J Cell Biol* 71:1–13

Omelchenko T, Fetisova E, Ivanova O, Bonder EM, Feder H, Vasiliev JM *et al.* (2001) Contact interactions between epitheliocytes and fibroblasts: formation of heterotypic cadherin-containing adhesion sites is accompanied by local cytoskeletal reorganization. *Proc Natl Acad Sci USA* 98:8632–7

Onken MD, Ehlers JP, Worley LA, Makita J, Yokota Y, Harbour JW (2006) Functional gene expression analysis uncovers phenotypic switch in aggressive uveal melanomas. *Cancer Res* 66:4602–9

Paffenholz R, Kuhn C, Grund C, Stehr S, Franke WW (1999) The arm-repeat protein NPRAP (neurojungin) is a constituent of the plaques of the outer limiting zone in the retina, defining a novel type of adhering junction. *Exp Cell Res* 250:452–64

Patel SD, Chen CP, Bahna F, Honig B, Shapiro L (2003) Cadherin-mediated cell–cell adhesion: sticking together as a family. *Curr Opin Struct Biol* 13:690–8

Peitsch WK, Grund C, Kuhn C, Schnölzer M, Spring H, Schmelz M *et al.* (1999) Drebrin is a widespread actin-associating protein enriched at junctional plaques, defining a specific microfilament anchorage system in polar epithelial cells. *Eur J Cell Biol* 78:767–78

Peitsch WK, Hofmann I, Bulkescher J, Hergt M, Spring H, Bleyl U *et al.* (2005) Drebrin, an actin-binding, cell-type characteristic protein: induction and localization in epithelial skin tumors and cultured keratinocytes. *J Invest Dermatol* 125:761–74

Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK (2001) Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *J Biol Chem* 276:24661–6

Qi J, Chen N, Wang J, Siu CH (2005) Transendothelial migration of melanoma cells involves N-cadherin-mediated adhesion and activation of the beta-catenin signaling pathway. *Mol Biol Cell* 16:4386–97

Qi J, Wang J, Romanyuk O, Siu CH (2006) Involvement of Src family kinases in N-cadherin phosphorylation and beta-catenin dissociation during transendothelial migration of melanoma cells. *Mol Biol Cell* 17:1261–72

Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR (2004) E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J* 23:1739–48

Robert G, Gaggioli C, Bailet O, Chavey C, Abbe P, Aberdam E *et al.* (2006) SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. *Cancer Res* 66:7516–23

Sanders DS, Blessing K, Hassan GA, Bruton R, Marsden JR, Jankowski J (1999) Alterations in cadherin and catenin expression during the biological progression of melanocytic tumours. *Mol Pathol* 52:151–7

Sandig M, Voura EB, Kalnins VI, Siu CH (1997) Role of cadherins in the transendothelial migration of melanoma cells in culture. *Cell Motil Cytoskeleton* 38:351–64

Schäfer S, Koch PJ, Franke WW (1994) Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. *Exp Cell Res* 211:391–9

Schmelz M, Duden R, Cowin P, Franke WW (1986) A constitutive transmembrane glycoprotein of Mr 165,000 (desmoglein) in epidermal and non-epidermal desmosomes.I. Biochemical identification of the polypeptide. *Eur J Cell Biol* 42:177–83

Schmidt A, Jäger S (2005) Plakophilins – hard work in the desmosome, recreation in the nucleus? *Eur J Cell Biol* 84:189–204

Schmidt A, Langbein L, Prätzel S, Rode M, Rackwitz HR, Franke WW (1999) Plakophilin 3 – a novel cell-type-specific desmosomal plaque protein. *Differentiation* 64:291–306

Shan WS, Tanaka H, Phillips GR, Arndt K, Yoshida M, Colman DR *et al.* (2000) Functional *cis*-heterodimers of N- and R-cadherins. *J Cell Biol* 148:579–90

Shimoyama Y, Tsujimoto G, Kitajima M, Natori M (2000) Identification of three human type-II classic cadherins and frequent heterophilic interactions between different subclasses of type-II classic cadherins. *Biochem J* 349:159–67

Silye R, Karayiannakis AJ, Syrigos KN, Poole S, van Noorden S, Batchelor W *et al.* (1998) E-cadherin/catenin complex in benign and malignant melanocytic lesions. *J Pathol* 186:350–5

Simonneau L, Kitagawa M, Suzuki S, Thiery JP (1995) Cadherin 11 expression marks the mesenchymal phenotype: towards new functions for cadherins? *Cell Adhes Commun* 3:115–30

Smalley KS, Brafford P, Haass NK, Brandner JM, Brown E, Herlyn M (2005) Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. *Am J Pathol* 166:1541–54

Stark HJ, Baur M, Breitkreutz D, Mirancea N, Fusenig NE (1999) Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J Invest Dermatol* 112: 681–91

Tang A, Eller MS, Hara M, Yaar M, Hirohashi S, Gilchrest BA (1994) E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes *in vitro*. *J Cell Sci* 107:983–92

Tepass U, Truong K, Godt D, Ikura M, Peifer M (2000) Cadherins in embryonic and neural morphogenesis. *Nat Rev Mol Cell Biol* 1:91–100

Troyanovsky SM, Eshkind LG, Troyanovsky RB, Leube RE, Franke WW (1993) Contributions of cytoplasmic domains of desmosomal cadherins to desmosome assembly and intermediate filament anchorage. *Cell* 72: 561–74

Van Marck V, Stove C, Van Den Bossche K, Stove V, Paredes J, Vander Haeghen Y *et al.* (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. *Cancer Res* 65:8774–83

Volk T, Cohen O, Geiger B (1987) Formation of heterotypic adherens-type junctions between L-CAM-containing liver cells and A-CAM-containing lens cells. *Cell* 50:987–94

Wheelock MJ, Johnson KR (2003) Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* 19:207–35

Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U *et al.* (2007) Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. *Cell Tissue Res*, 20 March 2007 [E-pub ahead of print]

Cell Tissue Res
DOI 10.1007/s00441-008-0704-7

REGULAR ARTICLE

Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins

Steffen Rickelt · Werner W. Franke · Yvette Doerflinger · Sergij Goerdt · Johanna M. Brandner · Wiebke K. Peitsch

Received: 10 September 2008 / Accepted: 17 September 2008

Abstract In the tissue integration of melanocytes and melanoma cells, an important role is attributed to cell adhesion molecules, notably the cadherins. In cultured melanoma cells, we have previously described a more heterogeneous repertoire of cadherins than normal, including some melanoma subtypes synthesizing the desmosomal cadherin, desmoglein 2, out of the desmosomal context. Using biochemical and immunological characterization of junctional molecules, confocal laser scanning, and electron and immunoelectron microscopy, we now demonstrate homo- and heterotypic cell-cell adhesions of normal epidermal melanocytes. In human epidermis, both in situ and in cell culture, melanocytes and keratinocytes are connected by closely aligned membranes that are interspersed by small *puncta adhaerentia* containing heterotypic complexes of E- and P-cadherin. Moreover, melanocytes growing in culture often begin to synthesize desmoglein 2, which is dispersed over extended areas of intimate adhesive cell-cell associations. As desmoglein 2 is not found in melanocytes in situ, we hypothesize that its synthesis is correlated with cell proliferation. Indeed, in tissue microarrays, desmoglein 2 has been demonstrated in a sizable subset of nevi and primary melanomas. The biological meanings of these cell-cell adhesion molecule arrangements, the possible diagnostic and prognostic significance of these findings, and the implications of the heterogeneity types of melanomas are discussed.

Keywords Melanocyte · Melanoma · Cell-cell junctions · Cadherins · Desmogleins

Electronic supplementary material The online version of this article (doi:10.1007/s00441-008-0704-7) contains supplementary material, which is available to authorized users.

This work was supported in parts by grants from the Deutsche Forschungsgemeinschaft to W. K. Peitsch (project PE 896/1) and the Deutsche Krebshilfe to W. W. Franke (project 10-2049).

S. Rickelt · W. W. Franke · Y. Doerflinger · W. K. Peitsch
Helmholtz Group for Cell Biology,
German Cancer Research Center,
Heidelberg, Germany

Y. Doerflinger · S. Goerdt · W. K. Peitsch (✉)
Department of Dermatology, University Hospital Mannheim,
University of Heidelberg,
Theodor-Kutzer-Ufer 1-3,
68135 Mannheim, Germany
e-mail: wiebke.peitsch@haut.ma.uni-heidelberg.de

J. M. Brandner
Department of Dermatology and Venerology,
University Hospital Hamburg-Eppendorf,
Hamburg, Germany

Introduction

In the epidermis, melanocytes reside in the basal layer, forming the "epidermal melanin unit" (Montagna and Parakkal 1974; Jimbow et al. 1986). This cell-type homeostasis and pattern is maintained by cell-cell adhesion structures between the melanocytes and the keratinocytes. Disturbances of this pattern may contribute to uncontrolled proliferation of the melanocytes and the development of aberrant structures such as nevi and ultimately malignant melanomas (for recent reviews, see, e.g., Haass et al. 2004, 2005). Cell adhesion molecules of special importance in this respect are the cadherins, calcium-dependent transmembrane glycoproteins, which can mediate intercellular

adhesion by both homophilic and heterophilic cis- and trans-interactions, and which specifically can establish homotypic contacts of punctate adhering junctions (*puncta adhaerentia*) between cells of the same type or heterotypic adhering junctions between different kinds of cells (Niessen and Gumbiner 2002; Duguay et al. 2003; Foty and Steinberg 2005; Troyanovsky 2005).

So far, more than 80 members of the larger cadherin superfamily have been identified, comprising the "classical" type I and type II cadherins as components of adherens junctions (*zonulae adhaerentes*), the desmosomal cadherins as transmembrane proteins of desmosomes, and the atypical cadherins (e.g., T- and Li-cadherin), protocadherins and cadherin-related proteins (Niessen and Gumbiner 2002; Patel et al. 2003; Goodwin and Yap 2004; Troyanovsky 2005). The type I cadherins include E-cadherin, which is regarded as typical of epithelial cells, N-cadherin, characteristically occurring on mesenchymal and neuronal cells, and P-cadherin, first identified in the placenta, whereas the human type II cadherins are a group of 24 members, including the mesenchymal cadherin 11 and cadherin 5, also termed VE-cadherin. The subfamily of desmosomal cadherins can be subdivided into the cell-type-specific desmoglein isoforms Dsg 1–4 and the desmocollins occurring in three isoforms (Dsc 1–3), each with two splice variants (Koch et al. 1990, 1991, 1992; Buxton et al. 1993; Yin and Green 2004; Troyanovsky 2005).

In normal epidermis, melanocytes and keratinocytes are mostly connected via E-cadherin or P-cadherin (Tang et al. 1994; Nishimura et al. 1999), and the ratio between the two cadherins has been reported to be essential for the location of the melanocytes: whereas melanocytes in the basal layer of the epidermis seem to contain predominantly E-cadherin, those residing in hair follicles are rich in P-cadherin (Nishimura et al. 1999). However, the ultrastructure of the various kinds of heterotypic adherens junctions between melanocytes and keratinocytes has not yet been clarified, either in situ or in co-culture.

Dependent on the developmental stage and the specific microenvironment, the cadherin repertoire of melanocytes can be remarkably variable. During embryonic development, melanocyte precursors provide striking examples of cells migrating over long distances from the neural crest, "homing" to specific epidermal tissues, and en route may also change their character (Le Douarin 1984). In the neural crest, they have been shown to contain cadherin 6B and N-cadherin (Hatta and Takeichi 1986; Nakagawa and Takeichi 1995), which later, i.e., after the onset of migration, can be replaced, at least in part, by cadherin 7. The patterns and the amounts of these three cadherins appear to be important for correct segregation from the neural crest, as alterations have been reported to inhibit the emigration of melanocyte precursors and their migration pathway (Nakagawa and Takeichi 1998; Moore et al. 2004). The general importance of the cadherin-catenin septum in the embryonal migration and homing processes is also indicated by the cell-type-targeted gene ablation study of Hari et al. (2002).

When melanocytes have reached their final position in the epidermis and are in contact with keratinocytes, synthesis of E- and P-cadherin is induced (Nishimura et al. 1999; Jouneau et al. 2000). However, during malignant transformation this process may be reversed; E-cadherin, and often apparently also P-cadherin, have been reported to be downregulated and substituted by N-cadherin (Hsu et al. 1996, 2000a; Sanders et al. 1999; Perlis and Herlyn 2004). This change, also known as the "cadherin switch", is widely discussed as an important prerequisite not only for the pathogenesis of malignant melanomas, but also for some tumors derived from epithelial cells and has several implications (Hazan et al. 2000; Christofori 2003; Cavallaro and Christofori 2004; Haass et al. 2004, 2005; Perlis and Herlyn 2004). First, it results in a drastic reduction of the E-cadherin-mediated coupling of melanocytic and melanoma precursor cells to keratinocytes, a reduction that then will further result in a reduction of gap junctions between these cell types (Hsu et al. 2000b). Second, it provides the melanoma cells with a novel adhesive repertoire for interaction with new, mostly mesenchymally derived neighbours such as fibroblasts and endothelial cells of blood and lymphatic vessels (Sandig et al. 1997; Li et al. 2001a; Qi et al. 2005; Haemmerling et al. 2006). Third, N-cadherin is widely assumed to promote the survival and migration of melanoma cells and also to provide proliferative and migratory signals (Li et al. 2001a; Kuphal et al. 2004; Qi et al. 2005, 2006; Kuphal and Bosserhoff 2006). Conversely, re-expression of E-cadherin has been reported to restore keratinocyte-mediated growth control and to reverse malignancy (Hsu et al. 2000a; Li et al. 2004). Certain UV-light components have also been reported to reduce E-cadherin not only in melanoma cells, but also in normal human melanocytes (see, e.g., Jamal and Schneider 2002; Perlis and Herlyn 2004).

The functional consequences of the cadherin switch have, for the most part, been studied in cell culture systems and in animal models (Hsu et al. 1996, 2000a; Li et al. 2001a, 2004; Liu et al. 2006). However, immunohistochemical examination of primary melanomas and their metastases has revealed that a proportion of melanoma cells are still E-cadherin-positive and present little, if any, N-cadherin (Danen et al. 1996; Hsu et al. 1996; Silye et al. 1998; Sanders et al. 1999). Therefore, the cadherin switch as an obligatory prerequisite of malignant behaviour and a prognostic marker is still controversial and might also depend on the subtype of melanoma examined (Andersen et al. 2004; Onken et al. 2006).

Similar to E-cadherin, P-cadherin has also been reported to promote adhesion and to counteract the migration of metastatic melanoma cells (Van Marck et al. 2005). This concept is also supported by observations that P-cadherin is frequently lost in advanced melanomas and melanoma metastases (Bachmann et al. 2005; Bauer et al. 2006).

We have recently examined the repertoire of adhesive molecules in cultured melanoma cell lines and found that their cadherin patterns might be more variable than hitherto thought (Schmitt et al. 2007). In particular, we have found that a number of melanoma cell lines synthesize, in the absence of desmosomes, the desmosomal cadherin desmoglein 2 (Dsg2) as a frequent plasma membrane glycoprotein that is not assembled into any junction but is dispersed over large parts of the cell surface. Therefore, we have analyzed the molecular composition and the ultrastructure of the junctions between normal melanocytes and between melanocytes and keratinocytes in situ in human epidermis and in co-cultures. We here demonstrate new forms of close adhesive membrane alignments over extended cell surface areas and small plaque-bearing *puncta adhaerentia* that connect melanocytes and keratinocytes and that contain E- and P-cadherin and the plaque proteins typical of adherens junctions. Moreover, we show that, when taken into culture, melanocytes might begin to synthesize Dsg2 as a frequent solitary glycoprotein out of the desmosomal context; Dsg2 is dispersed over large surface regions, similar to the distribution patterns recently described in certain types of melanomas (Schmitt et al. 2007).

Materials and methods

Antibodies

Murine monoclonal antibodies (mabs) specific for N-, E-, or P-cadherin, α- and β-catenin, and protein $p120^{ctn}$ were purchased from BD Biosciences Pharmingen (Heidelberg, Germany). Mabs directed against vinculin (clone 11-5) and α-actinin (clone BM-75.2) and rabbit antibodies to α- or β-catenin were obtained from Sigma (Deisenhofen, Germany). Mabs recognizing cadherin 11 and Dsg2 (clone 6D8) and polyclonal rabbit antibodies against proteins ZO-1 and ZO-2 were from Invitrogen (Karlsruhe, Germany). A rabbit antiserum to N-cadherin was obtained from Abcam (Cambridge, UK), and a pan-Dsc rabbit antiserum (clone AHP 322) from Serotec (Duesseldorf, Germany). The following antibodies against junction- and cytoskeleton-associated proteins were purchased from Progen Biotechnik (Heidelberg; see also Schmitt et al. 2007): mabs against desmoplakin 1 and 2 (DP; clones DP-2.15, DP-2.17, and DP-2.20), plakoglobin (clone PG 5.1), plakophilin 1, 2, or 3 (PKP; clones PP1-2D6, PP2/86, and PKP3-270.6.2, respectively), Dsc1 or 3 (clones Dsc1-U100 and Dcs3-U114), Dsg1 (clone P23) or Dsg1 and 2 (clone DG 3.10) , Dsg3 (clone Dsg-G194), vimentin (clone VIM 3B4), and drebrin (clone MX823). As a marker for melanocytes, a mab against Melan A from Progen Biotechnik was used.

A mab against cadherin 5 (VE-cadherin; clone BV9) was a kind gift from Elisabetta Dejana (Department of Biomolecular and Biotechnological Sciences, School of Sciences, University of Milan, Italy; Lampugnani et al. 1992). For the demonstration of plakoglobin, a mab (clone 11E4) generously provided by Margaret J. Wheelock (University of Nebraska Medical Center, Omaha, Neb., USA) was used. Rabbit antibodies against Dsg2 (clone rb5) were kindly provided by Lutz Langbein, and guinea pig antibodies against vimentin by Ilse Hofmann (German Cancer Research Center). Secondary antibodies were as described by Schmitt et al. (2007).

Cell culture

Normal human epidermal melanocytes from foreskin (NHEM-f) and from adult skin (NHEM-a) were obtained from PromoCell (Heidelberg) and cultured in melanocyte growth medium M2 (MGM-M2; PromoCell), which is free of serum and of mitogens such as phorbol-myristate-acetate. For passaging, cells were treated with 0.025% trypsin and 0.01% EDTA, followed by incubation in trypsin neutralization solution (PromoCell). Human melanoma cells of the line MeWo were provided by the American Type Culture Collection (ATTC; Manassas, Va., USA). Simian virus (SV40)-transformed human ("SV80") fibroblasts and human U333 glioma cells were as previously described (Franke et al. 1979; Achtstaetter et al. 1986). HaCaT keratinocytes were a gift from Petra Boukamp (Genetics of Skin Carcinogenesis, German Cancer Research Center; Boukamp et al. 1988). All of these lines were maintained in Dulbecco's minimal essential medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) and 2 mM glutamine. The isolation and cell culture of human umbilical vein endothelial cells (HUVEC) was as reported by Peitsch et al. (1999). For melanocyte-keratinocyte co-cultures, NHEM-f cells and HaCaT keratinocytes were trypsinized, counted in a Neubauer chamber, and seeded onto glass coverslips contained in plastic dishes. Co-cultures were maintained in MGM-M2 plus 5% FCS for 7 days and were then processed for immunofluorescence or immunoelectron microscopic analysis.

Tissues

Normal human epidermis was obtained during routine pathological diagnoses from the Departments of Dermatol-

ogy and Pathology of the University Hospital Mannheim, Germany. Samples were either fixed with 4% formaldehyde and embedded in paraffin or snap-frozen in isopentane precooled in liquid nitrogen and stored at −80°C. All procedures were approved by the Medical Ethical Committee of the University Hospital Mannheim, University of Heidelberg, and were performed with the patients' informed consent. Tissue microarrys were purchased from US Biomax (via BioCat, Heidelberg). The specimens were subjected to heat-induced antigen retrieval (see below) and double-labeled with antibodies to cadherins and to vimentin for cell-type identification, and the proportion of cadherin-positive tumor cells was determined.

Gel electrophoresis, immunoblotting, immunoprecipitation, sucrose gradient centrifugation, and MALDI-TOF analyses

Electrophoresis of total cell proteins in the presence of sodium dodecylsulfate (SDS) was performed in polyacrylamide gels (SDS-PAGE). Immunoblotting of gel-electrophoretically separated polypeptides and immunoprecipitation were conducted as reported (Peitsch et al. 1999, 2005; Schmitt et al. 2007). For immunoprecipitation, a "Triton X-100 immunoprecipitation buffer", containing 1% Triton X-100, 150 mM NaCl, and 20 mM HEPES (pH 7.4), supplemented with a protease inhibitor cocktail (Complete Mini Inhibitor Tabs, EDTA-free; Roche Diagnostics, Mannheim), was used. MALDI-TOF analyses were performed by Martina Schnoelzer and Tore Kempf (Protein Analysis Facility, German Cancer Research Center; cf. Peitsch et al. 1999).

For fractionation on sucrose gradients, NHEM-f cells were extracted with 1% Triton X-100 buffer. Supernatants obtained after centrifugation at 14,000 rpm for 10 min were loaded on linear 5%–30% sucrose gradients (Peitsch et al. 2001). Bovine serum albumin (BSA), catalase, and thyroglobulin (all from Sigma) were used as size markers and were fractionated on parallel gradients. The gradients were centrifuged in a SW40 rotor (Beckman Instruments, Munich, Germany) at 35,000 rpm for 18 h (4°C). Fifteen fractions of 800 μl were collected from top to bottom and were either supplemented with three-fold-concentrated sample buffer and analyzed by SDS-PAGE or were subjected to immunoprecipitation. For the latter, fractions (F) 5–7, comprising a peak of E- and P-cadherin-containing material, were pooled and diluted to a sucrose concentration of 5%, as determined by using a Zeiss refractometer (Carl Zeiss, Jena, Germany).

RNA isolation, cDNA synthesis, and polymerase chain reaction

Isolation of total RNA from cultured cells and human split skin and the synthesis of cDNA and PCR were conducted as described by Schmitt et al. (2007). RNA was extracted with TriPure Isolation Reagent from Roche Diagnostics according to the manufacturer's instructions. For polymerase chain reaction (PCR), primers specific for human Dsg1 (forward primer: 5′ AAT ACC AAG GAA CGA TTC 3′; reverse primer: 5′ CTC CTG ATG TGT CAA TGC 3′), for human Dsg2 (forward primer: 5′ GCC AAG AAA GTA CCA GTG TGC TGC 3′; reverse primer: 5′ CTT TCA TCG TGG CTT CCT TGG CCA 3′), and as a control, for the actin-binding protein drebrin (forward primer: 5′ TTT AGA TCT GCC GGC GTC AGC TTC AGC GGC 3′; reverse primer: 5′ CGC ACT TGC GGG CAT CAG GCA CAT 3′) were used. PCR fragments were analyzed on 2% agarose gels. The fragments obtained with Dsg2-specific primers were cloned into the *Eco*R1 restriction sites of a pCR2.1-TOPO vector by using a TOPO TA Cloning Kit (Invitrogen) and were verified by sequencing.

Transient transfection of NHEM-f cells

The full length human Dsg2 cDNA, generated in this laboratory, was cloned into the *Not*I sites of a eukaryotic p163/7 expression vector (Schaefer et al. 1994, 1996). This vector contains a major histocompatibility class I H2-2 promotor, which is identical to p164/7 (Niehrs et al. 1992). Transient transfection of NHEM-f cells was performed with Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's recommendations. Cells were analyzed 24 h and 48 h after transfection by using immunostaining with Dsg2 antibodies.

Immunofluorescence and electron and immunoelectron microscopy

Cultured cells grown on glass coverslips were fixed either in 2% formaldehyde in phosphate-buffered saline (PBS) for 5–7 min, followed by permeabilization with 0.1% Triton-X for 2–3 min or with methanol for 5 min at −20°C, followed by 20 s in −20°C acetone. Procedures for immunostaining were as reported by Schmitt et al. (2007). For immunolocalization on paraffin-embedded tissue samples, sections of human epidermis or tissue microarrays were deparaffinized according to standard techniques. To achieve heat-induced antigen retrieval, sections were pretreated by microwaving in 100 mM TRIS-HCl buffer containing 5% urea (pH 9.5, 10 min, 120°C or pH 11, 30 min, 120°C) or in citrate buffer (82 mM sodium citrate and 18 mM citric acid, pH 6, 10 min, 120°C). This was followed by a wash with PBS (5 min, room temperature), incubation with PBS containing 2% milk powder (10 min), and blocking with 10% goat serum and 2% milk powder in PBS (15 min). Primary antibodies were applied for 2 h, and secondary antibodies for 30 min. Microscopic images were recorded with an

Axiophot II photomicroscope (Carl Zeiss) equipped with an AxioCam HR (Carl Zeiss), and confocal images with a Zeiss LSM 510 UV microscope.

Electron and immunoelectron microscopy was performed as described (Langbein et al. 2002; Schmitt et al. 2007). For immunoelectron microscopy, NHEM-f cells, NHEM-f-HaCaT co-cultures, and cryostat sections of human epidermis were fixed in 2% formaldehyde (5–7 min), freshly prepared from paraformaldehyde, and permeabilized with 0.1% saponin (1–2.5 min), followed by incubation with primary antibodies for 2 h. After washing steps, the samples were incubated with secondary antibodies conjugated with 1.4-nm gold particles (Nanogold, Biotrend, Cologne, Germany) for 2–4 h, followed by silver enhancement (Langbein et al. 2002). Electron micrographs were taken at 80 kV by using an EM 910 (Carl Zeiss).

Results

Composition and ultrastructure of heterotypic melanocyte-keratinocyte connections in human epidermis

To determine the composition of the heterotypic cell adhesions connecting melanocytes and keratinocytes in situ, we immunostained cryostat and paraffin sections through normal human epidermis with antibodies to the constituents of adherens junctions and desmosomes in combination with Melan A or vimentin as melanocyte markers. Analyses of such sections by confocal microscopy showed an enrichment of E- and P-cadherin and of the plaque proteins of adherens junctions, i.e., α- and β-catenin and protein $p120^{ctn}$, both at homotypic keratinocyte contacts and at heterotypic adhesions between melanocytes and keratinocytes in the basal epidermis (for examples of E-cadherin and β-catenin, see Fig. 1a,a′,b,b′). By contrast, desmosomal cadherins and the desmosomal plaque protein desmoplakin exhibited the typical punctate staining pattern of desmosomes but appeared to be absent from melanocytes and from melanocyte-keratinocyte contacts. These findings were also obtained with antibodies to Dsg2 (Fig. 1c,c′), the desmosomal cadherin previously identified in a subset of melanoma cells (Schmitt et al. 2007). For comparison, sections of human scalp, showing hair follicles, and of human basal cell carcinomas were double-labeled and examined by confocal microsopy. Here, corresponding observations were made, i.e., an enrichment of α- and β-catenin and E-cadherin at contact sites between melanocytes and follicular epithelia or basal carcinoma cells. Desmosomal proteins, however, including Dsg2, were never detected at these sites (data not shown; for localization of desmosomal proteins in hair follicles, see Franke and Heid 1989; Kurzen et al. 1998).

For a detailed characterization of the heterotypic melanocyte-keratinocyte adhesions, electron and immunoelectron microscopy was performed on cryostat sections of healthy human epidermis. In such electron micrographs, small junctions with an electron-dense plaque, resembling *puncta adhaerentia*, were observed at sites of contacts between melanocytes and keratinocytes (Fig. 2a,d, arrows). Moreover, the plasma membranes of the melanocytes and keratinocytes were closely aligned to each other over remarkably long distances, with a consistently narrow intercellular space and occasionally a thin cytoplasmic coat (Fig. 2b,c, arrowheads). When the sections were labeled with antibodies to adherens junction proteins such as α- or β-catenin, significant label was seen at the plaques of *puncta adhaerentia* (e.g., Fig. 2e). Moreover, some catenin-positive reactions were also noted along the plasma membrane alignments of melanocytes and keratinocytes (data not shown). Using mabs reactive with Dsg2 for immunoelectron microscopy, marked enrichment was observed at the intercellular spaces of the desmosomes between keratinocytes. Corresponding to our confocal microscopical observations, the melanocytes and the melanocyte-keratinocyte contacts appeared free of Dsg2 (not shown).

Cadherins and cadherin complexes in cultured melanocytes

The synthesis and assembly patterns of junctional components were also analyzed in cultured normal human melanocytes, derived from foreskin (NHEM-f). Cell cultures were grown in medium free of serum and of mitogens to avoid transformation, and total protein lysates were prepared for immunoblot analyses after the cultures had reached 50%–70% confluence. Human HaCaT keratinocytes, SV80 fibroblasts, and cultured human melanoma cells of the line MeWo, which previously had been found to contain Dsg2 as a solitary plasma membrane protein (Schmitt et al. 2007), were used for comparison. Immunoblotting indeed demonstrated E- and P-cadherin in NHEM-f melanocytes (Fig. 3, Table 1). By contrast, N-cadherin, endothelial VE-cadherin, which had been reported to occur in a subset of highly aggressive melanoma lines (Hendrix et al. 2001; Hess et al. 2006), and cadherin 11, synthesized by osteoblasts, myofibroblasts, and various other mesenchymally derived cells, including stem cells (Simonneau et al. 1995; Hinz et al. 2004; Wuchter et al. 2007) and also found in one of our melanoma lines (Schmitt et al. 2007), were totally absent. However, the melanocytes contained the set of plaque proteins typical of adherens junctions, i.e., α- and β-catenin, protein $p120^{ctn}$, vinculin, α-actinin, plus proteins ZO-1 and ZO-2 (Itoh et al. 1993, 1999; Smalley et al. 2005; for the specific problem regarding the complexity of protein $p120^{ctn}$ isoforms, see Aho et al. 2002).

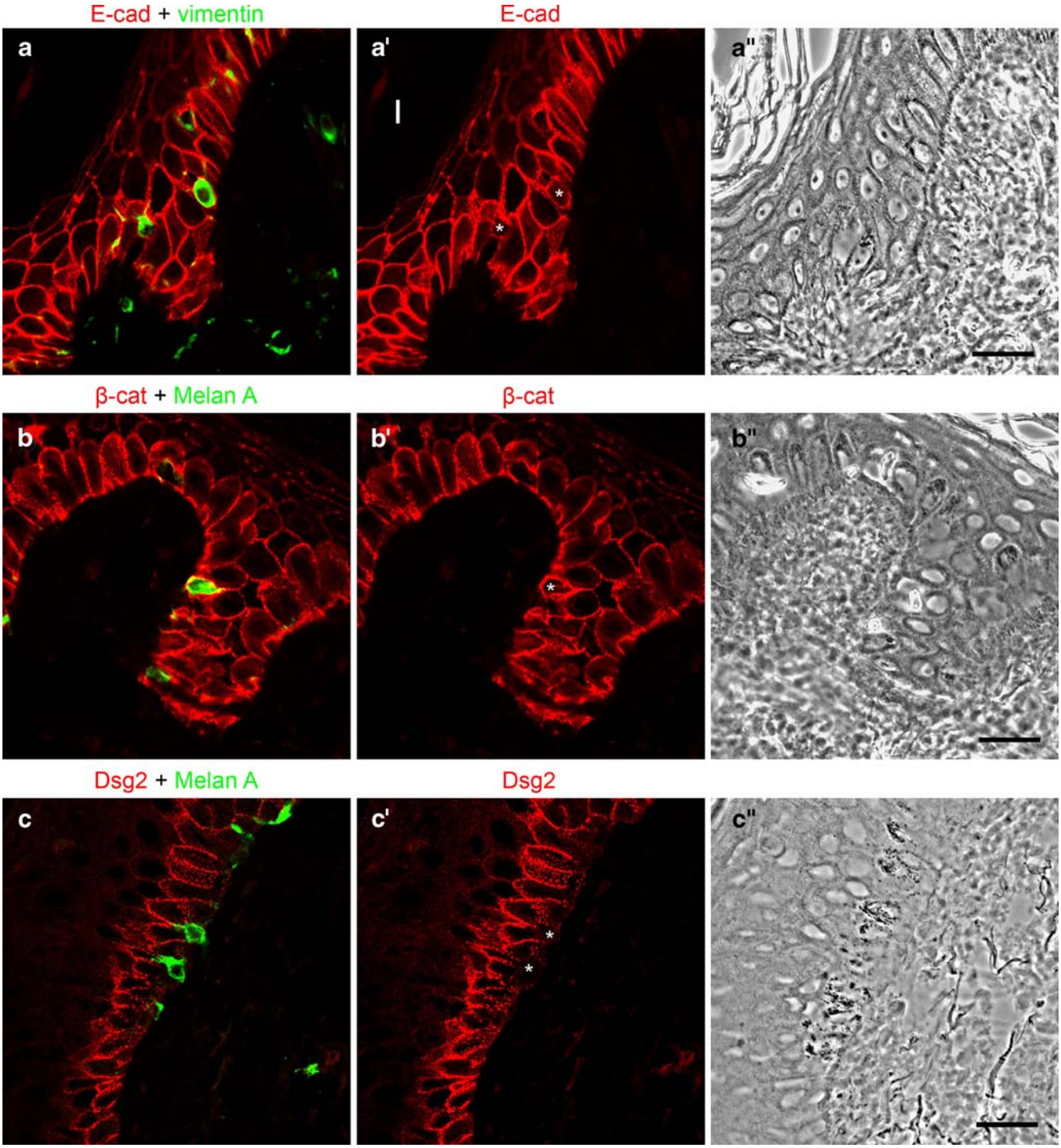


Fig. 1 Double-label confocal microscopy of sections through paraffin-embedded human epidermis, showing cell adhesion proteins at heterotypic melanocyte-keratinocyte contacts. Sections were double-immunostained with antibodies to E-cadherin (*red* in **a**, **a′**), β-catenin (*red* in **b**, **b′**), or Dsg2 (*red* in **c**, **c′**) in combination with antibodies to vimentin (*green* in **a**) or Melan A (*green* in **b**, **c**) as markers for melanocytes. E-cadherin and β-catenin are enriched at intercellular junctions between keratinocytes throughout the epidermis and also at the borders between melanocytes and keratinocytes in the basal layer (**a′**, **b′**, *stars*). By contrast, Dsg2 is found at the desmosomes of the basal keratinocytes but is absent from melanocytes and from melanocyte-keratinocyte contacts (**c′**, *stars*). **a″–c″** Phase-contrast images. *Bars* 20 μm

On immunoblotting with antibodies to desmosomal proteins, Dsg2 was seen in both the MeWo and the NHEM-f cells (Fig. 3). By contrast, none of the other desmosomal cadherins or plaque proteins was detected in these experiments, with the exception of small amounts of plakoglobin, a protein known to occur in both adherens junctions and desmosomes (Table 1; Cowin et al. 1986). Thus, we concluded that, as in certain melanoma cell lines (Schmitt et al. 2007), Dsg2 also often occurs in considerable amounts in proliferative human melanocytes, obviously as a solitary plasma membrane glycoprotein, without any of the other known desmosome-specific components.

To clarify whether other types of melanocytes also contained Dsg2, immunoblotting was performed with total cell lysates of cultured melanocytes derived from adult skin (NHEM-a). Indeed, these cells were also Dsg2-positive, as

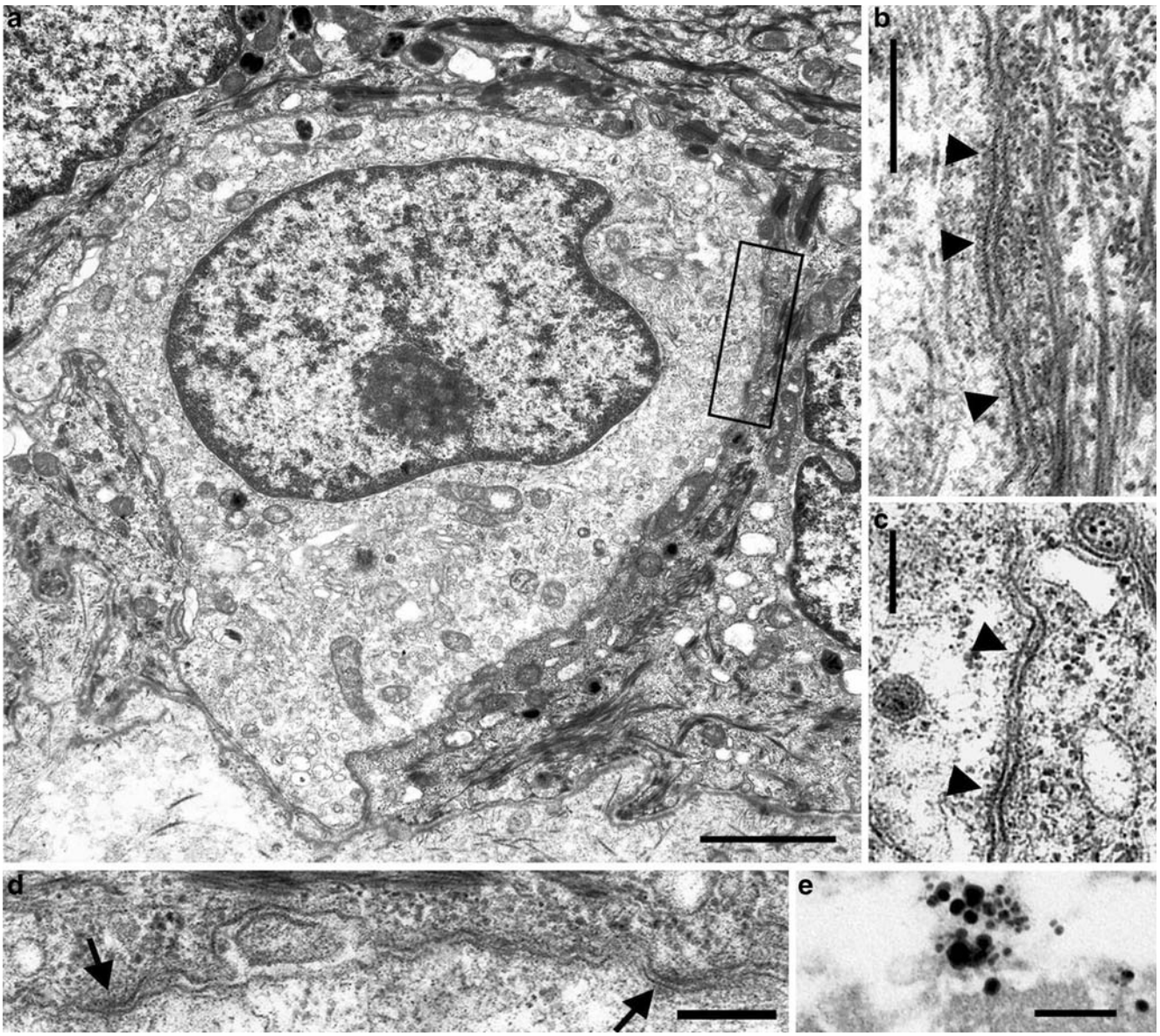


Fig. 2 Electron and immunoelectron microscopy showing adhesions between keratinocytes and melanocytes in the basal human epidermis. **a** Survey of a melanocyte surrounded by keratinocytes in the basal epidermal layer. **b–d** Conventional electron micrographs of cell-cell contacts between melanocytes and keratinocytes. Both cell types are connected by small, plaque-bearing junctions reminiscent of *puncta adhaerentia* (*arrows* in **d**). **d** Higher magnification of the *boxed region* in **a**). In regions devoid of such *puncta*, the plasma membranes of melanocytes and keratinocytes are often also closely aligned over long distances, with a narrow intercellular space (**b**, **c**, *arrowheads*). **e** Immunoelectron microscopy of melanocyte-keratinocyte contacts, demonstrating that β-catenin accumulates at the plaques of *puncta adhaerentia*. *Bars* 2 μm (**a**), 0.25 μm (**b–d**), 0.125 μm (**e**)

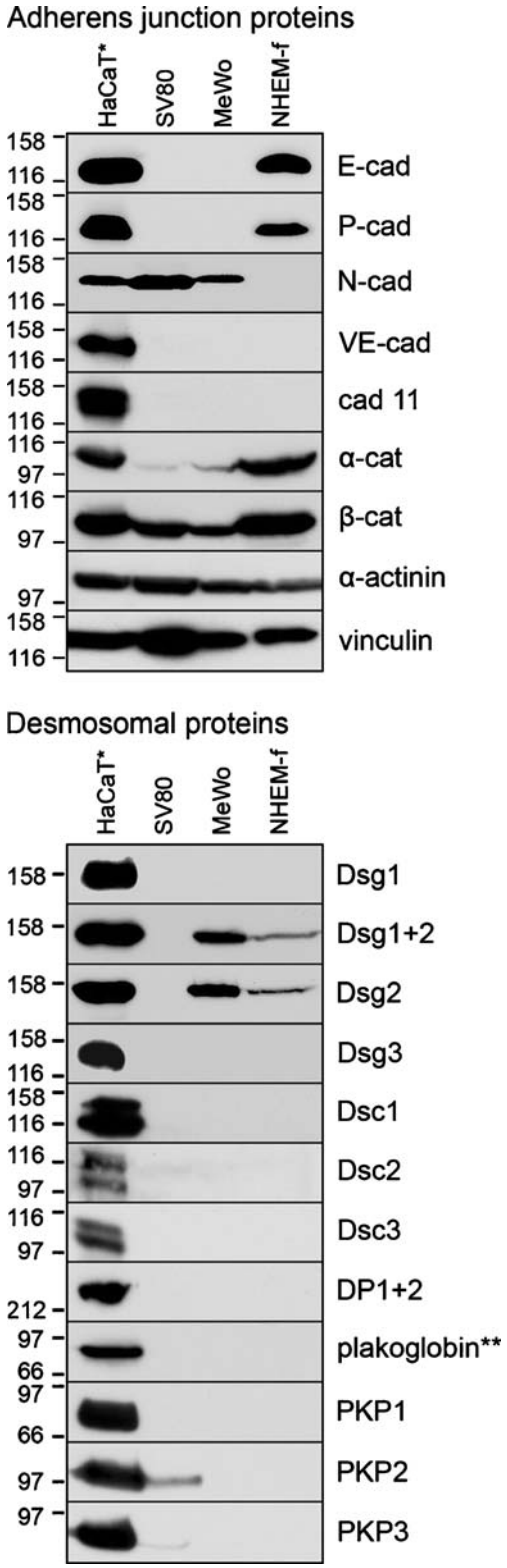


Fig. 3 Immunoblot detection of adherens-junction-associated and desmosomal proteins in cultured human melanocytes. Equal amounts of total proteins from human HaCaT keratinocytes (*HaCaT*), SV80 fibroblasts (*SV80*), melanoma cells of the line MeWo (*MeWo*), and normal human epidermal melanocytes from foreskin (*NHEM-f*) were applied to SDS-polyacrylamide gels, and the separated polypeptides were probed with antibodies against classical cadherins (E-, P-, N- and VE-cadherin, and cadherin 11 [*cad* cadherin]), plaque proteins of adherens junctions (α- and β-catenin [*cat* catenin], α-actinin, and vinculin), desmosomal cadherins (Dsg1-3 and Dsc1-3 [*Dsg* desmoglein, *Dsc* desmocollin]) and desmosomal plaque proteins (DP1 and 2 [*DP* desmoplakin], plakoglobin, and PKP1-3 [*PKP* plakophilin]). NHEM-f cells contain E- and P-cadherin and the plaque proteins typical of adherens junctions. Interestingly, these cells also synthesize the desmosomal cadherin Dsg2, whereas all other desmosomal proteins are absent. This finding has been confirmed by immunoblotting with two different Dsg2 antibodies, one recognizing both Dsg1 and Dsg2 (clone DG3.10) and one specific for Dsg2 (clone 6D8). *For the immunoblot identification of VE-cadherin, whole cell lysates of human umbilical vein endothelial cells (HUVEC) were loaded as a positive control, instead of HaCaT cells. For the detection of cadherin 11, total proteins from astrocytic glioma cells were applied, and for the detection of Dsg1 and Dsc1-3, total proteins of human epidermis were loaded. **After prolonged exposure, trace amounts of plakoglobin are detectable in SV80 and MeWo and in NHEM-f cells

confirmed by immunoblot experiments with two different Dsg2 antibodies (Fig. 4a). Correspondingly, Dsg2 mRNA was demonstrated in NHEM-f and NHEM-a cells (Fig. 4b), and sequencing of the PCR products showed full identity with the human Dsg2 sequence (Schaefer et al. 1994). As another group had reported the occurrence of Dsg1 in melanocytes and melanoma cells (Li et al. 2001b; see, however, also Sanders et al. 1999), we also conducted PCR experiments with primers specific for Dsg1 (Fig. 4b). However, amplification of Dsg1 mRNA was observed neither in NHEM-f nor in NHEM-a cells, in agreement with our immunoblot results (for a discussion, see Schmitt et al. 2007).

To identify possible interaction partners of Dsg2, extracts of NHEM-f cells were subjected to immunoprecipitation, followed by SDS-PAGE, Coomassie Blue staining, and MALDI-TOF analyses of the specifically enriched proteins. Following immunoprecipitation with Dsg2 antibodies, three bands were observed at about 212, 160, and 42 kDa, which corresponded to non-muscle myosin heavy chain, Dsg2, and actin (Fig. 5a). For comparison, immunoprecipitation was performed with β-catenin antibodies, revealing bands at ~130, ~100, ~90 and 42 kDa, which represented E- and P-cadherin, α- and β-catenin, and actin (Fig. 5b). When polypeptides of β-catenin immunoprecipitates were reacted with antibodies to Dsg2, no significant amounts of co-precipitation products were seen (Fig. 5c). Vice versa, neither α- or β-catenin (Fig. 5c′) nor E- and P-cadherin (not shown) were enriched in Dsg2 immunoprecipitates. By contrast, when the material precipitated with Dsg2 antibodies was probed for plakoglobin, specific enrichment was noted; however, the plakoglobin-specific band seen after the immunoblot reaction was usually weak (data not shown). As Dsg2 is normally linked to intermediate filaments, Dsg2 immunoprecipitates were also ana-

Table 1 Cell-adhering junction molecules in cell-cell contacts of cultures of human melanocytes (*NHEM-f*), as determined by SDS-polyacrylamide gel immunoblotting (*cad* cadherin, *Cat* catenin, *Dsg* desmoglein, *Dsc* desmocollin, *PKP* plakophilin, + presence of tested molecule, − absence of tested molecule, *(+)* small amounts of plakoglobin were detectable after prolonged exposure of the X-ray film)

Proteins and glycoproteins	NHEM-f
Transmembrane molecules	
N-cad	−
E-cad	+
P-cad	+
VE-cad	−
cad-11	−
Dsg1	−
Dsg2	+
Dsg3	−
Dsc1	−
Dsc2	−
Dsc3	−
Plaque proteins	
α-cat	+
β-cat	+
Plakoglobin	(+)
Protein p120^{ctn}	+
Vinculin	+
α-Actinin	+
Drebrin[a]	+
Protein ZO-1	+
Protein ZO-2	+
Neurojungin[b]	−
PKP1	−
PKP2	−
PKP3	−
Desmoplakin 1	−
Desmoplakin 2	−

[a] For details on adherens junction associations of the actin-binding protein drebrin, see Peitsch et al. 1999, 2005)

[b] Neurojungin is an arm-repeat protein previously described as a constituent of heterotypic adhering junctions at the outer limiting zone of the retina (Paffenholz et al. 1999) and has also been found in one of our Dsg2-positive melanoma cell lines (MeWo cells; Schmitt et al. 2007)

lyzed for vimentin, and indeed, this protein showed co-precipitation with Dsg2 (Fig. 5c″).

Further to characterize the cadherin complexes present, NHEM-f cell lysates were fractionated by sucrose gradient centrifugation. Immunoblot analyses then showed a peak of E-cadherin-, P-cadherin-, and β-catenin-containing material in F5–F8, with a maximum in F6, corresponding to complexes with a mean S value of ~8 (Fig. 6a). When F5–F7 were pooled and subjected to immunoprecipitation with E-cadherin antibodies, E-cadherin, P-cadherin, and β-catenin were specifically enriched, indicative of heterotypic complexes of E- and P-cadherin (Fig. 6b). By contrast, Dsg2 was observed in gradient F3–F6, corresponding to a monomeric form, but also in ~13S-complexes in F10 and F11 (Fig. 6a). Actin was revealed mostly in monomers and in small complexes, but a portion did co-distribute with Dsg2 in F10 and F11 (Fig. 6a). Taken together with the immunoprecipitation results, these observations indicate that NHEM-f cells contain hetero-complexes of E- and P-cadherin, associated with the plaque proteins of adherens junctions and actin. In addition, they seem to contain two sets of Dsg2 complexes: one in which Dsg2 is linked to vimentin filaments, and another presenting Dsg2 together with actin and non-muscle myosin.

Localization of cadherins in NHEM-f monocultures and NHEM-f-HaCaT co-cultures

The subcellular distribution of cadherins in cultures of NHEM-f cells and in NHEM-f-keratinocyte co-cultures was studied by immunostaining, confocal laser scanning, and immunoelectron microscopy. In NHEM-f monocultures, E- and P-cadherin and the typical plaque proteins of *puncta adhaerentia* appeared (as expected) predominantly at intercellular junctions, but with relatively lower intensity; such immunoreactions were also seen on free plasma membrane regions (not shown).

To specify the localization of Dsg2, NHEM-f cells were transfected with a eukaryotic expression vector containing the full-length human Dsg2 cDNA, followed by Dsg2 immunostaining and confocal microscopy. Indeed, in the transfected cells, the protein was enriched at the cell periphery, both at cell-cell contacts and along free cell margins (Fig. 7a), reminiscent of the localization seen in the Dsg2-positive melanoma cell lines (Schmitt et al. 2007).

When co-cultures of NHEM-f melanocytes and HaCaT keratinocytes were analyzed, an enrichment of E-cadherin (Fig. 7b,b′) and β-catenin (Fig. 7c,c′) not only at homotypic keratinocyte and homotypic melanocyte junctions, but also at heterotypic contacts between NHEM-f and HaCaT cells was seen. To study the intercellular adhesion sites of cultured melanocytes in greater detail, we also examined these homo- and heterotypic cell cultures by electron and immunoelectron microscopy. In monocultures of NHEM-f melanocytes, electron micrographs revealed numerous plaque-bearing junctions of the *punctum adhaerens* type (Fig. 8a–d, arrows). By immunoelectron microscopy, E-cadherin (e.g., Fig. 8e) and β-catenin (Fig. 8f,g) were seen at both kinds of cell-cell contacts, i.e., at adherens junctions with plaques and at the extended adhesive associations without noticeable plaques.

When co-cultures of NHEM-f and HaCaT cells were studied by electron microscopy, small heterotypic cell adhesions were observed that revealed thin dense plaques

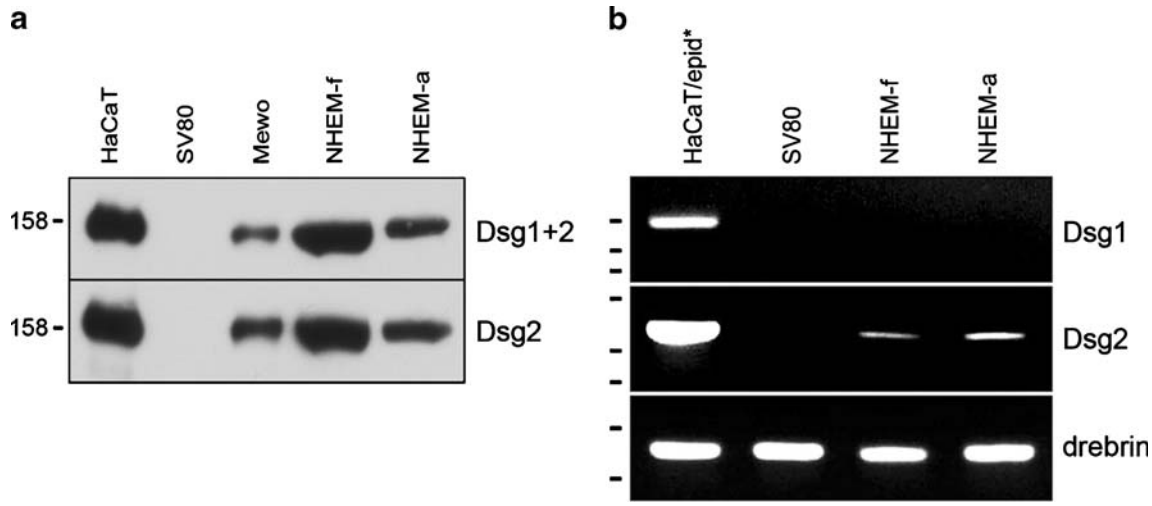


Fig. 4 Immunoblot and polymerase chain reaction (PCR) identification of Dsg2 in cultured human melanocytes. **a** Immunoblot analysis of total protein lysates from HaCaT keratinocytes (*HaCaT*), SV80 fibroblasts (*SV80*), MeWo melanoma cells (*MeWo*), and normal human epidermal melanocytes from newborn foreskin (*NHEM-f*) and from adult skin (*NHEM-a*). An intense Dsg2-specific band is detected in MeWo cells and in NHEM-f and NHEM-a cells, both with mab DG3.10 and mab 6D8. **b** PCR analysis with primers specific for Dsg1 and 2 and for the near-ubiquitous actin-binding protein drebrin (Peitsch et al. 1999, 2001, 2005). Dsg2 mRNA is seen both in NHEM-f and NHEM-a cells, whereas Dsg1 mRNA is not. *Human split skin was employed as a positive control for the Dsg1 PCR, HaCaT keratinocytes served as positive control for the Dsg2 PCR, and SV80 fibroblasts were used as a negative control. Size marker bars (*left*): 517, 396, and 356 bp (Dsg1 and Dsg2 PCR) or 356 and 247 bp (drebrin PCR)

on either side (Fig. 9a–e), reminiscent of the *puncta adhaerentia* connecting melanocytes and keratinocytes in human epidermis in situ. Again, immunoelectron microscopy demonstrated enrichment of the junctional markers at these sites (data not shown).

Cadherin patterns in tissue microarrays of primary melanomas and nevi

As Dsg2 was detected in melanocytes growing in cell culture but not in those residing in situ in the basal epidermis, we hypothesized that its advent and continual synthesis might be correlated with proliferation. To examine this, tissue microarrays comprising 56 primary melanomas and 24 nevi were immunolabeled with antibodies to Dsg2 in combination with vimentin antibodies for the unequivocal identification of the tumor cells (Fig. 10a,a′, Table 2; a survey presenting all immunostaining results in detail is given in Table S1 as Supplementary Material). Indeed, Dsg2-positive reactions were observed in seven primary melanomas (13.5%) and seven nevi (30.4%). In the Dsg2-positive tumor cells, the protein was again seen at cell-cell contacts. Remarkably, however, the staining patterns within the tumors were heterogeneous, with Dsg2-positive tumor cell clusters next to Dsg2-negative cell groups, and the percentage of Dsg2-containing tumor cells ranged from ~10% to 100% (Table 2).

In parallel, the tissue microarrays were immunolabeled for N-, E-, and P-cadherin in combination with vimentin (Fig. 10b–d, Tables 2, S1). Most of the primary melanomas (68.5%) and nevi (90.9%) contained N-cadherin accumulated at cell-cell boundaries (Fig. 10b,b′, Table 2). In another subtype of such tumors, N-cadherin-positive reactions seemed to occur exclusively in the cytoplasm, an observation difficult to explain on a cell biological basis. Here, however, the specificity and significance of the reaction sites remain to be determined.

A remarkably large group of primary melanomas (65.5%) and nevi (62.5%) was also positive for E-cadherin (see, e.g., Fig. 10c,c′, Table 2), whereas a smaller subset synthesized P-cadherin (27.8% of the primary melanomas and 37.5% of the nevi; Fig. 10d,d′). As noted for Dsg2 (Table 2), E- and P-cadherin often showed strikingly heterogeneous reaction patterns, in that E-cadherin- and P-cadherin-positive and -negative groups of tumor cells occurred next to each other (Table 2; for an example of P-cadherin, see Fig. 10d,d′). Within nevi, the E-cadherin-positive immunoreactions often decreased from epidermal to deeper dermal melanocyte nests. Interestingly, both E- and P-cadherin could occur in the same tumors as N-cadherin, and in a certain subset of primary melanomas, all three classical cadherins were even seen to occur simultaneously (6 of 56, 16.1%), without or with Dsg2 (3 of 56, 5.4%). By contrast, another relatively rare subtype was negative for all of the four cadherins tested (7 of 56, 12.5%; Table S1). In the 24 nevi examined, all three classical cadherins together were found in five tumors (20.8%); two of them were also positive for

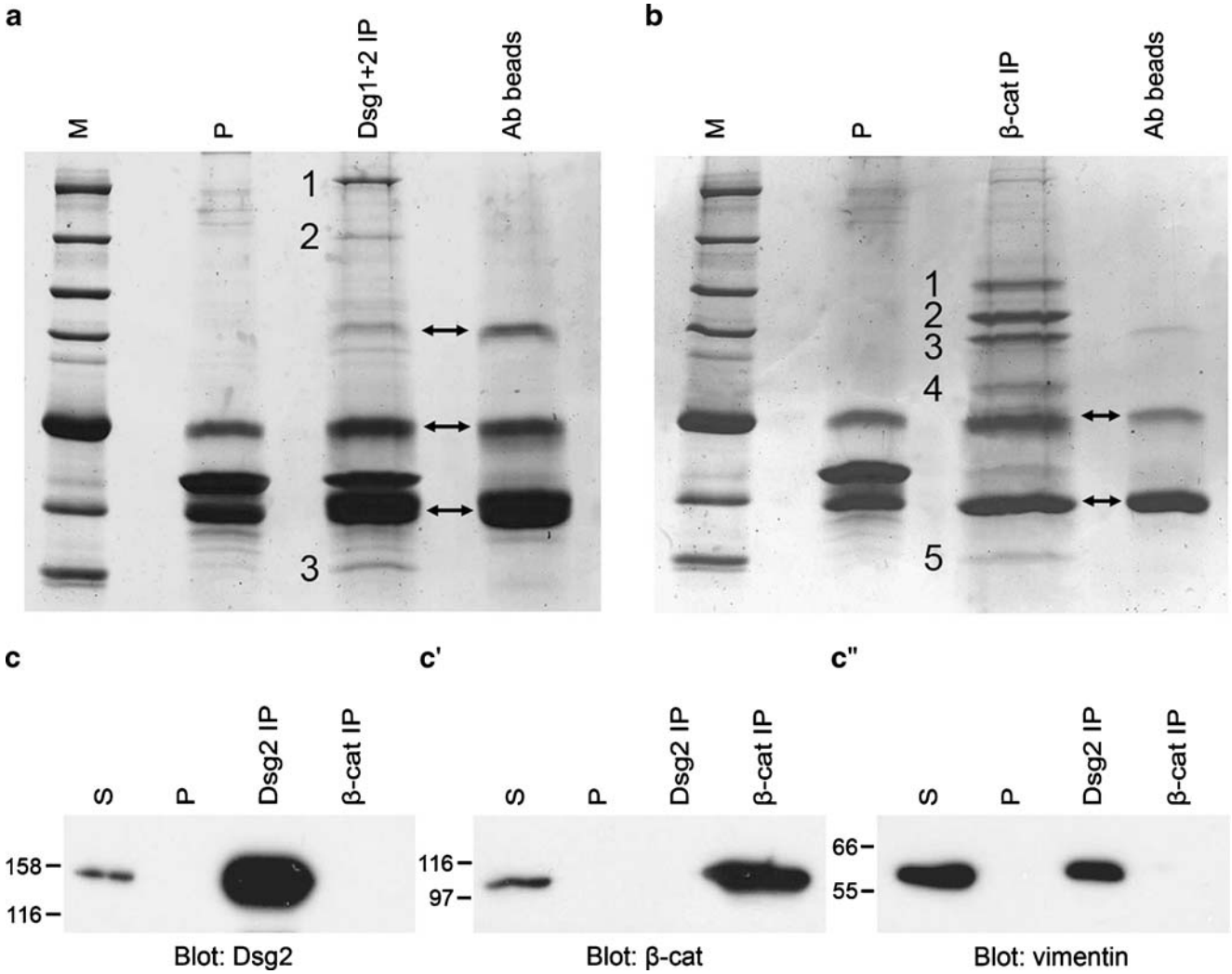


Fig. 5 Immunoprecipitation and MALDI-TOF analyses, showing cadherin complexes in NHEM-f melanocytes. Proteins immunoprecipitated from NHEM-f lysates with antibodies specific for Dsg2 (**a**) or for β-catenin (**b**) were separated on 8% acrylamide gels and stained with Coomassie Blue. In the Dsg2 immunoprecipitate (**a**), a band of ~160 kDa was seen (*band 2*), which was identified as Dsg2 by MALDI-TOF analysis. Further bands at ~212 kDa (*band 1*) and at ~42 kDa (*band 3*) represent non-muscle myosin and actin. In the β-catenin immunoprecipitate (**b**), *band 1* at ~130 kDa includes both E-cadherin and P-cadherin, with further bands containing α-catenin (*band 2*), β-catenin (*band 3*), bovine serum albumin (BSA; *band 4*), and actin (*band 5*). *Arrows* heavy chains of immunoglobulins and BSA (~66 kDa), *P* material of the preclearing step, *IP* immunoprecipitate, *Ab beads* Dynabeads loaded with primary antibodies, *M* molecular weight markers denoting (*top to bottom*): 212, 158, 116, 97, 66, 55, and 42 kDa. **c–c″** Immunoprecipitates (*IP*) from NHEM-f cells, obtained with antibodies to Dsg2 and to β-catenin and immunoblotted for Dsg2 (**c**), β-catenin (**c′**) and vimentin (**c″**). Dsg2 and β-catenin do not co-precipitate (**c**, **c′**), whereas vimentin is specifically enriched in the Dsg2 immunoprecipitate (**c″**)

Dsg2 (8.3%; Table S1). Taken together, these results obtained in tissue microarrays show that the cadherin profile can be highly variable not only among different primary melanomas and nevi, but also within the same tumor.

Discussion

Researchers studying the cell and molecular biology of proliferative or even malignantly transformed melanocytes are often confronted with "unusual" gene expression patterns combining, for example, certain epithelial and mesenchymal cell-type marker molecules or subtype patterns of proteins and structures that make it difficult to assign a distinct cell-type character to the specific cell colony or tumor. For instance, certain melanoma cells and proliferatively active melanocytes, although usually subsumed under neuroectoderm-derived mesenchymal-type cells, are able to synthesize a set of molecules that are characteristic of epithelial-type adhering junctions, such as E-cadherin (Tang et al.

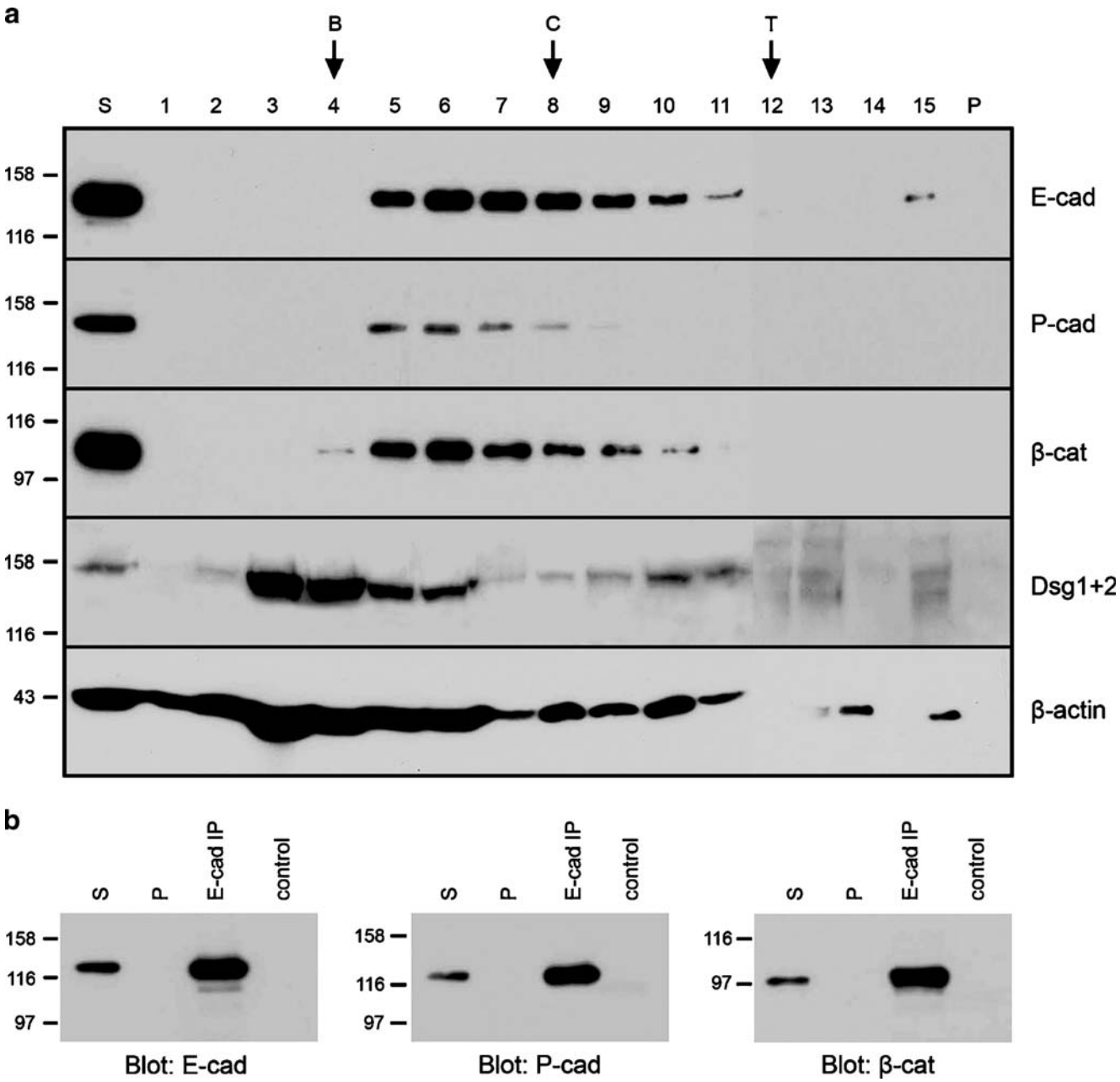


Fig. 6 Sucrose gradient fractionation of molecular complexes present in lysates from cultured melanocytes. **a** Extracts of proteins from cultured human melanocytes (NHEM-f) were centrifuged on linear 5%–30% sucrose gradients, and the fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to E-cadherin, P-cadherin, β-catenin, Dsg1+2 (clone DG3.10), and β-actin (*S* proteins of the supernatant before fractionation, *P* pellet). E- and P-cadherin are found, together with β-catenin, in fractions (F) 5–8, with a maximum in F6, i.e., in particles of a mean value of ~8S. Dsg2 immunoreactivity is seen in F3-F6, indicative of Dsg2 monomers but, interestingly, also with a peak in F10 and F11, corresponding to ~13S. Actin appears with a broad peak from F2 to F6, suggestive of a monomer, but is also co-distributed with Dsg2 in F10 and F11, i.e., ~13S. References are: BSA (*B*: 4.3S), catalase (*C*: 11.5S), and thyroglobulin (*T*: 16.5S). **b** Sucrose gradient F5–F7 were pooled and immunoprecipitated with antibodies to E-cadherin and to the tight junction protein occludin as a control. Note that both P-cadherin and β-catenin are specifically enriched in E-cadherin immunoprecipitates (*S* supernatant of the pooled F5–F7, *P* material of the preclearing step, *IP* immunoprecipitate)

1994; Hsu et al. 2000a; Li et al. 2001a) and Dsg2 (Schmitt et al. 2007). Furthermore, melanocytes and melanoma cells often form heterotypic adhering junctions with keratinocytes or with other epithelial and non-epithelial cells, and some are even able to adhere intimately to each other or to other kinds of cells over extended plasma membrane associations without distinct junctional structures.

Desmoglein-containing adhesive cell-cell alignments of proliferative melanocytes and melanoma cells

We have recently reported that certain melanoma cell culture lines regularly synthesize the desmosomal cadherin Dsg2, which hitherto had been assumed to be absent from melanocytes and melanomas (Schmitt et al. 2007). Remarkably, however, we have now detected Dsg2 as a major and frequent cadherin also in normal, i.e., not malignantly transformed, cultured melanocytes. Like the Dsg2-positive melanoma cell lines (Schmitt et al. 2007), the cultured melanocytes contain no other typical desmosomal proteins, except for occasional small amounts of plakoglobin, in general a widespread junctional plaque protein of both desmosomes and adherens junctions (Cowin et al. 1986).

Desmosomal cadherins, i.e., members of the desmoglein and desmocollin subfamilies of cadherin glycoproteins, have so far been identified only in adhering junction structures such as in desmosomes (Koch et al. 1990, 1991, 1992; Buxton et al. 1993; Godsel et al. 2004; Yin and Green 2004) and in the composite junctions (*areae compositae*) of mammalian heart muscle cells (Franke et al. 2007; Pieperhoff et al. 2008) or as integral molecules of "half-desmosomes". The latter have been described in processes of desmosome formation such as exocytosis, or in Ca^{2+}-deficiency-induced junctional splitting and in endocytic vesicle uptake (Cowin et al. 1984; Duden and Franke 1988; Demlehner et al. 1995; Schaefer et al. 1996).

The Dsg2 molecules that we have observed as frequent cell surface components in proliferative melanocytes and in a certain category of melanoma cells are evenly dispersed and do not seem to assemble into distinct adhering junctional structures. Nevertheless, they often appear to be intimately aligned with cell proteins on the surface of a neighboring cell, thus forming a novel homogeneous kind of cell-cell-adhesive association, which often extends over large areas. The importance of these solitary surface-exposed Dsg2 molecules, which are not co-assembled with any other detectable desmosome-specific component or integrated into any distinct junction structures, for cell-cell associations in normal and in pathologically altered tissues, notably in metastatic processes, remains to be determined.

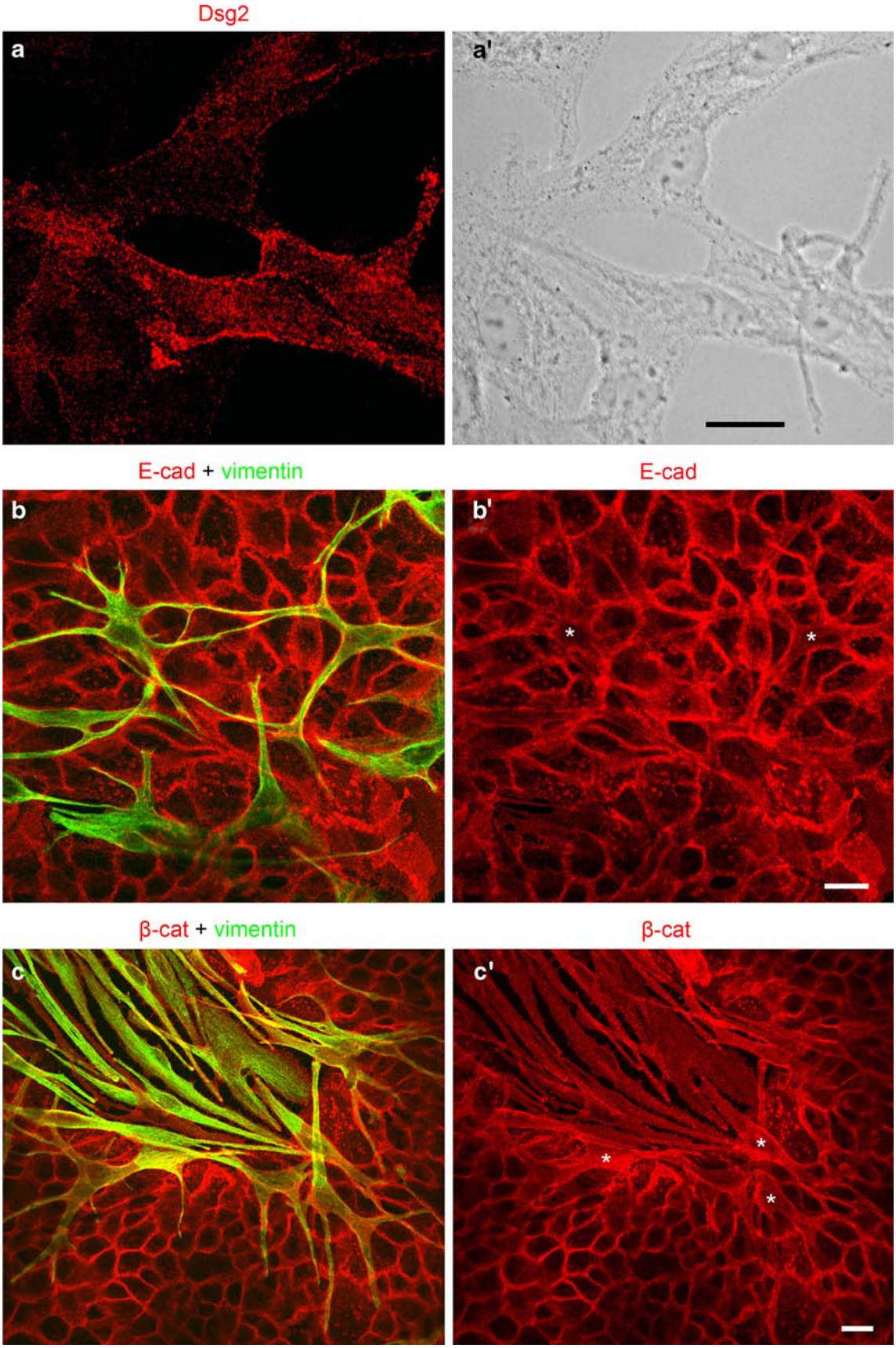
The Dsg2-presenting melanocytes and melanoma cell subtypes described in this and in our previous report (Schmitt et al. 2007) are not the only cells found to synthesize this desmosomal glycoprotein as a solitary molecule and to expose it over large areas of the cell surface. A subline of human fibrosarcoma cells has also been reported to synthesize Dsg2 continuously and to export this protein to the cell surface where it is seen in relatively large regions (Chitaev and Troyanovsky 1997). Only upon the addition of further desmosomal components by injection or cDNA transfection does an organized co-assembly of this pre-existing Dsg2 with the other partners into desmosome-like junctions take place (Koeser et al. 2003).

Obviously, the cell-cell trans-interactions between proliferative melanocytes or melanoma cells with adjacent host tissue cells, e.g., keratinocytes in the case of the epidermis, are stable enough to maintain their direct cross-talk in the architectonic context of the specific tissue or tumor. Indeed, in view of the surprisingly strong adhesive trans-interaction forces of individual desmosomal cadherins (Troyanovsky 2005), these numerous and rather widely spread Dsg2 molecules may be essentially involved in cell-cell recognition and attachment processes, thus also representing a significant factor in the metastatic process. Clearly, the cell-cell interaction strengths of such non-junction-integrated cadherin molecules will have to be experimentally determined in the future. We also propose to consider this and other types of cell-cell attachment forms in diagnoses of melanomas and other melanocyte-related disorders.

E- and P-cadherin heterodimers in melanocyte cultures

Our biochemical analyses of detergent-solubilized cadherins from cultured melanocytes cells have revealed heterotypic complexes of E- and P-cadherin, reminiscent of our previous studies on cultured melanoma cell lines (Schmitt et al. 2007), which have indicated the existence of such E-P-cadherin hetero-complexes. Whereas the binding specificities of cadherins have been traditionally considered as homotypic, it has recently become evident that their interactions can be more promiscuous (Volk et al. 1987; Shan et al. 2000; Shimoyama et al. 2000; Omelchenko et al. 2001; Duguay et al. 2003; Patel et al. 2003; Foty and Steinberg 2005). Specifically, heterodimers of E- and P-cadherin have been identified in cultures of human carcinoma cells of line A431 (Klingelhoefer et al. 2000).

When interacting in cell cultures, cadherins can form both cis-dimers, i.e., lateral dimers in adhering junctions of the same cell (Shan et al. 2000), or trans-dimers between two adjacent cells (Duguay et al. 2003). Obviously, heterotypic trans-cellular cadherin interactions are more frequent than previously thought (Shimoyama et al. 2000; Omelchenko et al. 2001; Duguay et al. 2003; Patel et al. 2003). The heterodimers of E- and P-cadherin described to predominate in cultures of A431 cells are of the cis-type (Klingelhoefer et al. 2000). On the other hand, many of the E- and P-cadherin molecules introduced into fibroblasts (L-cells) by cDNA transfections seem to form "trans E-P-hetero-cadherin" complexes between adjacent cells (Duguay et al. 2003; Foty and Steinberg 2005). Such trans-cellular hetero-cadherin complexes appear to be of a similar strength as the corresponding trans-cellular homo-cadherin complexes. Whether the immunoprecipitable


Dsg2
a
a'
E-cad + vimentin
E-cad
b
b'
β-cat + vimentin
β-cat
c
c'

◀**Fig. 7** Localization of cell junction proteins in NHEM-f monocultures and in keratinocyte-melanocyte co-cultures. **a**, **a′** NHEM-f cells were transfected with a eukaryotic expression vector containing human Dsg2 cDNA and immunoreacted with Dsg2 antibodies (clone DG3.10). Note Dsg2 accumulations at the contact sites between two melanocytes and at the free cellular margins. **b–c′** Co-cultures of NHEM-f and HaCaT keratinocytes labeled for E-cadherin (*red* in **b**, **b′**) or β-catenin (*red* in **c**, **c′**) in combination with vimentin as a melanocyte marker (*green* in **b**, **c**): E-cadherin and β-catenin are seen at homotypic plasma membrane adhesion sites between melanocytes, on the one hand, and keratinocytes, on the other, but also at heterotypic contacts between the two cell types (*stars* in **b**, **c**). *Bars* 20 μm

complexes of E- and P-cadherin found in the melanocytes and the melanoma cell cultures in the present and the preceding study (Schmitt et al. 2007) are of the cis- or of the trans-type remains to be examined. Moreover, these complexes will have to be further characterized with respect to their adhesive strength.

Heterotypic adhering junctions between melanocytic cells and keratinocytes

Melanocytes are known for their frequent (often obligatory) heterotypic cell-cell junctions. It is thus all the more surprising to note that the ultrastructure of such heterotypic adhering junctions has not yet been clarified, even for the abundant melanocyte-keratinocyte junctions, other than the repeated statements that they do not include desmosomes (Breathnach 1974; Montagna and Parakkal 1974; for the related problem of the association, often via invaginations, with keratinocytes and melanin transfer between these cells see, e.g., Jimbow et al. 1986).

In this study, we have presented, for the first time, the ultrastructure of the heterotypic adhering junctions connecting normal melanocytes and keratinocytes in the epidermis. Both in the human epidermis in situ and in co-cultures, melanocytes and keratinocytes are connected by small plaque-bearing structures that contain the protein and glycoprotein ensemble typical of adherens junctions; thus, in molecular terms, they represent typical *puncta adhaerentia* as known from a wide range of other cells (see, e.g., Wuchter et al. 2007 and references cited therein). In *puncta adhaerentia*, one or two of the classical cadherins can usually be identified that, on the cytoplasmic side, insert into a thin and indistinct coat formed by plaque proteins including α- and β-catenin, protein $p120^{ctn}$, plakoglobin, and (depending on the specific cell type) a few other, mostly actin-binding proteins. Morphologically, these "mini-junctions" are relatively inconspicuous and only sometimes can be demonstrated to anchor filament bundles on one or both cytoplasmic plaques. In the *puncta*-type adherens junctions of diverse subtypes of melanoma cells, the junctional plaque can exhibit various cadherin patterns, i.e., N-, E-, or P-cadherin or cadherin 11 or combinations of two or three of these cadherins (Schmitt et al. 2007 and references therein). Some melanoma subtypes, notably of uveal origin, have also been reported to contain VE-cadherin (Hendrix et al. 2001, 2003; Seftor et al. 2002; Hess et al. 2006). In addition, we have now made clear that not only melanoma cells (whether grown in situ or in cell culture), but also proliferative melanocytes in culture can synthesize the desmosomal cadherin, Dsg2. However, this protein is, for the most part, not integrated into any particular junction but is dispersed over the cell surface, without any obvious cytoplasmic coat of anchoring proteins.

Our electron-and immunoelectron-microscopic results indicate that the heterotypic *puncta* junctions between keratinocytes and melanocytes are primarily based on complexes between E- and P-cadherin, both synthesized in basal keratinocytes and in melanocytes (Tang et al. 1994; Nishimura et al. 1999). Moreover, we have noticed that, in the cells studied, some cadherins, including melanocytic E-cadherin, are not restricted to *puncta adhaerentia* structures but may also occur at plaque-free plasma membranes, both in tissue-bound and in cultured melanocytes. This indicates that two forms of cadherins should generally be distinguished here: a junction-bound form and a non-junction-bound form. A similar distribution has been reported by some authors for N-cadherin in endothelial cells; here, this cadherin can be enriched at intercellular junctions, apparently often together with VE-cadherin, but may also occur outside of the junctions on the free endothelial surface (for controversial discussions, see, e.g., Salomon et al. 1992; Alexander et al. 1993; Schulze and Firth 1993; Navarro et al. 1998; Jaggi et al. 2002; Luo and Radice 2005; for a review, see Dejana 2004).

Different cadherin profiles in primary melanomas and nevi as determined in tissue microarrys

As we had detected Dsg2 in certain melanomas and in cultured melanocytes but not in melanocytes in situ, we reasoned that its synthesis out of the context with the other desmosomal components might be correlated with cell proliferation or might be induced by some kind of activation characteristic of proliferating melanocytes. The results obtained in tissue microarrays of melanocytic tumors are to a certain degree compatible with this hypothesis. Indeed, Dsg2 has been found in a subset of such tumors, i.e., in 30% of the nevi and in 13% of the primary melanomas, in which it appears enriched at cell-cell contacts, essentially in agreement with a small number of melanoma metastases immunostained for Dsg2 (Schmitt

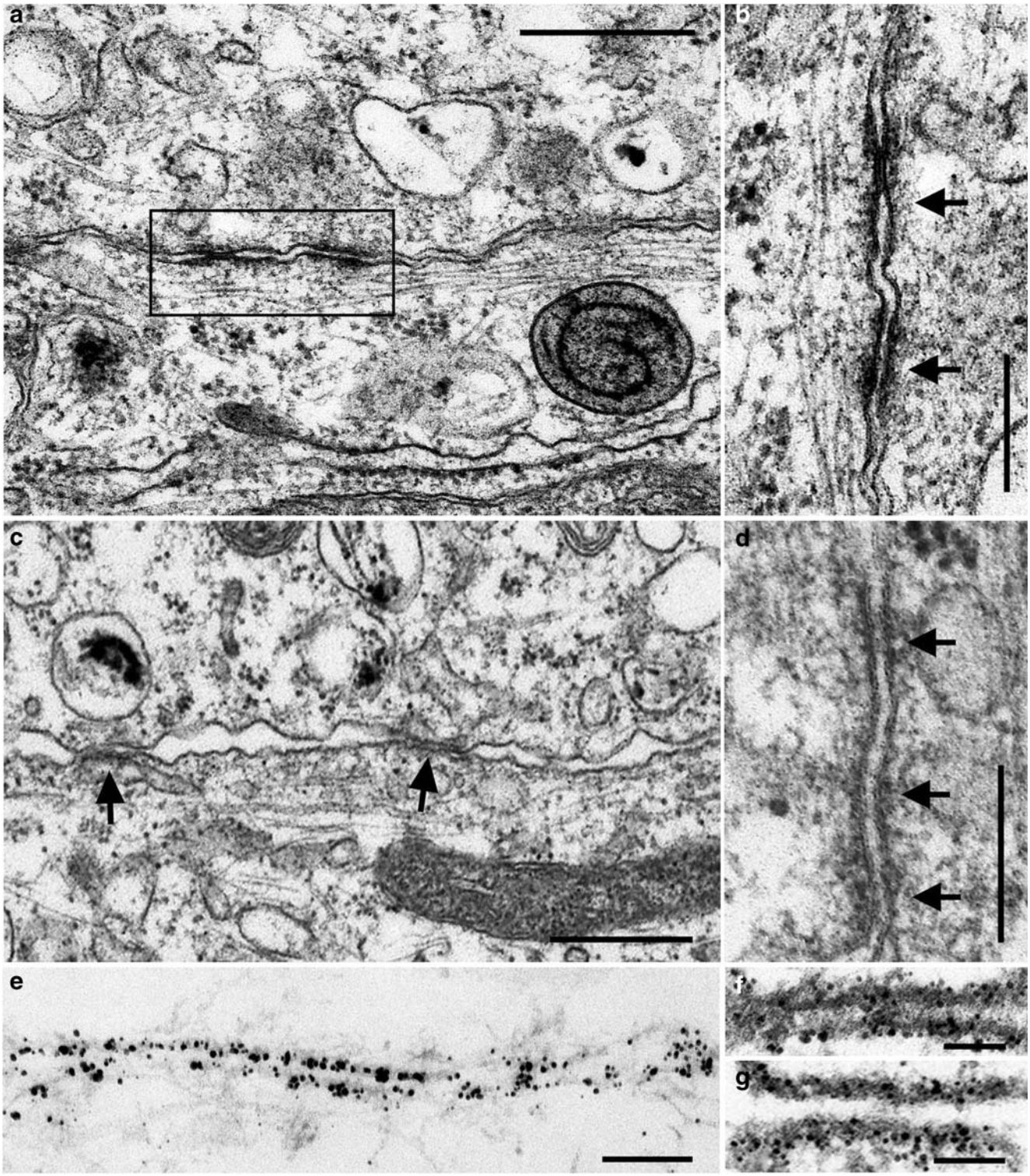


Fig. 8 Electron and immunoelectron microscopy of cultured NHEM-f melanocytes. **a–d** As seen by conventional electron microscopy, closely parallel cell-cell adhesive alignments between two melanocytes often consist of several distinct small plaque-bearing adhering junctions (*arrows* in **b**; **b** presents a higher magnification of the *boxed area* in **a**). Note, at higher magnification (**c**, **d**), the equidistance and parallel character of the extended plasma membrane intercepts, in places frequently revealing *puncta adhaerentia* (*arrows*) coated by a mostly thin, densely stained plaque. **e–g** Immunoelectron microscopy of NHEM-f cells, labeled with antibodies to E-cadherin (**e**) or β-catenin (**f**, **g**). Here, enrichment of β-catenin is observed at the plaques of the *puncta adhaerentia* (**f**, **g**), but both E-cadherin (**e**) and β-catenin (not shown) are also detectable along plaque-free plasma membranes connecting the two cells over long distances, i.e., in a non-junction-bound form. *Bars* 1 μm (**a**), 0.25 μm (**b**, **d**, **e**), 0.5 μm (**c**), 0.125 μm (**f**, **g**)

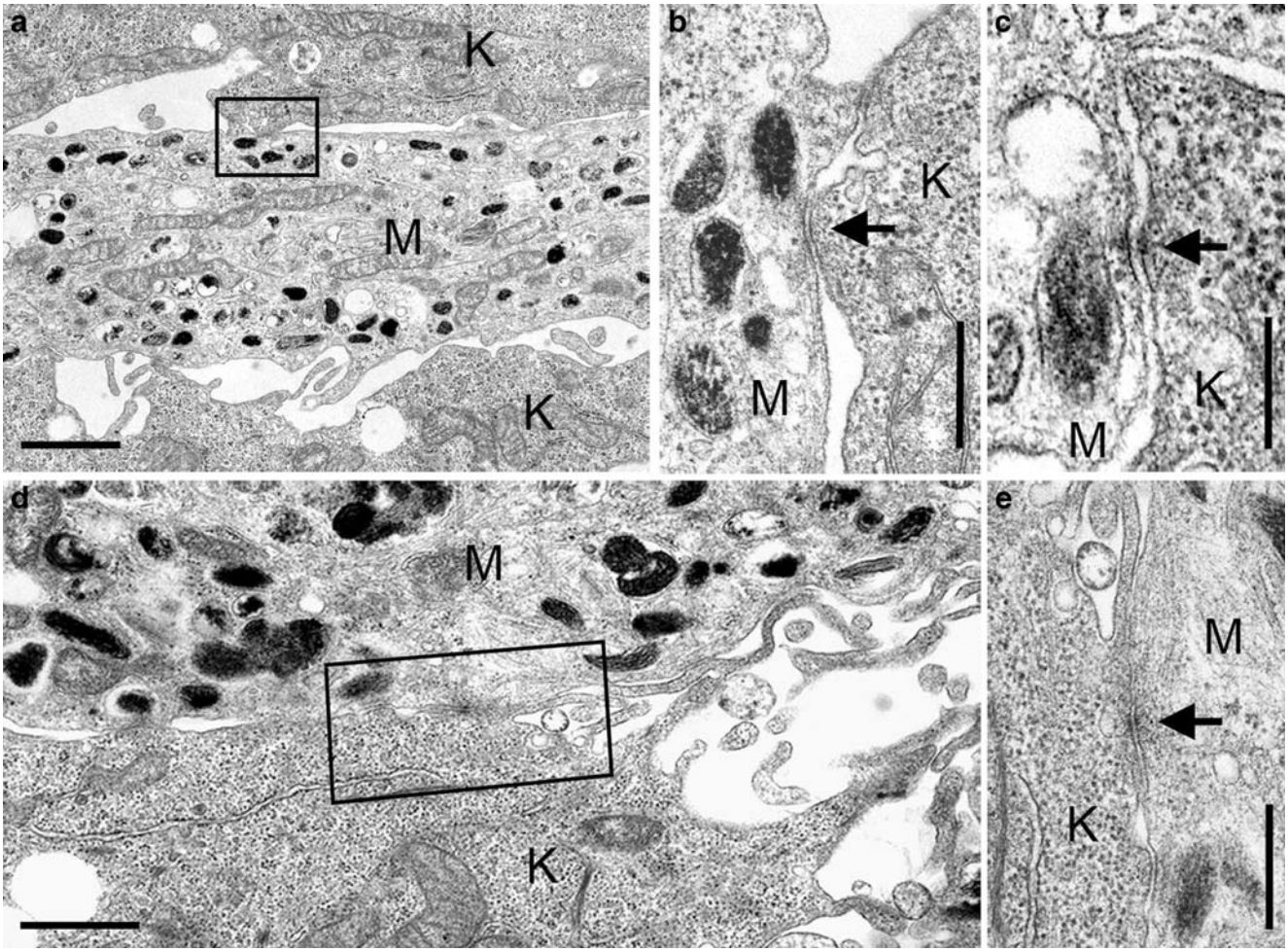
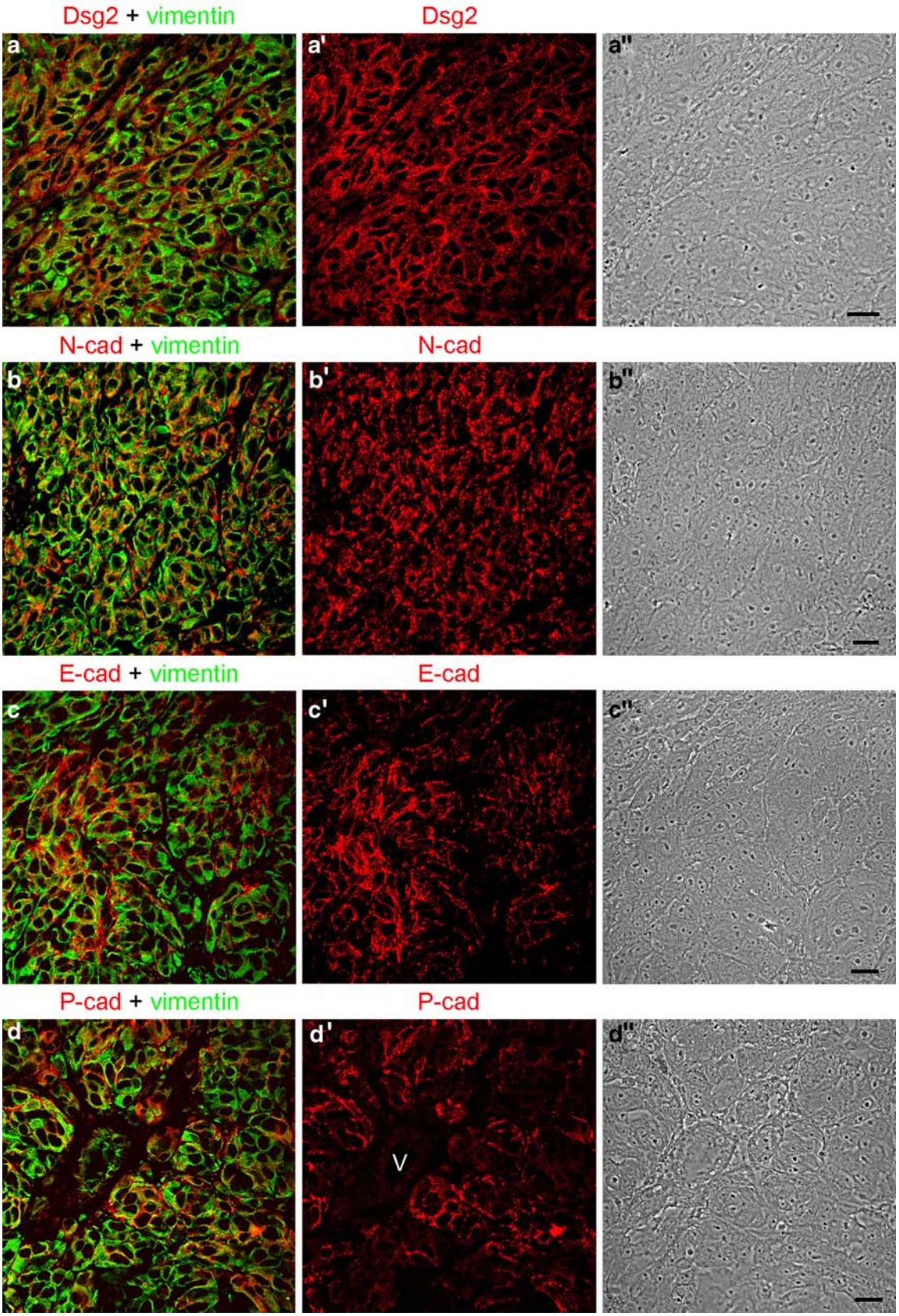


Fig. 9 Electron microscopy, presenting heterotypic cell adhesions in melanocytes (*M*) co-cultured with keratinocytes (*K*). **a**, **d** Survey micrographs of NHEM-f melanocytes co-cultured with HaCaT keratinocytes. Note the numerous cell protrusions in adjacent keratinocytes and melanocytes and the local contacts between the two cell types. **b**, **c**, **e** Details presenting regions of heterotypic cell-cell adhesions: NHEM-f and HaCaT cells are connected by small *puncta adhaerentia*-type junctions (*arrows*). **b** Higher magnification of the *boxed region* in **a**. **e** Detail of the *boxed area* in **d**). *Bars* 1.5 μm (**a**, **d**), 0.5 μm (**b**, **e**), 0.25 μm (**c**)

et al. 2007). This subtype of Dsg2-positive melanocytic tumors will have to be specified in the future. In this context, a minor but especially aggressive subtype of melanomas is characterized by the addition of VE-cadherin to normal cadherin complexes and by the appearance of certain kinases (Hendrix et al. 2001, 2003; Seftor et al. 2002; Hess et al. 2006). Whether the presence of Dsg2 in nevi and melanomas allows any prognostic conclusions will have to be examined in future clinically based studies on larger numbers of samples, in which Dsg2 expression will also have to be correlated with the Breslow and the Clark level of the melanomas.

On the other hand, however, marked and systematic heterogeneity has also been noted for P-cadherin. When the microarrays were labeled with antibodies to this cadherin, about 38% of the nevi and 28% of the primary melanomas exhibited P-cadherin-positive reactions along the cell boundaries. This is in correspondence with observations of membrane P-cadherin staining in benign nevi and in initial melanomas, both correlated with a favourable prognosis (Bachmann et al. 2005; Bauer et al. 2006). In contrast to other authors, however, we have not observed "cytoplasmic" immunoreactions for P-cadherin, probably because of differences of the antibodies employed or the protocols used for antigen retrieval and immunostaining.

Both E- and N-cadherin have been detected in a high percentage of the nevi and melanomas (cf. Tables 2, S1). Often these two cadherins occur together in the same tumor, a finding in accordance with our previous observations of melanoma metastases (Schmitt et al. 2007). Plasma membrane E-cadherin immunostaining has also been noted in a high percentage of advanced primary melanomas and of melanoma metastases by other groups (Silye et al. 1998; Sanders et al. 1999). This appears, at first glance, at variance with the prevailing hypothesis that a switch from E- to N-cadherin is essential for the progression of highly


Dsg2 + vimentin
Dsg2
a
a'
a''
N-cad + vimentin
N-cad
b
b'
b''
E-cad + vimentin
E-cad
c
c'
c''
P-cad + vimentin
P-cad
d
d'
d''
V

◀ **Fig. 10** Cadherin patterns of a primary melanoma, as determined by double-label immunofluorescence confocal microscopy. Tissue microarray samples of a primary melanoma from the left leg (no. 13, cf. Table S1) stained with antibodies to Dsg2 (**a**, **a′**, *red*), N-cadherin (**b**, **b′**, *red*), E-cadherin (**c**, **c′**, *red*), and P-cadherin (**d**, **d′**, *red*), in combination with antibodies to vimentin to identify the tumor cells (*green* in **a–d**). Note that most of the melanoma cells contain Dsg2, N-cadherin, and E-cadherin, all accumulated at the cell periphery. By contrast, P-cadherin-positive reactions were observed only in ~20% of the tumor (**d**, **d′**). A blood vessel (*V*) was P-cadherin-negative (**d′**). **a″–d″** Phase-contrast images. *Bars* 20 μm

malignant melanomas (see Introduction). On the other hand, a possible explanation for this result might be that E-cadherin may only temporarily be down-regulated during certain steps of tumor cell segregation and invasion and then re-expressed in the advanced metastatic tumor.

To our surprise, we have also frequently noted, for E- and P-cadherin and for Dsg2, heterogeneous staining patterns within the same tumor, i.e., groups of tumor cells strongly positive for the specific cadherin next to tumor regions negative for this glycoprotein (cf. Tables 2, S1, Fig. 10d). In nevi labeled for E-cadherin, the immunoreaction often shows a gradual decrease from the epidermal layers to deeper dermal nests, reminiscent of other reports (Krengel et al. 2004). However, in the melanomas of our microarray studies, such gradients have not been observed, possibly because the samples were taken from the very centers of the melanomas. Here, the heterogeneous cadherin patterns suggest that one and the same melanoma can contain multiple small cell colonies with strikingly different adhesion protein profiles. Mosaic patterns of junctional proteins have also been observed for desmosomal cadherins (Kurzen et al. 2003), the desmosomal plaque protein plakophilin 1 (Moll et al. 1997), the adherens junction-associated drebrin (Peitsch et al. 2005), and several tight junction molecules (Langbein et al. 2003) in other kinds of skin tumors. Such regionalization and subtype differences might contribute to chemoresistancy, a notorious problem in the therapy of malignant melanomas. Hence, this is another reason for mentioning, in diagnostic evaluation, the degree of regionalization of cell junctions.

Table 2 Cadherin (*cad*) profiles of primary melanomas and nevi, as determined in tissue microarrays (*Dsg* desmoglein). Microarrays were immunoreacted with antibodies to cadherins, in combination with vimentin, and analyzed by confocal microscopy. The samples were classified according to the specific percentage of cadherin-positive cells (− few or no reactive tumor cells, + 5%–24% reactive cells, ++ 25%–49% reactive cells, +++ 50%–74% reactive cells, ++++ more than 75% reactive cells)

	–	+	++	+++	++++	Total[a]	Total positive[b]
Primary melanomas							
Dsg2	45	0	2	5	0	52	7 (13.5%)
N-cad	17	0	0	1	36	54	37 (68.5%)
E-cad	19	9	2	5	20	55	36 (65.5%)
P-cad	39	4	0	3	8	54	15 (27.8%)
Nevi							
Dsg2	16	1	1	2	3	23	7 (30.4%)
N-cad	3	0	1	0	19	22	20 (90.9%)
E-cad	9	5	3	1	6	24	15 (62.5%)
P-cad	15	3	0	4	2	24	9 (37.5%)

[a] The microarrays comprised 56 primary melanomas and 24 nevi. Some specimens were insufficiently preserved for adequate interpretation

[b] Total number of tumors containing cadherin-reactive cells, independent of their percentage

Conclusions and recommendations

All three forms, proliferative normal melanocytes growing in culture, certain cells of nevi in situ, and malignant melanoma cells, are highly proliferative and markedly heterogeneous with respect to their cell-cell adhesion molecule profiles, their junction assemblies, and in the variety of their surface cluster- and domain-forming regionalization patterns. Our findings reported here have made it clear that such heterogeneity patterns are not restricted to malignant melanomas, but can also be seen in normal, i.e., non-malignant melanocytes and in nevus cells. They also suggest that the emergence and widespread regeneration of heterogeneous cell-cell adhesion structures is a feature intrinsic to the proliferative melanocyte, and not a special feature of melanomas. As such diversities, subtypes, or special regional domains may be of general importance not only for tissue patterning, but also for pathogenic processes (notably in melanoma metastasis formation), we propose to characterize the specific adhesion molecule pattern of a given cell colony or tumor in initial diagnosis, in particular in cases in which this may be relevant for the metastatic process. We also postulate that potent "factors" exist that can interfere with the adhesion of melanocytes or melanocyte precursors with each other or to other kinds of cells, and that such factors obviously play important roles in normal development (Le Douarin 1984; see also Hari et al. 2002) and in melanoma metastasis.

Acknowledgements We thank Martina Schnoelzer and Tore Kempf (Protein Analysis Facility, German Cancer Research Center) for performing the MALDI-TOF analyses.

References

Achtstaetter T, Moll R, Anderson A, Kuhn C, Pitz S, Schwechheimer K, Franke WW (1986) Expression of glial filament protein (GFP) in nerve sheaths and non-neural cells re-examined using monoclonal antibodies, with special emphasis on the co-expression of GFP and cytokeratins in epithelial cells of human salivary gland and pleomorphic adenomas. Differentiation 31:206–227

Aho S, Levänsuo L, Montonen O, Kari C, Rodeck U, Uitto J (2002) Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells. J Cell Sci 115:1391–1402

Alexander JS, Blaschuk OW, Haselton FR (1993) An N-cadherin-like protein contributes to solute barrier maintenance in cultured endothelium. J Cell Physiol 156:610–618

Andersen K, Nesland JM, Holm R, Florenes VA, Fodstad O, Maelandsmo GM (2004) Expression of S100A4 combined with reduced E-cadherin expression predicts patient outcome in malignant melanoma. Mod Pathol 17:990–997

Bachmann IM, Straume O, Puntervoll HE, Kalvenes MB, Akslen LA (2005) Importance of P-cadherin, beta-catenin, and Wnt5a/frizzled for progression of melanocytic tumors and prognosis in cutaneous melanoma. Clin Cancer Res 11:8606–8614

Bauer R, Wild PJ, Meyer S, Bataille F, Pauer A, Klinkhammer-Schalke M, Hofstaedter F, Bosserhoff AK (2006) Prognostic relevance of P-cadherin expression in melanocytic skin tumours analysed by high-throughput tissue microarrays. J Clin Pathol 59:699–705

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 106:761–771

Breathnach AS (1974) An atlas of the ultrastructure of human skin. Churchill, London

Buxton RS, Cowin P, Franke WW, Garrod DR, Green KJ, King IA, Koch PJ, Magee AI, Rees DA, Stanley JR, Steinberg MS (1993) Nomenclature of the desmosomal cadherins. J Cell Biol 121:481–483

Cavallaro U, Christofori G (2004) Multitasking in tumor progression: signaling functions of cell adhesion molecules. Ann N Y Acad Sci 1014:58–66

Chitaev NA, Troyanovsky SM (1997) Direct Ca^{2+}-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. J Cell Biol 138:193–201

Christofori G (2003) Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J 22:2318–2323

Cowin P, Mattey D, Garrod D (1984) Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab'. J Cell Sci 70:41–60

Cowin P, Kapprell HP, Franke WW, Tamkun J, Hynes RO (1986) Plakoglobin: a protein common to different kinds of intercellular adhering junctions. Cell 46:1063–1073

Danen EH, Vries TJ de, Morandini R, Ghanem GG, Ruiter DJ, Muijen GN van (1996) E-cadherin expression in human melanoma. Melanoma Res 6:127–131

Dejana E (2004) Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol 5:261–270

Demlehner MP, Schaefer S, Grund C, Franke WW (1995) Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated Sisyphus cycle. J Cell Biol 131:745–760

Duden R, Franke WW (1988) Organization of desmosomal plaque proteins in cells growing at low calcium concentrations. J Cell Biol 107:1049–1063

Duguay D, Foty RA, Steinberg MS (2003) Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. Dev Biol 253:309–323

Foty RA, Steinberg MS (2005) The differential adhesion hypothesis: a direct evaluation. Dev Biol 278:255–263

Franke WW, Heid H (1989) Desmosomal proteins and cytokeratins in the hair follicle. In: Rogers GE, Reis PJ, Ward KA, Marshall RC (eds) The biology of wool and hair. Chapman and Hall, London New York, pp 403–416

Franke WW, Schmid E, Winter S, Osborn M, Weber K (1979) Widespread occurrence of intermediate-sized filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp Cell Res 123:25–46

Franke WW, Schumacher H, Borrmann CM, Grund C, Winter-Simanowski S, Schlechter T, Pieperhoff S, Hofmann I (2007) The area composita of adhering junctions connecting heart muscle cells of vertebrates. III. Assembly and disintegration of intercalated disks in rat cardiomyocytes growing in culture. Eur J Cell Biol 86:127–142

Godsel LM, Getsios S, Huen AC, Green KJ (2004) The molecular composition and function of desmosomes. In: Behrens J, Nelson WJ (eds) Cell adhesion. Handbook of experimental pharmacology. Springer, Berlin Heidelberg New York, pp 137–193

Goodwin M, Yap AS (2004) Classical cadherin adhesion molecules: coordinating cell adhesion, signaling and the cytoskeleton. J Mol Histol 35:839–844

Haass NK, Smalley KS, Herlyn M (2004) The role of altered cell-cell communication in melanoma progression. J Mol Histol 35:309–318

Haass NK, Smalley KS, Li L, Herlyn M (2005) Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res 18:150–159

Haemmerling B, Grund C, Boda-Heggemann J, Moll R, Franke WW (2006) The *complexus adhaerens* of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought. Cell Tissue Res 324:55–67

Hari L, Brault V, Kléber M, Lee HY, Ille F, Leimeroth R, Paratore C, Suter U, Kemler R, Sommer L (2002) Lineage-specific requirements of β-catenin in neural crest development. J Cell Biol 159:867–880

Hatta K, Takeichi M (1986) Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature 320:447–449

Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 148:779–790

Hendrix MJ, Seftor EA, Meltzer PS, Gardner LM, Hess AR, Kirschmann DA, Schatteman GC, Seftor RE (2001) Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. Proc Natl Acad Sci USA 98:8018–8023

Hendrix MJ, Seftor EA, Hess AR, Seftor RE (2003) Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 3:411–421

Hess AR, Seftor EA, Gruman LM, Kinch MS, Seftor RE, Hendrix MJ (2006) VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry. Cancer Biol Ther 5:228–233

Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ (2004) Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell 15:4310–4320

Hsu MY, Wheelock MJ, Johnson KR, Herlyn M (1996) Shifts in cadherin profiles between human normal melanocytes and melanomas. J Investig Dermatol Symp Proc 1:188–194

Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE, Herlyn M (2000a) E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol 156:1515–1525

Hsu MY, Andl T, Li G, Meinkoth JL, Herlyn M (2000b) Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J Cell Sci 113:1535–1542

Itoh M, Nagafuchi A, Yonemura S, Kitani-Yasuda T, Tsukita S, Tsukita S (1993) The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J Cell Biol 121:491–502

Itoh M, Morita K, Tsukita S (1999) Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. J Biol Chem 274:5981–5986

Jaggi M, Wheelock MJ, Johnson KR (2002) Differential displacement of classical cadherins by VE-cadherin. Cell Commun Adhes 9:103–115

Jamal S, Schneider RJ (2002) UV-induction of keratinocyte endothelin-1 downregulates E-cadherin in melanocytes and melanoma cells. J Clin Invest 110:443–452

Jimbow K, Fitzpatrick TB, Quevedo WC Jr (1986) Formation, chemical composition and function of melanin pigments. In: Bereiter-Hahn J, Matoltsy AG, Richards KS (eds) Biology of the integument 2. Springer, Berlin Heidelberg New York, pp 278–292

Jouneau A, Yu YQ, Pasdar M, Larue L (2000) Plasticity of cadherin-catenin expression in the melanocyte lineage. Pigment Cell Res 13:260–272

Klingelhoefer J, Troyanovsky RB, Laur OY, Troyanovsky S (2000) Amino-terminal domain of classic cadherins determines the specificity of the adhesive interactions. J Cell Sci 113:2829–2836

Koch PJ, Walsh MJ, Schmelz M, Goldschmidt MD, Zimbelmann R, Franke WW (1990) Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. Eur J Cell Biol 53:1–12

Koch PJ, Goldschmidt MD, Walsh MJ, Zimbelmann R, Franke WW (1991) Complete amino acid sequence of the epidermal desmoglein precursor polypeptide and identification of a second type of desmoglein gene. Eur J Cell Biol 55:200–208

Koch PJ, Goldschmidt MD, Zimbelmann R, Troyanovsky R, Franke WW (1992) Complexity and expression patterns of the desmosomal cadherins. Proc Natl Acad Sci USA 89:353–357

Koeser J, Troyanovsky SM, Grund C, Franke WW (2003) De novo formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. Exp Cell Res 285:114–130

Krengel S, Grotelueschen F, Bartsch S, Tronnier M (2004) Cadherin expression pattern in melanocytic tumors more likely depends on the melanocyte environment than on tumor cell progression. J Cutan Pathol 31:1–7

Kuphal S, Bosserhoff AK (2006) Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. Oncogene 25:248–259

Kuphal S, Poser I, Jobin C, Hellerbrand C, Bosserhoff AK (2004) Loss of E-cadherin leads to upregulation of NFkappaB activity in malignant melanoma. Oncogene 23:8509–8519

Kurzen H, Moll I, Moll R, Schaefer S, Simics E, Amagai M, Wheelock MJ, Franke WW (1998) Compositionally different desmosomes in the various compartments of the human hair follicle. Differentiation 63:295–304

Kurzen H, Munzing I, Hartschuh W (2003) Expression of desmosomal proteins in squamous cell carcinomas of the skin. J Cutan Pathol 30:621–630

Lampugnani MG, Resnati M, Reiteri M, Pigott R, Pisacane A, Houen G, Ruco LP, Dejana E (1992) A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol 118:1511–1522

Langbein L, Grund C, Kuhn C, Praetzel S, Kartenbeck J, Brandner JM, Moll I, Franke WW (2002) Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. Eur J Cell Biol 81:419–435

Langbein L, Pape UF, Grund C, Kuhn C, Praetzel S, Moll I, Moll R, Franke WW (2003) Tight junction-related structures in the absence of a lumen: occludin, claudins and tight junction plaque proteins in densely packed cell formations of stratified epithelia and squamous cell carcinomas. Eur J Cell Biol 82:385–400

Le Douarin N (1984) Pigment cells. In: Le Douarin N (ed) The neural crest. Cambridge University Press, Cambridge, pp 108–133

Li G, Satyamoorthy K, Herlyn M (2001a) N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. Cancer Res 61:3819–3825

Li G, Schaider H, Satyamoorthy K, Hanakawa Y, Hashimoto K, Herlyn M (2001b) Downregulation of E-cadherin and desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene 20:8125–8135

Li G, Fukunaga M, Herlyn M (2004) Reversal of melanocytic malignancy by keratinocytes is an E-cadherin-mediated process overriding beta-catenin signaling. Exp Cell Res 297:142–151

Liu ZJ, Xiao M, Balint K, Smalley KS, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M (2006) Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res 66:4182–4190

Luo Y, Radice GL (2005) N-cadherin acts upstream of VE-cadherin in controlling vascular morphogenesis. J Cell Biol 169:29–34

Moll I, Kurzen H, Langbein L, Franke WW (1997) The distribution of the desmosomal protein, plakophilin 1, in human skin and skin tumors. J Invest Dermatol 108:139–146

Montagna W, Parakkal PF (1974) The structure and function of skin. Academic Press, New York London

Moore R, Champeval D, Denat L, Tan SS, Faure F, Julien-Grille S, Larue L (2004) Involvement of cadherins 7 and 20 in mouse embryogenesis and melanocyte transformation. Oncogene 23:6726–6735

Nakagawa S, Takeichi M (1995) Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. Development 121:1321–1332

Nakagawa S, Takeichi M (1998) Neural crest emigration from the neural tube depends on regulated cadherin expression. Development 125:2963–2971

Navarro P, Ruco L, Decana E (1998) Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. J Cell Biol 140:1475–1484

Niehrs C, Huttner WB, Ruether U (1992) In vivo expression and stoichiometric sulfation of the artificial protein sulfophilin, a polymer of tyrosine sulfation sites. J Biol Chem 267:15938–15942

Niessen CM, Gumbiner BM (2002) Cadherin-mediated cell sorting not determined by binding or adhesion specificity. J Cell Biol 156:389–399

Nishimura EK, Yoshida H, Kunisada T, Nishikawa SI (1999) Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. Dev Biol 215:155–166

Omelchenko T, Fetisova E, Ivanova O, Bonder EM, Feder H, Vasiliev JM, Gelfand IM (2001) Contact interactions between epitheliocytes and fibroblasts: formation of heterotypic cadherin-containing adhesion sites is accompanied by local cytoskeletal reorganization. Proc Natl Acad Sci USA 98:8632–8637

Onken MD, Ehlers JP, Worley LA, Makita J, Yokota Y, Harbour JW (2006) Functional gene expression analysis uncovers phenotypic switch in aggressive uveal melanomas. Cancer Res 66:4602–4609

Paffenholz R, Kuhn C, Grund C, Stehr S, Franke WW (1999) The arm-repeat protein NPRAP (neurojungin) is a constituent of the plaques of the outer limiting zone in the retina, defining a novel type of adhering junction. Exp Cell Res 250:452–464

Patel SD, Chen CP, Bahna F, Honig B, Shapiro L (2003) Cadherin-mediated cell-cell adhesion: sticking together as a family. Curr Opin Struct Biol 13:690–698

Peitsch WK, Grund C, Kuhn C, Schnoelzer M, Spring H, Schmelz M, Franke WW (1999) Drebrin is a widespread actin-associating protein enriched at junctional plaques, defining a specific microfilament anchorage system in polar epithelial cells. Eur J Cell Biol 78:767–778

Peitsch WK, Hofmann I, Praetzel S, Grund C, Kuhn C, Moll I, Langbein L, Franke WW (2001) Drebrin particles: components in the ensemble of proteins regulating actin dynamics of lamellipodia and filopodia. Eur J Cell Biol 80:567–579

Peitsch WK, Hofmann I, Bulkescher J, Hergt M, Spring H, Bleyl U, Goerdt S, Franke WW (2005) Drebrin, an actin-binding, cell-type characteristic protein: induction and localization in epithelial skin tumors and cultured keratinocytes. J Invest Dermatol 125:761–774

Perlis C, Herlyn M (2004) Recent advances in melanoma biology. Oncologist 9:182–187

Pieperhoff S, Schumacher H, Franke WW (2008) The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes. Eur J Cell Biol 87:399–411

Qi J, Chen N, Wang J, Siu CH (2005) Transendothelial migration of melanoma cells involves N-cadherin-mediated adhesion and activation of the beta-catenin signaling pathway. Mol Biol Cell 16:4386–4397

Qi J, Wang J, Romanyuk O, Siu CH (2006) Involvement of Src family kinases in N-cadherin phosphorylation and beta-catenin dissociation during transendothelial migration of melanoma cells. Mol Biol Cell 17:1261–1272

Salomon D, Ayalon O, Patel-King R, Hynes RO, Geiger B (1992) Extrajunctional distribution of N-cadherin in cultured human endothelial cells. J Cell Sci 102:7–17

Sanders DS, Blessing K, Hassan GA, Bruton R, Marsden JR, Jankowski J (1999) Alterations in cadherin and catenin expression during the biological progression of melanocytic tumours. Mol Pathol 52:151–157

Sandig M, Voura EB, Kalnins VI, Siu CH (1997) Role of cadherins in the transendothelial migration of melanoma cells in culture. Cell Motil Cytoskeleton 38:351–364

Schaefer S, Koch PJ, Franke WW (1994) Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. Exp Cell Res 211:391–399

Schaefer S, Stumpp S, Franke WW (1996) Immunological identification and characterization of the desmosomal cadherin Dsg2 in coupled and uncoupled epithelial cells and in human tissues. Differentiation 60:99–108

Schmitt CJ, Franke WW, Goerdt S, Falkowska-Hansen B, Rickelt S, Peitsch WK (2007) Homo- and heterotypic cell-cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein. J Invest Dermatol 127:2191–2206

Schulze C, Firth JA (1993) Immunohistochemical localization of adherens junction components in blood-brain barrier microvessels of the rat. J Cell Sci 104:773–782

Seftor EA, Meltzer PS, Schatteman GC, Gruman LM, Hess AR, Kirschmann DA, Seftor RE, Hendrix MJ (2002) Expression of multiple molecular phenotypes by aggressive melanoma tumor cells: role in vasculogenic mimicry. Crit Rev Oncol Hematol 44:17–27

Shan WS, Tanaka H, Phillips GR, Arndt K, Yoshida M, Colman DR, Shapiro L (2000) Functional cis-heterodimers of N- and R-cadherins. J Cell Biol 148:579–590

Shimoyama Y, Tsujimoto G, Kitajima M, Natori M (2000) Identification of three human type-II classic cadherins and frequent heterophilic interactions between different subclasses of type-II classic cadherins. Biochem J 349:159–167

Silye R, Karayiannakis AJ, Syrigos KN, Poole S, Noorden S van, Batchelor W, Regele H, Sega W, Boesmueller H, Krausz T, Pignatelli M (1998) E-cadherin/catenin complex in benign and malignant melanocytic lesions. J Pathol 186:350–355

Simonneau L, Kitagawa M, Suzuki S, Thiery JP (1995) Cadherin 11 expression marks the mesenchymal phenotype: towards new functions for cadherins? Cell Adhes Commun 3:115–130

Smalley KS, Brafford P, Haass NK, Brandner JM, Brown E, Herlyn M (2005) Up-regulated expression of zonula occludens protein-1 in human melanoma associates with N-cadherin and contributes to invasion and adhesion. Am J Pathol 166:1541–1554

Tang A, Eller MS, Hara M, Yaar M, Hirohashi S, Gilchrest BA (1994) E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci 107:983–992

Troyanovsky S (2005) Cadherin dimers in cell-cell adhesion. Eur J Cell Biol 84:225–233

Van Marck V, Stove C, Van Den Bossche K, Stove V, Paredes J, Vander Haeghen Y, Bracke M (2005) P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma. Cancer Res 65:8774–8783

Volk T, Cohen O, Geiger B (1987) Formation of heterotypic adherens-type junctions between L-CAM-containing liver cells and A-CAM-containing lens cells. Cell 50:987–994

Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U, Seckinger A, Peitsch WK, Ho AD, Franke WW (2007) *Processus adhaerentes*: giant cell processes studded with special adherens junctions attract and connect mesenchymal stem cells. Cell Tissue Res 328:499–514

Yin T, Green KJ (2004) Regulation of desmosome assembly and adhesion. Semin Cell Dev Biol 15:665–677

Table S1. Survey of the results of the tissue microarrays of human melanomas and nevi: clinical data and detailed immunofluorescence microscopical results.

No.	Sex	Age	Organ	Pathology	N-cad	E-cad	P-cad	Dsg2
1	F	80	Skin	Malignant melanoma, pate	++++	+	-	-
2	M	60	Skin	Malignant melanoma, right buttocks	++++	++++	-	-
3	F	16	Skin	Malignant melanoma, back	-	++++	+	-
4	M	74	Skin	Malignant melanoma, left foot	++++	++++	++++	-
5	M	55	Skin	Malignant melanoma, sole	++++	++++	++++	-
6	F	38	Skin	Malignant melanoma, left upper arm	+++	++	-	-
7	F	63	Skin	Malignant melanoma,right heel	++++	+	++++	-
8	M	27	Skin	Malignant melanoma, right thigh	-	++++	-	-
9	F	35	Skin	Malignant melanoma, waist	n.d.	n.d.	-	n.d.
10	M	42	Skin	Malignant melanoma, left heel	++++	+	+	+++
11	M	64	Skin	Malignant melanoma, sole	++++	-	-	-
12	M	64	Skin	Malignant melanoma, left shoulder	++++	-	-	-
13	M	59	Skin	Malignant melanoma, left leg	++++	++++	+	+++
14	F	88	Skin	Malignant melanoma, left sole	++++	++++	++++	-
15	M	57	Skin	Malignant melanoma, left shoulder	++++	++++	-	-
16	F	54	Skin	Malignant melanoma, left sole	++++	++++	-	-
17	F	42	Skin	Malignant melanoma, left thumb	-	-	++++	-
18	F	58	Skin	Malignant melanoma, left buttock	-	-	-	-
19	M	38	Skin	Malignant melanoma, right abdominal wall	-	++++	-	-
20	M	62	Skin	Malignant melanoma, heel	++++	+++	-	-
21	M	40	Skin	Malignant melanoma, right chest wall	-	-	n.d.	-
22	F	47	Skin	Malignant melanoma, perianal	++++	-	-	-
23	F	77	Skin	Malignant melanoma, left sole	++++	+++	+++	-
24	F	62	Skin	Malignant melanoma, right thumb	n.d.	-	n.d.	n.d.
25	F	66	Skin	Malignant melanoma, chest wall	++++	-	-	-
26	M	25	Skin	Malignant melanoma, heel	-	++++	-	-
27	F	38	Skin	Malignant melanoma, right toe	-	++++	-	-
28	F	52	Skin	Malignant melanoma, cunnus	++++	-	-	n.d.
29	M	52	Skin	Malignant melanoma, right heel	++++	+++	-	-
30	M	49	Skin	Malignant melanoma, shoulder	++++	-	-	-
31	M	36	Skin	Malignant melanoma, back	++++	+	-	-
32	M	71	Skin	Malignant melanoma, thigh	-	++++	-	-
33	M	51	Skin	Malignant melanoma, left oxter	++++	-	-	-
34	M	35	Skin	Malignant melanoma, left instep	-	-	-	-
35	M	66	Skin	Malignant melanoma, right thigh	++++	+++	-	++
36	F	81	Skin	Malignant melanoma, left arm	++++	-	-	-
37	M	65	Skin	Malignant melanoma, scalp	++++	++++	-	-
38	M	45	Skin	Malignant melanoma, perianal	-	+	-	-
39	M	48	Skin	Malignant melanoma, left thumb	++++	++++	++++	-

No.	Sex	Age	Organ	Pathology	N-cad	E-cad	P-cad	Dsg2
40	F	45	Skin	Malignant melanoma, cunnus	++++	+	++++	-
41	F	72	Skin	Malignant melanoma, cunnus	-	-	-	-
42	M	57	Skin	Malignant melanoma, right cheek	-	-	-	-
43	F	53	Skin	Malignant melanoma, right thumb	-	-	-	-
44	F	47	Skin	Malignant melanoma, right upper arm	-	+	-	-
45	M	53	Skin	Malignant melanoma, right sole	++++	-	-	-
46	M	79	Skin	Malignant melanoma, left leg	++++	++++	-	-
47	M	70	Skin	Malignant melanoma, right sole	++++	++	+++	-
48	M	65	Skin	Malignant melanoma, right thumb	++++	+	+++	-
49	M	76	Skin	Malignant melanoma, right medial malleolus	-	-	-	-
50	F	54	Skin	Malignant melanoma, left heel	++++	++++	-	+++
51	M	62	Skin	Malignant melanoma, left thumb	++++	-	-	-
52	M	55	Skin	Malignant melanoma, right forearm	++++	++++	-	-
53	M	74	Skin	Malignant melanoma, left sole	++++	++++	-	n.d.
54	M	65	Skin	Malignant melanoma, right sole	++++	+++	++++	++
55	M	31	Skin	Malignant melanoma, scalp	++++	++++	-	++++
56	F	41	Skin	Malignant melanoma, scalp	-	+	+	++++
57	M	45	Skin	Pigmented mole (sparse)	++++	-	-	++++
58	F	29	Skin	Atypical melanophoric nevus, left shoulder	++++	++++	-	-
59	F	36	Skin	Intradermal nevus, left abdominal wall	++++	++	-	-
60	F	35	Skin	Intradermal nevus, chest wall	-	-	-	-
61	F	23	Skin	Compound nevus, right waist	++++	+	-	+
62	M	25	Skin	Compound nevus, left leg	++++	++++	-	-
63	M	20	Skin	Intradermal nevus, left shoulder	++	-	+	-
64	M	19	Skin	Intradermal nevus, right cheek	++++	++++	+++	++++
65	F	10	Skin	Atypical melanophoric nevus, right foot dorsum	++++	++++	+++	-
66	F	2	Skin	Intradermal nevus, frontal region	++++	++++	++++	-
67	M	6	Skin	Compound nevus, left face	++++	-	-	-
68	M	0.5	Skin	Intradermal nevus, face	++++	+++	++++	-
69	M	25	Skin	Intradermal nevus, scalp	++++	+	-	-
70	M	46	Skin	Intradermal nevus, back	++++	-	-	-
71	F	1.5	Skin	Intradermal nevus, right leg	++++	-	--	-
72	M	42	Skin	Intradermal nevus of face	++++	++	+	n.d.
73	M	11	Skin	Junctional nevus, left thigh (sparse)	-	+	-	-
74	F	30	Skin	Compound nevus, right buttock	++++	-	-	+++
75	M	62	Skin	Junctional nevus	n.d.	-	-	-
76	M	39	Skin	Compound nevus, occiput	++++	+	-	-
77	F	7	Skin	Junctional nevus, right forearm	++++	++++	+	++++
78	M	50	Skin	Compound nevus, upper arm	++++	++	+++	-
79	M	53	Skin	Sebaceous nevus (sparse)	++++	-	-	+++
80	M	32	Skin	Sebaceous nevus, right elbow (sparse)	n.d.	+	+++	++

Int. J. Cancer: **125,** 2036–2048 (2009)

Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell–cell-attachment characteristic for transformed mesenchymal cells

Steffen Rickelt, Stefanie Winter-Simanowski, Edeltraut Noffz, Caecilia Kuhn and Werner Wilhelm Franke*

[1]*Helmholtz Group for Cell Biology, German Cancer Research Center, Heidelberg, Germany*

In contrast to the desmosome-containing epithelial and carcinoma cells, normal and malignantly transformed cells derived from mesenchymal tissues and tumors are connected only by adherens junctions (AJs) containing N-cadherins and/or cadherin-11, anchored in a cytoplasmic plaque assembled by α- and β-catenin, plakoglobin, proteins p120 and p0071. Here, we report that the AJs of many malignantly transformed cell lines are characterized by the additional presence of plakophilin-2 (Pkp2), a protein hitherto known only as a major component of desmosomal plaques, *i.e.*, AJs of epithelia and carcinomatous cells. This massive acquisition of Pkp2 and its integration into AJ plaques of a large number of transformed cell lines is demonstrated with biochemical and immunolocalization techniques. Upregulation of Pkp2 and its integration into AJs has also been noted in some soft tissue tumors *in situ* and some highly proliferative colonies of cultured mesenchymal stem cells. As Pkp2 has recently been identified as a functionally important major regulatory organizer in AJs and related junctions in epithelial cells and cardiomyocytes, we hypothesize that the integration of Pkp2 into AJs of "soft tissue tumor" cells also can serve functions in the upregulation of proliferation, the promotion of malignant growth in general as well as the close-packing of diverse kinds of cells and the metastatic behavior of such tumors. We propose to examine its presence in transformed mesenchymal cells and related tumors and to use it as an additional diagnostic criterion.

Key words: adherens junctions; cadherins; malignant cells; mesenchymal cells; plakophilin-2

Among the diverse kinds of normal and transformed cells those of epithelial and carcinomatous origin are characterized by bundles of intermediate-sized filaments (IFs) containing cytokeratins which are attached to special cell–cell-connecting adhering junctions, the desmosomes (*maculae adhaerentes*). These are assemblies of glycoproteins of the larger cadherin family, desmogleins (Dsg1–4), and desmocollins (Dsc1–3), the cytoplasmic tails of which are anchored in dense cytoplasmic plaques formed by desmoplakin, plakoglobin and 1 or 2 plakophilins (Pkp1–3).[1,2] In addition, epithelia and carcinomas contain plaque-bearing junctions of the adherens-category which can appear in diverse morphological forms known as *zonulae* or *fasciae adhaerentes* or as *puncta adhaerentia* and are commonly subsumed under the term adherens junctions (AJs).

Remarkably, such AJs are the only adhering junctions that occur in mesenchymally-derived tissues and in tumors grouped by pathologists as "soft tissue tumors" (*e.g.*, for review refer[3,4]). They contain special cadherins (for reviews refer[5–7]) in cell-type-specific combinations, mostly N- and/or P-cadherin and cadherin-11, anchored in a cytoplasmic plaque formed by α-catenin and a variety of combinations of *armadillo* proteins such as plakoglobin, β-catenin as well as proteins p120 and p0071, which in turn are associated with further actin microfilament binding proteins such as vinculin, α-actinin, afadin and formin.[8–12]

In addition, a number of cell type-specific AJ forms have been identified that cannot be readily subsumed under these 2 major categories, desmosomes and AJs. These include, for example the cardiomyocyte-connecting composite junctions (*areae compositae*) of mature mammalian myocardium,[13,14] the *complexus adhaerens* of the endothelial and virgultar cells of lymph node sinus which is characterized by its additional desmoplakin content[15] (for review refer[16]) and locally can also include certain tight junction typical proteins (*e.g.*,[16]), and as an extreme situation the extended *cortex adhaerens* of eye lens tissue.[17]

In general, the molecular composition of desmosomes and AJs is essentially identical in normal proliferative tissues and in tumors derived therefrom, and this is the main reason for the application of such molecules as cell type markers in tumor diagnosis, notably of metastases.[18–20] More recently, however, we have noted that cells of certain culture lines and in tumors of mesenchymal origin are connected by AJs which in addition to their typical molecular ensemble contain plakophilin-2 (Pkp2) as a major and stable plaque component. Once this Pkp2-modified AJ type has appeared in such non-epithelial cell types it seems to be a stable, frequent and characteristic plaque component typical of a novel AJ subtype.

The integration of Pkp2 into AJ plaques, however, is not only eye-catching as a phenomenon but also thought-provoking in view of the functional importance of this molecule in cell–cell adhesion as well as in some nuclear functions which—just like β-catenin—has also been identified in certain nucleoplasmic complexes, albeit in relatively low concentrations.[21–23] Moreover, the importance of Pkp2 as a major regulator of AJ composition and positioning has not only been reported for desmosomes but also for specific AJ proteins not restricted to epithelia such as β-catenin and α-T-catenin.[14,24] In recent years, the outstanding role of Pkp2 in the assembly, maintenance and functional stability of the myocardial composite junctions (*areae compositae*) has also been demonstrated in gene abrogation and siRNA-mediated knock-out and knock-down experiments[25–28] as well as in numerous studies of human Pkp2-gene modifications resulting in arrhythmogenic cardiomyopathies, including cases of "sudden death" (*e.g.*,[29–33]). Most strikingly, the absence of the Pkp2-gene or even marked down-regulation of Pkp2-mRNA in cardiomyocytes can result in drastic changes, *i.e.*, in deficient plaques with little or no desmoplakin attached and in a loss of cell–cell adhesion.[25,28]

We have, therefore, decided to study systematically the frequent and spontaneous changes resulting from the *de novo* acquisition of Pkp2 to the molecular cell–cell adhesion ensemble of transformed mesenchymal cells in general.

Material and methods

Cell culture

The cells used were cultured in Dulbeccos's Minimal Essential Medium (DMEM; Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine.

Abbreviations: AJ, adherens junction; cad, cadherin; cat, catenin; DFSP, dermatofibrosarcoma protuberans; IP, immunoprecipitation; mab, monoclonal antibody; Pkp, plakophilins.

Grant sponsor: Deutsche Krebshilfe; Grant number: 10-2049-Fr1. Grant sponsor: German Ministry for Research and Technology (Program Regenerative Medicine, START-MSC).

***Correspondence to:** Helmholtz Group for Cell Biology, German Cancer Research Center, Im Neuenheimer Feld 581, Building TP4-Room S3.222, D-69120 Heidelberg, Germany. Fax: +49-6221-423404.
E-mail: w.franke@dkfz.de

Received 23 December 2008; Accepted after revision 29 April 2009
DOI 10.1002/ijc.24552
Published online 4 May 2009 in Wiley InterScience (www.interscience.wiley.com).

Normal human fibroblasts (hFB, primary and secondary cultures) were obtained from several sources, including breast dermal tissue (provided by Dr. P. Angel, this Center), human diploid lung fibroblasts of line WI38 (ATCC No. CCL-75) or line LL24 (ATCC No. CCL-151), grown in 85% Ham's F12 medium containing 15% FCS, human fibroblasts of line Hs295.SK derived from healthy skin parts of a patient suffering from *dermatofibrosarcoma protuberans* (DFSP; ATCC No. CRL-7232) as well as human HG261 skin fibroblasts (ATCC No. CCL-122).

In addition, human ''mesenchymal stem cells'' (MSC) derived from bone marrow or umbilical cord blood kindly provided by Dr. K. Bieback (Institute of Transfusion Medicine and Immunology, Mannheim, Germany) and mouse bone marrow cells of line OP9 (ATCC No. CRL-2749), kept in MEM containing 10% FCS were examined as well as mouse 3T3 embryonic mesenchymal cells (ATCC No. CCL-92) and their SV40-transformants, SV3T3 cells, in comparison with mouse fibroblasts of strain L-929 (ATCC No. CCL-1), rat fibroblasts of line Rat2 (ATCC No. CRL-1764) and bovine dermal fibroblasts of line B1, all grown in 80% DMEM containing 20% FCS. For comparison, we studied SV40-transformed human fibroblasts of line "SV80," SV40-transformed human WI38 fibroblasts (line WI38VA13, subline 2RA; ATCC No. CCL-75.1) and mouse SV40-transformed embryonic cells of line VLM (German Collection of Microorganisms and Cell Cultures No. ACC 429).

The human tumor cell lines examined included liposarcoma cells of line SW-872 (ATCC No. HTB-92), fibrosarcoma SW-684 cells (ATCC No. HTB-91), both grown in Leibovitz L-15 Medium containing 5% FCS, and DFSP cells of line Hs63.T (ATCC No. CRL-7043), as well as rhabdomyosarcoma-derived cells of lines RD (ATCC No. CLL-136) and HS-729 (ATCC No. HTB-153). Moreover, we examined human astrocytoma cells of line U 333 CG/343 MG (*c.f.*,[34]) and promyelocytic leukemia cells of line HL60 (ATCC No. CCL-240).

For controls, various epithelial cells including human HaCaT-keratinocytes were used.[35]

For cell cloning experiments, individual cells or daughter pairs of cells were picked and grown as purified clonal cultures as previously described in detail.[36,37]

Antibodies

For immunofluorescence microscopy and immunoblotting analyses of gel-electrophoretically separated polypeptides, the monoclonal mouse antibodies (mabs) against N-, E-, P- (clone 56) and R-cadherin, α- and β-catenin and protein p120, all purchased from BD Biosciences Pharmingen (Heidelberg, Germany) as well as mabs against cadherin-11 from Zymed Laboratories (South San Francisco, CA) were used, while mabs against vinculin (clone 11–5) and α-actinin (clone BM-75.2) were obtained from Sigma (St. Louis, MO). A mab directed against VE-cadherin (clone BV9) was kindly provided by Elisabetta Dejana (School of Sciences, University of Milan, Italy), and the cadherin-6 mab used was from US Biologicals Swampscott, MA (obtained through Acris, Hiddenhausen, Germany). For the demonstration of plakoglobin, mab 11E4 (from M.J. Wheelock, University of Nebraska Medical Center, Omaha, NE) and clone PG 5.1 (from Progen Biotechnik, Heidelberg; *c.f.*,[8]) were used.

Rabbit polyclonal antibodies routinely used were directed against N-cadherin (QED Bioscience, San Diego, CA), α- or β-catenin, N-cadherin* ("Pan-cadherin," a wider spectrum of epitopes, for reference refer[38]), protein p120, α-actinin, l/s afadin or JAM-C (from Sigma) or against cadherin-11, protein ZO-1, ZO-2, connexin Cx 43, ponsin or to occludin, claudins 1–5 or 7 (from Zymed Laboratories). In addition, we used antibodies specific for protein p0071 (*c.f.*,[39]).

The following antibodies against desmosomal and other cytoskeletal proteins were purchased from Progen Biotechnik, including mabs against desmoplakins 1 and 2 (clones DP-2.15, DP-2.17 and DP-2.20;[40]), plakophilin-1 (clones PP1-2D6 and PP1-5C2;[41]), plakophilin-2 (PP2-62, PP2-86, PP2-150;[21]) and plakophilin-3 (clone PP3-270.6.2;[42]) as well as mabs against desmocollins (Dsc) 1 and 3[43] and desmogleins (Dsgs) 1 and 2 (clone DG3.10) or Dsg 3 (*c.f.*,[44,45]). For comparison, a mab against Dsc 2 (mab 7G6) obtained from Zymed Laboratories and a plakophilin-2-specific IgG fraction from guinea-pigs[21] were also used. To examine the mesenchymal differentiation state, murine mabs and guinea pig antisera against vimentin, desmin or keratin 8 and 18 were used (all obtained from Progen Biotechnik).

Primary antibody complexes were visualized with secondary antibodies coupled to Cy3 (Dianova, Hamburg, Germany) or Alexa 488 (MoBiTec, Göttingen, Germany). For immunoblot analysis, horseradish peroxidase-conjugated secondary antibodies were applied (Dianova).

Cell fractionation, gel-electrophoresis of polypeptides, and immunoblotting

Cells grown to confluence in 10 cm culture dishes, were rinsed several times with pre-cooled PBS and immediately scraped off in 1.0 ml pre-heated SDS sample buffer (250 mM Tris-HCl, 10% SDS, 20% glycerol, 100 mM DTT; pH 6.8), using a rubber policeman. Then the sample was heated at 95°C for 5 min and subsequently cooled on ice. Samples were then incubated with Benzonase (1:1000; Merck, Darmstadt, Germany) and homogenized by sucking up and down in a pipette for several times.

Methods used for SDS-PAGE, 2-dimensional non-equilibrium pH-gradient electrophoresis (2D-NEPHGE) and immunoblotting were essentially as described.[46] The polypeptides separated were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and stained with Coomassie Brilliant Blue before immunochemical detection. For NEPHGE analysis, the material obtained by chloroform/methanol-precipitation from total cell lysates dissolved in SDS sample buffer, we used "lysis buffer" (9.5 M urea, 2% NP-40, 2% ampholyte 3–10, 20 mM DTT; from Serva, Heidelberg) to reduce the final SDS concentration in the sample to 0.2%. For protein separation in the first dimension electrophoresis, a focusing time of 4 hr at 400 V was chosen, followed by SDS-PAGE and transfer to PVDF membranes.

For immunoblotting, background reactions due to non-specific binding had to be reduced by brief incubation in 5% low-fat dry milk in PBS containing 0.05% Tween (PBS-T) for 30 min. Blots were then subsequently incubated with the primary antibody (diluted to appropriate concentrations in PBS; 1:10 to 1:1000) for ~1 h, washed thrice in PBS-T and incubated for at least 30 min with HRP-conjugated secondary antibodies, followed by several washes in PBS. For detection of secondary antibodies bound to proteins, the "enhanced chemiluminescence" (ECL, Amersham-Buchler, Braunschweig, Germany) was used.

Immunoprecipitation

For Immunoprecipitation (IP), cells grown to confluence were lysed in IP-buffer (20 mM HEPES, pH 7.5, 1% NP-40, 0.5 mM $CaCl_2$, 5 mM EDTA, 150 mM NaCl) containing a protease inhibitor cocktail (Complete Mini Inhibitor Tabs, Roche Diagnostics, Mannheim, Germany) for 15 min on ice. The lysate obtained was centrifuged at 14,000 rpm for 10 min (4°C). The supernatant was then pre-cleared with "pan mouse IgG" Dynabeads (Dynal, Hamburg, Germany) for 2 hr on a rotating wheel at 4°C. After centrifugation, the supernatant was incubated overnight at 4°C on a rotating wheel with IgG Dynabeads coated with specific antibodies (Pkp2, N-cadherin and β-catenin) in 50 mM Tris-HCl, pH 7.5. As a control, unrelated mouse antibodies were processed in parallel. The beads were washed 4 times in ice-cold IP-buffer, then boiled in 60 µl of SDS sample buffer, processed by SDS-PAGE and stained either with a silver sodium solution or blotted to PVDF membranes. Immunoblotting was performed as described earlier.

Sucrose gradient centrifugation

Confluent cells were homogenized in 5:1 buffer (83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 3 mM DTT and 0.5% NP-40) containing protease inhibitors, on ice. After treatment with a Dounce homogenizer (30 strokes) and centrifugation at 16,000 rpm (10 min, 4°C) supernatants were loaded on top of a 10 to 40% sucrose gradients and centrifuged at 35,000 rpm in a Beckman SW40 rotor (Beckman Instruments, München, Germany) for 18 hr at 4°C. Sixteen density fractions of 0.8 ml each were collected from the gradient, and BSA (4.3S), catalase (11.3S) and thyroglobulin (16.5S) were used as markers in parallel gradients. In addition, 40S and 60S yeast ribosomal subunits (kindly provided by Dr. Rakwalska, Centre for Molecular Biology, Heidelberg) were used as S-value references. Single or pooled fractions were then analyzed by SDS-PAGE and immunoblotting.

Reverse transcriptase-PCR

RNA from various cell culture lines was isolated with the "TriPure Isolation Reagent" (Roche Diagnostics) according to the manufacturer's protocol. For reverse transcription assays, 10 μg samples of total RNA were used. The PCR primers for human N-cadherin were chosen as follows: a forward primer 5′-GGCTTCTGGTGAAATCGC-3′ and reverse 5′-TGTAGGTGGC-CACTGTGC-3′ to amplify a 392 bp fragment. To amplify a 521 bp fragment of human Pkp2 the forward 5′-TTTGAATTCGAC-CAATGCCGACATCAGTGG-3′ and reverse 5′-TTTGAAT

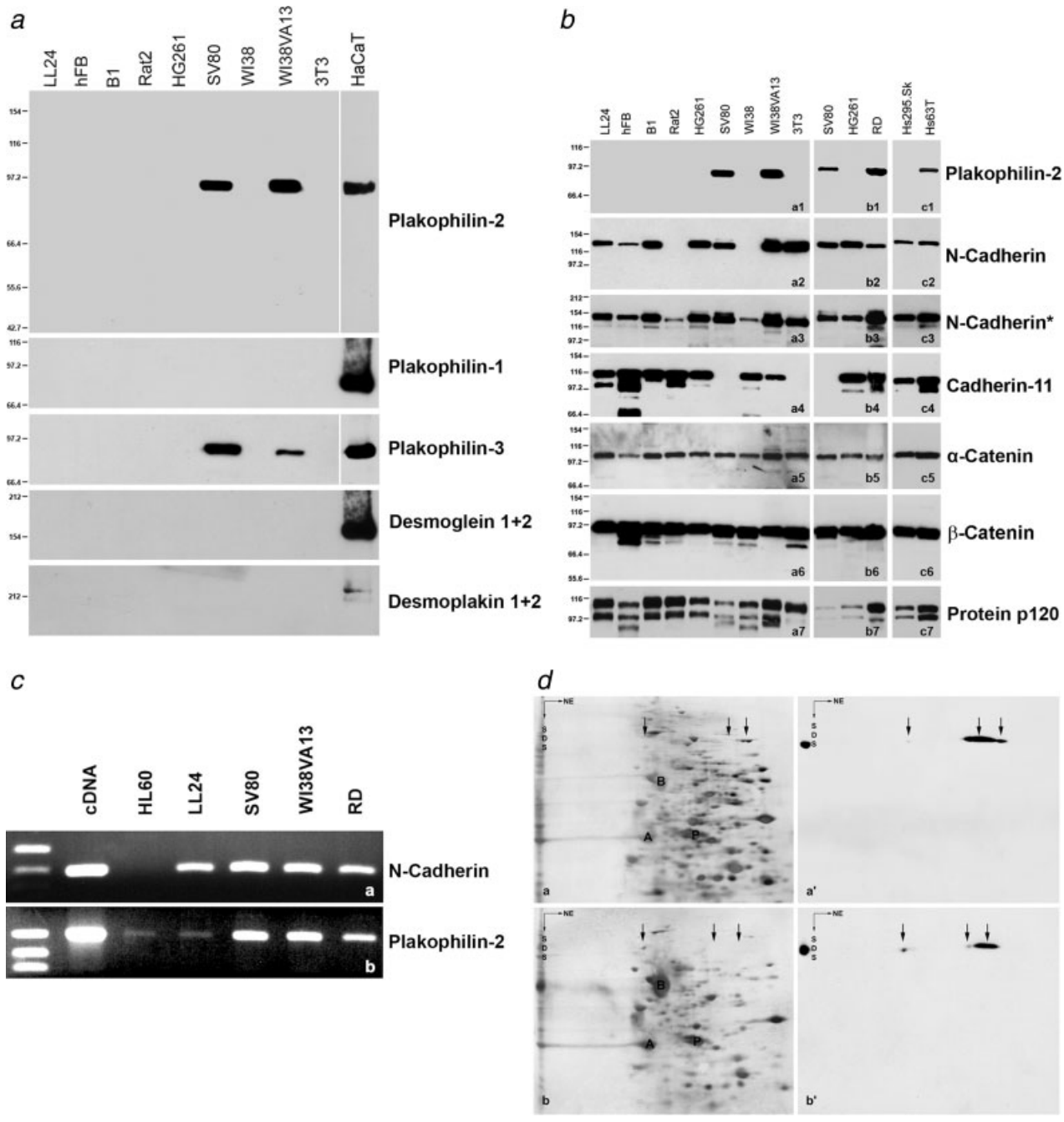


FIGURE 1

TCGTCTTTAAGGGAGTGGTAGGC-3′ primers were used. The PCR protocols performed for 30 cycles were as follows: 3 min initial denaturation at 94°C, per cycle 30 sec denaturation at 93°C, 30 sec annealing at 54°C, 1 min elongation at 72°C plus final elongation 10 min at 72°C for semi-quantitative analysis. PCR fragments were analyzed on 2% agarose gels.

Immunofluorescence microscopy

Cells grown on glass cover slips were briefly rinsed in PBS and fixed for 5 min either with 2% formaldehyde in PBS at room temperature or in methanol (5 min) followed by acetone (20 s), both at −20°C. The samples were then briefly air-dried and either stored at −25°C or rehydrated in PBS just prior to the immunostaining procedure. After permeabilization in 0.2% Triton X-100 for 5 min, cells were washed several times in PBS. Primary antibodies were applied for 1 hr at RT, followed by 3 washes in PBS (5 min each), followed by incubation with secondary antibody (30 min, RT), washing with PBS (3 × 5 min), a short rinse in distilled water and finally dehydration in 100% ethanol (1 min). After air-drying, specimens were mounted with Fluoromount-G (Southern Biotech, obtained through Biozol Diagnostica, Eching, Germany). Immunofluorescence microscopic images were recorded with an Axiophot II photomicroscope (Carl Zeiss, Jena, Germany), equipped with an AxioCam HR (Carl Zeiss). For confocal laser scanning microscopy, a Zeiss LSM 510 Meta microscope was used.

For immunolocalizations on paraffin-embedded tissue samples, sections were deparaffinized and heat-induced antigen retrieval was performed according to protocols published in the anthology of Shi[47] (for specific details refer also[48]).

Electron microscopy

For conventional electron microscopy (EM), cells and tissue sections were briefly rinsed in PBS, fixed in 2.5% glutaraldehyde (50 mM sodium cacodylate, pH 7.2) for 20 min and then washed thrice in the same buffer. Post-fixation was performed with 2% OsO_4 in cacodylate buffer for 2 hr on ice, followed by several washes in distilled water and heavy metal staining (0.5% uranylacetate) overnight at 4°C. After 3 washes in distilled water, samples were dehydrated in an ethanol series and propylenoxide before embedded in Epon. Ultrathin sections for EM were made with a Reichert-Jung microtome (Utracut, Leica, Bensheim, Germany). For contrast enhancement, the sections were stained with 2% uranylacetate in methanol for 15 min and lead citrate for 5 min.

For immunoelectron microscopy, cells grown on coverslips were fixed in 2% formaldehyde in PBS (5 min, RT), followed by incubation in 50 mM NH_4Cl (5 min), 2 washes in PBS (5 min each) and permeabilization in either 0.1% saponin in PBS for 2.5 min or 0.1% Triton X-100 in PBS for 1 min, followed by 2 washes in PBS. Incubation with primary antibodies was performed for at least 2 hr. After 3 washes in PBS, antibodies conjugated with 1.4 nm-gold particles (Nanogold, Biotrend, Cologne, Germany) were used as secondary reagents and incubated for 2 to 4 hr. Secondary antibodies not stably bound were removed by washing in PBS. Samples were then post-fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (15 min, RT), briefly rinsed in the same buffer and twice incubated in 200 mM sucrose in 50 mM HEPES buffer (pH 5.8) for 10 min. This was followed by silver enhancement (Nanoprobes, Stony Brooks, New York) for 7 to 10 min, 2 washes in 250 mM sodium thiosulfate, buffered with 50 mM Hepes (pH 5.8; 5 min each), and up to 10 washes in distilled water. After fixation with 0.2% OsO_4 in cacodylate buffer for 30 min on ice, samples were dehydrated, embedded in Epon, sectioned and stained as described earlier. Electron micrographs were taken at 80 kV, using an EM 910 (Carl Zeiss, Oberkochen, Germany).

Results

In protein chemical and immunolocalization analyses of diverse cell culture lines of non-epithelial origin, we have made the observation that some of them were connected by junctions strikingly positive for plakophilin-2 (Pkp2) but that such Pkp2-positive adherens junctions (AJs) lacked other obligatory desmosomal components such as desmoplakin and both kinds of desmosomal cadherins. A special complication was the finding that some sublines derived from the same original cell type or line differed with respect to the presence or absence of Pkp2-positive AJs. As we had not treated the plus and minus Pkp2 cell cultures of related origin in different ways, we hypothesized that such an apparently spontaneous advent of a major junctional protein may somehow resemble the spontaneous appearance of other cytoskeletal proteins such as certain cytokeratins or specific actin isoforms in individual cells of mesenchymally-derived cell cultures (*e.g.*,[36,37,49]). Because of this apparently spontaneous and frequent change of the cell–cell adhesion character and also because such changes may have far-reaching consequences in tumor spread and in diagnoses, we have decided to examine this phenomenon of a spontaneous and stable change of the molecular composition of AJs in detail.

Biochemical analyses

The gel-electrophoretic and immunoblot analyses of cytoskeletal proteins from a wide range of established mesenchymal cell

Figure 1 – Identification of constituent proteins of adherens junctions in a series of cell culture lines of mesenchymally-derived cells: The addition of plakophilin-2. (*a* and *b*) Immunoblot reactions of SDS-PAGE-separated total and cytoskeletal proteins from different fibroblastoidal cell culture lines. Proteins were probed with antibodies specific for desmosomal proteins (*a*), including plakophilin-1, plakophilin-2 (*a* and *ba*1–*bc*1), plakophilin-3, desmoglein 1+2 and desmoplakin 1+2 as well as for known adherens junction proteins (*b*) such as N-cadherin (*a*2–*c*2, polyclonal antibodies; *a*3–*c*3, broadly reactive antibodies of the "pan-cadherin"-type), cadherin-11 (*a*4–*c*4), α-catenin (*a*5–*c*5), β-catenin (*a*6–*c*6) and protein p120 (*a*7–*c*7). Names of cell lines are given on the upper margin of the specific lanes, including human (LL24, hFB, HG261, SV80, WI38, WI38VA13), bovine (B1), rat (Rat2) and murine (3T3) cells. Note that in addition to HaCaT-keratinocytes plakophilin-2 and plakophilin-3 are here only seen in the 2 SV40-protein transformed cell lines SV80 and WI38VA13 whereas the desmosomal marker proteins desmoglein or desmoplakin are not detectable. (*b*) SV80 and HG261 cells in direct comparison with human rhabdomyosarcoma cells of line RD (*b*1, Lane 3) and (*c*1) direct comparison with fibroblastoidal cells of lines Hs295.SK and Hs63.T, both from *dermatofibrosarcoma protuberans* (DFSP) patients. Additional junctional plakophilin-2 (*a*1–*c*1) is detected not only in SV40-transformed fibroblasts (SV80, WI38VA13) and in RD cells but also in the DFSP-derived cell culture line Hs63.T. The absence in murine 3T3 cells may be due to the specific antibody used. (*c*) RT-PCR analyses of RNAs from different human cell lines such as promyelocytic leukemia cells of line HL60, lung fibroblasts of line LL24, SV40-transformed fibroblasts of lines SV80 and WI38VA13 and rhabdomyosarcoma-derived cells of line RD, using primers specific for N-cadherin (*a*) or plakophilin-2 (*b*). As positive controls cDNA sequences of N-cadherin and plakophilin-2 PCR were used (Lane 1). Note that N-cadherin mRNA is detected in all cell lines examined, except of HL60. Plakophilin-2 mRNA is detected as a major component in some cell lines but only as minor component in lines HL60 and LL24. Size markers used were 517 and 397 bp for N-cadherin and 517, 396 as well as 356 bp for plakophilin-2. (*d*) Two-dimensional gel-electrophoresis of cultured human SV40-transformed fibroblastoidal cells and identification of one major and 2 minor forms of plakophilin-2 by immunoblotting. Non-equilibrium pH gradient electrophoresis (NEPHGE; horizontal arrows labeled NE) was used in the first and SDS-PAGE (vertical arrows labeled SDS) in the second dimension. Coomassie Brilliant Blue-stained proteins of the total lysates of the human SV80 (*a*) or WI38VA13 (*b*) cells were transferred to PVDF membranes. Positions of plakophilin-2 polypeptide variants are indicated by vertical arrows at a molecular weight of ~97 kDa (*a′* and *b′*). As reference proteins, rabbit skeletal muscle α-actin (*A*), bovine serum albumin (*B,* BSA) and rabbit phosphoglycerol phosphokinase (*P*) were used. Plakophilin-2 migrates as 3 components at pH 7.4 and 8.2 (right arrows) and a more acidic form at approximately pH 6.4 (left arrows).

culture lines of human or animal origin showed that some of them were distinctly rich in Pkp2 while other, even closely related cell lines were devoid of Pkp2 (*e.g.*, Figs. 1*a* and 1*b*). Two of these cell lines were also positive for Pkp3, a closely related junctional plaque protein, that is known often to coexist together with Pkp2.[42,50] Whereas a high amount of additional Pkp2 was also found in typical cytoskeletal residues resistant to extractions with buffers of high and low ionic strength and with various concentrations of detergent, the amounts of Pkp2 found in nuclear and cytosolic complexes were lower by several orders of magnitude and readily extractable during cell lysis using moderate detergent buffers of near-physiological ionic strength (*c.f.*,[21,23,39,42,51]). Other typical components of AJ structures such as N-cadherin, cadherin-11, α- and β-catenin, proteins p120 and p0071 as well as afadin and protein ZO-1 were also readily identified in such residual junction structures (*e.g.*, Figs. 1*b*–1*d*). A number of our analytical results are summarized in Table I.

Particularly surprising was the observation that SV40-transformed cell lines such as the "SV80 fibroblasts" and the WI38VA13 embryonic cells were intensely Pkp2-positive while the corresponding non-transformed lines were totally negative (*e.g.*, Figs. 1*a*, 1*b* and Table I). Such drastic differences between closely related cell lines could also be observed with different lines established from the same kind of human tumor such as those derived from *dermatofibrosarcoma protuberans* (DFSP) tumor samples (*e.g.*, Fig. 1*bc*1) or certain rhabdomyosarcoma sublines such as RD (*e.g.*, Fig. 1*bb*1).

These results were generally confirmed by RT-PCR analyses of mRNAs (for an example refer Fig. 1*c*), except for some cell lines devoid of—or very poor in—AJs (Fig. 1*c*, cell lines HL60 and LL24), as well as in 2-dimensional gel-electrophoreses in which mostly 1 major and 2 minor isoelectric variants of Pkp2 were detected (Fig. 1*d*; refer also[21]; for similar patterns of other plakophilins refer[41]).

The Pkp2 detected by RT-PCR and in the immunoblot analyses of gel-electrophoretic separations of cytoskeletal proteins was identified as a component of junctional complexes of the AJ-type, as also demonstrable in co-immunoprecipitates obtained with antibodies against N-cadherin or β-catenin (Fig. 2*a*) or with α-catenin antibodies (not shown).

We also characterized the possible Pkp2-containing protein complexes by gel-filtration and sucrose gradient centrifugation and noticed significant recoveries of Pkp2 in 2 kinds of particles, one with a mean S-value of a 14S and a larger particle category around 50S (Fig. 2*b*). The smaller complex form co-sedimented with N-cadherin and β-catenin (Fig. 2*b*) and a corresponding association was also demonstrable in co-immunoprecipitates from the pooled fractions 5–7 (not shown). Such results further suggested that the larger (~50S) Pkp2-containing complex category may also contain some not yet identified plaque proteins, a hypothesis that is currently under study in our laboratory.

In 2 of the human cell lines examined, SV80 and WI38VA13, we also noted the spontaneous but also stable advent of appreciable amounts of Pkp3, another plaque protein originally discovered in desmosomes which later was also identified in certain nuclear and cytoplasmic particles.[42,50,51]

TABLE I – ADVENT OF PLAKOPHILINS IN JUNCTIONS OF MALIGNANTLY TRANSFORMED MESENCHYMAL CELL LINES

	Human cells												Animal cells					
	LL24	hFB	HG261	SV80	WI38	WI38 VA13	Hs295.Sk	Hs63.T	Glioma U333	SW-684	SW-872	Hs-729	RD	B1 (b)	Rat2 (r)	L-929 (m)	VLM (m)	3T3 (m)	SV3T3 (m)	OP9 (m)
Transmembrane proteins																				
N-Cadherin	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	(+)	–	+	+	+	+
Cadherin-11	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	+
Desmoglein 1+2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Plaque proteins																				
α-catenin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+
β-catenin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Plakoglobin	+	(+)	+	+	+	(+)	+	+	+	(+)	+	+	+	+	+	(+)	+	+	+	(+)
Protein p120	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Plakophilin-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Plakophilin-2	–	–	–	+	–	+	–	+	+	–	+	–	+	–	–	+	+	(+)	–	–
Plakophilin-3	–	–	–	±	–	+	–	(–)	–	–	–	–	–	–	–	–	(–)	–	–	–
Desmoplakin 1+2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Adhering junction molecules of cell–cell contacts demonstrated in cultures of normal and malignantly transformed lines of mesenchymally derived cells as determined by SDS-PAGE and immunoblotting.

Brackets indicate cell lines in which the specific protein was either only weakly seen or detected only in some groups of cells. Symbol ± indicates that with positive as well as negative sublines have been noted (see also reference 42).

b, bovine; r, rat; m, mouse.

Localization of plakophilin-2 in adherens junctions

When the various mesenchymally-derived cells under question were examined by immunofluorescence microscopy using rigorous conditions to optimize antigen accessibility (for special short and "gentle" preparation conditions that also allow to demonstrate the more readily extractable nuclear plakophilins refer, *e.g.*,[21–23,42]), Pkp2 was distinctly localized to cell–cell junctions, for the most part in co-localization with N-cadherin and β-catenin (Fig. 3), with α-catenin, proteins p120 (Figs. 4*aa*–4*ad*) and protein p0071 (not shown; refer also[50]). Significant co-localization of Pkp2 with N-cadherin as well as with α-catenin and the AJ-typical

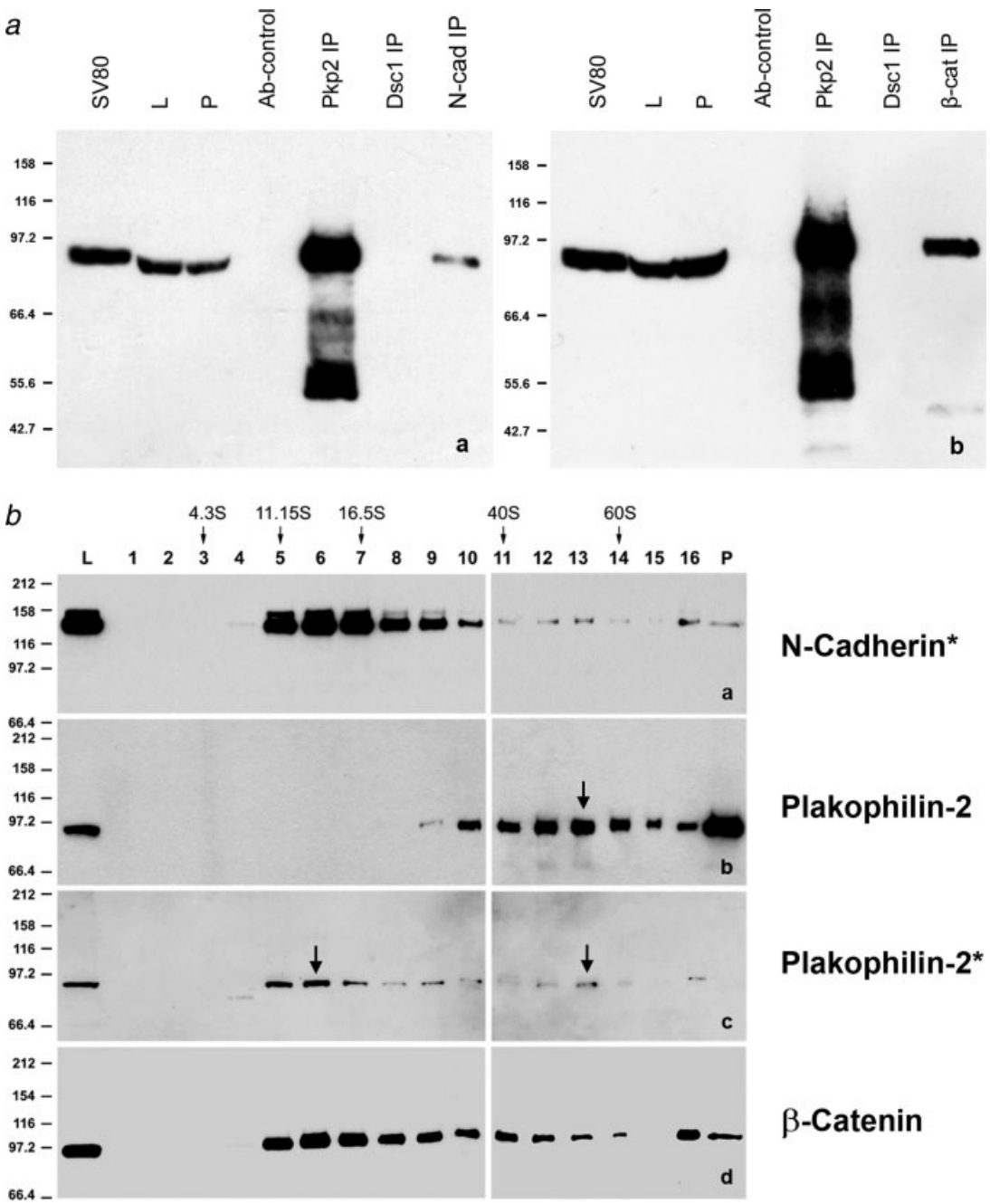


FIGURE 2 – Analyses of complexes of plakophilin-2 with adherens junction proteins. (*a*) Immunoblots of the SDS-PAGE-separated proteins of immunoprecipitates from total cell lysates of human SV40-transformed (SV80) fibroblasts. Antibodies used for immunoprecipitations (IP) were against plakophilin-2, desmocollin Dsc1, N-cadherin (*a*) and β-catenin (*b*). As positive controls, total cell lysates of SV80 cells, lysates as supernatant before IP (L) and material of the pre-clearing preparation step (P) were used. Immunoblots were performed, using monoclonal antibodies (mabs) specific for plakophilin-2. Note that beside the positive controls, IPs with plakophilin-2, N-cadherin (*a*) and β-catenin (*b*) clearly show immunoreactivity of plakophilin-2. As negative controls, the beads used after pre-clearing (Ab-control) and IP using mabs specific for desmocollin Dsc1 clearly showed an absence of plakophilin-2. Molecular weight markers (top to bottom) in kDa: 158, 116, 97.2, 66.4, 55.6, 42.7. (*b*) Proteins of lysates from SV40-transformed fibroblastoidal cells of line "SV80" were centrifuged on a linear 10–40% sucrose density gradient, fractions were collected from top to bottom and analyzed by SDS-PAGE and immunoblotting, using antibodies specific for N-cadherin (*a*), plakophilin-2 (*b* and *c*) and β-catenin (*d*). Most of the N-cadherin appears in particles with a maximum between fraction 5 and 7, corresponding to ~14S, and with a second minor peak at ~50S. Moreover plakophilin-2 appeared in 2 major particle forms which reacted somewhat differently with the 2 forms of antibodies used: Immunoreactivity in fractions with a peak between fractions 12 and 14 (mab) and with another peak around fraction 8, corresponding to mean values of ~14S and ~50S (arrows). Most of β-catenin is also recovered in fractions 5–8, corresponding to a mean value of ~14S, although here again a broad distribution of particles up to 60S was also noted for a minor proportion. Lanes labeled L and P contains total lysate (L) or the pellet (P) obtained after fractionation. The relative positions of marker proteins examined in parallel are indicated: BSA (4.3S), catalase (11.15S) and thyroglobulin (16.5S) as well as yeast ribosomal subunits (40S and 60S). N-cadherin*: Polyclonal antibodies of the preparation "pan-cadherin"; Plakophilin-2*: Guinea pig antibodies of the serum HP1.[21]

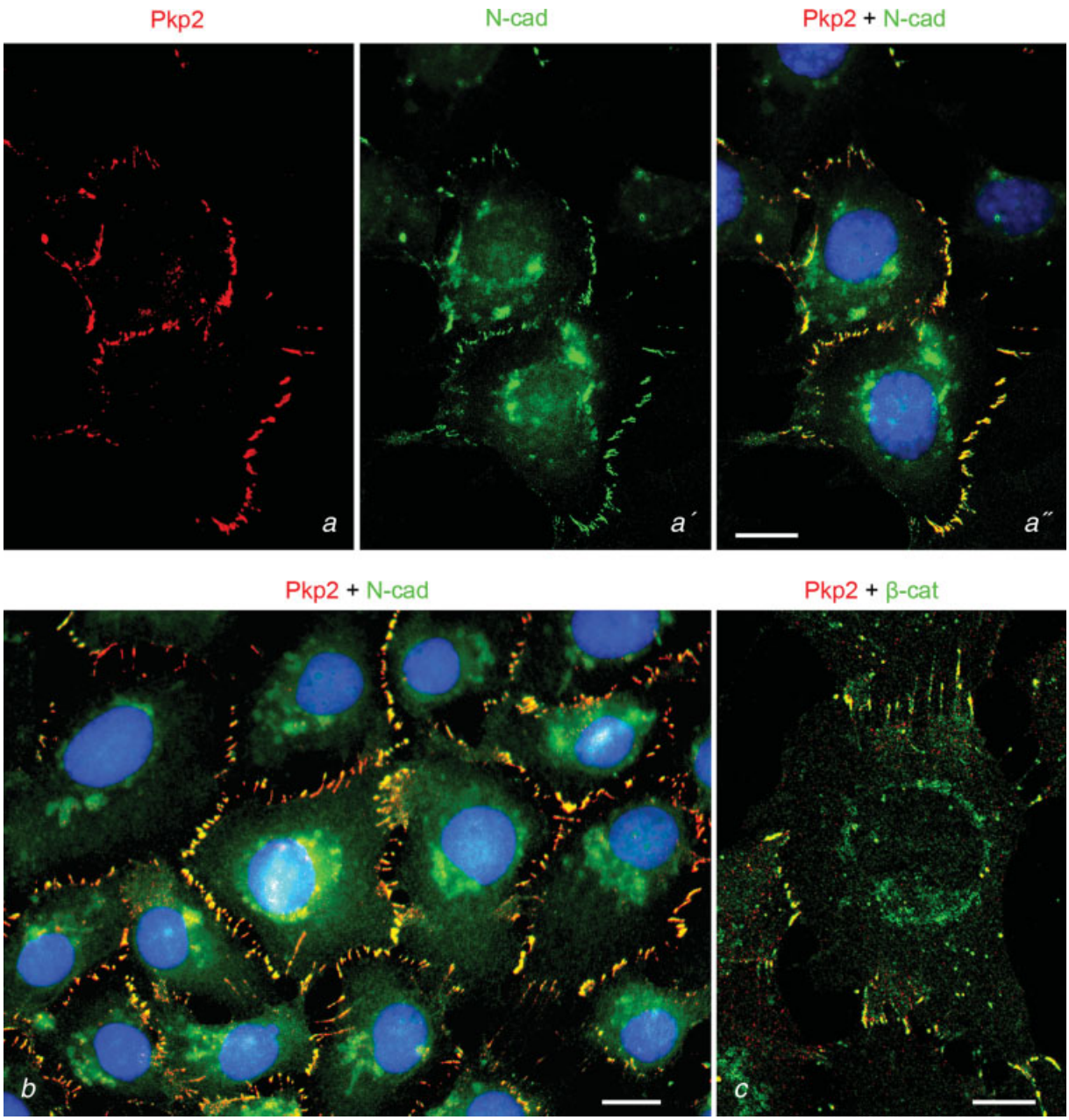


Figure 3 – Double-label immunofluorescence microscopy of SV40-transformed human fibroblastoidal cells, showing plakophilin-2 in adherens junctions. Double-label immunofluorescence microscopy images of SV40-transformed human fibroblasts (line SV80), after reaction with antibodies to plakophilin-2 (Pkp2; red), in comparison with constitutive proteins of AJs (green) such as N-cadherin (N-Cad; *a* and *b*) and β-catenin (β-cat; *c*). In both cells grown at low density (*a*, *a′*) and to confluency plakophilin-2 (*a*) and N-cadherin (*a′*) clearly co-localized in AJs (*a″*; merge colors: yellow–orange; standard fluorescence microscopy in *b* and confocal laser scanning microscopy in *c*). Note the frequency of such plakophilin-2-positive AJs in the confluent colonies of near-isodiametric cells. DAPI stain (blue) was used to visualize nuclei. Scale bars represent 10 μm.

armadillo proteins such as β-catenin was also seen in all the other Pkp2-positive cells, including the WI38VA13-cells, the rhabdomyosarcoma RD- and the DFSP-derived cells of line Hs63.T (Fig. 4*b*). In general, our immunolocalization studies have allowed us to conclude that the Pkp2 occurring in the mesenchymal cell lines found positive on gel-electrophoresis and RT-PCR is highly enriched in AJs, including the small AJs located in slender processes connecting such cells in non-confluent cultures (Fig. 4*ad*; for detailed electron microscopy of such junctions appearing on *processus adhaerentes* of mesenchymal cells refer, *e.g.*,[52]).

Electron microscopy then allowed the demonstration of the ultrastructural details of these AJs, which mostly displayed a rather thin electron-dense plaque, often laterally attached with actin microfilament bundles (*e.g.*, Figs. 5*ab*–5*ah*) or groups of microtubules (Figs. 5*ac* and 5*af*). Particularly, in subconfluent cultures, rather long filopodia-like processes were frequently seen that were dominated by a microfilament bundle core (Fig. 5*aa*) and could form small AJ-type connections with processes of other cells (Fig. 5*ab*). In certain cell lines, we also noted that several small AJs appeared to be laterally clustered as if they were approaching

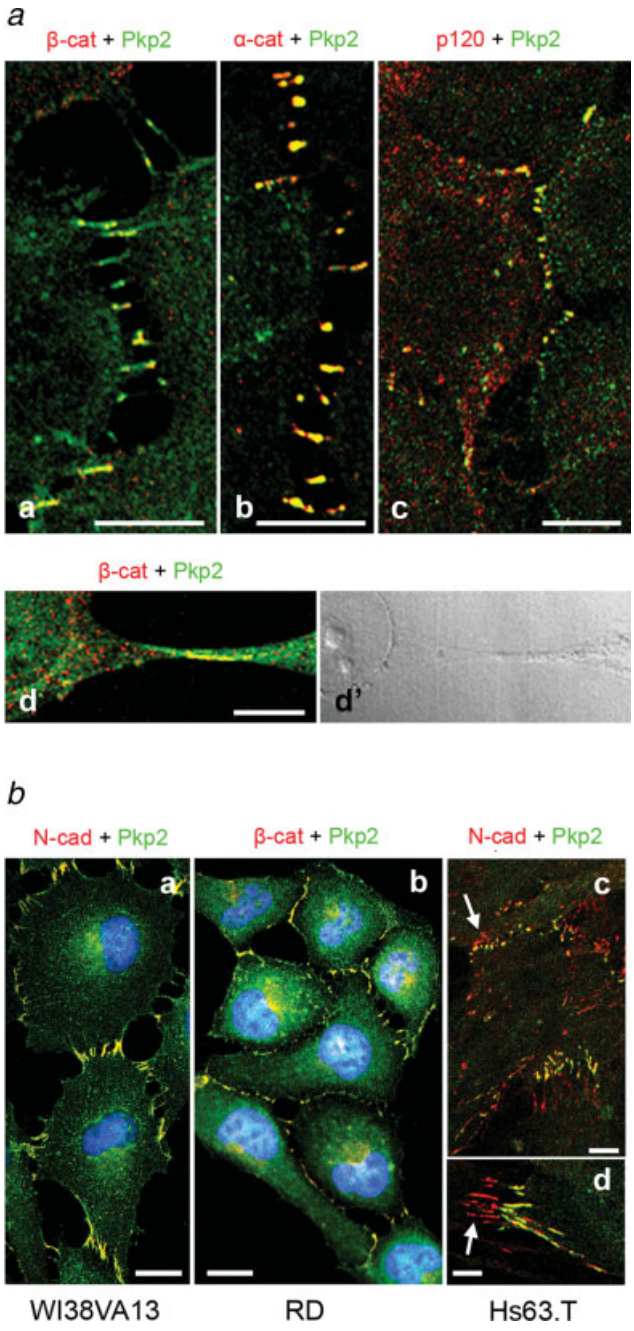


Figure 4 – Co-localization of adherens junction proteins and plakophilin-2 in human cell lines. (*a*) Double-label confocal immunofluorescence microscopy of SV40-transformed human fibroblasts (line SV80), comparing the localization of plakophilin-2 (Pkp2; green) with that of known AJ components (red). Note the co-localizations (yellow merge color) of plakophilin-2 with β-catenin (β-cat; *a* and *d*), α-catenin (α-cat; *b*) and protein p120 (*c*). Such co-localization is also seen in the small cell–cell junctions connecting slender cell processes (*d*). Phase-contrast image of *d* is indicated in *d'*. Scale bars represent 10 μm. (*b*) Double-label immunofluorescence microscopy of SV40-transformed fibroblasts of line WI38VA13 (*a*), rhabdomyosarcoma cells of line RD (*b*) and cells of the DFSP-derived line Hs63.T (*c* and *d*), labeled for N-cadherin (N-Cad; red; *a*, *c* and *d*) or β-catenin (β-cat; red; *b*) in combination with plakophilin-2 (Pkp2; green). All these malignantly transformed cell lines show co-localization of the specific AJ component with plakophilin-2. Note that not all of the intercellular junctions and bridges in cells of line Hs63.T contain plakophilin-2 (arrows in *c* and *d*). DAPI has been used to visualize nuclei. Scale bars represent 10 μm.

fusion to larger junctional structures (arrows and arrowheads in Figs. 5*ae* and 5*af*).

Using immunogold-labeling electron microscopy, we identified Pkp2 as an AJ component located in junctional complexes together with "classic" AJ plaque residents such as β-catenin (Figs. 5*ba–5bj* show a series of AJ examples of variable sizes). From such observations it became clear that the plaque-bound Pkp2 occurred in a remarkably symmetrical pattern: Pkp2 present in a given plaque of a certain cell obviously could induce the assembly of Pkp2 in the equivalent plaque of the AJ-half in the adjacent cell, and this phenomenon was visible in different lines, ranging from human fibroblasts (SV80, WI38VA13) to rhabdomyosarcoma RD cells and DFSP-derived cells (line Hs63.T).

We also examined some of the cell culture lines found positive for Pkp3 in SDS-PAGE and immunoblots (refer previous section) by immunofluorescence microscopy and noted a moderate number of groups of cells with immunostained AJs (not shown).

Occurrence of plakophilin-2-positive junctions in cultures of non-malignant mesenchymal stem cells

In primary and secondary cultures of "mesenchymal stem cells" (MSCs) derived from bone marrow or umbilical cord blood,[52] we were also able to identify sparse but clear signs of a novel kind of Pkp2-positive AJs in certain individual cells and cell groups, typically in regions of otherwise Pkp2-AJ-negative cells. Figures 6*a–6a‴* and 6*b* present such arrays of Pkp2-positive cells, and Figure 6*c* shows that Pkp2-positive AJs can also be enriched in the extended cell processes deeply protruding into—and tightly fitting in—the invaginated cell surface pockets of adjacent cells (*manubria adhaerentia*; *c.f.*,[52]). This also underscores our conclusion that the Pkp2-containing AJs do not represent a special junction newly added but that Pkp2 can be integrated in otherwise cell type-specific subforms of AJs. This sporadic appearance may also be taken as an indication that the *de novo* formation of Pkp2-AJs is not necessarily a change following a certain general signal of transformation to permanent growth or even malignancy. On the other hand, however, at present we cannot exclude that this phenomenon may just occur as a rather early change, specific for certain highly proliferative mesenchymal cells, but not necessarily for malignant growth.

The advent of plakophilin-2-containing adherens junctions in soft tissue tumors in situ

When we had noticed in many of our cell cultures of mesenchymally-derived cell lines the increased amounts of Pkp2 and its assembly with other proteins into AJ plaques, we also examined a variety of sections through samples containing "soft tissue tumor" portions. The example shown in Figure 7 presents evidence that Pkp2 can also selectively appear in some AJs connecting cells of such non-epithelial tumors, here in a rhabdomyosarcoma, whereas all other desmosomal markers examined have been negative (desmoplakin, desmogleins, desmocollins, Pkp1 and Pkp3). By double-label immunolocalization techniques, Pkp2 has been identified to co-localize in the typical N-cadherin-positive AJs characteristic of these tumors. In addition, Figure 7*b* presents groups of tumor cells in which Pkp2 is seen in practically all visible AJs. Co-localization experiments have again confirmed that the AJs connecting such Pkp2-positive AJs are also positive for other typical AJ plaque marker proteins such as α- and β-catenin (not shown).

The problem of clonal stability of cells containing plakophilin-2-positive adherens junctions

Although the spontaneous and randomly occurring cytoskeletal changes such as the advent of cytokeratin IFs in certain mesenchymally-derived cell lines, including some widely used "soft tissue tumor" reference lines, appeared to be clonally stable so that pure sublines of cytokeratin IF-rich cells could be cloned,[36,37] our results with AJs of cloned mesenchymal cells were somewhat

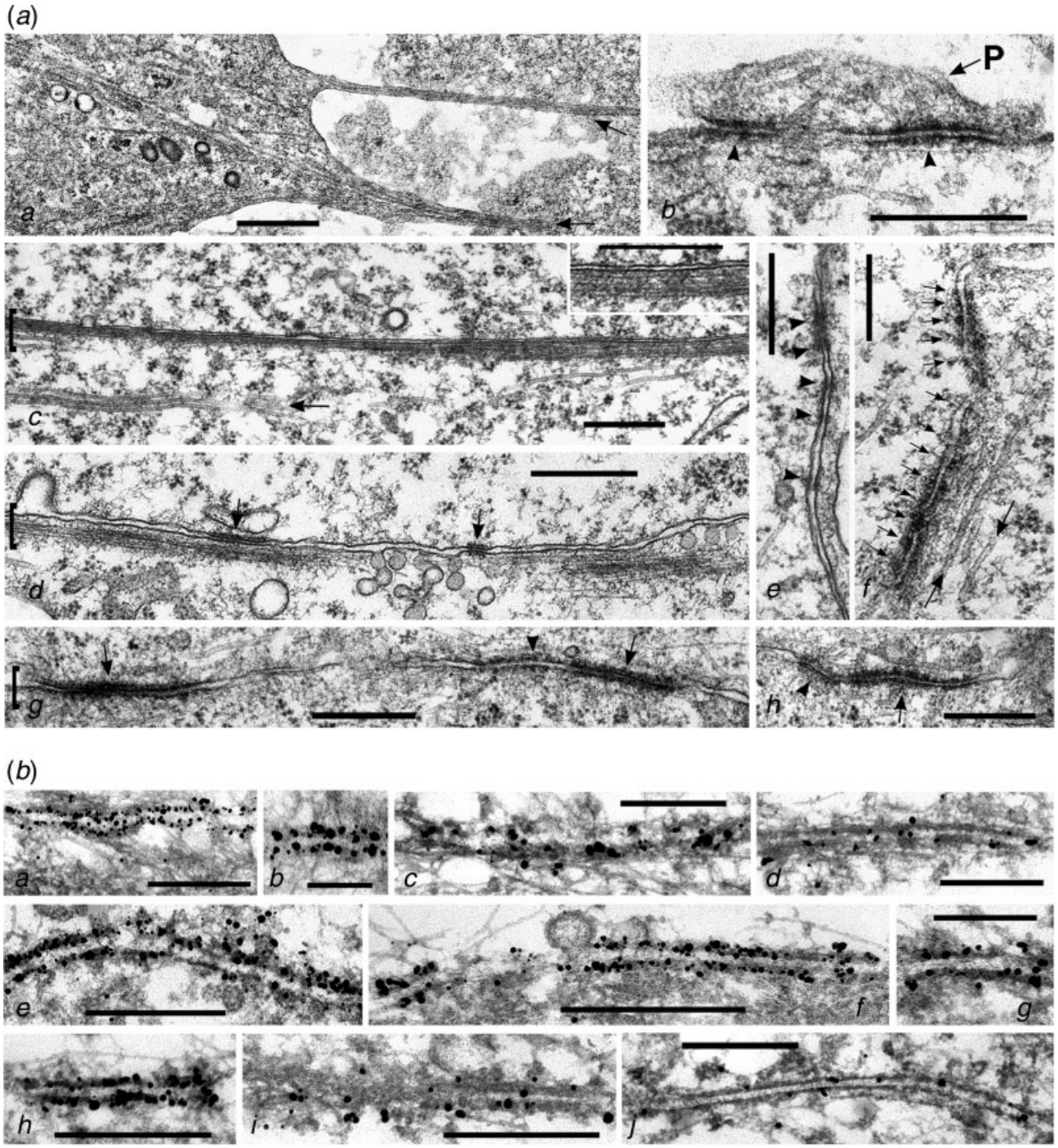


FIGURE 5 – Electron and immunoelectron microscopy of adherens junctions in mesenchymally-derived human cell culture lines. (*a*) Electron micrographs of ultrathin sections through conventionally fixed and embedded monolayer cell cultures, showing neighboring cells of the SV40-transformed human fibroblastoidal line SV80 (*a–f*) and of cells of the rhabdomyosarcoma-derived line RD (*g*, *h*), also presenting long cell processes (denoted by arrows in *a* and by the letter P in *b*) with small AJs (refer arrowheads in *b*). Near confluency, cells show numerous cell–cell contact regions with AJ morphology, including plaque-like structures and often also associated microfilament bundles (denoted by brackets and arrowheads in *c–g*; for higher magnification refer also the insert in the upper right of *c*) and cortical microtubules (*e.g.*, some are denoted by big arrows in *c* and *f*) as well as distinct junctional areas of different sizes and shapes (*d–h*; arrows and arrowheads). These cells not only show AJs of heterogeneous sizes (refer, *e.g.*, arrows in *d*), they can also occur as local clusters of separate junctions (*e*, arrowheads) composed of a series of small plaques (*f*, arrows) or as local close aggregates of very small plaque-like structures (arrows and arrowheads in *e* and *f*). By comparison, larger AJs are relatively rare (refer, *e.g.*, arrow and arrowheads in *g* and *h*). Scale bars represent 1 μm (*a*), 0.5 μm (*b–h*). (*b*) Immunoelectron micrographs of DFSP-derived cells of line Hs63.T (*a–d*), showing the specificity of the silver-enhanced immunogold grain reaction of antibodies specific for β-catenin (*a*, *b*) as well as for plakophilin-2 (*c*, *d*). Note that the various forms of AJs are denoted by the nanogold particles. (*e–j*) Cells of the rhabdomyosarcoma-derived line RD also show the localization of β-catenin (*e–g*), N-cadherin (*h*, *i*) and plakophilin-2 (*j*) in AJs. Scale bars represent 0.5 μm (*a–f* and *h–j*) and 0.25 μm (*g*).

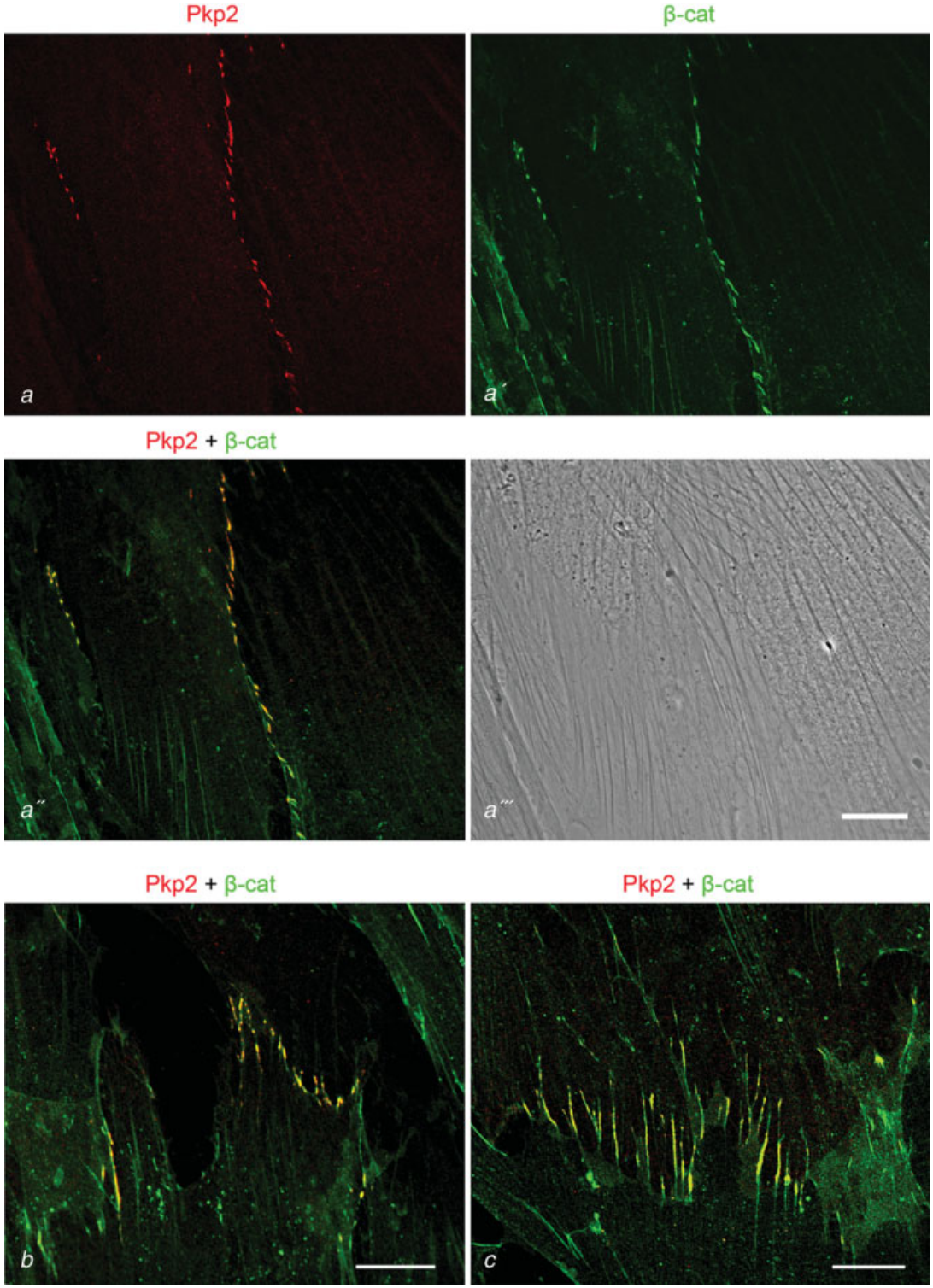


FIGURE 6 – Advent of plakophilin-2 in adherens junction connecting individual cells or small groups of cultured human bone marrow-derived mesenchymal cells. Double-label immunofluorescence microscopy of cultured human bone marrow-derived mesenchymal cells labeled for plakophilin-2 (Pkp2; red; *a*) in combination with the AJ reference protein, β-catenin (β-cat; green; *a′*). The merged picture is shown in *a″*; *a‴*[presents the phase contrast appearance of the confluent cell culture. In a limited region of cell–cell contacts, AJs show co-localization (yellow–orange) of the 2 plaque proteins. (*b*) Often the plakophilin-2-containing AJs are locally restricted, small and isolated; (*c*) presents an example of extended cell–cell bridges rich in AJs (refer the *manubria adhaerentia* of[52]) containing plakophilin-2. Scale bars represent 20 μm.

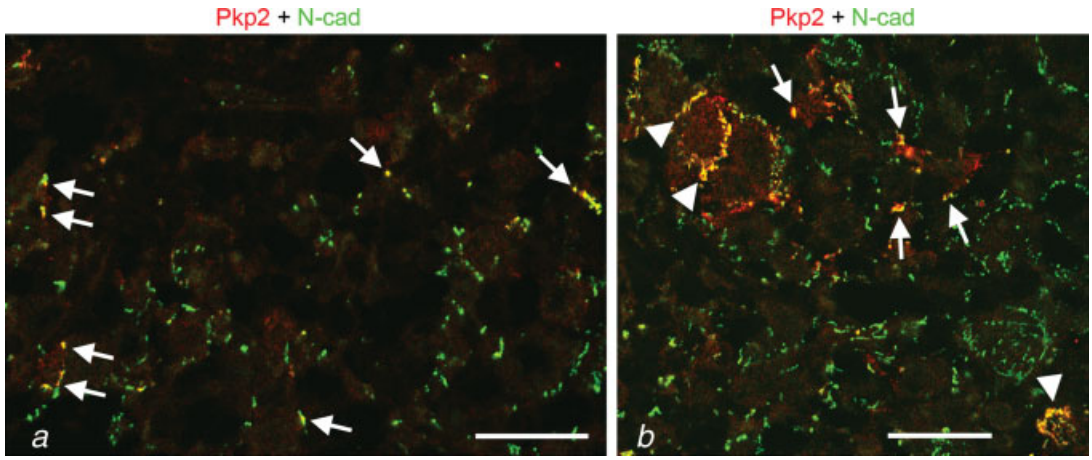


Figure 7 – Advent of plakophilin-2-containing adherens junctions in a human rhabdomyosarcoma *in situ* as demonstrated by immunostaining using double-label confocal immunofluorescence microscopy. Paraffin-embedded tissue of a formalin-fixed sample of human pleomorphic rhabdomyosarcoma of the *corpus uteri* has been treated using the microwave "antigen retrieval"—technique and reacted with antibodies specific for plakophilin-2 (Pkp2; red) in combination with antibodies to N-cadherin (N-cad; green). Note that all of the AJs detectable contain N-cadherin, whereas only some of them are also positive for plakophilin-2 (arrows; yellow–orange merge color). In some tumor regions such plakophilin-2-positive AJs appear widely dispersed (*a*) whereas in other parts of the tumor plakophilin-2 can be enriched in certain colonies (arrowheads) of cells (*b*). Scale bars represent 20 μm.

different: Clonal progeny of Pkp2-positive fibroblastoid cell types such as human SV80 cells was rather stable whereas most other clonally obtained cell lines showed some re-segregation into Pkp2-positive and Pkp2-negative subforms (data not shown). As this phenomenon of a tendency of a certain percentage of cells to switch back to the original Pkp2-negative form is presently a still unexplainable phenomenon, we have decided to elucidate the molecular patterns and mechanisms of such segregations in experimental detail in the future.

Discussion

In contrast to epithelial and myocardial cells, the cells of the mesenchymal tissues of the mammalian body are interconnected only by a single and simple type of AJs, mostly characterized by the transmembrane glycoprotein N-cadherin, with or without one of the other cadherins such as cadherin-11, anchored in a submembranous dense cytoplasmic plaque containing α- and β-catenin, mostly together with at least 3 further *armadillo*-type proteins, *e.g.*, plakoglobin as well as proteins p120 and p0071 (for the latter refer[39]). Therefore, our finding that AJs of many transformed cell culture lines of mesenchymal origin constitutively contain an additional *armadillo* protein, Pkp2, so far only known as a constituent of desmosomes in epithelia and a few other types (for references refer[13,14,21,22,28]) as well as tumors derived from such tissues (*e.g.*,[21,22]), represents a fundamental change in the molecular ensemble of a prominent cell–cell adhesion structure. Indeed, Pkp2 has so far never been detected in cell–cell junctions of normal mesenchymal tissues. The observation of this addition of Pkp2 to the AJ-plaque protein ensemble is all the more important as this molecule has been shown to be a molecule capable of binding and stably complexing a series of other junctional proteins.[24,53] In addition, Pkp2 is complexed to several cadherins such as desmogleins and desmocollins and N-cadherin as reported for cardiomyocytes and in the present report (for reviews refer also[2,54]).

Finally, the importance of Pkp2 for cell–cell adhesions in interphase as well as in mitosis is also indicated by gene mutation analyses as well as gene abrogation and mRNA knock-down experiments, which all have shown that the reduction of Pkp2 alone for an extended period of time can be sufficient for loosening cell–cell contacts and promoting the separation of cells (*e.g.*,[25–28]) and that even small mutations in the Pkp2-gene can result in lethal dysfunctions of cell–cell adhesion (*e.g.*,[29]; for further references refer Introduction). Therefore, we may hypothesize that the additional integration of Pkp2 into AJ plaques of mesenchymally-derived cells and tumors can markedly enhance the stability or the adhesive potential of these AJs.

The general increase of cellular Pkp2 and its integration into AJ plaque structures probably has also important effects on other cell structures and functions. As junctional plaque components have been shown to be able to compete for plaque binding capacity[24] (for reviews refer also[54,55]) we presently cannot exclude the possibility that the addition of sizable amounts of Pkp2 to the plaque ensemble results in a compensatory release of other plaque components, in particular of β-catenin, which in turn is known to be involved in a series of important regulatory functions in the nucleus. Of course, such possible mutual effects of junction-bound β-catenin and plakophilins on nuclear complexes and functions (refer also[23]) will have to be examined in special experiments.

In general, stability and adhesive force of AJs are also critical for contact formations, interactions and architectural connections of mesenchymal cells with each other as well as with other kinds of cells and tissues, either in normal development or a result of malignant transformation. Thus, rather small AJs have been shown to form the initial contacts as well as semistable connections—even over long distances and for remarkable periods of time—as shown in special detail for bone marrow- or umbilical cord blood-derived human mesenchymal stem cells growing in culture.[52] It is also clear that mesenchymally-derived cells can form extended, thin, AJ-studded cytoplasmic processes some of which can deeply insert into invaginations of neighboring cells, thus producing a tight-fitting interdigitation system with extended, laterally fused AJ-structures (*manubria adhaerentia*). Further special plaque-bearing internalizing AJ complexes are also the essential cell contact structures of the engulfment and destruction system named "entosis," which is also based on cadherin-mediated adhesion, although here so far only examples of epithelial cells have been reported in the literature.[56]

Mesenchymal cells are a highly heterogeneous group of cell types and this diversity is further magnified by the fact that during—and as a result of—malignant transformation the diversity of each cell type may even increase further (for some examples including tumor cell-stromal tissue refer, *e.g.*,[57–60]). This suggests that the morphological and the molecular typological diversity in the mesenchymal kingdom of differentiations, including their AJ

systems, may be almost as great as it has been reported for epithelia and their junction systems (for reviews refer, *e.g.*,[61]). From the present report, it is now also clear that among soft tissue tumor cells, 2 categories can—and have to—be distinguished, *i.e.*, the Pkp2-positive and the Pkp2-negative forms. Elucidation of these molecular differences will certainly provide valuable additional diagnostic information and the regulatory key protein Pkp2 may become an important molecular addition to the histodiagnostic armamentarium for soft tissue tumors.

Acknowledgements

The authors thank Ms. Heiderose Schumacher, Ms. Michaela Hergt and Ms. Christine Grund for excellent technical assistance. We also thank Prof. Roland Moll (Institute of Pathology, Philipps University Marburg) for the gift of the tumor sample shown in Figure 7. This work was supported by a grant from the Deutsche Krebshilfe and the German Ministry for Research and Technology (W.W.F.).

References

1. Koch PJ, Franke WW. Desmosomal cadherins: another growing multigene family of adhesion molecules. Curr Opin Cell Biol 1994;6:682–7.
2. Godsel LM, Getsios S, Huen AC, Green KJ. The molecular composition and function of desmosomes. In: Behrens J, Nelson WJ, eds. Cell adhesion, vol.165. Berlin, Heidelberg, New York: Springer Verlag, 2004:137–93.
3. Weiss SW, Goldblum JR. Soft tissue tumors, 4th edn. St. Louis: Mosby, 2001.
4. Miettinen M. Diagnostic soft tissue pathology, 1st edn. New York, Edinburgh, London, Philadelphia: Churchill Livingstone, 2003.
5. Takeichi M. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 1995;7:619–27.
6. Nollet F, Kools P, van Roy F. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. J Mol Biol 2000;299:551–72.
7. Perez TD, Nelson WJ. Cadherin adhesion: mechanisms and molecular interactions. In: Behrens J, Nelson WJ, eds. Cell adhesion, vol.165. Berlin, Heidelberg, New York: Springer Verlag, 2004:3–21.
8. Cowin P, Kapprell HP, Franke WW, Tamkun J, Hynes RO. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. Cell 1986;46:1063–73.
9. Herrenknecht K, Ozawa M, Eckerskorn C, Lottspeich F, Lenter M, Kemler R. The uvomorulin-anchorage protein alpha catenin is a vinculin homologue. Proc Natl Acad Sci USA 1991;88:9156–60.
10. Hirano S, Kimoto N, Shimoyama Y, Hirohashi S, Takeichi M. Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. Cell 1992;70:293–301.
11. Takai Y, Nakanishi H. Nectin and afadin: novel organizers of intercellular junctions. J Cell Sci 2003;116:17–27.
12. Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell 2005;123:903–15.
13. Franke WW, Borrmann CM, Grund C, Pieperhoff S. The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. Eur J Cell Biol 2006;85:69–82.
14. Goossens S, Janssens B, Bonne S, De Rycke R, Braet F, van Hengel J, van Roy F. A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs. J Cell Sci 2007;120:2126–36.
15. Schmelz M, Franke WW. Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: the syndesmos connecting retothelial cells of lymph nodes. Eur J Cell Biol 1993;61:274–89.
16. Moll R, Sievers E, Haemmerling B, Schmidt A, Barth M, Kuhn C, Grund C, Hofmann I, Franke WW. Endothelial and virgultar cell formations in the mammalian lymph node sinus: endothelial differentiation morphotypes characterized by a special kind of junction (complexus adhaerens). Cell Tissue Res 2009;335:109–41.
17. Straub BK, Boda J, Kuhn C, Schnoelzer M, Korf U, Kempf T, Spring H, Hatzfeld M, Franke WW. A novel cell-cell junction system: the cortex adhaerens mosaic of lens fiber cells. J Cell Sci 2003;116:4985–95.
18. Franke WW, Moll R, Mueller H, Schmid E, Kuhn C, Krepler R, Artlieb U, Denk H. Immunocytochemical identification of epithelium-derived human tumors with antibodies to desmosomal plaque proteins. Proc Natl Acad Sci USA 1983;80:543–7.
19. Moll R, Cowin P, Kapprell HP, Franke WW. Desmosomal proteins: new markers for identification and classification of tumors. Lab Invest 1986;54:4–25.
20. Folpe AL, Gown A. Immunohistochemistry for analysis of soft tissue tumors. In: Weiss S, Goldblum J, eds. Soft tissue tumors, 4th edn. St. Louis: Mosby, 2001:199–245.
21. Mertens C, Kuhn C, Franke WW. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J Cell Biol 1996;135:1009–25.
22. Mertens C, Kuhn C, Moll R, Schwetlick I, Franke WW. Desmosomal plakophilin 2 as a differentiation marker in normal and malignant tissues. Differentiation 1999;64:277–90.
23. Mertens C, Hofmann I, Wang Z, Teichmann M, Sepehri Chong S, Schnoelzer M, Franke WW. Nuclear particles containing RNA polymerase III complexes associated with the junctional plaque protein plakophilin 2. Proc Natl Acad Sci USA 2001;98:7795–800.
24. Chen X, Bonne S, Hatzfeld M, van Roy F, Green KJ. Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and beta -catenin signaling. J Biol Chem 2002;277:10512–22.
25. Grossmann KS, Grund C, Huelsken J, Behrend M, Erdmann B, Franke WW, Birchmeier W. Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. J Cell Biol 2004; 167:149–60.
26. Oxford EM, Musa H, Maass K, Coombs W, Taffet SM, Delmar M. Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. Circ Res 2007;101:703–11.
27. Fidler LM, Wilson GJ, Liu F, Cui X, Scherer SW, Taylor GP, Hamilton RM. Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations. J Cell Mol Med, in press.
28. Pieperhoff S, Schumacher H, Franke WW. The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference. RNA-mediated knockdown in cultured rat cardiomyocytes. Eur J Cell Biol 2008;87:399–411.
29. Gerull B, Heuser A, Wichter T, Paul M, Basson CT, McDermott DA, Lerman BB, Markowitz SM, Ellinor PT, MacRae CA, Peters S, Grossmann KS, et al. Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. Nat Genet 2004;36:1162–4.
30. Dalal D, James C, Devanagondi R, Tichnell C, Tucker A, Prakasa K, Spevak PJ, Bluemke DA, Abraham T, Russell SD, Calkins H, Judge DP. Penetrance of mutations in plakophilin-2 among families with arrhythmogenic right ventricular dysplasia/cardiomyopathy. J Am Coll Cardiol 2006;48:1416–24.
31. Dalal D, Molin LH, Piccini J, Tichnell C, James C, Bomma C, Prakasa K, Towbin JA, Marcus FI, Spevak PJ, Bluemke DA, Abraham T, et al. Clinical features of arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in plakophilin-2. Circulation 2006;113:1641–9.
32. Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ. Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. Circulation 2006; 113:356–64.
33. van Tintelen JP, Entius MM, Bhuiyan ZA, Jongbloed R, Wiesfeld AC, Wilde AA, van der Smagt J, Boven LG, Mannens MM, van Langen IM, Hofstra RM, Otterspoor LC, et al. Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. Circulation 2006;113:1650–8.
34. Boda-Heggemann J, Regnier-Vigouroux A, Franke WW. Beyond vessels: occurrence and regional clustering of vascular endothelial (VE-) cadherin-containing junctions in non-endothelial cells. Cell Tissue Res 2009;335:49–65.
35. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761–71.
36. Knapp AC, Bosch FX, Hergt M, Kuhn C, Winter-Simanowski S, Schmid E, Regauer S, Bartek J, Franke WW. Cytokeratins and cytokeratin filaments in subpopulations of cultured human and rodent cells of non-epithelial origin: modes and patterns of formation. Differentiation 1989;42:81–102.
37. Knapp AC, Franke WW. Spontanous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occuring at different levels of regulation. Cell 1989;59:67–79.

38. Geiger B, Volberg T, Ginsberg D, Bitzur S, Sabanay I, Hynes RO. Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. J Cell Sci 1990;97:607–14.
39. Hofmann I, Schlechter T, Kuhn C, Hergt M, Franke WW. Protein p0071—an armadillo plaque protein that characterizes a specific subtype of adherens junctions. J Cell Sci 2009;122:21–4.
40. Cowin P, Kapprell HP, Franke WW. The complement of desmosomal plaque proteins in different cell types. J Cell Biol 1985;101:1442–54.
41. Heid HW, Schmidt A, Zimbelmann R, Schafer S, Winter-Simanowski S, Stumpp S, Keith M, Figge U, Schnoelzer M, Franke WW. Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). Differentiation 1994;58:113–31.
42. Schmidt A, Langbein L, Pratzel S, Rode M, Rackwitz HR, Franke WW. Plakophilin 3-a novel cell-type-specific desmosomal plaque protein. Differentiation 1999;64:291–306.
43. Nuber UA, Schafer S, Stehr S, Rackwitz HR, Franke WW. Patterns of desmocollin synthesis in human epithelia: immunolocalization of desmocollins 1 and 3 in special epithelia and in cultured cells. Eur J Cell Biol 1996;71:1–13.
44. Schmelz M, Duden R, Cowin P, Franke WW. A constitutive transmembrane glycoprotein of Mr 165,000 (desmoglein) in epidermal and non-epidermal desmosomes. II. Immunolocalization and microinjection studies. Eur J Cell Biol 1986;42:184–99.
45. Kurzen H, Moll I, Moll R, Schafer S, Simics E, Amagai M, Wheelock MJ, Franke WW. Compositionally different desmosomes in the various compartments of the human hair follicle. Differentiation 1998;63:295–304.
46. Achtstaetter T, Hatzfeld M, Quinlan RA, Parmelee DC, Franke WW. Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. Methods Enzymol 1986;134:355–71.
47. Shi SR. Antigen retrieval techniques: immunohistochemistry and molecular morphology. Natick: Eaton Publishing, 2000.
48. Rickelt S, Franke WW, Doerflinger Y, Goerdt S, Brandner JM, Peitsch WK. Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. Cell Tissue Res 2008;334:401–22.
49. Ho AD, Wagner W, Franke W. Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 2008;10:320–30.
50. Hofmann I, Casella M, Schnoelzer M, Schlechter T, Spring H, Franke WW. Identification of the junctional plaque protein plakophilin 3 in cytoplasmic particles containing RNA-binding proteins and the recruitment of plakophilins 1 and 3 to stress granules. Mol Biol Cell 2006;17:1388–98.
51. Bonné S, van Hengel J, Nollet F, Kools P, van Roy F. Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells. J Cell Sci 1999;112:2265–76.
52. Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U, Seckinger A, Peitsch WK, Spring H, Ho AD, Franke WW. Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res 2007;328:499–514.
53. Koeser J, Troyanovsky SM, Grund C, Franke WW. De novo formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. Exp Cell Res 2003; 285:114–30.
54. Hatzfeld M. The p120 family of cell adhesion molecules. Eur J Cell Biol 2005;84:205–14.
55. Schmidt A, Jaeger S. Plakophilins-hard work in the desmosome, recreation in the nucleus? Eur J Cell Biol 2005;84:189–204.
56. Overholtzer M, Mailleux AA, Mouneimne G, Normand G, Schnitt SJ, King RW, Cibas ES, Brugge JS. A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell 2007;131:966–79.
57. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. J Pathol 2003;200:429–47.
58. Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ. Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell 2004;15:4310–20.
59. Kiener HP, Brenner MB. Building the synovium: cadherin-11 mediates fibroblast-like synoviocyte cell-to-cell adhesion. Arthritis Res Ther 2005;7:49–54.
60. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006;6:392–401.
61. Strumane K, Berx G, Roy FV. Cadherins in cancer. In: Behrens J, Nelson WJ, eds. Cell adhesion, vol.165. Berlin, Heidelberg, New York: Springer Verlag, 2004:69–103.

Cell Tissue Res (2009) 338:1–17
DOI 10.1007/s00441-009-0849-z

REVIEW

The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind

Werner W. Franke · Steffen Rickelt · Mareike Barth · Sebastian Pieperhoff

Received: 12 June 2009 / Accepted: 16 July 2009 / Published online: 14 August 2009

Abstract Immunocytochemical, electron-, and immunoelectron-microscopical studies have revealed that, in addition to the four major "textbook categories" of cell-cell junctions (gap junctions, tight junctions, adherens junctions, and desmosomes), a broad range of other junctions exists, such as the tiny *puncta adhaerentia minima*, the taproot junctions (*manubria adhaerentia*), the plakophilin-2-containing adherens junctions of mesenchymal or mesenchymally derived cell types including malignantly transformed cells, the composite junctions (*areae compositae*) of the mature mammalian myocardium, the *cortex adhaerens* of the eye lens, the interdesmosomal "sandwich" or "stud" junctions in the subapical layers of stratified epithelia and the tumors derived therefrom, and the *complexus adhaerentes* of the endothelial and virgultar cells of the lymph node sinus. On the basis of their sizes and shapes, other morphological criteria, and their specific molecular ensembles, these junctions and the genes that encode them cannot be subsumed under one of the major categories mentioned above but represent special structures in their own right, appear to serve special functions, and can give rise to specific pathological disorders.

Keywords Junctions · Desmosomes · Area composita · Filopodium · Plaque

Abbreviations

AJ	adherens junction
JAM	junction adhesion molecule
MAGUK	membrane-associated guanylate kinase
TJ	tight junction
MSCs	mesenchymal stem cells
PAM	*puncta adhaerentia minima*
ARVC	arrhythmogenic ventricular cardiomyopathies

The authors thank the "Deutsche Krebshilfe" (grant 10-2049-Fr I and II to W.W.F.) and the German Ministry for Research and Technology (Program Regenerative Medicine, START-MSC, to W.W.F.) for financial support. Sebastian Pieperhoff is grateful to the Canadian Government (DFAIT) for a Postodoctoral Research Fellowship (PDRF; 03/2008-03/2009) and the German Science Foundation (DFG) for a Postodctoral Research Fellowship (from 04/2009).

W. W. Franke (✉) · S. Rickelt · M. Barth · S. Pieperhoff
Helmholtz Group for Cell Biology,
German Cancer Research Center,
Im Neuenheimer Feld 581, Building TP4 - Room S3.222,
69120 Heidelberg, Germany
e-mail: w.franke@dkfz.de

S. Pieperhoff
Department of Zoology and Faculty of Land and Food Systems,
University of British Columbia,
2357 Main Mall,
Vancouver, BC V6T 1Z4, Canada

Introduction

An essential development in the evolution of multicellular organisms with a variety of tissues serving different functions has obviously been the formation of specific semi-stable and dynamic cell-cell junctions, i.e., architectonically positioned structures of limited size that connect cells of the same or different types into higher order organs. Laterally, i.e., in the same plasma membrane, such assemblies can be homophilic or heterophilic and are generally oriented head-to-head, usually with distinct substructures.

Major junctional types

In present textbooks of cell biology, four major categories of cell-cell junctions are distinguished (Table 1; for a historic review, see Franke 2009):

(1) Gap junctions (nexus) appear as densely packed hemichannels composed of tetraspan membrane proteins, which belong to the connexin family and which are symmetrically oriented into channels that allow cell-cell exchange of small molecules.
(2) Tight junctions (TJ; *zonulae* or *fascial adhaerentes*) are arrays of tetraspan transmembrane proteins forming tight-sealing bands of various lengths, often branched or ornamentally woven. These proteins are arranged head-to-head into membrane barrier structures containing cell-type-specific combinations of the claudin and the occludin families of proteins, mostly in association with specific immunoglobulin-like proteins of the junction adhesion molecule (JAM) group spanning the membrane once.
(3) Adherens junctions (AJ) are a group of variously sized and shaped cell-type-specific assemblies of glycoproteins of the cadherin family spanning the membrane once and capable of forming a continuous cell-surrounding belt (*zonula adhaerens*) or streak-like *fascia adhaerens*, or local near-isodiametric *puncta adhaerentia*.
(4) Generally the thickest and most robust junction type is represented by the desmosomes (*maculae adhaerentes*) formed by special subsets of cadherins (desmogleins, desmocollins).

In addition, these junctions are associated, on their cytoplasmic face, with specific ensembles of "coating" proteins, which again display similarities and junction-type-specific differences:

(1) Gap junctions do not reveal a distinct, i.e., electron microscopically demonstrable cytoplasmic plaque, but their connexins are complexed with cytoplasmic proteins of the membrane-associated guanylate kinase (MAGUK) family, which in turn can interact with microtubules or actin filaments (see anthology by Peracchia 2000).
(2) TJs are also associated with a thin and barely visible coat containing MAGUK proteins, specifically proteins ZO-1 – ZO-3, plus cingulin and a series of other proteins (see anthology by Cereijido and Anderson 2001, notably therein the review by Citi 2001).
(3) AJs are characterized by clearly demonstrable plaque structures of varying thickness, made up of cell-type-specific combinations of *armadillo* (*arm*)-type proteins, e.g., plakoglobin, β-catenin, proteins p120, p0071, and ARVCF, and neurojungin, together with vinculin-like or other actin-binding proteins such as α-catenin, vinculin, and afadin (for reviews, see the anthologies of Behrens and Nelson 2004; LaFlamme and Kowalczyk 2008).
(4) The plaques of desmosomes, the cadherins of which can project into (and even through) the mostly prominent and dense plaque, also contain plakoglobin, but in addition plakophilin-2 or combinations of two plakophilins, together with the special plaque protein, desmoplakin (for reviews, see the aforementioned anthologies and Holthöfer et al. 2007; Waschke 2008).

Other junctional types

In recent years, a series of conspicuous cell-type-specific forms of symmetrical cell-cell junctions with diverse shapes, sizes, and unusual molecular ensembles or complexities have been ultrastructurally and analytically characterized to a considerable degree. These studies have strengthened the conclusion that the structures under question are special junctions in their own right. Their characteristic structures and molecular ensembles known so far will be briefly described here and their possible functional significance will be discussed.

1. *Puncta adhaerentia minima* (minimal dot junctions)

Extremely small AJs have been found on the surfaces of several kinds of mesenchymally derived cells grown in cell culture, in particular in cultures of specific subsets of bone-marrow-, placenta-, or adipose-tissue-derived mesenchymal stem cells (MSCs) and in cultures of interstitial cells derived from specific organs such as the matrix of cardiac valves. Sparse cultures of such mesenchymally derived cells are characterized by the frequent occurrence of filopodia-like cell processes of widely variable lengths, including some that may even exceed 400 μm and that are studded in varying frequencies and patterns with punctate, often extremely small (20–50 nm diameter) AJs (Fig. 1a–c; see, e.g., Wuchter et al. 2007; Barth et al. 2009). In other words, the diameters of the smallest of these AJs are not much greater than those of nearby microtubules. These "minimal-size" AJs (*puncta adhaerentia minima*; PAM) are clearly different from the AJ-like structures located in the shorter "zipper" bridge structures connecting cultured murine keratinocytes (Vasioukhin et al. 2000). Light- and electron-microscopic immunolocalization, supported by the analytical biochemistry of total cell junctional proteins, have allowed the identification of N-cadherin and cadherin-11 in these PAM, together with α- and β-catenin, protein p120, and afadin as regular components (e.g., Fig. 1d; cf.

Table 1 Molecular components of the major categories (*I–IV*) and several other forms (*1–7*) of mammalian symmetrical (homotypic) junctions (*JAM* junction adhesion molecule, *brackets* not regularly seen in all cells, *nd* not decided as yet)

Type	Occurrence	Associated filaments	Transmembrane proteins and glycoproteins	Specific plaque proteins (selection of hallmark representatives)
I. Desmosomes				
Maculae adhaerentes	Epithelial cells	Intermediate-sized filaments (keratins, vimentin, desmin)	Desmogleins Dsg 1–3[a]	Plakoglobin
	Cardiomyocytes			Desmoplakins
	Meningothelial cells Reticulum cells of thymus and lymph follicles		Desmocollins Dsc 1–3[a]	Plakophilins 1–3[a]
II. Adherens junctions				
Zonulae adhaerentes	Epithelial cells Endothelial cells	Microfilaments (actin)	Typical cadherins[a] (e.g., E-cadherin, N-cadherin, VE-cadherin, cadherin-11) nectin	α- and β-Catenin, plakoglobin, protein p120, protein ARVCF, protein p0071, neurojungin[b], proteins ZO-1-3, afadin, vinculin
Fasciae adhaerentes	Various types of cardiomyocytes			
Puncta adhaerentia	Mesenchymal and neural cells			
III. Tight junctions				
Zonulae occludentes	Epithelial cells	– [d]	Occludin Claudins 1–24[a]	Proteins ZO-1-3
	Endothelial cells		Tricellulin(s)[c] JAM proteins	Cingulin
IV. Gap junctions				
Nexus	All kinds of tissue-forming cells	–	Connexins 1-21[a]	Proteins ZO-1-3
1. Minimal dot junctions				
Puncta adhaerentia minima	Mesenchymal cells	Microfilaments (actin)	N-cadherin, cadherin-11	α- and β-Catenin, proteins p120, p0071, ARVCF, (plakoglobin[e]), afadin
2. Taproot adherens junctions				
Manubria adhaerentia	Mesenchymal cells in culture	Microfilaments (actin)	N-cadherin, cadherin-11	α- and β-Catenin, (plakoglobin[e]), proteins p120, p0071, ARVCF, proteins ZO-1-3, afadin, vinculin
3. Plakophilin-2-containing adherens junctions				
Coniunctiones adhaerentes	Mesenchymally derived cells of high proliferative activity	Microfilaments (actin)	N-cadherin, cadherin-11 (nectin)	α- and β-Catenin, plakoglobin[e], proteins p120 and p0071[a], plakophilin-2, (plakophilin-3[f]), proteins ZO-1-3, afadin, vinculin
4. Composite junctions				
Areae compositae	Cardiomyocytes of maturing and adult heart	Microfilaments (actin)	N-cadherin Cadherin-11	Desmoplakin, α- and β-catenin, proteins p120, ARVCF and p0071, plakophilin-2, proteins ZO-1–3
		Intermediate-sized filaments	Desmoglein-2 Desmocollin-2	
5. Adherens cortex				
Cortex adhaerens	Eye lens interior	nd[d]	N-cadherin, cadherin-11	α- and β-Catenin, plakoglobin, protein p120, ezrin, periplakin, periaxin

Table 1 (continued)

Type	Occurrence	Associated filaments	Transmembrane proteins and glycoproteins	Specific plaque proteins (selection of hallmark representatives)
6. Sandwich junctions				
Iuncturae structae	Epidermal stratum spinosum or equivalent layers of other stratified epithelia	nd	Occludin, claudins	nd
7. Complex junctions				
Complexus adhaerentes	Endothelial and virgultar cells of lymph node sinus	nd	N-cadherin Cadherin-11 VE-cadherin Claudin-5 JAM proteins	Desmoplakin, α- and β-catenin, protein p120, plakoglobin, proteins ZO-1–3, afadin

[a] One isoform or combinations of a few representatives, often with cell-type and cell-layer specificities

[b] Only cell-type-specific combinations of the *armadillo*-type proteins underlined

[c] There are two mRNA splice products of which only one protein has so far been localized

[d] Actin microfilaments are seen near the junctions, but their specific association is not clear

[e] Plakoglobin has been demonstrated only in some cells and with markedly differing intensities, even in the same culture

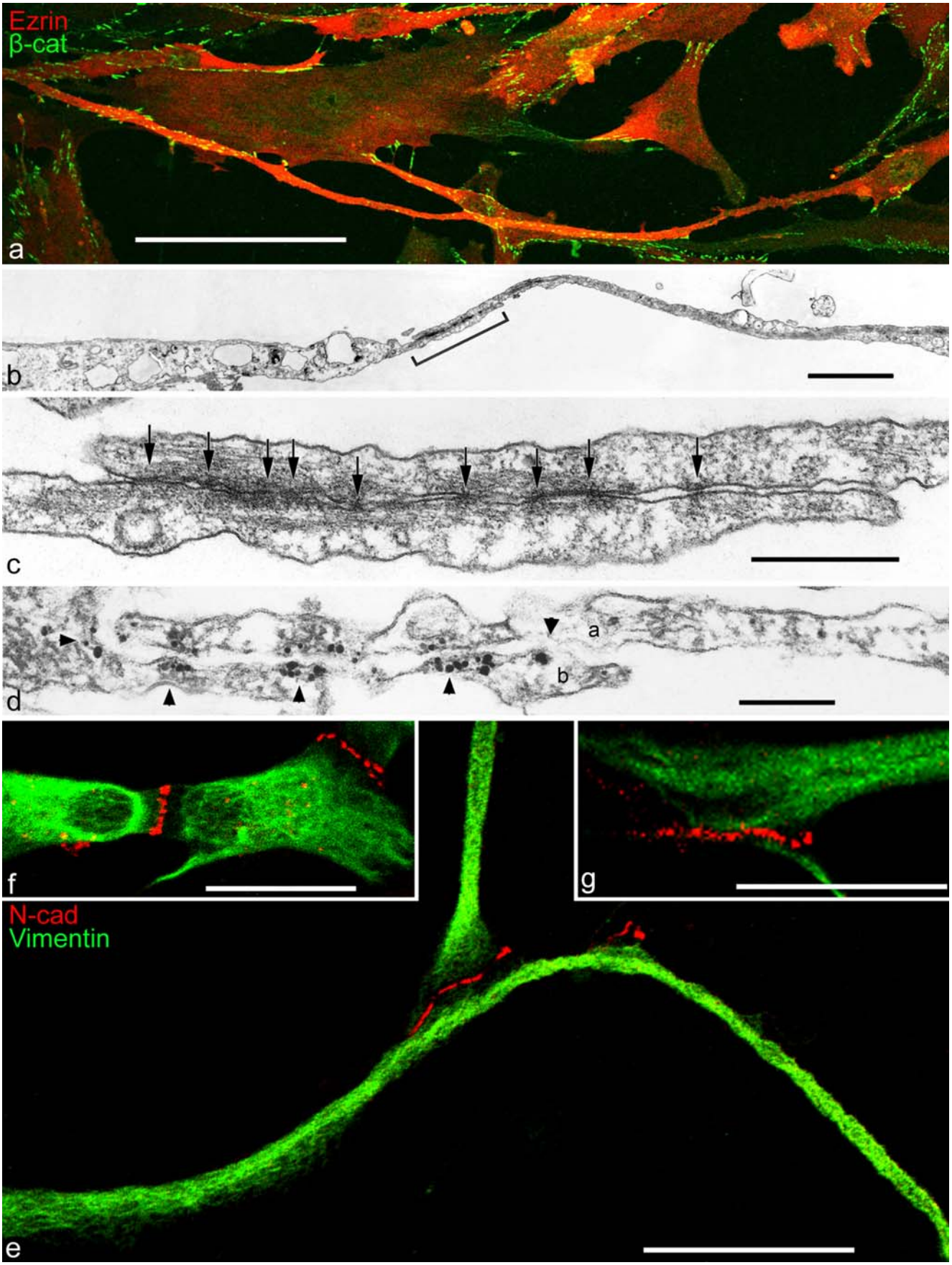
[f] Plakophilin-3 has been seen only in a portion of plakophilin-2-positive cells

Wuchter et al. 2007; for "normal-size" AJs of mesenchymal cells in culture, see, e.g., Hinz et al. 2004; Kiener et al. 2006). In recent experiments, we have also localized the *arm*-protein p0071 in such PAM, whereas plakoglobin has been repeatedly seen in some of cell cultures but only sporadically noted in others (cf. Rickelt et al. 2009).

Small junctions of the AJ type, including PAM, have also been frequently observed on long processes and on other surface regions of cells in primary and secondary cultures of mesenchymal cells derived from other tissues such as the interstitial cells of the interior of cardiac valves from various mammalian species, including rat, sheep, cow, and human (e.g., Fig. 1e-g; for details see Barth et al. 2009; and references cited therein). In such interstitial cell cultures, the small AJs are often clustered in specific regions of the filopodia, in particular at their tips, but may also occur on the central cell bodies (Fig. 1e–g). Again, the AJs of such cells, including PAM, have been found to be positive for N-cadherin and cadherin-11, for the *arm*-proteins β-catenin, plakoglobin, proteins p120, ARVCF, and p0071, and for α-catenin, afadin, and proteins of the ZO-1 group.

These *puncta*-studded long cell processes have to be distinguished from other long, thin and cylindrical filopodia-like actin-filament-rich cell-cell connections such

Fig. 1 Double-label immunofluorescence (**a**, **e-g**) and electron (**b**, **c**) and immunoelectron (**d**) microscopy showing cell processes of cultured human mesenchymal stem cells (MSCs), originally isolated from bone marrow (**a-d**) or ovine cardiac valve matrix (**e-g**). **a** Note that some of the cell processes are extremely long. The giant process extending in the lower part, for example, exceeds 450 μm in length and forms adherens junctions (AJs) of the *puncta adhaerentia minima* type (PAM) with at least five other cells. The microfilament-rich cell process is immunostained for the actin-binding protein, ezrin (*Ezrin*, *red*), and the numerous AJs have reacted with antibodies specific for β-catenin (*β-cat*, *green*). **b** Electron micrograph of the overlapping contact region of two cytoplasmic MSC processes that partly overlap in the contact region (*bracket*). **c** Higher magnification of the contact region demarcated in **b** showing a series of extremely small PAM (*arrows*; e.g., the diameter of the junction denoted by the *arrow right* is below 40 nm). **d** Immunoelectron microscopy of a similar region as that shown in **c** showing an overlap contact of processes of two cells (*a*, *b*); the processes are studded with PAM decorated with silver-enhanced immunogold-label for β-catenin (*arrowheads*). For details, see Wuchter et al. (2007). **e–g** Clusters of AJs at the tips of cell processes of cardiac valvular interstitial cells as visualized by immunostaining with antibodies to N-cadherin (*N-cad*; for details, see Barth et al. 2009). N-cadherin-positive (*red*) AJs connecting valvular interstitial cells (*green*, vimentin) are present as terminal punctate clusters at the tips of filopodium-like processes (e.g., the segment shown *bottom* in **e** exceeds 100 μm in length). Note the clusters of small AJs connecting the central bodies of three valvular interstitial cells (**f**) and the relatively large region densely studded with AJs connecting the terminal portions of two cell processes (**g**). For details, see Barth et al. 2009. *Bars* 100 μm (**a**), 2 μm (**b**), 0.5 μm (**c**), 0.2 μm (**d**), 25 μm (**e**, **f**), 20 μm (**g**)


Ezrin
β-cat
a
b
c
a
b
d
f
g
N-cad
Vimentin
e

as the cytonemata ("cytonemes") described in *Drosophila* and other invertebrate cells (Ramirez-Weber and Kornberg 1999 and further references therein) and from the "tunnelling nanotubes" of various vertebrate cell systems (Rustom et al. 2004; Gurke et al. 2008a, 2008b; Sowinski et al. 2008; Gerdes 2009; Gousset et al. 2009). Apparently, the presence of AJs, normal size-range or PAM, provides a good criterion for distinguishing the aforementioned cell-cell junction-based contact systems from cytonemes and nanotubes and possibly from other cell-connecting filopodial structures.

2. *Manubria adhaerentia* (taproot adherens junction)

In cultures of mesenchymally derived cells, we have also frequently noted a category of cell-cell junctions that has a highly conspicuous morphology and that often represents vast cell-cell contact areas (Wuchter et al. 2007). These cells are characterized by processes that do not make distinct small AJ contacts with the main cell bodies or with processes of other cells but deeply and tight-fittingly insert into special recesses of adjacent cells. Such taproot-like AJs (*manubria adhaerentia*) often occur in batteries of closely spaced structures of widely variable lengths (the more frequently observed manubrium-type of short-to-medium lengths is seen in Fig. 2a), occasionally with intracellular channel lengths of up to 50 μm (e.g., Fig. 2b). In such long filopodia-filled invaginations, both membranes (that of the filopodial process and that of the invagination recess) are in close contact and are coated on the cytoplasmic and on the filopodial side by an apparently continuous plaque. In some regions, this electron microscopically dense coat in some regions shows clustered, regularly spaced, extremely short spike-like projections into the cytoplasm (see, e.g., Fig. 2c). Thus, even at the electron-microscopic level, these taproot junctions often can be traced as essentially uninterrupted cylindrical AJ-like structures with cell-cell contact surfaces of up to ca. 100 μm^2, corresponding to 10^3 μm^2 and more per total cell, i.e., a gigantic cell-cell contact area.

That these manubrial cell-cell adhesion systems are indeed true AJ structures is evident from their positive immunostaining reaction for both N-cadherin and cadherin-11, together with a plaque structure positive for α- and β-catenin and proteins p120, p0071, and ARVCF, whereas only weak and variable reactions for plakoglobin have been seen, and MAGUK proteins of the ZO-1—3 group have not yet been identified with any significance (Table 1; see also Wuchter et al. 2007). By contrast, afadin and vinculin have generally been immunoreaction-positive. Moreover, the *manubria*-filling filopodia typically are intensely reactive for actin and with antibodies to ezrin, moesin, myosin, and α-actinin (for the general α-actinin-richness of the microfilament bundles, including the filopodia, of such cultured MSCs, see also Fig. 7 of Wuchter et al. 2007).

We have found it impressive to follow the fate of these taproot junction structures as the cell-packing density increases with cell culture time. Such studies have demonstrated that the lengths of the cell processes and, correspondingly, of the invaginations dynamically decrease in a spectacular way so that, in cultures of extremely high density, only short residual *manubria* structures are seen (see, e.g., Fig. 11 of Wuchter et al. 2007). The changes of the molecular packing in these AJ-related *manubria* junctions during this foreshortening phase will have to be studied in future experiments by using fluorescent-marker-coupled molecules in living cells.

For the sake of clarity, we wish finally to emphasize in this connection that the *manubria adhaerentia* structures only superficially resemble other kinds of "invaginations of cell processes" such as the filopodia-like "zippers" of Vasioukhin et al. (2000), the E-cadherin-based *Listeria* engulfment structures (Hamon et al. 2006), and the E-cadherin-AJ-based cell-in-cell "entosis" structures described by Brugge and collaborators (e.g., Overholtzer et al. 2007). However, that such filopodia-like processes may also occur in the body, at least at certain stages of development, is suggested by the observations of mesenchymal cells during and after mesoderm formation in mammalian embryos (see, e.g., Franke et al. 1983; Hashimoto and Nakatsuji 1989; Tam et al. 1993). Following such processes in their three-dimensional complexity *in situ* will clearly be difficult.

3. *Coniunctiones adhaerentes* (plakophilin-2-containing adherens junctions)

Recently, we have found that a certain subset of AJs of mesenchymally derived cells grown in culture or as tumors *in situ* is markedly modified by the selective acquisition of plakophilin-2, i.e., an *arm*-group protein hitherto only known as a constituent of desmosomes of proliferatively active epithelial or epithelium-derived cells (Barth et al. 2009; Rickelt et al. 2009). As in epithelia, this additional plaque protein in AJs seems to appear in a symmetrical fashion, i.e., in both plaques of the two cells connected by the specific AJ. Although AJs with the additional plakophilin-2 so far have been frequently seen in tumor-derived cell lines, this plakophilin-2-modified type of AJ is clearly not restricted to cultures of malignantly transformed cells (for non-transformed cells, see also Rickelt et al. 2009), as is shown with special clarity by the advent of this *arm*-protein in the AJs of cells growing in primary cultures of cardiac valvular interstitial cells (Fig. 3; Barth et al. 2009).

In this context, however, we consider it worth emphasizing that plakophilin-2 in general is a widespread near-ubiquitous component of all kinds of cells, i.e., of cells lacking any desmosomes. This protein appears to occur, albeit in low concentrations, as a component of certain

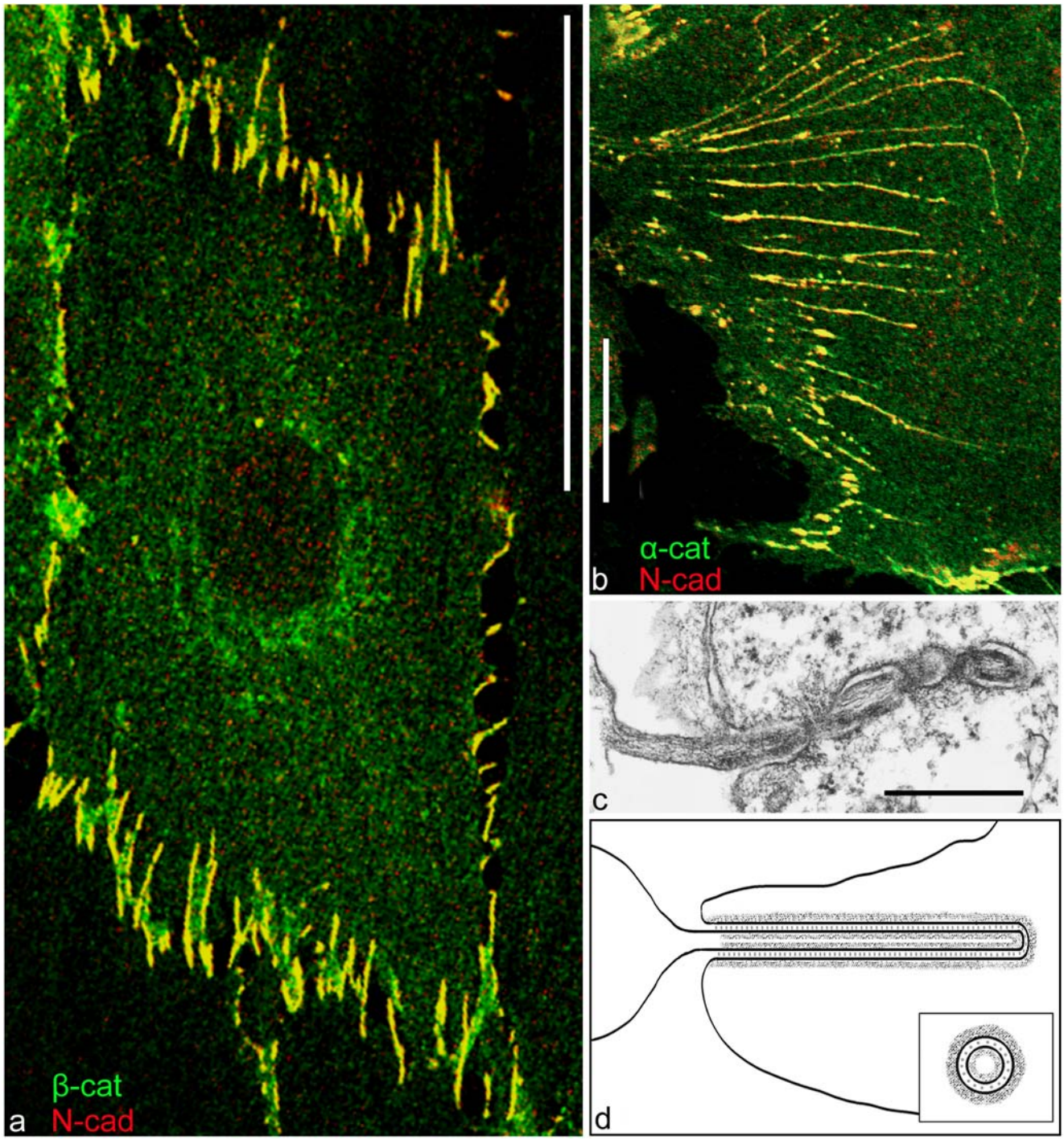


Fig. 2 Double-label immunofluorescence microscopy (**a**, **b**) and conventional ultrathin section transmission electron microscopy (**c**) showing connections of mesenchymal human-bone-marrow-derived stem cells (MSCs), including filopodia-like cytoplasmic processes of widely variable lengths that either form direct intercellular bridges. **a** Note that the cell shown here is connected to five other cells or deeply and tight-fittingly inserts into plasma membrane invaginations of an adjacent cell (*manubrium adhaerens*). **b** A series of such manubrial-type junctions of widely variable lengths, including examples up to 50 μm long (e.g., *top*). Most of these taproot junction formations are almost continuously positive for N-cadherin (*N-cad*, *red* in **a**, **b**) and α-catenin (*α-cat*, *green* in **b**), resulting in the *yellow* merge color. The same structures are also positive for β-catenin (*β-cat*, *green* in **a**), protein p120 (not shown here), and cadherin-11 (see also Wuchter et al. 2007). **c** Electron micrograph of a section through such a deep invagination tightly filled with a cell process from a neighboring cell forming a continuous plaque-like dense cytoplasmic coat over the entire length. **d** Representation showing a cell-cell junction of the *manubrium adhaerens* type and the resulting interlocking structure. Note that this form of structure essentially represents an extended AJ structure in a special form (*inset* cross-sectional image). Note also the continuous plaque system in the whole region. For further details, see Wuchter et al. (2007). *Bars* 50 μm (**a**), 20 μm (**b**), 0.2 μm (**c**)

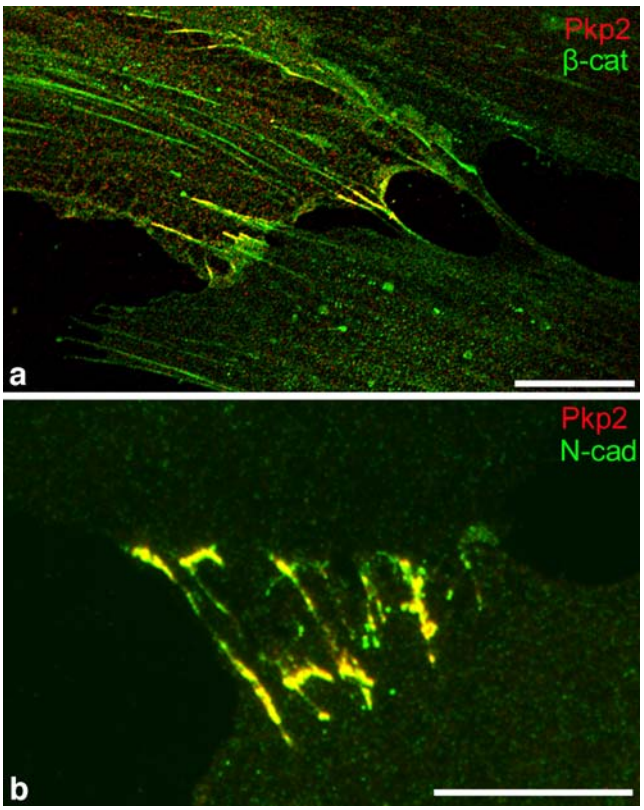


Fig. 3 Demonstration of the acquisition of plakophilin-2 (*Pkp2*) by some of the AJ-related cell-cell junctions between human mesenchymal cells in culture. **a** Double-label immunofluorescence microscopy of cultured human bone-marrow-derived mesenchymal cells (same culture as shown in Fig. 1a-d) immunostained for plakophilin-2 (*red*), in combination with the AJ protein, β-catenin (*β-cat*, *green*). Co-localization of the two plaque proteins appears in *yellow* in limited regions of some of the cell-cell contacts. **b** Plakophilin-2 also shows co-localization with the AJ-typical proteins, here with N-cadherin (*N-cad*, *green*), in cells of cultures of cardiac valvular interstitial cells of human origin. *Bars* 20 μm (**a**), 100 μm (**b**)

nuclear complexes, including regulatory complexes (Mertens et al. 1996, 2001). Consequently, the advent of plakophilin-2 as an additional AJ-plaque protein in mesenchymally derived cells does not reflect *de novo* synthesis but appears to be merely the result of an upregulation of the synthesis and stabilization of the protein product, perhaps only of certain posttranslational modifications. Obviously, the functional meaning of this dramatic increase of plakophilin-2 and its "anomalous" integration into AJs will have to be elucidated in the future, and we should also keep in mind that, in some of the cells with plakophilin-2-positive plaques, plakophilin-3 can also be detected as a junction plaque protein (Table 1; see also Rickelt et al. 2009).

4. *Areae compositae* (composite junctions)

In non-mammalian vertebrates and during fetal stages of mammalian development, the cardiomyocytes of the heart are connected, for the most part, in regions rich in typical AJ structures accompanied by a low proportion of desmosomes or at least desmosome-like-looking structures, representing about 10% or less of the cardiomyocyte contact surface area (e.g., McNutt 1970; Forbes and Sperelakis 1985). However, mammalian heart development continues postnatally with the desmosomal and the AJ structures clustering polarly into "intercalated disks" (IDs), and their two molecular ensembles mix and amalgamate (Fig. 4; Franke et al. 2006; Hirschy et al. 2006; Pieperhoff and Franke 2007).

Consequently, in the IDs of the mature mammalian heart, these junctional proteins and glycoproteins exist in almost a completely hybrid structure that has therefore been termed a "composite junction" (*area composita*, Table 1, Fig. 5). In these junctions, desmosomal molecules are no longer restricted to distinct structures but are major elements occurring in the entire plaque-coated region at which

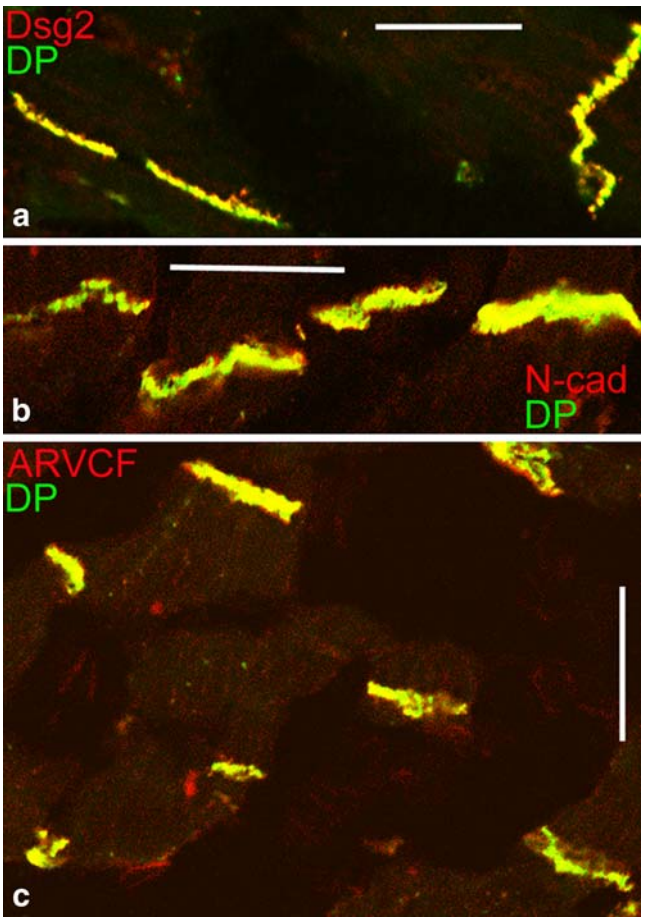


Fig. 4 Double-label immunofluorescence microscopy of cryostat sections through myocardium of an adult human heart, as seen after reactions with antibodies to desmoplakin (*DP*, *green*), in combination with antibodies to (*red* in each case) desmoglein 2 (*Dsg2*, **a**), N-cadherin (*N-cad*, **b**), or the plaque protein ARVCF (**c**). Only the merged color (*yellow*) is seen presenting near-complete colocalization in the composite junctions (*areae compositae*) of the intercalated disks and thus representing the amalgamated form containing both desmosomal and AJ proteins. *Bars* 20 μm

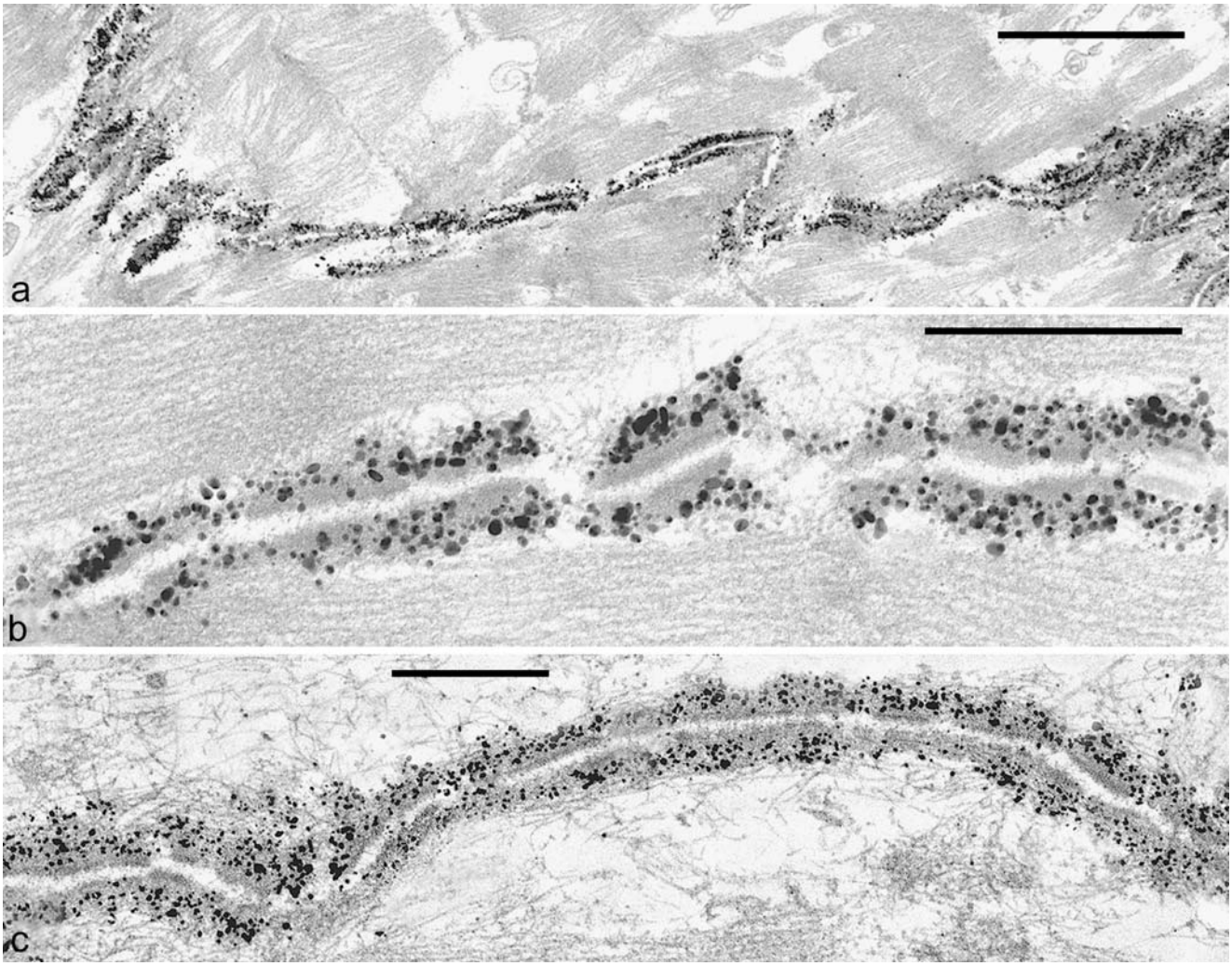


Fig. 5 Immunoelectron micrographs of sections through intercalated disks (IDs) of adult human heart. **a** Survey image showing the localization of a desmosomal protein, desmoplakin, by an immunogold-silver enhancement reaction in the entire ID plaque of the *area composita*. **b** Details of the intense plaque reaction in both small and large ID subdivisions. **c** An extended, continuous, completely plaque-covered, desmoplakin-rich junction (for details, see Franke et al. 2006). *Bars* 2 μm (**a**), 0.5 μm (**b**, **c**)

bundles of contractile myofilaments and of the desmin-rich intermediate filaments anchor (Kartenbeck et al. 1983; Borrmann et al. 2006; Franke et al. 2006; for protein p0071, see Hofmann et al. 2009). This special merger of two major junction ensembles and the resulting hybrid character is also seen in the specific interaction of the desmosomal protein, plakophilin-2, with the myocardium-typical AJ plaque protein, α-T-catenin (Goossens et al. 2007). The importance of plakophilin-2 for ID assembly and function has also been demonstrated in mouse embryogenesis by using gene knock-out experiments (Grossmann et al. 2004) and in cardiomyocyte cultures by means of experiments involving short interfering mRNA (Oxford et al. 2007; Fidler et al. 2008; Pieperhoff et al. 2008).

The recognition of a special composite junction in the IDs of mature mammalian hearts has been valuable in finding a compelling explanation for the recently increasing number of reports that mutations, even small ones, in desmosomal proteins are highly correlated with (and apparently causal for) the so-called arrhythmogenic cardiomyopathies (ARVC), including major causes of "sudden death", in young human beings, notably athletes (Table 2). As about two thirds of the ARVC cases genetically analyzed have been associated with specific mutations in genes encoding desmosomal proteins occurring in the composite junction ensemble (for specific reviews, see also Perriard et al. 2003; Herren et al. 2009), we are tempted to speculate that other mutations in ID proteins are responsible for the other third of ARVC cases still to be elucidated.

5. *Cortex adhaerens* (adherens cortex)

An extreme situation of a systemic and near-complete AJ-type integration of almost the entire cell-cell border is

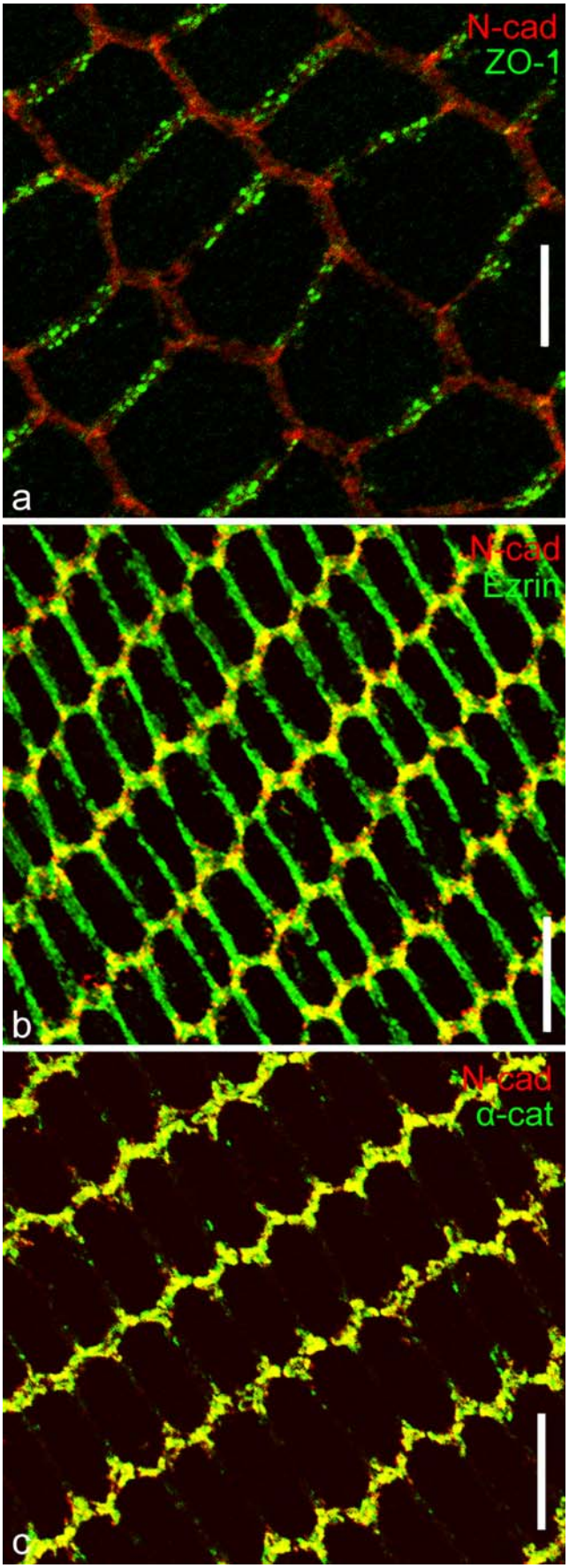
Table 2 Recent references reporting that certain mutations in human genes encoding desmosomal proteins and glycoproteins result in arrhythmogenic ventricular cardiomyopathies (ARVC) and references to related topics and reviews

Molecule	References	Molecule	Reference	Related topics/reviews	Reference
Plakophilin-2	Gerull et al. 2004 Antoniades et al. 2006	Desmoplakin	Norgett et al. 2000 Rampazzo et al. 2002	First animal model (boxer dogs)	Oxford et al. 2007
	Calkins 2006		Alcalai et al. 2003		
	Dalal et al. 2006		Norman et al. 2005		
	Kannankeril et al. 2006		Sen-Chowdhry et al. 2005		
	Nagaoka et al. 2006		Sen-Chowdhry et al. 2007		
	Syrris et al. 2006a		Tsatsopoulou et al. 2006		
	Tsatsopoulou et al. 2006 Van Tintelen et al. 2006		Yang et al. 2006		
	Lahtinen et al. 2007 Otterspoor et al. 2007	Desmoglein-2	Awad et al. 2006 Pilichou et al. 2006	Recent reviews	Bazzi and Christiano 2007. For an anthology of review articles, see Marcus et al. 2007; Awad et al. 2008; Herren et al. 2009; Corrado et al. 2009
	Fidler et al. 2008 Joshi-Mukherjee et al. 2008		Tsatsopoulou et al. 2006		
	Ram and Van Wagoner 2008 Tandri et al. 2008		Syrris et al. 2007		
	Wu et al. 2009 Qiu et al. 2009 (5 cases)		Yu et al. 2008		
Plakoglobin	Garcia-Gras et al. 2006 Asimaki et al. 2007	Desmocollin-2	Heuser et al. 2006 Syrris et al. 2006b	Presentation of a specific plakoglobin test for diagnosis of human ARVC	Asimaki et al. 2009
			Beffagna et al. 2007		

provided by the lens fibers, i.e. the internal tissue of the vertebrate eye, in which all the anucleate cell bodies are densely packed, leaving little "free" intercellular space and thus also contributing to the optical homogeneity of the lens. Here, the cytoplasmic sides of the large plasma membrane contacts are coated by a giant cortical plaque-bearing structure, which, however, shows marked regional differences. In some regions, in particular at the short polar sides, this cortical complex represents a junction-equivalent that contains not only N-cadherin and cadherin-11, but also classic plaque-components such as α- and β-catenin, plakoglobin, and protein p120, although it seems to lack proteins p0071 and ARVCF, afadin, and all desmosomal components. In addition, various other proteins generally occurring on cell contact structures of the lens interior, such as ezrin, periplakin, and periaxin, are also seen in this part of the cortex (Fig. 6). In some regions, a large proportion of the "long side" is also positive for AJ markers, including N-cadherin, with local exceptions of some gap junctions (see, e.g., Fig. 6a), whereas in other parts of the lens, only the "short sides" are markedly immunostained for such AJ molecules (e.g., Fig. 6a, c; for details and references, see Straub et al. 2003). By contrast, some other markers, in particular actin and actin-binding proteins such as ezrin, are present along the entire plasma membrane (e.g., Fig. 6c).

6. *Iuncturae structae* (sandwich junctions)

A true and trivial assertion is that TJs are recognized by localizations of TJ molecules. The reverse general conclusion, viz., that the localization of known TJ molecules identifies a TJ, cannot be upheld as a general dogma (cf. Table 1; Cereijido and Anderson 2001). Findings of TJ protein reactions in various epithelial tissues, such as the *stratum spinosum* of stratified squamous epithelia and histologically related tissues of thymic Hassall bodies and in squamous cell carcinomas, have been published but, until today, cannot be reconciled with a *zonula occludens* or with related "occluding" structures, which to date in normal stratified epithelia have only been demonstrated in the



◀**Fig. 6** Double-label immunofluorescence microscopy of cryostat sections through bovine lens tissue presenting details of the *cortex adhaerens*. A comparison of the reaction for N-cadherin (*N-cad*, *red*) with that for the prominently gap-junction-associated protein ZO-1 (**a**, *green*), the actin-filament-associated protein ezrin (**b**, *green*), and the AJ plaque protein α-catenin (**c**, *α-cat*, *green*). Only the merged images are shown. Note that here the ZO-1 reaction appears to be restricted to a limited region in the longer lateral wall, whereas N-cadherin and α-catenin are highly enriched at junction-like structures in the short wall elements. Ezrin is seen in the entire cell cortex. For further details, see Straub et al. (2003). *Bars* 10 μm

uppermost living cell layer, the *stratum granulosum* (e.g., Morita et al. 1998; Brandner et al. 2002; Furuse et al. 2002; Langbein et al. 2002, 2003; Schlüter et al. 2004, 2007). In contrast, several authors have shown that such TJ proteins can also occur in *strata spinosa*, but that their immunoreactions often do not colocalize. For example, some TJ markers such as claudin-1 occur practically throughout the spinous layer of the epidermis and other stratified epithelia and in tissues lacking any lumen such as thymic Hassall corpuscules and certain cell aggregates in squamous cell carcinomas, notably the so-called "horn-pearls" (Langbein et al. 2002, 2003). Indeed, corresponding immunoelectron microscopy has revealed that, in many of the interdesmosomal regions of these cell layers and tumors, an intense claudin-1 reaction is seen rather generally (Fig. 7; Langbein et al. 2002, 2003). In the uppermost *strata*, some of these sites are also positive for occludin but not for other TJ markers.

Whereas the *stratum spinosum* structures positive for specific TJ markers are often small and inconspicuous, a special heavy metal staining reaction is recognized in some of them, resulting in the appearance of an electron-dense layer between the two plasma membrane domains (Fig. 7; see also, e.g., Figs. 9–11 of Langbein et al. 2002). Depending on the thickness and the extent of this electron-dense middle layer in cell-cell contacts, such structures have been classified as "lamellated junctions" (*coniunctiones laminosae*) or as *iunctura structa* (sandwich junctions).

Finally, extremely small, i.e. punctate, TJ-resembling structures have been seen in freeze-fractures preparations and have been tentatively termed *puncta occludentia* (stud junctions; cf. Schlüter et al. 2007).

As the existence of such TJ-related structures in *stratum spinosum* structures and probably related layers in other stratified epithelia and in pathologically altered tissues derived therefrom now seems established, it is high time to characterize these TJ-protein-positive structures that are not TJs in both structural and molecular terms.

7. Complex junctions

As early as 1990, certain kinds of lymphatic endothelial cells, in particular those of the lymph node

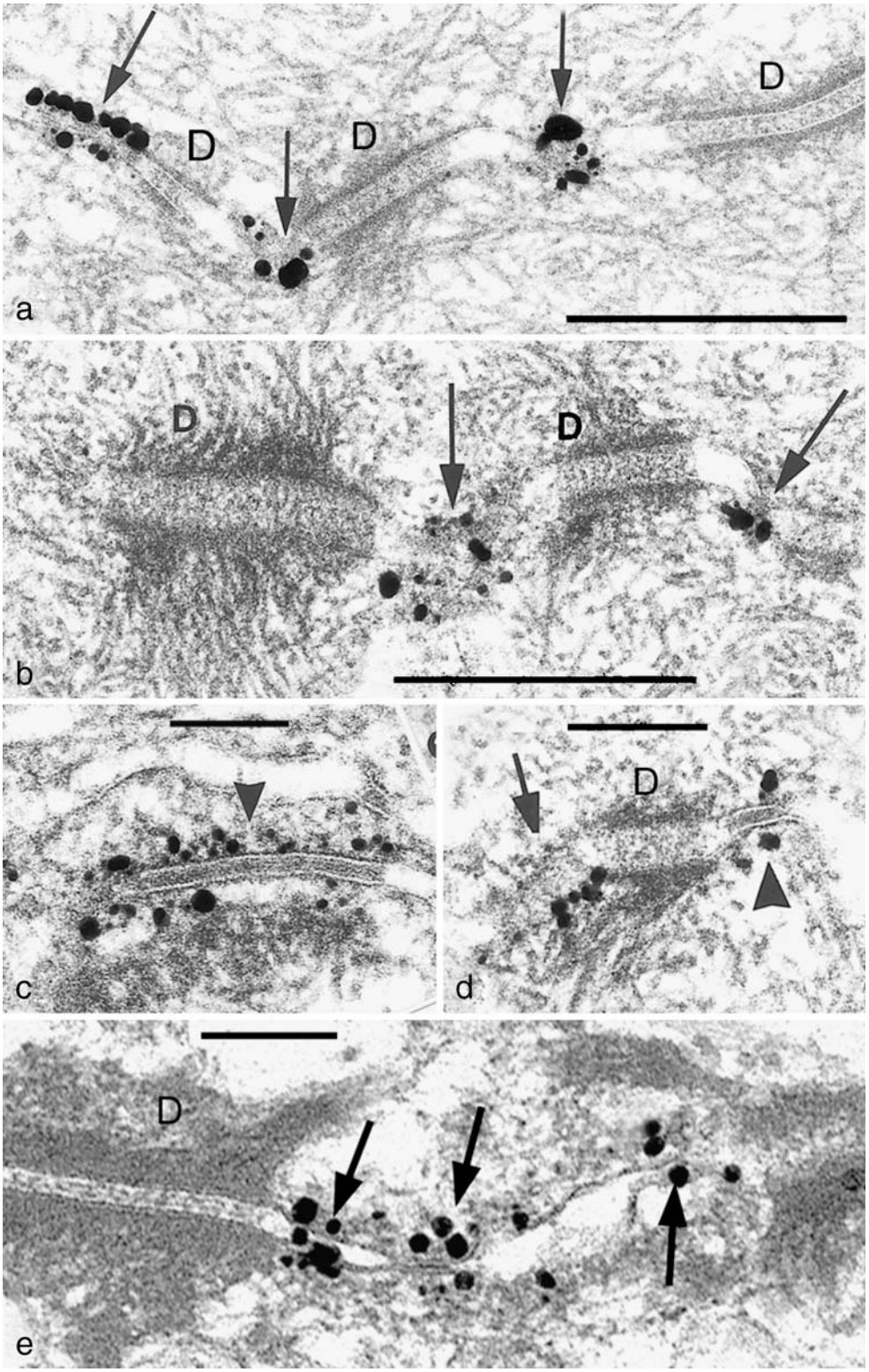


Fig. 7 Immunoelectron microscopy of ultrathin sections through the stratified squamous epithelium of bovine tongue mucosa (**a-d**) or a Hassall corpuscle of bovine thymus (**e**), as seen after reaction with antibodies to occludin. Immunogold label is not only seen in the uppermost living cell layer, the *stratum granulosum*-equivalent (for details see, e.g., Brandner et al. 2002; Langbein et al. 2002, 2003; Schlüter et al. 2004), but also in inconspicuous interdesmosomal regions (*arrows* in **a**, **b**, **d**, **e**) and in special junctions (*iuncturae structae*) with an electron-dense middle layer (*arrowheads* in **c**, **d**). Tight junction (TJ) proteins are not restricted to typical TJs but at least some of them also occur in additional, yet insufficiently characterized junctions (*D* desmosomes). *Bars* 0.2 μm (**a**, **b**), 0.1 μm (**c-e**)

sinus, were noted to be characterized by special, highly unusual kinds of cell-cell junction, collectively referred to as *complexus adhaerentes*. These junctions varied remarkably in their size and junctional architecture, including some excessively large structures. They contained VE-cadherin, often in co- or almost co-localization with N-cadherin, and were not only positive for other typical endothelial junction markers such as α- and β-catenin, plakoglobin, p120 protein and afadin, but were also strongly positive for desmoplakin and for some

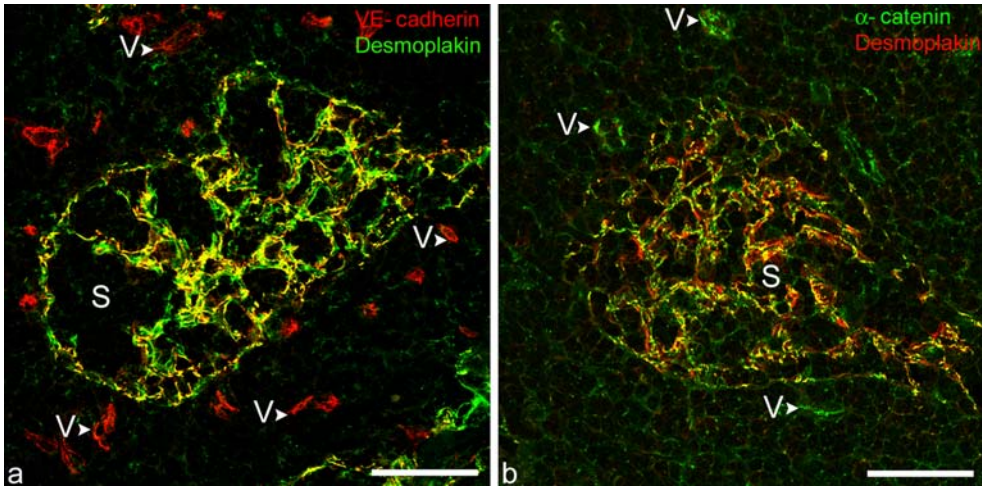


Fig. 8 Double-label immunofluorescence laser-scanning microscopy images of cryostat cross sections through human lymph nodes, showing the specific, mutually exclusive localization of VE-cadherin in the endothelium of small blood vessels (*V*) and the desmoplakin and α-catenin immunoreactions in the *complexus adhaerentes* of the endothelial and virgultar cells (SEVCs) of the sinus (*S*). **a** Colocalization of desmoplakin and VE-cadherin in the *complexus adhaerentes* of SEVCs cells in the sinus (*S*) can be seen with special clarity in the *yellow* merged image (*VE-cadherin*, *red* versus *Desmoplakin*, *green*). **b** Corresponding merged image showing co-localization (*yellow*) of desmoplakin (*red*) and α-catenin (*green*) at distinct small junctional structures (for details, see the review of Moll et al. 2009). *Bars* 50 μm

typical TJ proteins including, in certain positions, claudin-5 and JAM-A (Fig. 8, Table 1; cf. Schmelz and Franke 1990, 1993; Schmelz et al. 1994; Hämmerling et al. 2006; Moll et al. 2009). The unusual locations of, e.g., desmoplakin in these lymphatic endothelial junctions was then confirmed and extended in several ways for other lymph node structures and for other parts of the lymphatic vascular system (e.g., Valiron et al. 1996; Ebata et al. 2001; Baluk et al. 2007; Pfeiffer et al. 2008). The "strange" morphology of the complex virgultar meshwork of the intrasinusoidal endothelial cell types and the close association of cytoplasmic "wraps" with collagen fiber bundles has been presented in detail elsewhere (Moll et al. 2009). However, the functional relevance of the different cell-cell junction ensembles in different parts of the lymphatic system (subtypes of lymphatic endothelia are also positive for protein p0071; Hofmann et al. 2008) remains to be elucidated. The obvious importance of desmoplakin in angiogenesis during embryogenesis and in experimental systems (Kowalczyk et al. 1998; Gallicano et al. 2001; Zhou et al. 2004) also indicates that regional and developmental differences exist with regard to the influence of such *complexus adhaerens*-typical molecules.

Concluding remarks

The list of "special" junctions summarized in this review is certainly not complete. In particular, we have left out all those AJ-like junctions that couple two apparently highly different cell types, i.e., "heterophilic" or "asymmetric" junctions, simply because the two half-junctions might contain different molecular components from those in "symmetric" junctions. We have also omitted the AJs originally introduced as "contact junctions" (*contactus adhaerentes*), i.e., highly specialized AJs that have been identified to connect the granular cells of the cerebellar glomeruli. These AJ-type plaque-bearing structures contain N-cadherin and M-cadherin (Rose et al. 1995). Interestingly, however, M-cadherin in these structures obviously does not seem to be essential for life, as abrogation of the gene encoding M-cadherin does not result in major defects but apparently is compensated by an upregulation of N-cadherin (Hollnagel et al. 2002). Thus, irrespective of the molecular organization in the M-cadherin-containing junctions, the special contribution of this glycoprotein to the function of the junction will have to be defined in comparison with N-cadherin.

Therefore, although this review has in general to be considered incomplete, it serves primarily as a mind-opener indicating that further kinds of junctions may well lie just around the corner.

Acknowledgements The authors thank Caecilia Kuhn, Christine Grund, and Stefanie Winter-Simanowski for their enthusiastic technical assistance, and Dr. T. Keenan (Virginia Tech. University, Blacksburg, Va., USA) for competent correction and polishing of the text.

References

Alcalai R, Metzger S, Rosenheck S, Meiner V, Chajek-Shaul T (2003) A recessive mutation in desmoplakin causes arrhythmogenic right ventricular dysplasia, skin disorder, and woolly hair. J Am Coll Cardiol 42:319–327

Antoniades L, Tsatsopoulou A, Anastasakis A, Syrris P, Asimaki A, Panagiotakos D, Zambartas C, Stefanadis C, McKenna WJ, Protonotarios N (2006) Arrhythmogenic right ventricular cardiomyopathy caused by deletions in plakophilin-2 and plakoglobin (Naxos disease) in families from Greece and Cyprus: genotype-phenotype relations, diagnostic features and prognosis. Eur Heart J 27:2208–2216

Asimaki A, Syrris P, Wichter T, Matthias P, Saffitz JE, McKenna WJ (2007) A novel dominant mutation in plakoglobin causes arrhythmogenic right ventricular cardiomyopathy. Am J Hum Genet 81:964–973

Asimaki A, Tandri H, Huang H, Halushka MK, Gautam S, Basso C, Thiene G, Tsatsopoulou A, Protonotarios N, McKenna WJ, Calkins H, Saffitz JE (2009) A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. N Engl J Med 360:1075–1084

Awad MM, Dalal D, Cho E, Amat-Alarcon N, James C, Tichnell C, Tucker A, Russell SD, Bluemke DA, Dietz HC, Calkins H, Judge DP (2006) DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy. Am J Hum Genet 79:136–142

Awad MM, Calkins H, Judge DP (2008) Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Nat Clin Pract Cardiovasc Med 5:258–267

Baluk P, Fuxe J, Hashizume H, Romano T, Lashnits E, Butz S, Vestweber D, Corada M, Molendini C, Dejana E, McDonald DM (2007) Functionally specialized junctions between endothelial cells of lymphatic vessels. J Exp Med 204:2349–2362

Barth M, Schumacher H, Kuhn C, Akhyari P, Lichtenberg A, Franke WW (2009) Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. Cell Tissue Res 337:63–77

Bazzi H, Christiano AM (2007) Broken hearts, woolly hair, and tattered skin: when desmosomal adhesion goes awry. Curr Opin Cell Biol 19:515–520

Beffagna G, De Bortoli M, Nava A, Salamon M, Lorenzon A, Zaccolo M, Mancuso L, Sigalotti L, Bauce B, Occhi G, Basso C, Lanfranchi G, Towbin JA, Thiene G, Danieli GA, Rampazzo A (2007) Missense mutations in desmocollin-2 N-terminus, associated with arrhythmogenic right ventricular cardiomyopathy, affect intracellular localization of desmocollin-2 in vitro. BMC Med Genet 8:65

Behrens J, Nelson WJ (2004) Cell adhesion. Springer, Heidelberg

Borrmann CM, Grund C, Kuhn C, Hofmann I, Pieperhoff S, Franke WW (2006) The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk. Eur J Cell Biol 85:469–485

Brandner JM, Kief S, Grund C, Rendl M, Houdek P, Kuhn C, Tschachler E, Franke WW, Moll I (2002) Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. Eur J Cell Biol 81:253–263

Calkins H (2006) Arrhythmogenic right-ventricular dysplasia/cardiomyopathy. Curr Opin Cardiol 21:55–63

Cereijido M, Anderson J (2001) Tight junctions. CRC Press, Boca Raton

Citi S (2001) The cytoplasmic plaque proteins of the tight junction. In: Cereijido M, Anderson J (eds) Tight junctions. CRC Press, Boca Raton

Corrado D, Basso C, Thiene G (2009) Arrythmogenic right ventricular cardiomyopathy: an update. Heart 95:766–773

Dalal D, Molin LH, Piccini J, Tichnell C, James C, Bomma C, Prakasa K, Towbin JA, Marcus FI, Spevak PJ, Bluemke DA, Abraham T, Russell SD, Calkins H, Judge DP (2006) Clinical features of arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in plakophilin-2. Circulation 113:1641–1649

Ebata N, Nodasaka Y, Sawa Y, Yamaoka Y, Makino S, Totsuka Y, Yoshida S (2001) Desmoplakin as a specific marker of lymphatic vessels. Microvasc Res 61:40–48

Fidler LM, Wilson GJ, Liu F, Cui X, Scherer SW, Taylor GP, Hamilton RM (2008) Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations. J Cell Mol Med (in press)

Forbes MS, Sperelakis N (1985) Intercalated discs of mammalian heart: a review of structure and function. Tissue Cell 17:605–648

Franke WW (2009) Discovering the molecular components of intercellular junctions—a historical view. In: Nelson J, Fuchs E (eds) Cell-cell junctions. Cold Spring HarborLaboratory, Cold Spring Harbor, NY (in press)

Franke WW, Grund C, Jackson BW, Illmensee K (1983) Formation of cytoskeletal elements during mouse embryogenesis. IV. Ultrastructure of primary mesenchymal cells and their cell-cell interactions. Differentiation 25:121–41

Franke WW, Borrmann CM, Grund C, Pieperhoff S (2006) The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. Eur J Cell Biol 85:69–82

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156:1099–1111

Gallicano GI, Bauer C, Fuchs E (2001) Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature. Development 128:929–941

Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, Marian AJ (2006) Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. J Clin Invest 116:2012–2021

Gerdes HH (2009) Prions tunnel between cells. Nat Cell Biol 11:235–236

Gerull B, Heuser A, Wichter T, Paul M, Basson CT, McDermott DA, Lerman BB, Markowitz SM, Ellinor PT, MacRae CA, Peters S, Grossmann KS, Drenckhahn J, Michely B, Sasse-Klaassen S, Birchmeier W, Dietz R, Breithardt G, Schulze-Bahr E, Thierfelder L (2004) Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. Nat Genet 36:1162–1164

Goossens S, Janssens B, Bonne S, De Rycke R, Braet F, Hengel J van, Roy F van (2007) A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs. J Cell Sci 120:2126–2136

Gousset K, Schiff E, Langevin C, Marijanovic Z, Caputo A, Browman DT, Chenouard N, Chaumont F de, Martino A, Enninga J, Olivo-Marin JC, Mannel D, Zurzolo C (2009) Prions hijack tunnelling nanotubes for intercellular spread. Nat Cell Biol 11:328–336

Grossmann KS, Grund C, Huelsken J, Behrend M, Erdmann B, Franke WW, Birchmeier W (2004) Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. J Cell Biol 167:149–160

Gurke S, Barroso JF, Gerdes HH (2008a) The art of cellular communication: tunneling nanotubes bridge the divide. Histochem Cell Biol 129:539–550

Gurke S, Barroso JF, Hodneland E, Bukoreshtliev NV, Schlicker O, Gerdes HH (2008b) Tunneling nanotube (TNT)-like structures facilitate a constitutive, actomyosin-dependent exchange of endocytic organelles between normal rat kidney cells. Exp Cell Res 314:3669–3683

Hämmerling B, Grund C, Boda-Heggemann J, Moll R, Franke WW (2006) The complexus adhaerens of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought. Cell Tissue Res 324:55–67

Hamon M, Bierne H, Cossart P (2006) *Listeria monocytogenes*: a multifaceted model. Nat Rev Microbiol 4:423–434

Hashimoto K, Nakatsuji N (1989) Formation of the primitive streak and mesoderm cells in mouse embryos—detailed scanning electron microscopical study. Dev Growth Differ 31:209–218

Herren T, Gerber PA, Duru F (2009) Arrythmogenic right ventricular cardiomyopathy/dysplasia: a not so rare "disease of the desmosome" with multiple clinical presentations. Clin Res Cardiol 98:141–158

Heuser A, Plovie ER, Ellinor PT, Grossmann KS, Shin JT, Wichter T, Basson CT, Lerman BB, Sasse-Klaassen S, Thierfelder L, MacRae CA, Gerull B (2006) Mutant desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy. Am J Hum Genet 79:1081–1088

Hinz B, Pittet P, Smith-Clerc J, Chaponnier C, Meister JJ (2004) Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell 15:4310–4320

Hirschy A, Schatzmann F, Ehler E, Perriard JC (2006) Establishment of cardiac cytoarchitecture in the developing mouse heart. Dev Biol 289:430–441

Hofmann I, Kuhn C, Franke WW (2008) Protein p0071, a major plaque protein of non-desmosomal adhering junctions, is a selective cell-type marker. Cell Tissue Res 334:381–399

Hofmann I, Schlechter T, Kuhn C, Hergt M, Franke WW (2009) Protein p0071—an armadillo plaque protein that characterizes a specific subtype of adherens junctions. J Cell Sci 122:21–24

Hollnagel A, Grund C, Franke WW, Arnold HH (2002) The cell adhesion molecule M-cadherin is not essential for muscle development and regeneration. Mol Cell Biol 22:4760–4770

Holthöfer B, Windoffer R, Troyanovsky S, Leube RE (2007) Structure and function of desmosomes. Int Rev Cytol 264:65–163

Joshi-Mukherjee R, Coombs W, Musa H, Oxford E, Taffet S, Delmar M (2008) Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations. Heart Rhythm 5:1715–1723

Kannankeril PJ, Bhuiyan ZA, Darbar D, Mannens MM, Wilde AA, Roden DM (2006) Arrhythmogenic right ventricular cardiomyopathy due to a novel plakophilin 2 mutation: wide spectrum of disease in mutation carriers within a family. Heart Rhythm 3:939–944

Kartenbeck J, Franke WW, Moser JG, Stoffels U (1983) Specific attachment of desmin filaments to desmosomal plaques in cardiac myocytes. EMBO J 2:735–742

Kiener HP, Stipp CS, Allen PG, Higgins JM, Brenner MB (2006) The cadherin-11 cytoplasmic juxtamembrane domain promotes alpha-catenin turnover at adherens junctions and intercellular motility. Mol Biol Cell 17:2366–2376

Kowalczyk AP, Navarro P, Dejana E, Bornslaeger EA, Green KJ, Kopp DS, Borgwardt JE (1998) VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellular junctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions. J Cell Sci 111:3045–3057

LaFlamme SE, Kowalczyk AP (2008) Cell junctions. Wiley-VCH, Weinheim

Lahtinen AM, Lehtonen A, Kaartinen M, Toivonen L, Swan H, Widen E, Lehtonen E, Lehto VP, Kontula K (2007) Plakophilin-2 missense mutations in arrhythmogenic right ventricular cardiomyopathy. Int J Cardiol 126:92–100

Langbein L, Grund C, Kuhn C, Praetzel S, Kartenbeck J, Brandner JM, Moll I, Franke WW (2002) Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. Eur J Cell Biol 81:419–435

Langbein L, Pape UF, Grund C, Kuhn C, Praetzel S, Moll I, Moll R, Franke WW (2003) Tight junction-related structures in the absence of a lumen: occludin, claudins and tight junction plaque proteins in densely packed cell formations of stratified epithelia and squamous cell carcinomas. Eur J Cell Biol 82:385–400

Marcus FI, Nava A, Thiene G (2007) Arrythmogenic right ventricular cardiomyopathy/dysplasia. Springer, Heidelberg

McNutt NS (1970) Ultrastructure of intercellular junctions in adult and developing cardiac muscle. Am J Cardiol 25:169–183

Mertens C, Kuhn C, Franke WW (1996) Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J Cell Biol 135:1009–1025

Mertens C, Hofmann I, Wang Z, Teichmann M, Sepehri Chong S, Schnolzer M, Franke WW (2001) Nuclear particles containing RNA polymerase III complexes associated with the junctional plaque protein plakophilin 2. Proc Natl Acad Sci USA 98:7795–7800

Moll R, Sievers E, Hämmerling B, Schmidt A, Barth M, Kuhn C, Grund C, Hofmann I, Franke WW (2009) Endothelial and virgultar cell formations in the mammalian lymph node sinus: endothelial differentiation morphotypes characterized by a special kind of junction (complexus adhaerens). Cell Tissue Res 335:109–141

Morita K, Itoh M, Saitou M, Ando-Akatsuka Y, Furuse M, Yoneda K, Imamura S, Fujimoto K, Tsukita S (1998) Subcellular distribution of tight junction-associated proteins (occludin, ZO-1, ZO-2) in rodent skin. J Invest Dermatol 110:862–866

Nagaoka I, Matsui K, Ueyama T, Kanemoto M, Wu J, Shimizu A, Matsuzaki M, Horie M (2006) Novel mutation of plakophilin-2 associated with arrhythmogenic right ventricular cardiomyopathy. Circ J 70:933–935

Norgett EE, Hatsell SJ, Carvajal-Huerta L, Cabezas JC, Common J, Purkis PE, Whittock N, Leigh IM, Stevens HP, Kelsell DP (2000) Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. Hum Mol Genet 9:2761–2766

Norman M, Simpson M, Mogensen J, Shaw A, Hughes S, Syrris P, Sen-Chowdhry S, Rowland E, Crosby A, McKenna WJ (2005) Novel mutation in desmoplakin causes arrhythmogenic left ventricular cardiomyopathy. Circulation 112:636–642

Otterspoor LC, Reichert CL, Cramer MJ, Bhuiyan ZA, Wilde AA, Hauer RN (2007) Arrhythmogenic right ventricular cardiomyopathy: asymptomatic to life threatening as illustrated by the cases of two sisters. Neth Heart J 15:348–353

Overholtzer M, Mailleux AA, Mouneimne G, Normand G, Schnitt SJ, King RW, Cibas ES, Brugge JS (2007) A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell 131:966–979

Oxford EM, Musa H, Maass K, Coombs W, Taffet SM, Delmar M (2007) Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. Circ Res 101:703–711

Peracchia C (2000) Gap junctions. Molecular basis of cell communication and health and disease. Academic Press, San Diego

Perriard JC, Hirschy A, Ehler E (2003) Dilated cardiomyopathy: a disease of the intercalated disc? Trends Cardiovasc Med 13:30-38

Pfeiffer F, Kumar V, Butz S, Vestweber D, Imhof BA, Stein JV, Engelhardt B (2008) Distinct molecular composition of blood and lymphatic vascular endothelial cell junctions establishes specific functional barriers within the peripheral lymph node. Eur J Immunol 38:2142–2155

Pieperhoff S, Franke WW (2007) The area composita of adhering junctions connecting heart muscle cells of vertebrates. IV: Coalescence and amalgamation of desmosomal and adhaerens junction components. Late processes in mammalian heart development. Eur J Cell Biol 86:377–391

Pieperhoff S, Schumacher H, Franke WW (2008) The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes. Eur J Cell Biol 87:399–411

Pilichou K, Nava A, Basso C, Beffagna G, Bauce B, Lorenzon A, Frigo G, Vettori A, Valente M, Towbin J, Thiene G, Danieli GA, Rampazzo A (2006) Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. Circulation 113:1171–1179

Qiu X, Liu W, Hu D, Zhu T, Li C, Li L, Guo C, Liu X, Wang L, Zheng H, Wang C, Diao Q, Shi D, Zhan P, Deng Y, Liu K, Wang Y, Liu B, Liu H, Zhang L (2009) Mutations of plakophilin-2 in Chinese with arrhythmogenic right ventricular dysplasia/cardiomyopathy. Am J Cardiol 103:1439–1444

Ram R, Van Wagoner DR (2008) Plakophilin-2 mutations as a cause of arrhythmogenic right ventricular cardiomyopathy: progress toward linking structural with functional changes. Heart Rhythm 5:1724–1725

Ramirez-Weber FA, Kornberg TB (1999) Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. Cell 97:599–607

Rampazzo A, Nava A, Malacrida S, Beffagna G, Bauce B, Rossi V, Zimbello R, Simionati B, Basso C, Thiene G, Towbin JA, Danieli GA (2002) Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy. Am J Hum Genet 71:1200–1206

Rickelt S, Winter-Simanowski S, Noffz E, Kuhn C, Franke WW (2009) Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer (in press)

Rose O, Grund C, Reinhardt S, Starzinski-Powitz A, Franke WW (1995) Contactus adherens, a special type of plaque-bearing adhering junction containing M-cadherin, in the granule cell layer of the cerebellar glomerulus. Proc Natl Acad Sci USA 92:6022–6026

Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH (2004) Nanotubular highways for intercellular organelle transport. Science 303:1007–1010

Schlüter H, Wepf R, Moll I, Franke WW (2004) Sealing the live part of the skin: the integrated meshwork of desmosomes, tight junctions and curvilinear ridge structures in the cells of the uppermost granular layer of the human epidermis. Eur J Cell Biol 83:655–665

Schlüter H, Moll I, Wolburg H, Franke WW (2007) The different structures containing tight junction proteins in epidermal and other stratified epithelial cells, including squamous cell metaplasia. Eur J Cell Biol 86:645–655

Schmelz M, Franke WW (1990) A new type of intercellular junction: desmosomal proteins in the extended junctions of certain endothelial cells of the lymphatic system. Cell Biol Int Rep 14:54

Schmelz M, Franke WW (1993) Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: the syndesmos connecting retothelial cells of lymph nodes. Eur J Cell Biol 61:274–289

Schmelz M, Moll R, Kuhn C, Franke WW (1994) Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells. II. Different types of lymphatic vessels. Differentiation 57:97–117

Sen-Chowdhry S, Syrris P, McKenna WJ (2005) Genetics of right ventricular cardiomyopathy. J Cardiovasc Electrophysiol 16:927–935

Sen-Chowdhry S, Syrris P, Ward D, Asimaki A, Sevdalis E, McKenna WJ (2007) Clinical and genetic characterization of families with arrhythmogenic right ventricular dysplasia/cardiomyopathy provides novel insights into patterns of disease expression. Circulation 115:1710–1720

Sowinski S, Jolly C, Berninghausen O, Purbhoo MA, Chauveau A, Kohler K, Oddos S, Eissmann P, Brodsky FM, Hopkins C, Onfelt B, Sattentau Q, Davis DM (2008) Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. Nat Cell Biol 10:211–219

Straub BK, Boda J, Kuhn C, Schnoelzer M, Korf U, Kempf T, Spring H, Hatzfeld M, Franke WW (2003) A novel cell-cell junction system: the cortex adhaerens mosaic of lens fiber cells. J Cell Sci 116:4985–4995

Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ (2006a) Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. Circulation 113:356–364

Syrris P, Ward D, Evans A, Asimaki A, Gandjbakhch E, Sen-Chowdhry S, McKenna WJ (2006b) Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2. Am J Hum Genet 79:978–984

Syrris P, Ward D, Asimaki A, Evans A, Sen-Chowdhry S, Hughes SE, McKenna WJ (2007) Desmoglein-2 mutations in arrhythmogenic right ventricular cardiomyopathy: a genotype-phenotype characterization of familial disease. Eur Heart J 28:581–588

Tam PP, Williams EA, Chan WY (1993) Gastrulation in the mouse embryo: ultrastructural and molecular aspects of germ layer morphogenesis. Microsc Res Tech 26:301–328

Tandri H, Asimaki A, Dalal D, Saffitz JE, Halushka MK, Calkins H (2008) Gap junction remodeling in a case of arrhythmogenic right ventricular dysplasia due to plakophilin-2 mutation. J Cardiovasc Electrophysiol 19:1212–1214

Tintelen JP van, Entius MM, Bhuiyan ZA, Jongbloed R, Wiesfeld AC, Wilde AA, Smagt J van der, Boven LG, Mannens MM, Langen IM van, Hofstra RM, Otterspoor LC, Doevendans PA, Rodriguez LM, Gelder IC van, Hauer RN (2006) Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. Circulation 113:1650–1658

Tsatsopoulou AA, Protonotarios NI, McKenna WJ (2006) Arrhythmogenic right ventricular dysplasia, a cell adhesion cardiomyopathy: insights into disease pathogenesis from preliminary genotype-phenotype assessment. Heart 92:1720–1723

Valiron O, Chevrier V, Usson Y, Breviario F, Job D, Dejana E (1996) Desmoplakin expression and organization at human umbilical vein endothelial cell-to-cell junctions. J Cell Sci 109:2141–2149

Vasioukhin V, Bauer C, Yin M, Fuchs E (2000) Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell 100:209–219

Waschke J (2008) The desmosome and pemphigus. Histochem Cell Biol 130:21–54

Wu SL, Wang PN, Hou YS, Zhang XC, Shan ZX, Yu XY, Deng M (2009) Mutation of plakophilin-2 gene in arrhythmogenic right ventricular cardiomyopathy. Chin Med J (Engl) 122:403–407

Wuchter P, Boda-Heggemann J, Straub BK, Grund C, Kuhn C, Krause U, Seckinger A, Peitsch WK, Spring H, Ho AD, Franke WW (2007) Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res 328:499–514

Yang Z, Bowles NE, Scherer SE, Taylor MD, Kearney DL, Ge S, Nadvoretskiy VV, DeFreitas G, Carabello B, Brandon LI, Godsel LM, Green KJ, Saffitz JE, Li H, Danieli GA, Calkins H, Marcus F, Towbin JA (2006) Desmosomal dysfunction due to mutations in desmoplakin causes arrhythmogenic right ventricular dysplasia/cardiomyopathy. Circ Res 99:646–655

Yu CC, Yu CH, Hsueh CH, Yang CT, Juang JM, Hwang JJ, Lin JL, Lai LP (2008) Arrhythmogenic right ventricular dysplasia: clinical characteristics and identification of novel desmosome gene mutations. J Formos Med Assoc 107:548–558

Zhou X, Stuart A, Dettin LE, Rodriguez G, Hoel B, Gallicano GI (2004) Desmoplakin is required for microvascular tube formation in culture. J Cell Sci 117:3129–3140

A novel kind of tumor type-characteristic junction: Plakophilin-2 as a major protein of adherens junctions in cardiac myxomata

Steffen Rickelt[1*], Stefania Rizzo[2*]; Yvette Doerflinger[1,3]; Hanswalter Zentgraf[4] Cristina Basso[2]; Gino Gerosa[5]; Gaetano Thiene[2]; Roland Moll[6]; Werner W. Franke[1,7]

[1] Helmholtz Group for Cell Biology, German Cancer Research Center, Heidelberg, Germany; [2] Department of Medical-Diagnostic Sciences, University of Padua Medical School, Padua, Italy; [3] Department of Dermatology, University Hospital Mannheim, University of Heidelberg, Mannheim, Germany; [4] Monoclonal Antibody Facility, German Cancer Research Center, Heidelberg; [5] Department of Cardiac, Thoracic and Vascular Sciences, University of Padua Medical School; [6] Institute of Pathology, Philipps University Marburg, Germany; [7] Progen Biotechnik, Heidelberg

*Steffen Rickelt and Stefania Rizzo contributed equally to this work.

Corresponding author
Prof. Dr. Werner W. Franke
Helmholtz Group for Cell Biology, German Cancer Research Center
Im Neuenheimer Feld 581, Building TP4 - Room S3.222
D-69120 Heidelberg, Germany
Phone: +49-6221-423212; Fax: +49-6221-423404
E-mail: w.franke@dkfz.de

Running title: Plakophilin-2 in cardiac myxomata.

Sources of support: This work was supported by grants from the Deutsche Krebshilfe (grants 10-2049-Fr1 and 106976 to W.W.F.). Stefania Rizzo was a visiting fellow at the German Cancer Research Center, supported by the Registry of Cardio-Cerebro-Vascular Pathology, Veneto Region, Venice, Italy.

Abstract

Using novel antibodies of high avidity to – and specificity for – the constitutive desmosomal plaque protein, plakophilin-2 (Pkp2), in a systematic study of the molecular composition of junctions connecting the cells of soft tissue tumors we have discovered with immunocytochemical, biochemical and electron microscopical methods in all 32 cardiac myxomata examined, a novel type of adherens junctions (AJ). These AJs contain cadherin-11 as their major transmembrane glycoprotein, which we could repeatedly show in colocalization with N-cadherin, anchored in a cytoplasmic plaque formed by α- and β-catenin, together with the further *armadillo* (*arm*)-type proteins plakoglobin, p120, p0071 and ARVCF. Surprisingly, all AJs of these tumors contained in addition another major *arm*-protein, plakophilin Pkp2, hitherto known as a constituent of desmosomes in epithelium-derived tumors. We have not detected Pkp2 in a series of non-cardiac myxomata studied in parallel. Therefore we conclude that this acquisition of Pkp2, which we recently have also observed in some mesenchymally-derived cells growing in culture, can also occur in tumorigenic transformations *in situ*. We propose to examine the marker value of Pkp2 in clinical diagnoses of cardiac myxomata and to develop Pkp2-targeted therapeutic reagents.

Key words: myxoma; adherens junctions; plakophilin-2; *puncta adhaerentia*; differential histodiagnosis

Introduction

The identification of the specific types of transformed cells and of the kind and level of their differentiation is an essential part of tumor diagnosis, not only with respect to the histogenic origin but also as a basis for prognoses and therapeutic decisions. Therefore molecular markers for cell typing and differentiation have become almost obligatory criteria in pathological diagnoses, in particular of epithelium-derived tumors, for which reliable immunohistochemical marker antibodies are used worldwide (e.g., [1-4]; for a review see [5]). However, in the field of mesenchymally-derived tumors, the armamentarium of diagnostic markers is still relatively poor and problems of correct identification of certain soft tissue tumors are obvious.[6, 7] Therefore, we have recently begun systematically to determine the molecular composition of potential marker molecules of mesenchymal cell types and the diverse non-epithelial tumors derived therefrom (see, e.g., [6-9]).

Surprisingly, already in our initial series of experiments we have recognized unexpected changes in the molecular ensembles of cell-cell junctions of the adherens-category. An especially eye-catching finding has been the phenomenon that a series of mesenchymally-derived cells growing in culture, including human tumor cell lines, contain adherens junctions (AJs) of a drastically altered composition. They are based on clusters of N-cadherin or cadherin-11 anchored in a subplasmalemmal plaque which contains α- and β-catenin plus several further proteins of the *armadillo* (*arm*)-family such as plakoglobin and proteins p120, p0071 and ARVCF but in addition also contain another major *arm*-type protein, plakophilin-2 (Pkp2).[10, 11] This rapid acquisition of Pkp2 is perplexing as plakophilins hitherto have only been known as obligatory molecules specific for desmosomes of epithelial and myocardiac cells as well as of epithelium-derived tumors, including all carcinomas.

We then have also noted in sections through some tumors of mesenchymal origin occasional groups of cells which in immunohistochemistry show AJs positive for Pkp2, indicating that this change of molecular composition of the AJs may also take place during tumor formation *in situ*. Consequently, we have examined the molecular composition of AJs connecting the cells of various soft tissue tumors. Here we present as a first result our finding that all AJs connecting cardiac myxoma cells show exactly such a general acquisition of Pkp2.

Materials and Methods

Antibodies

To generate highly sensitive antibodies specific for plakophilin-2 (Pkp2), that could also be used on aldehyde-fixed and paraffin-embedded tissues, we have used peptides derived from promising, epitope-containing regions of the human Pkp2 amino acid (aa) sequence (aa positions 820-837, representing the carboxy-terminal aa sequence KTDFVNSRTAKAYHSLKD, and aa positions 611-625; i.e. VKEQYQDVPMPEEKS, representing a segment at the border between *arm-repeats* 5 and 6), conjugated them to keyhole limpet hemocyanin for the immunization of guinea pigs (for details of the molecule and of immunization protocols see reference [12]). The polyclonal antibodies selected, termed PP2-hCT (for the carboxyl terminal sequence) and PP2-hM (for the arm-repeat 5/6 border domain), were of excellent stability and accessibility in biochemical and immunological experiments.

Murine monoclonal antibodies (mAbs) to Pkp2 were generated by immunization of BalbC mice, using peptides derived from human Pkp2 aa position 527-872 (for method see [13]). The supernatants of the resulting monoclonal hybridoma cell cultures were screened by immunofluorescence microscopy using methanol/acetone-fixed epithelial cell cultures (for protocol see Supplementary Information) and tested by immunoblotting of total cellular proteins separated by SDS-PAGE. In addition, we specifically screened for immunostaining reactivity on formaldehyde-fixed cultured cells and tissue blocks (see below). From a total of ca. 2000 different hybridomas, 3 mAbs (Pkp2-402, Pkp2-407 and Pkp2-518) were prepared in sufficient amounts and characterized in detail. All antibodies routinely used are listed in the Supplementary Table 1 (see also [11, 14]).

Tissues

Thirty-two frozen and formaldehyde-fixed and paraffin-embedded samples of human tissues, including a large series of different myxoma types (see Supplementary Information) have been examined. Special diagnostic care was taken that no malignant heart tumors were included in the study.[15] Samples of heart tissues obtained from various mammalian, avian, amphibian and fish species (cf. [10, 16]) were examined in parallel.

Gel electrophoresis and immunoreactions of proteins

For preparations of control cell lysates, monolayer cell cultures were briefly rinsed twice with phosphate-buffered saline (PBS), suspended in sample buffer [250 mM Tris-HCl, 10 % SDS, 20 % glycerol, 100 mM dithiotreitol (DTT); pH 6.8] containing benzonase (1:1000; Merck, Darmstadt, Germany), and scraped off the dishes using a rubber policeman. Small pieces of frozen myxoma and control tissues were also homogenized in sample buffer containing benzonase. After vigorous vortex-homogenization, lysates were heated for 5 min at 95°C, briefly centrifuged and subjected to SDS-PAGE, followed by blot transfer to PVDF membranes (Millipore, Bedford, MA, USA), and reacted with horseradish peroxidase-conjugated secondary antibodies, applied in combination with a chemiluminescence system (ECL, Amersham-Buchler, Braunschweig, Germany). [11]

Results

Generation of highly sensitive plakophilin-2 antibodies

To detect plakophilin-2 (Pkp2) deep in AJ plaques, notably epitopes masked by obscuring complexes (for nuclei see [12]), we have generated improved antibodies for immunohistochemistry. Figures 1 A-C present two such reagents, a mono- and a polyclonal one, showing intense and specific immunoblot reactions as well as immunostaining of the Pkp2 located in the composite junctions of the intercalated disks of human heart (cf. [17]). For comparison, Figures 1 D-E show the very intense and desmosome-specific Pkp2-immunostaining a section through formaldehyde-fixed and paraffin-embedded tissue sample of human colon (Figure 1D) and on a monolayer of human breast carcinoma cells (line MCF-7) grown in culture (Figure 1E and E´). The latter illustration also demonstrates that the vast majority of the Pkp2-positive sites colocalize with desmoplakin, the hallmark component of desmosomal plaques. Similarly brilliant results were obtained with numerous other tissues and cell cultures of human or other mammalian origins.

We have recently reported that Pkp2 is not only a permanent constituent of true desmosomes of epithelial, myocardiac or meningeal tissues and tumors derived therefrom and the composite junctions of cardiomyocytes (e.g., [12, 17, 18]) but can also

be found in diverse mesenchymally-derived cell cultures.[11] Therefore, we have decided to examine the possible presence of Pkp2 in AJs of mesenchymal tumors.

The adherens junctions connecting cardiac myxoma cells

Cardiac myxomata are considered as benign tumors generally characterized by "stellate" cells with variously-long cell processes embedded in a jelly-like mucoid extracellular matrix.[19] They present a predominantly mesenchymal marker protein profile with abundant bundles of intermediate-sized filaments (IFs) of the vimentin type. Only in a minor proportion of cells in some of these tumors, we have noted small groups of cells that are also positive for the IF protein desmin, whereas IFs containing any keratins, glia filament or neurofilament proteins have not been seen. Non-muscle type actin microfilaments are prevalent, but in a minor proportion of myxoma cells we have also detected α-smooth muscle-type actin filaments but no sarcomeric α-actins. All our general immunocytochemical observations in the 32 myxomata studied are *grosso modo* in agreement with most previous reports indicating a derivation from cardiac mesenchymal cells ([20-29]; for occasional claims of special cardiac myxoma tumor cells showing keratin-reactions, mostly in glandular elements, see, e.g., [24, 29, 30])

Most of the myxoma cells formed numerous cell processes and were interconnected into a loose irregular meshwork by *punctum adhaerens*-type AJs. Consequently, myxoma cells can occur in relatively close apposition, as shown in Figure 2, or distant from each other, connected only by rather thin, variously-long, tentacle-like filopodial cytoplasmic processes.[24, 25] As the very short processes of perinuclear cytoplasm are adequately resolved only by electron microscopy we show (Figure 2) an example of such an interaction via short processes and their AJs, characterized by a thin (ca. 15 nm) dense plaque (e.g., insert in Figure 2B), i.e. myxoma structures that have repeatedly been described in the literature as "desmosome-like" (e.g., [8, 28, 31-34]).

Biochemical analyses of adhering junction proteins

Our systematic analyses of cryo-dissected tissue samples of various snap-frozen myxomata by SDS-PAGE and immunoblotting revealed consistent but surprising results (Figure 3). The predominant transmembrane AJ glycoprotein identified was

cadherin-11. Only in limited regions of some of the tumors this glycoprotein was accompanied by N-cadherin. VE-cadherin, on the other hand, was clearly seen in the *zonulae adhaerentes* of the endothelial structures of adjacent vascular elements but was totally absent from the myxoma cells proper (see also below). On the other hand, we found all major AJ-plaque proteins such as α- and β-catenin, plakoglobin, protein p120 (Figure 3) as well as proteins p0071 and ARVCF (not shown).

Unexpectedly, however, we identified in all cardiac myxomata examined the plaque protein Pkp2 (Figure 3 presents examples from six different tumors analyzed in parallel in the same SDS-PAGE), often together with varying proportions of an immunoblot-positive polypeptide of ca. 70 kDa, obviously a distinct proteolytic breakdown-product. In a few tumor samples we have also noted the additional presence of minor amounts of Pkp3 (not shown) but we have never detected Pkp1. Tests for the most predominant protein in desmosomal plaques, desmoplakin, were negative in all myxoma cells of all cases (not shown).

Immunolocalization Studies

The mostly rather small AJs which connect the myxoma cells in their nucleus-containing cell bodies as well as in their slender processes, are recognized by the colocalization of Pkp2 with typical AJ markers such as α- and β-catenin, plakoglobin and proteins p120, p0071 or ARVCF, as demonstrated by their yellow to orange merge color (see, e.g., Figure 4, A and B). This is in distinct contrast to the absence of Pkp2 from the AJs connecting the vascular endothelial cells (note the green immunostaining for β-catenin in Figure 4A). In the myxoma cell processes the AJs often are clustered, resulting in continuously yellow immunostaining (Figure 4B) or in closely-spaced Pkp2-reactive punctate or “beaded” arrays (Figure 4, B-E).

Colocalization of Pkp2 was also obtained with both cadherins mentioned. Cadherin-11 generally colocalized with Pkp2 in serial arrays of yellow dots or beaded chains of AJ structures (e.g., Figure 5, B and C), in the same way as it reacted with β-catenin and other plaque proteins (Supplementary Figure 1, A-E). Similar observations were made with N-cadherin which appeared only in AJs of certain limited regions in some of the tumors (e.g., Figure 5A). Again all these AJ reactions fully contrasted to those of the endothelium of the adjacent vessels (see the red β-catenin reaction in both Figures 5, A and B). In control experiments, VE-cadherin

reacted only with the endothelial AJs which otherwise contained all the plaque proteins mentioned above, with the exception of Pkp2 (not shown). As a positive colocalization control for junctional plaque immunostaining in the *zonulae adhaerentes* of vascular endothelium an example is shown in the Supplementary Figure 1F.

Discussion

The molecular analysis of the cell-cell junctions in cardiac myxomata has led to the unexpected identification of a novel adherens junction (AJ) type, the plakophilin-2 (Pkp2)-containing AJ, hitherto only known as *coniunctio adhaerens* of certain cell cultures (e.g., [10, 11]; for review see [35]). Obviously this AJ type represents a cell-cell connecting structure of its own kind which is characteristic for certain tumors as shown here for cardiac myxomata. So far AJs of this type have only sporadically been noted in some isolated cells or cell groups in a rhabdomyosarcoma.[11]

The mostly rather small, roundish–to–oval AJs identified as the major myxoma cell-cell contacts represent typical mesenchymal structures albeit with a special molecular composition. In these AJs we have detected cadherin-11 as the only ubiquitous transmembrane glycoprotein, while additional N-cadherin was seen only in restricted regions of a few tumors, which we also take as an indication of the existence of two myxoma subtypes, one with cadherin-11 only and the other containing both cadherin-11 and N-cadherin (for related observations see, e.g., [35-41]). At present, however, we cannot rigorously exclude the alternative explanation that all myxoma AJs may also contain some N-cadherin which tends to be rapidly degraded by some of the proteolytic enzymes known to occur in tissue preparations from these tumors (see e.g., [42]). None of the other cadherins has ever been detected in cardiac myxoma cells.

The molecular pattern of the AJ plaques of the cardiac myxoma cells is remarkably complex and specific. While most components identified, including α- and β-catenin, together with further *arm*-proteins such as plakoglobin and proteins p120, p0071 and ARVCF, have also been found in other non-epithelial cells (for reference see e.g., [11, 35-37]), it has been a surprise to find that the AJ plaques of all 32 myxomata examined contain an additional major *arm*-protein, Pkp2, which so far has been considered to be a protein exclusive to desmosomes and the composite

junctions of cardiomyocytes.[12, 17,.35, 43, 44] As shown by Goossens et al. (2007) the latter, cardiomyocyte-specific plaque integration of Pkp2 is based on its binding to myocardial α-T-catenin [45], a protein, however, that does not seem to be present in cardiac myxoma cells. Obviously, the specific molecular binding-partners of Pkp2 and the mechanisms and functions of its acquisition to the myxoma AJs will have to be determined in future experiments.

The stable integration of Pkp2 in the AJs of cardiac myxomata is functionally remarkable, since this protein is known as the only member of the plakophilin subfamily that occurs in all proliferatively active epithelial cells and is also an essential architectonic and cytoskeletal filament-anchoring molecule in the desmosomes and composite junctions of myocardial cells. In the latter it is necessary for heart formation as well as for the onset and coordination of rhythmic heart beat (e.g., [18, 44-48]), be it directly or indirectly (for further involvements of sodium channels or gap junctions see also recent knock-down experiments.[49-52] Most impressive in the discussions about possible functional roles of Pkp2 are certainly the recent reports that cardiac Pkp2 is by far the most sensitive protein which in mutated forms can contribute to arrhythmogenic ventricular cardiomyopathies ARVCs (see, e.g., [46-53]). Moreover, in Pkp2 gene knockdown experiments it has also been shown that its stabilizing effect on cell-cell adhesion in rat cardiomyocyte cultures is so important that a reduction in Pkp2 can result in a complete separation of the two junctional membranes.[49-52] Thus, in myxoma tumors growing in a very viscous, mucoid-gelatinous matrix the acquisition of Pkp2 to the AJs may have an important stabilizing effect and strengthen the cell-cell adhesions, in particular those connecting the long and thin cell processes.

Finally, our findings strongly support the hypothesis that cardiac myxoma cells originate from mesenchymal cells of the heart, as their similarity to the cardiac interstitial cells is remarkable.[10] The molecular AJ marker pattern of cardiac myxomata is indeed very similar to that reported for, e.g., valvular interstitial cells in culture, including the extensive and relatively rapid acquisition of Pkp2. Both cardiac interstitial and myxoma cells are also known for a certain spatial or developmental relationship to vascular endothelial cells, and synthesis and secretion of specific endothelial and angiogenic molecules have recently also been ascribed to vascular elements in myxomata.[54-56] By contrast, certain neural, neuroendocrine or glandular molecules seen in some elements of these tumors may be differentiation products of

some cells “entrapped” during development (for special discussions see, e.g., [24, 34, 40, 57-59]). A rather unexpected finding in our study has been the observation of Pkp2 in all AJs of all 32 cardiac myxomata whereas we have not detected this junction protein in any of the seven non-cardiac myxomata, including angiomyxomta and tumors grown in the vagina or the vulva. Differences of molecular markers and possible cell type-specific histological heterogeneities will be subject of a future extended study comparing cardiac and other myxomata as well as a comparison with some of the rare myxoma cases showing malignant behavior, i.e. recurrence and metastasis (for general discussions of these problems see, e.g., [20, 25, 29, 60, 61]).

It has also not escaped our attention that the general occurrence of cadherin-11 and Pkp2 in myxoma AJs may lead to new possible therapeutic concepts of molecular interference with these molecules and thus with cell-cell adhesion, and tumor growth. Although surgical excision is – and probably will remain – the standard therapy for the majority of myxoma cases there are also situations in which the value and availability of a pharmacological alternatives should not be underrated (see also e.g., [41, 56, 60, 61]). Certainly, recent strategies preclinical tests with reagents interfering with N-cadherin-mediated cell-cell interactions [62] and the aforementioned results using the siRNA-approach should now also encourage projects interfering with other AJ molecules.

Conflict of Interest

The authors declare no personal or financial conflict of interest.

Acknowledgements

We thank Dr. Hans Heid (German Cancer Research Center) for his help in the generation of improved polyclonal plakophilin-2 antibodies as well as Stefanie Winter-Simanowski and Caecilia Kuhn for excellent technical assistance. We also thank Dipl. Biol. Mareike Barth (German Cancer Research Center) for many discussions on cardiac interstitial cells.

List of abbreviations

arm – *armadillo*; AJ – adherens junction; cad – cadherin; Dsg – desmoglein; IF – intermediate-sized filament; mAb – monoclonal antibody; PBS – phosphate-buffered saline; Pkp – plakophilin

References

1. Franke WW, Schmid E, Weber K, Osborn M. HeLa cells contain intermediate-sized filaments of the prekeratin type. *Exp Cell Res* 1979 Jan; 118(1): 95-109.
2. Franke WW, Schmid E, Grund C*, et al.* Antibodies to high molecular weight polypeptides of desmosomes: specific localization of a class of junctional proteins in cells and tissue. *Differentiation* 1981; 20(3): 217-41.
3. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982; 31: 11-24.
4. Franke WW, Moll R, Mueller H*, et al.* Immunocytochemical identification of epithelium-derived human tumors with antibodies to desmosomal plaque proteins. *Proc Natl Acad Sci U S A* 1983 Jan; 80(2): 543-7.
5. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 2002 May; 40(5): 403-39.
6. Miettinen M. *Diagnostic Soft Tissue Pathology*, Vol. 1st edition, Churchill Livingstone: New York, Edinburgh, London, Philadelphia, 2003, 1-593pp.
7. Folpe AL, Gown A. Immunohistochemistry for analysis of soft tissue tumors. In: Weiss S, Goldblum J (eds). *Soft Tissue Tumors*, Vol. 4. Mosby: St.Louis, 2001, pp 199-245.
8. Ghadially FN. *Diagnostic Electron Microscopy of Tumors*, Butterworths & Co Ltd: London, Boston, 1980, 1-251pp.
9. Erlandson RA. *Diagnostic Transmission Electron Microscopy of Human Tumors*, Masson Publishing USA, Inc.: New York, Paris, Barcelona, 1981, 1-193pp.
10. Barth M, Schumacher H, Kuhn C*, et al.* Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. *Cell Tissue Res* 2009 Jul; 337(1): 63-77.
11. Rickelt S, Winter-Simanowski S, Noffz E, Kuhn C, Franke WW. Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. *Int J Cancer* 2009 Nov 1; 125(9): 2036-48.

12. Mertens C, Kuhn C, Franke WW. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. *J Cell Biol* 1996 Nov; 135(4): 1009-25.
13. Holmdahl R, Andersson M, Jansson L. A method for the analysis of a large number of specific and multispecific B cell hybridomas derived from primary immunized lymph nodes. *Hybridoma* 1987 Apr; 6(2): 197-204.
14. Wuchter P, Boda-Heggemann J, Straub BK*, et al.* Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. *Cell Tissue Res* 2007 Jun; 328(3): 499-514.
15. Neragi-Miandoab S, Kim J, Vlahakes GJ. Malignant tumours of the heart: a review of tumour type, diagnosis and therapy. *Clin Oncol (R Coll Radiol)* 2007 Dec; 19(10): 748-56.
16. Pieperhoff S, Franke WW. The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species. *Eur J Cell Biol* 2008 Jul; 87(7): 413-30.
17. Franke WW, Borrmann CM, Grund C, Pieperhoff S. The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. *Eur J Cell Biol* 2006 Feb; 85(2): 69-82.
18. Grossmann KS, Grund C, Huelsken J*, et al.* Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. *J Cell Biol* 2004 Oct 11; 167(1): 149-60.
19. Stout AP. Myxoma, the tumor of primitive mesenchyme. *Ann Surg* 1948 May; 127(4): 706-19.
20. Amano J, Kono T, Wada Y*, et al.* Cardiac myxoma: its origin and tumor characteristics. *Ann Thorac Cardiovasc Surg* 2003 Aug; 9(4): 215-21.
21. Jacot-Des-Combes E, Beyeler Y. Cardiac myxoma. Ultrastructural and immunohistochemical study of two cases. *Ann Pathol* 1982; 2(3): 223-8.
22. Takagi M. Ultrastructural and immunohistochemical characteristics of cardiac myxoma. *Acta Pathol Jpn* 1984 Sep; 34(5): 1099-114.
23. Schuger L, Ron N, Rosenmann E. Cardiac myxoma. A retrospective immunohistochemical study. *Pathol Res Pract* 1987 Feb; 182(1): 63-6.

24. Johansson L. Histogenesis of cardiac myxomas. An immunohistochemical study of 19 cases, including one with glandular structures, and review of the literature. *Arch Pathol Lab Med* 1989 Jul; 113(7): 735-41.
25. Basso C, Valente M, Poletti A, Casarotto D, Thiene G. Surgical pathology of primary cardiac and pericardial tumors. *Eur J Cardiothorac Surg* 1997 Nov; 12(5): 730-8.
26. Loire R, Tabib A. [Histopathologic study of cardiac myxoma. Apropos of 80 surgical cases]. *Arch Anat Cytol Pathol* 1991; 39(1-2): 5-13.
27. Govoni E, Severi B, Cenacchi G*, et al.* Ultrastructural and immunohistochemical contribution to the histogenesis of human cardiac myxoma. *Ultrastruct Pathol* 1988 Mar-Apr; 12(2): 221-33.
28. Orlandi A, Ciucci A, Ferlosio A*, et al.* Cardiac myxoma cells exhibit embryonic endocardial stem cell features. *J Pathol* 2006 Jun; 209(2): 231-9.
29. Curschellas E, Toia D, Borner M, Mihatsch MJ, Gudat F. Cardiac myxomas: immunohistochemical study of benign and malignant variants. *Virchows Arch A Pathol Anat Histopathol* 1991; 418(6): 485-91.
30. Pucci A, Bartoloni G, Tessitore E, Carney JA, Papotti M. Cytokeratin profile and neuroendocrine cells in the glandular component of cardiac myxoma. *Virchows Arch* 2003 Nov; 443(5): 618-24.
31. Ferrans VJ, Roberts WC. Structural features of cardiac myxomas. Histology, histochemistry, and electron microscopy. *Hum Pathol* 1973 Mar; 4(1): 111-46.
32. Valente M. Structural profile of cardiac myxoma. *Appl Pathol* 1983; 1(5): 251-63.
33. Stovin PG, Heath D, Khaliq SU. Ultrastructure of the cardiac myxoma and the papillary tumour of heart valves. *Thorax* 1973 May; 28(3): 273-85.
34. Kodama H, Hirotani T, Suzuki Y, Ogawa S, Yamazaki K. Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors. *Am J Pathol* 2002 Aug; 161(2): 381-9.
35. Franke WW, Rickelt S, Barth M, Pieperhoff S. The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind. *Cell Tissue Res* 2009 Oct; 338(1): 1-17.
36. Franke WW. Discovering the molecular components of intercellular junctions – a historical view. In: Nelson J, Fuchs E (eds). *Cold Spring Harbor Laboratory Press*, Vol. 1. Cold Spring Harbor Laboratory Press: New York, 2009, in press.

37. Strumane K, Berx G, Roy FV. Cadherins in Cancer. In: Behrens J, Nelson WJ (eds). *Cell Adhesion*, Vol. 165. Springer Verlag: Berlin, Heidelberg, New York, 2004, pp 69-103.
38. Butany J, Nair V, Naseemuddin A*, et al.* Cardiac tumours: diagnosis and management. *Lancet Oncol* 2005 Apr; 6(4): 219-28.
39. Reynen K. Cardiac myxomas. *N Engl J Med* 1995 Dec 14; 333(24): 1610-7.
40. Pucci A, Gagliardotto P, Zanini C*, et al.* Histopathologic and clinical characterization of cardiac myxoma: review of 53 cases from a single institution. *Am Heart J* 2000 Jul; 140(1): 134-8.
41. Burke AP, H. T, Gomez-Roman JJ*, et al.* Tumours of the Heart. Benign Tumors of Pluripotent Mesenchyme. Cardiac Myxoma. In: Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC (eds). *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*, Vol. IARC Press: Lyon, 2004, pp 260-3.
42. Orlandi A, Ciucci A, Ferlosio A*, et al.* Increased expression and activity of matrix metalloproteinases characterize embolic cardiac myxomas. *Am J Pathol* 2005 Jun; 166(6): 1619-28.
43. Bass-Zubek AE, Godsel LM, Delmar M, Green KJ. Plakophilins: multifunctional scaffolds for adhesion and signaling. *Curr Opin Cell Biol* 2009 Oct; 21(5): 708-16.
44. Borrmann CM, Grund C, Kuhn C*, et al.* The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk. *Eur J Cell Biol* 2006 Jun; 85(6): 469-85.
45. Goossens S, Janssens B, Bonne S*, et al.* A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs. *J Cell Sci* 2007 Jun 15; 120(Pt 12): 2126-36.
46. Gerull B, Heuser A, Wichter T*, et al.* Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy. *Nat Genet* 2004 Nov; 36(11): 1162-4.
47. van Tintelen JP, Entius MM, Bhuiyan ZA*, et al.* Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Circulation* 2006 Apr 4; 113(13): 1650-8.

48. Hall C, Li S, Li H, Creason V, Wahl JK, 3rd. Arrhythmogenic right ventricular cardiomyopathy plakophilin-2 mutations disrupt desmosome assembly and stability. *Cell Commun Adhes* 2009 Jun 16: 1-13.
49. Oxford EM, Musa H, Maass K*, et al.* Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. *Circ Res* 2007 Sep 28; 101(7): 703-11.
50. Sato PY, Musa H, Coombs W*, et al.* Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes. *Circ Res* 2009 Sep 11; 105(6): 523-6.
51. Joshi-Mukherjee R, Coombs W, Musa H*, et al.* Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations. *Heart Rhythm* 2008 Dec; 5(12): 1715-23.
52. Pieperhoff S, Schumacher H, Franke WW. The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes. *Eur J Cell Biol* 2008 Jul; 87(7): 399-411.
53. Marcus FI, Nava A, Thiene G. *Arrhytmogenic RV Cardiomyopathy/Dysplasia Recent Advances*, Springer Verlag: Milan, 2007.
54. Sakamoto H, Sakamaki T, Kanda T*, et al.* Vascular endothelial growth factor is an autocrine growth factor for cardiac myxoma cells. *Circ J* 2004a May; 68(5): 488-93.
55. Sakamoto H, Sakamaki T, Sumino H*, et al.* Production of endothelin-1 and big endothelin-1 by human cardiac myxoma cells--implications of the origin of myxomas. *Circ J* 2004b Dec; 68(12): 1230-2.
56. Gaumann A, Strubel G, Bode-Lesniewska B*, et al.* The role of tumor vascularisation in benign and malignant cardiovascular neoplasms: a comparison of cardiac myxoma and sarcomas of the pulmonary artery. *Oncol Rep* 2008 Aug; 20(2): 309-18.
57. Krikler DM, Rode J, Davies MJ, Woolf N, Moss E. Atrial myxoma: a tumour in search of its origins. *Br Heart J* 1992 Jan; 67(1): 89-91.
58. Terracciano LM, Mhawech P, Suess K*, et al.* Calretinin as a marker for cardiac myxoma. Diagnostic and histogenetic considerations. *Am J Clin Pathol* 2000 Nov; 114(5): 754-9.

59. Suvarna SK, Royds JA. The nature of the cardiac myxoma. *Int J Cardiol* 1996 Dec 13; 57(3): 211-6.
60. Keeling IM, Oberwalder P, Anelli-Monti M*, et al.* Cardiac myxomas: 24 years of experience in 49 patients. *Eur J Cardiothorac Surg* 2002 Dec; 22(6): 971-7.
61. Silverman NA. Primary cardiac tumors. *Ann Surg* 1980 Feb; 191(2): 127-38.
62. Blaschuk OW, Devemy E. Cadherins as novel targets for anti-cancer therapy. *Eur J Pharmacol* 2009 Dec 25; 625(1-3): 195-8.

Figure Legends

Figure 1. a-c: Specificity of two of the newly generated plakophilin-2 (Pkp2) antibodies in their immunoblot and immunofluorescence reactions on human heart tissue proteins. **a:** Lysates of total human myocardium, showing the reaction with a single polypeptide band of ca. 96 kDa, using monoclonal antibody (mAb) Pkp2-518 and guinea pig antibodies of antiserum PP2-hCT. Reference protein bands (on the left margin) give the molecular weight in kDa values.
b and **c:** Confocal laser scanning microscopy of paraffin-embedded human heart tissue, treated for antigen retrieval and reacted with mAb Pkp2-518 (red), shows a specific reaction of the composite junctions in the intercalated disks (**b**, fluorescence optics; **c**, fluorescence and phase contrast optics). **d-e´:** Confocal microscopy images showing the intense and specific reactions of mAb Pkp2-518 with desmosomes on a section through human intestinal tissue treated for antigen-retrieval (**d**) and on a monolayer of cultured human cells of the breast carcinoma line MCF-7 (**e, e´**).
d: Immunofluorescence and phase contrast optics demonstrating that the immunofluorescent reaction is specific for the desmosomes of the epithelium. **e:** The immunoreaction of Pkp2 (red) is detected in linear punctate arrays of small closely-spaced reaction sites, representing desmosomes, and in some sparse and irregularly distributed cytoplasmic dots, representing endo- or exocytotic vesicles coated with a Pkp2-containing plaque. **e`:** Interference contrast color image of the same region, demonstrating the colocalization of Pkp2 and desmoplakin (green) in desmosomal plaques (yellow merged color) and in some cytoplasmic desmosomal plaque structures. Scale bars: 20 µm

Figure 2. a and **b:** Electron micrographs of cross-sections through adjacent cell borders in an atrial human myxoma, presenting numerous cell-cell contacts at protrusions or variously-long filopodia-like processes, including highly folded and packed filopodia (an example is shown in the insert in the lower left). **a:** Extended contact regions of central cell bodies (N1 and N2: nuclei of the two cells; arrows denote the ends of the contact zone), embedded in a loose extracellular matrix. **b:** Higher magnification of a contact zone with numerous filopodia-like processes (arrows) which appear to be involved in cell-cell contacts, in specific places forming plaque-coated adherens junctions (arrowheads), one of which is shown in detail in

the insert in the upper left. Scale bars: 1 µm (**a**); 0.5 µm (**b** and insert in **a**), 0.25 µm (insert in **b**)

Figure 3. Immunoblot detection of adherens junction (AJ) proteins in human myxomata as revealed by SDS-PAGE-separated total cytoskeletal proteins and Western blots, in comparison with such proteins from cultured valvular human interstitial cells (hPK, *lane 1*; cf. [10]) or with junctional proteins from cultured human liver carcinoma cells of line PLC (primary liver carcinoma; *lane 1* with asterisk). The following tumors (numbers) are shown here: 1046-06 (*lane 2*), 1236-08 (*lane 3*) and 1334-08 (*lane 4*). Proteins were probed with antibodies specific for actin and vimentin, cadherin-11 (cad-11) and E-cadherin (E-cad), in comparison with the desmosomal cadherins desmogleins 1 and 2 (Dsg1+2) as well as AJ plaque proteins α- and β-catenin or protein p120. Note that, besides the IF protein vimentin, all AJ proteins tested, including cad-11, α- and β-catenin as well as protein p120 can be detected in the myxomata. Plakophilin-2 (Pkp2) is detected, at different intensities, as a polypeptide band of ca. 96 kDa (arrow at right hand margin); whereas other epithelial marker proteins such as E-cad and Dsg1+2 are absent in all myxoma samples. To show the general occurrence of Pkp2, samples from three different myxomata are presented here. Total proteins of tumor samples were applied as follows (tumor numbers given): hPK (*lane 1*), 1363-07 (*lane 2*) and 1334-08 (*lane 3*), 1236-08 (*lane 4*), 1046-06 (*lane 5*), 531-07 (*lane 6*) and 197-07 (*lane 7*). Positions of polypeptide molecular weights are indicated on the very left margin and correspond to 158, 116, 97.2, 66.4, 55.6 and 42.7 kDa (from top to bottom). Note also the appearance of a major proteolytic Pkp2 fragment at ca. 70kDa.

* For the immunoblot identification of E-cadherin and Dsg2 whole cell lysates of human liver carcinoma cells of line PLC were loaded as positive control, instead of interstitial cells from cardiac valves.

Figure 4. Localization of plakophilin-2 (Pkp2) in AJs of diverse cardiac myxomata. Sections through paraffin-embedded human myxomata were treated for antigen-retrieval and double-immunostained with antibodies to Pkp2 (red) in combination with antibodies to β-catenin (**a, b**, green) and vimentin (**c-e**, green). Note the extensive colocalization (yellow) of Pkp2 and β-catenin in AJs of intervascular myxomata (**a**) as well as in AJs connecting extended processes of myxoma cells (**b**), whereas the AJs

connecting the vascular endothelial cells are negative for Pkp2. Note also that all cells positive for Pkp2 contain IFs of the vimentin-type (**c-e**) and that the AJs connecting myxoma cell processes are often clustered and thus appear in some situations as “beaded” chains (**c-e**, red). V, vessel. Scale bars: 20 µm

Figure 5. Immunofluorescence microscopy of sections trough human myxomata treated for antigen retrieval and reacted with antibodies to the AJ plaque protein, β-catenin (**a-c**, red), and the transmembrane glycoproteins N-cadherin (**a**, green) or cadherin-11 (**b, c**, green. Note that both cadherins are prominent at cell-cell contact sites of the specific myxomata (yellow merged color) but not at the AJs of the endothelial cells of the vessels (V), which, however, are recognized here by their intense β-catenin immunostaining. **c:** Magnification showing the colocalization (yellow) of the AJ-plaque protein β-catenin (red) and the transmembrane cadherin-11 (green) in punctate series of distinct AJs. DAPI stain (blue in **b** and **c**) has been used to visualize nuclei. Scale bars: 20 µm

Supplementary Information

Supplementary Figure 1. - Immunofluorescence microscopy, with (**a, b**) or without (**c-f**) phase contrast optics of sections through routinely fixed, dehydrated and paraffin-embedded tissue samples of human cardiac myxomata, treated for antigen-retrieval and double-labeled for the adherens junction (AJ) plaque proteins β-catenin (**a-e**, red), α-catenin (**f**, red) and protein p120 (**f**, green), in combination with cadherin-11 (**a, c-e**, green) or N-cadherin (**b**, green), showing colocalizations in distinct cell-cell junctions (yellow merged color). By contrast, vascular endothelial cells as well as occasional stroma cells are intensely stained for β-catenin but are negative for cadherin-11. **a** and **b:** Merged images and phase contrast optics (**a**) demonstrate the web-like distribution of thin myxoma cell processes extending throughout the extracellular matrix, connected by numerous *puncta adhaerentia* AJs (yellow). **c-e:** Double-label immunostaining of AJs connecting myxoma cells and their processes (red, β-catenin; green, cadherin-11). Note the small yellow merge color-stained punctate junctions which in some places appear in linear beaded chain arrays (**d, e**). **f:** Double-label immunostaining of a section through a human myxoma, showing AJs positive for the plaque proteins α-catenin (red) and p120 (green) which colocalize in many places (yellow merged color). Note that in this combination junction colocalization is seen not only on myxoma cell AJs but also in the *zonulae adhaerentes* connecting the endothelial cells of the vessels (V). Scale bars: 20 µm

Supplementary Material

Cell cultures

The human mammary gland adenocarcinoma cell line MCF-7 (ATCC, HTB-22) and the hepatocellular carcinoma cell line PLC (ATCC, CRL-8024) were used for antibody screening. Primary cell cultures derived from the interstitial tissue of human pulmonary valves grown in culture were used for comparison.[1]

Tumors

The tumor cases studied (median age 57±17 years, age range: 14-80 years; 14 male and 18 female; 27 located in the left atrium, 5 in the right atrium; 17 with a smooth

surface, 15 villous) represented prototypic cardiac myxomata, with inflammatory infiltrates and blood vessel-like structures ("myxomatous endothelioma"). None of the cases included in this report showed glandular elements.

Immunofluorescence and electron microscopy

For antibody screenings, cultured cells grown on glass coverslips were fixed either in PBS containing 2 % formaldehyde for 5-7 min or with methanol for 5 min at -20°C, followed by incubation for 20 s in -20°C acetone. Alternatively, to optimize permeabilization, cells were exposed for 2-5 min in PBS containing either 0.2 % Triton X-100 or 0.1 % saponin. Protocols used for immunostaining were essentially as recently reported.[3, 4, 5] For immunolocalization experiments using paraffin-embedded tissue samples, sections obtained with a Rotary Microtome HM 355 S (Microm International GmbH, Walldorf, Germany) were deparaffinized and subjected to heat-induced antigen retrieval (AR) performed according to standard protocols.[6, 7] In most experiments, the sections were pre-treated in 100 mM Tris-HCl buffer containing 5 % urea (pH 11.0, 10 - 20 min, 120°C) or in citrate buffer (82 mM sodium citrate and 18 mM citric acid, pH 6.0, 10 - 20 min, 120°C) using a RHS "Rapid Microwave Histoprocessor" (Milestone, Sorisole, Italy). This AR-treatment was followed by several washes in PBS and a final 20 min incubation in PBS containing 2 % milk powder and 0.2 % Triton X-100. Primary antibodies were usually applied for 1 h, followed by three washes in PBS and exposure of the samples to secondary antibodies for 30 min.[5] In the present study primary antibody complexes were visualized with secondary anti-species IgG-directed antibodies coupled to Cy3 (Dianova, Hamburg, Germany) or Alexa 488 (MoBiTec, Goettingen, Germany). For immunoblot analyses, horseradish peroxidase-conjugated secondary antibodies were applied (Dianova).

Microscopic images were recorded with an Axiophot II photomicroscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam HR (Carl Zeiss), and confocal images were taken with a Zeiss LSM 510 UV microscope.

Electron microscopy has been described by Rickelt et al. (2009).[3] Electron micrographs were taken at 80 kV using an EM 910 (Carl Zeiss).

Supplementary References

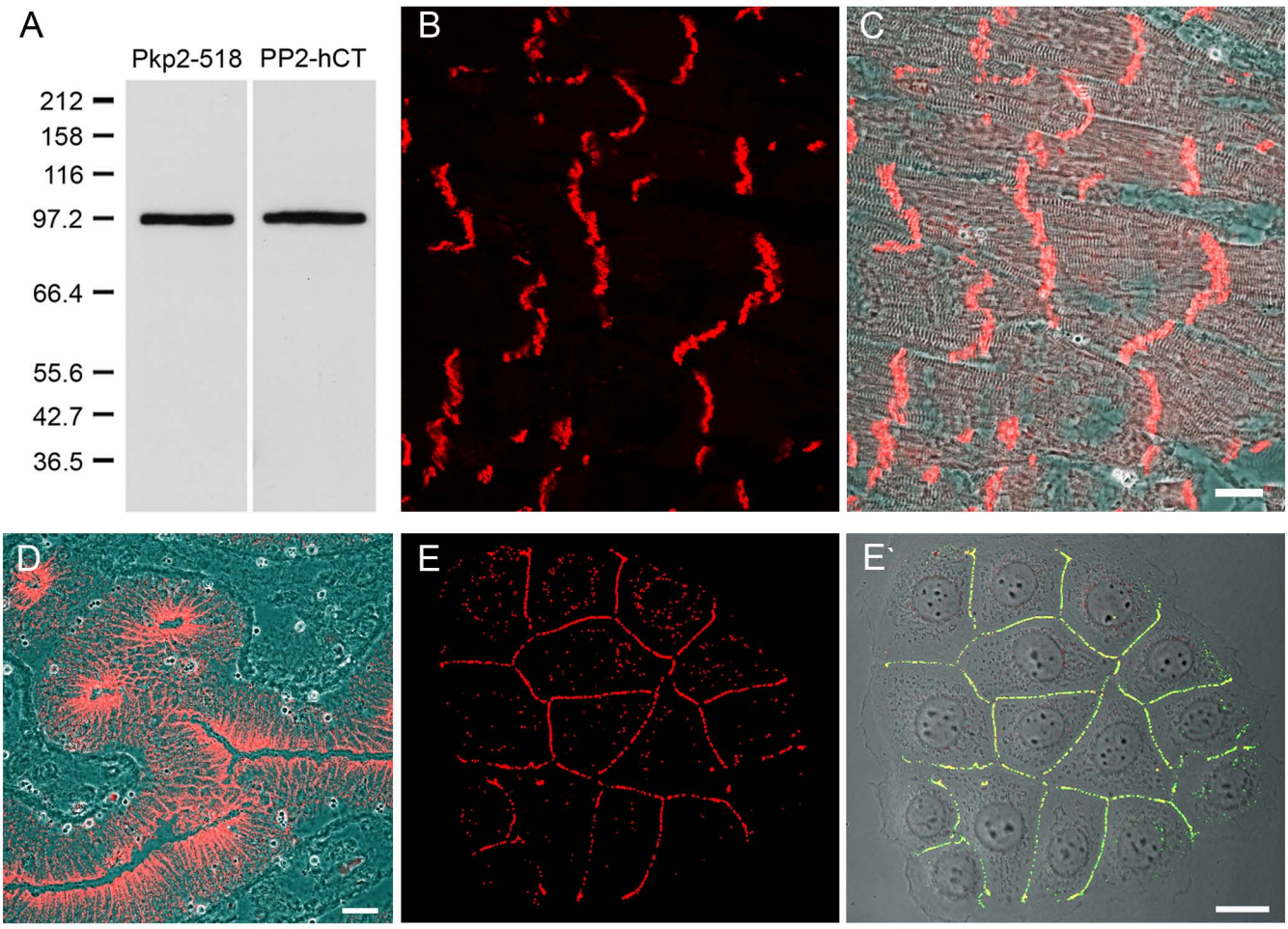
1. Barth M, Schumacher H, Kuhn C*, et al.* Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. *Cell Tissue Res* 2009 Jul; 337(1): 63-77.
2. Orr JW. Endothelioma (pseudomyxoma) of the heart. *J Pathol Bacteriol* 1942; 54: 125-8.
3. Rickelt S, Winter-Simanowski S, Noffz E, Kuhn C, Franke WW. Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. *Int J Cancer* 2009 Nov 1; 125(9): 2036-48.
4. Pieperhoff S, Franke WW. The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species. *Eur J Cell Biol* 2008 Jul; 87(7): 413-30.
5. Langbein L, Grund C, Kuhn C*, et al.* Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived therefrom. *Eur J Cell Biol* 2002 Aug; 81(8): 419-35.
6. Rickelt S, Franke WW, Doerflinger Y*, et al.* Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. *Cell Tissue Res* 2008 Oct 31; 334(3): 401-22.
7. Shi SR. *Antigen Retrieval Techniques: Immunohistochemistry and Molecular Morphology.*, Eaton Publishing: Natick, 2000, 1-353pp.

Supplementary Table 1. - Primary antibodies used in this study.

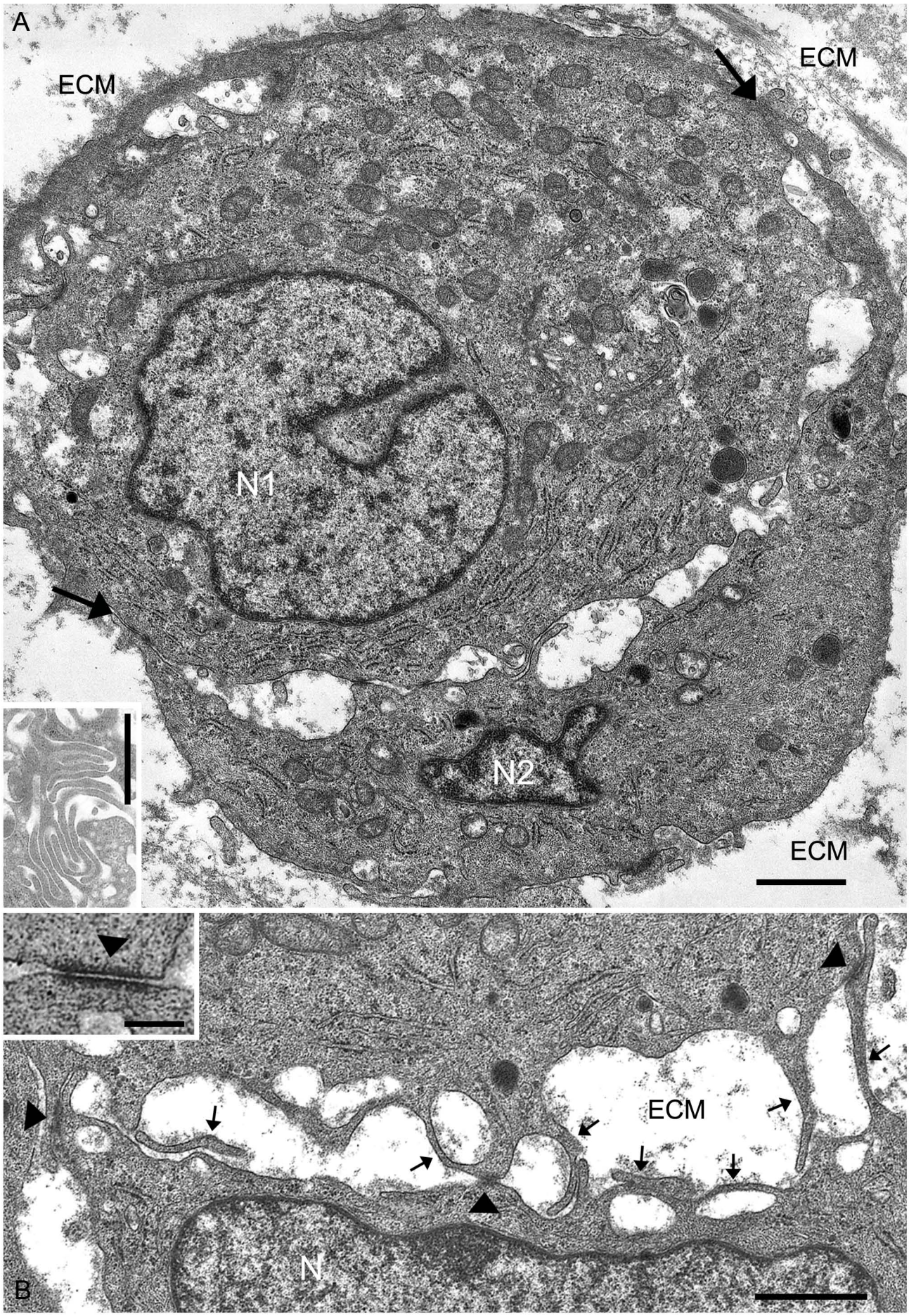
Antigen	Antibody type	Source (reference)
Transmembrane Proteins		
E-Cadherin	mAb, m	BD Biosciences Pharmingen (Heidelberg, Germany)
N-Cadherin	a) mAb, m	BD Biosciences
	b) As, rb	QED Bioscience Inc (San Diego, CA, USA)
P-Cadherin	mAb, m	BD Biosciences
VE-Cadherin	a) mAb, m (BV9)	Gift of E. Dejana (University of Milan, Italy)
	b) As, rb	Cayman Chemical Company (Ann Arbor, MI, USA)
Cadherin 11	a) mAb, m	Zymed Laboratories (San Francisco, CA, USA)
	b) As, rb	Zymed Laboratories
Desmoglein 1+2	mAb, (DG 3.10)	Progen Biotechnik (Heidelberg, Germany)
Desmocollin 1	mAb, m (U100)	Progen Biotechnik
Desmocollin 2	As, rb	Progen Biotechnik
Desmocollin 3	mAb, m (U114)	Progen Biotechnik
Plaque Proteins		
α-Catenin	a) mAb, m	Zymed Laboratories
	b) As, rb	Sigma (St. Louis, MO, USA)
β-Catenin	a) mAb, m	BD Biosciences
	b) As, rb	Sigma
Plakoglobin	a) mAb, m (11E4)	Gift of M.J. Wheelock (University of Nebraska, Omaha, NE, USA)
	b) mAb, m (PG 5.1)	Progen Biotechnik
Protein p120	a) mAb, m	BD Biosciences
	b) As, rb	Sigma
Protein p0071	a) mAb, m	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Protein ARVCF	a) mAb, m	Gift of I. Hofmann (German Cancer Research Center)
	b) AS, gp	Progen Biotechnik
Plakophilin-1	a) mAb, m (PP1-5C2)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Plakophilin-2	a) mAb, m (Pkp2-518)	Progen Biotechnik
	b) mAb, m (PP2/62, PP2/86, PP2/150)	Progen Biotechnik
	c) AS, gp	Progen Biotechnik

Plakophilin-3	a) mAb, m (PKP3-270)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Desmoplakin	a) mAb, m (DP-2.15, DP-2.17, DP-2.20)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Cytoskeletal filament proteins		
Vimentin	a) mAb, m (3B4)	Progen Biotechnik
	b) mAb, m (V9)	Progen Biotechnik
	c) AS, gp	Progen Biotechnik
Most keratins ("pan-Keratin")	mAb, m (Lu5)	Progen Biotechnik
Keratin 8	mAb, m (Ks8-17.2)	Progen Biotechnik
Keratin 18	mAb, m (Ks18.04)	Progen Biotechnik
Keratins 8 and18	AS, gp	Progen Biotechnik
Desmin	mAb, m	DAKO (Hamburg, Germany)
Smooth muscle α-Actin	mAb, m (ASM-1)	Progen Biotechnik
Cardiac/embryonic α-Actin	mAb, m (AC1-20.4.2)	Progen Biotechnik
Others		
Troponin T skeletal muscle	mAb, m	Sigma
Troponin T cardiac	AS, rb	Zytomed Systems (Berlin, Germany)
smooth muscle tropomyosin	AS, rb	Sigma
Myosin skeletal muscle heavy and light chain	AS, rb	Sigma
Protein Ki67	a) mAb, m	Zymed Laboratories
	b) As, rb	Zymed Laboratories

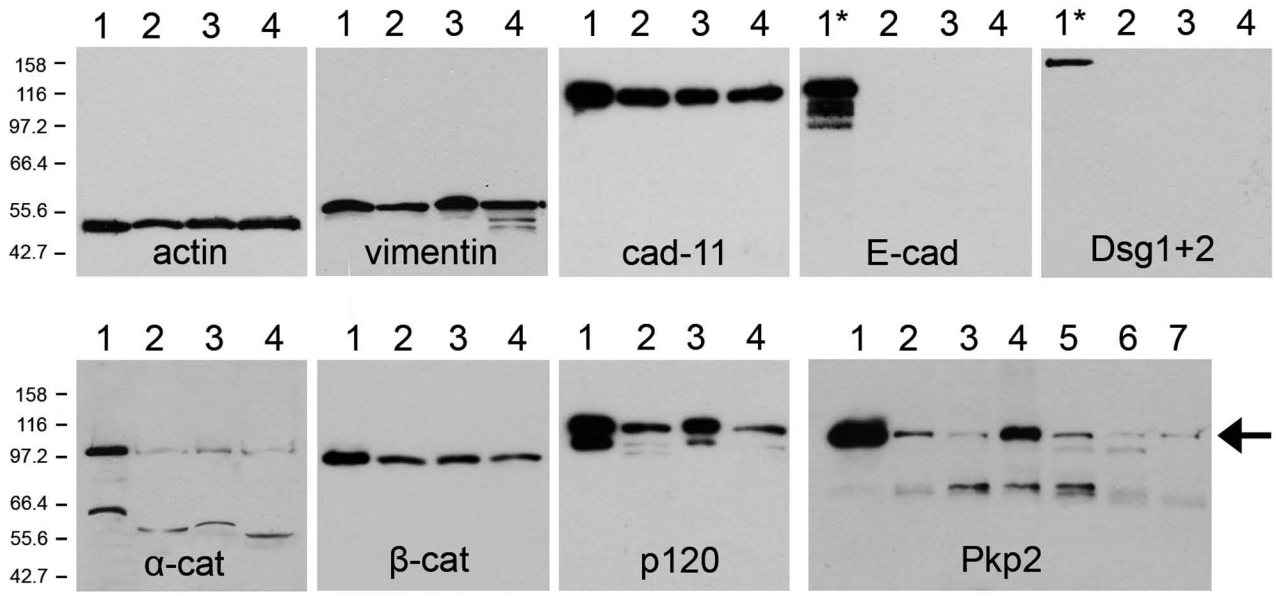
Supplementary Table 1 - Primary antibodies used in this study. As - antiserum or IgGs prepared therefrom; m - mouse; mAb - monoclonal antibody; rb - rabbit; gp - guinea pig



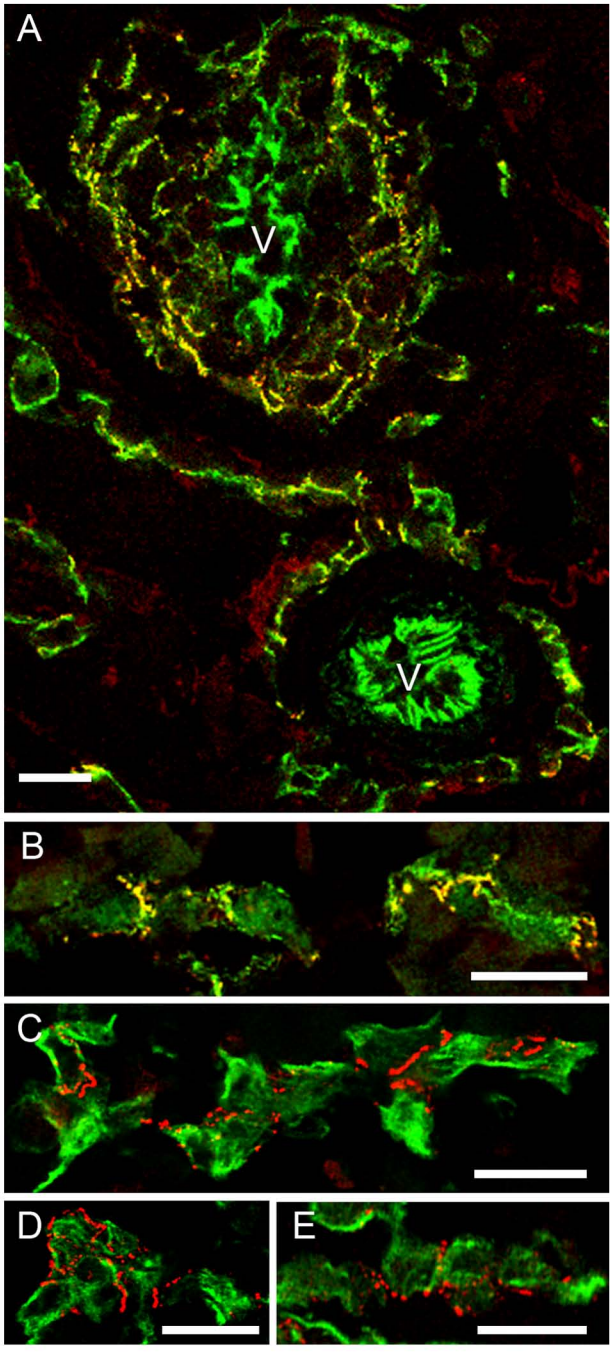
Rickelt et al. Figure 1



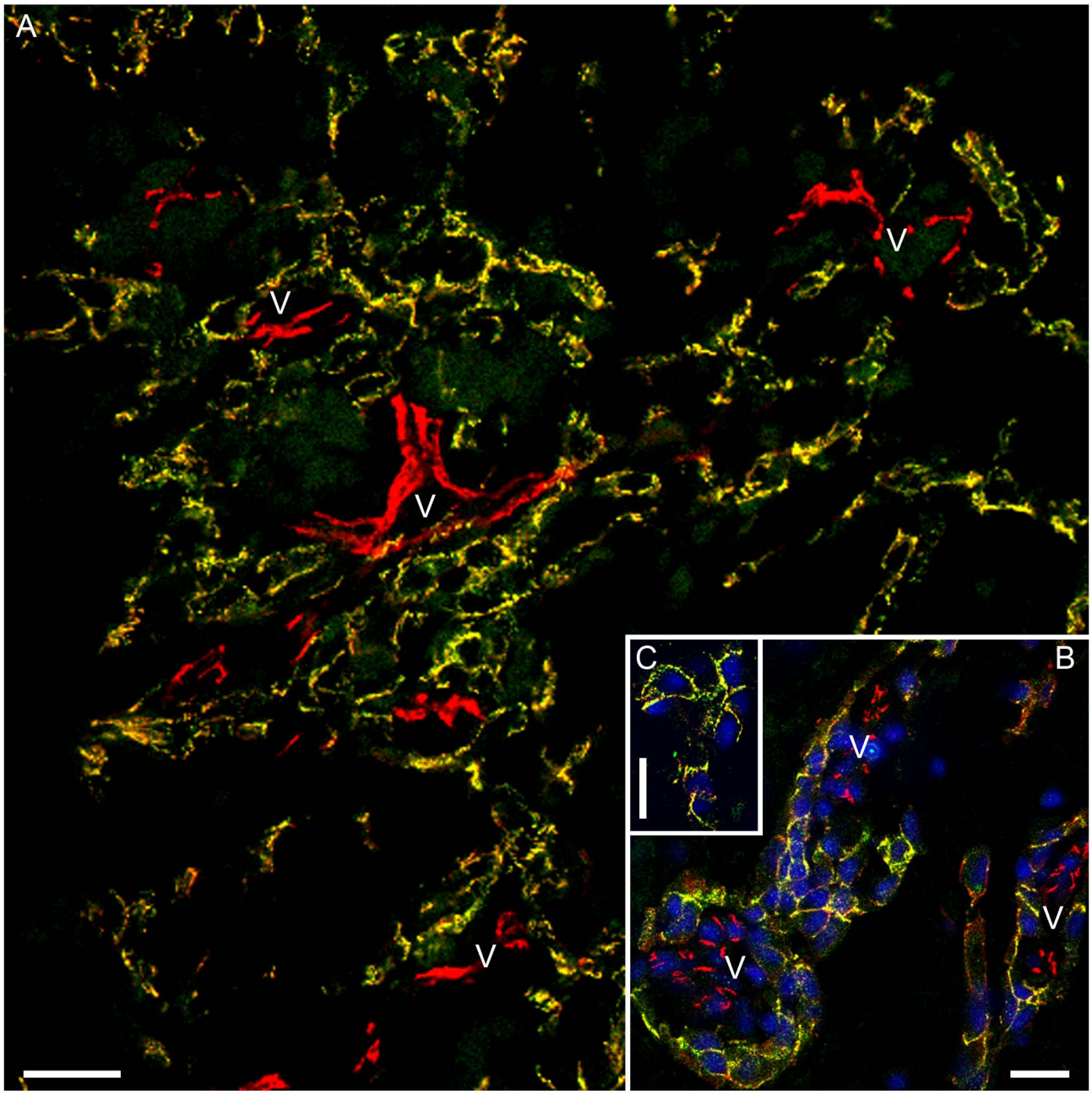
Rickelt et al. Figure 2



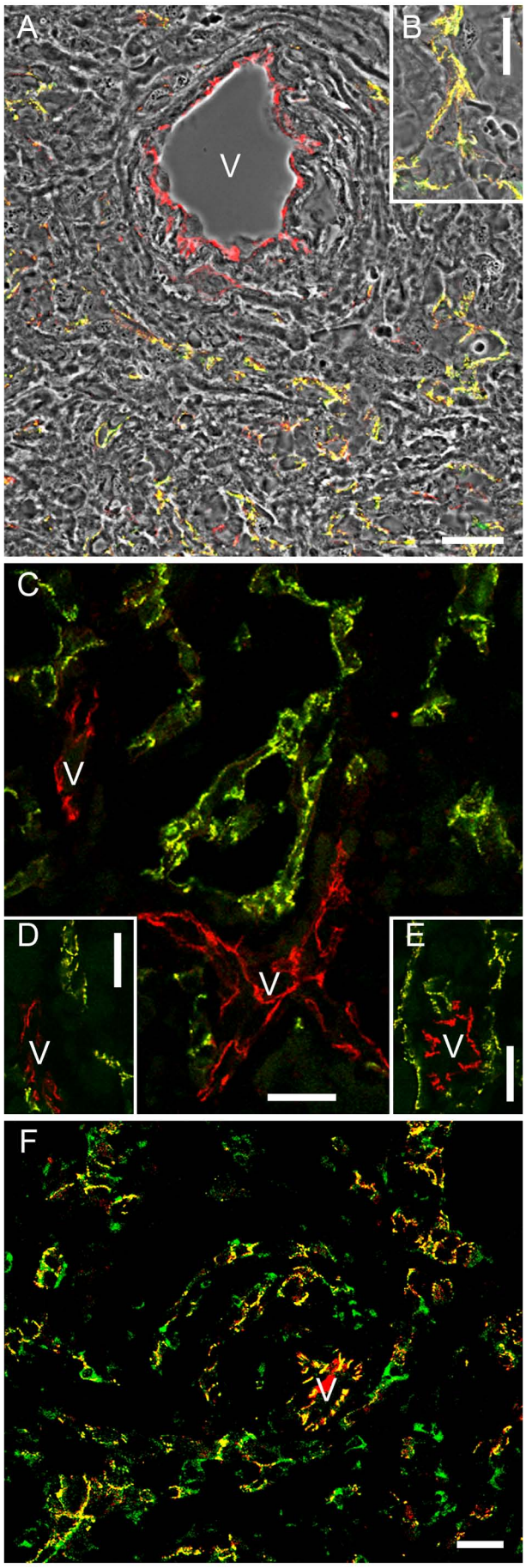
Rickelt et al. Figure 3



Rickelt et al. Figure 4



Rickelt et al. Figure 5



Rickelt et al. Supplementary Figure 1

Spontaneous and Cumulative Syntheses of Epithelial Proteins and Glycoproteins and Their Assemblies to Novel Cell-Cell Junctions in Malignantly Transformed Cells: I. Carcinomatoid Dysplasia Forms of Human Hematopoietic K562 Cells

Werner W. Franke[1,2] and Steffen Rickelt[1]

[1] Helmholtz Group for Cell Biology, German Cancer Research Center, Heidelberg, Germany; [2] Progen Biotechnik, Heidelberg

Corresponding author:
Prof. Dr. Werner W. Franke
Helmholtz Group for Cell Biology, German Cancer Research Center
Im Neuenheimer Feld 581, Building TP4
D-69120 Heidelberg, Germany
Phone: +49-6221-423212; Fax: +49-6221-423404
E-mail: w.franke@dkfz.de

Short title: Spontaneous Formation of Junctions in Hematopoietic Tumor Cells

Key words: hematopoietic cells; adhering junctions; cell differentiation; desmosomes; desmoglein

List of abbreviations: AJ – adhering junction; Dsc – desmocollin; Dsg – desmoglein; EpCAM – epithelial cell adhesion molecule; IF – intermediate-sized filament; mAbs – monoclonal antibodies

SUMMARY

Using biochemical as well as light- and electron-microscopic immunolocalization methods we have studied the phenomenon of spontaneous and cumulative syntheses of a series of epithelial proteins and glycoproteins and their assemblies to various kinds of small cell-cell junctions resembling adhering junctions (AJs) and half-AJ structures as well as punctate and extended cell-cell contact structures in various lines of human hematopoietic tumor cell cultures. We have enriched clonal cell culture colonies and sublines of multipotential human K562 tumor cells which are enriched in such newly formed junctions mostly based on cis- and/or trans-connected clusters of the epithelial-type cadherin, desmoglein Dsg2, anchored in a submembranous plaque containing plakoglobin and plakophilins (Pkp2 and Pkp3), with or without other *armadillo* (*arm*-) proteins or desmoplakin. A different mostly more extended junction system is based on another kind of transmembrane glycoprotein, epithelial cell adhesion molecule (EpCAM), which is associated with a cytoplasmic plaque rich in actin-microfilament-anchoring proteins such as afadin, α-actinin and vinculin. Both kinds of junctions are effective in connecting K562 cells to dense layers or even tissue-like three-dimensional structures. We discuss possible molecular mechanisms organizing these junction and their functions, in particular in the spread and metastasis of such tumor cells as well as possible diagnostic consequences and novel preventative and therapeutic possibilities.

SIGNIFICANCE

Provoked by observations that in various lines of hematopoietic tumor cells spontaneously novel kinds of small adhering junctions (AJs) and half-AJ structures appear, resulting in formations of tissue-like higher order structures, we determined the molecular composition, the ultrastructure and the stability of such junctions in cell cultures of one of the best studied multipotential leukemia lines, K562 cells. We have identified an epithelial desmosome-typical cadherin, desmoglein Dsg2, as the central cis-clustered transmembrane glycoprotein anchored in a cytoplasmic plaque formed by plakophilins and plakoglobin, in some AJ types together with certain other *armadillo*-type proteins or desmoplakin. Another – often extended – type of novel junctions is based on the epithelial type glycoprotein, EpCAM, anchored in a plaque rich in afadin, α-actinin and vinculin. These novel forms of non-genetic molecular heterogeneity of blood tumor cell-cell interactions not only provide new mechanistic insights into dysplasia and metastasis processes of specific hematopoietic tumor cells but also call for additional criteria and methods in diagnostics and therapy.

INTRODUCTION

Current views of tumorigenic cell transformation are dominated by concepts of specific gene mutations and alterations in the regulation of gene expression that result in developmentally uncontrolled proliferation, accompanied by changes of the specific cell's protein ensemble and changes of cell morphology and cell behavior, including invasive growth and metastasis. Correspondingly, determinations or estimations of the rate of cell proliferation, the cell type of origin of the primary tumor, the locations and types of differentiation of metastatic tumors are still the major basis of tumor diagnosis, prognosis and therapeutic proposals. In general, conservative mutation-genetic views prevail whereas potential non-genetically based but relatively stable changes of the cell protein pattern, the structural organization and the behavior are still only marginally considered (for reviews see e.g., Slack, 2007; Brock et al., 2009; Gilbert and Ross, 2009; Salk et al., 2010).

On the other hand, in certain tumors important and systematic non-genetic phenotype changes have been observed be they transient or even persistent. Such changes include alterations in the frequencies, patterns and forces of cell-cell adhesion structures, notably adhering junctions (AJs), which may result in rather radical, metaplastic changes of morphology as well as in metastatic spread and growth. Extensive changes of the molecular composition and higher order architecture of the cytoskeleton and AJs are as such not exclusive for malignant cells but resemble processes known from embryology, as for example the so-called "epithelial-mesenchymal transition" (EMT) of ectodermal cells. The current concept of EMT as an early and necessary step in the evasion of cells from the primary tumor, local lysis of the basal lamina or other boundary structures, invasion into a foreign tissue and metastatic growth of the resulting secondary tumor, is based on observations that at certain stages AJs of both categories, desmosomes and *adhaerens* junctions (*zonulae, fasciae* and *puncta*), can be weakened or even split so that malignant cells are released from the primary tumor and embark on the route of metastasis, often accompanied by a more or less extensive replacement of the original E-cadherin by N-cadherin ("cadherin switch"; Chen and Öbrink, 1991; Frixen et al., 1991; Navarro et al., 1991; Vleminckx et al., 1991; reviews: Takeichi et al., 1994; Mareel et al., 1994; Birchmeier, 1994; Brabletz, 2004; Strumane et al., 2004; Wheelock et al., 2008). In this scenario, it is also obvious that the docking of metastasizing cells to other cells, distant and foreign tissues included, involves

formations of novel AJ-type contacts. Thus, metastasis constitutively requires both, weakening old AJ contacts as well as establishing novel types of cell attachments to other (“host”) cells or to cells of its own kind.

In our studies of the molecular assemblies, changes and losses of cytoskeletal filaments and cell-cell contacts in normal and tumor cells (Franke et al., 1979a, b; Moll et al., 1983; Franke et al., 1983; review: Franke, 2009) we were baffled by repeated observations of the “sudden” appearance of new filament proteins which could then be followed as clonally stable properties over many generations (e.g., Knapp and Franke, 1989; Knapp et al., 1989). In addition, we have observed that certain cell-cell junction molecules and structures, including some of the AJ-category, spontaneously appeared *de novo* in colonies of certain cell culture lines and were then continuously seen in the specific cell progeny for decades, suggestive of a remanence of the underlying changes of gene expression and structure assembly. Therefore, we have decided to study this phenomenon, i.e., the spontaneous *de novo* formation of totally new kinds of cells and tissue-like structures in a systematic way over extended periods of time. Here we report the spontaneous emergence of diverse novel kinds of junctions, resulting in tissue-like cell assemblies, of the pluripotential human hematopoietic line K562.

RESULTS

The hematopoietic, myelogenous leukemia-derived cell line K562 represents one of the most widely used cell culture model systems for studies of hematopoiesis, displaying granulocytic, erythroid and megakaryocytic differentiation potential (e.g., Lozzio and Lozzio, 1975; Andersson et al., 1979; Lozzio and Lozzio, 1979; Rowley et al., 1981, 1985; reviews: Koeffler and Golde, 1980; Dimery et al., 1983; Drexler, 1994; Tsiftsoglou et al., 2003). In the course of our study, syntheses of such hematopoietic differentiation markers have repeatedly been induced using a diversity of agents, with results essentially similar to those previously reported by other authors (see Supplemental Experimental Procedures).

In our previous studies of rare but clonally stable and extensive cell alterations characterized by the *de novo* synthesis of specific cytoskeletal filament proteins we had noted that in a series of mesenchymally derived cell lines not only masses of bundles of endogenous vimentin intermediate-sized filaments (IFs) are assembled

but that in addition bundles of keratin IFs are formed as stable cell structures (Knapp and Franke, 1989; Knapp et al., 1989; for K562 cells see Zauli et al., 1986; Järvinen et al., 1990; Schäfer, 1995). As we had also noted the synthesis, structure assembly and correct topogenesis of large amounts of other unexpected cytoskeletal and junctional proteins in some K562 cells, we decided to examine these changes and their fate in detail.

Cadherin-based Junctions: Immunofluorescence Microscopy

Most current K562 cell cultures contain not only abundant bundles of vimentin IFs (Figure 1A) but also IF bundles formed in a stoichiometrically coordinated way by keratins 8 (Figure 1B), 18 (not shown) and 19 (Figure 1C) in practically all cells, i.e. in a ratio and pattern typical for endodermal epithelia and tumors derived therefrom (Franke et al., 1981; Moll et al., 1982). All fifteen K562 sublines established in the course of our study also showed smooth-muscle (sm)-α-actin-containing microfilament bundles in almost all cells that were compacted into a number of variously-sized and -shaped whorl aggregates (up to six could be counted per cell; Figure 1D). Since the appearance of microfilaments comprising sm-α-actin is widely regarded as an indication of a transformation to cells of marked contractility and motility such as myofibroblasts (e.g., Hinz et al., 2004, 2007), the massive synthesis and filament assembly of this protein in the rather sessile K562 cells seems to call for an alternative explanation.

In addition, we noted in a number of the K562 cell colonies and sublines groups of cells characterized by punctate structures at their surfaces, in particular at cell-cell contact sites positively immunostained for the desmosomal cadherin, desmoglein 2 (Dsg2), next to other cell colonies lacking this protein (Figure 2). Using various techniques of isolating such cells or small cell colonies, followed by growth in suspension or as substratum-adherent monolayer colonies (for methods see Knapp and Franke, 1989; Knapp et al., 1989), we selected Dsg2-positive cells and obtained clonal sublines in which many, in some cases all cells showed punctate Dsg2 reactions at cell-cell contacts or on free cell surfaces (Figure 3A-A''). We further confirmed the molecular identification of these Dsg2-positive "dot" structures by the specific and uniform reaction with different Dsg2-antibodies (e.g., Figure 3A-A''), including some reacting with surface-exposed, extracellular domains (cf. Schäfer et

al., 1996) and others reactive with cytoplasmic, plaque-bound epitopes (e.g., Schmelz et al., 1986a, b).

Such punctate cell surface reaction sites of Dsg2 were also frequently seen in co-localization with other desmosomal junctional plaque proteins such as plakophilin 2 (Pkp2, Figure 3B), plakophilin 3 (Pkp3; not shown) and plakoglobin (PG; Figure 3C). In two of the 15 cell sublines we observed cell colonies connected by punctate AJ structures positive for both desmosomal cadherins, i.e. Dsg2 and desmocollin, Dsc2, together with some of the plaque proteins mentioned before (Figure S1A-C). Two other sublines were established because they showed punctate cell-cell or cell surface reactions for Dsg2 and desmoplakin (Figure 3D). Moreover, similar dots showing co-localizations with Dsg2 were also observed, although at lower frequencies, for various non-desmosomal *armadillo*-type plaque proteins such as β-catenin as well as proteins p120 and p0071 (not shown). Extensive immunocytochemical examinations for other cadherins, including desmogleins Dsg 1, 3 and 4 as well as desmocollins Dsc1 and 3, and for α-catenin, neurojungin and protein ARVCF gave negative results in all sublines characterized.

Much to our surprise we also noted sublines in which different molecules of the desmosomal ensemble were present but clearly did not colocalize. Figure S1D presents such an example of cells in which Dsg2 and Dsc2 are assembled in strictly different “dots” whereas Figure S1E presents a cell subline in which Dsg2 does not colocalize in the same loci as the corresponding plaque partner protein, Pkp2, which in turn forms its own separate cluster. Such cell sublines showing differential localization of typical desmosomal molecules indicate that their normal co-assembly requires additional factors which are missing in the specific sublines.

Cadherin-based Junctions: Electron Microscopy

Normal as well as malignantly transformed hematopoietic or blood cells are believed to be unicellular and not connected by junctions of any kind (for certain N-cadherin–containing precursor cell types located in – or prepared from – the bone marrow see Wuchter et al., 2007; Wein et al., 2010). However, distinct AJ-type cell contact structures have been seen in K562 cell cultures by electron microscopy, in particular in densely-grown, substratum-adherent cell monolayers or in suspended spheroidal cell aggregates. In loose-packed cell associations such AJs were relatively sparse

and characterized by close membrane-to-membrane attachments with a dense and distinct, though mostly rather thin cytoplasmic plaque (Figure 4A-E).

Closer inspection, together with morphometric and immunocytochemical analyses, further showed that these AJ-type structures could markedly vary in size and ultrastructural details. Figure 5A-C presents survey pictures of an extended cell-cell contact region of two cells in a densely-grown monolayer culture (Figure 5A), comprising a number of different types of junctions. Partial magnifications (Figure 5B, C) demonstrate the marked structural heterogeneity of neighboring AJ structures, including variously-sized, highly-organized junctions of desmosome-like morphology, revealing straight, trilaminar membrane regions with connecting mesogloea-like ("midline") – elements and rather thick, densely-stained plaque structures (marked by brackets in Figure 5A-C). These typical AJs were interspersed with less distinct membrane-membrane associations of a similar wide range of sizes but different morphology, characterized by close membrane contact regions and rather thin cytoplasmic plaques (denoted by V-shaped symbols in Figure 5A).

We also frequently noted asymmetric AJ structures in which only one half of the junction was coated with a plaque (Figure 5D and E, arrowheads). Simple close membrane associations ("kisses") without a marked plaque layer were also seen (Figure 5F). Occasionally we have observed relatively deep plasma membrane invaginations with an extended plaque coating (Figure 5F, arrowhead), resembling the "taproot" AJs (*manubria adhaerentia*) described in cultures of human bone marrow-derived mesenchymal stem cells (Wuchter et al., 2007; Franke et al., 2009), and small spheroidal or flattened vesicles bearing desmosomal plaque-like coats, as repeatedly reported from epithelial cells (for review see Cowin et al., 1985b). Finally, we have regularly noted small regions of "free" cell surface (ca. 0.1-0.2 micrometer in diameter) characterized by a very dense cytoplasmic plaque (Figure 5G), i.e. plasma membrane domains similar to the half-desmosomal structures reported from various epithelial cell cultures (e.g., Duden and Franke, 1988; Demlehner et al., 1995; Schäfer, 1995).

Correspondingly, by immunoelectron microscopy we repeatedly noted rather small (0.05-0.2 micrometer) cell surface domains which on their outer surface showed cell coat material intensely immunogold-labeled with Dsg2-antibodies specific for epitopes known to be located on the extracellular domain (Figure 5H-J; for related electron microscopy with epithelial cells see, e.g., Demlehner et al., 1995).

As to the symmetrical desmosome-like junctions, their plaque structures were heavily labeled with antibodies to the cytoplasmic, i.e., carboxyterminal portion of Dsg2 (Figure 5K-M) and with antibodies to plakoglobin (Figure 5N) or Pkp2 (Figure 5O).

Cadherin-based Junctions: Biochemical Methods

For the identification of the molecular components of the AJs formed in K562 cell cultures, the proteins under question have been analyzed by SDS-PAGE or two-dimensional gel electrophoresis, followed by immunoblotting, in comparison with well-known epithelial and mesenchymal cell culture lines (see Supplemental Experimental Procedures). In such analyses (Figure 6 presents an example) the vast majority of the K562 cell sublines and their AJs contain desmoglein Dsg2 but not any of the desmocollins Dsc1-3, with the exception of some detectable desmocollin Dsc2 in two of the sublines (see below). We have also not identified any of the non-desmosomal cadherins (Figure 6, top panel) and of α-catenin whereas β-catenin as well as proteins p120 and p0071 were generally detected (Figure 6, second panel from top). Moreover, the desmosomal *armadillo*-type proteins plakophilins Pkp2 and Pkp3 as well as plakoglobin were consistently seen. By contrast, the other desmosomal cadherin, desmocollin Dsc2, and the major large desmosomal plaque protein, desmoplakin, have been identified only in two of the 15 different clonal K562 sublines examined (see also Figure S1).

The molecular neighborhood relationships of the various AJ components were biochemically analyzed by immunoprecipitation, followed by SDS-PAGE and immunoblot analysis. As shown in Figure 7, antibodies against Dsg2 co-immunoprecipitated plakoglobin as well as plakophilins Pkp2 and Pkp3, in some cell clones together with rather small amounts of proteins p120, p0071 and β-catenin (not shown). Plakoglobin and Pkp3 were also co-immunoprecipitated with plakophilin Pkp2, and desmoglein Dsg2 as well as both plakophilins, Pkp2 and Pkp3, were pulled down together with plakoglobin (Figure 7). Co-precipitation of very minor amounts of desmoplakin with Dsg2 was noted only in two sublines (see below).

Epithelial Membrane Cell Adhesion Molecules-based Junctions

Obviously, however, the Dsg2-positive punctate plasma membrane sites are not the only cell-cell contact structures that can be formed in K562 cultures. In very densely-grown, substratum-adherent cell monolayer colonies, a different kind of cell-cell

contact structure, extending along most of the cell periphery, is also often seen: These extended cell contact regions are characterized by a continuous cortical cytoplasmic plaque rich in afadin (Figure 8A), α-actinin (Figure 8A and B) as well as vinculin, protein ZO-1 and actin (not shown) but essentially negative for desmogleins (Figure 8B), desmocollins (not shown), α-catenin and all the *armadillo* proteins mentioned above (not shown). In these cortical layers the diverse actin-binding proteins are associated with the non-cadherin type transmembrane glycoprotein, epithelial cell adhesion molecule (EpCAM; Figure S2) and some other membrane components not yet identified. Figure 8B presents a direct comparison of the two fundamentally different cell-cell contact systems that can be seen in these cultures, the extended cortical layer and the punctate AJs.

As to the composition of the extended, non-AJ cortical plasma membrane layers we have consistently found in addition to α-actinin, afadin, vinculin and protein ZO-1, the transmembrane glycoprotein EpCAM (Figure S2).

Frequencies, Time Spans and Revertants

The appearance of the cell-cell junction and cell surface-exposed half-junction AJ-resembling structures in K562 cells is obviously not restricted to specific culture times and conditions but is a true *de novo* assembly process, again and again, in previously negative cell cultures and in clonal subcultures, including several that have initially been selected for the complete absence of such proteins and structures. In this context it is also noteworthy that Järvinen et al. (1990) have not detected any desmosomal protein-positive structure although they have also used some of our antibodies.

Obviously, in most cases of spontaneous, cumulative assembly of AJ components, desmoglein Dsg2 appears to act as a pacemaker and nucleator for the AJ-type structures while glycoprotein EpCAM is associated with the cortical structures containing actin-binding proteins. Rather infrequently have we been able to select cells with AJ structures showing reactions for desmoplakin or desmocollin Dsc2: Only two clonal sublines have been established which are rich in desmoplakin-positive junctions (clones K562-2001-DP1a, b; cf. Cowin et al., 1985a), and only one clone has been obtained that contains AJs positive for both Dsc2 and Dsg2 (Figure S1). Table 1 lists the major clonal sublines identified and grown in the entire period 1988-2009 (see also Schäfer, 1995, and Tian, 2000).

AJ structures based on cadherin molecules are notorious for their sensitivity to reduced Ca^{2+} concentrations (for Dsg2-containing desmosomes see, e.g., Hennings et al., 1980; Watt et al., 1984; Duden and Franke, 1988; Demlehner et al., 1995). At two time points, 1995 and 2008, we have also examined whether the formation of the AJ structures described was dependent on the Ca^{2+} content of the culture medium in the range from 0.1 mM -1.5 mM. However, in K562 cells exposed to low Ca^{2+} media the amounts of the two plakophilins, plakoglobin, β-catenin and the Ca^{2+}-binding protein, Dsg2, were only little reduced but, surprisingly, both the Triton X-100-extractability and the immunolocalization-positive punctate AJs were by and large lost (cf. Schäfer, 1995).

In the clonal cultures and sublines containing Dsg2-immunostaining-positive AJ structures we have found after a series of passages distinct colonies of K562 cells with multiple contact sited but without immunocytochemically demonstrable AJ-type reactions (not shown). It will have to be examined in single cell isolation and cloning experiments whether cell colonies showing such losses of AJ protein reactions reflect revertants of protein synthesis or of masking or of interference with structure assembly

DISCUSSION

Our initial observation that in cultures of simple unicellular systems such as the hematopoietic tumor cell line K562 novel, adhering junction (AJ) proteins and structures appear spontaneously, repeatedly and without specific inductions and that these can be maintained in the clonal progeny, was obviously in contrast to current textbook dogmata of blood cell histology, developmental biology and carcinogenesis. Even more surprising were our findings that in addition asymmetric half-AJ structures were frequently seen on free cell surfaces and that all these AJ-type structures were primarily based on desmosomal molecules which are commonly regarded as hallmarks of epithelial and myocardial differentiation and to be absent from blood cells. Therefore and because of the general importance of cadherin molecules and their changes in tumor spread and metastasis (see Introduction) we have studied this challenging phenomenon over a period of more than two decades, have repeatedly cloned and subcloned specific K562 cells and have compared substratum-adherent cell colonies with others grown as spheroidal colonies or as individual cells in

suspension. Moreover, we have repeatedly bought new batches of K562 cells from the suppliers to confirm the phenomenon.

The fifteen established K562 cell sublines studied in detail were characterized by the absence or presence of AJ molecules and AJ structures as demonstrable by immunocytochemistry and electron microscopy in many, but not necessarily all cells of a given subtype (Table 1). Moreover, the ensembles of the molecules synthesized and assembled in AJ-type structures often showed marked heterogeneity: Highly-organized, desmosome-resembling AJ forms, often presenting typical cell-cell connecting mesogloea-structures and dense cytoplasmic plaques, could occur next to AJ-type structures with a rather thin plaque and no mesogloea-like elements or even AJ structures in which only one half was plaque-coated.

In most cases the AJ-like structures seen were based on a single common type of transmembrane protein, a cadherin, desmoglein Dsg2, particularly known for the complex amino acid sequence subdomain pattern of its rather long carboxyterminal cytoplasmic extension (Schäfer et al., 1994; for comparisons with other desmogleins see Goodwin et al., 1990; Koch et al., 1990, 1991, 1992; Wheeler et al., 1991a, b; reviews: Holthöfer et al., 2007; Stokes, 2007; Garrod and Chidgey, 2008). Only in two of the fifteen clonal sublines established have we detected AJs which in addition contained desmocollin Dsc2, in normal desmosomes the isostoichiometric “twin” of Dsg2, whereas one clonal subline was characterized by the exclusive formation of separated AJ-types, one based on Dsg2 and the other on Dsc2. Obviously, our analyses also support earlier general conclusions from Dsg-cDNA transfections into desmosome-lacking cultured cells that heterophilic pairing with a desmocollin is not an absolute requirement of AJ formation and that desmocollins may even be wholly dispensable in the formation of certain types of junctions (Koeser et al., 2003; for partly controversial discussions on Dsc2-Dsc complexes see also Chitaev and Troyanovsky, 1997; Marcozzi et al., 1998; Tselepis et al., 1998; Syed et al., 2002; Troyanovsky, 2005; Stokes, 2007; Garrod and Chidgey, 2008). It is also noteworthy that this form of tightly cis-clustered, plaque-anchored Dsg2 molecules is also clearly distinguished from another form of Dsg2 distribution which has been described as dispersed, non-junction-integrated, “free” glycoprotein molecules in certain types of melanocytes and melanoma cells in cell cultures and *in situ* (Schmitt et al., 2007; Rickelt et al., 2008).

In all the Dsg2-positive AJ-like structures identified in this study (Table 1) the transmembrane Dsg2 clusters are anchored in cytoplasmic plaques constituted by the *armadillo* proteins, plakophilins Pkp2, Pkp3 and plakoglobin, i.e. a plaque ensemble well-known from the desmosomes of diverse epithelial cells. Only in a minority of the AJ subtypes noted specific non-desmosomal *armadillo* proteins such as β-catenin and proteins p120 and p0071 have also been detected. While co-assembly of plakophilins Pkp2 and Pkp3 is common in the desmosomes of diverse non-hepatocytic epithelial cells with proliferative potential (Bonné et al., 1999; Schmidt et al., 1999; reviews: Hatzfeld, 2007; Schmidt and Koch, 2008), Pkp2 co-localization with β-catenin and proteins of the p120 subgroup of *armadillo* proteins (cf. Anastasiadis and Reynolds, 2000) has previously only been shown for the composite junctions (*areae compositae*) of the intercalated disks connecting cardiomyocytes of mature mammalian hearts (e.g., Borrmann et al., 2006; Franke et al., 2006; Goossens et al., 2007; Pieperhoff et al., 2008; review: Franke et al., 2009) and for a special AJ-type of certain transformed, mesenchymally-derived cells (Barth et al., 2009; Rickelt et al., 2009). The pivotal architectonic role of Pkp2 – alone or together with Pkp3 – in plaque formations of desmosomes and related AJs is also indicated by its ultrastructural location (Mertens et al., 1996; North et al., 1999) and from experiments based on gene ablation (Grossmann et al., 2004) or siRNA-mediated reduction of Pkp2-mRNA (Oxford et al., 2007; Bass-Zubek et al., 2008; Pieperhoff et al., 2008; Hall et al., 2009; Li et al., 2009; review: Bass-Zubek et al., 2009).

Another surprise in our studies of AJ-type structures in K562 cell cultures has been the recognition that two different major plaque forms of AJs can be distinguished (Table 1): In most sublines we have found AJs without any detectable desmoplakin. However, we have also selected two sublines in which cells are connected by AJs containing desmoplakin in addition to plakoglobin and the plakophilins. This observation that in K562 cells junctional structures of similar sizes and ultrastructural appearance can be formed with and without desmoplakin seems puzzling in view of the important structural role that is generally ascribed to this large plaque protein (for review see Godsel et al., 2004). It is, however, compatible with the demonstrated desmoplakin-dependent occurrence of plaque-coated AJ-type structures in both epithelial and endothelial cells of early embryogenesis of mice lacking both alleles of the desmoplakin gene (Gallicano et al., 1998, 2001; Zhou et

al., 2004) and in early mouse embryo hearts lacking the Pkp2-gene in which desmoplakin aggregates are misplaced away from the plaques of the residual cell-cell junctions (Grossmann et al., 2004).

Our consistent finding that α-catenin has not been detectable in all fifteen subtypes of AJ-type ensembles of K562 cells is both surprising and telling. It reminds one of the series of reports on genetically defined losses of α-catenin in certain carcinomas and carcinoma-derived cell lines as well as reports on mutant α-catenins, all with the conclusion that α-catenin-deficient junctions are characterized by lost or highly weakened cell-cell adhesion, up to situations in which the cells are totally separated from each other (e.g., Hirano et al., 1992; Shimoyama et al., 1992; Aberle et al., 1994; Hülsken et al., 1994; Nagafuchi et al., 1994; Rubinfeld et al., 1995; Sacco et al., 1995; Pokutta et al., 2002; reviews: Kanai et al., 1994; Wheelock and Johnson, 2003; Benjamin and Nelson, 2008). In contrast to generalizing conclusions of some previous authors, our present results suggest that α-catenin is not absolutely needed for the formation of AJs with good membrane contact and a cytoplasmic plaque. Moreover, they are in agreement with the hypothesis that the α-catenin molecules – like the related vinculin and protein ZO-1 – are involved in the regulation of the anchorage of actin-microfilament bundles at AJ plaques and their functions (reviews: Drees et al., 2005; Benjamin and Nelson, 2008), as such filament attachments have only rarely been seen at the AJ-like structures of our K562 cell colonies. On the other hand, we have demonstrated that in confluent K562 cells vinculin and protein ZO-1 are abundantly present in the cortical layers of the extended, EpCAM-based cell-cell contacts. And finally, our observation of an apparent loss of α-catenin in a number of K562 sublines are certainly not in conflict with recent results interpreted to show that in several myeloid leukemia cell forms a decrease of α-catenin correlates with enhanced malignancy (e.g., Ulger et al., 2003; Desmond et al., 2007; review: Benjamin and Nelson, 2008).

The changes of the K562 cell character induced by the reported molecular assemblies and the generation of novel AJ-type junctions are profound in several ways: (1) Both the frequencies of formation and the stabilities of the molecular complexes and AJ structures formed can be rather high. (2) Synthesis and assembly of the AJ-line structures can occur spontaneously and randomly in the same environment, i.e., without a genetic change or a medium change or any addition of a specific inducing agent. (3) As is also the case for the K562 keratin IFs with their

isostoichiometric ensembles of keratins 8:18+19, the synthesis and the longevity of the AJ proteins are to a remarkable degree coordinated and cumulative so that these proteins can assemble into numerous AJ-like structures. (4) The AJs described can fundamentally change the cells higher order of organization and contribute to formations of epithelioid layers of interconnected cells or of three-dimensional arrays and thus of novel tissue-equivalents. (5) This also opens the possibility of associations with foreign cells displaying appropriate AJ-type attachment structures. Apparently, this new ability to form semistable, heterotypic AJ-type connections with complementary domains of other cells might also contribute to metastatic processes.

In conclusion, these formations of AJ-like junctions in a blood cell tumor system represent a case of non-genetic heterogeneity that combines a high proliferation rate with dramatically increased cell-cell adhesion potential (for discussion see Brock et al., 2009; cf. also Slack, 2007). Specifically, in K562 cells and similar cells *in situ* the cell-cell adhesive molecules and structures described in this report, together with the series of K562 surface molecules known to mediate cell adherence to basal lamina, stromal and endothelial components (e.g., Bendall et al., 1996; Turner et al., 1998; Gane et al., 2001; and references cited), certainly would contribute to the stability of tumor cell aggregates and to potential metastatic interactions.

The observations reported here, i.e. the synthesis, stabilization, accumulation and orderly structural assembly of a series of major epithelial “marker” molecules and structures far out of any epithelial developmental context are obviously worrisome challenges for current concepts of diagnoses of tumors based on histogenesis and differentiation markers. A blood tumor cell rich in keratin IF bundles, various subtypes of cell-cell junction markers and structures that are commonly regarded as to typical of desmosomes or epithelial cell adhesion molecule (EpCAM)-based structures will be of concern to developmental biologists as well as pathologists.

Although we have observed similar and even more complex changes of cell-cell junction systems also in other hematopoietic cell lines (unpublished work), we have selected for the present report cells of the K562 line as it has been the major reference system in this field of research for now more than three decades (for review see, e.g., Tsiftsoglou et al., 2003). The additions of masses of “foreign” molecules and structures to the cytoskeletal and junctional cell systems of these cells are obviously compatible with the notoriously high proliferation rate, the malignant

character and the isodiametric appearance of these cells. Now one of the logical consequences of our findings would have to be the systematic search for possible altered tumor cells which immunohistochemically might appear as specific “side populations” in hematological or histopathological diagnoses. It will also be important to determine the proliferation potential and the differentiation state of the various K562 subtypes and the effects of known growth-promoting or -inhibitory drugs on the induction or reduction of cell-cell attachment structures.

The fundamental practical diagnostic problems posed by the results of this study are also obvious: The epithelioid cell layers and AJ-mediated higher order structures of various subforms of K562 and other hematopoietic tumor cell lines, together with the large masses of bundles of sm-α-actin-containing microfilaments on the one hand and stoichiometrically correct simple epithelium-type bundles of keratin IFs on the other, and now also together with diverse desmosomal components and AJ structures and also the EpCAM junctions in immunocytochemical terms represent a scenario of potentially misleading, partly bizarre properties. Here only the parallel and combined use of haematological and epithelial cell type markers can lead to correct diagnostic identifications and classifications. On the other hand, the new findings presented in this report now allow one to think of developments of specific “anti-AJ molecule drugs” that bind to – and thus may block the binding of – the extracellular domains of the tumor cell Dsg2, i.e. a similar concept as it is already in promising use for N-cadherin of certain tumor cells (see e.g., Blaschuk and Devemy, 2009).

EXPERIMENTAL PROCEDURES

Cell Cultures

Cells of the human multipotential leukemia line K562 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) or the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were grown in Iscove´s Modified Dulbeccos´s Medium (IMDM; Invitrogen, Karlsruhe, Germany), supplemented with 10 % fetal calf serum (FCS) and 2 mM glutamine in suspension culture in small “cages” or as cells adherent to a substratum (plastic surface, slides or coverslips; coated with poly-L-lysine, collagen or fibronectin). Single cells or colonies were obtained by serial transfer essentially as previously reported

for other cell lines (e.g., Knapp and Franke 1989; Knapp et al., 1989) and grown as clonally derived sublines.

Antibodies

The primary antibodies used for immunofluorescence microscopy and for immunoblotting analyses of gel-electrophoretically separated polypeptides are listed in Table S1. Secondary antibodies for immunofluorescence microscopy were species-specific goat antibodies against immunoglobulins of mouse, rabbit or guinea pig, conjugated to Cy3 (Dianova, Hamburg, Germany) or Alexa 488 (MoBiTec, Göttingen, Germany). For immunoblot analyses, horseradish peroxidase (HRP)-conjugated secondary antibodies were used (Dianova).

Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (Rickelt et al., 2009) using total protein lysates of repeatedly washed cultured cells or cytoskeletal residue material obtained after treatments with non-denaturing detergents with or without high salt buffers (e.g., Rickelt et al., 2009; see also Achtstätter et al., 1986; Schäfer et al., 1994). For specific biochemical experiments cells were washed several times with pre-cooled PBS and then immediately dissolved in 1.0 ml pre-heated SDS sample buffer (250 mM Tris-HCl, 10 % SDS, 20 % glycerol, 100 mM dithiothreitol; pH 6.8), heated at 95℃ for 5 min and subsequently cooled on ice. The polypeptides separated were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and stained with Coomassie Brilliant Blue for immunochemical detection. Analyses of native protein complexes, exposed to different urea concentrations in the range of 4-9.5M urea, by two-dimensional gel electrophoresis were as described (e.g., Franke et al., 1983; Achtstätter et al., 1986; Schäfer et al., 1994). For immunoblotting protocols see Supplemental Experimental Procedures.

Immunoprecipitation

Immunoprecipitation (IP) was performed as reported by Rickelt et al. (2009) using “Triton X-100 IP buffer” (1 % Triton X-100, 150 mM NaCl, 20 mM HEPES; pH 7.4), supplemented with a protease inhibitor cocktail (Complete Mini Inhibitor Tabs, EDTA-free; Roche Diagnostics, Mannheim, Germany).

Phase Contrast, Immunofluorescence and Electron Microscopy

Cells grown on glass cover slips coated with poly-(L)-lysine, fibronectin or collagen, or cells grown in suspension and gently pelleted onto a planar substratum were briefly rinsed in PBS and fixed for 5 min in methanol, followed by acetone (20 s), both at -20℃. Prior to incubation with the first antibo dy solution for 1 h at room temperature, cells were permeabilized by 5 min treatment with 0.2 % Triton X-100 in PBS for 5 min. After several washes in PBS, cells were incubated for 30 min with the appropriate secondary antibodies, washed again in PBS, dehydrated in ethanol, air-dried, and mounted in Fluoromount (Biozol, Eching, Germany). Immunofluorescence microscopic images were recorded with an Axiophot II photomicroscope (Carl Zeiss, Jena, Germany), equipped with an AxioCam HR (Carl Zeiss). For confocal laser scanning microscopy a Zeiss LSM 510 Meta microscope was used.

The protocols used for conventional transmission- and immunoelectron microscopy were essentially as described for diverse other mesenchymally derived cells (cf. Rickelt et al., 2009; see also Barth et al., 2009). Electron micrographs were taken at 80 kV, using an EM 910 (Carl Zeiss, Oberkochen, Germany).

ACKNOWLEDGMENTS

We thank Caecilia Kuhn, Stefanie Winter-Simanowski, Christine Grund, Ralf Zimbelmann, Heiderose Schumacher and Edeltraut Noffz for three decades of excellent technical assistance. We also thank Drs. S. Schäfer and Q. Tian who have contributed to early studies of Dsg2-cDNA-cloning and expression control studies. The work was supported by a grant from the Deutsche Krebshilfe (grant 10-2049-Fr1 to WWF).

REFERENCES

Aberle, H., Butz, S., Stappert, J., Weissig, H., Kemler, R., and Hoschuetzky, H. (1994). Assembly of the cadherin-catenin complex in vitro with recombinant proteins. J Cell Sci *107 (Pt 12)*, 3655-3663.

Achtstätter, T., Hatzfeld, M., Quinlan, R. A., Parmelee, D. C., and Franke, W. W. (1986). Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. Methods Enzymol *134*, 355-371.

Anastasiadis, P. Z., and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. J Cell Sci *113 (Pt 8)*, 1319-1334.

Andersson, L. C., Nilsson, K., and Gahmberg, C. G. (1979). K562: a human erythroleukemic cell line. Int J Cancer *23*, 143-147.

Barth, M., Schumacher, H., Kuhn, C., Akhyari, P., Lichtenberg, A., and Franke, W. W. (2009). Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. Cell Tissue Res *337*, 63-77.

Bass-Zubek, A. E., Hobbs, R. P., Amargo, E. V., Garcia, N. J., Hsieh, S. N., Chen, X., Wahl, J. K., 3rd, Denning, M. F., and Green, K. J. (2008). Plakophilin 2: a critical scaffold for PKC alpha that regulates intercellular junction assembly. J Cell Biol *181*, 605-613.

Bass-Zubek, A. E., Godsel, L. M., Delmar, M., and Green, K. J. (2009). Plakophilins: multifunctional scaffolds for adhesion and signaling. Curr Opin Cell Biol *21*, 708-716.

Bendall, L. J., Bradstock, K. F., and Gottlieb, D. J. (1996). Identification of novel K562 membrane proteins that adhere to bone marrow fibroblasts. Blood *88*, 277-288.

Benjamin, J. M., and Nelson, W. J. (2008). Bench to bedside and back again: molecular mechanisms of alpha-catenin function and roles in tumorigenesis. Semin Cancer Biol *18*, 53-64.

Birchmeier, W. (1994). Molecular aspects of the loss of cell adhesion and gain of invasiveness in carcinomas. In: Molecular and cellular basis for cell to cell interaction: Its significance in cancer, S. Hirohashi, H.L. Moses, E. Ruoslahati, T. Sugimura, M. Takeichi, and M. Terada, eds. (Princeton Scientific Publishing Co., Inc., Princeton, New Jersey, U.S.A.), pp. 214-232.

Blaschuk, O. W., and Devemy, E. (2009). Cadherins as novel targets for anti-cancer therapy. Eur J Pharmacol *625*, 195-198.

Bonné, S., van Hengel, J., Nollet, F., Kools, P., and van Roy, F. (1999). Plakophilin-3, a novel armadillo-like protein present in nuclei and desmosomes of epithelial cells. J Cell Sci *112 (Pt 14)*, 2265-2276.

Borrmann, C. M., Grund, C., Kuhn, C., Hofmann, I., Pieperhoff, S., and Franke, W. W. (2006). The area composita of adhering junctions connecting heart muscle cells of vertebrates. II. Colocalizations of desmosomal and fascia adhaerens molecules in the intercalated disk. Eur J Cell Biol *85*, 469-485.

Brabletz, T. (2004). In vivo functions of catenins. In: Cell Adhesion, J. Behrens, and W.J. Nelson, eds. (Berlin, Heidelberg, New York: Springer Verlag), pp. 105-135.

Brock, A., Chang, H., and Huang, S. (2009). Non-genetic heterogeneity: a mutation-independent driving force for the somatic evolution of tumours. Nat Rev Genet *10*, 336-342.

Chen, W. C., and Obrink, B. (1991). Cell-cell contacts mediated by E-cadherin (uvomorulin) restrict invasive behavior of L-cells. J Cell Biol *114*, 319-327.

Chitaev, N. A., and Troyanovsky, S. M. (1997). Direct Ca2+-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. J Cell Biol *138*, 193-201.

Cowin, P., Franke, W. W., Grund, C., Kapprell, H.-P., and Kartenbeck, J. (1985a). The desmosome-intermediate filament complex. In: The Cell in Contact., G.M. Edelman, and J.P. Thiery, eds. (New York, Chichester, Brisbane, Toronto, Singapore: Neurosciences Research Foundation, Inc.; Published by John Wiley & Sons, Inc.), pp. 427-460.

Cowin, P., Kapprell, H. P., and Franke, W. W. (1985b). The complement of desmosomal plaque proteins in different cell types. J Cell Biol *101*, 1442-1454.

Demlehner, M. P., Schafer, S., Grund, C., and Franke, W. W. (1995). Continual assembly of half-desmosomal structures in the absence of cell contacts and their frustrated endocytosis: a coordinated Sisyphus cycle. J Cell Biol *131*, 745-760.

Desmond, J. C., Raynaud, S., Tung, E., Hofmann, W. K., Haferlach, T., and Koeffler, H. P. (2007). Discovery of epigenetically silenced genes in acute myeloid leukemias. Leukemia *21*, 1026-1034.

Dimery, I. W., Ross, D. D., Testa, J. R., Gupta, S. K., Felsted, R. L., Pollak, A., and Bachur, N. R. (1983). Variation amongst K562 cell cultures. Exp Hematol *11*, 601-610.

Drees, F., Pokutta, S., Yamada, S., Nelson, W. J., and Weis, W. I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell *123*, 903-915.

Drexler, H. G. (1994). Leukemia cell lines: in vitro models for the study of chronic myeloid leukemia. Leuk Res *18*, 919-927.

Duden, R., and Franke, W. W. (1988). Organization of desmosomal plaque proteins in cells growing at low calcium concentrations. J Cell Biol *107*, 1049-1063.

Franke, W. W., Schmid, E., Weber, K., and Osborn, M. (1979a). HeLa cells contain intermediate-sized filaments of the prekeratin type. Exp Cell Res *118*, 95-109.

Franke, W. W., Schmid, E., Winter, S., and Osborn, M. (1979b). Widespread occurrence of intermediate-sized filaments of the vimentin type in cultured cells from diverse vertebrates. Exp Cell Res *123*, 25-46.

Franke, W. W., Schiller, D. L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepier, R., and Platzer, B. (1981). Diversity of cytokeratins. Differentiation-specific expression of cytokeratin polypeptides in epithelial cells and tissues. J Mol Biol, 933-959.

Franke, W. W., Schiller, D. L., Hatzfeld, M., and Winter, S. (1983). Protein complexes of intermediate-sized filaments: melting of cytokeratin complexes in urea reveals different polypeptide separation characteristics. Proc Natl Acad Sci U S A *80*, 7113-7117.

Franke, W. W., Borrmann, C. M., Grund, C., and Pieperhoff, S. (2006). The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. Eur J Cell Biol *85*, 69-82.

Franke, W. W. (2009). Discovering the molecular components of intercellular junctions – a historical view. In: Cold Spring Harbor Perspectives in Biology., J. Nelson, and E. Fuchs, eds. (New York: Cold Spring Harbor Laboratory Press), p. 1:a003061.

Franke, W. W., Rickelt, S., Barth, M., and Pieperhoff, S. (2009). The junctions that don't fit the scheme: special symmetrical cell-cell junctions of their own kind. Cell Tissue Res *338*, 1-17.

Frixen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol *113*, 173-185.

Gallicano, G. I., Kouklis, P., Bauer, C., Yin, M., Vasioukhin, V., Degenstein, L., and Fuchs, E. (1998). Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. J Cell Biol *143*, 2009-2022.

Gallicano, G. I., Bauer, C., and Fuchs, E. (2001). Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature. Development *128*, 929-941.

Gane, P., Le Van Kim, C., Bony, V., El Nemer, W., Mouro, I., Nicolas, V., Colin, Y., and Cartron, J. P. (2001). Flow cytometric analysis of the association between blood group-related proteins and the detergent-insoluble material of K562 cells and erythroid precursors. Br J Haematol *113*, 680-688.

Garrod, D., and Chidgey, M. (2008). Desmosome structure, composition and function. Biochim Biophys Acta *1778*, 572-587.

Gilbert, C. A., and Ross, A. H. (2009). Cancer stem cells: cell culture, markers, and targets for new therapies. J Cell Biochem *108*, 1031-1038.

Godsel, L. M., Getsios, S., Huen, A. C., and Green, K. J. (2004). The molecular composition and function of desmosomes. In: Cell Adhesion., J. Behrens, and W.J. Nelson, eds. (Berlin, Heidelberg, New York: Springer Verlag), pp. 137-193.

Goodwin, L., Hill, J. E., Raynor, K., Raszi, L., Manabe, M., and Cowin, P. (1990). Desmoglein shows extensive homology to the cadherin family of cell adhesion molecules. Biochem Biophys Res Commun *173*, 1224-1230.

Goossens, S., Janssens, B., Bonne, S., De Rycke, R., Braet, F., van Hengel, J., and van Roy, F. (2007). A unique and specific interaction between alphaT-catenin and plakophilin-2 in the area composita, the mixed-type junctional structure of cardiac intercalated discs. J Cell Sci *120*, 2126-2136.

Grossmann, K. S., Grund, C., Hülsken, J., Behrend, M., Erdmann, B., Franke, W. W., and Birchmeier, W. (2004). Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation. J Cell Biol *167*, 149-160.

Hall, C., Li, S., Li, H., Creason, V., and Wahl, J. K., 3rd (2009). Arrhythmogenic right ventricular cardiomyopathy plakophilin-2 mutations disrupt desmosome assembly and stability. Cell Commun Adhes, 1-13.

Hatzfeld, M. (2007). Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? Biochim Biophys Acta *1773*, 69-77.

Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell *19*, 245-254.

Hinz, B., Pittet, P., Smith-Clerc, J., Chaponnier, C., and Meister, J. J. (2004). Myofibroblast development is characterized by specific cell-cell adherens junctions. Mol Biol Cell *15*, 4310-4320.

Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., and Gabbiani, G. (2007). The myofibroblast: one function, multiple origins. Am J Pathol *170*, 1807-1816.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. (1992). Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. Cell *70*, 293-301.

Holthöfer, B., Windoffer, R., Troyanovsky, S., and Leube, R. E. (2007). Structure and function of desmosomes. Int Rev Cytol *264*, 65-163.

Hülsken, J., Birchmeier, W., and Behrens, J. (1994). E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. J Cell Biol *127*, 2061-2069.

Järvinen, M., Andersson, L. C., and Virtanen, I. (1990). K562 erythroleukemia cells express cytokeratins 8, 18, and 19 and epithelial membrane antigen that disappear after induced differentiation. J Cell Physiol *143*, 310-320.

Kanai, Y., Oda, T., Shimoyama, Y., Ochiai, A., Oyama, T., Yoshiura, K., Akimoto, S., Yamada, T., and Hirohashi, S. (1994). Alterations of the cadherin-catenin cell adhesion system in cancers. In: Molecular and cellular basis for cell to cell interaction: Its significance in cancer, S. Hirohashi, H.L. Moses, E. Ruoslahati, T. Sugimura, M. Takeichi, and M. Terada, eds. (Princeton Scientific Publishing Co., Inc., Princeton, New Jersey, U.S.A.), pp. 51-62.

Knapp, A. C., Bosch, F. X., Hergt, M., Kuhn, C., Winter-Simanowski, S., Schmid, E., Regauer, S., Bartek, J., and Franke, W. W. (1989). Cytokeratins and cytokeratin filaments in subpopulations of cultured human and rodent cells of non-epithelial origin: modes and patterns of formation. Differentiation *42*, 81-102.

Knapp, A. C., and Franke, W. W. (1989). Spontanous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occuring at different levels of regulation. Cell *59*, 67-79.

Koch, P. J., Walsh, M. J., Schmelz, M., Goldschmidt, M. D., Zimbelmann, R., and Franke, W. W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. Eur J Cell Biol *53*, 1-12.

Koch, P. J., Goldschmidt, M. D., Walsh, M. J., Zimbelmann, R., and Franke, W. W. (1991). Complete amino acid sequence of the epidermal desmoglein precursor polypeptide and identification of a second type of desmoglein gene. Eur J Cell Biol *55*, 200-208.

Koch, P. J., Goldschmidt, M. D., Zimbelmann, R., Troyanovsky, R., and Franke, W. W. (1992). Complexity and expression patterns of the desmosomal cadherins. Proc Natl Acad Sci U S A *89*, 353-357.

Koeffler, H. P., and Golde, D. W. (1980). Human myeloid leukemia cell lines: a review. Blood *56*, 344-350.

Koeser, J., Troyanovsky, S. M., Grund, C., and Franke, W. W. (2003). De novo formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. Exp Cell Res *285*, 114-130.

Li, M. W., Mruk, D. D., Lee, W. M., and Cheng, C. Y. (2009). Connexin 43 and plakophilin-2 as a protein complex that regulates blood-testis barrier dynamics. Proc Natl Acad Sci U S A *106*, 10213-10218.

Lozzio, B. B., and Lozzio, C. B. (1979). Properties and usefulness of the original K-562 human myelogenous leukemia cell line. Leuk Res *3*, 363-370.

Lozzio, C. B., and Lozzio, B. B. (1975). Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood *45*, 321-334.

Marcozzi, C., Burdett, I. D., Buxton, R. S., and Magee, A. I. (1998). Coexpression of both types of desmosomal cadherin and plakoglobin confers strong intercellular adhesion. J Cell Sci *111 (Pt 4)*, 495-509.

Mareel, M., Vleminckx, K., Vermeulen, S., Yan, G., Bracke, M., and van Roy, F. (1994). Downregulation in vivo of the invasion-suppressor molecule E-cadherin in experimental and clinical cancer. In: Molecular and cellular basis for cell to cell interaction: Its significance in cancer, S. Hirohashi, H.L. Moses, E. Ruoslahati, T.

Sugimura, M. Takeichi, and M. Terada, eds. (Princeton Scientific Publishing Co., Inc., Princeton, New Jersey, U.S.A.), pp. 63-80.

Mertens, C., Kuhn, C., and Franke, W. W. (1996). Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomal plaque. J Cell Biol *135*, 1009-1025.

Moll, R., Franke, W. W., Schiller, D. L., Geiger, B., and Krepler, R. (1982). The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell *31*, 11-24.

Nagafuchi, A., Ishihara, S., and Tsukita, S. (1994). The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-alpha catenin fusion molecules. J Cell Biol *127*, 235-245.

Navarro, P., Gomez, M., Pizarro, A., Gamallo, C., Quintanilla, M., and Cano, A. (1991). A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. J Cell Biol *115*, 517-533.

North, A. J., Bardsley, W. G., Hyam, J., Bornslaeger, E. A., Cordingley, H. C., Trinnaman, B., Hatzfeld, M., Green, K. J., Magee, A. I., and Garrod, D. R. (1999). Molecular map of the desmosomal plaque. J Cell Sci *112 (Pt 23)*, 4325-4336.

Oxford, E. M., Musa, H., Maass, K., Coombs, W., Taffet, S. M., and Delmar, M. (2007). Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. Circ Res *101*, 703-711.

Pieperhoff, S., Schumacher, H., and Franke, W. W. (2008). The area composita of adhering junctions connecting heart muscle cells of vertebrates. V. The importance of plakophilin-2 demonstrated by small interference RNA-mediated knockdown in cultured rat cardiomyocytes. Eur J Cell Biol *87*, 399-411.

Pokutta, S., Drees, F., Takai, Y., Nelson, W. J., and Weis, W. I. (2002). Biochemical and structural definition of the l-afadin- and actin-binding sites of alpha-catenin. J Biol Chem *277*, 18868-18874.

Rickelt, S., Franke, W. W., Dörflinger, Y., Goerdt, S., Brandner, J. M., and Peitsch, W. K. (2008). Subtypes of melanocytes and melanoma cells distinguished by their intercellular contacts: heterotypic adherens junctions, adhesive associations, and dispersed desmoglein 2 glycoproteins. Cell Tissue Res *334*, 401-422.

Rickelt, S., Winter-Simanowski, S., Noffz, E., Kuhn, C., and Franke, W. W. (2009). Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a

novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer *125*, 2036-2048.

Rowley, P. T., Ohlsson-Wilhelm, B. M., Farley, B. A., and LaBella, S. (1981). Inducers of erythroid differentiation in K562 human leukemia cells. Exp Hematol *9*, 32-37.

Rowley, P. T., Ohlsson-Wilhelm, B. M., and Farley, B. A. (1985). K562 human erythroleukemia cells demonstrate commitment. Blood *65*, 862-868.

Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S., and Polakis, P. (1995). The APC protein and E-cadherin form similar but independent complexes with alpha-catenin, beta-catenin, and plakoglobin. J Biol Chem *270*, 5549-5555.

Sacco, P. A., McGranahan, T. M., Wheelock, M. J., and Johnson, K. R. (1995). Identification of plakoglobin domains required for association with N-cadherin and alpha-catenin. J Biol Chem *270*, 20201-20206.

Salk, J. J., Fox, E. J., and Loeb, L. A. (2010). Mutational heterogeneity in human cancers: origin and consequences. Annu Rev Pathol *5*, 51-75.

Schäfer, S., Koch, P. J., and Franke, W. W. (1994). Identification of the ubiquitous human desmoglein, Dsg2, and the expression catalogue of the desmoglein subfamily of desmosomal cadherins. Exp Cell Res *211*, 391-399.

Schäfer, S. (1995) Molekularbiologische Charakterisierung des desmosomalen Cadherins Desmoglein-2., Ph.D. Thesis, Faculty of Biology, University of Heidelberg, Heidelberg, Germany, pp.1-150.

Schäfer, S., Stumpp, S., and Franke, W. W. (1996). Immunological identification and characterization of the desmosomal cadherin Dsg2 in coupled and uncoupled epithelial cells and in human tissues. Differentiation *60*, 99-108.

Schmelz, M., Duden, R., Cowin, P., and Franke, W. W. (1986a). A constitutive transmembrane glycoprotein of Mr 165,000 (desmoglein) in epidermal and non-epidermal desmosomes. II. Immunolocalization and microinjection studies. Eur J Cell Biol *42*, 184-199.

Schmelz, M., Duden, R., Cowin, P., and Franke, W. W. (1986b). A constitutive transmembrane glycoprotein of Mr 165,000 (desmoglein) in epidermal and non-epidermal desmosomes. I. Biochemical identification of the polypeptide. Eur J Cell Biol *42*, 177-183.

Schmidt, A., Langbein, L., Prätzel, S., Rode, M., Rackwitz, H. R., and Franke, W. W. (1999). Plakophilin 3 - a novel cell-type-specific desmosomal plaque protein. Differentiation *64*, 291-306.

Schmidt, A., and Koch, P. J. (2008). Desmosomes in development and disease. In: Cell Junctions, S.E. La Flamme, and A.P. Kowalczyk, eds. (Weinheim: Wiley-VCH), pp. 235-249.

Schmitt, C. J., Franke, W. W., Goerdt, S., Falkowska-Hansen, B., Rickelt, S., and Peitsch, W. K. (2007). Homo- and heterotypic cell contacts in malignant melanoma cells and desmoglein 2 as a novel solitary surface glycoprotein. J Invest Dermatol *127*, 2191-2206.

Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S., and Hirohashi, S. (1992). Cadherin dysfunction in a human cancer cell line: possible involvement of loss of alpha-catenin expression in reduced cell-cell adhesiveness. Cancer Res *52*, 5770-5774.

Slack, J. M. (2007). Metaplasia and transdifferentiation: from pure biology to the clinic. Nat Rev Mol Cell Biol *8*, 369-378.

Stokes, D. L. (2007). Desmosomes from a structural perspective. Curr Opin Cell Biol *19*, 565-571.

Strumane, K., Berx, G., and Roy, F. V. (2004). Cadherins in Cancer. In: Cell Adhesion, J. Behrens, and W.J. Nelson, eds. (Berlin, Heidelberg, New York: Springer Verlag), pp. 69-103.

Syed, S. E., Trinnaman, B., Martin, S., Major, S., Hutchinson, J., and Magee, A. I. (2002). Molecular interactions between desmosomal cadherins. Biochem J *362*, 317-327.

Takeichi, M., Watabe, M., Shibamoto, S., and Ito, F. (1994). Cadherin-dependent organization and disorganization of epithelial architecture. In: Molecular and cellular basis for cell to cell interaction: Its significance in cancer, S. Hirohashi, H.L. Moses, E. Ruoslahati, T. Sugimura, M. Takeichi, and M. Terada, eds. (Princeton Scientific Publishing Co., Inc., Princeton, New Jersey, U.S.A.), pp. 28-37.

Tian, Q. (2000) Molekularbiologische Charakterisierung des menschlichen Desmoglein 2 (DSG2)-Gens und Ausschaltung dieses Gens bei der Maus durch homologe Rekombination, Ph. D. Thesis, Faculty of Biology, University of Heidelberg, Heidelberg, Germany, pp.1-157.

Troyanovsky, S. (2005). Cadherin dimers in cell-cell adhesion. Eur J Cell Biol *84*, 225-233.

Tselepis, C., Chidgey, M., North, A., and Garrod, D. (1998). Desmosomal adhesion inhibits invasive behavior. Proc Natl Acad Sci U S A *95*, 8064-8069.

Tsiftsoglou, A. S., Pappas, I. S., and Vizirianakis, I. S. (2003). Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther *100*, 257-290.

Turner, M. L., Masek, L. C., Hardy, C. L., Parker, A. C., and Sweetenham, J. W. (1998). Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. Br J Haematol *100*, 112-122.

Ulger, C., Toruner, G. A., Alkan, M., Mohammed, M., Damani, S., Kang, J., Galante, A., Aviv, H., Soteropoulos, P., Tolias, P. P.*, et al.* (2003). Comprehensive genome-wide comparison of DNA and RNA level scan using microarray technology for identification of candidate cancer-related genes in the HL-60 cell line. Cancer Genet Cytogenet *147*, 28-35.

Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W., and van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell *66*, 107-119.

Watt, F. M., Mattey, D. L., and Garrod, D. R. (1984). Calcium-induced reorganization of desmosomal components in cultured human keratinocytes. J Cell Biol *99*, 2211-2215.

Wein, F., Pietsch, L., Saffrich, R., Wuchter, P., Walenda, T., Bork, S., Horn, P., Diehlmann, A., Eckstein, V., Ho, A. D., and Wagner, W. (2010). N-cadherin is expressed on human hematopoietic progenitor cells and mediates interaction with human mesenchymal stromal cells. Stem Cell Res *4*, 129-139.

Wheeler, G. N., Buxton, R. S., Parker, A. E., Arnemann, J., Rees, D. A., King, I. A., and Magee, A. I. (1991a). Desmosomal glycoproteins I, II and III: novel members of the cadherin superfamily. Biochem Soc Trans *19*, 1060-1064.

Wheeler, G. N., Parker, A. E., Thomas, C. L., Ataliotis, P., Poynter, D., Arnemann, J., Rutman, A. J., Pidsley, S. C., Watt, F. M., Rees, D. A.*, et al.* (1991b). Desmosomal glycoprotein DGI, a component of intercellular desmosome junctions, is related to the cadherin family of cell adhesion molecules. Proc Natl Acad Sci U S A *88*, 4796-4800.

Wheelock, M. J., and Johnson, K. R. (2003). Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol *19*, 207-235.

Wheelock, M. J., Shintani, Y., Maeda, M., Fukumoto, Y., and Johnson, K. R. (2008). Cadherin switching. J Cell Sci *121*, 727-735.

Wuchter, P., Boda-Heggemann, J., Straub, B. K., Grund, C., Kuhn, C., Krause, U., Seckinger, A., Peitsch, W. K., Spring, H., Ho, A. D., and Franke, W. W. (2007). Processus and recessus adhaerentes: giant adherens cell junction systems connect and attract human mesenchymal stem cells. Cell Tissue Res *328*, 499-514.

Zauli, D., Gobbi, M., Crespi, C., Tazzari, P. L., Miserocchi, F., Magnani, M., and Testoni, N. (1986). Vimentin and keratin intermediate filaments expression by K562 leukemic cell line. Leuk Res *10*, 29-33.

Zhou, X., Stuart, A., Dettin, L. E., Rodriguez, G., Hoel, B., and Gallicano, G. I. (2004). Desmoplakin is required for microvascular tube formation in culture. J Cell Sci *117*, 3129-3140.

FIGURE LEDGENDS

Figure 1. Identification of Diverse Cytoskeletal Filament Proteins in Cultures of Multipotential Human Hematopoietic K562 Tumor Cells

Immunofluorescence microscopy of cultured human K562 cells grown in suspension and allowed spontaneously to adhere to the substratum, showing the abundance and coexistence of bundles or whorly aggregates of filaments containing vimentin (A, green) or keratins 8 (B, red), 18 (not shown) and 19 (C, red) or smooth-muscle-type α-actin (D, red). DAPI staining (blue) was used to visualize nuclei. Note the frequent densely aggregated forms of bundles of all three kinds of filaments. Scale bars: 20 µm

Figure 2. Coexistence of Two Major Kinds of Colonies of Human Hematopoietic K562 Cells With and Without Adhering Junctions (AJs)

Light microscopy showing two small colonies of substratum-adherent K562 cells (A, phase contrast image) which differ in their reaction with antibodies to the desmosomal glycoprotein, desmoglein Dsg2 clustered to small punctate structures, mostly located at cell-cell contacts in the colony shown in the right (B, immunofluorescence microscopy image), whereas the left hand colony is totally negative. Scale bars: 20 µm

Figure 3. Identification and Localization of Desmosomal Proteins in Hematopoietic K562 Cells in Clusters of Adhering Junctions and Hemi-Junctions by Immunofluorescence Microscopy

(A-A'') Laser-scanning confocal immunofluorescence microscopy of a double-labeling experiment, comparing the reactions of two antibodies against different epitopes of the desmosomal glycoprotein, desmoglein Dsg2 (A, red: Dsg2 rabbit antibodies Dsg2 - rb8; B, green: mAb Dsg2 - clone 10G11). The corresponding merged picture (A'') presented with a differential interference contrast background in A'', shows a complete and intense co-localization of both kinds of Dsg2 antibodies in small punctate structures (yellow) which either represent cell-cell-contacts sites or asymmetric "free" (half-junctional) plasma membrane domains.

(B-D) Double-label immunolocalization micrographs, on a differential interference contrast optical background, showing co-localizations of glycoprotein Dsg2 (green, B-

D) with the red-labeled cytoplasmic plaque proteins, plakophilin 2 (B), plakoglobin (C) and desmoplakin (D). Note that most, but not all of the punctate Dsg2-positive structures show co-localization (yellow) with the specific desmosomal plaque protein. Scale bars: 20 µm

Figure 4. Electron Micrographs Demonstrating Intercellular Contacts of the Adhering Junction-Type Connecting Cultured K562 Cells

Survey electron micrographs of ultrathin sections through K562 cells (A and C), showing cell-cell junction structures, as indicated by arrows (A, C) and shown in higher magnifications in B, D and E. Note that these junctions are defined by thin but densely heavy metal-stained plaque structures (B, D, E). N, nucleus. Scale bars: 2 µm (A, C) and 0.25 µm (B, D, E)

Figure 5. Electron and Immunoelectron Microscopy of Cell-Cell Adhering Junctions (AJs) and Surface-Exposed Half-Junctions in Cultures of K562 Cells

Survey electron micrograph (A) and partial magnifications (B, C) show a series of variously-sized and variously-developed AJs formed between these cells (indicated by brackets and pairs of bars in A-C). Note that in the example shown here an extended region of two cells in contact is presented which comprises AJs of widely varying sizes and plaque thicknesses (compare, e.g., in B and C the four junctions with “desmosome-like” morphology numbered D1-D4). Note also junctional structures showing a well-developed plaque only in one of the two cells in contact (D and E, arrowheads), with small plaque-lacking contact sites (E), deep invaginations of plaque-coated plasma membrane (an example is denoted by the arrowhead in F) and relatively small, surface-exposed half-junctions with a marked dense plaque (G).

The restriction of the desmosomal molecules to AJs is shown by the intense immunogold labeling of Dsg2 as seen in half-junctions (H-J) as well as in AJs (K-M). The identity of the plasma membrane domains representing half-junction equivalents is best demonstrated by immunoelectron microscopy using antibodies recognizing an epitope located in the aminoterminal, glycosylated extracellular part of Dsg2 (H-J), including structures located at the tips of filopodial cell processes (J). On the other side, the cytoplasmic portion of Dsg2 id demonstrated by the intense labeling of typical AJ structures with antibodies recognizing the carboxyterminal, plaque-integrated part of Dsg2 (K-M). Note also that the left junction shown in M presents

little, if any significant Dsg2-label, indicating that in these cells additional junction-like, cell-cell contact structures without Dsg2 and a conspicuous plaque may also occur. The Dsg2-positive ones are generally coated with junction plaques that are immunolabeled with antibodies against plakophilin 2 (N) and plakoglobin (O). M, mitochondrion (A). Scale bars: 1 µm (A), 0.5 µm (F, H, J), 0.25 µm (B-E, K, L, N, O) and 0.1 µm (G, I, M)

Figure 6. Gel-Electrophoresis and Immunoblot Detection of Adhering Junction (AJ) Proteins in Cultured Human Hematopoietic K562 Cells

Immunoblot reactions of near-equal amounts of SDS-PAGE-separated cytoskeletal proteins from cultured human HaCaT keratinocytes (*lane 1*), SV80 fibroblasts (*lane 2*) and K562 - hematopoietic cells (*lane 3*), probed with (A) antibodies specific for AJ cadherins (E-, P-, N-cadherin and cadherin 11) or for one of the plaque proteins, α- and β-catenin, protein p120 or protein p0071 in comparison with known desmosomal plaque proteins such as (B) plakophilins Pkp1, Pkp2 and Pkp3, plakoglobin (PG) and desmoplakin (DP) or with antibodies specifically recognizing the desmosomal cadherins desmoglein Dsg1 and Dsg2, Dsg2 and desmocollin Dsc2. Note that only the desmosomal cadherin Dsg2 is detectable in K562 cells whereas all the other cadherins are absent. This finding has been confirmed by immunoblotting with two different monoclonal antibodies (mAbs) to Dsg2, one recognizing both Dsgs1 and 2 (clone DG3.10) and the other exclusively Dsg2 (clone 6D8). In addition the plaque proteins Pkp2, Pkp3, plakoglobin as well as β-catenin and protein p120 have been detected and after prolonged exposure and only in trace amounts, some protein p0071 has been also found (*arrow*). Polypeptide molecular weight marker bands (on the left margin) correspond to 212, 158, 116, 97.2, 66.4, 55.6, 42.7 and 34.6 kDa (from top to bottom). * For the immunoblot identification of N-cadherin, cadherin 11 as well as α- and β-catenin whole cell lysates of murine 3T3-L1 cells were used for the loading of *lane 1* instead of HaCaT cells.

Figure 7. Biochemical Demonstration of Complexes of Desmoglein 2 (Dsg2) with Specific Other AJ Proteins in Cultures of K562 Cells

For direct identification of complex partner proteins of desmoglein Dsg2 immunoprecipitations (IP) from total lysates of K562 cells were performed, and the precipitated proteins were separated by SDS-PAGE and examined by

immunoblotting. Antibodies used for IP were directed against plakophilin 2 (Pkp2), VE-cadherin (VE-cad), desmogleins Dsg1 and Dsg2 (Dsg1+2) and plakoglobin (PG). As positive controls, total cell lysates of K562 cells, the supernatant fraction from the lysate before IP (L), and material of the specific pre-clearing preparation step (P) were used. Immunoblots were performed using monoclonal antibodies (mAbs) reacting with Dsg1+2 (A), PG (B), Pkp2 (C) and Pkp3 (D). Note that besides the positive controls, IPs with Dsg2 antibodies clearly show immunoreactivity (arrows) with antibodies specific for Dsg1+2, plakoglobin as well as Pkp2 and Pkp3. As negative controls, the antibody-coated beads used after pre-clearing (Ab-control) and IPs using antibodies specific for VE-cadherin clearly have been used. The lower immunoreactive positive band (~50 kDa) represents the heavy chains of the antibodies used for IP. Protein marker bands (on the left margin) correspond to 212, 158, 116, 97.2, 66.4, 55.6, 42.7 and 34.6 kDa (from top to bottom).

Figure 8. Localization of Non-Desmosomal Proteins with Respect to Adhering Junctions (AJs) and Other Cortical Structures of Dense-Grown, Substratum-Adherent K562 Cells

(A and B) Double-label laser-scanning confocal immunofluorescence microscopy comparing the localization of afadin (A, red) and desmoglein Dsg2 (B, red) with that of the actin-binding, cortical protein, α-actinin (A, green; B, green). Note extended regions of co-localization of afadin and α-actinin, as shown by the yellow merge color in A, whereas B shows that the mostly linear localization of α-actinin (green) is completely different from the punctate reaction sites of desmoglein Dsg2. Scale bars: 10 µm

Molecular Characterizations of Novel Types of Adhering Junctions (AJs) Containing Desmosomal Proteins Which Have Spontaneously Formed in Clonal Sublines of Human Hematopoietic K562 Tumor Cells

	AJ Subtype					
Transmembrane	**I**	**II**	**III**	**IV**	**V**	**VI**
AJ Cadherins						
E-Cadherin	-	-	-	-	-	-
P-Cadherin	-	-	-	-	-	-
VE-Cadherin	-	-	-	-	-	-
N-Cadherin	-	-	-	-	-	-
Cadherin 11	-	-	-	-	-	-
Desmoglein 1	-	-	-	-	-	-
Desmoglein 2	++	++	++	++	-	+
Desmoglein 3	-	-	-	-	-	-
Desmoglein 4	-	-	-	-	-	-
Desmocollin 1	-	-	-	-	-	-
Desmocollin 2	-	-	-	+	-	+
Desmocollin 3	-	-	-	-	-	-
AJ Plaque Proteins						
α-Catenin	-	-	-	-	-	-
β-Catenin	-	-	+	-	+	+*
Plakoglobin	++	++	++	++	++	+
Protein p120	-	-	+	-	+	+*
Protein p0071	-	-	+	-	+	+*
Plakophilin 1	-	-	-	-	-	-
Plakophilin 2	+	++	++	++	++	+*
Plakophilin 3	+	+	+	+	+	+*
Desmoplakin	-	++	-	+	-	-

Table 1. Molecules identified in AJs of the fifteen clonal sublines of human hematopoietic K562 tumor cells as determined by SDS-PAGE and immunoblotting as well as by immunocytochemistry. Symbols: +, moderate intensity of the reaction for the molecules tested; ++ high intensity of reaction; - not detected. Six major different subtypes have so far been distinguished with respect to AJ formations: **(I)** AJs comprising desmosomal molecules, with the exception of any desmocollin and desmoplakin; **(II)** AJs with all desmosomal molecules, including desmoplakin, but without any desmocollin; **(III)** AJs comprising the desmosome-type molecules as in type (I) plus the *armadillo* plaque proteins β-catenin, p120 and p0071; **(IV)** AJs with a full desmosomal molecule complement, including desmocollin Dsc2; **(V)** AJ-type assemblies similar to that presented in type (III) but without detectable desmocollins and desmogleins; **(VI)** punctate AJ-type assemblies positive for desmosomal cadherins, Dsg2 and Dsc2, and the plaque proteins as specified; *asterisks* denote reactions of low and sometimes variable intensity.

SUPPLEMENTAL DATA

Figure S1. Coincident and Differential Immunofluorescence Microscopic Localization of the Two Desmosomal Cadherins and Plakophilin, Pkp2, in Different Types of Adhering Junctions (AJs) and Half-Junctions in Human Hematopoietic K562 Tumor Cells

(A) Survey micrograph showing a colony of cells with punctate AJs positive for desmocollin Dsc2 (A; rabbit antibodies, red) and (B) partial magnification of the upper part of (A) shown in double-label laser-scanning confocal immunofluorescence microscopy a differential interference contrast background, together with the reaction for desmoglein Dsg2 (B; mAb, green). Only the merged color picture is shown here. Note that in this AJ-type (subtype VIa) most reaction sites are positive for both Dsg2 and Dsc2 (yellow merge color) but that there are also some additional punctate sites which are positive for Dsc2 only (red dots, denoted by triangular marks). (C) Double-label immunolocalization on a differential interference contrast background as in (B), showing complete co-localization of both Dsg2 and Dsc2 in desmosome-like AJs (subtype VIb) of varying sizes (only the yellow merge color picture is shown), representing either cell-cell AJs or surface-exposed half-AJs, characteristic of a specific type of subline (VIa). (D) Double-label immunolocalization (optical conditions as in C), presenting a subtype (VIc) showing complete differential localization of desmoglein Dsg2 (green arrows) and desmocollin Dsc2 (red, triangular marks), illustrating that here the molecular organization of AJ-type junctions is regulated differently from the colocalization noted in the other two subtypes and in regular desmosomes. (E) Differential topogenesis of AJ-related clusters of desmoglein Dsg2 (green, small arrows) and plakophilin Pkp2 (red, triangular marks), indicating that in this specific subtype both, the cadherin and the plaque protein, are synthesized but not co-assembled. Scale bars: 5 μm (E); 10 μm (A-D)

Figure S2. Immunolocalization and Molecular Identification of the Transmembrane Glycoprotein, Epithelial Cell Adhesion Antigen (EpCAM), in Adhering Junctions (AJs) of Colonies of Hematopoietic K562 Cell Cultures

(A) Double-label laser-scanning confocal immunofluorescence microscopy on differential interference contrast background, comparing the localization of EpCAM (red) with that of desmoglein Dsg2 (green, denoted by triangular marks). Note the extended and near-continuous cell-cell associations as well as distinct dots. (B) Higher magnification immunofluorescence microscopy of EpCAM (red), showing clearly the punctate EpCAM-containing junction structures as well as punctate EpCAM sites in free plasma membrane regions. (C) Immunoblot reactions of similar amounts of SDS-PAGE-separated cytoskeletal proteins from cultured human HaCaT keratinocytes, liver carcinoma cells of line PLC and hematopoietic K562 cells, probed with an antibody specific for EpCAM. Note that besides the epithelial reaction in the HaCaT and PLC cells, typical “twin band” immunoreactivity is also seen in K562 cell proteins, using antibodies specific for EpCAM (in this case mAb 33.2 has been used). After somewhat prolonged exposure, the intensity of the EpCAM-reaction indicates that EpCAM is indeed a regular frequent and major plasma membrane component of K562 cells (lane K562*). Scale bars: 10 μm. Co-electrophoresed reference protein bands (on the left margin) indicate molecular weights 158, 116, 97.2, 66.4, 55.6, 42.7 and 34.6 kDa (from top to bottom).

Table S1. - Primary antibodies used in this study.

Antigen	Antibody type	Source (reference)
Transmembrane Proteins		
E-Cadherin	a) mAb, m	BD Biosciences Pharmingen (Heidelberg, Germany)
	b) As, rb	Epitomics Inc. (Burlingame, CA, USA.)
N-Cadherin	a) mAb, m	BD Biosciences
	b) As, rb	QED Bioscience Inc (San Diego, CA, USA)
P-Cadherin	mAb, m	BD Biosciences
VE-Cadherin	a) mAb, m (BV9)	Gift of E. Dejana (University of Milan, Italy)
	b) As, rb	Cayman Chemical Company (Ann Arbor, MI, USA)
Cadherin 11	a) mAb, m	Zymed Laboratories (San Francisco, CA, USA)
	b) As, rb	Zymed Laboratories
Desmoglein 1	a) mAb, (P23)	Progen Biotechnik (Heidelberg, Germany)
Desmoglein 1+2	mAb, (DG 3.10)	Progen Biotechnik
Desmoglein 2	a) mAb, m (10G11)	Progen Biotechnik
	b) mAb, m (G96)	Progen Biotechnik
	c) mAb, m (G129)	Progen Biotechnik
	d) As, rb	Progen Biotechnik
	e) As, gp	Progen Biotechnik
Desmoglein 3	mAb, m (G194)	Progen Biotechnik
Desmoglein 4	As, gp	Progen Biotechnik
Desmocollin 1	mAb, m (U100)	Progen Biotechnik
Desmocollin 2	As, rb	Progen Biotechnik
	As, gp	Progen Biotechnik
Desmocollin 3	mAb, m (U114)	Progen Biotechnik
Occludin	a) mAb, m	Zymed Laboratories
	b) As, rb	Zymed Laboratories
Claudin 1	As, rb	Zymed Laboratories
Claudin 2	As, rb	Zymed Laboratories
Claudin 3	As, rb	Zymed Laboratories
Claudin 4	mAb, m	Zymed Laboratories
Nectin 3	As, rb	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Human Epithelial Antigen (EpCAM)	a) mAb, m (HEA125)	Progen Biotechnik
	b) mAb, m (MOC-31)	Progen Biotechnik
	c) mAb, m (33.2)	Gift of G. Moldenhauer (German Cancer Research Center)
Junctional Adhesion Molecule (JAM-A)	As, rb	Zymed Laboratories

AJ Plaque Proteins

α-Catenin	a) mAb, m	Zymed Laboratories
	b) As, rb	Sigma (St. Louis, MO, USA)
β-Catenin	a) mAb, m	BD Biosciences
	b) As, rb	Sigma
Plakoglobin	a) mAb, m (11E4)	Gift of M.J. Wheelock (University of Nebraska, Omaha, NE, USA)
	b) mAb, m (PG 5.1)	Progen Biotechnik
Protein p120	a) mAb, m	BD Biosciences
	b) As, rb	Sigma
Protein p0071	a) mAb, m	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Protein ARVCF	a) mAb, m	Gift of I. Hofmann (German Cancer Research Center)
	b) AS, gp	Progen Biotechnik
Plakophilin-1	a) mAb, m (PP1-5C2)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Plakophilin-2	a) mAb, m (Pkp2-518)	Progen Biotechnik
	b) mAb, m (PP2/62, PP2/86, PP2/150)	Progen Biotechnik
	c) AS, gp	Progen Biotechnik
Plakophilin-3	a) mAb, m (PKP3-270)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Desmoplakin	a) mAb, m (DP-2.15, DP-2.17, DP-2.20)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik

Intermediate Filament Proteins

Vimentin	a) mAb, m (3B4)	Progen Biotechnik
	b) mAb, m (V9)	Progen Biotechnik
	c) AS, gp	Progen Biotechnik
Most keratins (“pan-keratin”)	mAb, m (Lu5)	Progen Biotechnik
Keratin 8	mAb, m (Ks8-17.2)	Progen Biotechnik
Keratin 18	mAb, m (Ks18.04)	Progen Biotechnik
Keratins 8 and18	AS, gp	Progen Biotechnik
Keratin 19	a) mAb, m (Ks19.10)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Keratin 20	a) mAb, m (Ks20.10)	Progen Biotechnik
	b) AS, gp	Progen Biotechnik
Desmin	mAb, m	DAKO (Hamburg, Germany)

Actin - Microfilament and Associated Proteins		
Afadin	AS, rb	Sigma
α-Actinin	a) mAb, m	Sigma
	b) AS, rb	Sigma
Protein ZO-1	a) mAb, m	Zymed Laboratories
	b) AS, rb	Zymed Laboratories
Smooth muscle α-actin	mAb, m (ASM-1)	Progen Biotechnik
Cardiac/embryonic α-actin	mAb, m (AC1-20.4.2)	Progen Biotechnik
Troponin T skeletal muscle	mAb, m	Sigma
Troponin T cardiac	AS, rb	Zytomed Systems (Berlin, Germany)
Smooth muscle tropomyosin	AS, rb	Sigma
Myosin skeletal muscle heavy and light chain	AS, rb	Sigma

Table S1 - Primary antibodies used in this study. As - antiserum or IgGs prepared therefrom; m - mouse; mAb - monoclonal antibody; rb - rabbit; gp - guinea pig.

For characterizations of more recently described antibodies see, e.g., Franke et al., 2006; Hofmann et al., 2008; Barth et al., 2009; Moll et al., 2009; Rickelt et al., 2009.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Special Cell Cultures and Treatments

Various K562 cell culture sublines (see also Table 1) were repeatedly treated with differentiation-promoting or -inhibiting agents added to the culture medium, mostly for periods of 3-6 days. In particular, sublines with different AJ patterns were treated with inducers or promotors of erythroid or megakaryocytic differentiation, essentially using the agents and protocols published in the literature such as hemin, sodium butyrate (e.g., Järvinen et al., 1990; Rowley et al., 1985), diverse phorbolesters (e.g., Alitalo et al., 1990; Hickstein et al., 1993; Shelly et al., 1998; Cheng et al., 1994), cytosine arabinoside, 5-azacytidine or related drugs (e.g., Darmon et al., 1984; Bianchi et al., 1999; Huang et al., 2002), transforming growth factor-β1 or erythropoietin (EPO) and EPO-mimicking peptides (e.g., Burger et al., 1994; Debili et al., 1996; Lutomski et al., 1997; Stopka et al., 1998; for review see Tsiftsoglou et al., 2003).

For comparison we studied other hematopoietic and mesenchymally derived, human cell lines such as HEL, HL-60, BV173, KG-1a and RPMI 8226, SV40-transformed human fibroblasts of line “SV80”, and some malignantly transformed or untransformed human epithelium-derived cell lines such as human HaCaT-keratinocytes and hepatocellular carcinoma cells of line PLC (cf. Rickelt et al., 2009), all kept in Dulbeccos´s Minimal Essential Medium (DMEM; Invitrogen), supplemented with 10 % FCS and 2 mM glutamine.

Immunoblotting

For immunoblotting of the proteins transferred to PVDF membranes, background reactions due to non-specific binding were usually reduced by prior 30 min incubation in 5 % low-fat dry milk in PBS containing 0.05 % Tween (PBS-T). Blots were then incubated with the specific primary antibodies for 1 h, washed thrice in PBS-T and incubated for at least 30 min with HRP-conjugated secondary antibodies, followed by at least three washes of the blot paper in PBS. For the detection of the secondary antibodies bound to reactive proteins, enhanced chemiluminescence (ECL; Amersham-Buchler, Braunschweig, Germany) was applied.

SUPPLEMENTAL REFERENCES

Alitalo, R., Partanen, J., Pertovaara, L., Holtta, E., Sistonen, L., Andersson, L., and Alitalo, K. (1990). Increased erythroid potentiating activity/tissue inhibitor of metalloproteinases and jun/fos transcription factor complex characterize tumor promoter-induced megakaryoblastic differentiation of K562 leukemia cells. Blood *75*, 1974-1982.

Barth, M., Schumacher, H., Kuhn, C., Akhyari, P., Lichtenberg, A., and Franke, W. W. (2009). Cordial connections: molecular ensembles and structures of adhering junctions connecting interstitial cells of cardiac valves in situ and in cell culture. Cell Tissue Res *337*, 63-77.

Bianchi, N., Osti, F., Rutigliano, C., Corradini, F. G., Borsetti, E., Tomassetti, M., Mischiati, C., Feriotto, G., and Gambari, R. (1999). The DNA-binding drugs mithramycin and chromomycin are powerful inducers of erythroid differentiation of human K562 cells. Br J Haematol *104*, 258-265.

Burger, P. E., Dowdle, E. B., Lukey, P. T., and Wilson, E. L. (1994). Basic fibroblast growth factor antagonizes transforming growth factor beta-mediated erythroid differentiation in K562 cells. Blood *83*, 1808-1812.

Cheng, T., Wang, Y., and Dai, W. (1994). Transcription factor egr-1 is involved in phorbol 12-myristate 13-acetate-induced megakaryocytic differentiation of K562 cells. J Biol Chem *269*, 30848-30853.

Darmon, M., Nicolas, J.-F., and Lamblin, D. (1984). 5-azacytidine is able to induce the conversion of teratocarcinoma-derived mesenchymal cells into epithelial cells. EMBO *3*, 961-967.

Debili, N., Coulombel, L., Croisille, L., Katz, A., Guichard, J., Breton-Gorius, J., and Vainchenker, W. (1996). Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. Blood *88*, 1284-1296.

Franke, W. W., Borrmann, C. M., Grund, C., and Pieperhoff, S. (2006). The area composita of adhering junctions connecting heart muscle cells of vertebrates. I. Molecular definition in intercalated disks of cardiomyocytes by immunoelectron microscopy of desmosomal proteins. Eur J Cell Biol *85*, 69-82.

Hickstein, D. D., Grunvald, E., Shumaker, G., Baker, D. M., Back, A. L., Embree, L. J., Yee, E., and Gollahon, K. A. (1993). Transfected leukocyte integrin CD11b/CD18 (Mac-1) mediates phorbol ester-activated, homotypic cell:cell adherence in the K562 cell line. Blood *82*, 2537-2545.

Hofmann, I., Kuhn, C., and Franke, W. W. (2008). Protein p0071, a major plaque protein of non-desmosomal adhering junctions, is a selective cell-type marker. Cell Tissue Res *334*, 381-399.

Huang, M., Wang, Y., Collins, M., Gu, J. J., Mitchell, B. S., and Graves, L. M. (2002). Inhibition of nucleoside transport by p38 MAPK inhibitors. J Biol Chem *277*, 28364-28367.

Järvinen, M., Andersson, L. C., and Virtanen, I. (1990). K562 erythroleukemia cells express cytokeratins 8, 18, and 19 and epithelial membrane antigen that disappear after induced differentiation. J Cell Physiol *143*, 310-320.

Lutomski, D., Fouillit, M., Bourin, P., Mellottee, D., Denize, N., Pontet, M., Bladier, D., Caron, M., and Joubert-Caron, R. (1997). Externalization and binding of galectin-1 on cell surface of K562 cells upon erythroid differentiation. Glycobiology *7*, 1193-1199.

Moll, R., Sievers, E., Hammerling, B., Schmidt, A., Barth, M., Kuhn, C., Grund, C., Hofmann, I., and Franke, W. W. (2009). Endothelial and virgultar cell formations in the mammalian lymph node sinus: endothelial differentiation morphotypes characterized by a special kind of junction (complexus adhaerens). Cell Tissue Res *335*, 109-141.

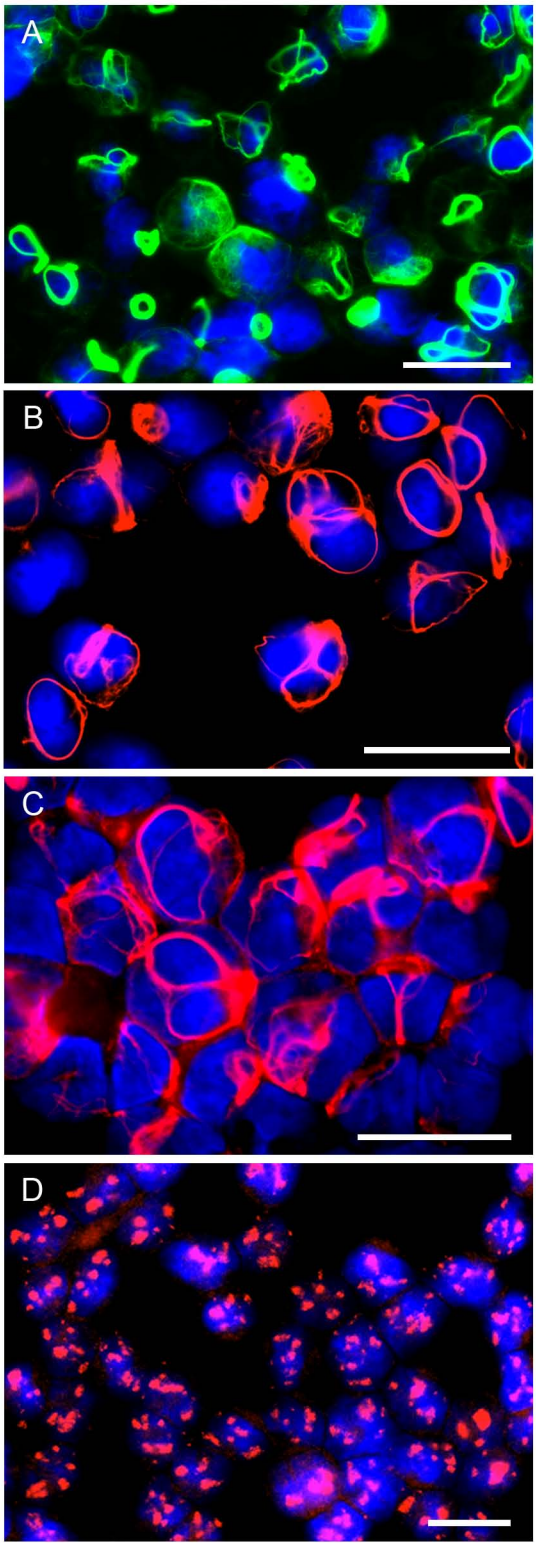
Rickelt, S., Winter-Simanowski, S., Noffz, E., Kuhn, C., and Franke, W. W. (2009). Upregulation of plakophilin-2 and its acquisition to adherens junctions identifies a novel molecular ensemble of cell-cell-attachment characteristic for transformed mesenchymal cells. Int J Cancer *125*, 2036-2048.

Rowley, P. T., Ohlsson-Wilhelm, B. M., and Farley, B. A. (1985). K562 human erythroleukemia cells demonstrate commitment. Blood *65*, 862-868.

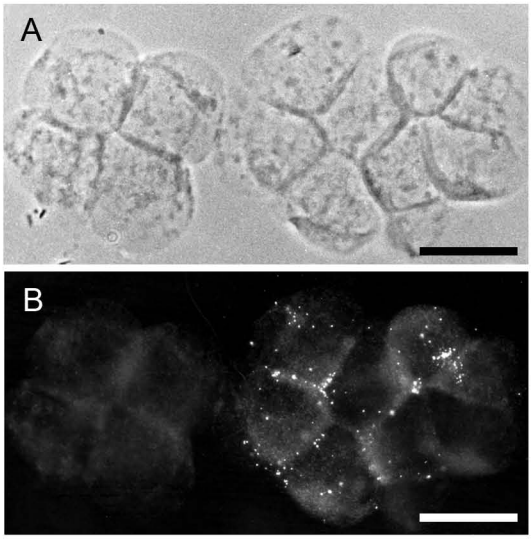
Shelly, C., Petruzzelli, L., and Herrera, R. (1998). PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events. Leukemia *12*, 1951-1961.

Stopka, T., Zivny, J. H., Stopkova, P., Prchal, J. F., and Prchal, J. T. (1998). Human hematopoietic progenitors express erythropoietin. Blood *91*, 3766-3772.

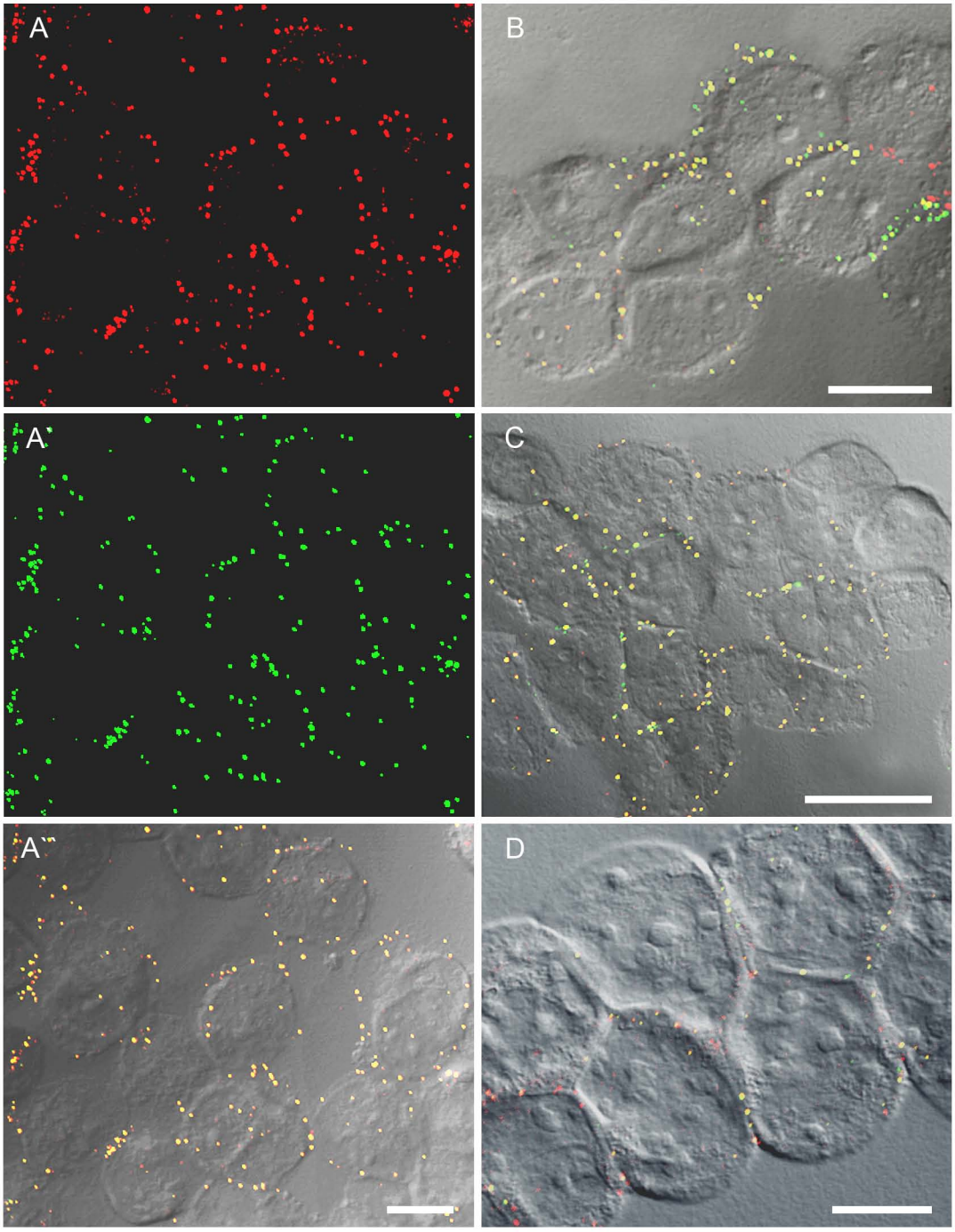
Tsiftsoglou, A. S., Pappas, I. S., and Vizirianakis, I. S. (2003). Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther *100*, 257-290.



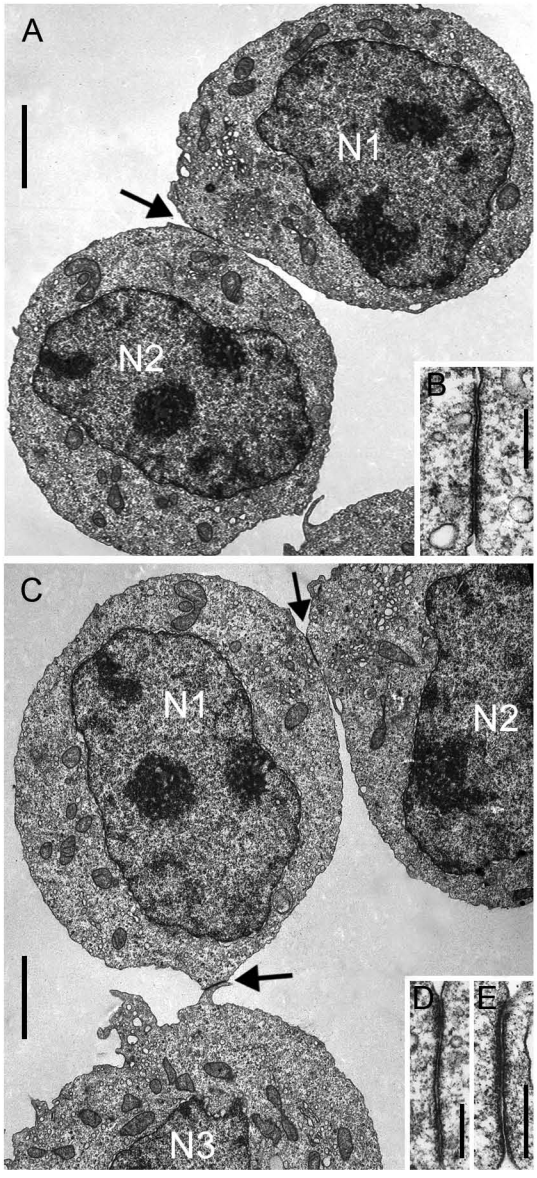
Franke and Rickelt - Figure 1



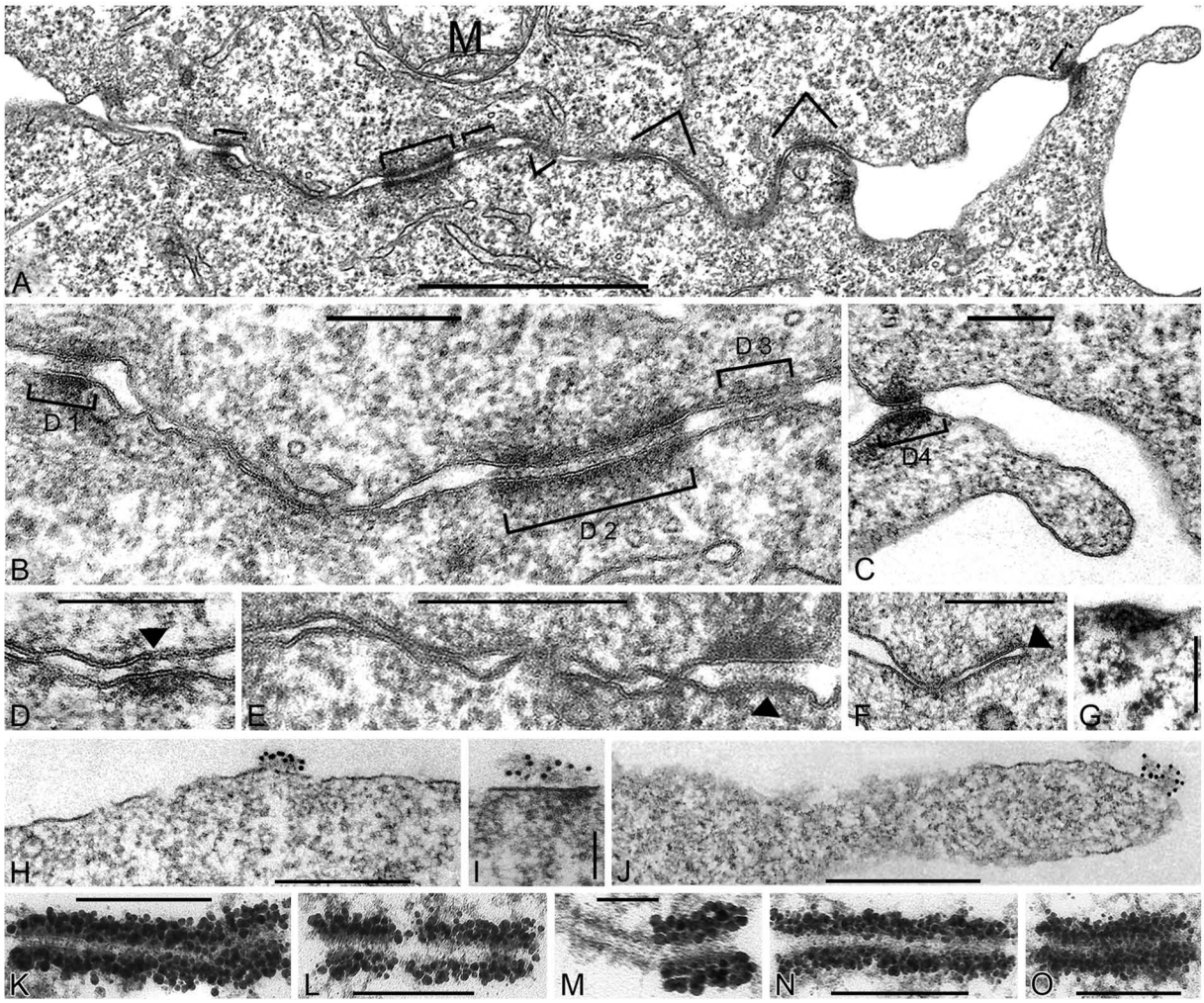
Franke and Rickelt - Figure 2



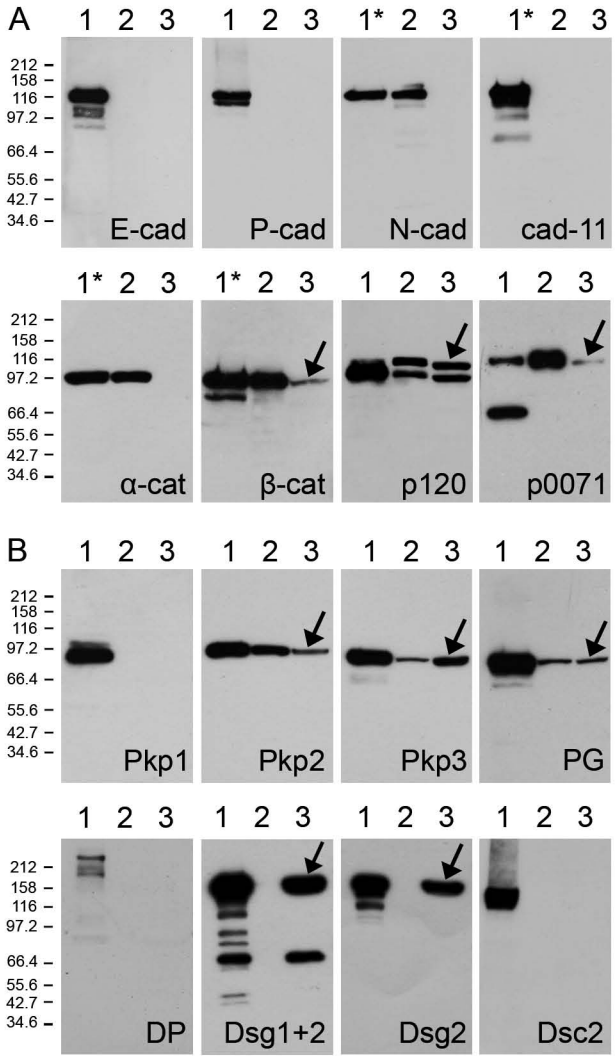
Franke and Rickelt - Figure 3



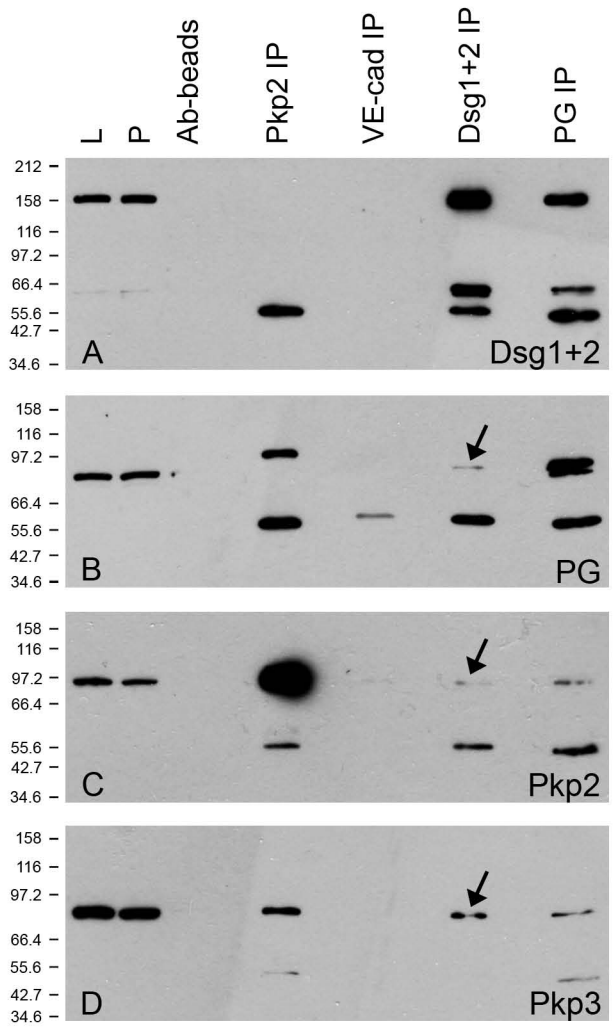
Franke and Rickelt - Figure 4



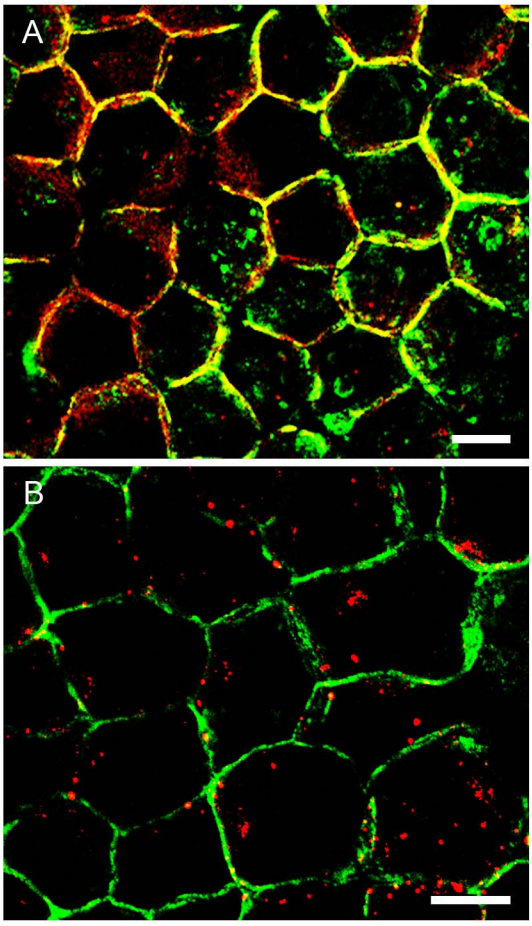
Franke and Rickelt - Figure 5



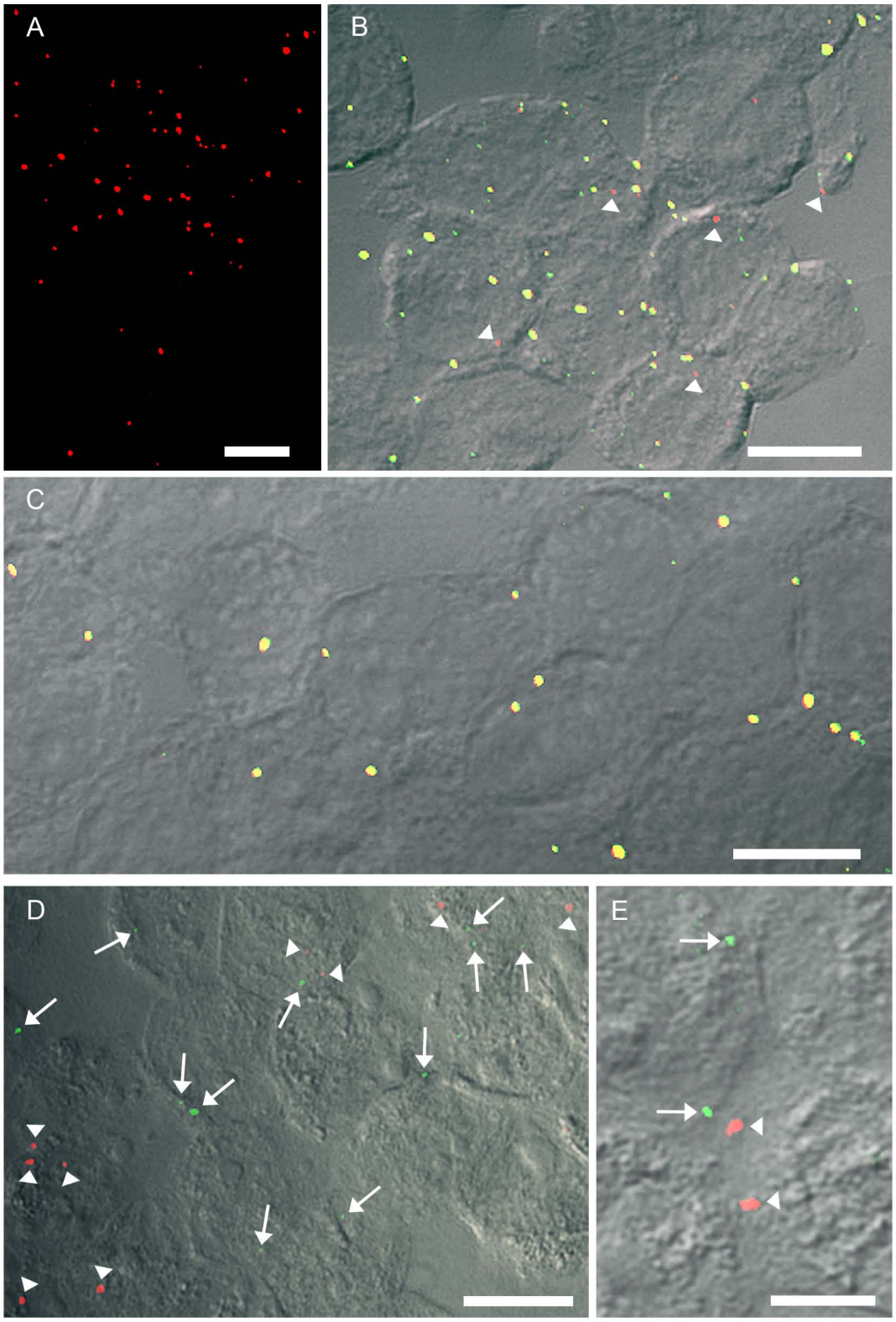
Franke and Rickelt - Figure 6



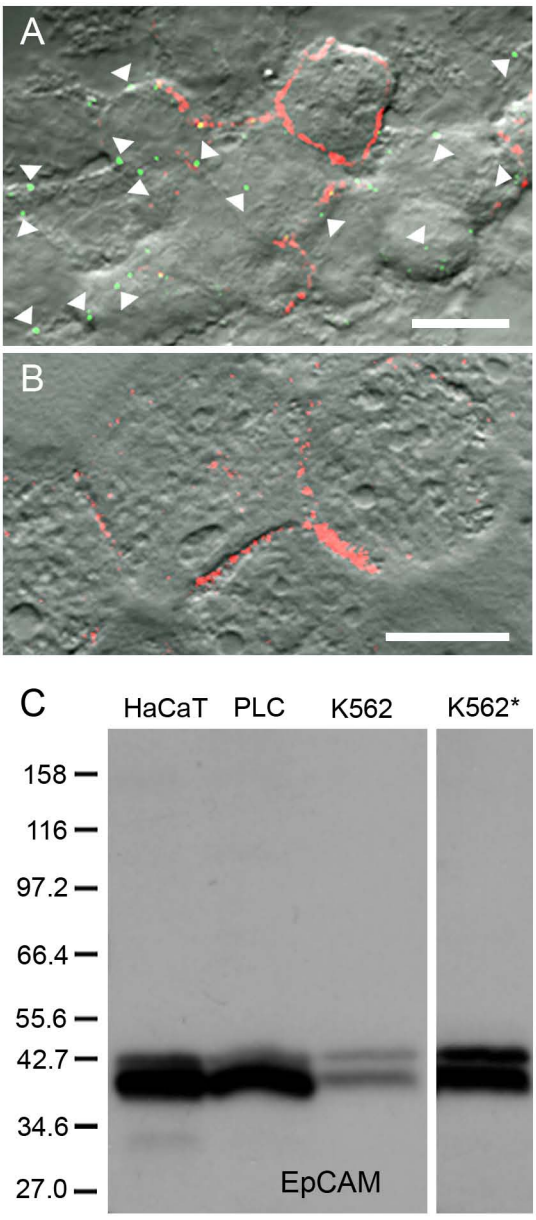
Franke and Rickelt - Figure 7



Franke and Rickelt - Figure 8



Franke and Rickelt - Figure S1



Franke and Rickelt - Figure S2