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New regulatory molecules in growth and invasion of melanoma and breast cancer: cadherins, heregulin and follistatin

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Summary

Cancer is often the result of a multi-step process in which normal cells have acquired the ability to survive and proliferate limitlessly and can invade the surrounding tissue, which may lead to metastasis. In this study, we addressed the role that growth factor systems and cell-cell adhesion molecules may play in melanoma and breast cancer.

Crucial in the process of carcinogenesis is the mutual cross-talk between the tumor and the micro-environment, an interaction which may be exerted by soluble factors. Based on the identification of two glycoproteins that were secreted by melanoma cells, we characterized two new growth factor systems in human melanoma. Both of these may contribute to malignant progression. The use of mass spectrometry as a biochemical approach led to the identification of follistatin as a factor secreted by several melanoma cell lines. Since we found that follistatin can protect melanocytes against the growth-inhibitory and pro-apoptotic effects of activin (a transforming growth factor-β-like ligand), its secretion may promote the survival of transformed melanocytic cells. Using a biological approach as a starting point, we found that a melanoma conditioned medium can induce activation of a 185kD protein in a test cell line. This activity was attributed to the presence of heregulin, an epidermal growth factor-like ligand for receptor tyrosine kinases. Subsequent evaluation of the heregulin-human epidermal growth factor receptor system in a panel of melanoma cell lines revealed the presence of multiple deregulations. These included both autocrine activation and ligand insensitivity owing to the absence of (functional) receptors.

When heregulin was tested in an *in vitro* model of invasion and aggregation, using a breast cancer cell line with a functionally deficient cell-cell adhesion complex, we found that invasion is counteracted, whereas aggregation is promoted. Crucial for cell-cell contacts between epithelial cells are cadherins, calcium-dependent cell-cell adhesion molecules that are involved in the regulation of cell motility, growth, differentiation and survival. E(epithelial)-cadherin, the prototype member of this family, has a well-described invasion-suppressor function. Functional inactivation of E-cadherin may occur at the genomic, transcriptional, and post-transcriptional level. An example of the latter is proteolytic cleavage of E-cadherin, resulting in release of its ectodomain, which we found to be capable of promoting invasion and counteracting aggregation.

In contrast to E-cadherin, which has been extensively studied in cancer, little is known about the role of the highly related P-cadherin in cancer. We found that P-cadherin may either function as an invasion-suppressor or as an invasion promoter, depending on the context. Melanoma cells lose P-cadherin expression during progression. Reconstituting this expression led to improved cell-cell adhesion and counteraction of invasion. In breast cancer, in contrast, upregulation of P-cadherin correlates with estrogen receptor negativity and poor survival. We established a direct link between P-cadherin expression and the lack of estrogen receptor signaling and found that P-cadherin, via its intracellular membrane-proximal domain, promotes invasion of breast cancer cells.

Samenvatting

Kanker is vaak het resultaat van een meerstapsproces, waarbij normale cellen de eigenschap verworven hebben te overleven, ongebreideld te delen en naburig weefsel te invaderen, wat kan leiden tot metastase. In deze studie hebben we onderzocht welke rol groeifactor- systemen en cel-celadhesiemoleculen kunnen spelen in melanoom en borstkanker.

Een cruciale factor in het proces van carcinogenese is de wederzijdse communicatie tussen de tumor en de micro-omgeving die tot stand kan worden gebracht door oplosbare factoren. Op basis van de identificatie van twee glycoproteïnes die vrijgesteld werden door melanoomcellen, karakteriseerden we twee nieuwe groeifactor-systemen in humaan melanoom die beide kunnen bijdragen tot maligne progressie. Het gebruik van massaspectrometrie als een biochemische benadering leidde tot de identificatie van follistatine als een factor die werd vrijgesteld door verscheidene melanoomcellijnen. Vermits we zagen dat follistatine melanocyten kon beschermen tegen de groeiremmende en pro-apoptotische effecten van activine (een transformerende groeifactor-β-achtige ligand), kan secretie ervan mogelijk bijdragen tot het overleven van getransformeerde melanocytische cellen. Uitgaand van een biologische benadering vonden we dat een door melanoom geconditioneerd medium activering van een 185 kD proteïne kon induceren in een testcellijn. Deze activiteit kon toegeschreven worden aan de aanwezigheid van hereguline, een epidermale groeifactor-achtige ligand voor receptor tyrosine kinases. De daaropvolgende evaluatie van het hereguline-humaan epidermale groeifactor receptorsysteem in een set van melanoomcellijnen toonde de aanwezigheid van meerdere ontregelingen aan, gaande van autocriene activering tot ligandongevoeligheid te wijten aan de afwezigheid van (functionele) receptoren.

Wanneer hereguline getest werd in een *in vitro* model van invasie en aggregatie, gebruik makend van een borstkankercellijn met een functioneel deficiënt cel-celadhesiecomplex, vonden we dat invasie werd tegengegaan, terwijl aggregatie bevorderd werd. Cruciaal voor cel-celcontacten tussen epitheliale cellen zijn cadherines, calcium-afhankelijke cel-celadhesie-moleculen die betrokken zijn in de regeling van de beweeglijkheid, groei, differentiatie en overleving van cellen. E(epitheliaal)-cadherine, het prototype van deze familie, bezit een goed beschreven invasie-onderdrukkende functie. Functionele inactivering van E-cadherine kan ontstaan op zowel genomisch, transcriptioneel, als post-transcriptioneel niveau. Een voorbeeld van dit laatste is de proteolytische klieving van E-cadherine, met een vrijstelling van zijn ectodomein als resultaat, waarbij we vaststelden dat dit ectodomein invasie bevorderde en aggregatie tegenwerkte.

In tegenstelling tot E-cadherine, dat reeds intensief werd bestudeerd in kanker, is er slechts weinig gekend over de rol van het sterk verwante P-cadherine in kanker. Wij stelden vast dat P-cadherine, afhankelijk van de context, zowel als een invasie-onderdrukker als een invasie-promotor kan functioneren. Melanoomcellen verliezen P-cadherine tijdens progressie. Het herstel van deze expressie resulteerde in een verbeterde cel-celadhesie en een opheffing van hun invasief vermogen. In borstkanker daarentegen, correleert de opregulatie van P-cadherine met oestrogeenreceptor negativiteit en zwakke overleving. We legden een rechtstreekse link tussen P-cadherine expressie en het gebrek aan oestrogeenreceptorsignalering en vonden dat P-cadherine, via zijn intracellulair membraan-proximaal domein, invasie bevorderde in borstkankercellen.

Résumé

Le cancer est souvent le résultat d'un processus multi-étapes dans lequel les cellules normales ont acquis la capacité de survivre, de proliférer de façon illimitée et d'envahir le tissu environnant, lequel peut conduire aux métastases. Dans cette étude, nous avons adressé le rôle que les systèmes de facteur de croissance et des molécules d'adhérence cellule-cellule peuvent jouer dans le mélanome et le cancer du sein.

Le cross-talk mutuel entre la tumeur et l'environnement est crucial dans le processus de cancérologie, lequel peut être exercé par des facteurs solubles. Basé sur l'identification de 2 glycoprotéines qui sont sécrétées par les cellules du mélanome, nous avons caractérisé deux nouveaux systèmes de facteurs de croissance dans les mélanomes humains, lesquels peuvent contribuer à la progression maligne. L'utilisation de la spectrométrie de masse comme une approche biochimique a conduit à l'identification de la follistatine comme un facteur sécrété par de nombreuses lignées de cellules de mélanome. Depuis, nous avons trouvé que la follistatine pourrait protéger les mélanocytes contre les effets inhibiteurs de croissance et pro-apoptique de l'activine, un ligand de la famille TGF-β ('transforming growth factor β'). Ainsi, la sécrétion de follistatine peut contribuer à la survie de cellules mélanocytes transformés. En utilisant une approche biologique comme point de départ, nous avons trouvé que un milieu conditionné de mélanome pouvait induire l'activation d'une protéine de 185 kDa dans une lignée de cellules expérimentale. Cette activité pourrait être attribué à la présence de l'héréguline, un ligand de la famille de l'EGF ('epidermal growth factor') pour les récepteurs à activité tyrosine kinase. Une évaluation ultérieure du système héréguline-EGFR dans un panel de lignées cellulaires de mélanome a révélé la présence de dérégulations multiples, allant de l'activation autocrine à l'insensibilité du ligand autorisée par l'absence de récepteurs fonctionnels.

Quand l'héréguline était mis dans un modèle d'invasion et d'agrégation, utilisant des cellules de cancer du sein qui renferment un complexe E-cadhérine fonctionnellement inactif, l'agrégation améliorait et l'invasion était contre carré. Crucial pour les contacts entre des cellules épithéliales sont les cadhérines, des molécules d'adhésion cellule-cellule calcium dépendantes qui sont impliquées dans la régulation de la motilité cellulaire, la croissance, la différenciation et la survie. La E(épithéliale)-cadhérine, membre prototype de cette famille, possède une fonction suppresseur de tumeur très bien décrite. L'inactivation fonctionnelle de la E-cadhérine peut se produire à la fois au niveau génomique, transcriptionnel, ainsi qu'au niveau post-transcriptionnel. Un exemple de cette dernière est le clivage protéolique de la E-cadhérine, conduisant à la libération de son ectodomaine, lequel nous avons trouvé d'être capable de promouvoir l'invasion et de contre carrer l'agrégation.

Au contraire de la E-cadhérine, qui a été largement étudiée dans le cancer, peu de choses sont connues à propos du rôle de la P-cadhérine dans cette pathologie. Nous avons trouvé que la P-cadhérine peut, en fonction du contexte, fonctionner comme un suppresseur d'invasion ou comme un promoteur d'invasion. Les cellules de mélanome ont perdu l'expression de la P-cadhérine durant leur progression tumorale. La restauration de cette expression a conduit à améliorer l'adhérence cellule-cellule et à contrecarrer l'invasion. Dans le cancer de sein, au contraire, l'augmentation de l'expression de la P-cadhérine corrobore avec la négativité du récepteur de l'oestrogène et une pauvre survie. Nous avons établi un lien direct entre l'expression de la P-cadhérine et le manque de la signalisation du récepteur de l'oestrogène, et nous avons trouvé que la P-cadhérine, via son domaine intracellulaire membrane-proximale, promouvoit l'invasion des cellules de cancer du sein.

General Introduction and Objectives

In this thesis we have combined the study of cell-cell adhesion molecules and growth factors in two different types of cancer, melanoma and breast cancer. Although, at first sight, the different themes that are presented may seem unrelated, they all result from a same starting point. One interesting observation that had been done long before I entered the laboratory was the phenotypical difference between two sub-lines of the MCF-7 mammary cancer cell line. This estrogen receptor positive cell line was isolated in 1970 from a pleural effusion of a mammary cancer patient by Dr. Soule, working at the Michigan Cancer Foundation (Soule *et al.*, 1973). This cell line was distributed over the entire world and was used as a model for studying breast cancer by many thousands of researchers. However, different culture conditions and/or spontaneous alterations led to the generation of different variants of this cell line. Two such variants are the MCF-7/AZ ('Academisch Ziekenhuis') and MCF-7/6 cell line, obtained from Dr P. Briand from Copenhagen en Dr H. Rochefort from Paris, respectively. Although these cell lines share the same genetic background and have many common characteristics, they diverge in several biochemical and functional aspects. When tested in some *in vitro* aggregation and invasion assays, the MCF-7/6 cell line poorly aggregates and invades, in contrast to the non-invasive behavior and strong cell-cell adhesion of the MCF-7/AZ cell line in these assays (De Bruyne *et al.*, 1988). Since exogenous factors may direct the behavior of one cell line to that of the other, this couple represents a useful *in vitro* system for studying compounds/conditions that influence cell-cell adhesion and invasion and the mechanism underlying these changes (Bracke *et al.*, 1996).

Among the many conditions that promoted cell-cell adhesion of MCF-7/6 cells and negatively influenced their invasive behavior in a chick heart invasion assay, was a medium that had been conditioned by a melanoma cell line (Bowes melanoma). However, the nature of the factor(s) that was (were) responsible for these effects remained unknown. **Therefore, our study had two aims**. The first one was to characterize the factors that are released by the melanoma cells and to analyze their potential effects on these cells themselves. Using biological (induction of phosphorylation) and biochemical (mass spectrometry) approaches, this led to the identification and characterization of two new growth factor systems in human melanoma. The second aim was to gain knowledge about conditions that may alter the aggregation and invasion status of cells, with special focus on the role of cell-cell adhesion molecules.

Critical for cell-cell adhesion of MCF-7 cells is the functionality of the cadherin cell-cell adhesion molecules. In these cells, the transmembrane E(epithelial)-cadherin molecules form homophilic complexes between molecules on adjacent cells. Cancers have developed many ways to overcome the pro-aggregating and anti-invasive effects of these molecules. In this part of our work, new aspects of the role of these molecules were studied. Proteolytical cleavage of E-cadherin may result in the release of the extracellular domain, which, as such, turned out to be capable of promoting invasion and counteracting aggregation of cells *in vitro*. The second mechanism studied was based upon the observation that aberrant expression of another cadherin, P(placental)-cadherin, in breast cancer correlates with estrogen receptor negativity of the tumor and is a bad prognostic factor. Using MCF-7/AZ as a model system, we found that abrogation of estrogen receptor signaling results in decreased aggregation, increased invasion and increased P-cadherin expression. Ectopic expression of P-cadherin in these cells can mimic the effect of the anti-estrogen on invasion, providing evidence that aberrant expression of P-cadherin may provide cells with a pro-invasive signal, in the presence of their endogenous cadherin(s). However, P-cadherin expression is not always aberrant: in some cell types, like melanocytes, it is normally expressed but is lost during malignant progression. Ectopic expression of P-cadherin in melanoma cell lines confirmed a role for this protein in maintaining cell-cell contacts of melanoma cell lines, inhibiting invasion of these cells both *in vitro* and *in vivo*.

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Part I. Identification of new growth factor systems in human melanoma

I.1. Introduction

I.1.1. Melanoma: general aspects

Human skin is a multi-layered, cohesive tissue with a unique functional architecture, providing the primary barrier to the outside environment. The pigment-producing cells of the skin are called melanocytes and their activity is the major determinant of the color of the hair and skin. Melanocytes originate in the neural crest and migrate to the basal layer of the epidermis and the hair matrices during embryogenesis (Dupin and Le Douarin, 2003). These neural crest-derived cells also populate the inner ear (cochlea, stria vascularis), uveal tract (choroid, retina, sclera), central nervous system (leptomeninges, substantia nigra, locus ceruleus, dorsal root ganglia, cranial ganglia) and mesentery. A failure of melanocytes to migrate to these locations explains several clinical features, such as white spotting of the skin (piebaldism), heterochromia of the iris and congenital deafness in Waardenburg syndrome. Within the epidermis, melanocytes reside in the basal layer, undergoing controlled self-replication, resulting in a stable, life-long ratio with basal keratinocytes of approximately 1 melanocyte:5-8 keratinocytes. Within its 'epidermal melanin unit', each melanocyte supplies via its dendrites pigment (melanin) to approximately 30 nearby keratinocytes, thus providing protection against the deleterious effects of ultraviolet light.

Among the most common cancers are malignant neoplasms of the skin (Geller and Annas, 2003; Hall *et al.*, 2003). Whereas melanoma ranks as the third most common form of skin cancer, after basal and squamous cell carcinoma, it is far more notorious since it affects a relatively younger population and causes far more deaths because of its propensity to metastasize and its poor response to therapeutic treatment. Melanoma, a malignant tumor, derived from melanocytes is one of the fastest rising malignancies. In 2002, approximately 53.000 individuals developed melanoma in the U.S. and more than 7000 died of the disease. For the moment, more than 1 of 70 Americans is expected to develop melanoma over his/her lifetime (Geller and Annas, 2003). Despite worldwide efforts in prevention, diagnosis and treatment, melanoma incidence continues to rise at an alarming rate. Fortunately, the increasing incidence exceeds the mortality rate, apparently because of faster detection of biologically early primary melanomas which are curable through surgery. However, despite significant improvements in diagnosis and surgical, local and systemic therapy, reducing the mortality from melanoma metastases remains a major challenge.

Although most melanomas arise in the skin, they may also arise from mucosal surfaces or at other sites to which neural crest cells migrate. Melanoma occurs predominantly in adults, and more than half of the cases arise in apparently normal areas of the skin. As main risk factors for melanoma are considered: the total number of nevi, number of dysplastic nevi, color of hair, skin, and eyes, and sun exposure during childhood. Clinical staging and prognosis are affected by clinical and histological factors and by anatomic location of the lesion. Among factors that affect the prognosis are: thickness and/or level of invasion of the melanoma, mitotic index, presence of tumor infiltrating lymphocytes, number of regional lymph nodes involved, and ulceration or bleeding at the primary site (Slominski *et al.*, 1995; Tucker and Goldstein, 2003; Beddingfield III, 2003).

I.1.2. Molecular events in melanoma

During melanoma development and progression, profound histological alterations take place: aberrant cell growth and migration lead to cluster formation, dermal invasion and, eventually, distant metastasis (Clark, 1991). This is typically a multi-step process, in which melanoma develops and progresses in a sequence of events (Meier *et al.*, 1998). However, as in any neoplastic system, individual melanomas can skip steps in their development or alternatively, may arise from malignant transformation of precursor cells.

The progression from a melanocyte to a **common acquired nevus** is very common and does not appear to be dictated by major genetic changes. This is supported by the fact that nevus cells isolated from common acquired nevi 1) have a finite lifespan and 2) generally do not carry cytogenetic abnormalities. However, this view has recently been challenged by the high frequency of mutations in an oncogene, *BRAF*, in nevi (see below) (Pollock *et al.*, 2003). At that time, cells must receive a (second) genetic "hit" in order to progress, and then cytological and architectural atypia may follow. The nature of these changes is not fully clear, yet. Two models are currently proposed to explain the genesis of **melanocytic dysplastic nevi** (MDN) (Hussein and Wood, 2002). The first one considers that MDN arise by inactivation of one allele of melanoma suppressor genes, while subsequent loss of the second allele leads to malignant transformation of the MDN. The second model relies on the presence of at least two genes working independently. Therefore, alterations of one of these may cause dysplasia in melanocytes, while the other results in malignant transformation. Both models rely on the "two-hit" hypothesis, suggesting that at least two genetic events are required for inactivation of tumor suppressor genes. In familial forms of cancer, one mutation is believed to be germline and the other somatic, whereas in sporadic cancers, both mutations are somatic (Knudson, 1971). Whatever the mechanisms that may be involved, from here on the cells do not regress anymore, but persist. This persistence can last for years before they begin to proliferate to form **radial growth phase (RGP) melanoma**.

Progression from dysplasia to *in situ* and invasive RGP melanoma is gradual and spontaneous, and may not require additional molecular changes. The transition from RGP to **vertical growth phase (VGP) melanoma** is a biologically and clinically critical step, likely accompanied by additional genetic changes. At this step, cells begin to proliferate more rapidly and stroma induction and angiogenesis occurs. Unlike RGP melanomas, VGP cells are metastasis-competent and are easily adapted to growth in culture. They are less dependent on exogenous growth factors and have characteristics that are similar to metastatic cells, such as anchorage-independent growth and tumorigenesis in immunodeficient mice. VGP primary melanomas display numerous cytogenetic abnormalities, suggesting considerable genomic instability. They can rapidly adapt to selective pressure, allowing them to survive even under the most unfavorable circumstances. No major additional genetic changes may be required for further progression to **metastatic dissemination**, since most VGP melanomas can be readily adapted to a metastatic phenotype through selection in growth factor-free medium or induction of invasion through artificial basement membranes. This final step, metastasis, is dictated by endogenous oncogenes and tumor suppressor genes, and by exogenous interactions with the host, through micro-environmental factors, such as cell-matrix and cell-cell signaling (Meier *et al.*, 1998; Bogenrieder and Herlyn, 2003).

Growth autonomy appears as the result of multiple mechanisms by which growth regulatory pathways are perturbed and deregulated. It can occur at various steps of signal transduction: **1**) excessive production of autocrine growth factors, which substitutes for exogenous growth factor requirements; **2**) resistance to physiologically inhibitory exogenous growth factors by excessive production of ligand traps; **3**) mutation or altered expression of stimulatory and/or inhibitory receptors; **4**) mutation or altered expression of second messengers. Table I.1 summarizes the current information on genes that have been found to be mutated in melanoma. The possible contribution of these mutant proteins to melanomagenesis is depicted schematically in Figure I.1.

A family of genes that is frequently found to harbor a mutation in human tumors is that of the ***ras*** genes. This family consists of three functional genes, H(Harvey)-ras, K(Kirsten)-ras and N-ras, which encode highly similar 21 kD proteins with GTPase function (Bos *et al.*, 1989). Whereas mutation of *K-* or *H-ras* is rare in melanoma, mutations of *N-ras* are found in 5-30% of cases (Albino *et al.*, 1984; Raybaud *et al.*, 1988; Ball *et al.*, 1994; Demunter *et al.*, 2001a,b; Omholt *et al.*, 2003; Alsina *et al.*, 2003). These are mostly missense mutations, affecting codons 61 (most frequently Q61R and Q61K), 12 or 13. The resulting protein has a defective GTPase activity and can no longer be switched off by GTPase activating proteins (GAPs), rendering it constitutively active.

A major breakthrough of The Cancer Genome Project, an initiative launched in 1999 to identify new cancer-causing genes, was the identification of ***BRAF*** (v-raf murine sarcoma viral oncogene homolog B1) as a new oncogene (Davies *et al.*, 2002). As is the case for *N-ras*, mutations in *BRAF* lead to a constitutively

active protein, causing aberrant activation of the Ras-Raf-MEK-ERK or MAPK pathway (Mercer and Pritchard, 2003) (Figure I.1). *BRAF* is mutated in a variety of cancer types, including gliomas, cholangiocarcinomas, ovarian, lung and colorectal cancers, although for some of these mutation is a rare event. Cancers where it is mutated at high rate (up to 70%) include thyroid cancer and cutaneous melanoma (Davies *et al.*, 2002; Brose *et al.*, 2002; Pollock *et al.*, 2003; Dong *et al.*, 2003; Kumar *et al.*, 2003; Gorden *et al.*, 2003; Omholt *et al.*, 2003; Alsina *et al.*, 2003; Tsao *et al.*, 2004). In melanoma, the vast majority of mutations comprises a transversion at codon 599, exchanging valine for glutamic acid. This mutation most likely interferes with negative regulatory phosphorylation at sites Thr598 and Ser601, resulting in a higher kinase activity (Davies *et al.*, 2002; Mercer and Pritchard, 2003). *BRAF* mutation in melanoma is an acquired event, occurring early during melanocytic dysplasia, as it is already frequently found in nevi and early during melanoma pathogenesis (Pollock *et al.*, 2003; Omholt *et al.*, 2003). Strikingly, *BRA*F and *NRAS* mutations almost never co-exist in the same lesion, supporting the hypothesis that these mutations are complementary and may have similar effects during tumor

Mutated Gene	Remark	Selected references
B-Raf	Gene most commonly mutated (70-90%) in melanoma, from early stages on See text for details	Davies *et al.*, 2002 Brose *et al.*, 2002 Alsina *et al.*, 2003 Dong *et al.*, 2003 Gorden *et al.*, 2003 Kumar *et al.*, 2003 Tsao *et al.*, 2004
CDKN2A *(p16INK4A)* *(p14ARF)*	Germline mutations are most common cause of inherited susceptibility to melanoma (mutations on average in approximately 25% of melanoma-prone families). The frequency of mutations in probands increases with: • the number of affected relatives • the presence of multiple melanomas in the same patient • a history of pancreatic cancer cases in the family Mutations are much less frequent in sporadic melanoma.	Kamb *et al.*, 1994 Hussussian *et al.*, 1994 Piepkorn *et al.*, 2000 Bishop *et al.*, 2002 Soufir *et al.*, 2004
N-Ras	Relatively common (5-24%) in malignant melanoma, only occasionally found in dysplastic nevi	Albino *et al.*, 1984 Raybaud *et al.*, 1988 Ball *et al.*, 1994 Demunter *et al.*, 2001a,b Omholt *et al.*, 2003 Alsina *et al.*, 2003
p53	Relatively few mutations (1-5%, higher in cell lines), when compared to many other cancers Typically found at dipyrimidine sites (C→T and CC→TT transitions)	Levine *et al.*, 1991 Hollstein *et al.*, 1991 Oren *et al.*, 1992 Hussein *et al.*, 2003
β-catenin	Relatively few mutations	See chapter II
PTEN	Phosphatase that downregulates the activity of PKB/AKT. PKB/AKT becomes activated by PIP3 and participates in e.g.: *activation of p70S6K (stimulation of protein synthesis) *suppression of apoptosis by phosphorylating (inactivating) Bad and caspase-9 *CREB (cAMP response element binding protein) activation (→targeting gene expression)	Guldberg *et al.*, 1997b Robertson *et al.*, 1998 Tsao *et al.*, 1998
PKCα	Mutation of protein kinase Cα has only been shown in a cell line	Linnenbach *et al.*, 1988
c-myb	Mutation of this transcription factor has only been shown in a cell line Overexpression rather than mutation may be of importance in melanoma	Linnenbach *et al.*, 1988
CDK-4	Mutations in cyclin dependent kinase-4 are a rare cause of inherited susceptibility to melanoma. Only four germline alterations have been described to date, in four melanoma-prone kindreds and in one melanoma patient with no known family history.	Zuo *et al.*, 1996 Bartkova *et al.*, 1996 Flores *et al.*, 1997 Guldberg *et al.*, 1997a Soufir *et al.*, 1998
NF-1	Mutations in neurofibromin (NF-1 gene product), a protein with GTPase-activating activity for Ras molecules, are found in patients with type 1 neurofibromatosis. Disruption of the *NF1* gene is rare in melanoma in general, but is frequently found in subtypes such as desmoplastic neurotropic melanoma	Andersen *et al.*, 1993 Johnson *et al.*, 1993 Gomez *et al.*, 1996 Gutzmer *et al.*, 2000
APC	Mutations in adenomatous polyposis coli (APC) have only been found in cell lines (2/27)	Rubinfeld *et al.*, 1997

Table I.1. Genes that have been found to be mutated in melanoma. In grey are genes that, upon mutation, become oncogenes, in white are tumor suppressor genes.

development (Davies *et al.*, 2002; Omholt *et al.*, 2003). Taken together, a single genomic event, leading to mutational activation of the MAPK pathway, takes place in up to 90% of melanoma cases, rendering it a likely early key step in the initiation of melanocytic dysplasia (Cohen *et al.*, 2002; Omholt *et al.*, 2003). However, since the frequency of mutation is similar throughout all stages, including metastases, these mutations seem insufficient to trigger melanoma progression, but may be required for melanoma maintenance. Consistent with this hypothesis is the observations that *BRAF* and *NRAS* mutations don't show a significant correlation to any clinical parameter (Omholt *et al.*, 2003). An additional genetic hit may be mutation of ***PTEN*** (phosphatase and tensin homolog deleted in chromosome ten), as suggested by Tsao *et al.* (2004). The PTEN protein functions as a phosphatase for both lipids and proteins. Best documented in its involvement in tumorigenesis is its lipid phosphatase activity, which decreases the levels of phosphatidylinositol-3-phosphate and downstream protein kinase B (PKB/Akt) activity. As a result, proliferation is inhibited and apoptosis is promoted. As a protein phosphatase, PTEN is involved in inhibition of focal adhesion formation, cell spreading and migration. Inactivating mutations of PTEN, mostly a late event in melanoma, abolish these effects (Wu *et al.*, 2003). Upon testing a large panel of melanoma cell lines and a small cohort of patient samples for the co-existence of *NRAS*, *BRAF* and/or *PTEN* mutations, Tsao *et al.* found that nearly all cases with a *PTEN* mutation had a BRAF mutation as well. This suggests the existence of a possible cooperation between BRAF activation and loss of PTEN in melanoma development. However, extending this observation to large patient groups is necessary. In addition, constitutive MAPK activation could render immortalized melanocytes tumorigenic, with increased production of downstream target genes as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) (Govindarajan *et al.*, 2003), both known to be involved in malignant progression.

In conclusion, based upon the high rate of MAPK activation in melanoma (either by mutated *NRAS* or *BRAF*, or by autocrine growth factor loops, see further), this pathway seems THE pathway to be targeted in melanoma (Satyamoorthy *et al.*, 2003;

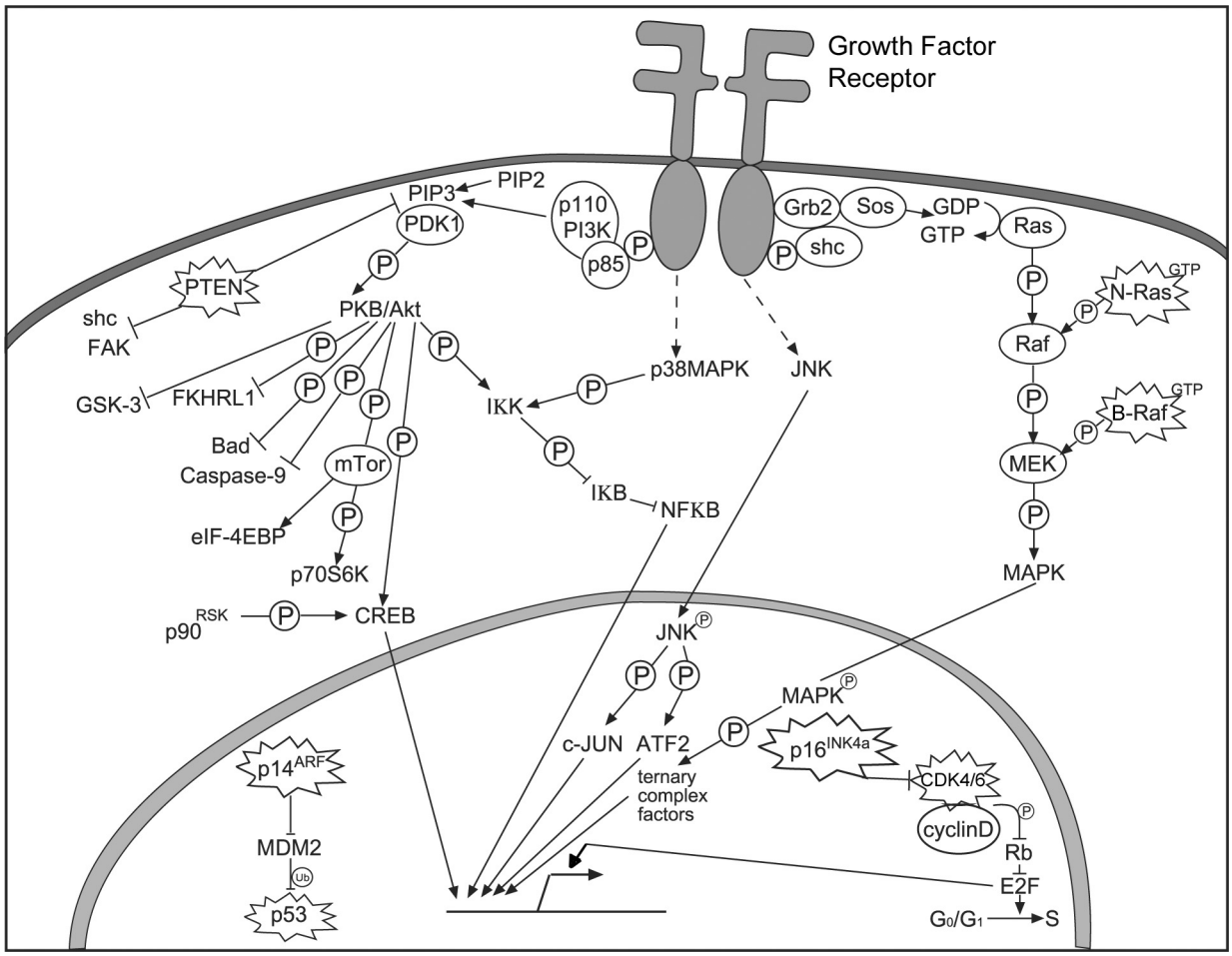


Figure I.1. Involvement of oncogenes and tumor suppressor genes in signal transduction pathways. Genes that have been found to be mutated in human melanoma are represented in star-shaped forms. See text for details.

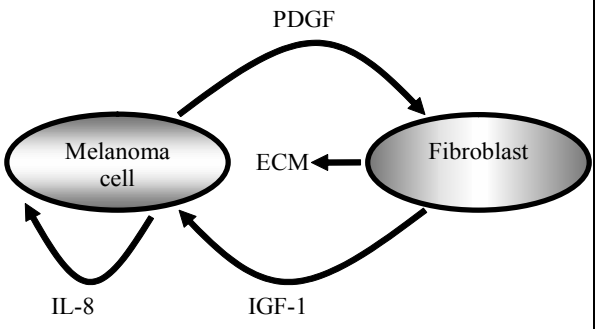
Tuveson *et al.*, 2003). As a consequence, the development of potent and selective kinase inhibitors may represent a boost in melanoma therapy.

Familial melanomas comprise from 8% to 12% of all cutaneous malignant melanoma cases (Greene, 1999; Hayward, 2003). Two highly penetrant melanoma-predisposing genes have been identified to date, ***INK4a-ARF*** (Inhibitor of Cyclin dependent kinase (CDK) 4 – Alternative reading frame) and ***Cdk4*** (Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Zuo *et al.*, 1996). The INK4a-ARF gene on chromosome 9p21 encodes two structurally distinct tumor-suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, by virtue of different 5' exons (1α and 1β, respectively), spliced in different reading frames to common exons 2 and 3. $p16^{INK4a}$ is part of the G1-S cell cycle checkpoint mechanism that involves the retinoblastoma-susceptibility tumor suppressor protein (pRb) (Figure I.1). pRB, in its unphosphorylated state, inhibits G1→S cell cycle progression by sequestering the transcription factor E2F1. Phosphorylation of pRB by the cyclin-dependent kinases CDK4 and CDK6, in complex with cyclin D, releases E2F1, allowing progression through the G1-S checkpoint (Serrano *et al.*, 1995; Zhang *et al.*, 1996). Since $p16^{INK4a}$ binds to and inhibits CDK4 and CDK6 by preventing their association with cyclin D, mutations in both $p16^{INK4a}$ and CDK4 that abolish this inhibitory binding will result in constitutively hyper-phosphorylated pRB, allowing the cell to escape from cell cycle arrest in G1.
The other product of the *INK4A-ARF* locus, $p14^{ARF}$ (ARF in mice), also acts as a tumor suppressor, inducing cell cycle arrest or apoptosis. This protein mediates G1 and G2 arrest, at least partly by its interaction with MDM2, a protein that binds both p53 and pRB. MDM2 targets p53 for degradation by ubiquitination and also inhibits pRB growth-regulatory function (Sharpless and Chin, 2003). ARF binds to MDM2 and promotes MDM2 degradation. As a result, p53 is not degraded anymore and accumulates, leading to stimulation of its downstream target genes, such as p21, an inhibitor not only of CDK4 and CDK6, but also of other CDK's. Thus, inactivating mutations of $p14^{ARF}$ result in reduced levels of p53 and p21, again relieving a brake on cell cycle progression (Zhang *et al.*, 1998; Pomerantz *et al.*, 1998).
Although for these genes it is not clear at which point the second allele is lost, their frequent mutation in familial melanoma suggests that their loss is directly involved in melanoma progression (Kamb *et al.*, 1994).

Melanoma cells express a variety of growth factors and cytokines and their receptors (see Figure I.2 and references therein). Depending on the stage of tumor progression, melanocytic cells have different growth factor requirements. Unlike normal melanocytes, melanoma cells often constitutively express a variety of growth factors and cytokines and their respective receptors, enabling the cells to progress to a more aggressive phenotype. **Autocrine growth factors** (e.g. basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), hepatocyte growth factor (HGF), platelet derived growth factor A (PDGF-A)) act on the producing melanoma cells themselves, stimulating proliferation and/or migration. **Paracrine growth factors** produced by the melanoma cells (e.g. PDGF, bFGF, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF)) modulate the micro-environment, especially stromal fibroblasts, and are involved in stroma induction and angiogenesis and/or interaction with the host defense mechanism (i.e. specific T-cells and non-specific inflammatory cells). Melanocytes and melanoma cells are also subject to the paracrine action of stimulatory (e.g. insulin-like growth factor 1 (IGF-1), PDGF, HGF) and inhibitory (e.g. IL-1 and -6, TGF-β, interferons, tumor necrosis factor-α) factors, released by keratinocytes, endothelial cells,

An example of the complex cross-talk between melanoma cells and the micro-environment

Melanoma cells secrete PDGF, which may stimulate growth in an autocrine way or may activate fibroblasts in a paracrine way. Besides its mitogenic activity on fibroblasts, PDGF stimulates the production of ECM components and induces the production of growth factors, such as HGF, bFGF, endothelin-3 (ET-3) and insulin-like growth factor 1 (IGF-1) by the activated fibroblasts. IGF-1, in turn, may act on the IGF-1 receptor, which is expressed by all melanocytic cells, promoting their survival, growth and migration, either directly, or indirectly through the upregulation of other growth factors (such as VEGF and CXCL8/IL-8). CXCL8, besides having an autocrine function (growth stimulatory, inducing haptotactic migration, inducing MMP production and the selection of metastatic tumor clones), may also act on nearby cells, thereby extending the cascade of events taking place (Satyamoorthy *et al.*, 2001, 2002).



fibroblasts, lymphocytes, monocytes and granulocytes. These exogenous paracrine factors may either inhibit tumor growth and cause differentiation or enhance tumor growth, angiogenesis, adhesion or motility or promote dissemination. As progression occurs, disturbance of the balance between inhibitory and stimulatory factors increases. In some cases, a growth factor can switch from a paracrine or autocrine inhibitor to an autocrine stimulator (e.g., interleukin-6, TGF-β). Of particular importance in melanoma are the growth factors influencing the stromal fibroblasts. These melanoma-derived paracrine growth factors act as mitogens for the fibroblasts and/or stimulate the deposition and secretion of extracellular matrix (ECM) and growth factors by the latter. In turn, the deposited ECM provides a scaffold for the melanoma cells, enhancing their survival, while fibroblast-derived growth factors contribute to the growth and motility of melanoma cells. Figure I.2 presents the main autocrine and paracrine growth factors and cytokines involved in the growth regulation of melanoma cells. Recently, the group from Dr. Herlyn found that, although many growth factors promote growth and/or survival of melanocytes, overexpression of a single growth factor, combined with ultraviolet B (UVB) irradiation or with other growth factors could not induce melanoma in xenograft studies. However, when overexpression of several growth factors was combined with intermittent UVB exposure (presumably resulting in acquired mutations), a melanoma phenotype was found in these models (Berking *et al.*, 2004). Although these lesions regressed spontaneously upon withdrawal of the growth factors (likely because they did not acquire enough stable mutations), they highlight the causative role that growth factors may play in melanomagenesis.

The two articles in this chapter describe our identification of two new growth factor systems that may play a role in human melanoma. These are preceded by a review on the role of neuregulins, ligands for receptor tyrosine kinases, in cancer.

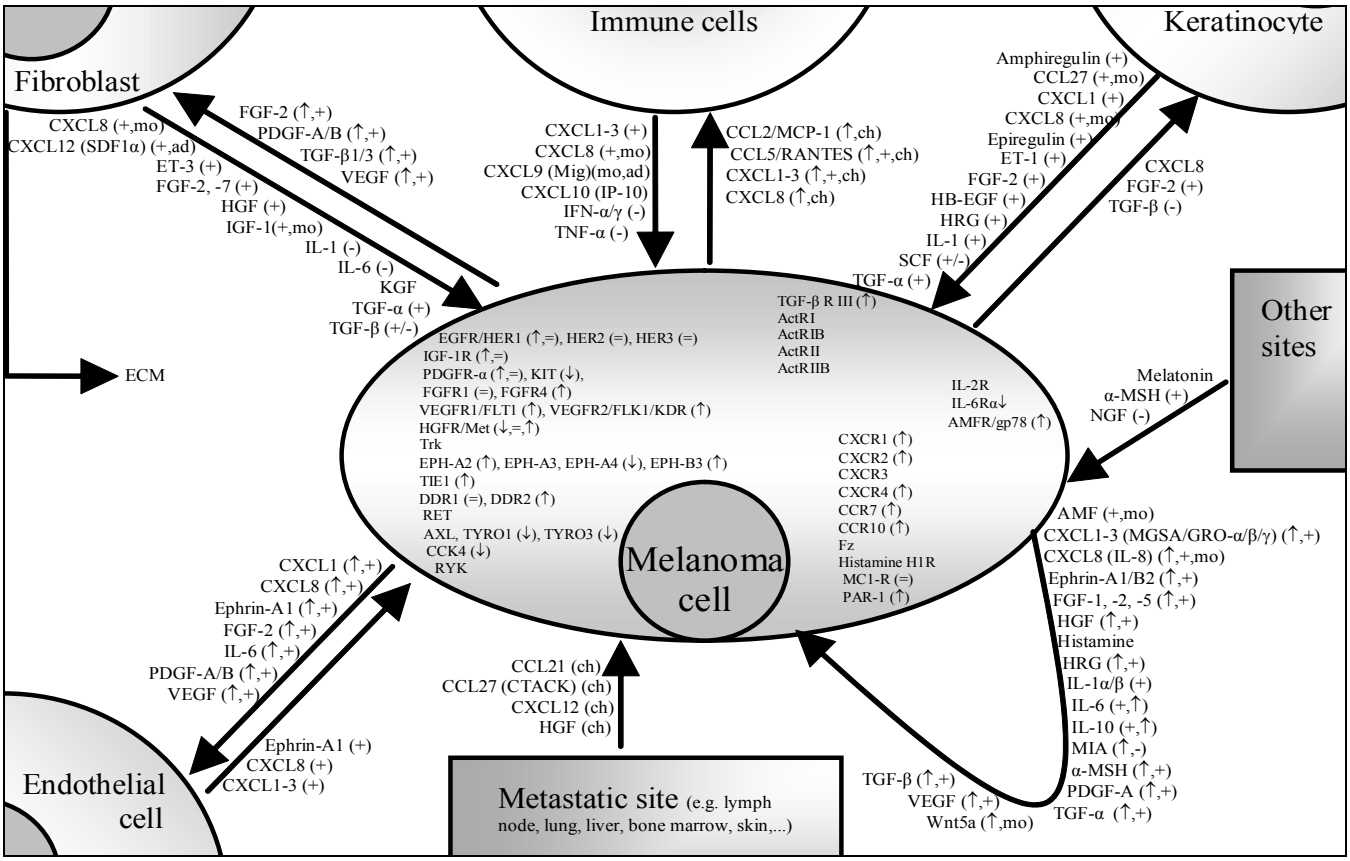


Figure I.2. Regulation of melanoma cells by autocrine and paracrine factors. Figure was based upon Plaisance *et al.*, 1993; Werner *et al.*, 1994; Rodeck *et al.*, 1994; Easty *et al.*, 1997; Rodeck *et al.*, 1999; Halaban, 2000; Easty and Bennett, 2000; Lázár-Molnár *et al.*,,2000; Biitner *et al.*, 2000; Schelfhout *et al.*, 2000; Shirakata *et al.*, 2000; Strachan *et al.*, 2000; Meier *et al.*, 2000; Müller *et al.*, 2001; Robledo *et al.*, 2001; Satyamoorthy *et al.*, 2001, 2002; Payne and Cornelius, 2002; Ruiter *et al.*, 2002; Schelfhout *et al.*, 2002; Murakami *et al.*, 2002, Bogenrieder and Herlyn, 2002; Hsu *et al.*, 2002; 2003; Meier *et al.*, 2003; Gruss *et al.*, 2003; Li *et al.*, 2003; Tellez and Bar-Eli, 2003; Streit and Detmar, 2003 and references in these articles. The indications between brackets indicate the following: ↑, increased expression/secretion in melanoma (compared to normal melanocytes); +/-, stimulation/inhibition of growth and/or survival; mo, stimulation of motility (migration, metastasis); ch, chemotactic factor; ad: stimulation of adhesion.

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I.2. Roles for neuregulins in human cancer

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Roles for neuregulins in human cancer

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ABSTRACT

The human epidermal growth factor (EGF) receptor (HER) family of receptor tyrosine kinases has frequently been implicated in cancer. Apart from overexpression or mutation of these receptors, also the aberrant autocrine or paracrine activation of HERs by EGF-like ligands may be important in cancer progression. Neuregulins constitute a family of EGF-like ligands that bind to HER3 or HER4, preferably forming heterodimers with the orphan receptor HER2. Mesenchymal neuregulin typically serves as a pro-survival and pro-differentiation signal for adjacent epithelia. Disruption of the balance between proliferation and differentiation, because of autocrine production by the epithelial cells, increased sensitivity to paracrine signals or disruption of the spatial organization, may lead to constitutive receptor activation, in the absence of receptor overexpression. Consequently, the analysis of ligand expression and/or activated receptors in tumor samples may broaden the group of patients that can benefit from targeted therapies.

The human epidermal growth factor receptor family

On the basis of their sequence homology and structural characteristics, growth factor receptors can be classified into different groups. The human epidermal growth factor (EGF)[1] receptor (HER) family of receptor tyrosine kinases consists of four members: HER1 (EGFR, ERBB1), HER2 (neu, ERBB2), HER3 (ERBB3) and HER4 (ERBB4). They are widely expressed in epithelial, mesenchymal and neuronal tissues, playing fundamental roles during development and being implicated in the regulation of a variety of biological processes, including cell proliferation, apoptosis and differentiation [1, 2]. Structurally, they consist of a ligand-binding extracellular domain, a single transmembrane domain and an intracellular tail. The latter contains the kinase domain and multiple tyrosine residues that become phosphorylated upon activation and may serve as docking sites for signal transduction molecules. Although all family members share considerable homology, two members deviate from this general description: the extracellular domain of HER2 has not been shown to be involved in ligand binding (orphan receptor) and the HER3 intracellular tail is devoid of kinase activity (kinase dead receptor) [3, 4]. This receptor family has been implicated frequently in human malignancies. This is particularly the case for HER1 and HER2. HER2 overexpression has been described in a wide variety of cancer types (e.g. breast and ovarian) and correlates with poor prognosis [5, 6]. Overexpression of this receptor leads to ligand-independent homodimerization, resulting in constitutive receptor activation, sufficient to induce transformation. Alternative mechanisms leading to ligand-independent constitutive HER2 signaling include a point mutation in the transmembrane domain (only found in rodents) and truncation of non-catalytic sequences [7].

Neuregulins

Based on distinct biological activities, neuregulins were initially purified as related factors released by a variety of cell types, and were subsequently given different names (NDF, HRG, GGF, ARIA), leading to a confusing nomenclature. Using HER2 activating potential as a read-out, Peles et. al. and Wen et. al. identified neu differentiation factor (NDF) as a factor released by H-Ras-transformed rat fibroblasts [8, 9], whereas Holmes et al. isolated heregulin (HRG) from medium conditioned by the MDA-MB-231 human mammary cancer cell line [10]. Other groups used neuronal tissue extracts as a source for protein purification: glial growth factor (GGF) was purified from bovine pituitary glands as a Schwann cell mitogen [11] and acetylcholine receptor inducing activity (ARIA) was purified from chicken brain extracts [12]. All these factors, identified in different

[1] Abbreviations used: ARIA, acetylcholine receptor-inducing activity; CDK, cyclin-dependendent kinase; COX-2, cyclooxygenase-2; EGF, epidermal growth factor; E2, estradiol; ER, estrogen receptor; FAK, focal adhesion kinase; GAP, GTPase-activating protein; GGF, glial growth factor; HER, human EGF-like receptor; HRG, heregulin; MAPK/ERK, mitogen-activated protein kinase/extracellular-regulated kinase; MMP, matrix metalloproteinase; MMTV, murine mammary tumor virus; MTA1, metastasis and tumor-associated protein 1; NDF, neu differentiation factor; NRG, neuregulin; Pak1, p21-activated kinase 1; PGE2, prostaglandin E2; PI3K, phosphoinositide-3 kinase; PKB/Akt, protein kinase B/a kinase on threonine; PR, progesterone receptor; RAFTK, Related Adhesion Focal Tyrosine Kinase; SMDF, sensory and motor neuron-derived factor; VEGF, vascular endothelial growth factor.

species, are derived from alternative splicing of a single gene, *neuregulin-1* (NRG-1) [11, 13]. Although it was initially thought that NRG-1 would directly bind to and activate HER2, the observation that certain cell lines which express a functional HER2 did not respond to NRG, indicated that another component was needed to mediate the tyrosine phosphorylation of HER2. This was confirmed by the cloning of HER3 and HER4 and their subsequent identification as the primary NRG-binding receptors [14-16].

NRG-1 isoforms are widely expressed in numerous tissues, including brain, heart, skeletal muscle, breast, liver and lung [17, 18] Based on sequence homology with NRG-1, neuregulin-2 (also termed Divergent of neuregulin-1 (Don-1) or Neural- and thymus-derived activator of ErbB kinases (NTAK)), was cloned [19-21]. Later, two additional neuregulin families (neuregulins-3 and -4) were identified [22, 23]. The latter two only induce activation of HER4, whereas NRGs-1 and -2 bind and activate both HER3 and HER4.

Structural features of neuregulin-1

At present, at least 26 different neuregulin-1 isoforms have been identified in different species. Ten of these were identified in human, all resulting from alternative splicing of a single gene, located in the short arm of chromosome 8 [24] (Figure 1). Based on the N-terminal domain, they are subdivided into three types: HRG (Type I) [10], GGF (Type II) [11] and sensory and motor neuron derived factor (SMDF, Type III) [25] [reviewed in 13]. Although the N-terminal domain of all three types is involved in cell-surface association, it does so in different ways. HRG and GGF can be distinguished from SMDF by the presence of a heparin-binding immunoglobulin-like sequence in their N-terminal domain. This feature was shown to improve receptor binding by association with cell surface heparin sulphate binding proteoglycans, and may function to sequester these factors in the extracellular matrix as well [26]. Instead, the N-terminal domain of SMDF contains an additional hydrophobic domain that may function as a transmembrane sequence. Insertion of this sequence into the membrane results in the formation of a type II transmembrane protein, with the receptor-activating domain exposed [27-29]. The only exon that is shared by all isoforms is the one encoding the major part of the EGF-like domain. It encodes five of the six cysteines present in the EGF-like domain, the sixth encompassed in the sequence encoded by either the α or β exon. This EGF-like domain is both necessary and sufficient for receptor activation, with β-isoforms having higher receptor affinity and generally being more potent than α-isoforms [30].

In transmembrane isoforms, the EGF-like domain is connected to the transmembrane domain by a juxtamembrane domain of variable length (α/β 1 or 2), which is susceptible to proteolytic cleavage, releasing the ectodomain [30, 31]. Alternatively, read-through of the β-encoding exon results in isoforms lacking the transmembrane domain, which are presumably retained intracellularly. Further alternative splicing leads to different lengths of intracellular tails, a, b and c, the a- and c-tail being the longest and shortest, respectively [32]. The role of this intracellular tail is mainly unknown. The two interacting molecules that have been identified are both zinc finger proteins: LIM kinase 1 [33], a serine/threonine kinase that blocks the actin depolymerizing activity of cofilin and has been implicated in cancer cell invasion [34], and a PLZF-related protein of undetermined function [35]. However, the functional relevance of these interactions has not been established, yet. In addition, the cytoplasmic tail was shown to be necessary for the apoptosis-inducing properties of neuregulins (see below) and may play a role in 'reverse' signaling. The latter occurs following binding of the extracellular domain of the HRG type III precursor to HER2/4 dimers (reverse juxtacrine signaling) and involves the γ-secretase-mediated proteolytic release of the HRG

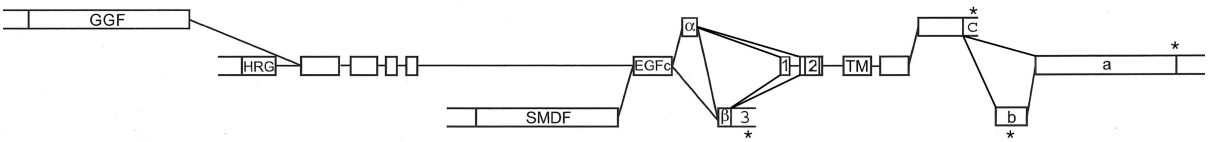

Figure 1. Schematic representation of the exon organization and possible alternative splicing of the NRG1 gene. Closed boxes indicate exons (on scale), lines between these indicate possible splicing events, open boxes represent untranslated regions. Introns, which may be very large, are not on scale. An asterisk indicates the position of stop codons. Three different start codons may be used, generating glial growth factor (GGF), heregulin (HRG) or sensory and motor neuron derived factor (SMDF). The epidermal growth factor (EGF)-like domain is composed of a common exon and an α or β exon. A combination of an α and β exon, found infrequently, leads to the generation of a premature stop codon within the β exon (not indicated). Readthrough of the β-exon leads to the generation of a stop codon, while further splicing generates α/β-1 or -2 isoforms. Following the sequence encoding the transmembrane (TM) domain, alternative splicing leads to isoforms having a-, b- or c-type intracellular tails.

intracellular domain and its subsequent nuclear translocation, where it may influence the transcription of target genes [35]. This nuclear translocation was dependent on the presence of a nuclear localization sequence in the HRG juxtamembrane domain [35] and has been observed in several types of cancer [36-38] Other roles for the transmembrane/cytoplasmic domain include the regulation of the proteolytic release of mature HRG [39-41] and the correct trafficking and subcellular targeting of neuregulins to e.g. rafts, specialized membrane platforms rich in cholesterol [32, 42, 43].

Transmembrane heregulins (with an a-tail) are synthesized as transmembrane precursor proteins of ± 105kDa. Following cleavage in the juxtamembrane domain by metalloproteinases [44, 45], they are released as ±45kDa secreted factors that may bind to nearby receptors and thus function as autocrine/paracrine factors [42, 31]. Alternatively, intact transmembrane HRG molecules may activate HERs, leading to juxtacrine signaling [46], which may be bi-directional: 'forward', leading to HER activation and 'reverse', leading to modulation of intracellular pathways by the HRG cytoplasmic tail, as described above.

Based upon its expression *in vivo*, heregulin is often referred to as a neuronal (β isoforms) or mesenchymal (α isoforms) factor [17, 18, 32]. In the latter case, it has been suggested to function as a paracrine factor for the neighboring cells, such as glia, muscle or epithelia, which express HER3 and/or HER4 [17]. The importance of such paracrine signaling is exemplified in *NRG-1* knockout mice, where targeting of the EGF-like domain results in disruption of all NRG-1 isoforms. These mice die *in utero* at embryonic day 10.5 due to a lack of trabeculation, a developmental process involved in ventricular differentiation during heart formation. This process requires the activation of ErbB2 and ErbB4 in the myocytes by HRG, which is normally released from the nearby endocardium [47].

Signaling induced by neuregulins

Based upon the recent elucidation of the crystal structures of HER1, HER2 and HER3, a unique model has been proposed for HRG-induced receptor activation. In this model, binding of HRG to HER3 leads to a ligand-driven conformational change of HER3, allowing a back-to-back association with HER2 [reviewed in 48]. On its turn, HER2 will auto-phosphorylate and trans-phosphorylate HER3, resulting in the generation of multiple docking sites for signal transduction molecules in their cytoplasmic tails [reviewed in 1]. Thus, this represents a unique system in which an orphan receptor (HER2) and a kinase-dead receptor (HER3) cooperate to generate a potent signal [reviewed in 49]. Following HER2 activation, many phosphotyrosines in its carboxy-terminal tail serve as docking sites for Src homology-2- and phosphotyrosine-binding-domain containing proteins, such as Shc, Grb2 and Grb7, generating a highway leading to mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK) activation [50]. Activated HER3, in contrast, harbors multiple docking sites for the p85 subunit of phosphoinositide-3-kinase PI3K [51]. Thus, it is not surprising that the combined activation of both HER2 and HER3 by HRG provides the cells with a very potent signal. In addition to the 'classical' MAPK and PI3K pathways, multiple other pathways have been shown to be activated by HRG. Examples include the p38MAPK, ERK5, protein kinase C, phospholipase Cγ and signal transducer and activator of transcription (STAT) pathways. These have been reviewed recently and will not be discussed into detail here [49,52]. In addition, HRG-mediated activation of HERs may result in the nuclear localization of full-length or cleaved receptors, suggesting direct, receptor-mediated signaling [53, 54]. Moreover, adding an additional level of complexity to the system, also trans-activation of other receptors may contribute to HRG's actions [52]. For example, HRG has been shown to trans-activate the progesterone receptor, in a HER2- and MAPK-dependent way, leading to enhanced proliferation of mammary cancer cells [55]. The combined action of all or of several of these pathways on transcriptional and post-transcriptional (mRNA stability, translation, post-translational modifications, and protein stability) events determines the final outcome in a given system.

In cancer, aberrant receptor activation may be the result of truncation, mutation, association with other cell-surface proteins, transactivation via other receptors or the presence of autocrine loops [7,56]. Recently, an interesting physiological control mechanism for the HRG-HER system was described in human differentiated airway epithelia, in which both HRG-α and its receptors are expressed. In these epithelia, HRG, which is apically expressed, is physically segregated from the basolaterally expressed receptors, preventing HER activation. However, upon disruption of the integrity of the tight junctions, HRG may locally activate its receptors [57]. Thus, this model suggests an additional mechanism for abnormal receptor activation in diseases, such as inflammation and cancer, in which increased epithelial permeability occurs.

Although the exact mechanism(s) by which NRG-1 may lead cells towards malignancy may be distinct for different cell types, some general aspects hold for the majority of these. Cancer cells that aberrantly produce NRG-1 are likely to use it in an autocrine manner, resulting in constitutive activation of HERs and the downstream signaling molecules. This, in turn, leads to increased proliferation, alterations in the phosphorylation state of molecules implicated in the

cytoskeleton, and modulation of neuregulin-responsive genes. All these events may drive the cell towards a more malignant phenotype.
Since far most studies related to the potential role of HRGs in regulating normal and oncogenic signals through its receptors have focused on its effects on mammary (cancer) cells, we have made a distinction between the studies focusing on the involvement of HRG in normal mammary gland physiology and breast cancer and its involvement in other cell types.

Distinct roles for heregulin in mammary epithelial cells

Heregulin as a mammary gland mitogen regulating differentiation

In vivo, in the mouse mammary gland, the only neuregulin-1 identified is heregulin-α. Its expression by the mammary mesenchyme, adjacent to lobuloalveolar structures, is suggestive for a role in the morphogenesis and ductal migration of mammary epithelial cells [58]. Expression is virtually absent in virgin glands, peaks at mid to late pregnancy and then sharply decreases after several days of lactation, becoming undetectable again during involution [58]. A crucial role for HRG in the differentiation of the mammary epithelium into secretory lobuloalveoli was confirmed by studies using mammary gland organ cultures [58] or mammary implants containing HRG [59]. This differentiation is characterized by an increase in nuclear size, large cytoplasmic vesicles, synthesis of milk protein (β-casein) and appearance of cytoplasmic fat droplets [58-60]. HRG-mediated upregulation and increased membrane targeting of Rab3A, a low molecular weight GTPase involved in vesicular trafficking, has been suggested to play a role in these processes [61]. Direct evidence for a role of HRG in lobuloalveolar development came from the generation of HRG-α knockout mice. In contrast to HRG-null mice, these mice survive to adulthood, but have transient defects in lobuloalveolar development, showing abrogated proliferation of luminal mammary epithelial cells during pregnancy and lactation. During lactation, this was accompanied by a dramatic reduction in β-casein expression [62]. Similar defects in lobuloalveolar development and lactation were observed in mice expressing dominant negative mutants of HER2 or HER4 in the mammary gland [63, 64].
In conclusion, HRG-α expression in the mammary gland may have a dual function, supporting survival and proliferation, while at the same time inducing differentiation. Deregulation of this balance between proliferation and differentiation, by alterations in expression levels of ligands or receptors, may contribute to transformation, tumor progression and metastasis. The dual action of HRG is also evident from its effects *in vitro* on cell lines: whereas sometimes proliferation is induced [10, 65-69], HRG treatment may lead cells to differentiation as well [8, 60, 66, 67, 70, 71]. Important determinants for these distinct effects likely include the system/cell line examined, methodology used, HRG isoform(s) involved, the concentration used, receptor expression profile, receptor-receptor interactions, as well as the intensity and duration of activation.

Heregulin as a growth-inhibiting, pro-apoptotic differentiation factor for mammary cancer cells

Heregulin has been shown to exert differentiating effects on breast cancer cell lines in *in vitro* cell culture systems. As was observed in mammary glands, HRG may induce a differentiated phenotype of cultured cell lines, characterized by a flattened morphology, increased nuclear size, large cytoplasmic vesicles, synthesis of milk protein (β-casein), appearance of fat droplets and increased ICAM-1 expression [66, 72, 73]. In 3-D cultures inside collagen type I of immortalized non-tumorigenic mammary epithelial cells and SKBR-3 and T47D mammary carcinoma cell lines, HRG treatment induced a more differentiated phenotype [74]. When combined with retinoids, which are known inducers of differentiation in breast cancer cell lines [75], this differentiation was even more pronounced, possibly due to the induction of retinoid acid receptor α by HRG in these cells [76]. The increase in differentiation was associated with elevated cell adhesion to cell surfaces, brought about by alterations in the level and distribution of integrins α2 and β1 [74]. Increased cell-cell adhesion, dependent on PI3K activity, has been observed in several breast cancer cells following exposure to HRG [77]. Since this increase in cell-cell adhesion was present in E-cadherin negative cell lines and could take place at 4°C, it is cadherin-independent. Thus, also here, HRG-mediated modulation of integrins may play a role. In addition, using an MCF-7 variant with a functionally deficient E-cadherin, we found that HRG could stimulate E-cadherin mediated adhesion as well. This was accompanied by decreased invasion of these cells into a precultured chick heart fragment (Stove *et al.*, manuscript submitted). On solid substratum, NRG-1 may induce a morphogenetic effect, rearranging epithelial islands into ring-shaped arrays with internal lumens [78], which may be a reflection of its function in lobuloalveolar morphogenesis in vivo [58]. Primary human mammary epithelial cells produce endogenous HRG [69], which possibly contributes to spontaneous differentiation of these cells.
HRG is involved in the development of the mammary gland, possibly through the regulation of the apoptotic process. In general, apoptosis may result from the net impact on pro- versus anti-apoptotic pathways. A critical determinant in this balance may be the

expression levels of HER2, resulting in increased proliferation in cells that express low levels and leading to growth inhibition in HER2 overexpressing cells. This is evident from the fact that HRG is able to inhibit the growth of several breast and ovarian cancer cell lines that overexpress HER2 [8, 70, 73, 76, 79-83]. However, no growth inhibition of HER2-overexpressing cell lines was seen by others [69], indicating the important influence the test conditions may have on the outcome. HRG-mediated growth inhibition of the HER2 overexpressing SKBR3 breast cancer cell line relates to its induction of apoptosis, cell cycle G2-M arrest and cell differentiation [70, 79, 82]. These effects were suggested to be mediated via a mitochondrial pathway, involving downregulation of the anti-apoptotic protein Bcl-2 and activation of caspase-7 and -9, and being potentiated by inhibition of protein kinase Cα [83]. Alternative pathways by which HRG may induce apoptosis include the p70S6K/mTOR pathway, sustained activation of the JNK or MAPK pathway and late activation of p38 MAPK (day2-3) [70, 80, 82]. Again, the pathway used may differ among different cell lines tested and even with a given cell line, may depend on the assay conditions. Although HRG-mediated induction of apoptosis and differentiation often coincide, different pathways seem to be involved, as is evident from the involvement of the PI3K pathway in HRG-mediated induction of differentiation, but not apoptosis, of SKBR-3 cells [70].

In contrast to the above mentioned mechanisms, in which HER activation leads to growth inhibition and apoptosis in selected cell lines, another mechanism for the pro-apoptotic activity of HRG has been suggested. This was based upon the identification of HRG-β2b as a dominant apoptosis-inducing gene, the transmembrane domain and intracellular tail being important for this effect [81, 84, 85]. The fact that this HRG isoform could induce apoptosis in the absence of HER-binding, illustrates the dual function HRG might have: while cleavage may lead to secretion of HRG, which may have a transforming activity on HER-expressing cells (see below), the producing cell may undergo apoptosis when intracellular levels of the HRG cytoplasmic tail become too high. This selective killing of HRG-overexpressing cells might represent an auto-regulatory physiological mechanism protecting the organism against tumorigenesis. Consequently, loss of this sensitivity to apoptosis in HRG-overexpressing cells allows the continuous secretion of high amounts of HRG and may represent a step towards tumorigenesis. Consistent with these observations, epithelial cells from MMTV/heregulin-induced tumors exhibit high levels of apoptosis, in contrast to tumors induced in transgenic mice by HRG lacking the intracellular tail [81, 85, 86] Overexpression of HRG-β2b in MCF-7 cells induced apoptosis as well, which depended on caspase activation and was accompanied by a downregulation of Bcl-2 [81]. In addition, constitutive overexpression of rHRG-β2 in MCF-7 cells has been shown to markedly increase their sensitivity to doxorubicin and etoposide treatment [87].

Finally, it should be noted that inhibition of proliferation does not necessarily coincide with a less aggressive phenotype. This is evident from the effects of HRG and agonistic anti-HER2 antibodies on SKBR-3 mammary cancer cells: although proliferation of these cells is inhibited following receptor activation, invasion of these cells is increased [73].

Heregulin as a mitogenic, pro-invasive and metastatic factor in breast cancer

As already mentioned above, the role of HRG in cancer has been particularly investigated in breast cancer. More than 60% of human breast cancers express estrogen receptor (ER) and hormone therapy is a commonly applied adjuvant therapy for breast cancer patients. Although ER expression is used to predict which patients will respond to hormone therapy, not all patients with positive ER will benefit from endocrine therapy [88]. It is being increasingly recognized that altered expression of a variety of growth factors and their receptors may activate signaling pathways that influence ER signaling [89-91]. One of such factors is HRG. Elevated expression of HRG was found in a subset of breast cancer cell lines and in 25-30% of human primary breast cancers [92, 93], a percentage similar to that of HER2 overexpression in breast cancer [5, 6]. HRG expression in mammary tumors was found both in the stroma as in the tumor cells themselves, with stromal expression correlating with increased recurrence [94] or with activated protein kinase B (PKB/Akt), a downstream intermediate of PI3K, whose activation predicts a worse outcome among breast cancer patients [95]. Interestingly, HRG is particularly present in cell lines and tumor biopsies that do not overexpress HER2, inversely correlating with expression of estrogen receptor-α [93, 95]. The fact that the HRG-overexpressing population of breast tumors is distinct from that overexpressing HER2, combined with the ability of HRG to promote tumor formation in the absence of overexpression of HER2 (see below), suggests an important role for the HRG/HER system in the etiology of breast cancer. This may be of clinical significance, since nowadays only HER2 expression levels are being evaluated in tumor samples. Therefore, assessment of both receptor and ligand levels, or alternatively of receptor activation status [96], may provide a better basis for evaluation of the role these molecules play in particular cancers. This may result in a better identification of patients that may benefit from targeted therapies.

HRG is a potent mitogen for most breast cancer cell lines, provided they express HERs. Using a panel of different assays applied on a series of breast cancer cell lines with varying HER2 levels, Lewis *et al.* [68] and Aguilar *et al.* [69] demonstrated a clear growth-stimulatory role for HRG, both *in vitro* and *in vivo*. Supporting these findings, the mitogenic action of exogenous HRG was less pronounced or absent in cells producing HRG in an autocrine way, although increasing HER2 levels in these cells increased their responsiveness. Its potency is evident from its ability to act as a dual specificity growth factor for the human mammary epithelial cell line MCF-10A, in which it drives signal transduction pathways that normally require both EGF and insulin-like growth factor-I [97]. The mitogenic action of HRG is likely to be achieved through the combined action of multiple pathways, including PI3K, MAPK and p38MAPK pathways [98, 99]. These affect proteins involved in cell cycle progression, as exemplified by the promotion of expression of c-Myc, A-, E- and D-cyclins and $p21^{cip1}$, activation of cyclin-dependent kinases (CDK) and phosphorylation of the retinoblastoma protein (pRB) [98, 100]

Multiple studies suggest that HRG induces breast cancer progression towards an aggressive phenotype, as determined by hormone independence, antiestrogen resistance (loss of ER function and response), tumorigenicity [101], invasion [102-104], and metastasis [105-107]. It does so directly by increasing cell motility or indirectly, via the regulation of genes that regulate and control malignant progression (Table 1, see below).

In breast cancer cells, HRG can block both estradiol (E2) action and ER function. HRG antagonizes the E2-mediated downregulation of HER2 and is capable of enhancing tamoxifen-induced stimulation of the receptor [101, 108, 109]. HRG supplementation of ER-positive breast cancer cell lines results in resistance to the growth inhibitory effects of antiestrogens, which is more pronounced with partial than with full antiestrogens [110]. In addition, treatment/transfection of the ER-positive, E2-dependent MCF-7 breast cancer cell line with HRG-β1/2 resulted in a loss of E2 dependence and acquisition of antiestrogen resistance both *in vitro* and *in vivo* [101, 109, 106, 111]. Inoculation of HRG-supplemented or HRG-producing MCF-7 cells in the mammary glands of ovariectomized nude mice resulted in spontaneous, E2-independent tumor formation [69, 106]. This model mimics what is seen in many breast cancer patients: ER-positive tumors acquire antiestrogen resistance during the course of the treatment, while still retaining some levels of ER. The fact that other growth factors, such as transforming growth factor α, which are also mitogenic for MCF-7 cells, cannot support this E2-independent growth, pleads for the strength of HRG as a transforming factor [112]. Depending on the conditions applied, both suppression [101, 108, 113] and activation [109, 114] of the ER signaling pathway have been described. Both scenarios, likely resulting in differences in activation of several pathways, may lead to E2-independency.

In the first scenario, low concentrations of HRG decrease ERα levels, activity and binding to its response element in a DNA mobility shift assay, as well as estradiol-induced growth of breast cancer cell lines [101, 108, 113]. A molecular mechanism, involving upregulation of metastasis-associated protein 1 (MTA1), has been proposed for the HRG-mediated suppression of E2 response elements (ERE) and disruption of E2 responsiveness [115]. In accordance with the correlation between MTA1 expression and the metastatic potential of several human cell lines and tissues [116] these authors found that MTA1 was increased in Harderian-gland tumors in MMTV-driven HRG transgenic mice and could induce invasion *in vitro* [115]. By physically interacting with the activation factor domain of ERα and with histone-deacetylase (HDAC) 1 and 2, MTA1 recruits HDAC activity to ERE's, suppressing transcription of target genes containing these ERE's. Alternatively, MTA1s, an MTA1 splice variant containing a unique RLILL motif, has been shown to prevent E2-induced nuclear translocation by sequestering ERα in the cytoplasm. Expression of this splice variant was higher in breast cancers and correlated with absence of nuclear ER [117]. Hyperactivation of MAPK, accompanied by loss of ERα without activation of ERα responsive genes, may be a cause of estrogen unresponsiveness as well [118]. Interestingly, cells expressing the MTA1 splice variant had increased levels of MAPK activation, suggesting that expression of this protein may confer unresponsiveness to E2 while stimulating other pathways at the same time [117].

For the second scenario leading to E2-independency, in which ligand-independent ER activation occurs, Stoica *et al.* [114] recently presented an interesting mechanism. Using MCF-7 cells, they showed that both the binding of HRG to HER3/HER2 ánd the presence and activity of membrane ERα (which may be physically associated with HER2 [119]) are required for PI3K/Akt activation. Akt activation, on its turn, leads to phosphorylation of ERα and a decrease of ERα mRNA and protein, coinciding with increased expression of progesterone receptor (PR) and pS2, two markers of ERα signaling [109, 114]. Interestingly, further demonstrating the intense cross-talk between HERs and ERα, they also showed that ERα-mediated PI3K/Akt activation and activation of PR and pS2 target genes was abrogated by blocking HER2. Also the MAPK pathway is thought to play an important role in mediating the effect of HRG on cell proliferation and ERα. A possible crosstalk between the ER and MAPK pathways is supported by studies

in which ERα was found to be phosphorylated by MAPK and in which inhibition of the MAPK pathway restored the sensitivity to antiestrogens [120, 121]. In conclusion, HRG may provide cells with a phenotype that depicts the passage from an initial E2-responsive and antiestrogen-sensitive tumor cell to a later step in carcinogenesis, resembling the common progress of many human breast tumors.

HRG promotes tumorigenicity and metastasis of breast cancer cells that do not overexpress any of the HERs [101, 106]. An increase in the invasive phenotype of MCF-7 cells exposed to HRG was found both *in vitro* and *in vivo*. In contrast to normal MCF-7 cells, HRG-expressing MCF-7 cells injected into the mammary fat pad of nude mice metastasize to the axillary lymph nodes and induce preneoplastic transformation of the adjacent mouse mammary epithelium [106]. Further substantiation for a role of HRG as a transforming and tumor progression factor was provided by transgenic mouse models in which HRGβ2c, either full length or lacking most of the cytoplasmic tail, was expressed under the control of the murine mammary tumor virus (MMTV) promoter. Overexpression of HRG in the mammary glands of these mice induces a persistence of the terminal end buds in the transgenic virgin females. More importantly, multiparous female mice have extensive mammary hyperplasia and develop mammary adenocarcinomas at 12-15 months of age [86, 122]. In addition, less apoptosis was observed in tumors from transgenic mice that overexpress the extracellular region of HRG, as compared with tumors from mice overexpressing full-length HRG, in line with a proposed role for the HRG cytoplasmic tail in apoptosis [86]. Conversely, upon treatment of HRG-overexpressing breast cancer cell lines with HRG blocking antibodies, *in vitro* growth, motility and invasion of these cells is decreased [111]. This was further confirmed by Tsai *et al.* [107], using stable HRG-antisense transfectants of MDA-MB-231 mammary carcinoma cells, endogenously expressing HRG. In these cells, reduction of HRG expression abrogated the pre-existing autocrine loop, resulting in decreases in proliferation, anchorage-independent growth, motility and invasion *in vitro*, the extent of these mirroring the decrease in HRG expression. Moreover, inoculation of these cells into the mammary fat pads of athymic nude mice resulted in tumors that were significantly smaller than those induced by the parental cells and that did not metastasize [107].

Expression and role of neuregulin in other cancers

Heregulin expression has been described in various normal epithelial and carcinoma cells. We recently reported that HRG may act as a potent paracrine growth factor for melanocytes and that multiple deregulations of the HRG/HER system are present in human **melanoma** cell lines. These included a lack of HRG responsiveness, owing to the absence of the HRG-receptor HER3, or owing to functionally inactive HER2, as well as autocrine growth stimulation by HRG. The latter resulted in constitutive activation of HER2 and HER3 and the downstream MAPK pathway [123]. Similarly, others have found that HRG may function as an autocrine and paracrine mitogenic factor for both normal lung epithelial cells and **lung cancer** cell lines. In normal human fetal lung, HRG expression is confined to the mesenchyme, suggesting a paracrine mechanism for stimulation of the adjacent epithelial cells. In contrast, in fetal lung explants, normal bronchial epithelial cell lines and lung cancer cell lines, HRG becomes expressed by the pulmonary epithelial cells as well, functioning as an autocrine, mitogenic ligand [124-130]. Also in **ovarian cancer** cell lines, HRG expression has been found, with the majority of these cell lines showing a mitogenic response following treatment with HRG [68, 69, 131, 132]. Expression of HRG-α1 and -β1, as measured by RT-PCR and immunohistochemistry, was present in about 80% of ovarian carcinomas, the levels being higher in serous carcinomas than in endometrioid carcinomas, indicating the potential of autocrine regulation. Examination of HRG expression in normal endometrium revealed higher HRG-α and -β levels in the secretory phase of the menstrual cycle as compared with the proliferative phase. Cytoplasmic HRG-α immunoreactivity was significantly higher in the stroma (which also sometimes displayed nuclear staining) than in the glands, whereas HRG-β expression was weak to moderate in both the glands and stroma [132]. In **endometrial cancer**, HRG-β levels remained unchanged, whereas decreased HRG-α levels paralleled loss of differentiation [133]. A similar loss of HRG-α expression in **prostatic adenocarcinoma** has been observed in one study [134], whereas another study on prostatic carcinoma reported upregulation of the protein [135]. HRG expression was absent in a panel of prostate carcinoma cell lines, but present in an immortalized non-transformed prostate epithelial cell line. Interestingly, similar to the differentiation effects that were seen in some breast cancer cell lines, HRG reduced growth of the LNCaP prostatic cancer cell line, which coincided with increased contact-formation of these cells [71]. In **colon cancer** cells, autocrine HRG may give rise to constitutively activated HER2 and HER3, protecting against apoptosis and generating growth factor independence [136, 137]. In addition, HRG is able to upregulate cyclooxygenase-2 (COX2) in these cells, resulting in increased prostaglandin E2 (PGE_2) secretion. The induction of COX-2 may have functional implications in colorectal cancer cells, as both the growth-stimulatory and pro-invasive effects of HRG could be

blocked by a specific COX-2 inhibitor [136]. As discussed below, HRG also upregulated both uPA (PGE_2-dependent and -independent) and uPAR (PGE_2-independent) in these cells, the uPA/uPAR system being necessary and sufficient for HRG-mediated invasion [138]. In addition, using a model of FET differentiated colon carcinoma cells plated on Matrigel, these authors showed that HRG affected colonic tissue remodeling, leading to the formation of three-dimensional, multiluminal structures. Formation of these lumen-containing structures was dependent on uPA/uPAR interaction as well and may also, at least in part, depend on upregulation of PGE_2. In **papillary thyroid carcinomas**, immuno-histochemistry revealed increased levels of HRG (α, β1, β2 and/or β3) in both primary tumors and lymph node metastases, as compared to normal tissue [38]. No association was found between HRG protein expression and clinical parameters in these patients. Interesting is that, apart from an increase in membrane and cytoplasmic HRG, the vast majority of tumors had positive nuclear immunostaining, whereas this was never seen in normal thyroid tissue or in diffuse hyperplasia. This nuclear staining was only observed with an antibody recognizing the cytoplasmic tail of the HRG precursor, rendering papillary thyroid carcinoma a candidate system for the recently described nuclear signaling function of the HRG cytoplasmic tail [35]. Nuclear HRG-β staining has also been observed in **medulloblastomas**, embryonal tumors of the cerebellum believed to arise from the cerebellar external granule cell layer. While newly acquired HRG-α expression was found in about one third of these tumors, almost all were positive for HRG-β, which is already present in normal human cerebellum during development. HRG was often co-expressed with HER4 and newly acquired HER2, suggesting the presence of an autocrine loop in the progression of medulloblastoma [37, 139]. Having in mind the widespread expression of HRG in neurons and glia, it is not surprising that HRG expression has also been found in **gliomas**, which are though to arise from central nervous system glial cells [140]. HRG treatment of glioma cells has been shown to increase both motility and invasion, which was accompanied by increased phosphorylation and association of focal adhesion kinase with HER2 [141]. HRG transcripts were detected in esophagus, stomach and duodenum, with no changes observed in **gastric cancers**. HRG-α, which is secreted by gastric fibroblasts, was shown to be mitogenic for gastric epithelial cells, which may be of relevance since the high expression of HER3 and HER4 in gastric cancers [142, 143]. **Activated monocytes** may serve as a source of HRG as well [144]. Since these cells cannot use the secreted HRG themselves, and since activation and infiltration of monocytes may be triggered by tumors, the secreted HRG may serve as a paracrine growth factor for the tumor cells. **Paget's disease** of the breast occurs in about 1% of the patients with breast cancer and is characterized by the invasion of large neoplastic cells originating in the mammary gland into the epidermis of the nipple. Normal keratinocyte-derived HRG-α has been suggested to play a crucial role in this process, by inducing chemotaxis of the mammary cancer cells, which frequently overexpress HER2 [145]. Conflicting reports have been published considering the correlation between HRG expression and the differentiation state of keratinocytes and its mitogenic effects on these cells [146-148]. The primary role of HRG production by keratinocytes does not seem to be growth stimulation [147, 149-151], but would rather involve the stimulation of motility, playing a central role in wound healing processes [146, 151, 152]. Elevated HRG expression has been suggested to play a role in hyperproliferative skin disorders [148], and may play a role in neoplasia as well, as suggested by its high expression in a skin **squamous cell carcinoma** cell line [123, 153]. Autocrine growth stimulation by HRG has been described in **head and neck squamous carcinomas**, where 11/14 cell lines displayed mRNA for HRG and secreted HRG in the medium [154]. The majority of these cell lines showed a mitogenic response to exogenously added HRG, which was accompanied by increased matrix metalloproteinase (MMP) production and invasion [155]. Low HRG expression was found in both normal human urothelium and a **transitional-cell carcinoma**. However, in contrast to primary cultures of human urothelial cells, which were growth stimulated by a variety of EGF-like growth factors, but not by HRG, the latter was a potent mitogen for the transitional-cell-carcinoma cell line [156, 157]. Finally, increased heregulin expression has been found in a **malignant melanoma of soft parts** cell line [158].

Involvement of neuregulin-responsive genes in malignant progression

Several neuregulin-responsive target genes are known candidate genes for malignant transformation. These genes may fulfill important roles in distinct steps of cancer progression. One of the important steps for tumor progression, invasion and eventually metastasis is the destruction of the extracellular matrix that separates the epithelial and stromal compartments. Several proteases have been involved in this process. Of these, the matrix metalloproteinases (MMP's), a family of more than 20 zinc-dependent endopeptidases, are involved in the breakdown of extracellular matrix during tissue remodeling. Aberrant expression of MMP's has been widely recognized in cancer progression, being involved in tumor cell invasion and metastasis [159]. One such enzyme is MMP-9 (gelatinase B), which plays a role in the degradation of type IV collagen (gelatin) and is

highly expressed in breast carcinomas, correlating with increased metastatic potential [160, 161]. HRG is known to upregulate MMP-9 expression and enzymatic activity [73, 104, 106, 107, 155]. Multiple signaling pathways, including PKC, p38MAPK and MAPK pathways, were found to contribute to this upregulation [104]. Moreover, using a specific MMP-9 inhibitor, the invasive phenotype of HRG-expressing cells can be blocked [106], thus providing evidence for an important role for MMP-9 in HRG-induced invasion. Other MMP's that were upregulated following treatment of head and neck squamous carcinoma cell lines with HRG included collagenases, stromelysins and matrilysins, but not gelatinase A (MMP-2) [155]. In both breast and colon cancer cell lines, HRG upregulates the serine protease urokinase plasminogen activator (uPA) and its receptor (uPAR) [103, 138]. Following HRG treatment, an increase in membrane-bound uPA was observed, being redistributed to focal adhesion points, most frequently at the leading edges of the cell. A role for the uPA/uPAR system in HRG-mediated invasion could be deduced from the fact that invasion could be counteracted by blocking uPAR [138]. These findings are consistent with the frequent expression of uPA/uPAR at the leading edge of tumor cells and its correlation with tumor cell invasiveness and aggressiveness [reviewed in 162]. Whether uPA plasminogenic activity or uPAR-induced signaling is involved in the effects of HRG, has not been studied. It should be noted that the induction of proteolytic enzymes by HRG is not observed in all cell lines under all conditions tested [163]. For instance, in the invasive LM3 mouse mammary adenocarcinoma cell line, HRG treatment resulted even in a decreased activity of uPA and MMP-9, correlating with decreased proliferation and migration [164].

Increased *de novo* formation of vascular and lymphatic systems contributes to several aspects of tumor progression: they do not only supply the tumor with fresh blood, which is a key factor for the growth and survival of solid tumors, they comprise systems by which tumor cells can easily spread to nearby or distant tissues as well. Critical growth factors known to mediate these processes belong to the vascular endothelial growth factor (VEGF) family. Overexpression of VEGF family members has been detected in tumor specimens and correlated with an advanced invasive phenotype [165]. HRG has been implicated in (lymph)angiogenesis by its mitogenic effects on endothelial cells and its induction of VEGF-A and -C in breast cancer cells *in vitro* and *in vivo* [166-172]. This induction was not observed in normal mammary or bronchial epithelial cells [168]. HRG treatment of head and neck squamous carcinoma cells resulted in an upregulation of VEGF-A, and –C, did not affect VEGF-B levels, and downregulated VEGF-D [173]. Interesting in this respect is that such differential regulation of different VEGF family members has been associated with malignant progression and lymph node metastasis in lung cancers [174]. Several mechanisms have been proposed for the HRG-mediated increase in VEGF-A and –C. For VEGF-A, upregulation may occur in a PI3K-dependent manner, by activation of p21-activated kinase 1 (Pak1); by increased translation of hypoxia inducible factor (HIF)-1α, which, together with HIF-1β, acts as a transcriptional activator of the VEGF-A gene; and/or by MAPK-dependent activation of Sp-1 and AP-2 transcription binding sites in the VEGF-A promoter [167, 170, 171]. Consistent with this, it has been shown that the Sp-1 transcription factor is stimulated by phosphorylation following HRG-induced HER activation [175]. Alternatively, or in combination, HRG-induced transient activation of p38MAPK results in enhanced VEGF-A and -C transcription and secretion of VEGF protein in breast cancer cells [169, 172]. For the lymphangiogenic VEGF-C, this was shown to depend on a p38MAPK-dependent phosphorylation and inactivation of IκBα, releasing the transcription factor NFκB, which, after nuclear translocation, binds to and activates a NFκB-binding site in the VEGF-C promoter [172].

Another angiogenic factor that is upregulated by HRG is Cyr61, a cysteine-rich ligand that associates with the cell surface and extracellular matrix and functions as a ligand for αvβ3 integrin, promoting cell adhesion and migration. Cyr61 was overexpressed in about 30% of breast tumor specimens, its expression in breast cancer cell lines correlating with the presence and absence of HRG and ER, respectively [102]. Moreover, Cyr61 overexpression was shown to support estrogen-independent growth *in vitro* and *in vivo* [176]. Furthermore, angiogenic effects have also been attributed to MMP-9 and the uPA/uPAR system, extending the roles these molecules may play in HRG-induced tumorigenesis.

Tumor cells must adhere to endothelial cells and interact with components of the extracellular matrix to establish metastatic foci. In this context, the upregulation of cell adhesion molecules may contribute to increased invasion by HRG. Examples include ICAM-1 (CD54) and HCAM (CD44), which are both upregulated following HRG treatment of SKBR-3 breast cancer cells [72, 73]. HRG increased adhesion to plastic and induced invasion of SKBR-3 cells. This invasion could be partially blocked by either anti-CD44 or anti-CD54 antibodies, indicating a role for these molecules in the invasion process [73].

HRG treatment of breast cancer cells results in the upregulation of autocrine motility factor (AMF), a growth factor whose expression has been proposed to play a role in metastasis and correlates with disease progression in various cancers [177, 178]. Roles for HRG-induced AMF include the promotion of motility, since HRG-induced scattering and invasion

could be reduced upon blocking AMF function, as well as growth support, based on the additive growth inhibitory effect of a HER2-blocking antibody and AMF inhibitors.

Amplification of the HRG signal may occur by upregulation of proteins involved in transcription, since these, on their turn, may activate a subset of target genes. Among the proteins involved in transcription that have been shown to be upregulated by HRG are HIF-1α [170], heterogeneous nuclear ribonucleoprotein K (hnRNP K, a known activator of c-myc) [179] and the basic leucine zipper transcription factors activating transcription factor 4 (ATF4) [180] and GADD153 (growth-arrest and DNA-damage 153)[181]. Interestingly, the latter could activate transcription of the β-casein promoter in a Stat5a-dependent way. Since GADD153 expression in the mouse mammary gland is predominantly restricted to early lactation, it represents a good candidate gene for the induction of β-casein by HRG *in vivo*. Also translation may be enhanced, since elongation factor-1α (EF-1α), a ubiquitously expressed protein that is involved in the elongation cycle during translation, was identified as a HRG target gene as well. Upregulation of this protein was mediated by a Sp-1 transcription site in its promoter and involved increased chromatin acetylation [182]. Further upstream, upregulation of proteins with chaperoning function may facilitate correct protein folding and processing. Examples include the induction of heat shock protein-70 and calnexin [183, 184].

Stimulation of cells with HRG does not lead exclusively to upregulation of responsive genes. As already mentioned above, estrogen and progesterone receptor levels decrease following HRG treatment. BRCA1, a tumor suppressor gene whose inactivation is associated with a high incidence of familial breast and ovarian cancer, represents another HRG-repressed gene [185]. HRG treatment of breast cancer cells was shown to affect BRCA1 in two ways: first, receptor activation results in a phosphorylation of BRCA1 in a PI3K-dependent manner and by cyclin-dependent kinase 4; second, HRG stimulation causes a decrease in BRCA1 mRNA levels, which is dependent on protein synthesis. Both effects were enhanced upon culturing the cells on extracellular matrix.

Table 1. Genes, whose functional relevance has been described, that are regulated by HRG. Upregulated genes are indicated in the upper part of the table, downregulated genes are at the end of the table. Abbreviations used for cell types: BC, breast cancer, CC, colon cancer, HNSCC, head and neck squamous cell carcinoma, OC, ovarian cancer, KC, keratinocytes; for level of regulation: mRNA: altered mRNA levels, but mechanism not specified, TC: altered transcription; TL: altered translation, $\uparrow t^{1/2}$: increased halflife. When regulatory level or pathway is not indicated, it was not described in literature.

Gene	Cell-type	Level	Pathway	Function	Reference
ATF4 (activating transcription factor 4)	BC, CC	TC	MAPK	TRANSCRIPTION	[180]
GADD153 (growth-arrest and DNA-damage 153)	BC	TC protein ($\uparrow t^{1/2}$)		TRANSCRIPTION	[181]
HIF-1α (hypoxia inducible factor-1α	BC	TL	PI3K (AKT/FRAP)	TRANSCRIPTION	[170]
hnRNP K (heterogeneous nuclear ribonucleoprotein K)	BC	mRNA		TRANSCRIPTION	[179]
MTA1 (Metastasis and tumor associated protein 1)	BC	TC		TRANSCRIPTION REPRESSOR	[115]
EF-1α (elongation factor- 1alpha)	BC	TC	p38MAPK MAPK	TRANSLATION	[182]
G3BP (GTPase-activating protein SH3 domain-binding protein)	BC	TC		RNASE AND HELICASE ACTIVITIES	[186]
HSP70 (heat shock protein-70)	BC	mRNA		CHAPERONE	[183]
Calnexin	BC	TC		CHAPERONE	[184]
VEGF-A (vascular endothelial growth factor A)	BC, HNSCC	TC Post-TC	PI3K (Pak1)(HIF-1α) MAPK P38MAPK	ANGIOGENESIS	[166-171, 173]
VEGF-C	BC, HNSCC	TC	P38MAPK (NFκB)	LYMPHANGIOGENESIS	[172, 173]

Cyr61	BC	mRNA		ADHESION MIGRATION ANGIOGENESIS	[102]
ICAM-1(CD54)	BC	mRNA	PKC	ADHESION	[72, 73]
HCAM (CD44)	BC			ADHESION	[73]
Integrin alpha 5/6	KC			ADHESION	[146]
Integrin alpha6beta4	KC			ADHESION	[147]
AMF (autocrine motility factor)	BC, CC	TC	P38MAPK MAPK	MOTILITY	[177]
Rab3A	BC	TC	PI3K	VESICULAR TRAFFICKING	[61]
COX-2 (Cyclooxygenase-2)	CC (not BC, OC)	TC		PROSTAGLANDIN SYNTHESIS	[136]
RARα (Retinoic acid receptor α)	BC	mRNA		TRANSCRIPTION	[76]
MMP-9 (matrix metalloproteinase - 9)	BC, HNSCC	TC	PKC p38MAPK (MAPK)	PROTEOLYSIS	[104, 106, 107, 155]
MMP-1, -3, -7, -10, -11, -13	HNSCC	TC Post-TC		PROTEOLYSIS	[155]
uPA (urokinase plasminogen activator)	BC, CC	TC	MAPK NFKB	PROTEOLYSIS	[103, 138]
uPAR (uPA receptor)	BC, CC	TC	MAPK p38MAPK	PROTEOLYSIS	[103, 138]
β-casein	BC	mRNA		DIFFERENTIATION MARKER	[58, 59, 60, 62]
c-Myc	BC, OC, CC	TC		CELL CYCLE	[98, 100, 136]
p21cip1	BC	TC	PI3K p38MAPK	CELL CYCLE	[98, 100]
Cyclin A	BC	TC		CELL CYCLE	[82, 100]
Cyclin B				CELL CYCLE	[82]
Cyclin E	BC	TC		CELL CYCLE	[100]
Cyclin D1	BC	TC	PI3K MAPK p38MAPK	CELL CYCLE	[98, 100]
Cyclin D2	BC	mRNA	PI3K MAPK p38MAPK	CELL CYCLE	[98]
Cyclin D3	BC	mRNA	PI3K MAPK	CELL CYCLE	[98]
DOWNREGULATED GENES:					
ERα (estrogen receptor α)	BC	TC	PI3K/AKT MAPK	TRANSCRIPTION	[109, 113, 114]
BRCA1	BC	mRNA protein	PI3K	TRANSCRIPTION UBIQUITIN POLYMERIZATION	
Keratin 1, 10	KC	mRNA		CYTOSKELETON	[147]
Involucrin, loricrin	KC	mRNA		DIFFERENTIATION MARKER	[147]

Non-genomic effects of HRG on cell motility

Members of the small GTPase superfamily have emerged as key regulators of the actin cytoskeleton, which has been implicated in many cellular functions, including motility, chemotaxis, cell proliferation, differentiation, endocytosis, secretion and cell polarization [187]. HRG stimulation of non-invasive breast cancer cells enhanced the conversion of globular actin to filamentous actin and the formation of membrane ruffles, stress fibers, filopodia, and lamellipodia, which was accompanied by increased cell migration [188]. These prominent cytoskeletal changes induced by HRG are accompanied by dramatic changes in phosphorylation status of several proteins present in focal adhesions, regions where cells make integrin-mediated contacts with the

extracellular matrix and that serve as anchorage points for actin stress fibers. Concomitantly, β1-integrin-mediated adhesion to extracellular matrices is rapidly upregulated [189]. A protein which is localized at focal contacts is paxillin, a cytoskeletal phosphoprotein that becomes phosphorylated on a serine residue following HRG treatment. This occurs in a p38MAPK-dependent manner and results in disassembly of paxillin from the focal adhesion complexes during HRG-induced cell shape alterations and motility [190]. In addition, HRG triggered a redistribution of paxillin to the perinuclear regions. Regulation of cytoskeletal reorganization and cell migration by HRG were found to be mediated by a PI3K-dependent activation of p21-activated kinase-1 (Pak1), which was redistributed to the leading edges of motile cells upon HRG treatment. Another target of HRG signaling includes Tiam1, a guanine nucleotide exchange factor that has been implicated in regulation of cell migration [191]. HRG stimulation of breast cancer cells induces phosphorylation and redistribution of Tiam1 to membrane ruffles. This is accompanied by loosening of intercellular junctions and tyrosine phosphorylation of β-catenin, which is redistributed from the membrane to the cytosol and nucleus, where it may serve as a transcription factor. Also focal adhesion kinase (FAK), a well-characterized protein in focal adhesion complexes that has been implicated in the regulation of cell motility, invasion, adhesion and anti-apoptotic signaling [192], is subject to regulation by HRG [193]. At low concentrations, in which increased cell adhesion and formation of well-defined focal points took place, phosphorylation of FAK was induced. At these concentrations, association of FAK with HER2 could be observed. High doses of HRG, which led to increased migratory potential of the cancer cells, resulted in a dephosphorylation of FAK. This was accompanied by a decreased association of FAK with HER2, but an increased association of the tyrosine phosphatase SHP-2 with the activated receptor, which may be responsible for the observed dephosphorylation of FAK. HRG treatment of breast cancer cells resulted in phosphorylation of RAFTK (Related Adhesion Focal Tyrosine Kinase), a cytoplasmic protein related to focal adhesion kinase, leading to its localization in a multiprotein complex containing HER2, Src, and the GTPase activating proteins (GAPs) p190 RhoGAP and RasGAP. Formation of this complex, in which p190RhoGAP becomes phosphorylated by Src, has been shown to play a role in various aspects of HRG function, including HRG-induced invasion and MAPK activation [194]. Alternatively, HRG treatment increased the association of RAFTK with Csk homologous kinase (CHK), which, via inhibition of RAFTK phosphorylation, may negatively regulate HRG-mediated signaling [195]. Consistent with this, CHK was shown to inhibit *in vitro* cell growth, transformation and invasion induced upon HRG stimulation [196].

Regulation of HRG expression

Relatively little is known about the regulation of HRG expression. This may be due to the complexity of the NRG-1 gene, where the expression of the distinct isoforms is most likely regulated through the usage of alternative promoters. However, upregulation of NRG expression has been described in several cell systems. As can be expected from its prominent increase in the mammary gland during pregnancy [58], HRG expression is under the influence of hormonal control. Thus, HRG not only influences steroid receptor signaling, it is itself subject to regulation by **steroid receptors**. Upregulation of HRG expression in epithelial tumor cells was observed in an *in vivo* model of mammary carcinogenesis induced by the synthetic progestin medroxyprogesterone acetate (MPA) [197]. This upregulation was found in MPA-dependent and –independent ER/PR-positive ductal tumors but was absent in ER/PR-negative lobular carcinomas. These findings are consistent with the suggestion of Jones *et al*. [59] that progesterone might contribute to the HRG-induced lobuloalveolar development *in vivo*. In the mouse mammary gland, stromal HRG expression required the presence of adjacent epithelia, suggesting a reciprocal interaction between these cell types, possibly mediated by secreted proteins [58]. Growth factors such as **keratinocyte growth factor** (KGF) and **hepatocyte growth factor** (HGF) and **EGF**, but not insulin-like growth factor-1 or transforming growth factor-β were able to upregulate HRG expression in keratinocytes, although the underlying mechanisms are not clear. This induction was confirmed in two *in vivo* models of tissue repair in mice: in full-thickness skin wounds, following locally increased KGF production, and in kidney after partial hepatectomy, following elevation of increasing HGF levels [152]. **Cross-induction**, i.e. upregulation of an EGF-like growth factor following stimulation with a family member, frequently occurs in human carcinomas. As a result, two or more EGF-like peptides are often co-expressed, leading to a sustained mutual co-amplification mechanism. Using a squamous carcinoma cell line as a model, O-charoenrat *et al.* found that HRG mRNA was strongly upregulated following treatment with any of the EGF-like ligands tested (EGF, transforming growth factor-α, betacellulin, heparin-binding-EGF, amphiregulin and HRG-β1 itself). This was suggested to involve both transcriptional and post-transcriptional mechanisms [154]. Consistent with its identification as a factor released by *ras*-transformed fibroblasts [8, 9], the insertion of **activated *H*-or *K-ras*** also resulted in upregulation of NRG-expression in breast cancer cells [198]. However, our analysis in melanoma cells, in which activated *N-ras* was present, could not

provide a role for Ras-signaling in upregulation of HRG expression (unpublished data). Another oncogene, ***cph***, was suggested to underlie the high HRG expression observed in a hamster embryo fibroblast cell line [199]. Regulation of HRG expression by its co-receptor **HER2** has been described. However, whereas in ovarian cancer cell lines HER2 overexpression induced HRG, its expression in both normal immortalized and cancer-derived breast cell lines significantly decreased or remained unchanged [69]. Stimulation of a monocytic cell line and normal peripheral blood monocytes with **phorbolester** resulted in increased HRG mRNA and protein levels [144]. Consistent with this, we found increased levels of HRG mRNA in primary human melanocytes cultured in the presence of phorbolester (unpublished data). In keratinocytes, a decrease in the level of HRG transcripts occurred upon attaining culture confluence, conversely correlating with the levels of HER2 and HER3 in these cells [147].
In addition to the several pathways that have been described to upregulate HRG expression, improvement of its release by the cells may contribute to its effects as well. This was evident from a study in which growth inhibition of squamous cell carcinoma cell lines by MMP inhibitors could be attributed to their inhibitory effects on the release of EGF-like ligands, including HRG [200]. Since the HRG precursor could not be cleaved anymore, no HRG was available anymore to function in an autocrine loop. Also in a physiological context, this metalloproteinase-mediated cleavage of neuregulins is important, as exemplified by Meltrin-β (ADAM19) knockout mice, in which heart development is compromised because of a lack of neuregulin-mediated paracrine signaling [201].

The *NRG1* locus as a target of recurrent chromosome translocation breakpoints

Recently, disruption of the 1.1 Mb-spanning *NRG1* locus at 8p12 was found in several human breast (5 out of 34) and pancreas cancer cell lines (2 out of 9) [202]. These breakpoints differed among the different cell lines. In the human breast cancer cell line MDA-MB-175, a t(8;11) translocation leads to a fusion product consisting of the N-terminal part of DOC4, a stress-induced gene of unknown function located on chromosome 11q13, and HRG [202-204]. Expression of this novel secreted chimeric ligand, coined γ-heregulin, is under the control of the DOC4 promoter, thus generating an autocrine loop which gives rise to constitutive activation of HERs in these cells [205]. A survey for this particular translocation in a series of 141 breast cancer patients did not reveal any abnormalities [206]. However, since in all of the cell lines with disruption of the *NRG1* locus the breakpoint differed, thorough examination of the entire *NRG1* locus rather than a screening for a specific breakpoint is needed. Although the MDA-MB-175 example likens NRG1 to be an oncogene, in the majority of the cell lines with a *NRG1* locus breakage the result of the translocation is unknown. Although no correlation could be found between the presence of a breakpoint and the (pattern of) secretion of NRG isoforms, it is interesting to mention that in the initial characterization of one of the affected cell lines, SUM-52PE, a juxtacrine HRG autocrine loop was described [207]. Yet, it remains to be determined whether in this and other cell lines the chromosomal translocation directly contributes to malignancy. If so, either activation by promoter swapping or protein fusion, or inactivation may contribute to tumor progression. Alternatively, it is possible that, depending on the site of breakage and the isoform(s) targeted, cell behavior is affected in a different way. The observation that loss of heterozygosity at microsatellite markers from region p11-21 of chromosome 8, which encompasses the *NRG1* locus, is a frequent event in microdissected breast tumor samples, but not in peritumoral cells [208], offers an intriguing possibility that disruption of this locus may indeed represent an important event in cancer. In conclusion, the evidence that the *NRG1* locus may encompass a fragile site in cancer, warrants further research, both in the clinic, verifying whether rearrangement of this locus is also present in fresh tumor samples, as in basic research, elucidating the mechanisms underlying the breakage and resulting from it.

Is there a role for neuregulins-2, -3 and -4 in cancer?

As is evident from the above, the vast majority of publications describing the role of NRGs in cancer focuses on members of the NRG-1 family. Recently, however, some attention is being given towards the other neuregulins and their possible role in malignant progression. Like neuregulin-1, also neuregulin-2, -3 and -4 were described to stimulate cell proliferation, provided the cells express the appropriate receptors [21, 23, 209, 210]. Moreover, suggesting a similar dual function as has been described for neuregulin-1, also neuregulin-2 can induce differentiation of breast cancer cells *in vitro*, the strength and biochemical characteristics of the effect depending on the isoform applied [211-213]. No reports have been published on aberrant expression or signaling of neuregulins-2, -3 or -4 in cancer, yet. Recently, an anti-angiogenic role has been attributed to a neuregulin-2/NTAK isoform, NTAKγ, on basis of its ability to inhibit proliferation of endothelial cells [214]. Since this effect was not mediated by its EGF-like domain but, instead, depended on its N-terminal domain, this may open a wide array of novel functions attributed to domains outside the EGF-like domain.

Conclusion

In conclusion, the neuregulin-HER system is of critical importance in regulating a variety of physiological events. A tightly regulated balance exists between differentiating and growth-promoting effects of neuregulins. Deregulation of this balance may contribute to malignant transformation. While most studies have focused on overexpression of HERs as a mechanism leading to constitutive receptor activation, it is becoming increasingly clear that, in the presence of normal receptor levels, aberrant constitutive signaling may occur as well, due to the presence of autocrine or paracrine loops. While the action of neuregulins *in vivo* is typically paracrine, being expressed in mesenchymal tissues adjacent to epithelia, epithelial tumors frequently show gain of expression, increased (sensitivity to) paracrine signaling and/or disruption of spatial control, all of which may contribute to the transformation process. This transformation is likely mediated by activation of a wide array of signaling events, leading to changes in gene expression, as well as rapid alterations in the actin cytoskeleton. Although relatively little is known about the mechanisms leading to increased HRG expression, the recent identification of the *NRG1* locus as a target of recurrent chromosome breakpoints in breast and gastric cancer cell lines offers an intriguing possible explanation. The characterization of HRG as a constitutive activator of HERs in a variety of tumors may offer opportunities for targeted therapies in these cancers.

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I.3. The Heregulin/Human Epidermal Growth Factor Receptor as a New Growth Factor System in Melanoma with Multiple Ways of Deregulation

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ORIGINAL ARTICLE
See related Commentary on page xi

The Heregulin/Human Epidermal Growth Factor Receptor as a New Growth Factor System in Melanoma with Multiple Ways of Deregulation

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In a screening for new growth factors released by melanoma cells, we found that the p185-phosphorylating capacity of a medium conditioned by a melanoma cell line was due to the secretion of heregulin, a ligand for the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. Expression of heregulin, including a new isoform, and secretion of functionally active protein was found in several cell lines. Receptor activation by heregulin, either autocrine or paracrine, resulted in a potent growth stimulation of both melanocytes and melanoma cells. Heregulin receptor HER3 and coreceptor HER2 were the main receptors expressed by these cells. Nevertheless, none of the cell lines in our panel overexpressed HER2 or HER3. In contrast, loss of HER3 was found in two cell lines, whereas one cell line showed loss of functional HER2, both types of deregulations resulting in unresponsiveness to heregulin. This implies the heregulin/HER system as a possible important physiologic growth regulatory system in melanocytes in which multiple deregulations may occur during progression toward melanoma, all resulting in, or indicating, growth factor independence. ***Key words: heregulin-neuregulin-1/autocrine-paracrine communication/receptor tyrosine kinases. J Invest Dermatol 121:802–812, 2003***

Growth of melanocytes and their malignant counterparts is regulated by a variety of cytokines and other polypeptides (Lázár-Molnár *et al*, 2000; Payne and Cornelius, 2002). Under physiologic conditions, melanocytes depend for their survival on paracrine stimulatory factors provided by the surrounding keratinocytes (Meier *et al*, 1998). Transformed melanocytes have a decreased dependence on paracrine stimulation, which facilitates their survival outside their natural environment, the epidermis. Changes in several growth factor systems contribute to this decreased dependence. Whereas overexpression of receptor tyrosine kinases (RTK) may lead to increased growth factor sensitivity and constitutive signaling, loss of expression may result in insensitivity to inhibitory factors or indicate growth factor independence (Easty and Bennett, 2000). Also, the profile of growth factors secreted by melanoma cells is frequently altered, compared to melanocytes. Whereas *de novo* expression of some growth factors by melanoma cells may stimulate proliferation of these cells in an autocrine loop, these factors may act on the surrounding cells as well, stimulating or inhibiting these cells in a paracrine way (Halaban, 2000; Lázár-Molnár *et al*, 2000; Ruiter *et al*, 2002).

The human epidermal growth factor receptor (HER) family of RTK consists of four members, epidermal growth factor receptor (EGFR)/erbB1/HER1, neu/erbB2/HER2, erbB3/HER3, and erbB4/HER4 (Olayioye *et al*, 2000; Yarden and Sliwkowski, 2001). Although constitutive activation of these receptors, owing to overexpression, frequently occurs in various types of cancers (Révillion *et al*, 1998), this does not seem to be common in melanoma (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Constitutive RTK signaling may also be the result of truncation, mutation, association with other cell-surface proteins, transactivation via other receptors, or the presence of autocrine loops (Blume-Jensen and Hunter, 2001; Gullick, 2001). The latter may result from the aberrant expression of HER ligands.

Neuregulin-1 is the term for a family of proteins derived by alternative splicing from a single gene, functioning as ligand for HER3 and HER4 (Holmes *et al*, 1992; Yarden and Sliwkowski, 2001). At present, at least 24 splice variants have been identified in different species, of which 10 were found in humans. Alternative splicing at the N-terminus results in three types of proteins: heregulins (HRG, type I) (Holmes *et al*, 1992), glial growth factors (type II) (Marchionni *et al*, 1993), and sensory- and motor-neuron-derived factors (type III) (Ho *et al*, 1995). Further alternative splicing of HRG at the EGF-like domain (α or β), the C-terminal part of the EGF-like domain (1, 2, or 3) (**Fig 1**), and/or at the intracellular tail (a, b, or c) gives rise to closely related proteins, differing in size and cellular localization and having distinct receptor activation potentials and functions (Wen *et al*, 1994; Pinkas-Kramarski *et al*, 1996; Meyer *et al*, 1997). Transmembrane

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Abbreviations: bFGF, basic fibroblast growth factor; CM, conditioned medium; EGF, epidermal growth factor; HER, human EGF receptor; HRG, heregulin; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; rHRG-β1, 7-kDa recombinant EGF-like domain of heregulin isoform b1; RTK, receptor tyrosine kinase.

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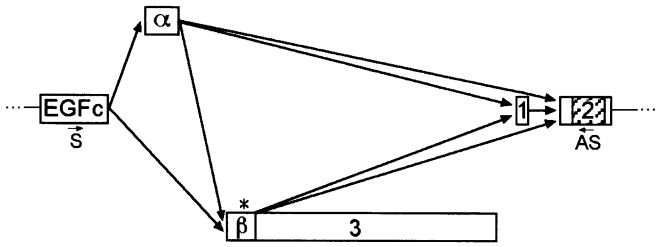


Possible exon combinations	Isoform	Expected PCR product length
(EGFc) - (α) - (2)	α2	202 bp
(EGFc) - (α) - (1) - (2)	α1	226 bp
(EGFc) - (β) - (2)	β2	193 bp
(EGFc) - (β) - (1) - (2)	β1	217 bp
(EGFc) - (α) - (β) - (2)	?	261 bp
(EGFc) - (α) - (β) - (1) - (2)	α4	285 bp
(EGFc) - (α) - (β) - (3)	α3	NA
(EGFc) - (β) - (3)	β3	NA

Figure 1. Neuregulin-1 splicing variation in the EGF-like domain. The scheme depicts the genomic organization of the exons encoding the region surrounding the variable part of the EGF-like domain of the NRG-1 gene. The locations of the sequences that are complementary to the primers used for RT-PCR are indicated with *S* and *AS* for the sense and antisense primers, respectively. The table indicates all possible splicing variants within this domain with their expected amplification product lengths in base pairs (bp), when using the indicated primer pair. *NA,* not amplified; *EGFc,* sequence common in the EGF-like domain of all HRG; *hatched,* coding sequence for the HRG transmembrane domain; *asterisk,* location of the stop codon in case of the (α)–(β) combination; *?,* putative isoform not yet characterized.

HRG typically function as precursor molecules that are subject to the action of metalloproteinases. This results in the release of the extracellular domain that may subsequently bind to nearby receptors (autocrine/paracrine action) (Montero *et al*, 2000; Shirakabe *et al*, 2001). Necessary and sufficient for receptor binding is the EGF-like domain; the roles of the various other domains have not been fully elucidated yet. Some regulation may be exerted by the cytoplasmic tail (Liu *et al*, 1998a, b; Han and Fischbach, 1999) or by interaction of the N-terminal heparin-binding motif with other molecules such as cell surface heparan sulfate proteoglycans (Li and Loeb, 2001). A recently proposed model for ligand-mediated HER activation proposes receptor conformational changes as the driving force for receptor activation (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002). For HRG, this would mean that binding of its EGF-like domain to HER3 or HER4 leads to an altered receptor conformation, thus promoting dimerization with another HER, preferentially HER2. Hetero- or homodimerization of the receptors leads to *trans*- and autophosphorylation, creating specific docking sites for signal transduction molecules (Dankort *et al*, 2001; Hellyer *et al*, 2001) and initiating further downstream signaling. When only HER2 and HER3 are present, this model of HRG-induced receptor activation implies that HER3, which lacks catalytic activity (Guy *et al*, 1994; Sierke *et al*, 1997), can only become phosphorylated *in trans* by heterodimerization with HER2 (Kim *et al*, 1998). Conversely, HER2, for which no direct ligand has been identified yet, only becomes activated after ligand binding to HER3.

Based on the initial observation that conditioned medium (CM) from a melanoma cell line induced a strong phosphorylation of HERs in MCF-7 mammary cancer cells, we decided to dissect the role of this putative ligand–receptor system in melanocytes and a panel of melanoma cell lines. Here, in 4 of a panel of 13 melanoma cell lines, we describe a number of deregulations in the HRG/HER system. Production and release of functionally active HRG in the medium were found in three cell lines and resulted in an autocrine loop in one case. Whereas exogenous HRG-stimulated growth of the majority of melanoma cell lines and melanocytes, three cell lines did not respond to HRG, owing to the absence of HER3 or owing to a functionally incompetent HER2.

MATERIALS AND METHODS

Cell lines The cell lines were obtained as follows: 530 and BLM melanoma cell lines from L. Van Kempen (University of Nijmegen, the Netherlands); A375 melanoma cell line from J. Hilkens (NKI, the Netherlands); Bowes melanoma from G. Opdenakker (Rega Institute, Belgium); DX3 and DX3azaLT5.1 melanoma cell lines from J. Ormerod (Imperial Cancer Research Fund, UK); FM3/D, FM3/p, FM45, and FM87 melanoma cell lines from J. Zeuthen (Danish Cancer Society, Denmark); HMB2, MeWo, and MJM melanoma cell lines from D. Rutherford (Rayne Institute, St Thomas Hospital, UK); MCF-7/6 mammary carcinoma cell line (further called MCF-7) from H. Rochefort (University of Montpellier, France); COLO-16 squamous skin carcinoma cell line and SK-BR-3 mammary carcinoma cell line from C. De Potter (Ghent University Hospital, Belgium); and MDA-MB-231 breast cancer cell line from American Type Culture Collection (Manassas, VA). Cell lines were routinely maintained in the following media (Gibco BRL, Belgium): RPMI 1640 (FM and COLO-16 cell lines), L15 (MDA-MB-231), 50% Dulbecco's modified Eagle's medium/50% Ham's F12 (MCF-7), or Dulbecco's modified Eagle's medium (all other cell lines). All media for routine culture contained 10% heat-inactivated fetal bovine serum (Greiner Bio-One, Belgium), 100 IU per mL penicillin, 100 μg per mL streptomycin, and 2.5 μg per mL amphotericin B. Epidermal melanocyte primary cultures were obtained from neonatal foreskins and established in M199 medium (Gibco BRL), supplemented with 2% fetal bovine serum, 10^{-9} M cholera toxin, 10 ng per mL basic fibroblast growth factor (bFGF), 10 μg per mL insulin, 1.4 μM hydrocortisone, and 10 μg per mL transferrin (all from Sigma, Belgium). Postprimary cultures were maintained in low-calcium (0.03 mM) M199 medium, supplemented with the same factors and 10% fetal bovine serum. The melanocytic origin of all melanoma cell lines was checked by immunocytochemistry using two antibodies against melanoma-specific proteins, HMB45 (Enzo Diagnostics, Farmingdale, NY) and NKI/C3 (Biogenex, San Ramon, CA). All melanoma cell lines were positive for at least one of these markers (data not shown). Because most of the experiments were carried out with Bowes melanoma cells, which were only positive for NKI/C3, additional electron microscopy was performed to confirm the presence of premelanosome-like structures in this nonpigmented cell line (data not shown).

Antibodies and reagents Primary antibodies used were: rabbit polyclonal anti-HER1, -2, -3, and -4 and anti-HRG precursor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antitubulin (Sigma), mouse antiphospho-mitogen-activated protein kinase (MAPK; Westburg, the Netherlands), and antiphosphotyrosine antibody RC20 conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY). Goat anti-HRG-α and recombinant HRG-β1, consisting of the EGF-like domain of HRG (rHRG-β1, used at 10 ng/mL unless indicated otherwise), was purchased from R & D Systems (Abingdon, UK). Full-length recombinant HRG-β1 was obtained from Laboratory Vision (Fremont, CA), whereas heparin, PD168393 (used at 2 μM, unless indicated otherwise), and PD98059 were from Calbiochem (Darmstadt, Germany).

Preparation of CM Subconfluent monolayers were washed three times with phosphate-buffered saline (PBS), incubated for 24 h with serum-free medium, and washed again three times with PBS, followed by a 48-h incubation with serum-free medium. The latter medium was cleared from cells by 5 min centrifugation at $250 \times g$. The resulting supernatant was centrifuged for an additional 20 min at $2000 \times g$ to remove cell debris, filtered through a 0.2-μm filter, and stored at –20°C until use. To isolate the heparin-binding fraction from the CM, the latter was depleted from heparin-binding factors by triple precipitations with heparin beads (Bio-Rad, Hercules, CA). Elution of the heparin-binding fraction was done with 1 M NaCl, followed by desalting and dilution in fresh serum-free medium.

Western blotting and (immuno)precipitation All lysates were made of cells of approximately 90% confluence. For phosphorylation experiments, cells were washed three times with PBS, serum-starved overnight, washed again three times with PBS, and treated with serum-free medium for the indicated times. Before making all lysates, the cells were washed three times with PBS. Cells were lyzed with PBS containing 1% Triton X-100, 1% Nonidet P-40 (Sigma), and the following protease inhibitors: aprotinin (10 μg/mL), leupeptin (10 μg/mL) (ICN Biomedicals, Costa Mesa, CA), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 μM), $NaVO_3$ (500 μM), and $Na_4P_2O_7$ (500 μg/mL) (Sigma). After clearing the lysates, protein concentration was determined using Rc Dc protein assay (Bio-Rad), and samples were prepared such that equal amounts of protein were to be loaded. For immunoprecipitation, equal amounts of protein were first incubated with protein A–Sepharose (Amersham Pharmacia Biotech, UK) for 30 min. After discarding the beads, the supernatant was incubated with primary antibody for 3 h at 4°C, followed by incubation with the added protein A–Sepharose beads for 1 h. For heparin and streptavidin precipitations, cell lysates were incubated with heparin beads (Bio-Rad) or streptavidin beads (Sigma). Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 min and separation of proteins by gel electrophoresis on a 8 or 12% polyacrylamide precast gel (Invitrogen, San Diego, CA) and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining of the blots were done in 5% nonfat dry milk in PBS containing 0.5% Tween 20, except for RC-20 and antiphospho-MAPK antibodies, where 4% bovine serum albumin in PBS containing 0.2% Tween 20 was used instead. The membranes were quenched for 1 h, incubated with primary antibody for 1 h, washed four times for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min, and washed six times for 10 min. Detection was done using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with antitubulin antibody was performed routinely (not shown). Quantification of bands was done using Quantity-One software (Bio-Rad).

RT-PCR, cloning, and sequencing Total RNA was extracted from approximately 5×10^6 cells using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). One microgram of total RNA was reverse transcribed with oligo(dT) primers using the Qiagen RT kit (Qiagen) according to the manufacturer's instructions. HRG cDNA encoding all transmembrane isoforms was amplified using the sense primer 5′-CTGTGTGAATGGAG-GGGAGTGC-3′ (complementary to a sequence encoding a conserved part of the EGF-like domain) and the antisense primer 5′-GACCACAAG-GAGGGCGATGC (complementary to a sequence encoding part of the transmembrane domain) (**Fig 1**). As a control (not shown), β2-microglobulin cDNA was amplified using the sense primer 5′-CATCCAGCGTACTC-CAAAGA-3′ and the antisense primer 5′-GACAAGTCTGAATGCTC-CAC-3′ to generate a 165-bp product. PCR was performed on 250 ng template cDNA using the Qiagen *Taq* PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were done in a Minicycler (Biozym, the Netherlands) with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 50 s (denaturation), 61°C for 50 s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 10 min. For cloning of the HRG amplification products, the HRG sense and antisense primers were extended at the 5′ end with GCCGGATCCG, creating a *Bam*HI restriction site, and with TCCGAATTC, creating a *Eco*RI restriction site, respectively. The resulting amplification products were either separated by agarose gel electrophoresis, followed by gel extraction using Qiagen gel extraction kit (Qiagen), or used directly for digestion with *Bam*HI and *Eco*RI restriction enzymes (Roche Diagnostics, Germany). Digested products were ligated into dephosphorylated *Bam*HI/*Eco*RI-digested pIRES2-EGFP vector (Clontech, Palo Alto, CA). After transformation of competent DH5α bacteria with the ligated product, the kanamycin-resistant clones were screened by PCR using primers complementary to sequences of the pIRES2-EGFP vector surrounding the insert. This resulted in PCR products of different lengths, corresponding to different HRG isoforms, which were subjected to sequencing (Applied Biosystems, Foster City, CA). The sequence of the α4-isoform was submitted to GenBank (Accession Number AY207002).

Scattering assay MCF-7 cells were seeded until small islands were formed. The cells were washed three times with PBS and were serum-starved overnight. The following day, the cells were washed again three times with PBS, after which the treatments (all in serum-free medium) were applied for 2 h. Pictures were taken with an Axiovert 200M microscope (Carl Zeiss Vision, Germany) on living cultures or after the cultures had been fixed with crystal violet (0.5% in 4% formaldehyde, 30% ethanol and 0.17% NaCl) for 15 min.

Cell proliferation assays A total of 12,500 melanocytes were seeded in the wells of a 96-well plate in 100 μL of Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% fetal bovine serum. After attachment, 100 μL of medium, supplemented with growth factors as indicated, was added. After 5 days, metabolic activity was measured with a colorimetric assay. Briefly, 100 μL of medium was taken off, followed by the addition of 20 μL of 5 mg per mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) in PBS. After a 2-h incubation and removal of all fluid, the colored formazan formed was dissolved in 100 μL of dimethyl sulfoxide and absorption was measured with an ELISA reader at 490 nm. Proliferation of melanoma and MCF-7 cells was measured with the sulforhodamine B colorimetric assay. A total of 5000 cells were seeded in 96-well plates, allowed to attach, and treated as indicated. After 5 days, 50 μL of 50% trichloroacetic acid was added to the culture medium, followed by an incubation of 1 h at 4°C. The wells were rinsed with water, dried, incubated with 100 μL of sulforhodamine B (0.4% in 1% acetic acid) for 30 min, rinsed with 1% glacial acetic acid, and dried again. Bound dye was dissolved in 200 μL of 10 mM Tris buffer, pH 10.5, and absorption was measured with an ELISA reader at 490 nm.

Annexin V staining Melanocytes were seeded on a collagen type I gel for 4 days. After taking photographs, adherent cells were detached with a swab and brought together with floating cells. Annexin V staining was performed with Annexin V–PE (Becton Dickinson Biosciences, Mountain View, CA), according to the manufacturer's instructions. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) with an argon–ion laser tuned at 488 nm and a helium–neon diode laser at 635 nm. Forward light scattering, orthogonal scattering, and two fluorescence signals were stored in list-mode data files. Data acquisition and analysis were done using the CellQuest software (Becton Dickinson). Additional propidium iodide (PI) staining was performed to rule out cells that were necrotic (Annexin V^+ and PI^+).

Statistics Differences between means were considered significant when the p value was less than 0.01, using Student's *t* test.

RESULTS

Expression of HERs in a panel of melanoma cell lines Expression of HERs by melanocytes and melanoma cell lines was examined by Western blotting and immunostaining with anti-HER1, -2, -3, and -4 antibodies. Neither melanocytes nor any of the melanoma cell lines showed expression of full-length HER1 or HER4, compared with the respective positive controls A431 and T47D (data not shown). Nevertheless, this does not necessarily mean that these receptors are completely absent. All melanoma cell lines, as well as melanocytes, expressed HER2 (**Fig 2**). HER2 levels in melanocytes and in all

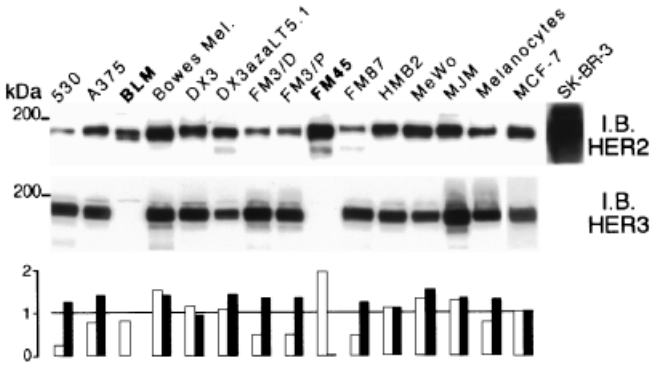


Figure 2. Analysis of HER2/HER3 expression in melanocytes and melanoma cell lines. Whole-cell lysates were analyzed by immunoblotting (*I.B.*) with anti-HER2 and anti-HER3 antibodies, as described under Materials and Methods. Quantification of the resulting bands was done relatively to the level of HERs in MCF-7 mammary carcinoma cells, set at 1. *Open and filled bars,* HER2 and HER3 expression, respectively.

melanoma cell lines were far below the level found in the HER2-overexpressing SK-BR-3 mammary carcinoma cell line (Press *et al*, 1993). Moreover, quantification showed that none of the melanoma cell lines had HER2 levels that were more than two times higher than that seen in MCF-7 mammary carcinoma cells, often used as a control for normal expression (Press *et al*, 1993; Aguilar *et al*, 1999). Comparable levels of HER3 were expressed by melanocytes and 11 of the melanoma cell lines. Two melanoma cell lines (BLM and FM45) did not show expression of HER3 protein (**Fig 2**). The absence of HER3 protein was due to a strongly reduced level of HER3 mRNA in these cell lines, compared with the other cell lines (data not shown).

Expression of HRG by melanoma cell lines Western blotting, followed by immunostaining of total lysates using an anti-HRG antibody directed against a cytoplasmic sequence conserved in all transmembrane HRG isoforms, revealed the presence of a ± 105-kDa band in Bowes melanoma, BLM, and MJM, the first two having the stronger expression (**Fig 3*A***, *top panel*). This band was also present in the positive controls MDA-MB-231 (although very faint) and COLO-16, previously described to secrete HRG (Holmes *et al*, 1992) or HRG-like activity (De Corte *et al*, 1994), respectively, and was not found in the MCF-7-negative control (Aguilar *et al*, 1999; Aguilar and Slamon, 2001). The size of this band indicates that it corresponds to the full-length HRG precursor (Burgess *et al*, 1995; Aguilar and Slamon, 2001). The 50-kDa band, also seen by others using this antibody (Aguilar and Slamon, 2001), probably represents an artifact, because it could also be found in the MCF-7 HRG-negative cells. Also two other bands (at ± 85 and 75 kDa), seen in some melanoma cell lines, are likely due to cross-reactivity of the antibody with other proteins. Because these bands were not consistently found in cell lines expressing HRG mRNA, and CM of these cells had no HRG-like activity (see below), they are unlikely to represent cleavage products of transmembrane HRG. The localization of the 105-kDa immunoreactive band at the plasma membrane was confirmed for Bowes melanoma cells by biotinylation (**Fig 3*B***, *lane 4*) and by precipitation using heparin beads (**Fig 3*B***, *lane 3*), which is consistent with the presence of a heparin-binding motif at the extracellular N-terminus of HRG.

RT-PCR analysis was carried out to verify the results obtained by western blotting and to detect which isoforms were expressed by the HRG-positive melanoma cell lines. Because alternative splicing of the HRG-encoding gene leads to multiple isoforms, with most variation in the EGF-like domain, primers were chosen so that different lengths of amplification products were obtained, depending on the isoform expressed. The sense and antisense primers chosen were complementary to the mRNA encoding a conserved part of the EGF-like domain and a sequence conserved in all transmembrane isoforms, respectively (**Fig 1**). RT-PCR using this primer set, with MDA-MB-231 and COLO-16 as positive controls and MCF-7 as a negative control, confirmed the results obtained by western blotting for Bowes melanoma, BLM, and MJM cells (**Fig 3*A***, *bottom panel*). In addition, lower levels of mRNA were found in some other melanoma cell lines, possibly resulting in HRG protein levels that were below the detection level in western blotting. Melanocytes had amounts of mRNA that were comparable to those found in MJM cells. Because we initially did not detect HRG protein in these cells (**Fig 3*A***), we loaded more protein and overexposed the film, which eventually resulted in the appearance of a weak band at ± 105 kDa (**Fig 3*C***). The pattern of PCR amplification products that was obtained from Bowes melanoma suggested the presence of multiple isoforms. Cloning and sequencing of these products revealed that α2, β1, and β2 isoforms were the most abundant transmembrane isoforms in this cell line (**Fig 3*D***). In addition, a new isoform, designated α_4, was identified. This isoform combines the sequences from

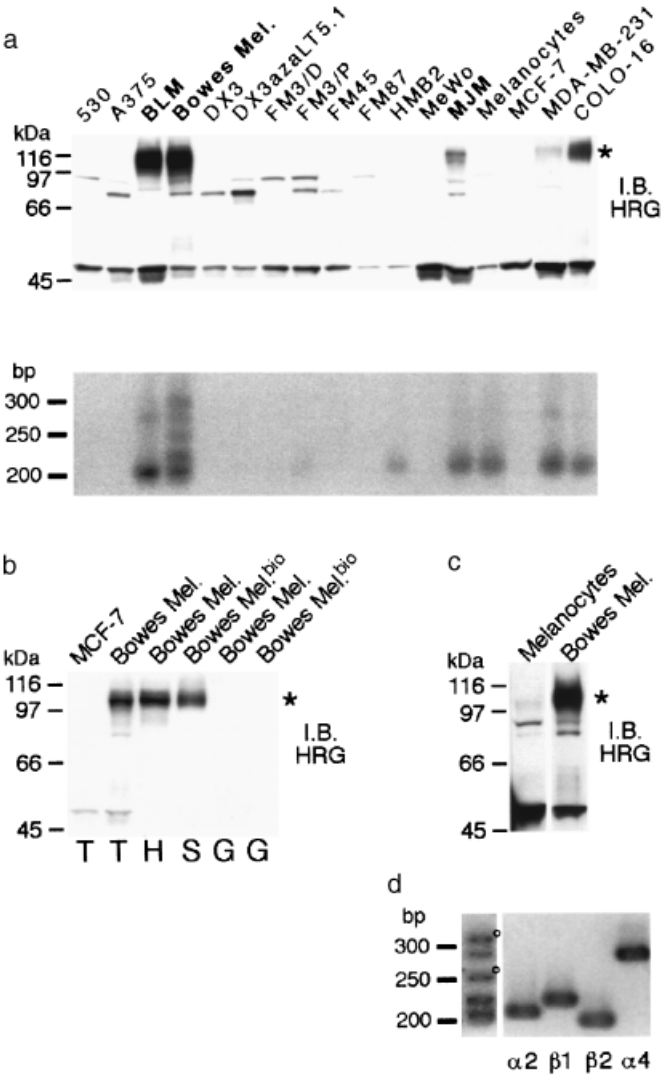


Figure 3. Presence of HRG protein and mRNA in melanocytes and melanoma cell lines. (*a, top panel; c*) HRG precursor expression was analyzed by immunoblotting (*I.B.*) of whole-cell lysates with an anti-HRG antibody. (*b*) Total lysates of MCF-7 and Bowes melanoma (*T*), a heparin precipitate (*H*), streptavidin precipitate (*S*), or control protein G–Sepharose precipitates of Bowes melanoma cells or biotinylated Bowes melanoma cells (*bio*) were analyzed by immunoblotting with an anti-HRG antibody. (*c*) Prolonged exposure after immunoblotting of total lysates of melanocytes and Bowes melanoma cells reveals a weak band in the former. *Asterisk,* the band of full-length HRG at 105 kDa. (*a, bottom panel*) RT-PCR analysis of HRG mRNA in the indicated cell lines using the primer panel indicated in the legend to **Fig 1**. (*d*) The *first lane* depicts HRG mRNA expression in Bowes melanoma as assessed by RT-PCR, using the primer panel indicated in the legend to **Fig 1**. *Lanes 2–5,* PCR analyses of clones derived from Bowes melanoma, representing the indicated HRG isoforms. *Open circle,* bands corresponding to PCR products formed by cross-annealing of two related isoforms and thus considered as aspecific.

both the exon leading to the α isoforms and the exons leading to the β_1 isoform (**Figs 1, 3*D***). Two bands (indicated with an *open circle*) did not correspond to a specific isoform because they were the result from cross-annealing of PCR products coming from β1–β2 or α2–β2 isoforms, presumably resulting in an imperfect double strand with slower migration on agarose gel (**Fig 3*D***).

Melanoma cells release functionally active HRG in the culture medium Following cleavage in the juxtamembrane

extracellular region, HRG are released into the culture medium as 40- to 45-kDa proteins, depending on the isoform and glycosylation level (Holmes *et al*, 1992; Lu *et al*, 1995a, b). Western blotting of 50 × concentrated CM, using an antibody directed against the HRG extracellular domain, revealed the presence of a broad band at the expected molecular weight (**Fig 4*A***, *lane 1*). This band was absent upon depletion of the concentrated CM from heparin-binding factors (**Fig 4*A***, *lane 2*).

To test whether the released HRG was functional, we verified whether CM from the melanoma cell lines was capable of activating HERs in MCF-7 cells. Prominent tyrosine phosphorylation of a 185-kDa protein was evident upon

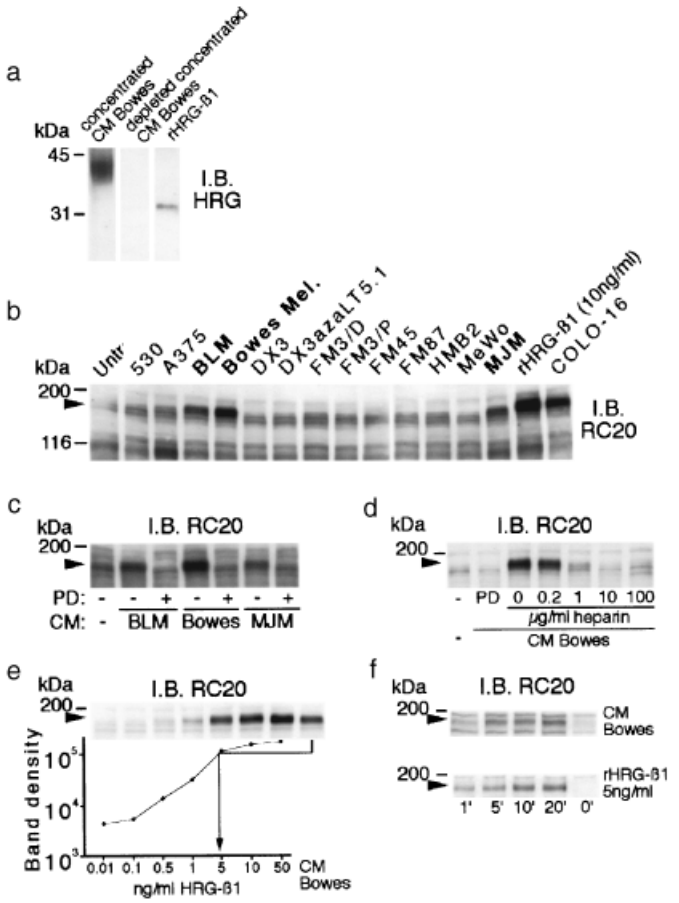


Figure 4. Melanoma cells release receptor activating HRG in the medium. (*a*) Immunoblotting (*I.B.*), using an anti-HRG antibody directed against the EGF-like domain, of concentrated CM of Bowes melanoma, before (*lane 1*) and after (*lane 2*) depletion of heparin-binding factors reveals the presence of a 45-kDa protein only in *lane 1.* Full-length recombinant HRG-β1 (*rHRG-β1*), produced in *Escherichia coli* (*lane 3*) migrates at 33 kDa owing to differences in glycosylation. (*b–e*) Analysis of tyrosine-phosphorylated proteins in serum-starved MCF-7 cells. (*b*) Cells treated for 30 min with serum-free medium (*Untr*), with rHRG-β1, or with CM from the indicated cell lines. (*c*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated for an additional 30 min with serum-free medium or CM from the indicated cell lines. (*d*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated with serum-free medium or with CM of Bowes melanoma to which heparin was added in the indicated concentrations. (*e*) Quantification of tyrosine phosphorylation, induced by treating MCF-7 cells with Bowes melanoma CM or by treating these cells with increasing concentrations of rHRG-β1. The *arrow* indicates that, by extrapolation, the phosphorylating capacity of Bowes melanoma CM is equivalent to that of ± 5 ng per mL rHRG-β1. (*f*) MCF-7 cells treated with 5 ng per mL rHRG-β1 or CM Bowes melanoma for the indicated periods of time. *Arrowheads,* ±185-kDa tyrosine-phosphorylated bands.

treatment with the positive controls recombinant HRG-β1 (rHRG-β1) and COLO-16 CM and was further restricted to CM from HRG-positive melanoma cell lines (**Fig 4*B***). This phosphorylation could be blocked by pretreating the MCF-7 cells for 30 min with the HER-specific irreversible inhibitor PD168393 (Fry *et al*, 1998) or by adding heparin to the CM (**Fig 4*C,D***). Heparin treatment did not interfere as such with the capability of HERs in MCF-7 cells to become activated, because the combination with rHRG-β1 (lacking a heparin-binding domain) still resulted in full phosphorylation of HERs in these cells (data not shown). To quantify the phosphorylating capacity of the Bowes melanoma CM, we made a comparison with the phosphorylation of MCF-7 cells that had been treated with different concentrations of rHRG-β1. As also shown by others (Aguilar and Slamon, 2001), phosphorylation of a 185-kDa protein could readily be detected using 0.5 ng per mL rHRG-β1 (**Fig 4*E***). Treatment with CM of Bowes melanoma cells resulted in a phosphorylation at 185-kDa equivalent to ±5 ng per mL (700 pM) rHRG-β1, correlating with a concentration of ±100 pM 45-kDa HRG in the CM. Also the kinetics of this phosphorylation were similar, with phosphorylation occurring already after 1 min of treatment, suggesting a similar mechanism of direct receptor activation (**Fig 4*F***).

One of the well-described biologic effects of HRG is the rapid induction of spreading/scattering of epithelial islands (Spencer *et al*, 2000), which led us to test the effect of the CM of the HRG-positive melanoma cell lines in this assay. We found that a 2-h treatment of serum-starved MCF-7 islands with melanoma cell line CM resulted in a disruption of epithelial islands similar to that of treatment with rHRG-β1. This scattering was blocked by pretreating the cells for 30 min with PD168393 (**Fig 5*A***).

Based on the fact that HRG contains an extracellular heparin-binding domain, we performed precipitations using heparin beads on 50 × concentrated CM. Three consecutive precipitations completely abolished the ability of the CM to induce phosphorylation (**Fig 5*B***, *lane 4*) or spreading/scattering of epithelial MCF-7 islands (**Fig 5*C***). In contrast, after eluting the heparin-binding factors from the heparin beads, desalting, and dilution of these factors in serum-free medium, used for treating MCF-7 cells, the phosphorylation of a 185-kDa band (**Fig 5*B***, *lane 3*) as well as the induction of spreading/scattering (**Fig 5*C***) were evident. Both effects could be blocked by pretreating the cells for 30 min with PD168393 (**Fig 5*C,B***, *lane 6*).

An autocrine loop in Bowes melanoma cells leads to constitutive HER phosphorylation, MAPK activation, and increased growth Total lysates from different melanoma cell lines were immunostained for tyrosine-phosphorylated proteins. This revealed the presence of a highly phosphorylated protein at 185 kDa, possibly reflecting activated HER2 and HER3, in Bowes melanoma cells, but not in the other melanoma cell lines tested (**Fig 6*A***). This band was also found in the HRG-positive COLO-16 cells, but not in the weaker HRG-positive MDA-MB-231 cells. By precipitating HER2 and HER3 from Bowes melanoma cells and staining for tyrosine phosphorylated proteins, we could show constitutive phosphorylation of HER2 and HER3 (**Fig 6*B***, *middle panel*). Treating the cells for 30 min with PD168393 resulted in a complete block of this phosphorylation (**Fig 6*B***, *left and middle panels*). This was not due to alterations in receptor levels, as immunostaining for HER2 and HER3 showed no differences between untreated cells and cells treated with PD168393 (**Fig 6*B***, *right panel*). In line with this, when tested in a 5-day growth assay, PD168393 gave a significant ($p < 0.01$) and concentration-dependent growth inhibition of Bowes melanoma cells (**Fig 6*C***). This effect was not due to general cytotoxicity because virtually no growth inhibition was seen of MCF-7 cells (HER2/3-positive and HRG-negative) or BLM cells (HER2/HRG-positive and HER3-negative).

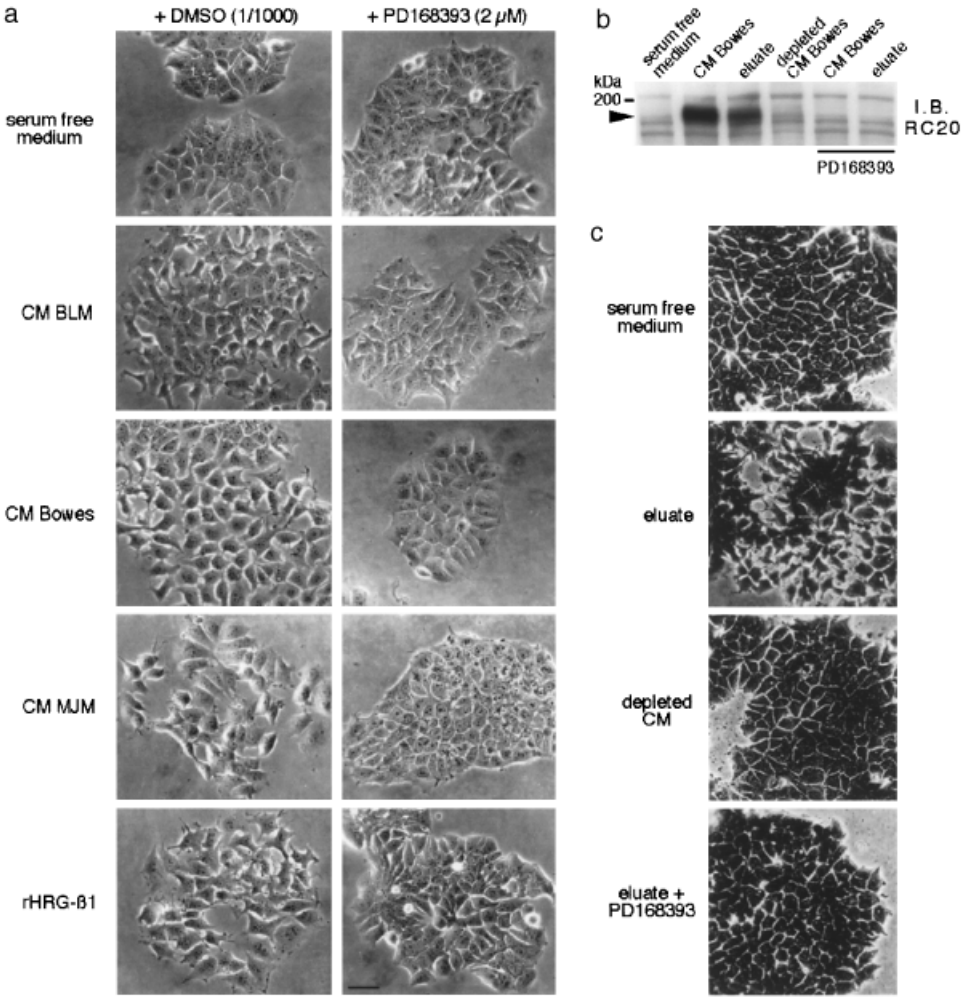


Figure 5. Scattering of MCF-7 epithelial islands by rHRG-β1 and by CM of HRG-positive cell lines. Serum-starved MCF-7 cells pretreated or not for 30 min with 2 μM PD168393, followed by an additional 30-min treatment and preparation of lysates (*b*) or followed by additional 2-h treatments before taking pictures of living cultures (*a*) or crystal-violet-fixed cultures (*c*). (*b*) Antiphosphotyrosine immunoblotting (*I.B.*) of whole-cell lysates of MCF-7 cells that were treated for 30 min with serum-free medium, CM of Bowes melanoma (*CM Bowes*), the heparin-binding fraction from CM Bowes (*eluate*) or CM Bowes depleted from heparin-binding factors. *Arrowhead,* ±185-kDa phosphorylated band. (*c*) Indicated conditions as in (*b*). *Bars,* 50 μm.

Constitutive receptor activation in Bowes melanoma cells could be inhibited not only by directly blocking its kinase activity, but also the interference with ligand binding resulted in this effect. This was evident from the rapid, concentration-dependent inhibition of HER phosphorylation and the consequent growth inhibition seen upon treatment with heparin (**Fig 6*D,E***). The MAPK pathway is a major pathway implicated in uncontrolled growth of melanomas (Govindarajan *et al*, 2003; Satyamoorthy *et al*, 2003). Because it is also a well known signaling pathway activated by HRG (Pinkas-Kramarski *et al*, 1998), we checked its activation status in Bowes melanoma cells by western blotting using a phospho-MAPK-specific antibody. Constitutive HER phosphorylation of Bowes melanoma cells was accompanied by a constitutively active MAPK pathway (**Fig 6*F***, *lane 1*). Blocking HER phosphorylation with PD168393 rapidly led to a block of MAPK activation (**Fig 6*F***, *lanes 2,3*), showing that continuous HER activation is the main cause of the constitutively activated MAPK pathway in these cells. The importance of the continuous activation of this pathway for the growth of Bowes melanoma cells was shown in experiments in which we used PD98059, a MAPK inhibitor. Bowes melanoma cells were particularly sensitive to this inhibitor and showed significant growth inhibition at concentrations that did not have any effect on growth of control BLM or MCF-7 cells (**Fig 6*G***). In conclusion, constitutive HER activation by autocrine HRG supports growth of Bowes melanoma cells via continuous MAPK activation.

Exogenous HRG stimulates growth of melanoma cells and melanocytes but does not protect melanocytes against apoptosis Melanocytes depend for their survival *in vitro* strongly upon the addition of extracellular stimuli. A prominent growth factor promoting growth and survival of these cells is bFGF (Halaban, 2000). To test whether HRG could have similar effects, we treated melanocytes with different concentrations of rHRG-β1, bFGF, or the combination of both. As is evident from **Fig 7*A***, rHRG-β1 stimulated HER phosphorylation of melanocytes and a variety of melanoma cell lines. rHRG-β1 concentration-dependently stimulated growth of melanocytes and could even provide an additive stimulus over bFGF (**Fig 7*B***). A significant growth stimulation was also seen when, e.g., MCF-7, MeWo, and A375 cells were treated with rHRG-β1 (data not shown). Because bFGF is also a potent antiapoptotic factor for melanocytes (Alanko *et al*, 1999), we next tested whether HRG might have a similar effect. Upon seeding of melanocytes on a collagen gel, these cells undergo apoptosis, round up, and become annexin V-positive owing to the exposure of phosphatidylserine at the outer surface of the cells. This can be inhibited by adding bFGF to the medium (Alanko *et al*, 1999) (**Fig 7*C,D***). Although a small decrease in the percentage of apoptotic cells was reproducibly seen upon treatment with rHRG-β1, this effect was negligible compared to the antiapoptotic effect of bFGF (**Fig 7*C,D***). Overall, the results from these assays show that HRG potently stimulates growth of melanoma cells and melanocytes, but does not protect melanocytes against collagen-induced apoptosis.

Defective HRG/HER system in various melanoma cell lines As shown in **Fig 8*A***, phosphorylation in Bowes melanoma cells was already maximal, because treatment with

rHRG-β1 did not result in an increase of phosphorylation. Consistent with this, no additional growth stimulation could be seen upon treatment with rHRG-β1 (data not shown). The observation that the HRG-positive BLM cells lack constitutive receptor activation (**Fig 6*A***) and cannot be activated upon addition of rHRG-β1 (**Fig 8*A***) is in agreement with the absence of the HRG-receptor HER3 (**Fig 2*A***) in these cells. The lack of HER3 also accounts for the unresponsiveness of FM45 cells to rHRG-β1 (**Fig 8*A***). MJM cells seem to have a defective HER-system as well: although HER2 and HER3 were present (**Fig 2*A***), treatment with rHRG-β1 led only to a minor phosphorylation, compared with treated MCF-7 cells (**Fig 8*A***). This minor phosphorylation was due to a small increase in phosphorylation of HER3, but not of HER2 (**Fig 8*B***, *lane 2*). To check whether HER2 can be activated in MJM cells, we treated them for 10 min with pervanadate, a phosphatase inhibitor. This led to phosphorylation of multiple proteins, including HER2 and HER3 (**Fig 8*B***, *lane 3*). Cotreatment with rHRG-β1 and pervanadate led to an additional increase in phosphorylation of only HER3 (**Fig 8*B***, *lane 4*), compared to treatment with pervanadate only. To verify whether mutations in HER2 could be responsible for the lack of activation in response to signaling from outside the cell, we sequenced all exons of the *HER2* gene. Apart from described polymorphisms in the sequences encoding the transmembrane domain (Ile^{655} to Val^{655}) (Ehsani *et al*, 1993) and the C-terminal tail (Pro to Ala), no mutations were found. Furthermore, biotinylation revealed that full-length HER2 was present at the plasma membrane of MJM cells (data not shown). So, despite the lack of mutations of HER2 and its localization at the plasma membrane in MJM cells, this receptor lacks the potential to become activated via stimulation with ligands.

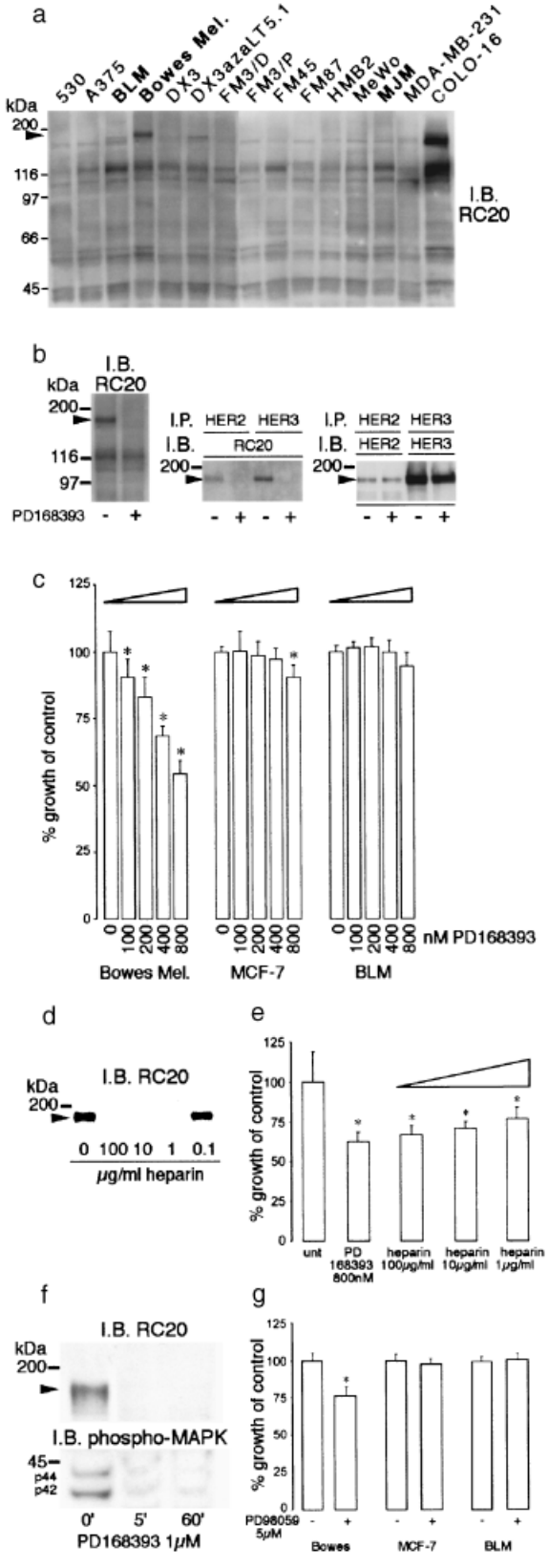


Figure 6. Correlation between constitutive HER activation in Bowes melanoma, continuous MAPK activation, and increased growth. (*a*) Whole lysates from 48-h serum-starved cell lines analyzed by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b,d*) Immunoblotting of tyrosine-phosphorylated proteins in serum-starved Bowes melanoma cells, treated or not with 2 μM PD168393 for 30 min (*b*) or with different concentrations of heparin for 10 min before making cell lysates (*d*). (*b*) The *left panel* indicates whole-cell lysates. *Middle and right panels,* equal amounts of protein were immunoprecipitated (*I.p.*) using anti-HER2 or anti-HER3 antibodies, before immunoblotting with anti-phosphotyrosine, anti-HER2, or anti-HER-3 antibodies. (*f*) Bowes melanoma cells treated with 1 μM PD168393 for different periods of time. Whole-cell lysates were analyzed by immunoblotting using antiphosphotyrosine (*top panel*) or antiphospho-MAPK antibodies (*bottom panel*). *Arrowheads,* ±185-kDa phosphorylated bands. (*c,e,g*) Growth, relative to vehicle-treated cells, as measured by sulforhodamine B assay. Cells were treated for 5 days with the indicated concentrations of PD168393 (*c,e*) or heparin (*e*) or with 5 μM PD98059 (*g*). *Asterisks,* differs significantly ($p < 0.01$) from controls.

DISCUSSION

In this report, we investigated the expression and function of the HRG/HER ligand–receptor system in 13 melanoma cell lines, compared to normal melanocytes. HER2 and HER3 were found to be the main members of the EGFR family expressed in these cells. Nevertheless, these receptors were not overexpressed, which is consistent with the analysis of both nevi and melanoma tumor material by others (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Similar amounts of HER3 protein were present in melanocytes and in 11 of the 13 cell lines. The fact that two melanoma cell lines (BLM and FM45) showed only low HER3 mRNA levels and even no detectable HER3 protein is a first example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Loss of HER3 may imply that transformed melanocytes no longer depend on HRG, normally provided by the surrounding keratinocytes (Schelfhout *et al*, 2000), for their survival. Downregulation or loss of other melanocytic RTK (e.g., c-Kit, protein-tyrosine kinase 4, ephrin receptor EphA4) during melanoma progression has been

described before (Easty and Bennett, 2000). Their loss may uncouple the melanocytes from certain physiologic regulatory mechanisms or may indicate an independence from the growth factor system involved. In the latter case, other systems (autocrine/paracrine) and/or activating mutations of other (intracellular) proteins may have substituted for the loss. In this context, it is noteworthy that we found a mutated N-*ras* allele in BLM, and also in MJM cells (see further), resulting in a constitutively active N-ras (data not shown), whereas FM45 cells have a mutation in the tumor suppressor PTEN (Guldberg *et al*, 1997), resulting in

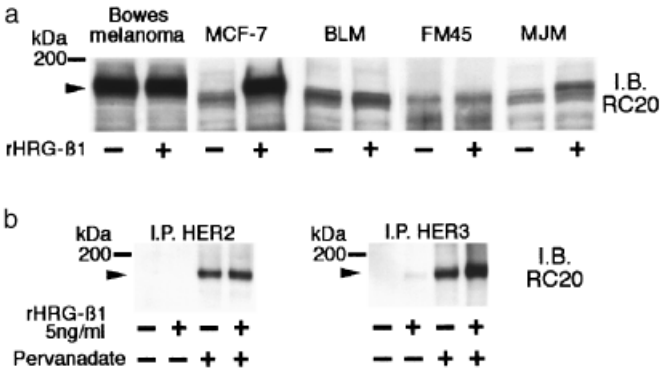
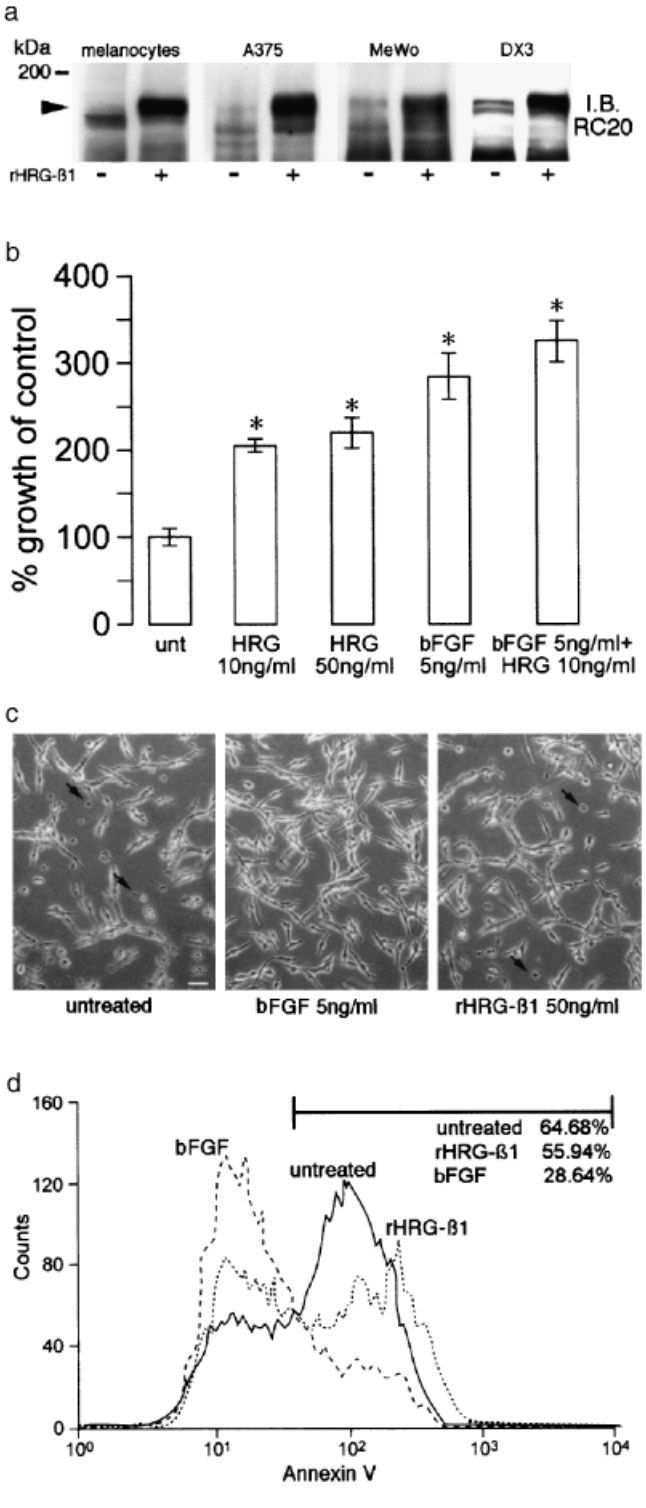


Figure 8. Deregulated HRG/HER system in several melanoma cell lines. (*a*) Serum-starved cells, treated with rHRG-β1 or not before the preparation of whole-cell lysates and analysis by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b*) Equal amounts of protein from cell lysates of MJM cells that had been treated for 10 min with rHRG-β1 and/or pervanadate were subjected to immunoprecipitation (*I.p.*) with anti-HER2 or anti-HER3 antibodies before immunoblotting using an anti-phosphotyrosine antibody. *Arrowhead,* ±185-kDa phosphorylated band.



constitutive phosphoinositide 3-kinase signaling. We have evidence (unpublished data) that the absence of HER3 in BLM and FM45 cells is accompanied by the loss of microphtalmia transcription factor M, a melanocyte-specific transcription factor necessary for melanocyte development. Interestingly, expression of both HER3 and microphtalmia transcription factor M has been described to be under the control of SOX-10, a transcription factor necessary for melanocyte development, thus making this protein a putative regulatory candidate (Verastegui *et al*, 2000; Britsch *et al*, 2001).

The MJM cell line represents a second example of a deregulated HRG/HER system in melanoma (**Fig 9**). Although it expresses HRG, HER2, and HER3, surprisingly, it cannot use the secreted HRG in an autocrine loop. Following treatment of MJM cells with exogenous HRG, HER2 is not activated at all, whereas HER3 becomes only weakly phosphorylated. The fact that a minute phosphorylation of HER3 still occurs is surprising, because HER3 lacks catalytic activity and needs HER2 for its phosphorylation in the absence of other HERs. We cannot exclude that HER1 or HER4, whose levels were below the detection limit of our western blotting experiments, account for this effect. We can exclude the possible (lack of) regulatory action of heparan sulfate proteoglycans to be responsible for the impaired response, because the used rHRG-β1 only consists of the EGF-like domain. Also the interference by circulating soluble HER2

Figure 7. Receptor-activating and growth-promoting, but no anti-apoptotic effects of exogenous HRG on melanoma cells and melanocytes. (*a*) Immunoblotting (*I.B.*), using an antiphosphotyrosine antibody, of lysates from serum-starved cells, treated or not with rHRG-β1. *Arrowhead,* ±185-kDa phosphorylated band. (*b*) Growth, relative to untreated cells, as measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Melanocytes were treated for 5 days with the indicated concentrations of growth factors. *Asterisks,* differs significantly ($p < 0.01$) from control. (*c*) Phase contrast photographs of melanocytes cultured on a collagen gel for 4 days with the treatments indicated. *Arrows,* rounded cells in melanocyte cultures that were left untreated or were treated with rHRG-β1. These rounded cells were not found in bFGF-treated cultures. *Bar,* 50 μm (*d*) Histogram, showing the profile of annexin V positivity in melanocytes that were cultured on a collagen gel in the absence of added growth factors (*solid line*) or in the presence of 5 ng per mL bFGF (*broken line*) or 50 ng per mL rHRG-β1 (*dotted line*). The percentage of annexin V-positive cells is indicated.

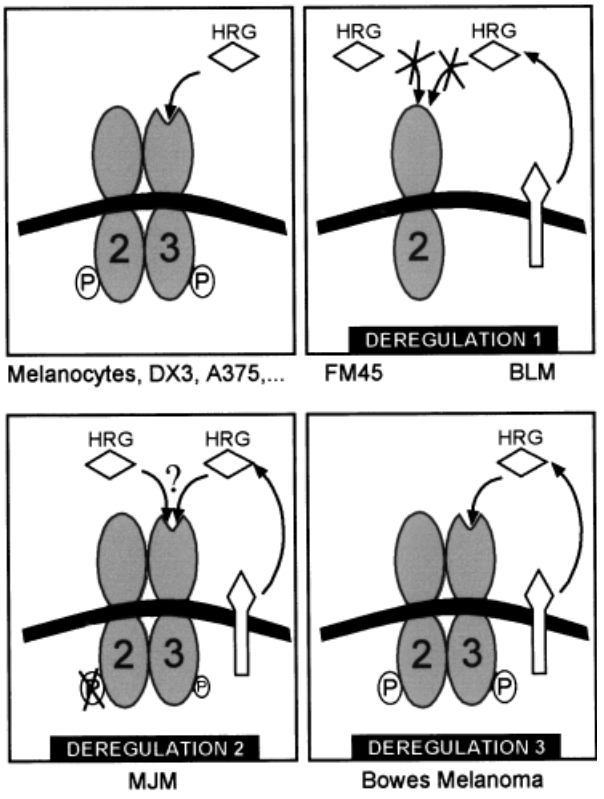


Figure 9. Schematic representation of the several deregulations of the HRG/HER system found in human melanoma cells. In melanocytes and the majority of melanoma cell lines, HERs can be activated by exogenous HRG. The absence of HER3 in BLM and FM45 cells or the presence of functionally inactive HER2 in MJM hampers HRG responsiveness. Bowes melanoma cells show constitutive HER activation, owing to the presence of an autocrine loop.

or HER3 ectodomains (Doherty *et al*, 1999; Aigner *et al*, 2001; Lee *et al*, 2001; Justman and Clinton, 2002) is unlikely, because CM from the HRG-positive MJM cells still induced activation of HERs in MCF-7 cells. Biotinylation experiments suggested a transmembrane localization of HER2 in MJM cells, whereas analysis of functional sequences failed to show mutations. Nevertheless, sequencing did reveal two polymorphisms, one of which (Ile to Val at position 655 in the HER2 transmembrane domain) was predicted to favor the formation of stable HER2 homodimers (Fleishman *et al*, 2002). Although it still needs to be shown whether the proposed model also holds for HER2 activation by heterodimerization (following ligand binding), it would mean that HER2 in MJM cells would be more easily activated than in, e.g., Bowes melanoma cells, which is in contrast to our findings. It thus seems unlikely that this polymorphism may provide an explanation for the observed lack of HER2 activation in MJM cells treated with HRG. A possible role for the other polymorphism we identified in MJM cells has not been established yet. Remaining explanations for the lack of HER2 activation following stimulation with HRG are the interference with ligand binding by sterical hindrance, the constitutive association or action of intracellular negative regulatory proteins or receptor mislocalization (although transmembrane).

Here, we have shown by western blotting, RT-PCR, cloning, and using functional assays the presence and function of HRG as new growth factors produced by human melanoma cells. In addition to three known HRG isoforms, we could identify a new isoform, designated α_4. This isoform combines the sequences that normally lead to either α- or β-isoforms. A similar combination was already described for the α_3-isoform, which differs from the α_4-isoform because the latter contains the coding sequence for the transmembrane domain (Wen *et al*, 1994) (**Fig 1**). Nevertheless, the resulting protein is the same, because this α–β combination leads to a frameshift, resulting in the generation of a stop codon upstream of the sequence encoding the transmembrane domain. This truncated protein is most likely cytosolic because the transmembrane domain functions as a signal peptide (Burgess *et al*, 1995). The presence of HRG in melanomas fits with the neuroectodermal origin of melanocytes and the fact that HRG are molecules typically expressed in neuroectodermal tissues (Marchionni *et al*, 1993; Meyer and Birchmeier, 1995). Although melanocytes showed HRG expression at the mRNA level, HRG could barely be detected at the protein level, suggesting the presence of (post)translational negative regulatory mechanisms in these cells. Activating mutations in H-*ras* have been shown to result in upregulation of HRG in mammary epithelial cells (Mincione *et al*, 1996). Although two of the HRG-producing cell lines (BLM and MJM) have an activating mutation in N-*ras*, transient transfections using dominant-negative and constitutively active N-ras constructs learned that this was unlikely to be the underlying cause of the increased HRG expression (data not shown). Thus, the molecular basis for the high HRG expression in some melanoma cell lines is not clear, yet. Although exogenous HRG did not exert a significant antiapoptotic effect, it potently stimulated growth of cultured melanocytes and melanoma cells and could even provide an additive growth stimulation over bFGF. Upregulation of HRG expression in melanomas may result in the generation of an autocrine loop and in the independence from HRG normally provided by the keratinocytes (Schelfhout *et al*, 2000). This decreased dependence from paracrine growth factors is one of the hallmarks of melanoma progression (Lázár-Molnár *et al*, 2000). Our data clearly show that in the Bowes melanoma cell line, in the absence of receptor overexpression, HER2 and HER3 are permanently activated, leading to continuous MAPK activation and stimulation of growth. This activation is due to continuous ligand–receptor interactions and not to, e.g., activating mutations. Arguments hereto are that the phosphorylation could be abolished by adding heparin to the culture medium and that refreshing of the culture medium led to a transient, gradual decrease in receptor phosphorylation, followed by a gradual recovery to the initial levels (data not shown). Thus, the Bowes melanoma cell line, with its autocrine loop, represents a third example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Expression of the HRG/HER system was also described in various other types of cancers (e.g., breast, lung, endometrium, thyroid, head and neck, colon, ovarium) (Ethier *et al*, 1996; Fernandes *et al*, 1999; Srinivasan *et al*, 1999; Fluge *et al*, 2000; O-Charoenrat *et al*, 2000; Gilmour *et al*, 2002; Venkateswarlu *et al*, 2002). Although in only some of these studies constitutive receptor activation, owing to an autocrine loop, was looked at, it may play a role in the other cases as well, rendering it a possible target for future therapies. In line with this is the increased attention that is being given toward receptor activation status in certain cancers, rather than only taking into account receptor levels as a criterium of malignancy (Thor *et al*, 2000).

Overall, it is striking that two of the three HRG-positive cell lines, BLM and MJM, cannot use the secreted HRG in an autocrine loop, because of the absence of HER3 or because of an impaired HER2 activation, respectively. Still, in a physiologic situation, the HRG secreted by such cells may have prominent effects on the surrounding cells, directly or indirectly contributing to malignant progression. Direct effects may include an increased motility of the surrounding keratinocytes (Schelfhout *et al*, 2002), possibly rendering the environment in which the melanocytes reside less tight. Indirect effects may be the stimulation of angiogenesis owing to a HRG-mediated upregulation of vascular endothelial growth factor or increased expression of other growth factors by the target cells (O-Charoenrat *et al*, 2000; Talukder *et al*, 2000; Yen *et al*, 2000; Ruiter *et al*, 2002).

In summary, we have shown the presence of HRG, including one new isoform, as new factors produced and secreted by human melanoma cell lines. The HRG/HER system is functional in melanocytes and in the majority of melanoma cell lines, leading to growth stimulation. Nevertheless, multiple deregulations in this growth factor system may release the melanocytes from their natural dependence on keratinocyte-derived factors and thus represent a step toward melanoma progression. Lack of stimulation by HRG in some melanoma cell lines is due to the loss of expression of HER3 protein or to a severely impaired HER2 activation. In contrast, the aberrant expression and secretion of HRG by melanoma cells may serve as an autocrine and/or paracrine signal, promoting cell growth and/or migration. These distinct types of deregulation of the HRG/HER system may contribute to the malignant phenotype of melanoma cells. In the future, it will be important to verify whether these deregulations are present in tumor samples of melanoma patients and may become a therapeutic target for this disease with ever increasing incidence.

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I.4. Melanoma cells secrete follistatin, an antagonist of activin-mediated growth inhibition

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ORIGINAL PAPER

Melanoma cells secrete follistatin, an antagonist of activin-mediated growth inhibition

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Using a proteomic approach to screen for new growth factors released by melanoma cells, we identified follistatin as a major heparin-binding factor in medium conditioned by the Bowes melanoma cell line. Since follistatin is primarily studied in relation to its neutralization of activin, a member of the transforming growth factor-β family of ligands, the expression and function of this receptor system was investigated in a panel of melanoma cell lines and melanocytes. All cell lines expressed activin receptors and showed phosphorylation of Smad signal transduction molecules upon treatment with activin. Secretion of follistatin, either native or after retroviral transduction, efficiently prevented Smad activation or activation of an activin-responsive luciferase reporter construct. In melanocytes, activin treatment led to growth inhibition and induction of apoptosis. These effects were counteracted by cotreatment with follistatin. In summary, we characterized the activin–activin receptor system in melanocytes and melanoma cell lines and found that secretion of follistatin by melanoma cells may represent an effective way to circumvent activin's negative regulatory effects.
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Keywords: follistatin; activin; Smad; melanoma; growth; apoptosis

Introduction

Melanocytes, the pigment-forming cells of the skin, are a quiescent cell population located in the basal layer of the epidermis. Their growth is controlled by an interplay with the surrounding keratinocytes (Meier *et al*., 1998; Lázár-Molnár *et al*., 2000). Disturbance of this tight control may result in an imbalance of stimulatory versus inhibitory factors and may represent a first step towards malignant transformation. The transforming growth factor-β (TGF-β) family of growth factors has been described as an important growth-regulatory system in melanocytes. Whereas TGF-β acts as a potent inhibitor of growth and inducer of apoptosis in normal melanocytes, these effects are often much less pronounced, absent or even inversed in melanoma cells (Rodeck *et al*., 1994, 1999; Alanko and Saksela, 2000).

Activins are TGF-β-like dimeric molecules that were initially isolated as stimulators of follicle-stimulating hormone secretion (hence the name) (Ling *et al*., 1986; Vale *et al*., 1986). They consist of two disulfide-linked β subunits, translated from one of three known (A, B or C) human inhibin β gene transcripts, the main activin proteins being the homodimeric activins A and B and the heterodimeric activin AB (Knight, 1996). They exert their biological effects by interacting with two types of transmembrane serine/threonine kinase receptors (types I and II). Following binding of the dimeric ligand to two type II receptors (II or IIB), two type I activin-like receptor kinases (ALK4 = activin receptor IB) are recruited and activated by phosphorylation in *trans*. On their turn, the type I receptors will phosphorylate cytoplasmic Smad proteins (Smad2 and 3) which, after forming a complex with Smad4, will translocate to the nucleus to regulate transcription of activin-responsive genes (Shi and Massagué, 2003). Activins have been implicated in the control of diverse cellular processes, ranging from tissue patterning during embryogenesis to the control of homeostasis, cell growth and differentiation in multiple adult tissues (Ball and Risbridger, 2001; Luisi *et al*., 2001; Risbridger *et al*., 2001; Chen *et al*., 2002; Welt *et al*., 2002). The effects of activin are cell-type specific: in some tissues or cell types, it stimulates proliferation, while in others it induces differentiation, growth inhibition and/or apoptosis. Disruption or deregulation of activin signaling is associated with multiple pathological states, including inflammation, reproductive disorders and carcinogenesis (Risbridger *et al*., 2001; Chen *et al*., 2002; Cho *et al*., 2003). Cells may escape from the growth inhibitory effects of activin

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in several ways: by mutation or downregulation of activin responsive genes or inhibition of their actions or alternatively, by alterations interfering with the activin signal transduction pathway. The latter may occur both by mutations or inhibitory interactions at the receptor level as well as at the level of the Smad signal transduction molecules (Chen *et al*., 2002). Alternatively, several factors (inhibins, cripto, follistatin) may interfere with the ability of activin to gain access to and/or assemble its signaling receptors (Gumienny and Padgett, 2002; Gray *et al*., 2003).

Follistatin (FS), a monomeric secreted glycoprotein that binds activin with high affinity, blocks the ability of activin to bind its cell-surface receptors (de Winter *et al*., 1996). FS was first isolated from ovarian follicular fluid on the basis of its ability to suppress the secretion of follicle-stimulating hormone from pituitary cells (Robertson *et al*., 1987; Ueno *et al*., 1987), an activity that later was found to be due to the sequestering of activin secreted by these cells (Nakamura *et al*., 1990). Up to now, almost all known biological effects of FS are due to its high affinity, nearly irreversible, binding to activin (Nakamura *et al*., 1990; Schneyer *et al*., 1994). Alternative splicing at the 3′ end of the *follistatin* gene results in two isoforms, FS-288 and FS-315, the latter having a 27 amino-acid extension at its C-terminus (Shimasaki *et al*., 1988). Both contain an N-terminal activin-binding domain and three consecutive 10-cysteine follistatin domains, the first of these containing a lysine-rich heparin-binding motif (Wang *et al*., 2000; Sidis *et al*., 2001; Innis and Hyvönen, 2003). FS-288 is primarily bound to cell surface heparan-sulfate proteoglycans, presumably creating a barrier that prevents activin from accessing its receptors (Delbaere *et al*., 1999; Sidis *et al*., 2002) and leading to endocytosis and degradation of surface-bound activin (Hashimoto *et al*., 1997). FS-315 contains an additional acidic C-terminal tail which interferes to some extent with heparin binding, rendering this the major isoform in solution (Schneyer *et al*., 1996). This feature may explain the different potencies of both isoforms in inhibiting the actions of endogenous versus exogenous activin. It is not fully clear what the relative importance of the different isoforms is. Although FS-315 transcripts, in general, are more abundant than FS-288 transcripts, the biologically active form of FS-315 *in vivo* may be a processed shorter form with properties similar to those of FS-288 (Inouye *et al*., 1991).

Based on the identification of FS as a new major heparin-binding factor released by melanoma cells, we characterized the activin/activin receptor system as a new growth factor system in melanoma and melanocytes and investigated the potential role of FS in controlling this system.

Results

Identification of follistatin as a factor secreted by melanoma cell lines

In a screening for new growth factors secreted by melanoma cell lines, conditioned medium (CM) from the Bowes melanoma cell line was prepared, concentrated 50 times, and subjected to a triple precipitation procedure using heparin beads in order to deplete it from heparin-binding factors. The initial and depleted concentrated CM and the resulting heparin-binding fraction were subjected to gel electrophoresis, followed by protein silver staining of the gel. This resulted in a very similar pattern of proteins in the concentrated CM and in the depleted concentrated CM, indicating that the vast majority of secreted proteins did not bind to the heparin beads (Figure 1a, lanes 1 and 2). However, a band at ± 45 kDa (indicated with an asterisk) was not present in the depleted CM. This band corresponded with the major band found in the heparin-binding fraction (Figure 1a, lanes 3 and 4). N-terminal sequencing identified this band as FS. The sequence obtained, GN(C)WLRQA, represents the N-terminus of mature FS after cleavage of its precursor form (Esch *et al*., 1987; Robertson *et al*., 1987; Ueno *et al*., 1987; Nakamura *et al*., 1990) (Figure 1b). To ascertain that this major band is due to FS and does not represent a major other, N-terminally blocked protein, we performed an in-gel digestion of this band, using trypsin. The resulting peptides were extracted and then submitted to mass spectrometric analysis. This confirmed that the major heparin-binding factor released by Bowes melanoma cells was indeed FS (Figure 1b). Western blotting and immunostaining with an anti-FS antibody confirmed the presence of FS in concentrated Bowes melanoma CM, but not in concentrated CM depleted of heparin-binding factors (Figure 1c). To verify whether the different molecular weight forms recognized by the antibody represent different FS isoforms or result from differential post-translational modifications (glycosylation, C-terminal processing), the Bowes melanoma cell line was treated for 24 h with the glycosylation inhibitor tunicamycin before harvesting the CM. Subsequent immunostaining of this concentrated CM with an anti-FS antibody revealed only one, faster migrating band (Figure 1d), meaning that the different bands seen in CM from the Bowes melanoma cell line represent differentially glycosylated forms of one FS isoform. As the tryptic peptides that had been identified by mass spectrometry did not allow us to conclude which isoform (FS-288 or FS-315) was produced by these cells, we focused further on the tryptic fragment that would be obtained from the C-terminus. However, although we screened specifically for masses of potential C-terminal peptides by mass spectrometry, we could not identify this peptide. This may be due to the highly hydrophilic nature of the C-terminus or, alternatively, may mean that the C-terminus of mature FS is close to the last trypsin cleavage site, and would thus give rise to a peptide that is too small to be detected.

As an alternative approach to identify which FS isoform was produced, RT–polymerase chain reaction (PCR) was performed. A primer set was designed such that different lengths of amplification products were obtained, depending on the isoform present. This primer set was used to amplify cDNA from Bowes melanoma cells, melanocytes and melanocytes that had been

cultured in the presence of the phorbolester PMA, a known upregulator of FS (Tano *et al*., 1995). As compared to nonstimulated melanocytes, high FS-315 mRNA levels were found in Bowes melanoma cells and in melanocytes cultured in the presence of PMA. In all cases, only an amplification product derived from the FS-315-encoding mRNA was obtained (Figure 1e). This isoform has been described to be the major FS isoform in solution (in contrast to FS-288, which is primarily cell surface bound), consistent with our isolation of FS as a factor secreted in the medium.

To verify whether FS production was also seen in other melanoma cell lines, we analysed concentrated CM from a panel of melanoma cell lines by Western blotting (Figure 1f). This revealed that, albeit lower than was found for the Bowes melanoma cell line, FS secretion was easily detectable in several cell lines (530, A375, DX3), whereas lower (FM45, FM87, HMB2) to faint signals were obtained in others (e.g. FM3/P, MeWo). None of the cell lines accumulated higher FS levels than those found for the Bowes melanoma cell line. Also here, RT–PCR analysis learned that FS-315 was the only isoform produced by melanoma cells (data not shown). In conclusion, several melanoma cell lines release FS-315, either as a full-length or a C-terminal-truncated form, in their medium.

Expression of activin and activin receptors in melanoma cell lines

Since nearly all known effects of FS are mediated via its binding to and neutralization of activin, we first characterized whether activin can act on melanoma cells. Therefore, we performed RT–PCR for activin receptors I, IB, II and IIB. Of these, activin receptors IB and IIB were highly expressed in all melanoma cell lines and melanocytes (Figure 2). To verify whether activin and inhibin were produced by melanocytes and the melanoma cells, we performed RT–PCR for the inhibin α-subunit and the β_A, β_B and β_C subunits (Figure 2). This revealed a high expression of the β_A subunit in the majority of the melanoma cell lines, suggesting that activin A, a homodimer of two β_A subunits, is the major activin expressed in this type of cells. Melanocytes cultured in the presence of PMA had higher mRNA levels of inhibin-α, -β_A and -β_B, consistent with the PMA-induced upregulation of activin expression and secretion in a variety of cells (Cho *et al*., 2003). To quantify activin A secretion by the melanoma cells, we set up an ELISA, which allowed us to detect activin A levels as low as 0.25 ng/100 μl. When 25-fold concentrated CM of the melanoma cell lines was tested in this ELISA, no specific signal could be obtained, meaning that activin A levels in the melanoma cell lines do not exceed 0.1 ng/ml (data not shown). Using a similar ELISA to detect FS, a signal was easily obtained using crude CM from the Bowes melanoma and FS-transduced FM3/P cell lines, whereas vector-transduced FM3/P cells were completely negative (data not shown). These data suggest that activin A is secreted in only very low amounts by the melanoma cells, or may remain cell-surface associated, leading to residual levels in the medium, or may, in contrast to FS, be subject to fast proteolytical breakdown.

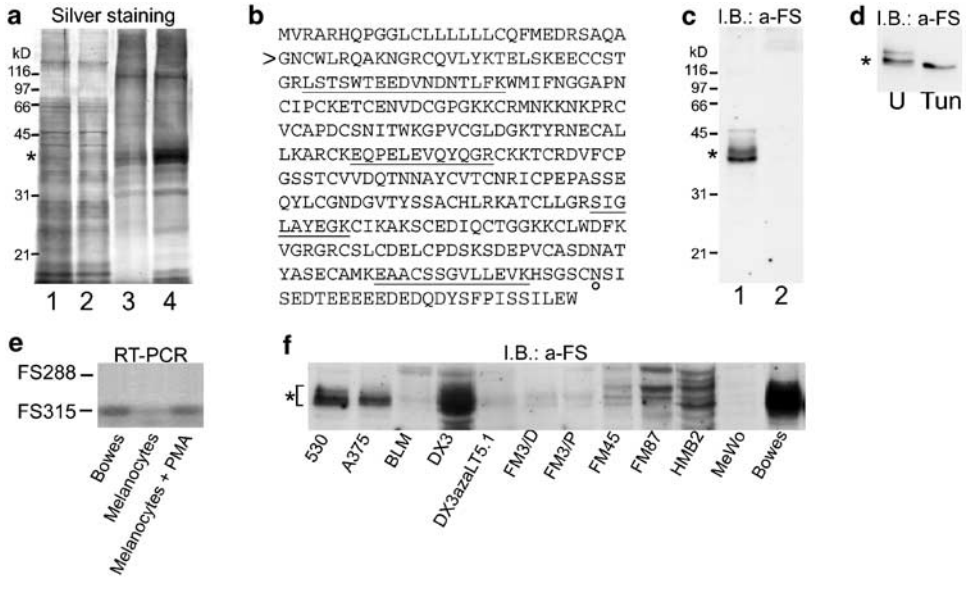

Figure 1 Follistatin secretion by melanoma cell lines. (**a**) Protein silver staining and (**c**) immunoblotting (I.B.) with an anti-FS antibody of 50 × concentrated CM of Bowes melanoma cells (lane 1), CM depleted from heparin-binding factors (lane 2), the heparin-binding fraction of the CM (lane 3) or a more concentrated heparin-binding fraction (lane 4). (**b**) Sequence of FS precursor with the sequences identified by mass spectrometry (underlined). > Indicates the N-terminus of mature FS that was identified by N-terminal sequencing. ° Indicates the C-terminus of FS-288. (**d**) Immunoblotting with anti-FS antibody of 50 × concentrated CM of Bowes melanoma cells, left untreated (U) or treated for 24 h with 10 μg/ml tunicamycin (Tun). (**e**) RT–PCR analysis of FS mRNA in the indicated cells. (**f**) Immunoblotting with anti-FS antibody of 50 × concentrated CM of the indicated cell lines. The asterisk indicates the band(s) corresponding to FS

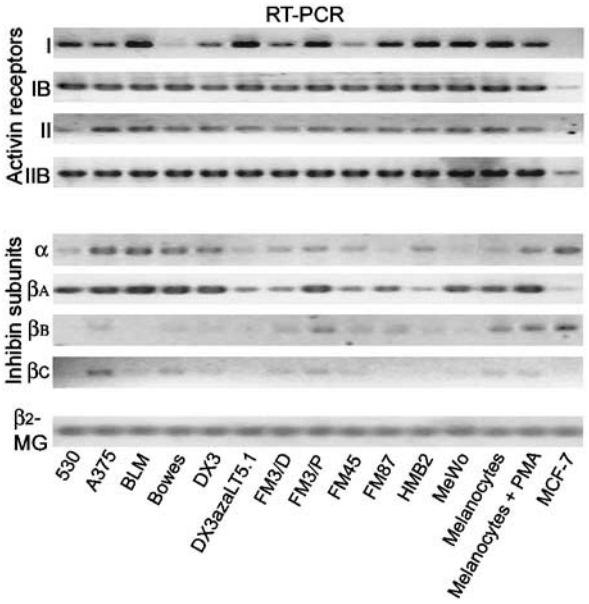

Figure 2 Expression of the activin–activin receptor system in melanoma cell lines and melanocytes. RT–PCR analysis of activin receptors and activin/inhibin subunits in the indicated cell lines. β2-microglobulin (β2-MG) was used as a control

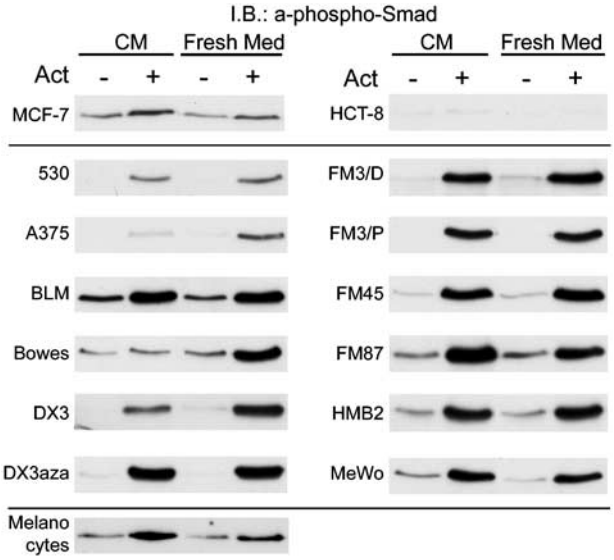

Figure 3 Activin treatment of melanoma cells and melanocytes induces Smad activation. Immunoblotting (I.B.), using an anti-phospho-Smad antibody of whole-cell lysates of the indicated cells. All cell lines were serum starved for 48 h before a 30-min treatment with activin (10 ng/ml) in their medium (CM) or in fresh serum-free medium that was added to cells that had been washed three times with PBS (Fresh Med). In the case of melanocytes, CM and Fresh Med indicate serum-containing medium

Activin-induced Smad-phosphorylation in melanoma cell lines

Receptor activation by members of the TGF-β family of growth factors is typically followed by activation (phosphorylation) of receptor Smads (Shi and Massagué, 2003). Using a phospho-Smad-specific antibody, we determined whether the addition of activin indeed resulted in activation of Smads in the cells. We therefore added activin to serum-free medium or to medium conditioned for 48 h by the melanoma cell lines. Positive and negative controls, respectively, were the human breast cancer cell line MCF-7 (Liu *et al*., 1996) and the human colon cancer cell line HCT-8, the latter having a nonsense mutation in activin receptor II (Hempen *et al*., 2003) (Figure 3, upper panel). Note that basal Smad activation levels seen in Figure 3 should not be compared between cell lines, since differences in band intensities between blots may be the result of different film exposure times. When activin was added in fresh serum-free medium, Smad phosphorylation increased in all melanoma cell lines tested, meaning that the first steps of the activin signal transduction (receptor binding and activation of signal transduction molecules) remained intact (Figure 3, middle panel, last two lanes). Of note, the Smad activation observed was much more pronounced than that seen in treated MCF-7 breast cancer cells, possibly due to the higher receptor levels in all of the melanoma cell lines (Figure 2). Smad phosphorylation following activin treatment in fresh, serum-containing medium was also seen in melanocytes (Figure 3, lower panel, last two lanes). Here, serum starvation was omitted, since the majority of melanocytes does not survive, or goes into apoptosis, after a 48-h serum depletion (unpublished observations). To verify whether the presence of secreted factors influences the response of the cells, activin was added to medium conditioned by the cells. In several melanoma cell lines, this resulted in a lower (e.g. A375, DX3) or even completely absent (Bowes) Smad activation, when compared to activin treatment in fresh medium (Figure 3, first two lanes). This means that, although these cell lines have retained the intrinsic capacity to respond to activin in terms of Smad activation, the secretion of (an) inhibitory factor(s) prevents this activation. Since the prevention of Smad activation following activin treatment in the CM correlated well with the presence of FS in this CM (Figure 1f), the action of FS, secreted by melanoma cells, was further investigated.

Follistatin-315 secretion by melanoma cell lines inhibits activin-induced Smad activation

In the Bowes melanoma cell line, secreting the highest amounts of FS (Figure 1f), Smad phosphorylation was not altered upon addition of activin to the CM (Figure 3 and 4a). We therefore chose this cell line to provide further evidence that activin's stimulatory effects are indeed blocked by secretion of FS into the medium. Bowes melanoma cells were first washed extensively before the addition of activin to CM or to fresh medium. When administered in the CM, activin treatment did not lead to an increase in Smad phosphorylation, irrespective of whether (Figure 4a, lanes 3 and 4) or not (lanes 1 and 2) the cells had been washed before the treatment. Addition of activin to serum-free medium that was added to washed cells did result in increased Smad

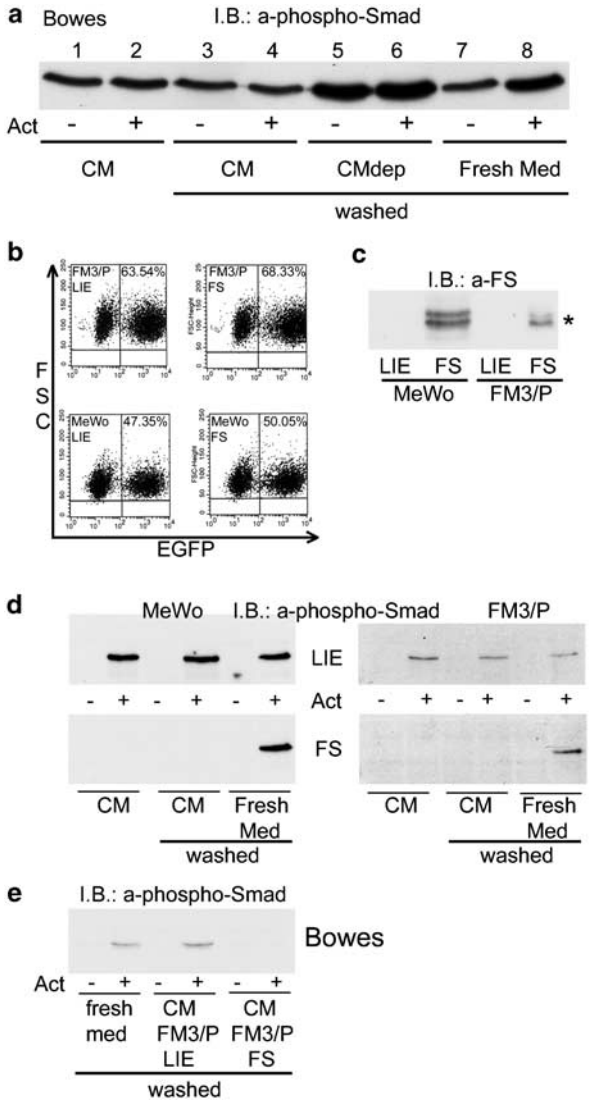

Figure 4 Follistatin secretion prevents activin-mediated Smad activation. (**a**) Immunoblotting (I.B.), using an anti-phospho-Smad antibody of total lysates of Bowes melanoma cells. The 48-h serum-starved cells were washed three times with PBS (washed), or were not, and treated with activin (10 ng/ml) for 30 min in their CM (CM), in this CM depleted from heparin-binding factors (CMdep) or in fresh serum-free medium (Fresh Med). (**b**) Dot plot analysis of EGFP expression by the indicated retrovirally transduced cell lines. Percent positivity is indicated in the upper right panel. (**c**) Immunoblotting, using an anti-FS antibody, of 50 × concentrated CM of the indicated retrovirally transduced cell lines. The asterisk indicates the band corresponding to FS. (**d**) and (**e**) Immunoblotting (I.B.), using an anti-phospho-Smad antibody of total lysates of the indicated cell lines. The 48-h serum-starved cells were washed three times with PBS (washed), or were not, and treated with activin (10 ng/ml) for 30 min in their CM (CM) or in fresh serum-free medium (Fresh Med). LIE indicates cells transduced with empty vector, FS indicates cells transduced with FS-315. In (**e**), also non-self serum-free CM was used

activation (lanes 7 and 8). Taken together, this shows that Bowes melanoma cells release a factor in their medium that prevents activin-induced Smad activation. Since FS is a heparin-binding factor, we next depleted Bowes melanoma CM from heparin-binding factors (CMdep). Interestingly, treatment with CMdep already leads to an increase in Smad activation (Figure 4a, lane5), and addition of activin to this medium does not result in an increase in Smad phosphorylation (Figure 4a, lane 6). This raises the possibility that, by depleting Bowes melanoma CM from FS, the balance between endogenous activin and FS is disrupted, readily leading to Smad activation. Finally, to provide proof that the production and secretion of the FS-315 isoform by melanoma cell lines can indeed fully prevent Smad activation by activin, two FS-negative cell lines, FM3/P and MeWo, were infected with the retroviral vector pLZRS-FS315-internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP). An IRES allows both FS315 and EGFP to be translated separately from the same mRNA transcript, with expression levels of FS315 and EGFP being directly proportional. As a control, retroviral transduction with pLZRS-IRES-EGFP, encoding only EGFP, was performed. As shown in Figure 4b, transduction with retrovirus containing either an empty vector (pLZRS-IRES-EGFP, LIE) or FS (pLZRS-FS315-IRES-EGFP, FS) resulted in an efficiency of around 50% for both melanoma cell lines. As evidenced by Western blotting, FS-transduced, but not empty-vector-transduced cells, secreted FS into their medium (Figure 4c). Owing to this secretion, we reasoned that further sorting of these cell lines would not be necessary to investigate its effects: FS produced by the transduced cell lines was likely to protect nontransduced cells as well. No differences in morphology or growth could be observed between the two transduced populations, either in the presence or absence of activin (data not shown). When tested for Smad activation, both the empty-vector- and FS-transduced FM3/P and MeWo cell lines were responsive to activin, when added in serum-free medium (Figure 4d, lanes 5 and 6). However, similar to what was observed in the Bowes melanoma cell line, no increase in Smad activation occurred when activin was added immediately to FS-transduced cells (lanes 1 and 2) or, alternatively, was added to 48-h conditioned medium that was added to washed cells (lanes 3 and 4). T his contrasts with the empty-vector-transduced cells, which were still fully responsive to activin (lanes 1, 2 and 3, 4). In agreement with this, treatment of washed Bowes melanoma cells with activin in CM from FM3/P FS did not lead to increased Smad activation (Figure 4e, lanes 5 and 6), in contrast to its addition in fresh serum-free medium or in CM from FM3/P LIE (lanes 1, 2 and 3, 4). In conclusion, FS secretion by melanoma cells is an effective way to prevent Smad activation by activin.

Follistatin secretion by melanoma cell lines inhibits their response to an activin-responsive reporter gene construct

Since the activation of receptor-Smads does not necessarily mean that the signal will lead to alterations in gene transcription, we performed luciferase-reporter assays using the TGF-β- and activin-responsive CAGA promoter construct (Dennler *et al*., 1998). Activin treatment of FM3/P cells resulted in a twofold increase

of luciferase activity that could be completely inhibited by the addition of exogenous FS (Figure 5a). When the transduced cells were tested, a similar increase was seen in empty-vector-transduced cells, whereas the FS-transduced FM3/P cells did not show any increase in promoter activity (Figure 5b). This confirms the results of Smad activation and, again, shows that the secretion of FS by melanoma cells is an effective mechanism to neutralize the effects of activin.

Activin inhibits melanocyte growth and induces apoptosis

Since TGF-β has been described as a proapoptotic and growth-inhibitory factor for melanocytes, we addressed the question whether activin may have similar effects. Melanocytes cultured in the presence of activin showed a marked decrease in growth, as measured by mitochondrial activity using the MTT assay (Figure 6a). This decrease could be counteracted in a dose-dependent way by cotreatment with FS, which, when administered alone, did not have an effect on the growth of melanocytes. To verify whether an increase in apoptosis can provide an explanation for the observed decrease in growth, we performed Annexin V labeling of serum-starved melanocytes that were treated or not with activin. Annexin V has a high affinity for phosphatidylserine, which is exposed at the outer surface of the cells in early apoptosis. Deprivation of melanocytes from growth factors readily renders them susceptible to apoptosis. As is evident from Figure 6b, a 20-h treatment with activin results in a significant increase in apoptosis, thus providing a likely explanation for the decreased 'growth' observed, as measured by mitochondrial activity. Again, this effect could be counteracted by coadministering FS. In conclusion, FS protects melanocytes against growth inhibition by activin, at least partly, by counteracting its proapoptotic effects.

Discussion

Although TGF-β-like molecules have been studied for more than a decade in melanocytes and their malignant derivatives, surprisingly, only the roles of TGF-β itself have been looked at (Rodeck *et al*., 1994, 1999; Alanko and Saksela, 2000). In the present paper, based on the identification of FS as a major heparin-binding growth factor released by melanoma cells, we studied the activin/FS system as a new regulatory growth factor system in melanocytes and melanoma cell lines.

Melanocytes are neural crest-derived cells that migrate to the skin during an early stage of development. They form a quiescent population in the basal layer of the epidermis, making extensive contacts with the surrounding keratinocytes, to which they deliver melanin. Conversely, keratinocytes are crucial in controlling normal proliferation of melanocytes. They do so in a contact-dependent manner by the formation of E-cadherin-mediated adherens junctions with the melanocytes, necessary for and leading to the formation of gap junctions (Meier *et al*., 1998). A delicate balance exists between stimulatory and inhibitory factors, released either by the melanocytes themselves or by the keratinocytes. Loss of control of keratinocytes over melanocytes may thus be the result of altered cell–cell adhesion and/or alterations in growth factor systems in either of both partners. A shift in the balance between positive (basic fibroblast growth factor, insulin-like growth factor-I, heregulin, etc.) and negative (TGF-β, interleukin-6, etc.) regulators of melanocyte growth may represent a first step towards uncontrolled proliferation (Lázár-Molnár *et al*., 2000; Stove *et al*., 2003a). Here, we have shown that activin may serve as a novel negative regulatory factor controlling melanocyte proliferation. Exogenous activin is able to inhibit proliferation and induce apoptosis in primary melanocytes. These effects resemble those of TGF-β (Alanko and Saksela, 2000). *In vivo*, activin may be provided by the keratinocytes,

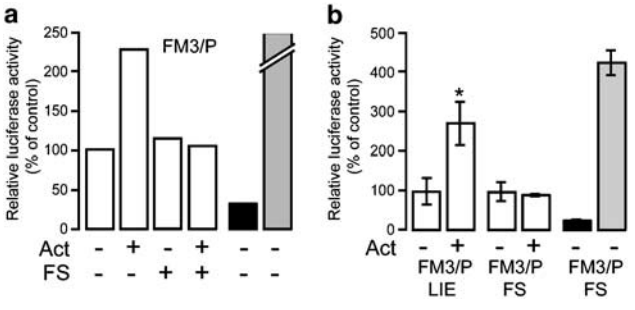

Figure 5 Follistatin prevents activin-mediated induction of a luciferase reporter construct. Activin (25 ng/ml) and/or follistatin (100 ng/ml) was added for 30 min to the indicated melanoma cell lines that had been transiently transfected with a β-galactosidase reporter construct and a luciferase-reporter construct with an activin-responsive promoter (open bars), without a promoter (black bars) or with a constitutively active promoter (gray bars). Bars represent normalized luciferase activities, relative to untreated cells. In (**a**), a representative experiment is shown, in (**b**), the asterisk indicates that the mean is significantly larger than the control ($P<0.05$)

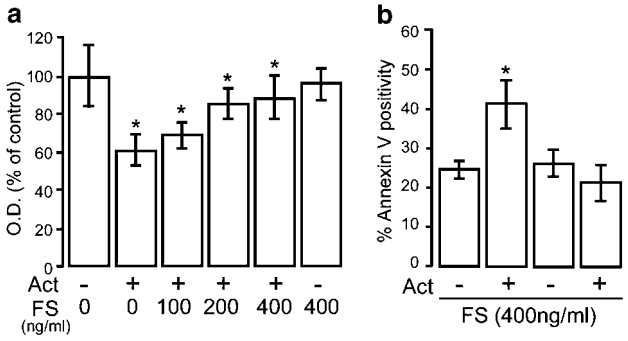

Figure 6 Follistatin counteracts activin-mediated growth inhibition and apoptosis in melanocytes. (**a**) Growth, relative to untreated cells, as measured by MTT assay. Melanocytes were treated for 4 days with activin (25 ng/ml) and the indicated concentration of follistatin. (**b**) % Annexin V positivity of serum-starved melanocytes that have been treated for 20 h with activin (25 ng/ml) and/or follistatin (400 ng/ml). Asterisks indicate means that differ significantly ($P<0.05$) from control

which are known producers of this molecule (Beer *et al*., 2000). Using transgenic mice, a role for keratinocyte-derived activin in the skin has been shown to exist in wound healing processes and in the regulation of cell matrix deposition (Munz *et al*., 1999; Wankell *et al*., 2001b). Although, *in vitro*, keratinocyte proliferation was shown to be decreased by activin, no decreased keratinocyte proliferation was observed *in vivo*, possibly because of the induction by activin of other factors that may act as prosurvival factors for the keratinocytes (Beer *et al*., 2000). Here, we found that both melanocytes and melanoma cells express mRNA for inhibin β subunits, primarily the βA subunit, presumably leading to the formation of the homodimeric activin A. Expression of this βA subunit was markedly higher in melanocytes cultured in PMA-containing medium, consistent with the fact that PMA is a known inducer of activin (Cho *et al*., 2003). However, surprisingly, measurement of activin A levels in melanoma CM revealed that activin A levels in none of the melanoma cell lines exceeded 0.1 ng/ml. Although it is possible that melanoma cells indeed secrete only residual amounts of activin A, we cannot exclude that it is rapidly degraded, or alternatively, remains associated with the cell surface, prohibiting measurement in the CM. Therefore, although several of the melanoma cell lines had higher expression of the βA subunit, when compared to the levels found in non-PMA treated melanocytes, a role for activin in melanomagenesis, as has been proposed for TGF-β (Shellman *et al*., 2000; Berking *et al*., 2001), remains speculative. Further *in vitro* and *in vivo* experiments, using activin-transduced cells, are needed to clarify this issue.

Early in malignant transformation, it is crucial for the survival of transformed cells to profit optimally from mitogenic stimuli, while escaping from inhibitory signals. Overexpression or mutation of receptor systems, or the gain of autocrine loops, may contribute to the first, while downregulation of inhibitory receptors or the secretion of ligand traps have been shown to contribute to the latter (Massagué and Chen, 2000; Blume-Jensen and Hunter, 2001; Gullick, 2001). Since activin acts as an inhibitory molecule for melanocytes, neutralization of its effects implies the release from a negative regulatory factor. As shown here, one way to do so is the production and secretion of FS. By binding to activin, FS sequesters it in the medium and prevents it from accessing its cell surface receptors. As a result, although these cell lines have an intact signaling machinery, no signaling occurs when activin is added to medium in which these cells have released FS previously. A similar activin-inhibitory effect has been described for the more recently described follistatin-related protein FLRG/FSTL3 (Sidis *et al*., 2002). Using RT–PCR, we found transcripts of the latter in melanocytes and in all melanoma cells (data not shown). However, we consider it unlikely that follistatin-related protein would play a major role in our system, since (1) no correlation could be found between its expression and the activin responsiveness of the cells, with FLRG/FSTL3-expressing cells still being fully responsive when activin was administered in their CM and (2) FLRG/FSTL3 lacks a heparin-binding site, while the activin-neutralizing effect of our CM can be abolished by depleting it from heparin-binding factors. FS is a known target gene of the canonical Wnt signaling pathway, in which β-catenin translocation to the nucleus results in transcriptional activation of target genes (Willert *et al*., 2002). In cancer, including melanoma, β-catenin mutations may lead to constitutive activation of this pathway, resulting in aberrant activation of these target genes (Rubinfeld *et al*., 1997). However, examination of β-catenin localization in our panel of melanoma cell lines did not reveal obvious nuclear β-catenin localization in any cell line (data not shown). Other factors capable of regulating FS secretion include retinoic acid, prostaglandin E_2, activators of protein kinase A and C, as well as growth factors such as keratinocyte growth factor and epidermal growth factor (Michel and Farnworth, 1992; Wankell *et al*., 2001a). Interesting in this respect is our recent identification of a potent autocrine loop of heregulin, an epidermal growth factor-like ligand, in the Bowes melanoma cell line, which secretes the highest amounts of FS (Stove *et al*., 2003a). Whether there is a crosstalk between receptor tyrosine kinase pathways and regulation of FS expression in melanoma cell lines is currently being investigated. We could not find a clear correlation between FS expression and the differentiation status of the cell lines in our panel: FS secretion was both present in cell lines that had lost several melanocytic markers (e.g. Bowes melanoma, A375 and DX3) as in cell lines that retained, at least partly, markers of differentiation (e.g. the pigmented 530 and FM87).

Surprisingly, although the first steps in the activin signaling pathway were intact in all cell lines tested, none of the melanoma cell lines showed significant growth inhibition following activin treatment, in contrast to what was seen for melanocytes. Several explanations may account for this. Important for activin's growth-inhibitory effects is the modulation of genes involved in cell-cycle regulation (Chen *et al*., 2002). These genes are frequently mutated in melanoma (Bartkova *et al*., 1996). Alternatively, activation of the MAPK pathway, either by mutation of signaling molecules (N-ras and B-raf) (Davies *et al*., 2002; Tuveson *et al*., 2003) or by autocrine loops (Lázár-Molnár *et al*., 2000; Stove *et al*., 2003a) was shown to be of major importance in many melanomas. Constitutive activation of this pathway was shown to result in insensitivity to growth inhibition by TGF-β, while still allowing other effects of these molecules to occur (Shellman *et al*., 2000).

In conclusion, the effects of the activin–follistatin system in melanoma may be dual: while early in progression the secretion of FS may be crucial as a protection against activin's inhibitory effects, cells in a later stage may use activin – either autocrine or paracrine – to maintain a state of dedifferentiation and to create a supportive microenvironment. It will be important to determine the presence of this growth factor system in tumor samples from melanoma patients

in different stages of progression, in order to evaluate whether it may become a therapeutic target.

Materials and methods

Cell lines

The cell lines were obtained and cultured as described before (Stove *et al*., 2003a). Epidermal melanocyte primary cultures were obtained from neonatal foreskins and established in M199 medium (Gibco BRL, Merelbeke, Belgium), supplemented with 2% fetal bovine serum, 10^{-9} M cholera toxin, 10 ng/ml basic fibroblast growth factor, 10 μg/ml insulin, 1.4 μM hydrocortisone and 10 μg/ml transferrin (all from Sigma, Bornem, Belgium). Postprimary cultures were maintained in low-calcium (0.03 mM) M199 medium, supplemented with the same factors and 10% fetal bovine serum. The melanocytic origin of all melanoma cell lines was checked by immunocytochemistry using two melanoma-specific antibodies, HMB45 (Enzo Diagnostics, Farmingdale, NY, USA) and NKI/C3 (Biogenex, San Ramon, CA, USA). All melanoma cell lines were positive for at least one of these markers (data not shown). As most of the experiments were carried out with Bowes melanoma cells, which were only positive for NKI/C3, additional electron microscopy was performed to confirm the presence of premelanosome-like structures in this nonpigmented cell line (data not shown).

Antibodies and reagents

Primary antibodies used were mouse monoclonal anti-follistatin (R&D Systems, Abingdon, UK) and rabbit polyclonal anti-phospho-Smad 2 (Cell Signaling Technology, Beverly, MA, USA). Recombinant activin and follistatin were purchased from R&D Systems, tunicamycin was obtained from Sigma.

Preparation of CM

Subconfluent monolayers were washed three times with phosphate-buffered saline (PBS), incubated for 24 h with serum-free medium, washed again three times with PBS, followed by a 48 h incubation with serum-free medium. The latter was cleared from cells by a 5-min centrifugation step at 250 *g*. The resulting supernatant was centrifuged for an additional 20 min at 2000 *g* to remove cell debris, filtered through a 0.2 μm filter, and stored at -20°C until use. To isolate the heparin-binding fraction from the CM, the latter was depleted from heparin-binding factors by triple precipitations with heparin beads (Bio-Rad, Hercules, CA, USA). Elution of the heparin-binding fraction was carried out with 1 M NaCl, followed by desalting using Microcon filters.

Western blotting

All lysates were made from cell cultures of approximately 90% confluence. For phosphorylation experiments, cells were washed three times with PBS and serum starved for 48 h. Then, the cells were treated without washing or, alternatively, after washing three times with PBS. Immediately after the treatments, the cells were washed three times with PBS before lysis with PBS containing 1% Triton X-100, 1% Nonidet P-40 (Sigma) and the following protease inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml) (ICN Biomedicals, Costa Mesa, CA, USA), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 μM), $NaVO_3$ (500 μM) and $Na_4P_2O_7$ (500 μg/ml) (Sigma). After clearing the lysates, protein concentration was determined using the Rc Dc Protein Assay (Bio-Rad), and samples were prepared such that equal amounts of protein were to be loaded. Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 min and separation of the proteins by gel electrophoresis on 8 or 12% polyacrylamide gels and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Quenching and immunostaining of the blots was performed in 5% nonfat dry milk in PBS containing 0.5% Tween-20. The membranes were quenched for 30 min, incubated with primary antibody for 2 h, washed four times for 5 min, incubated with horseradish peroxidase-conjugated secondary antibody for 30 min, and washed five times for 5 min. Detection was carried out using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with anti-tubulin antibody was performed routinely (not shown).

N-terminal sequencing and mass spectrometry

N-terminal sequence analysis of a PVDF electroblotted sample was performed on a 476A Protein sequencer. For mass spectrometric analysis, the gel slice containing the band of interest was excised and washed twice with 200 mM ammonium bicarbonate in 50% acetonitrile/water (20 min, at 30°C), and allowed to dry at room temperature. The tube was then chilled on ice and 8 μl of digestion buffer (50 mM ammonium bicarbonate, pH 7.8) containing 150 ng of modified trypsin was added. The sample was kept on ice for 45 min, after which 15 μl of digestion buffer was added, followed by overnight incubation at 37°C. The supernatant was recovered, and the peptides were extracted from the gel piece by washing twice with 60% acetonitrile/0.1% formic acid in water. The extracts were then combined, followed by drying in a Speedvac centrifuge. The sample was dissolved in 0.1% formic acid and injected in an automated nano-HPLC system (Dionex) (Devreese *et al*., 2002). The separated peptides were detected on-line by an ESI-Q-TRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) (Hager, 2002), equipped with a nanospray ion source (Protana, Odense, Denmark). In this method, an automated MS to MS/MS switching protocol was used for LC-MS/MS analysis of the peptides (Sandra *et al*., 2004). Briefly, first an enhanced MS scan as survey scan (m/z 400–1500), followed by an enhanced resolution scan of the two most intense ions was performed. If their charge state was +2 or +3, an enhanced product ion scan (MS/MS) of these ions was performed. The total cycle time of this set-up was approximately 4.5 s.

RT–PCR

Total RNA was extracted from approximately 5×10^6 cells using the Qiagen RNEASY kit (Qiagen, Chatsworth, CA, USA). In total, 1 μg of total RNA was reverse-transcribed with oligo-dT primers using the Qiagen RT kit (Qiagen) according to the manufacturer's instructions. PCR was performed on 250 ng template cDNA using the Qiagen Taq PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were carried out in a Minicycler (Biozym, The Netherlands) with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 50 s (denaturation), 61°C for 50 s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 10 min. Table 1 lists the primers that were used.

Table 1 Primers used for RT–PCR

Target cDNA	*Sense primer (5′–3′)*	*Antisense primer (5′–3′)*
Activin receptor I	ggaagatgagaagcccaagg	agagagaataatgaggccaacc
Activin receptor IB	cacatggagatcgtgggcac	ccgagggcataaaatatcagc
Activin receptor II	ggtgctatacttggtagatcag	tagggtggcttaggtgtaac
Activin receptor IIB	tgtcatggaaggccgtgatg	caggacaagcggctgcactg
Inhibin α	gtctccctctgctctgcgcc	ctctgcctttcctcccagctg
Inhibin β_A	ggcaggagcagatgaggaa	atgcggtagtggttgatgac
Inhibin β_B	gctctgcctcctcctttccacac	ctcaccccattctctccgac
Inhibin β_C	cgactgccaaggagggtcca	agatgctcaggaagagggagtc
Follistatin[a]	ccagcgagtgtgccatgaag	tcatcttcctcctcttcctcg
β2-microglobulin	catccagcgtactccaaaga	gacaagtctgaatgctccac

[a]Depending on the isoform, the primer set for FS gives rise to a PCR amplification product of 114 (for FS-315) or 378 (for FS-288) base pairs

Retroviral transduction

FS315 was *Tsp509*I digested from the FS315 plasmid construct, pSV2HF-315 (Inouye *et al*., 1991), and *Eco*RI subcloned into the retroviral vector LZRS-IRES-EGFP. This control vector expresses only EGFP from an IRES. The production of retroviral supernatant was carried out as described before (Stove *et al*., 2003b). Briefly, the Phoenix-Amphotropic packaging cell line (a kind gift from Dr GP Nolan, Stanford University School of Medicine, Stanford, CA, USA) was transfected with the LZRS-IRES-EGFP control vector, or with LZRS-FS315-IRES-EGFP, by using calcium-phosphate precipitation (Invitrogen, San Diego, CA, USA) to generate the retrovirus. The retroviral supernatant was spun (10 min for 350 *g*) and aliquots were stored at -70°C until use. For transduction of cell lines, cells were mixed with retroviral supernatant that was incubated for 10 min with Dotap (Roche Molecular Biochemicals, Mannheim, Germany). To increase transduction efficiency, cells were spun (90 min, 950 *g*, 32°C). Transduction efficiency was evaluated by flow cytometry.

Cell proliferation assay

A total of 12 500 melanocytes were seeded into the wells of a 96-well plate in 100 μl DMEM medium containing 10% fetal bovine serum. After 24 h, the cells were washed twice with serum-free DMEM and then treated with 200 μl serum-free medium, supplemented with growth factors as indicated. After 4 days, metabolic activity was measured with a colorimetric assay, using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), as described before (Stove *et al*., 2003a).

Annexin V staining

150 000 melanocytes were seeded in the wells of a 24-well plate in 1 ml DMEM medium containing 10% fetal bovine serum. After 24 h, the cells were washed twice with serum-free DMEM and then treated for 20 h with 500 μl serum-free medium, supplemented with growth factors as indicated. After detaching the cells, Annexin V staining and flow cytometric analysis were performed as described before (Stove *et al*., 2003a).

Luciferase reporter assay

A total of 100 000 cells were seeded per well of a 24-well plate. After 24 h, cells were transfected with 300 ng of pGL3basic, pGL3 control (Promega) or the CAGA luciferase reporter construct (Dennler *et al*., 1998), together with 100 ng pUT β-galactosidase reporter construct to normalize for transfection efficiencies. Some 24 h after transfection, cells were treated, or left untreated, for 20 h, after which they were lysed. After freezing the lysates for 1 h at -80°C and thawing, they were cleared from debris by centrifugation and used for measuring luciferase- and β-galactosidase activities.

Statistics

Differences between means were considered as significant when the *P*-value was <0.05, using Student's *t*-test.

Abbreviations

CM, conditioned medium; EGFP, enhanced green fluorescence protein; FS, follistatin; IRES, internal ribosomal entry site; LIE, LZRS-IRES-EGFP; PMA, phorbol 12-myristate 13-acetate; TGF-β, transforming growth factor-β.

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Part II: New aspects of the role of cadherins in cancer

II.1. Introduction: The cadherin/catenin system

Adhesion of cells to their neighbors determines cellular and tissue morphogenesis and regulates major cellular processes including motility, growth, differentiation and cell survival (Conacci-Sorrell *et al.*, 2002). Cadherins (Ca^{2+}-dependent adherent proteins) are a superfamily of single-pass transmembrane glycoproteins responsible for calcium-dependent cell-cell adhesion in a variety of tissues (Gumbiner, 1996). They are characterized by the presence of distinctive cadherin repeat sequences of ± 110 amino acids in their extracellular domains. Cadherins typically have several of these repeats tandemly organized in their extracellular domain, the connections between these repeats being rigidified by the specific binding of three Ca^{2+} ions (Figure II.1). They can be classified into several subfamilies: the type I (classical) and type II cadherins, linked to the actin cytoskeleton; desmosomal cadherins (desmocollins and desmogleins), which are linked to intermediate filaments; protocadherins, expressed primarily in the nervous system; and several 'atypical' cadherins, containing one or more cadherin repeats, but bearing no other hallmarks of cadherins. Type I 'classical' cadherins, which will be the main subject of the following discussions, include E-, N-, P- and R-cadherin (cadherins 1-4, respectively). They have 5 repeats (extracellular repeats EC1-5) in their extracellular domain, the first containing an HAV (histidine alanine valine) sequence in EC1. Via these extracellular domains, they mediate (primarily) homophilic protein-protein interactions. In 2002, Boggon *et al.* elucidated the crystal structure of the type I C(*C. elegans*)-cadherin ectodomain. The structure shows an adhesive interface involving the N-terminal cadherin repeats of opposing cadherin molecules, supporting the strand-dimer model of the

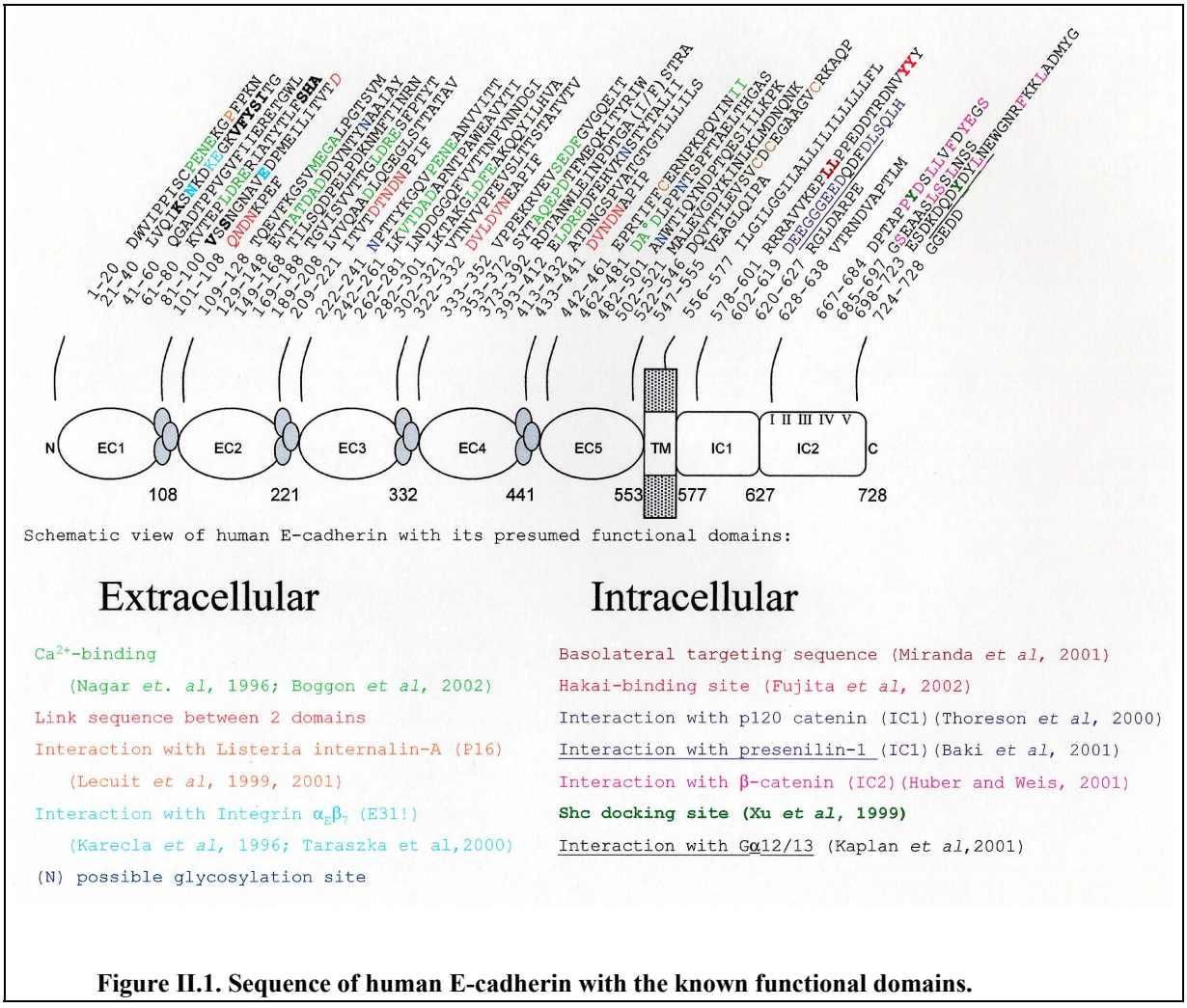

Figure II.1. Sequence of human E-cadherin with the known functional domains.

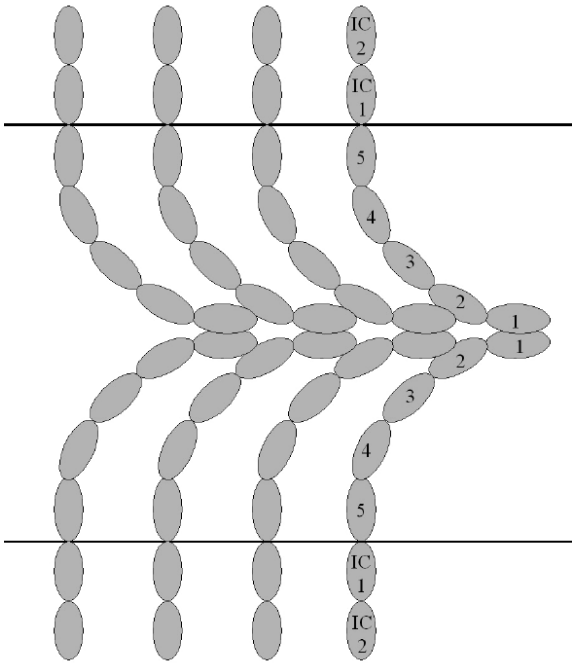

Figure II.2. Schematic representation of the strand dimer model, based on the model proposed by Boggon *et al* (2002). Represented are the *trans* interactions between cadherin molecules of opposing cells (EC1-EC1) and the *cis* interactions between cadherin molecules on the same cell (EC1-EC2)

binding interaction between cadherin molecules (see Figure II.2.). In this model, the interaction interface is mediated by the exchange of the N-terminal β-strands between the partner EC1 repeats, with as a central feature the docking of the conserved Trp2 side chain from one molecule into a hydrophobic pocket (with at its bottom the conserved HAV sequence) of the other.

Cell adhesion by cadherins is modulated by their conserved juxtamembrane (JMD) and catenin-binding (CBD) cytoplasmic domains, linking them to the actin cytoskeleton and cell-signaling pathways. Catenins, namely the β-, γ-, p120- and α-catenins are the best-documented interaction partners (Yap et al., 1997). β-catenin (βctn) (and perhaps also γ-catenin) serves as an important signaling molecule, playing a critical role in both tissue patterning during development and maintaining the normal cellular phenotype. Its signaling functions are regulated by its specific binding to the CBD of cadherins, and by interactions with receptor tyrosine kinases and transcription factors of the lymphocyte enhancer factor/T-cell factor family (Morin, 1999). P120-catenin (p120ctn) is also a member of the Armadillo/βctn gene family and is emerging as an important factor regulating cadherin function. Originally identified as a substrate for Src (Reynolds et al., 1992) and various receptor tyrosine kinases (Downing and Reynolds, 1991), p120ctn has subsequently been shown to interact directly with the JMD of cadherins (Reynolds et al., 1994, 1996) and has been implicated in cadherin clustering and cell motility, depending on the cell type and phosphorylation state (Daniel and Reynolds, 1997). The cadherin/catenin junctional complex is linked to the actin cytoskeleton via α-catenin (Rimm et al., 1995), thus strengthening its adhesive force. In general, differential expression of cadherins has been implicated in several aspects of embryonic development, including cell sorting during gastrulation and tissue morphogenesis (Gumbiner, 1996; Tepass et al., 2000), as well as the establishment of a differentiated cell identity. In addition, cadherins have been studied extensively with respect to their role in carcinogenesis (Conacci-Sorrell et al., 2002).

The best-characterized cadherin, E-cadherin (E-cad), has been studied as a prototype molecule for the whole cadherin superfamily. In many different cancers, including breast cancer, a relationship has been suggested between its reduced expression and neoplastic progression and metastasis (Berx and Van Roy, 2001; Strumane *et al.*, 2003). Bi-allelic inactivation of the E-cadherin gene by any combination of inactivating mutations in the coding region, loss of heterozygosity for 16q22.1, promoter hypermethylation and expression of trans-repressors (Snail, Slug, Twist, SIP1) have been demonstrated in various tumor types, including breast, diffuse gastric and hepatocellular carcinomas. Also mutation of other components of the cadherin/catenin system (e.g. mutation of α-catenin) may result in inactivation of the complex. Furthermore, posttranslational modifications of components of the cadherin/catenin system, including phosphorylation, glycosylation and proteolytical processing, may affect the functionality of the complex as well (reviewed by Strumane *et al.*, 2003). Finally, even when E-cadherin remains present in tumors, its function of an invasion suppressor may be overcome by the aberrant expression or upregulation of other cadherins, as has been shown for N-cadherin (N-cad) (Hazan et al., 2000) and cadherin-11 (Feltes et al., 2002). These cadherins have been associated with development or progression of breast carcinoma and are thought to interfere with E-cad mediated cell-cell adhesion, hamper its signaling and/or lead to activation of alternative signaling pathways (Yap and Kovacs, 2003).

The four manuscripts in this chapter describe novel features of the cadherin/catenin system in breast cancer and melanoma. A first manuscript, linking the previous chapter with the present, describes the pro-aggregating and anti-invasive effects of conditioned medium from a melanoma cell line on MCF-7/6 cells, harboring a functionally deficient E-cadherin/catenin complex. These effects could, at least partly, be attributed to the secretion of heregulin by the

melanoma cells. In a second manuscript, we studied the functional role of a classical cadherin, P-cadherin, in aggregation and invasion of melanoma cells. The last two manuscripts describe two novel ways of interference with the invasion-suppressor function of E-cadherin. In the first of these we studied the upregulation of P-cadherin in breast cancer cells in the absence of estrogen signaling and the subsequent promotion of invasion, mediated by the P-cadherin juxtamembrane domain. The last manuscript describes the action of a proteolytically generated E-cadherin ectodomain fragment, which is capable of counteracting cell-cell adhesion and inducing invasion.

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II.2. Bowes melanoma cells secrete heregulin, which can activate the E-cadherin/catenin invasion suppressor complex in human mammary cancer cells

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BOWES MELANOMA CELLS SECRETE HEREGULIN, WHICH CAN ACTIVATE THE E-CADHERIN/CATENIN INVASION SUPPRESSOR COMPLEX IN HUMAN MAMMARY CANCER CELLS

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SUMMARY

Invasiveness, the ability of cancer cells to migrate beyond the normal tissue boundaries, often leads to metastasis and thereby usually turns cancer into a fatal disease. At the molecular level, the E-cadherin/catenin complex is an example of a powerful invasion suppressor in epithelial cells. Since the absence of melanocytes has been associated with disturbances in epithelial organization, we decided to investigate the influence of molecules secreted by melanocytes on the function of the E-cadherin/catenin complex. We used the Bowes melanoma cell line as a source of such molecules. The conditioned medium of Bowes melanoma stimulated aggregation of human MCF-7/6 mammary adenocarcinoma cells at short (30 min) and at long (24 to 72 h) notice. This effect could be inhibited by MB2, an antibody against human E-cadherin. Conditioned medium of Bowes melanoma also inhibited invasion of MCF-7/6 cells into precultured chick heart fragments. Candidate molecules, such as insulin, insulin-like growth factor I, follistatin and interleukins were ruled out to be responsible for the effects, but heregulin mimicked some of the effects of the conditioned medium. Our data indicate that heregulin stimulates aggregation and inhibits invasion of MCF-7/6 cells *via* activation of the E-cadherin/catenin complex.

INTRODUCTION

The acquisition of invasive and metastatic behavior is the hallmark of cancer malignancy. Previous experiments have shown a key role for the E-cadherin/catenin complex in epithelial organization during normal development and in the regulation of the invasive behavior of cancer cells[1-3]. The cell-cell adhesion molecule E-cadherin is a 120 kD transmembrane glycoprotein linked to the actin cytoskeleton via β-catenin or plakoglobin and α-catenin[4]. β-Catenin is an intracellular 94-kDa molecule with 13 armadillo repeats[5]. These repeats are also present in plakoglobin. α-Catenin is an intracellular 102-kDa molecule with strong homology to vinculin[6]. Downregulation of the E-cadherin/catenin complex or of its function has proven to be a key element in the acquisition of the invasive phenotype in experimental and clinical cancer[7]. Up- and downregulation of the E-cadherin/catenin complex is sensitive to external factors and has been described previously[8]. Intercellular junctions may also be disturbed in non-cancerous epithelia, e.g. in Waardenburg's syndrome. This is an autosomal-dominant disorder characterized by sensorineural hearing loss, displacement of the inner canthus of the eyes and pigmentary disturbances due to absence of melanocytes[9]. The absence of melanocytes seems to interfere with the cochlear morphogenesis: in a mouse model ultrastructural cell-cell contact specializations in the epithelium are lacking and this absence is responsible for auditory dysfunctions[10]. We, therefore, wondered if melanocytes could produce factors that promote the epithelial organization. We also wanted to investigate if this effect on epithelia was E-cadherin/catenin-mediated and might, by consequence, also have implications for the invasive behavior of cancer cells. Since the culture of melanocytes requires the presence of insulin, a molecule already known to activate the E-cadherin/catenin complex[11], we chose an insulin-independent melanoma cell line, coined Bowes, as a possible source of such factors. As a target for the study of the effects on cell-cell adhesion and invasion, we used the human MCF-7/6 mammary adenocarcinoma cell line. This is a variant of the MCF-7 family that is invasive in the chick heart

Abbreviations used are: CM, conditioned medium; HER, human epidermal growth factor-like receptor; HRG, heregulin; IGF-I, insulin-like growth factor-I; IGFBP, IGF-binding protein

invasion assay, and displays poor cell-cell adhesion in aggregation assays in suspension, due to a defective function of the E-cadherin/catenin complex[12]. In this study, we present evidence that Bowes melanoma cells produce a factor that stimulates E-cadherin-dependent aggregation of MCF-7/6 cells, and abrogates the invasive potential of those cells. Different candidate molecules were considered, among which heregulin appeared to be one of the responsible factors.

MATERIALS AND METHODS

Cell lines and culture media

Human Bowes melanoma cells[13], a gift from G. Opdenakker (Department of Molecular Immunology, Rega Institute for Medical Research, Leuven, Belgium) and human MCF-7/6 breast adenocarcinoma cells (provided by H. Rochefort, Unité d' Endocrinologie Cellulaire et Moléculaire, Montpellier, France) were cultured in a 50:50 mixture of D-MEM and HAMF12 (Invitrogen, Merelbeke, Belgium) supplemented with 10 % fetal bovine serum (Invitrogen), 100 IU/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen) and 2.5 µg/mL amphotericin B (Bristol-Meyers Squibb, Brussels, Belgium) in an atmosphere of 10 % CO_2 in air. For subcultivation, Bowes melanoma cells were brought into suspension by vigorous shaking. All cells were tested for mycoplasma contamination by staining with 4',6-diamidino-2-phenylindole (DAPI) and found to be negative.

Conditioned medium from Bowes melanoma (CM Bowes melanoma) was obtained as follows. Cells were grown to confluence in 75 cm^2 tissue culture plastic vessels (Becton Dickinson, New Jersey). The medium was removed, and 10 mL of fresh D-MEM/HAM F12 culture medium was added. When serum-free CM was needed, the cells were first washed three times with phosphate buffered saline (containing calcium and magnesium) for 5 min. Cells were kept in culture for two additional days, and then the medium was harvested, centrifuged at 2,000 x g for 30 min and filtered through a 0.22 µm filter to eliminate any possible cell particles. This medium was used directly, or frozen at -20°C for later use.

Antibodies and other products

HECD-I (Takara, San Diego) and affinity purified MB2[11] are both murine monoclonal anti-E-cadherin antibodies. PY20 (ICN, Costa Mesa, California) is a murine monoclonal anti-phosphotyrosine antibody. αIR3 (Calbiochem, San Diego, California) is a murine monoclonal antibody against the insulin-like growth factor-(IGF)-I-receptor β-subunit. Secondary antibodies for immunoblotting were alkaline phosphatase-labeled rabbit polyclonal anti-mouse-IgG and goat polyclonal anti-rabbit-IgG (both from Sigma, St.-Louis, Missouri). IGF-I was obtained from Boehringer Bioproducts (Verviers, Belgium). A mixture of human interleukins and chemokines (a gift from Dr J. Van Damme, Department of Molecular Immunology, Rega Institute for Medical Research, Leuven, Belgium) was prepared by stimulation of Malavu cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) (100 ng/mL) and interleukin (IL)-1 (10 ng/mL). This resulted in secretion of IL-6 (25,000 U/mL) and IL-8 (1,300 ng/mL). Recombinant heregulin-β1, consisting of the epidermal growth factor-like domain of heregulin, was purchased from R&D Systems (Abingdon, U.K.). Polyclonal antibodies against chick heart were used as described earlier[14]. 5D10 is a murine monoclonal antibody against MCF-7 cells[15]. PD168393 was obtained from Calbiochem.

Aggregation Assays

Slow Aggregation Assay

In suspension: 100,000 cells per mL were added to 50 mL-Erlenmeyer flasks in 6 mL medium containing the test agents[16]. The flasks were incubated on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 72 rpm, and continuously gassed with humidified CO_2 (10 %) in air. Aggregate formation was evaluated under a Macroscope (Wild, Heerbrugg, Switzerland) at a magnification 25x after 4 days. On photographs the larger (**a**) and the smaller (**b**) diameter of 12 aggregates were measured, and apparent volumes (**v**) were calculated in accordance with the formula of Attia and Weiss[17]: $\mathbf{v} = 0.4 \times \mathbf{a} \times \mathbf{b}^2$. Statistical analysis was performed with StatView 4.1 for Macintosh software (Abacus Concepts, Inc., Berkeley, California).

On semi-solid substratum: 20,000 MCF-7/6 cells in 200 µL medium were seeded on solidified agar in a 96-well-plate, and treated with the test agents[18]. Aggregate formation was evaluated under an inverted microscope after 24 and 48 h.

Fast Aggregation Assay

A fast aggregation assay was used as described earlier[18]. Cells were pretreated with CM Bowes melanoma for 24 h and detached in accordance with an "E-cadherin-saving" procedure[19]. The cell suspension was then treated further with CM Bowes melanoma for 30 min at 4°C and incubated in BSA-coated wells at 37°C for 30 min under shaking. Untreated cells were incubated in fresh medium. Cells were fixed and the particle size distribution was measured with a Coulter Particle Size Counter LS 200 (Coulter Company, Miami, FL). The diameter of the particles can be considered as a measure for aggregate formation. Statistical analysis of differences between the particle size distribution curves was done with the Kolmogorov-Smirnov method.

In all aggregation assays, E-cadherin specificity of the aggregation was demonstrated with a functionally blocking antibody against E-cadherin (MB2 at 2 µg/mL)[20].

Chick heart invasion assay *in vitro*

The chick heart invasion assay was used as described earlier[16,21]. Briefly, heart fragments of 9-day old chick embryo's were precultured for 4 days (PHF) and then confronted with tumor cells under continuous Gyrotory shaking. Tumor cells were applied as monolayer fragments (Bowes melanoma) or as aggregates (MCF-7/6). For double confronting cultures, Bowes melanoma and MCF-7/6 cells were simultaneously confronted with the chick heart fragments. The individual confrontations were fixed in Bouin-Hollande's solution after 4, 7 or 11 days of incubation. They were afterwards embedded in paraffin, serially sectioned and stained with hematoxylin-eosin. Immunohistochemistry with antibodies against the chick heart and the MCF-7/6 cells was performed as described earlier[14].

Measurements of insulin and IGF-I

Insulin in CM Bowes melanoma was measured with the radioimmunoassay kit INS-RIA-100 (Medgenix Diagnostics, Brussels, Belgium). The minimal detectable concentration of insulin is 3.6 µU/mL. IGF-I in CM Bowes melanoma was determined with the IRMA kit Active™ IGF-I (Diagnostic Systems Laboratories, Webster, Texas). The minimal detectable concentration of IGF-I is 4 ng/mL.

Phosphorylation of the IGF-I receptor and analysis of total tyrosine phosphorylation

Phosphorylation of the IGF-I receptor was determined as described earlier[22]. Briefly, cells were labeled with 500 µCi/mL HCl-free ^{32}P-orthophosphate (ICN) and incubated with the test agents during 10 min up to 24 h. After washing in PBS, cells were treated with lysis buffer containing 1 % Triton X-100 and 1 % Nonidet P40 in PBS, with the protease inhibitors leupeptin (ICN), aprotinin (ICN) and phenylmethylsulfonyl fluoride (PMSF) (Sigma) and the phosphatase inhibitors $NaVO_3$, $Na_4P_2O_7$ and NaF (all from Sigma). Equal amounts of trichloroacetic acid (TCA)-precipitable radioactive protein material were precleared with Protein G Sepharose CL-4B beads (Pharmacia Biotech AB, Uppsala, Sweden) and antiphosphotyrosin antibody PY-20 was added, followed by Protein G Sepharose beads. After elution of tyrosine-phosphorylated proteins with excess phospho-L-tyrosine, αIR3 antibody was added to the elutes, followed by Protein G Sepharose CL-4B beads. After washing, the immune complexes were extracted with Laemmli buffer and boiled for 5 min. The centrifuged supernatants were analyzed by SDS-polyacrylamide

(7.5 %) gel electrophoresis under reducing (mercapto-ethanol) conditions. After electroblotting, Hyperfilm MP (Amersham, Buckinghamshire, UK) was exposed to the membranes and developed. Blot membranes were immunostained afterwards to confirm the identity of the bands appearing on the film.
For analysis of total tyrosine phosphorylation, Western Blotting and immunostaining was performed as described before[23].

Ligand blot assay for IGF-binding proteins
IGF-binding proteins (IGFBP) were evaluated by ligand blotting as described earlier[22]. Briefly, cells were washed 5 times with serum-free medium followed by incubation in serum-free medium for 48 h. The conditioned medium was dialyzed against 1.5 mM Tris, and concentrated 100 times by lyophilisation. Lyophilized proteins were denatured with sodium dodecyl sulphate under non-reducing conditions, separated on a 12 % polyacrylamide gel and electroblotted. IGFBP were visualized by autofluorography after binding of [^{125}I]-IGF-I (Amersham).

RESULTS

Effect of CM Bowes melanoma on aggregation

In slow aggregation assays in suspension or on semi-solid substratum, untreated MCF-7/6 cells formed small and irregular aggregates. This limited aggregate formation could be inhibited by MB2, an antibody against E-cadherin. Treatment of MCF-7/6 cells with CM Bowes melanoma stimulated aggregate formation in both types of assays. The diameter of the aggregates was larger in comparison with the untreated MCF-7/6 cells, and their aspect was more compact. In suspension, after 4 days, untreated MCF-7/6 cells formed aggregates with a mean apparent volume of 79.0 mm^3 (standard deviation = 23.6 mm^3). Cells treated with 20 % CM Bowes melanoma formed aggregates with a mean apparent volume of 827.5 mm^3 (standard deviation = 372.1 mm^3). Treatment with 50 % CM Bowes melanoma resulted in a mean apparent aggregate volume of 1553.4 mm^3 (standard deviation = 653.6 mm^3). Differences between all three groups were statistically significant ($P < .0001$, except for the comparison 20 % versus 50 % CM Bowes melanoma where $P = .0038$, Student's t-test). In presence of CM Bowes melanoma plus MB2, aggregate formation was abrogated and the cells remained single. Similar results were obtained in cultures on semi-solid substratum after 24 h (Figure 1). CM Bowes melanoma did not stimulate the aggregation of MDA-MB-231, an E-cadherin-negative cell line[24]. In line with this, the aggregation of the human colon carcinoma cell line HCT-8/E11, expressing the complete E-cadherin/catenin complex, was stimulated, whereas no effect was seen on the aggregation of HCT-8/E11R1, an α-catenin-negative variant of this cell line (data not shown)[25].

In a fast aggregation assay, the particle size distribution profiles showed limited spontaneous aggregation of MCF-7/6 cells after 30 min incubation in suspension. This aggregation was calcium-dependent and could be prevented by the monoclonal anti-E-cadherin antibody MB2. CM Bowes melanoma, diluted 1:3, stimulated aggregate formation of MCF-7/6 cells within 30 min. This was observed as a shift of the particle size distribution curve towards the larger particle diameters (Figure

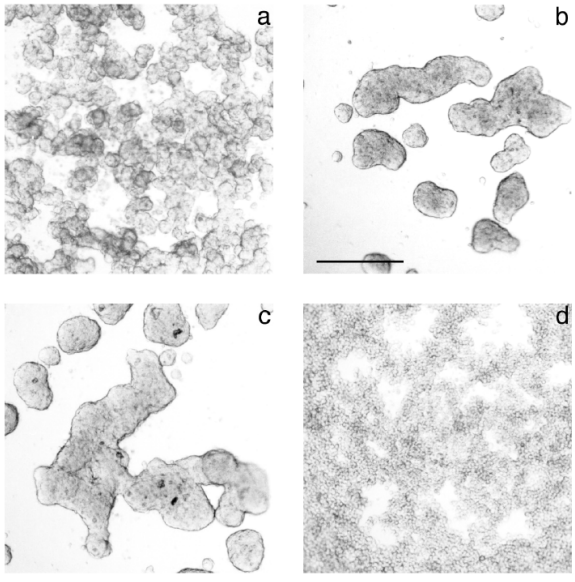

Figure 1. Phase contrast micrographs of MCF-7/6 aggregates on soft agar (after 24 h), untreated (**a**), treated with 20 % CM Bowes melanoma (**b**), treated with 50 % CM Bowes Melanoma (**c**) and treated with 50 % Bowes Melanoma plus MB2 diluted 1:20 (**d**). Scale bar = 200 µm

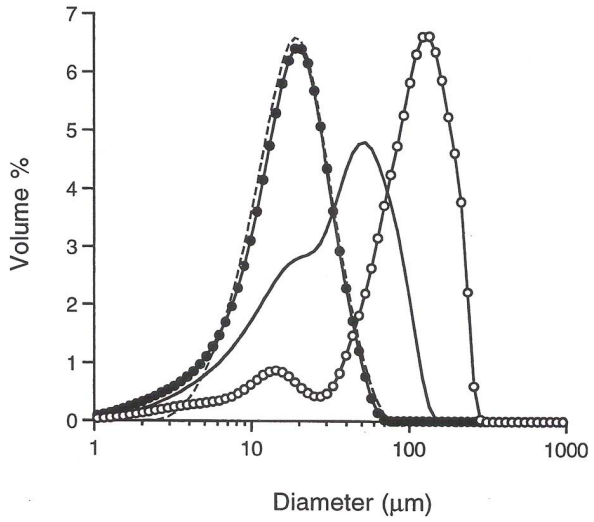

Figure 2. Volume percentage distribution plotted against the diameter of MCF-7/6 aggregates. Measurements were done after 0 min (broken line, ---) and after 30 min (full lines, —), either untreated (without symbol), treated with CM Bowes melanoma (open circles) or treated with CM Bowes melanoma and MB2 (filled circles). The curve of the cells treated with CM Bowes melanoma at 0 min coincided with the curve of the untreated cells at 0 min (not shown).

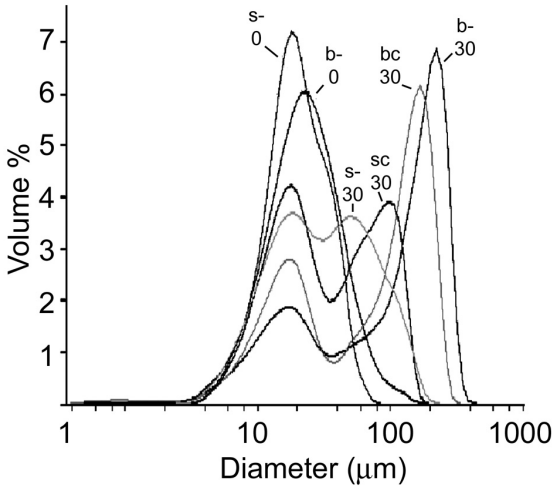

Figure 3. Volume percentage distribution plotted against the diameter of MCF-7/6 aggregates. Measurements were done after 0 min (0) and after 30 min (30) of MCF-7/6 aggregates pretreated (c) or not (-) with cycloheximide, and then treated either with serum free medium (s) or with CM Bowes melanoma (b)

2). The mean particle diameter of the untreated MCF-7/6 cells was 41.8 µm (standard deviation = 29.9 µm), while the mean particle diameter of the MCF-7/6 cells treated with CM Bowes melanoma was 110.4 µm (standard deviation = 63.8 µm). Kolmogorov-Smirnov statistics were applied to prove the statistical significance of this shift ($P < .001$). In presence of MB2, the effect of CM Bowes melanoma was absent. The rapid pro-aggregating effect of the CM (occurring within 30 minutes) suggested that protein synthesis did not play a role in the observed response. To confirm this, MCF-7/6 cells were pretreated for 1 hour with the translational inhibitor cycloheximide at 20 µg/ml, a concentration known to inhibit protein synthesis. Addition of Bowes melanoma CM to these cycloheximide-pretreated cells in the aggregation assay still resulted in an increase in cell-cell adhesion, suggesting that CM Bowes melanoma activates the E-cadherin/catenin complex in a post-translational way (Figure 3).

Effect of Bowes melanoma and CM Bowes melanoma on invasion of MCF-7/6 cells in chick heart fragments

In untreated confronting cultures, MCF-7/6 cells had invaded the PHF after 8 days of incubation (Figure 4a). Histological sections stained with haematoxylin-eosin showed MCF-7/6 cells occupying and replacing the PHF. Immunohistochemical staining with antibodies against the chick heart tissue and the MCF-7/6 cells (5D10) confirmed this observation. Treatment of the confrontations with CM Bowes melanoma in a dilution 1:3 resulted in absence of invasion by the

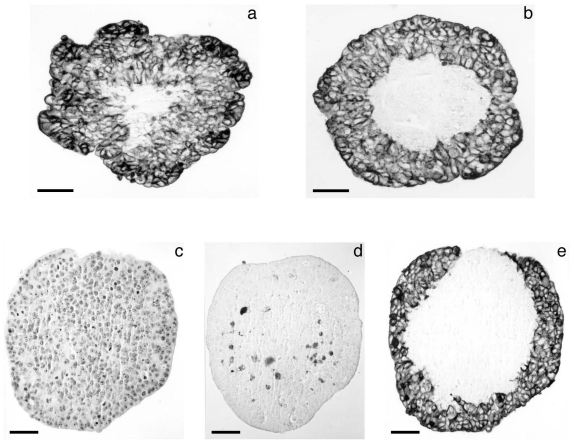

Figure 4. Light micrographs of sections from 8-day old confronting cultures between PHF and MCF-7/6 cells. An untreated confrontation (a) is compared with a confrontation treated with CM Bowes melanoma (diluted 1:3) (b) or simultaneous confrontations with Bowes melanoma cells (c, d and e). The sections were stained with hematoxylin-eosin (c), anti-chick heart antibody (d) or 5D10 against MCF-7/6 cells (a, b and e). Panel e shows the MCF-7/6 cells that are not invading the PHF which is totally destroyed (panel d) by the Bowes melanoma cells visible on the hematoxylin-eosin staining in panel c. Scale bar = 50 µm.

MCF-7/6 cells. The MCF-7/6 cells formed a regular epithelioid layer around the PHF and no MCF-7/6 cells were found inside the heart tissue (Figure 4b). Bowes melanoma cells confronted separately with the chick heart fragments invaded spontaneously. When MCF-7/6 and Bowes melanoma cells were simultaneously confronted with the chick heart fragments, in equal numbers, MCF-7/6 cells formed an epithelioid layer around the chick heart and did not invade, while Bowes melanoma were found inside the chick heart fragments (Figure 4c, d and e).

Responsible factors

We analyzed CM Bowes melanoma for the presence of factors that were proven to upregulate the function of the E-cadherin/catenin complex in MCF-7/6 cells in previous experiments[8]. With a Radioimmunoassay kit, having a detection limit of 3.6 µU/mL, no insulin was found in CM Bowes melanoma. With an IRMA-test we found small concentrations (around 10 ng/mL) of insulin-like growth factor I (IGF-I). These concentrations are slightly above the detection limit of the assay (4 ng/mL). To investigate if the IGF-I receptor could be activated at this concentration, we treated MCF-7/6 cells with CM Bowes melanoma or 10 ng/ml of pure IGF-I and checked the phosphorylation status of the IGF-I receptor after 24 or 48 hours. As a positive control, MCF-7/6 cells were treated for 10 minutes with 500 ng/mL of IGF-I[22]. Whereas phosphorylation was evident after both 24h and 48h treatment with 10

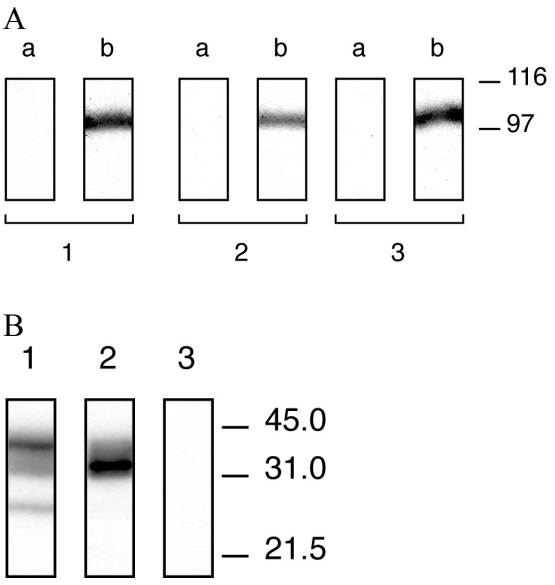

Figure 5.
(A) Fluorographs of a sequential immunoprecipitation with PY20 and _IR3 antibodies of the beta subunit of the IGF-IR in MCF-7/6 cells labelled with ^{32}P and treated with IGF-I or CM Bowes melanoma. Lanes **a** are from cells treated with CM Bowes melanoma, lanes **b** are from cells treated with IGF-I. Treatment periods were 10 min (lanes **1**), 24 h (lanes **2**) and 48 h (lanes **3**). The IGF-I concentration was 500 ng/mL for lane **1b** and 10 ng/mL for lanes **2b** and **3b**. Figures indicate molecular weight in kDa.
(B) Fluorographs of a ligand blot of IGFBPs detected with [^{125}I]-IGF-I. Examined samples are CM MCF-7/6 (lane **1**), CM Bowes Melanoma (lane **2**) and fresh D-MEM/HAM F12 medium (lane **3**). Figures indicate molecular weight in kDa.

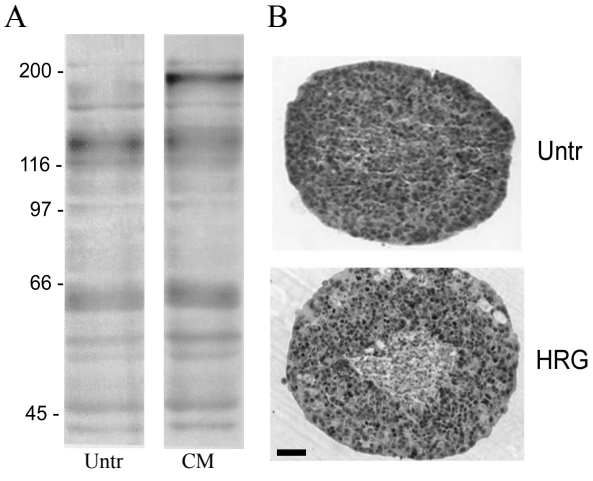

Figure 6. (A) Antiphosphotyrosine immunoblotting of total lysates of MCF-7/6 cells, left untreated (Untr) or treated for 30' with CM Bowes melanoma (CM). A prominent tyrosine-phosphorylated band appears at 185kD. (B) Light micrographs of hematoxyline-eosine stainings of sections from 8-day old confronting cultures between PHF and MCF-7/6 cells, left untreated (Untr) or treated with 50ng/ml HRG-β1. Scale bar = 50μm

ng/ml IGF-I, treatment with CM Bowes melanoma for 10 min, 24 or 48 h, did not result in phosphorylation of the IGF-IR in MCF-7/6 cells (Figure 5A). In a parallel experiment, this CM was found to be biologically active, since it stimulated aggregate formation of MCF-7/6 cells. The lack of IGF-I receptor phosphorylation by CM Bowes melanoma may be explained by the secretion of IGF-binding proteins (IGFBP) by these cells. Binding of IGF-I to IGFBP's usually results in prevention of IGF-I binding to its receptor and hence inhibition of receptor activation. We therefore checked IGFBP secretion by Bowes melanoma cells using a ligand blot assay with [^{125}I]-IGF-I. As a control, medium conditioned by MCF-7/6 cells was used. Based on molecular weight, MCF-7/6 cells were found to produce IGFBP-2 (34 kD), IGFBP-5 (29-32 kD) and IGFBP-4 (24 kD) (Figure 5B, lane 2), in accordance with findings by others[26,27]. In CM Bowes melanoma, IGFBP-4 could not be detected, there was a stronger band for IGFBP-5 and a less prominent band for IGFBP-2 than in CM MCF-7/6 (Figure 5B, lane 3). Fetal bovine serum showed a faint band representing IGFBP-5 (not shown) and no band was detected in fresh D-MEM/HAM F12. Thus, the lack of IGF-IR phosphorylation by CM Bowes melanoma may be attributed to the inactivation of IGF-I by the concomitant secretion of (mainly) IGFBP-5 by these cells. To exclude that certain interleukins were responsible for the effect, we added an interleukin-mix (containing interleukin-1, -6, -8) to MCF-7/6 cells in a slow aggregation assay. No stimulation of aggregation could be observed (data not shown).

Recently, we reported the secretion of heregulin by Bowes and other melanoma cell lines. Heregulin in CM Bowes melanoma was able to phosphorylate the 185 kD human epidermal growth factor-like receptor 2 and 3 (HER2 and 3) in MCF-7/6 cells (Figure 6A)[23]. Human recombinant heregulin-β1 (rHRG-β1) could mimic the effect of CM Bowes on invasion: treatment with 50 ng/ml rHRG-β1 inhibited invasion of MCF-7/6 cells into PHF, and yielded histological images similar to those obtained with CM Bowes (Figure 6B). Moreover, this heregulin concentration also stimulated cell aggregation of MCF-7/6 cells in the slow aggregation assay (Figure 7A and 7C, left panels), despite the fact that on solid substratum both rHRG-β1 and Bowes melanoma CM induced scattering of epithelial MCF-7/6 islands (Figure 7B). Since transfection of Bowes melanoma with an antisense HRG construct yielded no colonies and since none of the anti-HRG antibodies we tested could fully block HRG-induced phosphorylation (data not shown), we used a specific, irreversible HER inhibitor, PD168393[28], to evaluate whether heregulin was the only factor responsible for increased aggregation of MCF-7/6 cells. PD168393 fully prevented the rHRG-β1-mediated increase in aggregation of MCF-7/6 cells, without affecting control aggregation (Figure 7C, right panels). However, this inhibitor could nut fully prevent the increase in aggregation by CM Bowes melanoma, suggesting that additional pro-aggregating factors

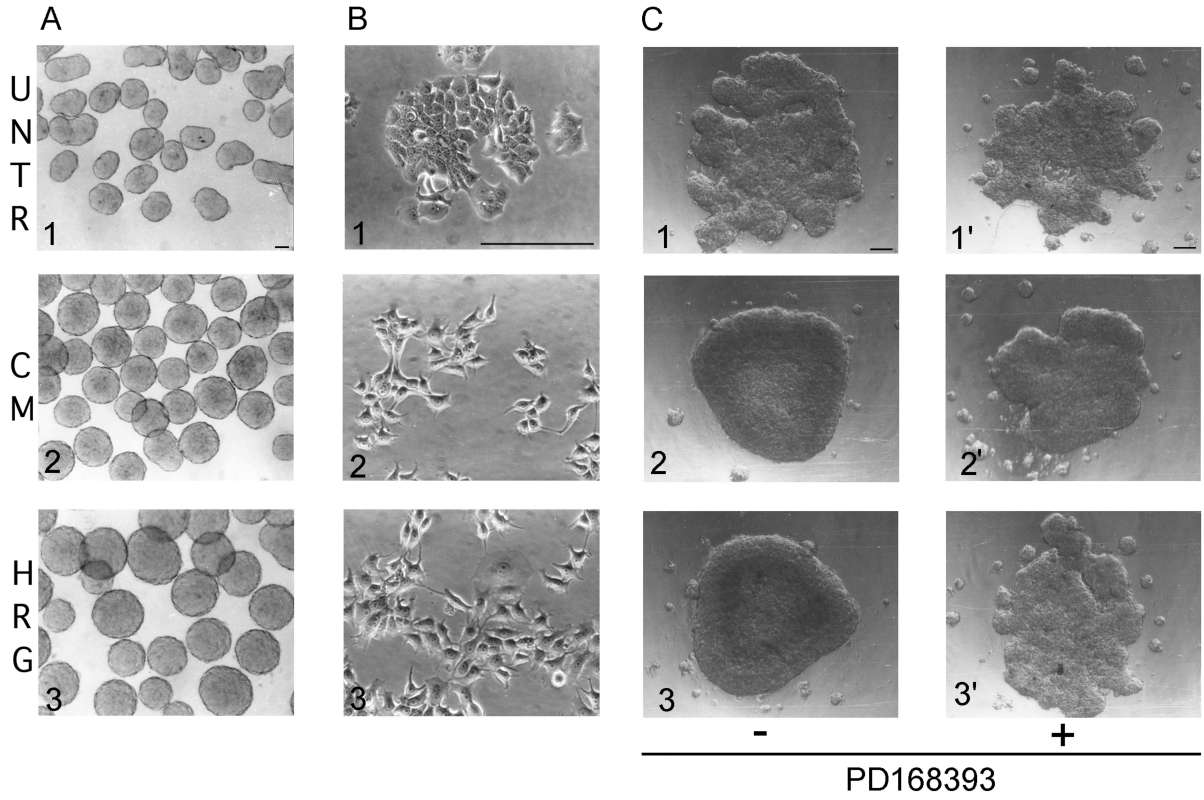

Figure 7. Phase contrast micrographs of MCF-7/6 aggregates (A) in suspension (after 3 days) or (C) on soft agar (after 48 h), left untreated (**untr**), treated with CM Bowes melanoma (**CM**), or treated with 50ng/ml rHRG-β1 (**HRG**), in combination or not with 2μM PD168393. (B) Phase contrast micrographs of serum-starved MCF-7/6 cells, treated for 2 hours with serum free medium (**untr**), with CM Bowes Melanoma (**CM**) or with 50ng/ml rHRG-β1. Scale bars = 100 μm.

may be present in the CM. In agreement with the presumption that HRG secretion by Bowes melanoma is not sufficient to explain all of its effects, is the inability of rHRG-β1 to promote cell aggregation of MCF-7/6 in the fast aggregation assay (Figure 8). In conclusion, although for some of the observed effects on MCF-7/6 cells the cooperation with a synergizing factor may be necessary, HRG secretion by Bowes melanoma cells is sufficient for its anti-invasive and pro-aggregating effects on long term.

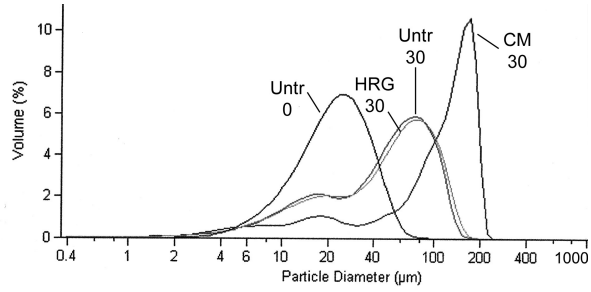

Figure 8. Volume percentage distribution plotted against the diameter of MCF-7/6 aggregates. Measurements were done after 0 min (0) and after 30 min (30) of MCF-7/6 aggregates treated either with serum free medium (Untr), with 50ng/ml rHRG-β1 in serum free medium (HRG) or with CM Bowes melanoma (CM).

DISCUSSION

Our data show that Bowes melanoma cells produce a factor that stimulates aggregation and abrogates invasion of human MCF-7/6 mammary cancer cells *in vitro*. Our experiments were inspired by the finding that melanocytes play a role in the normal development of the mammalian inner ear. In mutant mice, with a primary neural crest defect leading to absence of recognizable melanocytes, transmission electron microscopy revealed cell-cell-contact defects between the cells of the stria vascularis[10]. A similar syndrome has been found in humans and is known as Waardenburg's syndrome. There also, the absence of human melanocytes seems to be the key event. We chose Bowes melanoma cells as candidate-producers of factors that might modulate epithelial cell-cell interactions *in vitro*. In a number of aggregation assays we observed that CM Bowes melanoma was able to increase the aggregation of MCF-7/6 cells. These human mammary carcinoma cells are a variant of the MCF-7 family and express all the elements of the E-cadherin/catenin complex, but the function of the complex is impaired depending on the culture conditions[12]. When cultured on solid substratum, the epithelial organization of the cells is intact and the complex is evidently active. However, when the cells are detached from their substratum, they display poor aggregate formation

and clear cut invasive behavior in various *in vitro* invasion systems[11,12]. Interestingly, the complex is still active to some extent, since the remaining aggregation can be abrogated completely by MB2, a monoclonal antibody against E-cadherin. Treatment of MCF-7/6 with CM Bowes melanoma resulted in increased aggregate formation in the three types of aggregation assays mentioned. The effects on stimulation of aggregation were E-cadherin-mediated, as proven by inhibition experiments with MB2. These data show that CM Bowes melanoma increases aggregation of MCF-7/6 cells and that this effect is due to functional upregulation of the E-cadherin/catenin complex. By calculating apparent volumes, we were able to quantify and statistically prove this effect. The E-cadherin/catenin complex is not only implicated in cell-cell adhesion, but also has a powerful invasion-suppressor function in epithelial cells[2]. Our results demonstrate that upregulation of the complex with CM Bowes melanoma leads to inhibition of invasion, as evidenced in the chick heart assay. MCF-7/6 cells do no longer invade into the chick heart when they are treated with CM Bowes melanoma. Another explanation could be that CM Bowes melanoma enhances the resistance of the chick heart towards invading cells. However, the double confrontation cultures (with MCF-7/6 and Bowes melanoma cells used at the same time) render this possibility unlikely: the melanoma cells still invade the chick heart while the MCF-7/6 cells form an epithelioid layer around the chick heart, without any sign of invasion. This shows that the anti-invasive effect of CM Bowes melanoma is exerted by influencing the MCF-7/6 cells, and not the chick heart.

As far as the responsible factor is concerned, we can consider the implication of certain molecules as improbable. The effects presented resemble those that have been described for a number of molecules that are able to functionally up-regulate the E-cadherin/catenin complex. Examples are: tamoxifen[29], insulin and IGF-I[11], retinoic acid[22] and tangeretin[30]. However, the presence of any of those compounds was either unlikely (tamoxifen, tangeretin and retinoic acid) or undetectable (insulin). Although small amounts of IGF-I, or a molecule immunologically recognized as such, may be secreted by Bowes melanoma cells, no detectable phosphorylation of the IGF-I-receptor occurred following treatment with the CM. Most likely, this is due to the concomitant secretion of IGF binding proteins (IGFBP-5) by the melanoma cells, since an equivalent concentration of pure IGF-I did lead to receptor activation. Since phosphorylation of the receptor was previously found to be indispensable for the activation of the signal transduction pathway towards the E-cadherin/catenin complex[11], it is unlikely that IGF-I or IGF-I-like molecules are implicated. Previous studies[31] have described that melanomas can produce cytokines, like interleukins, tumor necrosis factor-alpha, transforming growth factor-beta, granulocyte-macrophage-colony stimulating factor and stem cell factor. However, most of these molecules do not alter the function of the E-cadherin/catenin complex in MCF-7 cells[32]. Furthermore, as treatment with an interleukin mixture did not result in stimulated aggregation of the cells, we further could exclude interleukin-1, -6 and -8 as responsible factors.

Heregulin (HRG) fits into the profile of the candidate factor in CM Bowes. Bowes melanoma cells synthesize at least 4 heregulin isoforms, heregulin was detected in their CM[23], and addition of rHRG-β1 to MCF-7/6 cells activated the E-cadherin/catenin complex, resulting in promotion of slow cell aggregation and inhibition of invasion in the chick heart assay. An aggregation-promoting effect of HRG has already been described by Tan et al[33]. However, since there the improved aggregation could take place at 4°C and was also seen in E-cadherin-negative breast cancer cells, it was independent of E-cadherin. Some data indicate that heregulin may not be the only anti-invasive factor in CM Bowes: in contrast to CM Bowes, recombinant heregulin failed to promote fast aggregation of MCF-7/6 cells, and a selective heregulin receptor tyrosine kinase inhibitor (PD168393)[24] was unable to fully block slow cell aggregation after treatment with CM Bowes, but did so upon heregulin treatment. Moreover, recent data indicate that E-cadherin can exert its anti-invasive effect without promotion of cell-cell adhesion[34]. We cannot exclude that other factors in CM Bowes act synergistically with heregulin, as it was recently shown for retinoids in a model for branching morphogenesis of breast cancer cells in collagen gels[35]. It should be noted that retinoic acid on itself is an anti-invasive agent for MCF-7/6 cells in the chick heart assay[12], although no evidence could be found that the activating effect on the E-cadherin/catenin complex was responsible for the inhibition of invasion[22].

As for a number of other molecules such as estradiol[36,37] and IGF-I[38], it is not evident to reconcile the established motogenic effect of heregulin with its anti-invasive effect. In particular, the inactivating and downregulating effects of heregulin on the E-cadherin/catenin complex leading to cell scattering appear to be in contradiction with its effect on aggregation and invasion. However, two points should be considered here. First, the effects are clearly dependent on the models used, since for MCF-7/6 cells heregulin induces scattering on a tissue culture plastic substratum (Figure 7B, [23]) and promotion of aggregation in suspension culture. Second, triggering of the E-cadherin/catenin complex can activate the small GTPase rac, and hence induce the formation of lamellipodia, a kind of motility meant to achieve additional cell-cell contacts that eventually result in the formation of compact epithelia[39].

In conclusion, the human melanoma cell line Bowes secretes one or more factors that can upregulate the function of the E-cadherin/catenin complex and inhibit invasion of the human breast carcinoma cells MCF-7/6. Most probably heregulin is responsible for these activities, although additional factors cannot be excluded.

ACKNOWLEDGEMENTS

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II.3. The role of cadherins in melanocyte physiology and in human melanoma

II.3.1. Cell adhesion molecules in human melanoma

Cell adhesion molecules belonging to the integrin, immunoglobulin and cadherin superfamilies have been implicated in cutaneous melanoma. Altered expression of many of these cell-cell or cell-matrix adhesion molecules is frequently observed during tumor progression.

Integrins are heterodimeric cell surface receptors formed by non-covalent association of an α chain with a β chain. At least 20 different α and 9 different β chains have been identified to date. These mediate cation dependent adhesion to extracellular matrix molecules and to cell surface ligands. Aberrant expression of integrins has been associated with cancer progression in multiple malignancies, including melanoma (Keely *et al.*, 1998; Seftor *et al.*, 1999; Hood and Cheresh, 2002). As compared to benign melanocytic lesions, malignant melanomas demonstrate a loss of expression of the laminin receptor α6β1 (Natali *et al.*, 1991). Increased expression of α4β1 (also known as CD49d/CD29 and VLA-4), which may mediate interaction of the tumor cells with VCAM-1 (vascular cell adhesion molecule-1) on vascular endothelium, has been associated with metastatic properties of melanoma cells Martin-Padura *et al.*, 1991; Schadendorf *et al.*, 1995). Upregulation of the β3 chain, leading to high levels of the vitronectin receptor αvβ3 integrin is another frequent event in melanoma progression (Albelda *et al.*, 1990). The β3 integrin chain is exclusively expressed in vertical growth phase and metastatic melanomas and correlates with proliferation (Felding-Habermann *et al.*, 1992), invasion (Seftor *et al.*, 1992; Van Belle *et al.*, 1999), recurrence and mortality (Hieken *et al.*, 1996). Expression of the β3 integrin allows the cells to interact with denatured collagen, improving survival and proliferation (Montgomery *et al.*, 1994; Petitclerc *et al.*, 1999), and leads to enhanced matrix metalloproteinase (MMP) expression (Hofmann *et al.*, 2000). Moreover, an important role for αvβ3 integrin in angiogenesis has been suggested (Friedlander *et al.*, 1995). Despite this extensively described role for β3 integrin, it cannot, when overexpressed alone, promote the progression of melanocytes to melanoma (Meier *et al.*, 2003).

Immunoglobulin superfamily cell adhesion molecules mediate cation independent adhesion with themselves or other members of this superfamily, but can act as receptors for integrins and extracellular matrix proteins as well. Two molecules, capable of mediating homophilic adhesion of melanoma cells, which are upregulated in melanoma cells include ALCAM (activated leukocyte cell adhesion molecule, also known as CD166, BEN/DM-GRASP, SC1) and Mel-CAM/MCAM (Melanoma cell adhesion molecule, also known as CD146 or MUC18)(Degen *et al.*, 1998; Shih *et al.*, 1994). More specifically, high MCAM expression has been shown to confer metastatic properties to cells in experimental metastasis assays and is frequently found in metastases of melanoma patients (Van Kempen *et al.*, 2000). MCAM expression leads to upregulation of matrix metalloproteinase (MMP) activity and appears to be involved in melanoma-endothelial cell interactions, suggesting a role in tumor cell migration, invasion, extravasation and angiogenesis (Johnson *et al.*, 1999). ICAM-1 (CD54) expression is stronger on malignant than on benign melanocytic lesions and has been correlated with significantly shorter disease free and overall survival of melanoma patients (Johnson *et al.*, 1989). However, the exact role of this molecule in progression of melanoma is not well known.

The role of cadherins, a large family of Ca^{2+}-dependent cell-cell adhesion molecules will be discussed into detail below.

II.3.2. Cadherins in normal melanocyte physiology

Cell-cell adhesion, migration and invasion are not only crucial events in cancer, they also fulfill a central role in normal melanocyte biology. Melanocytes are derived from the neural crest. Neural crest cells can be defined as a pluripotent population of cells that originate at the time of neural tube closure at its dorsal site. Neural crest cell precursors are located at the border of the neural ectoderm (neural plate) and the non-neural ectoderm. Neural crest cells emerge after an epithelial-mesenchymal transition, with disruption of the tight cell-cell contacts with other neuro-epithelial cells, a reorganization of the cytoskeletal network and the activation of specific genes (Knecht and Bronner-Fraser, 2002). Two related transcription factors, Snail and Slug (both known transcriptional repressors of the E-cadherin gene, see further) seem to play a key role in this process. At the trunk, neural crest cells proliferate extensively, and follow two main migration pathways: dorso-ventral or dorso-lateral. Cells migrating dorsolaterally, between the somites and the ectoderm, give rise to melanocytes.

Differential expression of cadherins has been suggested as a requirement for the correct migratory pathway to be followed (Furukawa *et al.*, 1997; Nishimura *et al.*, 1999; Jouneau *et al.*, 2000, Pla *et al.*, 2001). Table II.1 summarizes the current knowledge

Stage - species	Event/localization	Cadherins present	Cadherins absent
	Ectoderm	E	
	Neurulation: formation of the neural plate	E↓ N↑	
	Neural crest cell precursors are located at the border of the ectoderm and neural plate	E? (ectoderm), N? (neural plate)	
	Formation of the neural fold, leading to neural tube formation	N 6 (low)	
	Closure of neural tube	N (low) 6 (high)	
	Epithelial-mesenchymal transition, start of migration	N ↓ 6 (6B↓in chick) (7↑ in chick)	
E10.5 mouse embryonic skin	Dorsolateral migration of melanoblasts in dermis	6 (7 in chick) 11	E, N, P Desmoplakin 1/2
E12.5 mouse embryonic skin	Dermal melanoblasts	E (low)	N, P Desmoplakin 1/2
E14.5 mouse embryonic skin	Dermal (melanoblasts are migrating to epidermis)	E	N, P Desmoplakin 1/2
	Epidermal (melanoblasts migrated from the dermis)	E, P	N Desmoplakin 1/2
Newborn skin	Dermal melanocytes and melanoblasts (rare in regions covered with hair, but abundant in regions lacking hair, e.g. pinna)	N	E, P Desmoplakin 1/2
	Epidermal	E, P Desmoplakin 1/2	N
	Hair follicles	E, P (higher P than in epidermis)	N Desmoplakin 1/2

Table II.1. Expression of cadherins during melanocyte expansion and migration in development.

of cadherin expression in murine neural crest cell formation, migration and differentiation into melanocytes. Before migrating, the founder melanoblasts start to proliferate, producing precursor melanoblasts, on their turn giving rise to melanoblasts, a population of actively proliferating and migrating cells. In melanoblasts that have completed their migration in mesenchymal tissues, expression of P-, and particularly E-cadherin, is strongly elevated upon crossing the basement membrane, which separates the dermis from the epidermis (Nishimura *et al.*, 1999). During this 'invasion' of the epidermis, the melanocytes retain contact with the basement membrane on the epidermal side, eventually becoming interspersed among basal keratinocytes. In the end, skin melanocytes are found at three locations: at the basal side of the epidermis, dermal and in the hair follicles. In hair follicles, melanocytes have high levels of P-cadherin and little or no E-cadherin. The importance of P-cadherin expression in melanocytes is evident in juvenile hypotrichosis with macular dystrophy (JHMD, a syndrome found in humans with bi-allelic inactivation of P-cadherin that is characterized by early hair loss and progressive degeneration of the retina, culminating in blindness (Sprecher *et al.*, 2001; Indelman *et al.*, 2002, 2003). Although N-cadherin expression is absent or very weak in epidermal melanocytes *in vivo*, it is strongly expressed by dermal melanocytes. N-cadherin expression in the skin is further confined to the fibroblasts and endothelial cells in the dermis. Expression of N-cadherin in epidermal melanocytes is elevated upon *in vitro* culture of these cells and further increases with the number of passages (Hsu *et al.*, 1996).

In normal human skin, all epidermal cells, including melanocytes, keratinocytes (both basal and differentiated) and Langerhans cells, express E-cadherin, whereas P-cadherin expression is further only found in the basal keratinocytes. The latter are the dominant cellular partner of melanocytes and are able to control the growth, morphology and antigenic phenotype of melanocytes. Neither soluble factors nor extracellular matrix derived from the keratinocytes are able to recapitulate this control (Valyi-Nagy *et al.*, 1990, 1993). Instead, specific heterotypic gap-junctional communication was shown to play a crucial role in conserving epidermal homeostasis (Hsu *et al.*,

2000a). Gap junctions are composed of radially arranged integral membrane proteins, connexins, forming hemi-channels (connexons). Alignment of these half-channels between adjacent cells results in communication channels which allow the direct transport of small molecules between cells (reviewed by Segretain and Falk, 2004). The specificity of this gap-junctional communication is dependent on cadherin-driven cell sorting (Prowse *et al.*, 1997).

II.3.3. Cadherin switches in melanoma

In contrast to melanocytes, melanoma cells are often refractory to the normal contact-mediated phenotypic control of keratinocytes (Shih *et al.*, 1994). Normal melanocytes are positive for E- and P-cadherin (N-cadherin is absent or only very weak). During melanoma progression a shift in cadherin profile frequently occurs. This shift may confer new adhesive properties to the cells: while a progressive reduction or loss of E-and P-cadherin abolishes heterotypic contact formation with E- and P-cadherin positive basal keratinocytes, upregulation of N-cadherin allows a better homotypic communication among melanoma cells, as well as heterotypic communication with fibroblasts and endothelial cells (Tang *et al.*, 1994; Seline *et al.*, 1996; Hsu *et al.*, 1996, Danen *et al.*, 1996; Seline *et al.*, 1996; Silye *et al.*, 1998; Sanders *et al.*, 1999; Li *et al.*, 2001a,b; Krengel *et al.*, 2004). The important biological consequences of these interactions are evident from several observations:

1) restoration of E-cadherin expression in melanoma cells restored their ability to associate with keratinocytes, allowing the formation of gap junctions and resulting in growth retardation, inhibition of invasion and induction of apoptotic cell death in a three-dimensional skin reconstruct, and decreased tumorigenicity in mice (Hsu *et al.*, 2000a, 2000b);

2) N-cadherin-mediated cell adhesion promotes survival of melanoma cells via activation of the anti-apoptotic protein Akt/PKB (Li *et al.*, 2001a)

3) N-cadherin promotes adhesion of and stimulates migration of melanoma cells over dermal fibroblasts and vascular endothelial cells, which may improve their ability to migrate over the stroma and enter the vasculature (Li *et al.*, 2001a,b);

4) anti-N-cadherin antibodies can delay the transendothelial migration of melanoma cells (Sandig *et al.*, 1997; Voura *et al.*, 1998);

5) N-cadherin expression in a murine model of melanoma development is only found in melanocytic fractions isolated from deeper parts of the skin (dermis and basement membrane), implying that the cadherin shift occurred in the cells invading the skin (Berking *et al.*, 2004);

6) *in vivo*, invading melanoma cells show pagetoid distribution or nest formation, suggesting the existence of tight cell-cell adhesion in these cells (Berking *et al.*, 2004).

In conclusion, the E-to-N cadherin switching in melanoma cells not only frees melanocytic cells from the control of keratinocytes, but also provides growth and metastatic advantages to the melanoma cells. Thus, E- and N-cadherin seem to act in opposite ways in the melanoma system, as a tumor suppressor and tumor promoter, respectively. Several explanations may account for the striking different functional roles of these two highly related molecules. One plausible possibility is that the differences are a matter of the differential action of the partner cells: while 'good neighbors' (keratinocytes) serve as E-cadherin dependent master controllers of melanocytes, 'bad neighbors' (fibroblasts), via N-cadherin dependent contacts, may serve as positive stimulators for cancer cells by promoting proliferation, survival and migration. In this view, the initial cadherin-mediated contact selects the partner cell and represents a first step in the reciprocal communication between the partners. Alternatively, E- and N-cadherin, apart from functioning as 'glue keeping cells together', also participate in signal transduction pathways. As is known from other cancer types, the panel of molecules recruited by E- and N-cadherin may differ, resulting in quantitative and qualitative differences in the signaling pathways in which these molecules are involved. These differences may actively confer growth and motility advantages to N-cadherin positive cells (Suyama *et al.*, 2002; Fedor-Chaiken *et al.*, 2003).

The molecular basis for the decreased expression of E-cadherin does not seem to involve mutations in the E-cadherin gene or promoter methylation (Poser *et al.*, 2001; Li *et al.*, 2001b). Instead, upregulation of the zinc finger transcription factor Snail plays an important role in transcriptional repression of E-cadherin expression in melanoma (Poser *et al.*, 2001; Li *et al.*, 2003). No inverse correlation was found between the expression of E-cadherin and Slug, another Snail family member (Li *et al.*, 2003). Controversy exists about the role of another transcription factor, AP-2, in regulation of E-cadherin expression in melanoma. Although loss of expression of the transcription factor AP-2 during melanoma progression was proposed as one of the mechanisms leading to loss of E-cadherin (Nyormoi and Bar-Eli, 2003), others did not support this view (Poser *et al.*, 2001). Also the continuous exposure of melanocytic cells to HGF (hepatocyte growth factor) results in downregulation of E-cadherin and the desmosomal cadherin desmoglein (Li *et al.*, 2001b). In contrast to melanocytes, which only express the hepatocyte growth factor (HGF) receptor c-Met, many melanoma cell lines express HGF as well, which may act in an autocrine loop, leading to constitutive receptor activation. The presence of this autocrine loop may explain the reduced expression of these

cadherins in several melanoma cell lines (Li *et al.*, 2001b). Although c-Met, E-cadherin and desmoglein were all part of a multi-protein complex in these cells, and downregulation could be (partially) prevented by blocking PI3K and MAPK pathways, the exact mechanisms leading to downregulation of these cadherins was not elucidated. Downregulation of E-cadherin following HGF treatment has been shown in various epithelial cell types and may result from its internalization, driven by the ubiquitin ligase Hakai (Fujita *et al.*, 2001). However, no data are available concerning the expression or action of this protein in melanoma. Moreover, Hakai-mediated down-regulation is known as a relatively fast process, occurring within several hours (Fujita *et al.*, 2001), which contrasts with the prolonged exposure required in melanocytic cells (Li *et al.*, 2001b). These findings were confirmed in a transgenic mouse model, where keratin 14 promoter-driven HGF expression by keratinocytes resulted in dermal melanocytosis, accompanied by reduced E-cadherin expression (Kunisada *et al.*, 2000). Among other growth factors that have been reported to downregulate E-cadherin expression in melanocytic cells is endothelin-1, which is produced by keratinocytes upon UV induction and can downregulate E-cadherin in a caspase-8 dependent manner (Jamal and Schneider, 2002).

Relatively few studies have studied the expression of other cadherins in melanoma. For instance, although Tang *et al.* (1994) proposed that E-cadherin mediated cell adhesion dominated a possible role of P-cadherin, studies examining the function of the latter in melanoma are lacking. Also the mechanisms underlying the increased N-cadherin expression in melanoma cells remain unknown. Apart from N-cadherin, *de novo* expression of other cadherins, such as VE-cadherin and protocadherins, is found in melanoma cells (Hendrix *et al.*, 2001; Matsuyoshi *et al.*, 1997). Expression of VE-cadherin has been shown to play an important role in the ability of malignant melanoma cells to form tubular structures and patterned networks in 3D cultures, mimicking the embryonic vasculogenic networks formed by differentiating endothelial cells (so-called 'vasculogenic mimicry') (Maniotis *et al.*, 1999; Hendrix *et al.*, 2001). Although it is not clear what role proto-cadherins play, they may possibly contribute to the communication between melanoma cells themselves and/or with the micro-environment.

II.3.4. β-catenin: a player in melanoma?

Apart from shifts in the expression of cadherins in melanoma, other events involving this system may take place as well. A molecule whose localization is frequently affected in melanoma is β-catenin (Rimm *et al.*, 1999). Loss of membranous expression of β-catenin in melanomas has been associated with tumor progression (Kageshita *et al.*, 2001; Demunter *et al.*, 2002; Maelandsmo *et al.*, 2003). Apart from its role in cell-cell adhesion, β-catenin is also involved in signaling and gene transcription. More specifically, it has a central role in what is known as the 'canonical Wnt signaling pathway'. In this pathway, binding of a ligand, Wnt, to its receptor, Frizzled, leads to phosphorylation and inhibition of glycogen-synthase-kinase-3β (GSK-3β). This enzyme is part of a multi-protein complex, also containing APC (adenomatous polyposis coli) and axin, that drives non-cadherin-bound β-catenin via subsequent phosphorylation and ubiquitination towards proteasomal degradation. A blockage of β-catenin degradation may thus be the result from Wnt signaling, but may also arise from mutations in proteins involved in its degradation (e.g. axin or APC) or in β-catenin itself. As a result, β-catenin may accumulate, translocate to the nucleus and associate with Lef/Tcf (lymphocyte enhancer factor/T-cell factor) transcription factors to influence gene transcription (Lustig and Behrens, 2003).

Mutations in APC have been described in two melanoma cell lines (Rubinfeld *et al.*, 1997), but its relevance for the *in vivo* situation is unclear. In a small series of cutaneous melanoma samples, no loss of heterozygosity (LOH) of the APC gene could be detected (Ichihashi and Kitajima, 2000). **β-catenin mutations**, affecting GSK-3β phosphorylation sites, have been found both in melanoma cell lines and in a limited number of melanoma cases (See table II.2.). However, as is evident from table II.2, these only account for a small part of the melanoma cases where nuclear β-catenin staining is observed. Consequently, additional mechanisms must be present in melanoma cells, leading to nuclear β-catenin localization. These include a **reduction of cadherin expression** (resulting in a reduction of membranous β-catenin), as well as mutations in other components of the β-catenin degradation machinery. Among possible candidate molecules is the APC modifier **phospholipase A2-activating protein** (PLAP), a gene localized at 9p21, a chromosomal region that is frequently affected by LOH in cutaneous melanoma (Ruiz *et al.*, 1999). **Aberrant expression of Wnt ligands**, which may act in an autocrine way on the melanoma cells, may represent an alternative way leading to nuclear β-catenin. In this context, based upon gene expression analysis of cutaneous melanoma samples, expression of Wnt5a has been proposed as a robust marker of aggressive behavior (Bittner *et al.*, 2000)[5028]. However, subsequent functional analysis showed that, although Wnt5a expression could indeed increase melanoma cell motility and invasion, these effects were not accompanied by increased β-catenin nuclear localization and signaling, but required PKC activation instead (Weeratna *et al.*, 2002).

Overall, although from these studies it is evident that in a significant amount of melanoma

samples cytoplasmic and/or nuclear β-catenin staining is present, no clear role for this protein has been established in melanoma development and/or progression, yet.

Material analyzed	Technique	Mutations found	Mutation Frequency	Localization	Reference
Cell lines	DS	S37F (4) S45Y (1) Exons 2,3 and 2.3.4 deleted from mRNA (mutation??)(1)	6/26		Rubinfeld *et al.*, 1997
Biopsies metastases	DS	S45P	1/50	10/18 + 8/47 nuclear and/or cytoplasmic	Rimm *et al.*, 1999
Biopsies	MD & SSCP/DS	S45F	1/31 (primary) 1/37 (metastases) (mutation in same patient)	Primary: 17/28 excl membr 10/28 cytopl & nucl 1/28 reduced/absent Metastases: 30/36 excl membr 2/36 cytopl & nucl 4/36 reduced/absent	Omholt *et al.*, 2001
	DGGE/DS	S37P S37Y & B32Q D32G	2/43 (primary) 1/30 (metastases)	Primary: 37/48 membranous (more in RGP than in VGP) 28/48 cytoplasmic 11/48 nuclear Metastases: 7/32 membranous 21/32 cytoplasmic 6/32 nuclear	Demunter *et al.*, 2002
Cell lines	DS		1/62		Pollock and Hayward, 2002

Table II.2. Overview of studies assessing β-catenin mutational status and localization in melanoma samples and cell lines. One should take care while interpreting the data in this table, as they result from different protocols used for the detection of mutations and/or immunohistochemistry. Especially in the latter, considerable variation is possible, considering the different criteria (e.g. number of cells positive) and/or fixations protocols used (see e.g. Rimm *et al.*, 1999[2205]). MD= microdissection, DS = direct sequencing of PCR products, SSCP =single strand conformation polymorphism, DGGE = denaturing gradient gel electrophoresis.

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II.4. P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma

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Manuscript in preparation

P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma

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ABSTRACT

Malignant transformation of melanocytes frequently coincides with a loss of E- and P-cadherin expression. Using retroviral transduction of several melanoma cell lines with P-cadherin, we show here that ectopic expression of P-cadherin in P-cadherin negative cell lines (BLM and HMB2) promotes the formation of cell-cell contacts, both in two- and three-dimensional cultures, and counteracts invasion. These effects were not observed following introduction of P-cadherin in a cell line (MeWo) which already had P-cad expression. The pro-adhesive and anti-invasive effects of P-cadherin were abolished upon mutation of its intracellular juxtamembrane domain and by mimicking a known missense mutation in its extracellular domain which gives rise to hypotrichosis with juvenile macular dystrophy. In conclusion, this study establishes a potent pro-adhesive and anti-invasive effect of P-cadherin in melanoma cell lines.

INTRODUCTION

The development of metastatic disease is a complex process in which tumor cells separate from the primary tumor mass, migrate through the extracellular matrix, enter and survive in the vascular system, extravasate into a foreign environment and establish new foci of growth. This process often involves changes in adhesive preferences of the tumor cells, which dictate their interactions with the surrounding extracellular matrix and neighboring cells. A better understanding of these adhesive changes may help to identify key molecules involved in the various steps of tumor progression.

Human skin is a multi-layered, cohesive tissue with a unique functional architecture affording the primary barrier to the outside environment. In the epidermis, melanocytes reside at the dermal-epidermal border, lying on the basement membrane. There, they are surrounded by basal keratinocytes, to whom they deliver pigment as a protection against the deleterious effects of UV. Under normal circumstances, melanocytes form a very quiescent population, undergoing controlled self-replication, resulting in a stable, life-long ratio with basal keratinocytes of 1:5-6. During melanoma development and progression, profound changes take place. These include uncontrolled proliferation and derangement of cellular and morphological differentiation (dysplasia), resulting in a radial growth phase, which, following additional genetic changes, results in profound invasion (vertical growth phase) and colonization of distant organs (metastasis) (Clark, 1991). Heterotypic cell-cell adhesion between melanocytes and keratinocytes is crucial for their intercellular communication. Important molecules involved in these contacts are cadherins, which are capable of establishing Ca^{2+}-dependent cell-cell adhesion (Tang *et al.*, 1994). Via association with their cytoplasmic binding partners, the catenins, cadherins are coupled to the actin cytoskeleton and to several signaling pathways (Yap *et al.*, 1997; Peifer, 2003). β-catenin (βctn)[2] binds to the cadherin C-terminal intracellular tail, via α-catenin providing a stabilizing link with the actin cytoskeleton. P120 catenin (p120ctn), another member of this family, binds to the cadherin juxtamembrane domain and is involved in regulating cadherin intracellular trafficking, stability, adhesive capacity, as well as cadherin-controlled migration (Anastasiadis *et al.*, 2000, Davis *et al.*, 2003; Chen *et al.*, 2003). Epidermal melanocytes express two types of classical cadherins, E- and P-cadherin (cad). Both are expressed by the basal keratinocytes as well, whereas keratinocytes in the upper layers of the epidermis are only E-cadherin positive. The ratio between E- and P-cad in both keratinocytes and melanocytes depends on their localization: E-cad^{high}, P-cad^{low} cells are primarily found in the epidermis, whereas E-cad^{low}, P-cad^{high} cells reside at the hair follicles (Nishimura *et al.*, 1999). During melanoma progression, a shift in cadherin expression has been described: while cells lose both E- and P-cad expression, N-cad is elevated (Hsu *et al.*, 1996; Seline *et al.*, 1996; Furukawa *et al.*, 1997; Silye *et al.*, 1998; Sanders *et al.*, 1999; Krengel *et al.*, 2004). Whereas a role for both E- and N-cad has been shown in melanoma, respectively counteracting and promoting invasive growth (Hsu *et al.*, 2000, Li *et al.*, 2001a), studies addressing a possible role for P-cad are lacking. We therefore set out to investigate the function of P-cad in *in vitro* cell-cell adhesion and

[2] Abbreviations used are: βctn β-catenin; cad, cadherin; p120ctn, p120 catenin; wt, wild-type.

invasion assays and found that P-cadherin could counteract invasion and promote adhesion of P-cad negative melanoma cell lines.

RESULTS

Cadherin expression in melanoma cell lines

To investigate the role of P-cadherin in malignant melanoma, the expression of the major classical cadherins, E-, N- and P-cad was determined by Western Blotting in a panel of melanoma cell lines. This revealed three types of expression: cell lines that did not contain any classical cadherin (as measured with cadherin-specific antibodies and a pan-cadherin antibody), cell lines that only expressed N-cadherin, and cell lines that were E-, P- and N-cadherin positive. For our evaluation of P-cadherin expression, we selected a representative cell line from each of these three groups: BLM, HMB2 and MeWo (Table 1).

Cell line	E-cad	P-cad	N-cad
BLM	-	-	-
HMB2	-	-	+
MeWo	+/-	+	+

Table 1. Expression of classical cadherins in melanoma cell lines used in this study.

Ectopic de novo expression of P-cadherin induces an epithelioid morphology in melanoma cells

Since BLM did not express any of the classical cadherins examined, this cell line was of first choice to explore the functional effects of P-cad expression. For this purpose, BLM cells were infected with the retroviral vector pLZRS-P-cad-internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP). An IRES allows both P-cad and EGFP to be translated separately from the same mRNA transcript, with expression levels of P-cad and EGFP being directly proportional. As a control, retroviral transduction with pLZRS-IRES-EGFP, encoding only EGFP, was performed. As shown in Figure 1, transduction with retrovirus containing either an empty vector (pLZRS-IRES-EGFP, LIE) or P-cad (pLZRS-P-cad-IRES-EGFP, P-cad), followed by sorting of the cells, resulted in a population that was more than 90% positive for EGFP (Figure 1).
P-cad expression was verified in the BLM cell lines by Western blotting. Unlike in the total lysates of parental BLM and BLM LIE, a band of the expected 118kD size, reactive with a monoclonal anti-P-cad antibody was revealed in BLM P-cad (Figure 2, upper panel, first 3 lanes). Biotinylation experiments confirmed the presence of P-cad at the cell surface in P-cadherin transduced cells (Fig 2, middle panel, third lane).

As is evident from figure 3 (pictures 1-3), a striking change in morphology was seen in P-cad transduced BLM cells, as compared to non-transduced or vector-transduced cells. Parental and vector-transduced cells are polygonal or spindle-shaped, with only minimal cell-cell contacts. In contrast, BLM P-cad cells adopt a more flattened, epithelioid morphology and arranged in cell rows with extensive cell-cell contacts. These changes were particularly evident in sub-confluent cultures and got less pronounced when cultures reached higher grades of confluency. In order to examine whether this feature was specific for BLM cells, or could be extended to other melanoma cell lines, we transduced and sorted HMB2 and MeWo cells, similarly as described above for BLM. Evaluation of the morphology of these transduced cells revealed that P-cad expression in HMB2 cells also promoted an epithelioid phenotype, although less pronounced than seen in BLM cells, whereas no

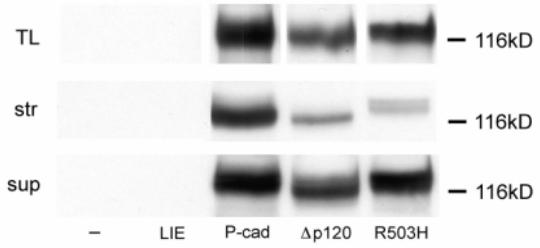

Figure 2. Western Blotting of total lysates (TL), streptavidin immunoprecipitates of lysates from biotinylated cells (str), or of the non-biotinylated fraction (sup) of BLM cells transduced with the indicated constructs

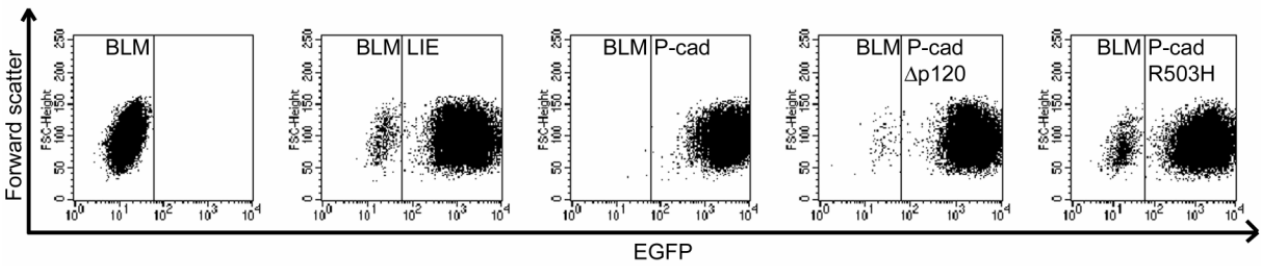

Figure 1. Flow cytometric evaluation of EGFP expression of transduced BLM cells.

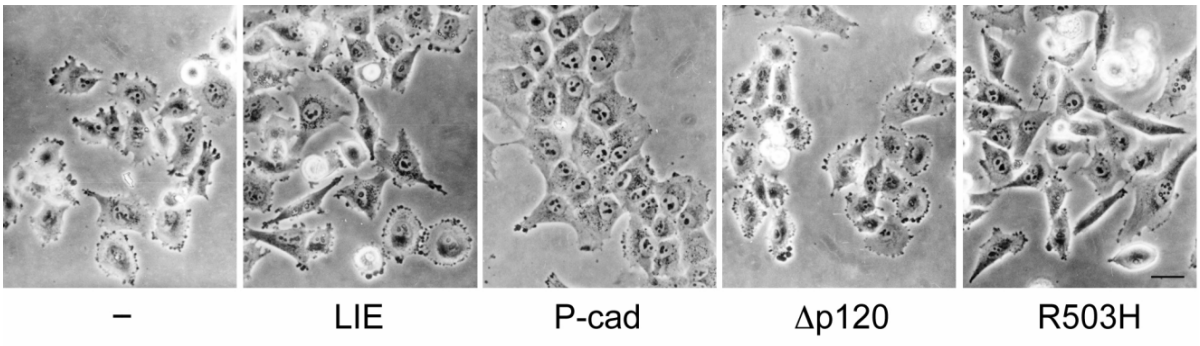

Figure 3. Phase-contrast micrographs of BLM cells transduced with the indicated constructs. Scalebar = 50µm

differences were observed in MeWo cells (data not shown). None of the transduced cell lines showed changes in growth, as measured by a colorimetric assay (data not shown). Upon testing in a wound healing migration assay, no differences could be observed in the speed of wound closure. However, the migration of P-cad transduced HMB2 and BLM cells differed from that of controls in that P-cad transduced cells migrated as sheets, whereas control cells migrated as single cells (data not shown).

In conclusion, these results suggest that P-cad may contribute to an increased epithelioid phenotype in melanoma cells that do not express this protein natively.

Ectopic de novo expression of P-cadherin stimulates cell-cell aggregation

In a slow aggregation assay on soft agar, parental and vector-transduced BLM cells are forming no (24 h) or loose aggregates (72h), while in BLM P-cad, cell aggregation is promoted leading to the formation of compact aggregates already after 24h (Figure 4, upper two panels). Similar to our observations in BLM cells, P-cad could confer increased aggregation to HMB2 cells. However, in the latter these changes were less evident at 24h, in which both parental, vector- and P-cad transduced cells formed small cell clusters (not shown), but became increasingly clear upon prolonged incubation times (Figure 4, second lower panel). In MeWo cells, which already express P-cad, no changes in cell-cell adhesion could be observed, neither at early (not shown) nor at late time points (Figure 4, lower panel). These cells already showed relatively high levels of aggregation, presumably mediated by their native N-, P- or E- cad. To verify these data, we used another aggregation assay, in which the different cell lines were incubated in suspension under continuous shaking. As is evident from a shift to the right of the particle size distribution curves, larger aggregates were formed by P-cad transduced BLM and HMB2 cells, while no

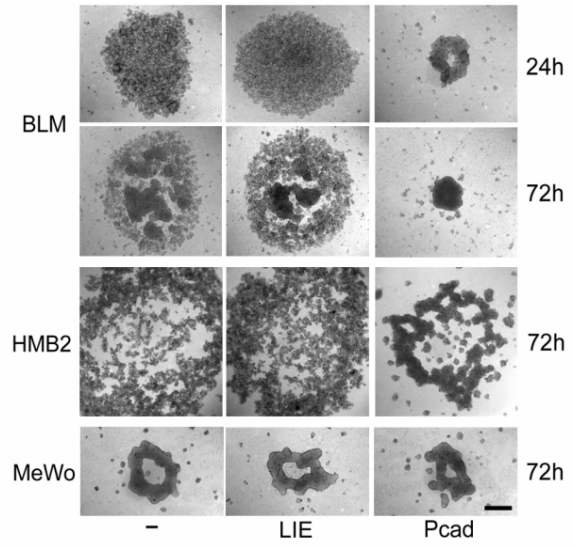

Figure 4. Slow aggregation assay on soft agar of the indicated cell lines, photographed after the indicated time points. Scalebar = 500µm

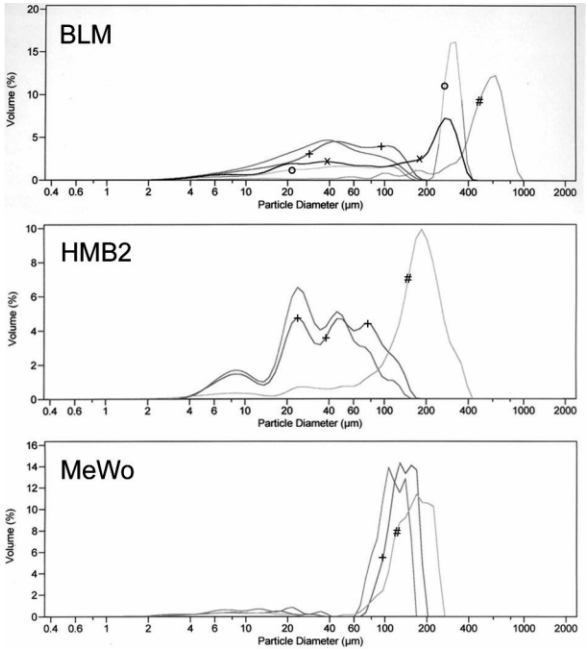

Figure 5. Particle size distribution of aggregates of the indicated cell lines. Aggregation times were 24h for BLM cells and 48h for HMB2 and MeWo cells. Curves without symbols indicate parental cell lines, for the transduced cell lines + indicates LIE, # indicates P-cad, x indicates P-cadΔp120, and o indicates P-cadR503H.

differences were observed in MeWo cells (Figure 5). Because the aggregates formed by BLM P-cad cells were too large to be measured at the 48h time point, the assay was stopped after 24h for these cells, while HMB2 and MeWo cells were allowed to form aggregates for 48h.

P-cadherin counteracts invasion of BLM and HMB2

To test the role of P-cadherin in the invasive phenotype of melanomas, the chick heart invasion assay was performed. As illustrated in Figure 6A, confrontation of BLM and BLM LIE cells (indicated with "T") with a precultured chick heart fragment (indicated with "N") for 4 (left panels) or 8 (right panels) days resulted in a marked invasion of tumor cells into the chick heart already after 4 days of coculture. In contrast, BLM P-cad cells only showed minimal invasion, rather surrounding (4d), and eventually compressing (8d) the chick heart fragment. This anti-invasive effect was also obvious after blind-coded acquisition of the invasion scores of independent confrontation cultures (Figure 6C). The counteraction of invasion by P-cad was also pronounced in HMB2 cells (Figure 6B, 6C). Whereas parental and vector-transduced HMB2 cells strongly invaded the chick heart, P-cad transduced cells nicely surrounded it. However, also in the confrontation cultures with P-cad transduced cells, one or some

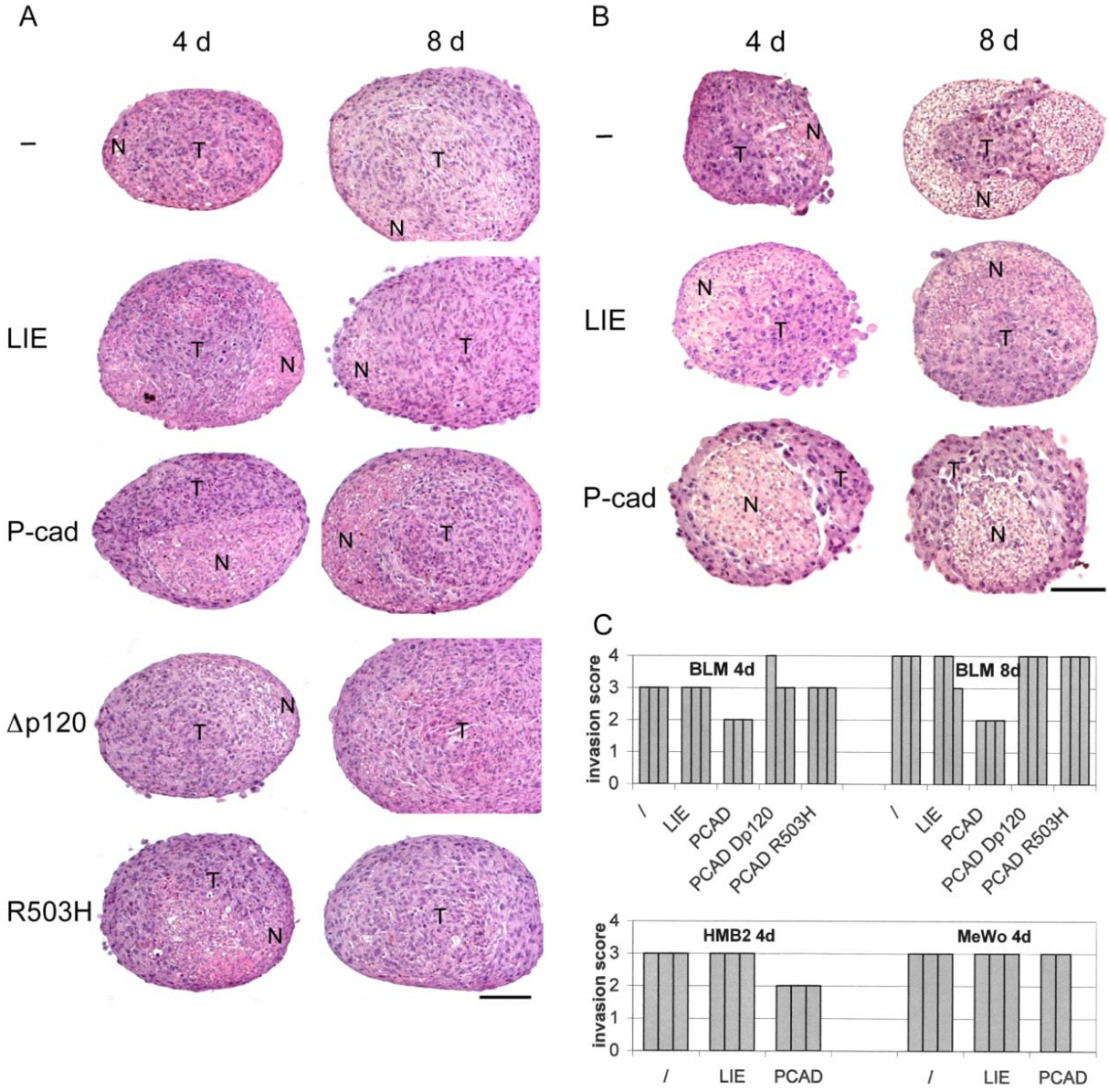

Figure 6. (A,B) Hematoxylin-eosin staining of chick heart confrontation cultures with the indicated BLM (A) or HMB2 (B) cell lines for the indicated time points. N stands for (remnants) of normal chick heart tissue, T stands for tumor cells. Scalebar = 100μm. (C) Invasion scores, obtained by scoring blindly independent confrontation cultures of the indicated cell lines for the indicated time points.

single cells could be found within the chick heart. Since our EGFP-based cell sorting resulted in population purities between 90 and 100%, we consider it very likely that these few single invasive cells represent non-transduced cells. The invasive behavior of MeWo cells was not affected by P-cad (Figure 6C). In conclusion, P-cad expression could counteract invasion of P-cad negative cells, paralleling its stimulating effects on aggregation.

Generation of BLM P-cad mutants

In order to identify which domains in P-cadherin were important for its pro-adhesive and anti-invasive activities, we constructed two P-cadherin mutants. We therefore targeted two different domains that can be expected to alter P-cad function. A first construct, P-cadΔp120, lacks 5 amino acids (EEGGG) in the presumed p120ctn binding site in the juxtamembrane domain of P-cadherin. The corresponding domain in E- and N-cadherin has been shown to be implicated in modulation of cell-cell adhesion and motility (Thoreson *et al.*, 2000, Fedor-Chaiken *et al.*, 2003). For the second construct, P-cadR503H, we targeted an arginine residue in the fourth extracellular domain (EC4) of P-cad. This R503H mutation represents the only known missense mutation of P-cad, which, in patients carrying this mutation, results in hypotrichosis with juvenile macular dystrophy (Indelman *et al.*, 2002, 2003). Since this mutation targets a conserved LDRE motif, which, on basis of sequence homology with E-cad, is likely implicated in Ca^{2+}-binding (Troyanovsky, 1999), it is expected to affect P-cad-mediated adhesion. BLM cells were transduced with these P-cad mutants and sorted to more than 90% purity (Figure 1). Integrity and localization of the constructs at the plasma membrane was confirmed by Western Blotting of total lysates (Figure 2, upper panel, last two lanes). As expected, P-cadΔp120ctn migrated slightly faster in SDS-PAGE than the other P-cad constructs. Cell surface expression of the P-cad mutants was verified by biotinylation. As compared to BLM P-cad, cell surface levels of both P-cad mutants were lower (Figure 2, middle panel), possibly reflecting decreased stability, which may be inherent, or may be the consequence of their altered capacity to form stable cell-cell contacts (see further). Overall, although significant amounts of cytoplasmic P-cad were found in all P-cad transduced cells, these were apparently not higher in cells expressing P-cad mutants (Figure 2, lower panel).

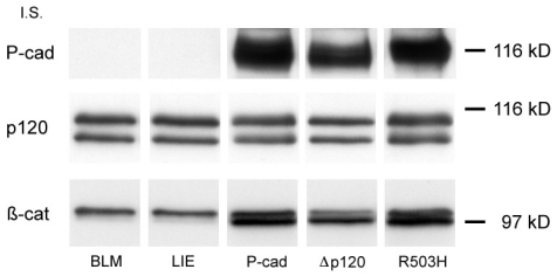

Figure 7. Analysis of the expression of P-cadherin, p120ctn and βctn in BLM-derived cell lines. Whole cell lysates of the indicated BLM-derived cell lines were analyzed by Western Blotting and immunostaining (I.S.) for the indicated proteins.

Influence of mutation of the p120 catenin binding domain or the extracellular domain on β- and p120ctn

To verify whether introduction of P-cad or P-cad mutants into BLM cells affected levels of β- and p120ctn, we performed Western Blotting of total lysates. As shown in figure 7, p120ctn levels remained unchanged upon expression of wild-type (wt) or mutated P-cad. In contrast, total levels of β-ctn were increased by all P-cad constructs, suggesting its stabilization by recruitment to the P-cad constructs. This was somewhat less pronounced in BLM P-cadΔp120 cells. Examination of the association of these proteins with the P-cad constructs showed that, whereas all constructs co-immunoprecipitated with βctn (Figure 8, upper panel), the P-cadΔp120 mutant failed to co-immunoprecipitate with p120ctn, thus confirming that this construct cannot efficiently couple to p120ctn (Figure 8, lower panel). In order to evaluate the localisation of β- and p120ctn, which are both typically localized at sites of cell-cell contact in cadherin expressing cells, we performed immunocytochemistry for these molecules in nearly confluent cultures, in which, also in the control and P-cad mutant cells, cell-cell contact formation is forced. As expected from their epithelioid morphology, BLM P-cad cells showed a nice honeycomb-like membranous staining pattern for both β- and p120ctn (Figure 9). In contrast, stainings in BLM (not shown) and BLM LIE were diffuse. Examination of the BLM P-cad mutants revealed striking differences with BLM P-cad. Whereas the staining pattern in BLM P-cadR503H did reveal membranous localisation (albeit to a much lower extent than in wt P-cad transduced cells), recruitment of β-catenin to the plasma membrane was absent in BLM P-cadΔp120ctn. In the latter, βctn staining was present in discrete puncta instead. Concerning p120ctn, recruitment to the plasma membrane was not seen in P-cadΔp120ctn transduced cells, as expected. As observed for βctn, P-cadR503H cells showed membranous p120ctn immunostaining, which was considerably weaker than in wt P-cad transduced cells.

Mutation of the p120 catenin binding domain or the extracellular domain of P-cadherin interferes with its pro-adhesive effects

Examination of subconfluent cultures revealed that the morphology of both BLM P-cadR503H and BLM P-cadΔp120 was indistinguishable from that of controls, meaning that in both instances, the promotion of cell-cell contact formation as seen with wt P-cad was completely abolished (Figure 3).
When tested in a 24h slow aggregation assay in suspension, both mutants could promote cell aggregation to some extent, although significantly less than P-cad (Figure 5). As is evident from Figure 10A, taken after 48h in suspension, these differences became even more pronounced upon prolonged culture (the same magnification is used for all pictures). A similar effect was seen in a slow aggregation assay on soft agar (Figure 10B). However, here the changes induced by the mutations were less drastic than in suspension, likely because in the latter, due to the continuous shaking, a more stringent condition is created in which weak cell-cell contacts are less easily maintained. The reduced aggregation on soft agar was clearly visible in BLM PCADΔp120 cells. The BLM P-cadR503H cells, on the other hand, showed significant aggregation, with the formation of a single aggregate, at first sight resembling the aggregate formed by the P-cad transduced cells. However, as indicated in the right panels of Figure 10B, this association remained loose. While the P-cad transduced BLM cells formed a very tight, compact aggregate, characterized by a smooth surface (indicated by arrowheads), this smooth surface ('compaction') was lacking in the aggregate formed by BLM P-cadR503H cells.

In conclusion, disruption of the extracellular domain or the p120ctn binding domain of P-cad interferes with its ability to establish tight, stable cell-cell contacts.

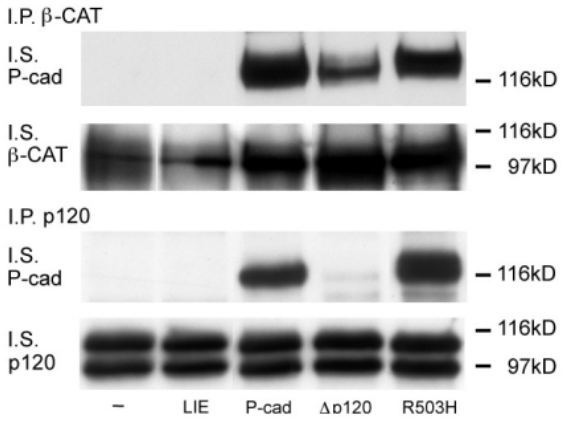

Figure 8. Association of βctn and p120ctn with P-cad and P-cad mutants. Equal amounts of whole cell lysates of the indicated cell lines were subjected to immunoprecipitation (I.P.), using anti-βctn antibody (upper 2 panels) or anti-p120ctn antibody (lower 2 panels), followed by immunostaining (I.S.) as indicated.

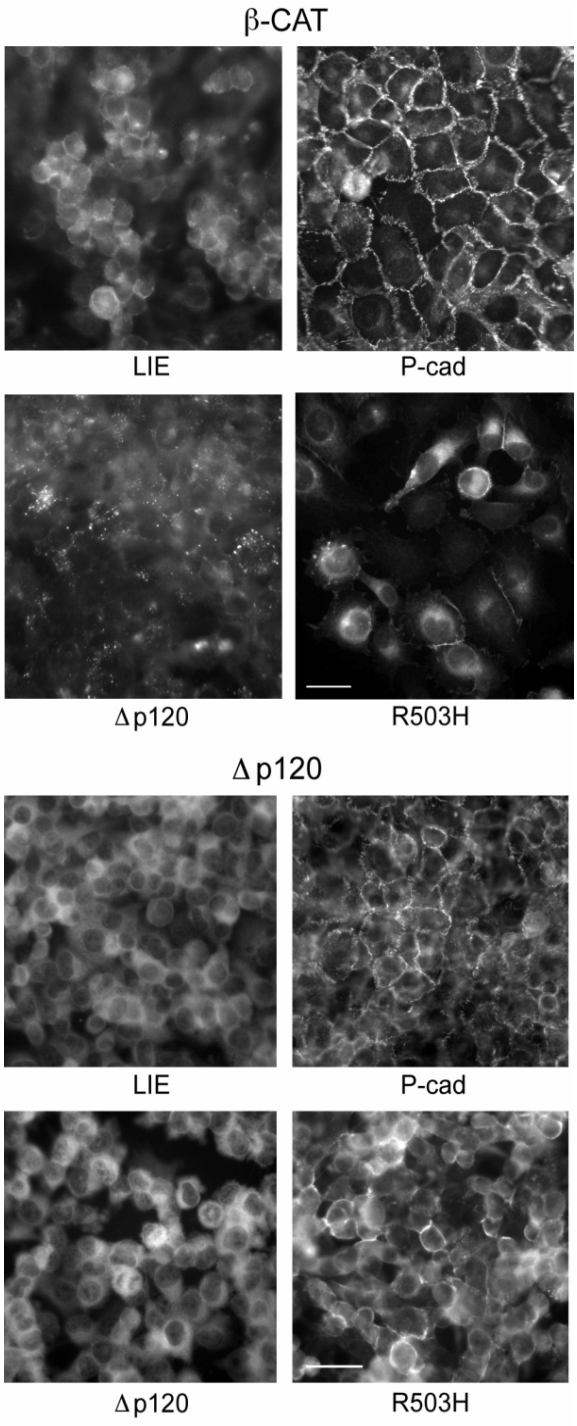

Figure 9. Immunocytochemistry for βctn and p120ctn in the indicated BLM cell lines. Nearly confluent cultures of the indicated BLM-derived cell lines were fixed and stained using anti-βctn (upper panel) or anti-p120ctn (lower panel) antibodies. Scalebar = 50μm.

Mutation of the p120 catenin binding domain or the extracellular domain of P-cadherin interferes with its anti-invasive effects

In order to determine the involvement of the p120ctn binding domain and the EC4 domain in the anti-invasive function of P-cadherin, the BLM P-cad mutants were examined in the chick heart invasion assay. As shown in the lower panels of Figure 6A, and as evaluated in Figure 6C, both mutations completely abrogated the anti-invasive activity of P-cad. The mutant cell lines showed invasion of the chick heart tissue at both time points and to the same extent as the BLM LIE control cells. These results indicate that both the p120ctn binding domain and an intact EC4 domain are implicated in the cell-cell adhesive and anti-invasive function of P-cadherin.

DISCUSSION

Altered expression of cell-cell adhesion molecules is a frequent event during malignant transformation of melanocytic cells (Johnson, 1999). Downregulation of two members of the cadherin family, E- and P-cadherin, has been observed during melanoma progression (Hsu *et al.*, 1996; Seline *et al.*, 1996; Furukawa *et al.*, 1997; Silye *et al.*, 1998; Sanders *et al.*, 1999; Li *et al.*, 2001b; Krengel *et al.*, 2004). The present study is the first to evaluate the possible role P-cadherin may play in cell-cell adhesion and invasion of melanoma cells, two processes involved in melanoma progression.

We found that ectopic expression of P-cadherin in P-cad negative melanoma cell lines promoted cell-cell contact formation and suppressed invasion. Importantly, these effects were not limited to cells lacking endogenous cadherin expression. Also the behavior of the HMB2 cell line, expressing N-cadherin endogenously, was redirected towards increased aggregation and reduced invasion. Ectopic expression of P-cad in MeWo cells, which already expresses endogenous P-cad, in addition to N-cad and minute amounts of E-cad, could not alter the adhesive and invasive behavior of these cells. We hypothesize that this cell line has acquired additional genetic changes, rendering it resistant to the protective effects of P-cad.

In order to evaluate the role selected domains in P-cad may play in the observed effects, we created two P-cad mutants. A first mutant we tested, P-cadR503H, was chosen based on its occurrence in a human syndrome, hypotrichosis with juvenile macular dystrophy. This is a rare autosomal recessive disorder characterized by early hair loss and severe macular degeneration, eventually leading to blindness in most patients (Souied *et al.*, 1995). This syndrome has recently been shown to result from mutations in *CDH3* (the gene encoding P-cadherin) on 16q21 (Sprecher *et al.*, 2001; Indelman *et al.*, 2002, 2003).

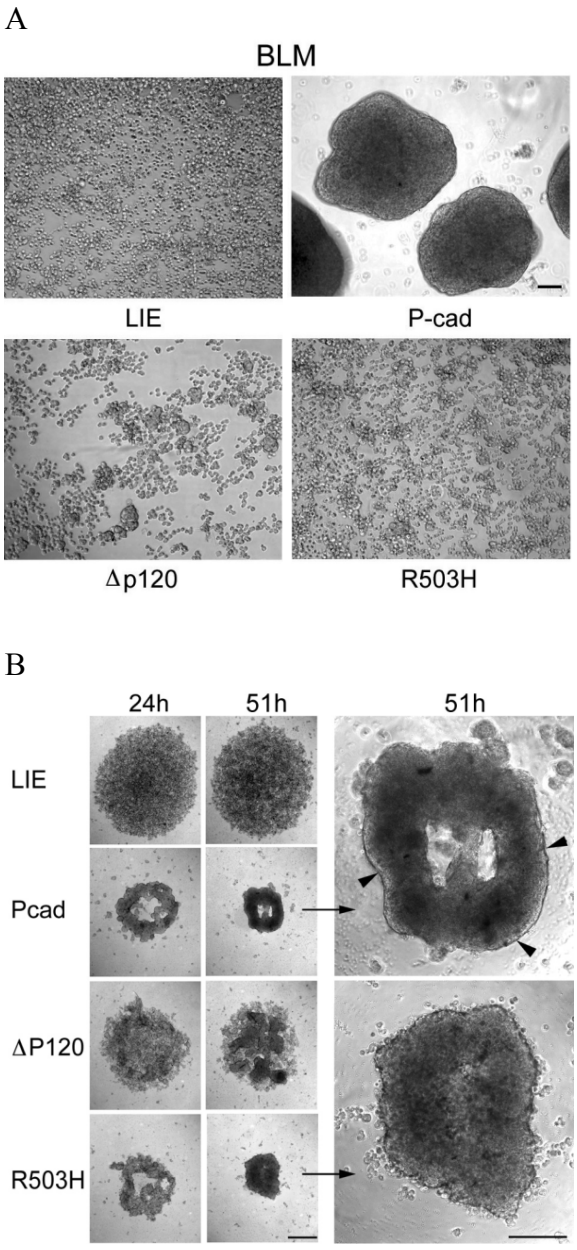

Figure 10. (A) Slow aggregation assay in suspension of the indicated BLM-derived cell lines, photographed after 48h. Scalebar = 100µm. (B) Slow aggregation assay on soft agar of the indicated BLM-derived cell lines, photographed after the indicated time points. Scalebar = 500µm in the left panels, 100µm in the right panel. Arrowheads indicate the smooth, compacted surface of the BLM P-cad aggregate.

Apart from deleterious frameshift mutations, disrupting the *CDH3* open reading frame, a more subtle mutation, resulting in the substitution of a single amino acid, was found as well (Indelman *et al.*, 2002, 2003). The crystal structure of the C-cadherin ectodomain (Boggon *et al.*, 2002) shows the involvement of the corresponding C-cad arginine residue, embedded in a highly conserved LDRE motif, in Ca^{2+}-binding. Thus, although not tested experimentally, it was suggested that this mutation may affect P-cad function by disrupting the P-cad conformation. Here we have mimicked the R503H mutation in a melanoma cell line and analyzed its

influence on P-cad function. The second domain we targeted in P-cadherin was its juxtamembrane domain. This domain, which contains the p120 ctn binding sequence, is highly similar to the corresponding domain in E- and N-cadherin, where it has been involved in regulation of cell-cell adhesion and motility (Thoreson *et al.*, 2000; Fedor-Chaiken *et al.*, 2003). The resulting mutant, P-cadΔp120, lacked p120ctn binding, as evidenced by co-immunoprecipitation and immunocytochemistry.

Evaluation of the function of PCADR503H and PCADΔp120 in BLM cells, lacking expression of any other classical cadherin, learned that both have a severely impaired ability to suppress invasion and support cell-cell adhesion. The latter effect was more pronounced for PCADΔp120. Although P-cadR503H, in non-stringent conditions, could support cell-cell adhesion in BLM cells, it failed in generating the compact aggregates that were formed in P-cad transduced cells. In contrast to wt P-cad, both mutants failed to form well-organized cell-cell contacts. In subconfluent cultures this was evident from the absence of cell islands, as seen in wt P-cad transduced cells. In more confluent cultures, in which cell-cell contacts are forced, the lack of organization was witnessed by the impaired recruitment of βctn to adherens junctions. Parallel with the effect of the mutations on aggregation, this impaired recruitment of βctn was less severe in P-cadR503H than in P-cadΔp120, in which it was almost completely absent. In the latter, βctn staining was confined to discrete cytoplasmic puncta. Contrasting with the failure of the P-cad mutants to recruit βctn to the cell membrane as wt P-cad did, βctn co-immunoprecipitated with all P-cad constructs. Moreover, expression of all mutants resulted in increased levels of βctn, suggesting stabilization of the latter. Of note, this increase was particularly seen in a band migrating slightly faster than the band corresponding to βctn in control or vector-transduced cells. This might represent a difference in phosphorylation status of βctn. Overall, these results suggest that P-cad mutants can still recruit and stabilize βctn, they largely fail to stabilize it at the membrane. In contrast to the increase of total βctn, p120ctn levels remained unchanged upon expression of P-cad. Recruitment of p120ctn to the plasma membrane was absent in BLM P-cadΔp120 (as expected) and was largely decreased in BLM P-cadR503H. The decreased ability of the mutants to counteract invasion, stabilize cell adhesion and recruit βctn and p120ctn to cell-cell contacts is likely due to their decreased cell surface expression. This was evident from biotinylation experiments, in which lower levels of the P-cad mutants were found at the cell surface, despite similar overall levels of P-cad. Consistent with this is our observation that P-cadherin shows the same subcellular distribution as βctn in transduced BLM cells: mainly membranous for wt P-cad; membranous (at sites of cell-cell contacts) and cytoplasmic for P-cadR503H; and in discrete puncta, with some membranous staining at cell-cell contacts for P-cadΔp120 (data not shown). Whether this increased cytoplasmic staining is the cause or the consequence of defective adhesion is not clear, yet. The recent involvement of p120ctn in kinesin-mediated transport of cadherin-catenin complexes to intercellular junctions (Chen *et al.*, 2003) may provide an explanation for the impaired functions of P-cadΔp120. However, using p120ctn-uncoupled N-cadherin, these authors reported a delay, rather than a general incapability in reaching cell-cell contacts. A more detailed study of the discrete areas where βctn and P-cadΔp120ctn were found may help to resolve this question.

In conclusion, we have demonstrated that P-cad may promote strong aggregation and counteract invasion of melanoma cells. Our functional analysis of two P-cad mutants, one lacking binding to p120ctn and the other representing the only known missense mutation of P-cad, revealed that both have a strongly impaired capacity to support cell-cell adhesion. This coincides with a decreased cell surface localization and likely explains the failure of these mutants to counteract invasion.

MATERIALS AND METHODS

Plasmids and cDNA Constructs, Retroviral Transduction and cell sorting. The hP-cad/pBR322-23-b expression vector, containing the 3.2kb cDNA encoding full-length human P-cad (13), was kindly provided by Prof. Keith R. Johnson (Department of Oral Biology, College of Dentistry and the Eppley Cancer Center, Nebraska Medical Center, Omaha, NE), with the permission from Prof. Yukata Shimoyama (Department of Surgery, International Catholic Hospital, Nakaochiai, Shinjuku, Tokyo, Japan). To create P-cadΔp120, P-cad was EcoRI subcloned into pIRES2-EGFP (Clontech, Palo Alto, CA), generating pP-cad-IRES2-EGFP. This was XhoI/SmaI digested and the removed fragment was cut with EarI, recognizing two nearby sites in the P-cad sequence. The small fragment between the two EarI restriction sites (encoding EEGGG) was removed with a PCR-purification column and the remaining two fragments (XhoI/EarI and EarI/SmaI) were ligated into the dephosphorylated XhoI/SmaI digested pP-cad-IRES2-EGFP. To create the P-cad point mutant (P-cadR503H), the P-cad cDNA was EcoRI subcloned into pBluescript and the sequence coding for a large part of the extracellular domain was BamHI/TaqI excised. A PCR product, encompassing the point mutation, was obtained, using the following primers: a sense primer (5'-GGCACCCTCGACCATGAGGATGAG-3'), with the TaqI restriction site underlined and the point mutation in bold; and the antisense primer 5'-CGTGCCGTCGACCTACAGGTTCTCAAT-3'. This product was TaqI/NdeI digested and ligated with the BamHI/TaqI fragment. After this, the resulting fragment was ligated into dephosphorylated BamHI/NdeI digested pP-cad-Bluescript. All these constructs were *EcoR*I transferred to the LZRS-IRES-EGFP (LIE) retroviral vector, to generate the LZRS-P-cad-IRES-EGFP vector. The LIE retroviral vector, encoding only EGFP, was used as a control. Direct sequencing (ABI, Perkin Elmer, Foster City, CA) was performed for all the constructs, in order to confirm their integrity.

For the production of retroviral supernatant, the Phoenix-Amphotropic packaging cell line (a kind gift from Dr. G.P. Nolan, Stanford University School of Medicine, Stanford, CA) was transfected with the LIE and the LZRS-P-cad-IRES-EGFP plasmids using calcium-phosphate precipitation (Invitrogen) to generate both retroviruses. The viral supernatant was spun (10 min at 350xg) and aliquots were stored at –70°C until use. Transduction, flow

cytrometrc evaluation and sorting of cell lines was performed as described before (Stove *et al.*, 2003b).

Cell lines, Restriction Enzymes, Antibodies and Chemical Reagents. Human cancer cell lines were obtained and cultured as described before (Stove et al., 2003a). All the restriction enzymes were purchased from New England BioLabs (Beverly, MA). Antibodies used were: monoclonal anti-P-cad (clone 56) and anti-p120ctn (clone 98) (BD Transduction Laboratories, Lexington, KY), polyclonal anti-βctn (Sigma-Aldrich, Bornem, Belgium) and a polyclonal rabbit antiserum generated against a 15-mer peptide, corresponding to the C-terminus of E-cadherin, which is cross-reactive with P-cadherin and was used for immunocytochemistry.

Biotinylation, Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting experiments were performed as published before, except that for preparation of cell lysates 0.7% Nonidet P-40 in PBS was used as lysis buffer (Stove *et al*., 2003a). For biotinylation, the cells were first incubated with 0.5 mg/ml of biotin, during 30 min, and washed four times with PBS, before lysing the cells.

Slow Aggregation Assays. On Semi-Solid Substratum: $2x10^4$ cells in 200 µl medium were seeded on solidified agar in a 96-well-plate (Boterberg et al., 2001). Aggregate formation was evaluated under an inverted microscope at indicated time points. In Suspension: $6x10^5$ cells were added to 50 ml-Erlenmeyer flasks in 6 ml medium. The flasks were incubated on a Gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 72 rpm, and continuously gassed with humidified 10% CO_2 in air. The particle size distribution of the aggregates was measured with a Coulter Particle Size Counter (LS2000, Coulter Company, Miami, FL). The diameter of the particles can be considered as a measure for aggregate formation. Statistical analysis of differences between the particle size distribution curves was done with the Kolmogorov-Smirnov method.

Chick Heart Invasion Assay. The chick heart invasion assay was used as described earlier (Bracke et al, 2001). Briefly, heart fragments of 9-day old chick embryo's were precultured for 4 days (PHF) and then confronted with tumour cells under continuous Gyrotory shaking. Tumour cells were applied as monolayer fragments. The individual confrontations were fixed in Bouin-Hollande's solution after 4 or 8 days of incubation. They were afterwards embedded in paraffin, serially sectioned and stained with haematoxylin-eosin.

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II.5. P-cadherin is upregulated by the anti-estrogen ICI 182,780 in breast cancer cells and promotes invasion via its juxtamembrane domain

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P-cadherin is Upregulated by the Antiestrogen ICI 182,780 in Breast Cancer Cells and Promotes Invasion via its Juxtamembrane Domain[1]

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ABSTRACT

P-cadherin expression in breast carcinomas has been associated with tumors of high histological grade and lacking estrogen receptor-α, suggesting a link between these proteins. In the MCF-7/AZ breast cancer cell line, blocking estrogen receptor-α signaling with the antiestrogen ICI 182,780 induced an increase of P-cadherin, which coincided with induction of *in vitro* invasion. Retroviral transduction of MCF-7/AZ cells, as well as HEK 293T cells, showed the pro-invasive activity of P-cadherin, which requires the juxtamembrane domain of its cytoplasmic tail. This study establishes a direct link between P-cadherin expression and the lack of estrogen receptor-α signaling in breast cancer cells, and suggests a role for P-cadherin in invasion, through its interaction with proteins bound to the juxtamembrane domain.

INTRODUCTION

Cell adhesion determines morphogenesis and regulates cell motility, growth, differentiation and survival (1). Classical cadherins are a superfamily of transmembrane glycoproteins responsible for calcium-dependent cell-cell adhesion (2), which mediate homophilic protein-protein interactions. These are modulated by their conserved cytoplasmic juxtamembrane domain (JMD)[3] and catenin-binding domain (CBD), linking them to the actin cytoskeleton. α-, β-, p120-, and γ-catenins are the best-documented interaction partners (3). β-catenin (βctn) (and perhaps also γ-catenin) is a signaling molecule, implicated in tissue patterning and maintenance of the cell phenotype, whose functions are regulated by binding to the CBD of cadherins, and by interactions with receptor tyrosine kinases and transcription factors of the lymphocyte enhancer factor/T-cell factor family (4). P120-catenin (p120ctn) was identified as a substrate for *Src* and several receptor tyrosine kinases (5), and interacts directly with the JMD of cadherins (6), modulating cadherin clustering and cell motility in a cell type and phosphorylation state-dependent way (7). The cadherin/catenin junctional complex is linked to the actin cytoskeleton via α-catenin (8), thus strengthening its adhesive force.

Reduced expression of E-cadherin (E-cad) is associated with tumor progression in many different cancers, including breast cancer (9), and may result from mutations, loss of heterozygosity, promoter hypermethylation, or upregulation of transcriptional repressors, as SIP1, Snail, Slug or Twist (1). Moreover, the invasion suppressor function of normally expressed E-cad may be overcome by the aberrant expression of N-cadherin (N-cad) (10) or cadherin-11 (11), which have been associated with progression of breast carcinoma through interference with E-cad function (12).

P-cadherin (P-cad), another classical cadherin, is expressed in ectodermal tissues, more specifically in the basal layers of stratified epithelia (13-15) and in myoepithelial cells of the breast (16-17). P-cad is implicated in growth and differentiation, as evidenced by knockout mice displaying precocious differentiation of the mammary gland (18), and is aberrantly expressed in mammary carcinomas of high histological grade and with a poor prognosis (19-24), as well as in other types of carcinomas and proliferative inflammatory lesions (25-29). It has been suggested that suppression of the P-cad gene is lost during carcinogenesis (14), but the nature of this mechanism and the biological role of the newly acquired P-cad remain to be investigated.

Since aberrant expression of P-cad identified a subgroup of estrogen receptor-α (ERα) negative breast carcinomas (23), we raised the hypothesis that the

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[4] The abbreviations used are: βctn, β-catenin; CBD, catenin binding domain; CHX, cycloheximide; E2, 17β-estradiol; E-cad, E-cadherin; ERα, estrogen receptor-α; HEK, HEK 293T cell line; ICI, ICI 182,780; JMD, juxtamembrane domain; LIE, LZRS-IRES (internal ribosome entry site) – EGFP vector; MCF7, MCF-7/AZ cell line; N-cad, N-cadherin ; p120ctn, p120-catenin; P-cad, P-cadherin.

expression of P-cad in mammary epithelial cells is hormonally regulated, as described for E-cad (30), N-cad (31) and cadherin-11 (32).

In mammary epithelial cells, ERα is a key regulator of proliferation and differentiation and a crucial prognostic indicator and therapeutic target in breast cancer (33). ERα is a ligand-dependent transcription factor (34) acting through direct transcriptional target activation (35). Estradiol acts as a potent mitogen for many breast cancer cell lines (36), and about 70% of breast carcinomas are ERα positive. This mitogenic effect is blocked by estrogen antagonists. Pure antiestrogens (like ICI 182,780 (ICI)) and selective estrogen receptor modulators (like tamoxifen) (37-38) are used for the treatment of osteoporosis, breast cancer and other diseases (39). In breast cancer, a high number of patients eventually develop antiestrogen resistance, for unknown reasons. Continuous exposure of steroid-hormone responsive breast cancer cell lines to ICI leads to resistant sub-lines, with signaling pathways alternative to ERα (40).

Using the antiestrogen ICI, we investigated a putative molecular and functional link between the absence of ERα signaling and P-cad expression in breast cancer cells. In order to understand the relationship between P-cad and the aggressive breast cancer phenotype, we studied the effect of wild-type P-cad and several mutants on cell aggregation and invasion. We report that aberrant expression of P-cad may result from a lack of ERα signaling and may induce cell invasion, in a JMD-dependent manner.

MATERIALS AND METHODS

Plasmids and cDNA Constructs. The hP-cad/pBR322-23-b expression vector, containing the 3.2kb cDNA encoding full-length human P-cad (13), was kindly provided by Prof. Keith R. Johnson (Department of Oral Biology, College of Dentistry and the Eppley Cancer Center, Nebraska Medical Center, Omaha, NE), with the permission from Prof. Yukata Shimoyama (Department of Surgery, International Catholic Hospital, Nakaochiai, Shinjuku, Tokyo, Japan). The cDNA encoding full-length mouse E-cad was kindly provided by Jolanda van Hengel (Department of Molecular Biology, Laboratory of Molecular Cell Biology, Ghent University and Flanders Interuniversity Institute of Biotechnology, Ghent, Belgium). Both cDNAs (PC-WT and mEC-WT) were transferred to the expression vector pIRES2-EGFP (Clontech, Palo Alto, CA), in order to facilitate the evaluation of transfection efficiencies due to co-expression of EGFP after transfection. To generate P-cad deletion mutants, P-cad was *Eco*RI subcloned into pBluescript (Promega, Madison, WI) and *Nde*I/*Sal*I digested to remove the region coding for the C-terminal part of this protein. PCR fragments corresponding to different lengths of the removed tail, flanked by *Nde*I/*Sal*I restriction enzyme digest sites at the 5' and 3' ends, respectively, were obtained using always the same sense primer (5'-AAGCAGGATACATATGACGTG-3') and different antisense primers for the following constructs: PC-CT682: 5'-CGTGCCGTCGACCTACTTCCGCTTCTT-3'; PC-CT702: 5'-CGTGCCGTCGACCTAGCCATAGTAGAA-3'; PC-CT711: 5'-CGTGCCGTCGACCTACTGGTCCTCTTC-3'; PC-CT719: 5'-CGTGCCGTCGACCTAGTGGAGCTGGGT-3'; PC-CT727: 5'-CGTGCCGTCGACCTACTCCGGCCTGGC-3'; and PC-CT762: 5'-CGTGCCGTCGACCTACAGGTTCTCAAT-3'. After *Nde*I/*Sal*I digestion, these products were ligated into dephosphorylated *Nde*I/*Sal*I digested pP-cad-Bluescript, and the resulting construct was *Eco*RI/*Sal*I transferred to pIRES2-EGFP. Additionally, a mutant with a small deletion in the p120ctn-binding sequence (lacking the nucleotides coding EEGGG), and retaining the intact CBD, was created (PC-Δ703-707). Therefore, pP-cad-IRES2-EGFP was *Xho*I/*Sma*I digested and the removed fragment was cut with *Ear*I, recognizing two nearby sites in the P-cad sequence. The small fragment between the two *Ear*I restriction sites (encoding EEGGG) was removed with a PCR-purification column and the remaining two fragments (*Xho*I/*Ear*I and *Ear*I/*Sma*I) were ligated into the dephosphorylated *Xho*I/*Sma*I digested pP-cad-IRES2-EGFP. To create the P-cad point mutant (PC-R503H), the P-cad cDNA was *Eco*RI subcloned into pBluescript and the sequence coding for a large part of the extracellular domain was *Bam*HI/*Taq*I excised. A PCR product, encompassing the point mutation, was obtained, using the following primers: a sense primer (5'-GGCACCCTCGAC**CAT**GAGGATGAG-3'), with the *Taq*I restriction site underlined and the point mutation in bold; and the antisense primer used for generating PC-CT762. This product was *Taq*I/*Nde*I digested and ligated with the *Bam*HI/*Taq*I fragment. After this, the resulting fragment was ligated into dephosphorylated *Bam*HI/*Nde*I digested pP-cad-Bluescript and the construct was *Eco*RI transferred to pIRES2-EGFP. Direct sequencing (ABI, Perkin Elmer, Foster City, CA) was performed for all the constructs, in order to confirm their integrity.

Restriction Enzymes, Antibodies and Chemical Reagents. All the restriction enzymes were purchased from New England BioLabs (Beverly, MA). Anti-human primary mouse monoclonal antibodies used were against: P-cad (clone 56) and p120ctn (clone 98) (BD Transduction Laboratories, Lexington, KY), N-cad (CH-19 and GC-4) and α-tubulin (B-5-1-2) (Sigma-Aldrich, Bornem, Belgium), E-cad (HECD-1) (Takara Biochemicals, Kyoto, Japan), and ERα (NCL-L-ER-6F11) (Novocastra, Newcastle, United Kingdom). 17β-estradiol (E2) was purchased from Sigma-Aldrich Química (Portugal) and ICI was kindly provided by AstraZeneca (Portugal). Both drugs were dissolved in 100% ethanol, and added to the culture media. The concentrations used were 10 nM for E2 and 100 nM for ICI, unless mentioned otherwise. Cycloheximide (CHX) was obtained from Sigma, and used at 25 μg/ml. For the control situations, cells were treated only with 100% ethanol.

Cells and transient transfection. Human cancer cell lines were obtained as follows: BT-20 from P. Coopman (Laboratory of Molecular Biology, Ghent University, Belgium), MCF-7/AZ (MCF7) from P. Briand (The Fibiger Institute, Copenhagen, Denmark), ZR-75.1 and T47D from American Type Culture Collection (Manassas, VA), and HEK 293T (HEK) cells from V. De Corte (Department of Biochemistry, Faculty of Medicine and Health Sciences, Flanders Interuniversity Institute for Biotechnology, Ghent University, Belgium). Cell lines were routinely maintained at 37ºC, 10% CO_2, in the following media (Invitrogen, Merelbeke, Belgium): 50% DMEM/50% HamF12 (MCF7), DMEM (BT-20, T47D, HEK) or RPMI-1640 (ZR-75.1). All media for routine culture contained 10% heat-inactivated FBS (Greiner bio-one, Wemmel, Belgium), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Invitrogen). To obtain transient transfectants, appropriate expression vectors (2.5 μg) were introduced into HEK cells ($2x10^5$) with Fugene® (Roche Molecular Biochemicals, Mannheim, Germany) and transfection efficiencies were evaluated by measuring EGFP expression by flow cytometry.

Retroviral Transduction. The P-cad coding sequence was EcoRI digested from pIRES2-EGFP, and EcoRI subcloned into the LZRS-IRES-EGFP (LIE) vector, to generate the LZRS-P-cad-IRES-EGFP vector. The LIE retroviral vector, encoding only EGFP, was used as a control. For the production of retroviral supernatant, the Phoenix-Amphotropic packaging cell line (a kind gift from Dr. G.P. Nolan, Stanford University School of Medicine, Stanford, CA) was transfected with the LIE and the LZRS-P-cad-IRES-EGFP plasmids using calcium-phosphate precipitation (Invitrogen) to generate both retroviruses. The viral supernatant was spun (10 min at 350xg) and aliquots were stored at –70°C until use. Transduction of cell lines was performed as described before (41).

Flow Cytometry Staining and Cell Sorting. For analysis of E- and N-cad surface expression, cells were detached under cadherin saving procedures (42) and approximately $1x10^5$ cells were used for staining. Cells were washed with cold PBS containing BSA and incubated for 30 min with the anti-E-cad HECD-1 or anti-N-cad GC-4 antibodies. This was followed by two washes with PBS/BSA, 30 min incubation with biotinylated rabbit anti-mouse monoclonal antibody, two washes with PBS/BSA, 20 min incubation with streptavidin-phycoerythrin and a final wash with PBS/BSA. For routine analysis of EGFP expression, cells were detached with trypsin/EDTA. Flow cytometric analysis and/or cell sorting were performed as described before (41).

Biotinylation, Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting experiments, and quantification of bands, were performed as published before (43). For biotinylation, the cells were first incubated with 0.5 mg/ml of biotin, during 30 min, and washed four times with PBS, before the cell lyses. To control for equal loading of total lysates, immunostaining with anti-α-tubulin was performed routinely (not always shown). Each immunoblot was done at least three times and the ones that were selected to show are representative experiments.
RT-PCR. RT-PCR experiments were done as previously described (43). Primers specific for P-cad cDNA were: sense 5'-ACGAAGACACAAGAGAGATTGG-3' and antisense 5'-CGATGATGGAGATGTTCATGG -3', to generate a 287-bp product. PCRs were done in a Minicycler™ (Biozym, The Netherlands) with an annealing temperature of 55°C.

Slow Aggregation Assays. On Semi-Solid Substratum: $2x10^4$ cells in 200 µl medium were seeded on solidified agar in a 96-well-plate (42). Aggregate formation was evaluated under an inverted microscope after 24h, 48h and 72h. In Suspension: $6x10^5$ cells were added to 50 ml-Erlenmeyer flasks in 6 ml medium. The flasks were incubated on a Gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 72 rpm, and continuously gassed with humidified 10% CO_2 in air. The particle size distribution of the aggregates was measured with a Coulter Particle Size Counter (LS2000, Coulter Company, Miami, FL). The diameter of the particles can be considered as a measure for aggregate formation. Statistical analysis of differences between the particle size distribution curves was done with the Kolmogorov-Smirnov method.

Invasion Assays. Collagen Type I Invasion Assay (44): Six-well plates were filled with 1.3 ml of neutralized collagen type I (0.09% w/v) (Upstate Biotechnology, Inc, Lake Placid, NY) and incubated for at least 1h at 37°C to allow gelification. $1x10^5$ cells of a single-cell suspension were seeded on top of the gel and cultures were incubated at 37°C during 24h. Using an inverted microscope controlled by a computer program (45), invasive and superficial cells were scored blind-coded in 12 fields of 0.157 mm^2. The invasion index expresses the percentage of cells invading into the gel over the total number of cells counted. Matrigel Invasion Assay: Transwell chambers with polycarbonate membrane filters (6.5 mm diameter, 8 µm pore size) (Costar, Corning, NY) were coated with 20 µl of a Matrigel solution (Becton Dickinson). $1x10^5$ cells were added to the upper compartment of the chamber. In the lower compartment, conditioned cell culture medium of the MRC-5 human embryonic lung fibroblast cell line was added as a chemo-attractant. After 24h of incubation at 37°C, the upper surface of the filter was cleared from non-migratory cells with a cotton swab and washes with serum-free DMEM. The remaining (invasive) cells at the lower surface of the filter were fixed with cold methanol and stained with DAPI (Sigma, 0.4 mg/ml). Invasive cells were scored by counting 50 fields/filter with a fluorescence microscope, at x250 of magnification. Rat myofibroblast DHD-FIB cells were routinely included as a positive control for invasion in both assays. Each experiment was repeated at least three times. For collagen invasion assay, data are expressed as mean ± SD; for matrigel invasion assay, a representative experiment is shown, with the SD for the number of cells scored on the 50 microscopic fields. Statistical significance was determined by t test, and differences between groups were analyzed using the ANOVA; $p<0.05$ was considered significant.

RESULTS

The antiestrogen ICI 182,780 upregulates P-cadherin in ERα positive breast cancer cell lines

To test the hypothesis that ERα negatively regulates P-cad, we examined the expression of ERα and cadherins in breast cancer cell lines by Western blot (Table I). Interestingly, higher levels of P-cad were found in ERα negative BT-20 cells.

A 24h treatment with the antiestrogen ICI [10^{-7} M] increased P-cad protein levels in MCF7 and ZR-75.1 cells, but not in BT-20 cells (Fig. 1). There were no significant changes in P-cad levels observed in T47D cells, bearing already higher pre-treatment levels of P-cad and lower levels of ERα than the responsive cell types. ICI-induced increase of P-cad was associated with a decline of ERα levels (Fig. 1).

For further investigation, we chose the MCF7 cell line, because it is ERα positive, highly responsive to estrogen and extensively investigated as a model of breast cancer (46). In these cells, ICI induced, respectively, up- and downregulation of P-cad and ERα in a time- and dose-dependent way (Fig. 2A and 2B). A decrease of ERα levels was already observed after 6h of treatment, whereas P-cad levels nearly doubled after 12h. After 24h of exposure to ICI,

	Type	E-cad[a,b]	P-cad[a,b]	N-cad[a,b]	ERα[a,b]	Invasion in Collagen Type I[c,d]
MCF-7/AZ	Breast	+++	+	—	+++	—
ZR-75.1	Breast	+++	+	+	+++	—
T47D	Breast	+++	++	—	++	+
BT-20	Breast	+++	+++	—	—	+
HEK 293T	Kidney	—	—	+++	—	—

Table I. Analysis of E-cad, P-cad, N-cad and ERα expression, and in vitro invasion into collagen type I, of the human cell lines used in this study. [a], determined by Western blot analysis; [b], —, negative; +, low; ++, moderate; +++, strong; [c], determined by Collagen Type I Invasion Assay; [d], —, not invasive; +, invasive

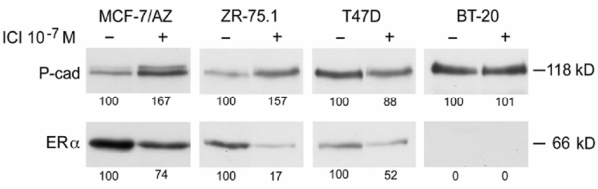

Figure 1. Effect of ICI 182,780 on P-cadherin and Estrogen Receptor-α expression in breast cancer cell lines. Immunoblotting, using anti-P-cad and anti-ERα mAb, of cell lysates derived from the breast cancer cell lines MCF7, ZR-75.1, T47D (50 µg of protein loaded) and BT-20 (30 µg), after a 24h treatment with ICI [10^{-7}M]. Band quantification was done relatively to the expression levels in control cells. An increase of P-cad expression in MCF7 and ZR-75.1 cells was observed, whereas the levels in ERα-negative BT-20 cells were not altered. P-cad levels were not changed in T47D cells, although ERα levels declined in all positive cell lines.

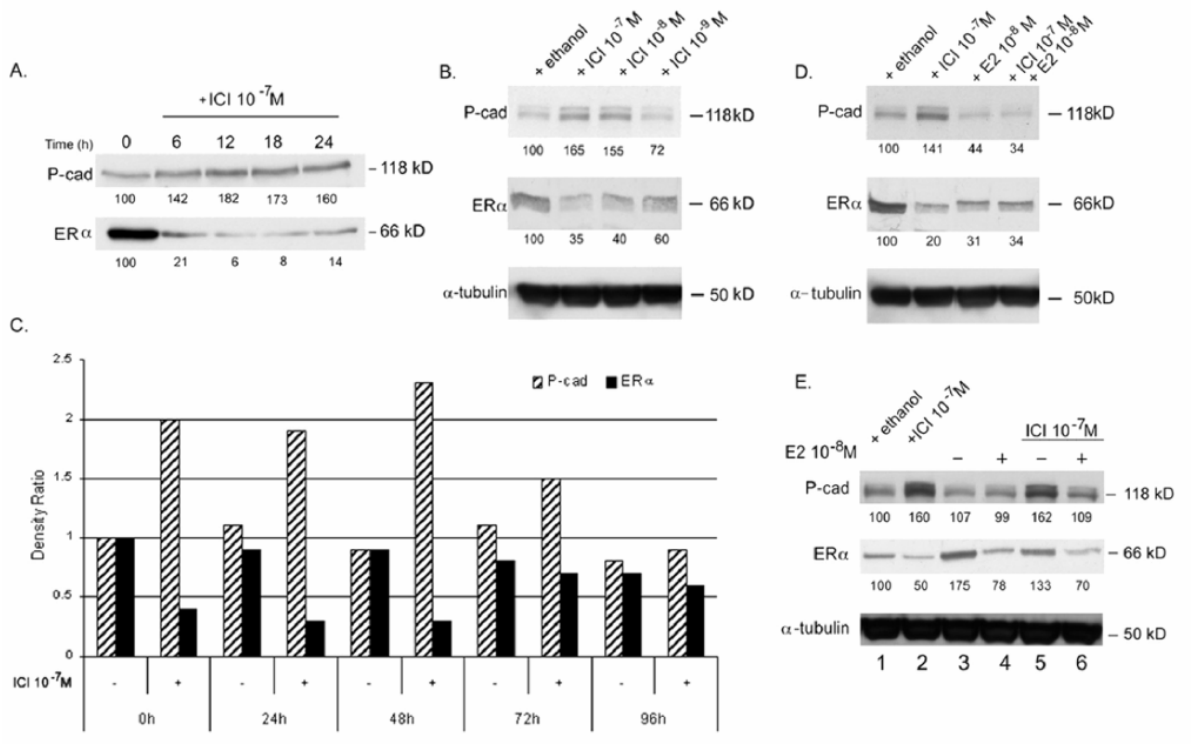

Figure 2. Regulation of P-cadherin expression by an ERα-dependent signaling pathway in MCF-7/AZ breast cancer cells. Immunoblotting, using anti-P-cad and anti-ERα mAb, of total lysates from MCF7 cells that had been treated with the indicated concentrations of ICI, for the indicated time points. Band quantification was done relatively to the expression levels in untreated cells. Immunostaining for anti-α-tubulin was done to control for equal loading. **(A)** ICI induces upregulation of P-cad and downregulation of ERα levels in a time-dependent manner, being maximal after 12h of treatment. **(B)** ICI induces upregulation of P-cad and downregulation of ERα levels in a dose-dependent way, the higher concentrations leading to a more pronounced effect. **(C)** MCF7 cells were grown in the presence of ICI for 24h. At time 0h, ICI was withdrawn and cell lysates were prepared at the indicated time points. Immunoblotting was performed to analyze P-cad (hatched bars) and ERα (black bars) expression. The levels of both proteins start to normalize again 96h after ICI withdrawal, showing the reversibility of the effect. **(D)** Cells were treated with ICI, E2 or a combination of both, for 24h. Although both ICI and E2 decreased ERα levels, the ICI-induced upregulation of P-cad was counteracted by estradiol. **(E)** MCF7 cells were grown in the presence of ICI for 24h (lanes 2, 5 and 6). After that, ICI was withdrawn and cells were treated (4 and 6) or not (3 and 5) with E2 [10^{-8}M] for additional 24h. E2 accelerated the reversion of P-cad expression to control levels in cells that had been treated with ICI.

higher P-cad and lower ERα levels persisted during several days, with normalization 96h after ICI withdrawal (Fig. 2C). To examine whether or not the effect of ICI on P-cad expression was mediated via ERα, we did a competition experiment. As already described (47), E2 readily decreased ERα levels, although to a lesser extent than ICI did (Fig. 2D). Importantly, the ICI-induced upregulation of P-cad was counteracted by E2 (Fig. 2D), as it also accelerated normalization of P-cad in cells treated for 24h with ICI (Fig. 2E). Together, these results suggest that not the decrease in ERα, but the lack of ERα signaling is responsible for the increase of P-cad by ICI.

RT-PCR revealed an increase of P-cad mRNA after ICI treatment, suggesting that the higher P-cad protein expression is based on an upregulation of P-cad transcripts (Fig. 3A). This was confirmed by a micro-array, performed on estradiol- or ICI-treated MCF-7 cells, in which estradiol did not alter P-cad mRNA levels, whereas ICI induced an eightfold increase. Finally, it remained to be determined whether induction of the P-cad gene (*CDH3*) was a direct effect of ICI or required prior induction of other genes. We addressed this question by blocking protein synthesis in cells, since the induction of primary target proteins (like ERα), or immediate early genes, should not be sensitive, whereas secondary targets should be blocked. The treatment of MCF7 cells with

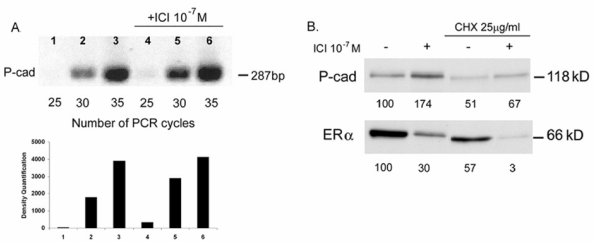

Figure 3. Effect of ICI 182,780 on P-cadherin mRNA levels in MCF-7/AZ cells and involvement of *de novo* protein synthesis in this process. (A) RT-PCR analysis of P-cad mRNA levels after ICI treatment of MCF7 cells for 24h. The analysis was done after the indicated No. of cycles of PCR amplification. P-cad mRNA increased in the presence of ICI (more evident at the 30 cycle point, in the exponential phase). Band quantification is presented in the graph shown below. **(B)** Immunoblotting, using anti-P-cad and anti-ERα mAb, of lysates from cells treated with CHX during 24h, alone or in combination with ICI. Band quantification was done relatively to the expression levels in untreated cells. CHX blocked P-cad upregulation induced by ICI, suggesting the involvement of *de novo* protein synthesis.

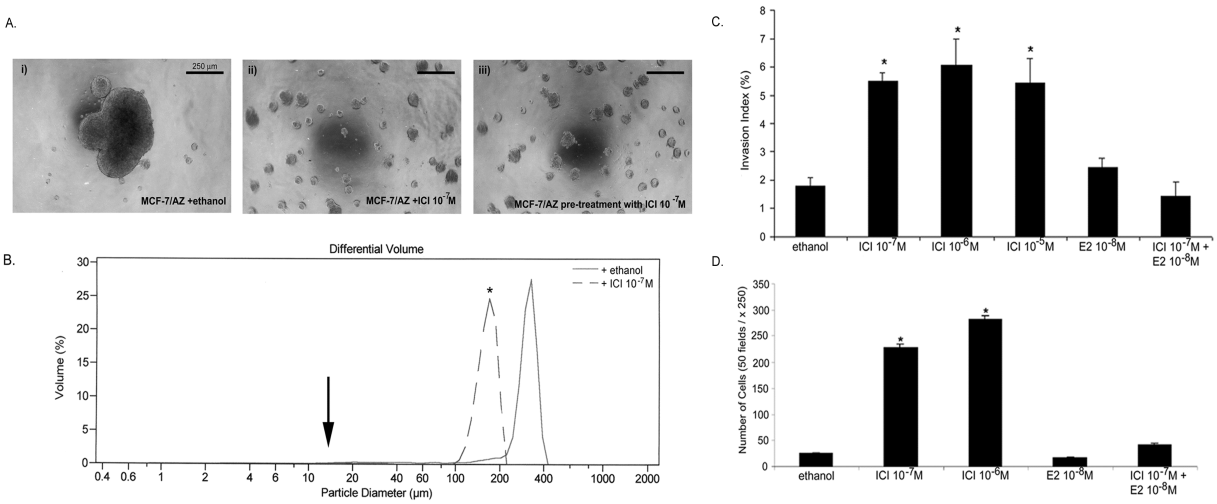

Figure 4. Effect of ICI on aggregation and on *in vitro* invasion of MCF7 cells. A: Pictures, after 72h, of the slow aggregation assay on semi-solid substratum. *(i)* Cells that were left untreated form compact aggregates, which are inhibited in cells cultured in presence of ICI *(ii)*, and in cells that have been pre-treated with ICI before seeding on top of agar *(iii)*. **B:** Slow aggregation assay in suspension: the cells were pre-treated with ICI for 24h, before incubation in Erlenmeyer flasks. A particle size distribution curve was generated using a particle size counter. Aggregates formed by ICI-treated cells were significantly smaller (*, $p=0.002$) than control aggregates. The arrow indicates the measurement of a single cell suspension at the beginning of the experiment. **C and D:** Cells that had been pre-treated for 24h with the indicated concentrations of ICI, E2 or the combination of both, were seeded as a single cell suspension on top of collagen type I gels (C) or on matrigel-coated filters (D). In collagen type I invasion assay, ICI-treated cells were significantly more invasive than control cells (*, $p=0.0081$, $p=0.02$ and $p=0.03$ for ICI [10^{-7}M], [10^{-6}M] and [10^{-5}M], respectively). In Matrigel, the differences were also statistically significant (*, $p<0.001$ for ICI [10^{-7}M] and [10^{-6}M]).

CHX, a de novo protein synthesis inhibitor, largely blocked P-cad upregulation by ICI (Fig. 3B), which is consistent with a requirement for newly synthesized proteins, probably induced by ICI, prior to *CDH3* activation. In contrast, as expected, this drug did not block ERα downregulation mediated by ICI (Fig. 3B).

ICI 182,780 decreases cell-cell adhesion and increases invasiveness of MCF-7/AZ cells

MCF7 cells formed compact aggregates on top of soft agar or when incubated in Erlenmeyer flasks under continuous shaking (Fig. 4A*(i)* and 4B), as previously shown (48). In presence of ICI, this effect was counteracted (Fig. 4A*(ii)* and 4B). Even a 24h pre-treatment with ICI, followed by testing these cells in the absence of ICI, still revealed inhibition of aggregation (Fig. 4A*(iii)*).

These same MCF7 cells failed to invade into collagen, as previously described (48-49). A 24h pre-treatment with ICI induced invasion of MCF7 cells, tested in the absence of ICI, which was also shown in the Matrigel assay (Fig. 4C and 4D). These pro-invasive effects of ICI were counteracted by E2 (Fig. 4C and 4D), indicating that they are mediated by interference with ERα-signaling.

Aggregation and invasion of MCF7 cells, in the presence of ICI, mimics the behavior of the poorly aggregating and invasive ERα negative and P-cad positive BT-20 cells (Table I), which remained unchanged upon treatment with ICI (Fig. 5).

P-cadherin expression increases invasiveness but does not alter cell-cell adhesion of MCF-7/AZ cells

Cells, retrovirally transduced to encode only EGFP (MCF7.LIE) or both P-cad and EGFP (MCF7.P-cad), were sorted to more than 90% EGFP positivity (Fig. 6A). P-cad levels were higher at the cell surface in P-cad transduced cells (Fig. 6B). The levels of cell-surface E-cad were the same in P-cad transduced cells, as in vector-transduced cells (Fig. 6A and 6B), excluding an effect of the exogenous cadherin on the levels of the major endogenous cadherin.

On plastic substratum, P-cad transduced MCF7 cells, like their parental or vector-transduced cells, formed epithelioid islands, showing no morphotype differences (data not shown). Transduction with P-cad did not interfere with E-cad mediated cell-cell adhesion (Fig. 6C and 6D). However, P-cad induced invasion, in collagen type I and in Matrigel, as was observed with ICI-treated MCF7 cells, but not with parental or vector (LIE) controls (Fig. 6E and 6F).

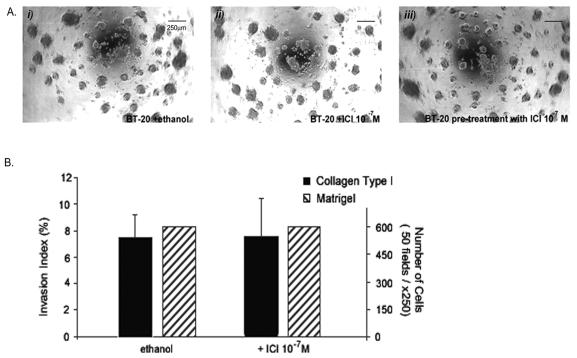

Figure 5. Effect of ICI on aggregation and on *in vitro* invasion of BT-20 cells. A: Pictures, after 72h, of the slow aggregation assay on semi-solid substratum. *(i)* Cells that were left untreated do not form compact aggregates; no alterations *(ii)* in cells cultured in presence of ICI, and *(iii)* in cells that have been pre-treated with ICI before seeding on top of agar, in the absence of ICI. **B:** Cells that had been pre-treated for 24h with ICI were seeded as a single cell suspension on top of collagen type I gels (black bars) and on matrigel (hatched bars). ICI-treated cells are as invasive as control cells.

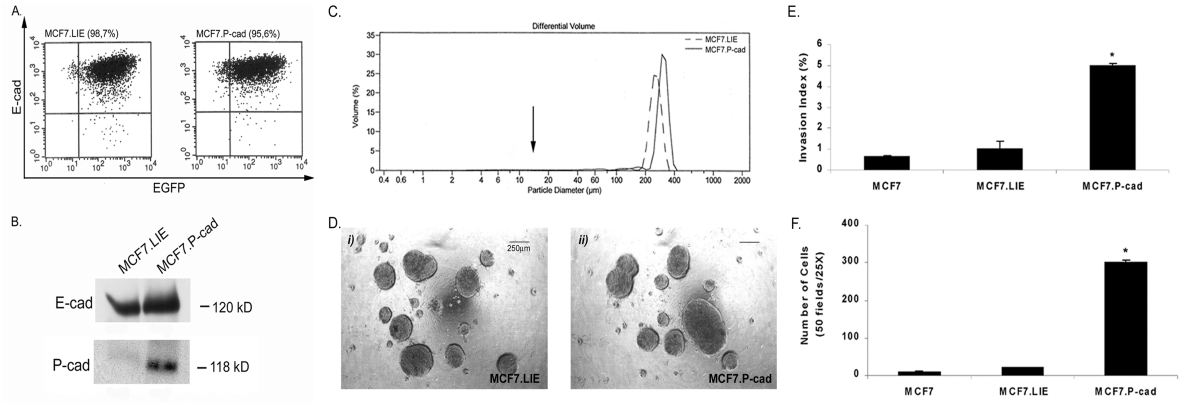

Figure 6. Cell aggregation and *in vitro* invasion of stably transduced MCF7 cells with P-cad cDNA. A: Flow cytometric evaluation of EGFP expression (x-axis) and E-cad expression (y-axis) in MCF7.LIE and MCF7.P-cad cells. P-cad expression did not induce alterations in E-cad expression levels (right plot). The percentage of EGFP positivity is indicated for each cell line. **B:** Immunoblotting, using anti-E-cad and anti-P-cad antibodies, of lysates from biotinylated MCF7.LIE and MCF7.P-cad cells. **C:** In the slow aggregation assay in suspension, both MCF7.LIE and MCF7.P-cad cells form similar compact aggregates, after 48h. The arrow indicates the measurement of a single cell suspension at the beginning of the experiment. **D:** Pictures, after 48h, of the slow aggregation assay on semi-solid substratum. MCF7.LIE (i) and MCF7.P-cad (ii) cells form round and compact aggregates, with no differences observed. **E:** In the collagen type I invasion assay, MCF7.P-cad cells invade significantly more (*, $p=0.0024$). **F:** Representative experiment of Matrigel invasion assay, where MCF7.P-cad cells invade significantly more than empty-vector transduced cells (*, $p<0.001$).

P-cadherin-induced invasion is not breast cancer cell or endogenous cadherin-specific

P-cad retroviral transduction was also performed with HEK cells, expressing at their surface low and high levels of E- and N-cad, respectively (50) (Table I and Fig. 7A), and being invasive neither into collagen type I nor into Matrigel (51). Sorting of vector- or P-cad-transduced cells resulted in populations having either moderate or high EGFP expression (HEK.LIE.Med, HEK.LIE.High, HEK.P-cad.Med and HEK.P-cad.High) (Fig. 7A and 7B). As for MCF7 cells, no differences in morphotype or aggregation were observed between parental and transduced cells (Fig. 7C and 7D). Although there was a downregulation of superficial N-cad in the highest P-cad expressing cells (Fig. 7B), this did not result in a significant decrease in total levels of N-cad (Fig. 7A). P-cad transduced cells were significantly more invasive into collagen type I or Matrigel than vector-transduced cells, with higher invasiveness of the cells expressing more P-cad (Fig. 7E and 7F). In both assays, the control cells with higher LIE expression levels showed an increased invasion index when compared to the ones with moderate levels of expression. This may be due to the insertion of viral promoters into the host genome, leading to the aberrant activation of host genes. However, although this observation highlights the care that should be taken when using these systems, it does not influence the interpretation of our results as such: the values of the P-cad transduced cells remain significantly different from those of the respective vector-transduced cells.

P-cadherin mediates invasion of HEK 293T cells via its juxtamembrane domain

To identify the P-cad domain(s) necessary for its pro-invasive effects, we used several P-cad constructs (Fig. 8A) for transient transfection of the HEK cell line. Biotinylation and immunoblotting confirmed expression of all constructs at the plasma membrane (Fig. 8B). Transient transfection with P-cad induced invasion into collagen type I, as observed with stably transduced HEK cells (Fig. 8C).

The P-cad point mutant, PC-R503H (Fig. 8A), representing the missense mutation in *CDH3*, found in hypotrichosis with juvenile macular dystrophy (52), failed to support strong cell-cell adhesion unlike wild-type P-cad (unpublished data). Most likely, the reason for this failure is the disruption of the strongly conserved LDRE Ca^{2+}-binding motif in the fourth extracellular domain of P-cad. Nevertheless, PC-R503H still induced invasion (Fig. 8C).

Mutants of the P-cad cytoplasmic tail were also generated (Fig. 8A). Transfection into HEK cells showed that PC-CT762, retaining the intact P-cad JMD, induced invasion like wild-type P-cad (Fig. 8C). Since this mutant is truncated just before the CBD, we assume that βctn, γ-catenin, or any other protein that binds to this region are not needed for P-cad-mediated invasion.

With mutants within the JMD (Fig. 8A), statistically significant invasion into collagen was seen only with the truncation mutants that still retained the intact JMD (PC-CT719 and PC-CT727) (Fig. 8C). The somewhat decreased ability of PC-CT719 to induce invasion (Fig. 8C) might be due to its lower expression levels (Fig. 8B). In line with the results obtained with the truncation mutants, and confirming that the CBD is not involved in the pro-invasive effects, the PC-Δ703-707 mutant (lacking EEGGG in the p120ctn binding site), with impaired p120ctn binding (Fig. 8D), was not able to induce invasion of HEK cells into collagen type I (Fig. 8C). So, P-cad needs to have an intact JMD to be able to induce invasion of HEK cells.

To exclude that the gain of any exogenous cadherin, retaining its JMD, would be sufficient for a

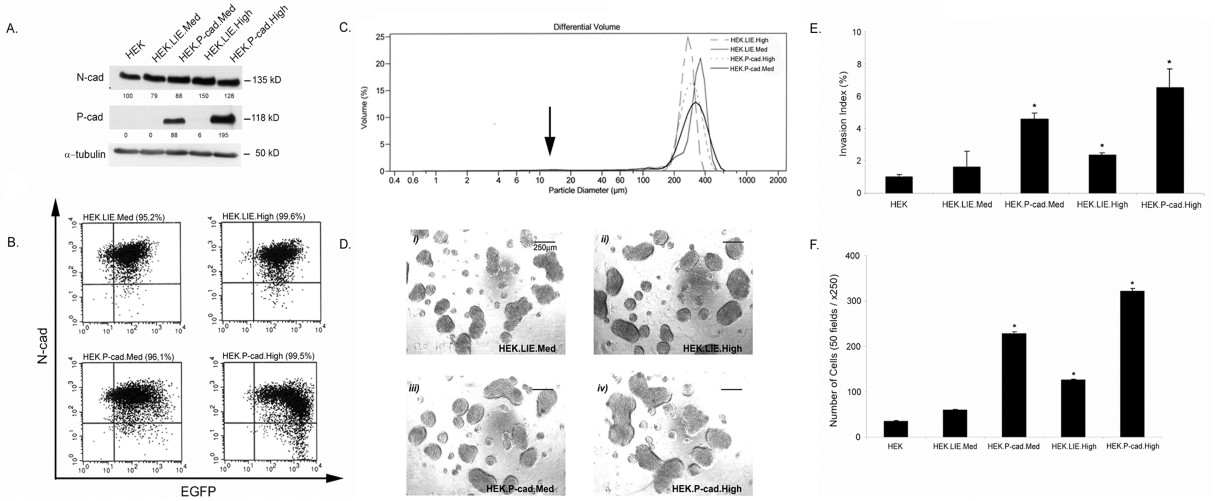

Figure 7. Cell aggregation and *in vitro* invasion of stably transduced HEK cells with P-cad cDNA. A: Immunoblotting, using anti-N-cad and anti-P-cad antibodies, of lysates from biotinylated HEK, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High and HEK.P-cad.High cells. Band quantification was done relatively to the expression levels in control cells. To control for equal loading, immunostaining with anti-α-tubulin was done. **B:** Flow cytometric analysis of EGFP expression (x-axis) and N-cad expression (y-axis) in the indicated cell lines. High levels of P-cad in the HEK.P-cad.High cell line induced downregulation of endogenous N-cad. The percentage of EGFP positivity is indicated for each cell line. **C:** In the slow aggregation assay in suspension, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High and HEK.P-cad.High cell lines form similar compact aggregates, after 48h. The arrow indicates the measurement of a single cell suspension at the beginning of the experiment. **D:** Pictures, after 48h, of the slow aggregation assay on semi-solid substratum. HEK.LIE.Med *(i)*, HEK.LIE.High *(ii)*, HEK.P-cad.Med *(iii)*, and HEK.P-cad.High *(iv)* cells form round and compact aggregates, with no differences observed. **E:** A single cell suspension of these cell lines was seeded on top of collagen type I gels. After 24h of incubation, HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more into the collagen type I matrix (*, $p=0.005$ and $p=0.02$, respectively). Although HEK.LIE.High was statistically different from non-transduced cells (*, $p=0.003$), the comparison with HEK.P-cad.High still shows the significant effect of P-cad ($p=0.03$). **F:** Representative experiment of Matrigel invasion assay. HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more than empty-vector transduced cells (*, $p<0.001$). Also here, although HEK.LIE.High differed significantly from control, the comparison with HEK.P-cad.High still shows the significant effect of P-cad on invasion ($p<0.001$).

pro-invasive effect, we demonstrated that HEK cells transfected with mouse wild-type E-cad cDNA (Fig. 8A) failed to invade into collagen type I (Fig. 8C). In conclusion, the juxtamembrane domain of P-cad confers to this molecule the specific ability to induce invasion of HEK cells, in the presence of the endogenously expressed cadherin.

DISCUSSION

The antiestrogen ICI increased time- and dose-dependently P-cad expression in ERα-positive breast cancer cells. This increase could be completely reverted by E2, categorizing *CDH3* as an estrogen-repressed gene and pointing to E2 as a key regulator of this cadherin. In addition to competing for ERα, ICI also increased its breakdown (37). As a result, ICI abrogates ERα signaling, and the subsequent regulation of E2 responsive genes (53). As the human P-cad promoter (GI:2950171) does not contain the consensus sequence 5'-GGTCAnnnTGACC-3' of the estrogen-responsive elements (54), E2 is unlikely to have a direct inhibitory effect on transcription of the *CDH3* gene. The increase of P-cad by ICI, some hours after the decrease of ERα, and its inhibition by CHX, pleads for the activation of a *CDH3* transcription factor, through ICI-mediated abrogation of ERα. Presence of this E2-regulated factor may account for the high P-cad levels in some breast cancer cell lines and for the inverse correlation between ERα and P-cad expression in mammary tumors.

In MCF7 breast cancer cells, ICI treatment led to a decreased cell-cell adhesion and promotion of invasion *in vitro*. This is in line with the finding that E2 (55-56), and even the unliganded receptor (57), may decrease *in vitro* invasiveness and motility of breast cancer cells, suggesting that some estrogen-regulated genes negatively control invasion. Since this control is lost in cells treated with high concentrations of ICI, which upregulate P-cad, the latter was further investigated in *in vitro* aggregation and invasion assays.

Surprisingly, retroviral transduction of MCF7 and HEK cells with P-cad did not change aggregation. These results suggest that P-cad does not shift the aggregation balance established by the other cadherins in these systems. By contrast, such balance may well be changed for invasion, as demonstrated with P-cad transduced cells. Furthermore, the pro-invasive effect of P-cad was not restricted to E-cad positive MCF7 cells, suggesting factors others than endogenous cadherins. A critical level of P-cad seems to be needed for its pro-invasive activity, as evidenced by a comparison between the highly invasive and highly P-cad positive BT-20 and T47D cells, and the non-invasive and weakly P-cad positive MCF7 and ZR-75.1 cells (Table I).

In contrast to its pro-invasive activity in our cells, transfection of other cell lines with P-cad inhibited

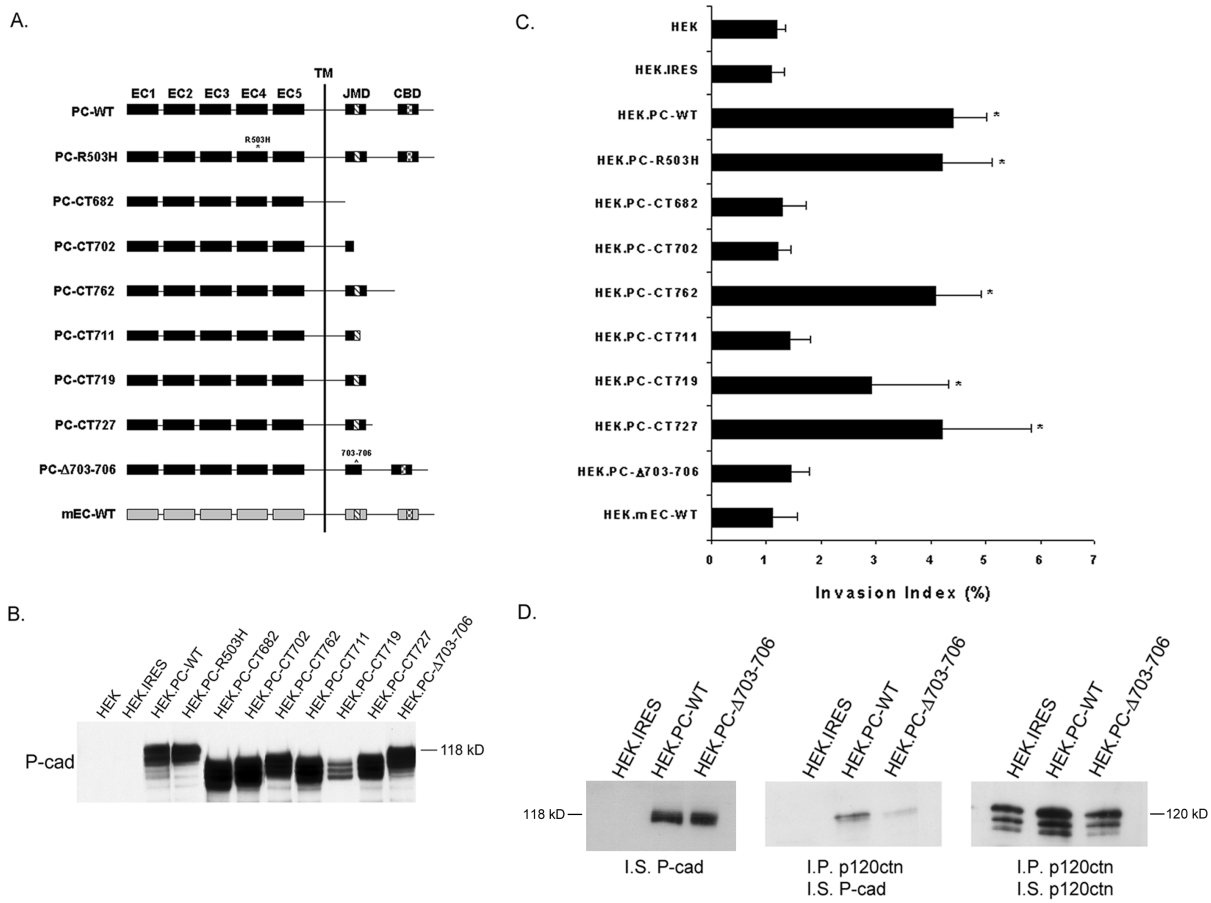

Figure 8. ***In vitro*** **invasion of HEK cells transiently transfected with P-cad constructs. A:** Schematic diagram of the various constructs used for transient transfection of HEK cells. **B:** Immunoblotting, using anti-P-cad antibody, of streptavidin precipitations of biotinylated HEK cells, transiently transfected with P-cad constructs. **C:** A single cell suspension of HEK cells, transiently transfected with the indicated constructs, was seeded on top of collagen type I gels. After 24h, invasive cells were scored. Besides HEK.PC-WT cells, also HEK.PC-R503H, HEK.PC-CT762, HEK.PC-CT719, and HEK.PC-CT727 invaded significantly more than empty-vector transfected cells (HEK.IRES) into the collagen (*, $p<0.001$ for HEK.PC-WT and HEK.PC-R503H, $p=0.003$ for HEK.PC-CT762, $p=0.015$ for HEK.PC-CT719, and $p=0.023$ for HEK.PC-727). **D:** Immunoprecipitation (I.P.), using anti-p120ctn antibody, of total lysates of HEK cells transiently transfected with the empty vector (IRES), with PC-WT and with PC-Δ703-706 constructs. Immunostaining (I.S.) of these blots with anti-P-cad and anti-p120ctn antibodies, showed that PC-WT strongly precipitates with p120ctn, as compared to PC-Δ703-706, where there is interference with this binding (right two blots). The staining of total lysates with P-cad indicates a similar expression of the transfected P-cad constructs (left blot).

invasion (58-59 and our unpublished data), suggesting that P-cad may act both as an invasion promoter and suppressor, depending on the cell type and its invasive status. Transgenic mice expressing high levels of P-cad in the normal mammary epithelium (60) contributed little to this issue, as they did not produce tumors, and as *neu* oncogene-induced mammary tumors were always P-cad negative.

In the present study, the pro-invasive action of P-cad is unlikely to be the result of alterations in cell-cell adhesion, since 1) the assays score invasion of single cells into or through a matrix, 2) the retroviral transduction of MCF7 and HEK cells with P-cad did not change aggregation, and 3) the point mutant PC-R503H, incapable of supporting strong P-cad mediated adhesion, still induced invasion. We presume that the pro-invasive activity of P-cad is due to changes in signaling pathways.

The cytoplasmic domain of classical cadherins is highly conserved, suggesting crucial roles for proteins binding to this region (3). Recently, Wong and Gumbiner (61) attributed the anti-invasive activity of wild-type E-cad to its interaction with βctn. An E-cad mutant, retaining the CBD, but with a point mutation that abolishes p120ctn binding, was still able to suppress invasion. By contrast, in P-cad, maintenance of the JMD domain is crucial for the induction of invasion, irrespective of the CBD. Ireton et al. (62) showed that restoration of p120ctn in a colon carcinoma cell line resulted in E-cad stabilization and rescue of its function, either directly or indirectly, by preventing other proteins of hampering E-cad. Although a related mechanism could account for the induction of invasion by P-cad, where destabilization of anti-invasive cadherin/catenin complexes would result from competition for the available p120ctn by the P-cad JMD, we consider this possibility less likely. Despite the fact that the downregulation of N-cad in HEK cells by high levels of the several P-cad constructs coincided with stimulation of invasion (Fig. 8C and 9), moderate P-cad expression levels, leaving the endogenous cadherin unchanged, were

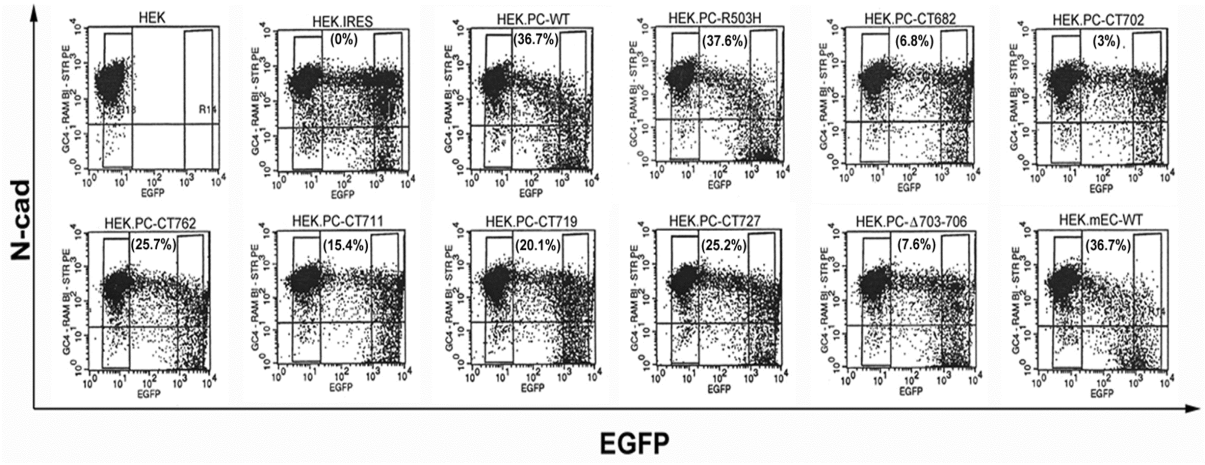

Figure 9. N-cadherin expression of HEK cells transiently transfected with P-cad constructs. Flow cytometric analysis of EGFP expression (x-axis) and N-cad expression (y-axis) in the indicated cell lines. High levels of the P-cad constructs, which maintain an intact JMD, induced downregulation of endogenous N-cad. The percentage of N-cad downregulation is indicated for each cell line, compared to HEK.IRES cell line (0%).

sufficient to induce invasion. Further, transfection of HEK cells with E-cad did not induce invasion (Fig. 8C), despite decreased endogenous cadherin in highly expressing cells (Fig. 9), and expected competition for cadherin-binding proteins. Alternatively, P-cad may generate a specific pro-invasive signal via its JMD. In this hypothesis, JMD binding of p120ctn, or other proteins, to P-cad has to differ from the binding to E- or N-cad, either by strength, conformation or recruitment of other members of the complex. This, in turn, may result in the activation of pathways that overcome the suppressive signals mediated by the endogenous cadherins.

Although the JMD of cadherins is highly conserved, its function is very context-dependent. It may both positively and negatively regulate cadherin activity. Cells expressing mutated E-cad JMD are weakly adherent (63-64), more motile (65), but still epithelioid. Upon formation of adhesive contacts, the JMD recruits and activates Rac, regulating the actin cytoskeleton (66-68). In another context, the JMD inhibits aggregation mediated by classical cadherins and induces cell motility (69-71). The JMD of VE-cadherin, for example, excludes endogenous N-cad from junctions, and regulates cell proliferation (72). Except from the presently demonstrated induction of invasion, no regulatory functions have been described for the P-cad JMD.

Although binding of proteins to the JMD of P-cad has just been documented for p120ctn (6), we cannot exclude the possibility that its disruption introduces conformational changes and/or uncouples the interaction of other proteins, which could be responsible for the observed effects. Striking examples of this were shown for E-cad, where functional differences have been noted between larger and minimal deletions of the JMD, with even the minimal changes disrupting binding of multiple molecules (71, 73).

Data about the role of p120ctn in normal and cancer cells are conflicting. Positive and negative regulation of cell-cell adhesion and motility possibly reflect differences in cell type, cadherins, p120ctn isoforms and shuttling between cadherin-bound and cytoplasmic pools (74). When overexpressed in the cytoplasm, p120ctn may regulate the actin cytoskeleton and cell motility, through Rho GTPases (75-77). E-cad would also bind p120ctn more tightly than N-cad, which is found in motile cells such as fibroblasts (78). Similarly, in our experiments, differences in binding strength between E-, N- and P-cad-bound p120ctn may influence its effect on the activity of the Rho GTPases, possibly making the cells more prone to invade. Alternatively, the panel of molecules recruited by p120ctn may differ depending on the cadherin it is bound to.

In addition, other molecules, like Hakai and presenilin-1 (PS-1) have been reported to bind to the JMD of classical cadherins, to a sequence adjacent to or overlapping the p120ctn-binding domain, thereby competing with p120ctn for binding (73, 79). However, the significance of these interactions is not well known, and the interaction of these proteins with P-cad still has to be confirmed.

In conclusion, our study establishes an as yet unknown role for P-cad in cancer: 1) P-cad expression is regulated through ERα-signaling, suggesting that the inverse *in vivo* correlation between these molecules stems from a causal relationship. 2) P-cad induces invasion, in the context of endogenous E- or N-cad expression; since P-cad expression in breast cancer is far more frequent than aberrant expression of N-cad, its physiological relevance is more likely to be higher (22, 24). 3) Invasion mediated by P-cad required its intact JMD. This establishes a novel role for this domain and distinguishes P-cad mediated invasion from invasion induced by N-cad, which depends on a physical interaction of its extracellular domain with the fibroblast growth factor receptor (80). Moreover, although the P-cad JMD differs in only few amino acids from the corresponding E-cad domain, yet it exerts an opposite function: whereas

the E-cad JMD suppresses motility (81), the P-cad JMD is necessary for induction of invasion. In order to understand why such related domains can have opposite functions, it will be crucial to identify new interaction partners and/or to study if the interaction of known partner molecules differs between cadherins.

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II.6. E-cadherin is a substrate for multiple proteases

One of the multiple mechanisms of functional downregulation of E-cadherin that have been described, is its post-translational inactivation via proteolytical cleavage. This processing may occur by various enzymes at distinct sites of the 120kD E-cadherin protein (see inset).

Calpain cleaves the cytosolic E-cadherin domain upstream of the β-catenin binding motif, generating a 100-kD transmembrane fragment that cannot bind β-, γ- or p120 catenin anymore. Consistent with the frequent overexpression of calpain in prostate cancer, the 100kD fragment was found to accumulate in prostate tumors (Rios-Doria *et al.*, 2003).

When epithelial cells go into apoptosis, a 97kD membrane-bound E-cadherin fragment is detected in the cells. This fragment derives from an intracellular proteolytic event, releasing part of its cytoplasmic tail, including the β-catenin binding domain (Vallorosi *et al.*, 2000). A subsequent study showed that this cleavage was mediated by caspase-3, a cysteine protease involved in apoptosis that cleaves its substrates after aspartate residues (Steinhusen *et al.*, 2001). In addition to this caspase-3 mediated cleavage, a second proteolytic event leads to release of the E-cadherin ectodomain (see further). Accumulation of the truncated 97kD fragment has been observed in prostate carcinomas, as compared to matched normal prostate tissue (Rashid *et al.*, 2001).

Presenilin-1 (PS1) is a polytopic transmembrane protein involved in most cases of early-onset familial Alzheimer's disease. It is part of a multi-protein complex, which is involved in the γ-secretase cleavage of multiple proteins (Selkoe, 2001). PS1 binds to the juxtamembrane intracellular domain of E-cadherin, to the same domain that is responsible for p120 catenin binding, and competes with p120 catenin for binding. PS1 binding to E-cadherin stimulates association of E-cadherin with β- and γ-catenin, promotes cytoskeletal association of the cadherin/catenin complex and increases Ca^{2+}-dependent aggregation (Baki *et al.*, 2001). However, PS1 has also been implicated in E-cadherin proteolysis. PS1-mediated cleavage of both full-length and truncated (after ectodomain shedding) E-cadherin at the membrane-cytoplasm interface has been shown to release the E-cadherin cytoplasmic tail into the cytosol, promoting disassembly of adherens junctions (Marambaud *et al.*, 2002). What role for PS1 prevails in a particular system may be dependent on the conditions. Under conditions promoting cell-cell adhesion, incorporation of PS1 into the E-cadherin/catenin complex may have a stabilizing effect. However, in conditions of stress, cell-cell dissociation or apoptosis, PS1 may facilitate cell separation by promoting disassembly of the adherens junctions. These two contrasting effects combined into one polypeptide may represent a quick and efficient way for the modulation of cell-cell contacts. Recently, PS1-mediated cleavage of N-cadherin was shown to release a cytoplasmic fragment that was capable of modulating signaling via binding to the transcription factor CBP (CREB binding protein; CREB, cyclic AMP response element binding protein) (Marambaud *et al.*, 2003). Association of the cytoplasmic N-cadherin fragment with CBP promoted degradation of the latter, resulting in suppression of CREB-mediated transcription. Whether the E-cadherin intracellular tail may have a similar function, remains to be determined.

Cleavage in the extracellular juxtamembrane domain of the 120kD E-cadherin gives rise to a released 80kD ectodomain, coined soluble E-cadherin (sE-CAD). E-cadherin ectodomain shedding was first suspected from the detection of a soluble 80kD fragment in medium conditioned by MCF-7 breast cancer cells (Wheelock *et al.*, 1987). E-cadherin cleavage and release of the 80kD sE-CAD is not necessarily a pathological process, as it also occurs during the preparation phase for uterine invasion in pregnant mice, where it is promoted by estradiol (Potter *et al.*, 1996). Distinct sE-CAD fragments may be formed, depending on the protease involved. E-cadherin ectodomain shedding following treatment with trypsin, in the presence of Ca^{2+}, results in a fragment that is somewhat smaller than that seen upon cleavage by metalloproteases (MMPs). The latter has been studied most extensively, with characterization of the cleavage site (Steinhusen *et al.*, 2001; Marambaud *et al.*, 2002). Several MMPs, including stromelysin-1 (MMP-3) and matrilysin (MMP-7), have been involved in the production of sE-CAD in cancer (Noë *et al.*, 2001; Davies *et al.*, 2001), as well as in injured epithelia (McGuire *et al.*, 2003). Interestingly, stromelysin-1 has been shown to induce EMT and acquisition of a premalignant phenotype, concomitant with cleavage of E-cadherin and release of sE-CAD by mammary epithelial cells (Lochter *et*

..VCRKAQPVEAGLQIPAILGILGGILALLILILLLLLFLRRRAVVKEPLLPPEDDTRDNVYYYDEEGGGEEDQDFDLS…

↑ MMP ↑ γ-secretase ↑ caspase-3 ↑ calpain

Amino acid sequence of the human E-cadherin juxtamembrane extracellular (left), transmembrane (shaded) and juxtamembrane intracellular domain (right), indicating the cleavage sites of proteases (based upon Steinhusen *et al.*,2001; Marambaud *et al.*, 2002, Rios-Doria *et al.*, 2003) .

al., 1997). In the study presented here, we show that exposure of E-cadherin expressing cells to the serine protease plasmin may result in the generation of sE-CAD.

Several studies comparing the circulating sE-CAD levels in biological fluids of patients suffering from various types of cancer with the levels found in healthy persons revealed higher sE-CAD levels in the cancer patients. This was shown for liver cancer (Katayama *et al.*, 1994), gastric cancer (Katayama *et al.*, 1994; Gofuku *et al.*, 1998; Chan *et al.*, 2001), colon cancer (Velikova *et al.*, 1998), bladder cancer (Banks *et al.*, 1995; Grifiths *et al.*, 1996, Protheroe *et al.*, 1999), lung cancer (Cioffi *et al.*, 1999), prostate cancer (Kuefer *et al.*, 2003), skin cancer (Shirahama *et al.*, 1996) and other cutaneous diseases (Matsuyoshi *et al.*, 1995) and ovarian cancer (Sundfeldt *et al.*, 2001), although controversy exists about the latter, since others did not find increased levels of sE-CAD in the cyst fluid from malignant ovarian tumors, as compared with benign cysts (Darai *et al.*, 1998a, b).

The release of an sE-CAD fragment may have several effects. First, the proteolytic release of the E-cadherin ectodomain may represent a mechanism that leads to a decrease of E-cadherin on the tumor cells, facilitating their release from the primary tumor, thus enhancing invasion and metastases. Second, the remaining transmembrane-intracellular fragment may compete with full-length cadherin for catenin binding (Hermiston and Gordon, 1995) or, alternatively, have a signaling function. Third, the released ectodomain may fulfill a biological role. sE-CAD has been shown to induce scattering of epithelial islands, which could be neutralized by adding an antibody against E-cadherin (Wheelock *et al.*, 1987). In contrast, neither a 84kD glycoprotein (gp84), trypsinized from murine embryonal carcinoma PCC4 cells (Hyafil *et al.*, 1980), nor a recombinant 80kD fragment (Herrenknecht and Kemler, 1993) had decompacting activity. Synthetic E-cadherin peptides containing the histidine-alanine-valine (HAV) sequence present in the first extracellular domain of E-cadherin, could interfere with E-cadherin function, as indicated by inhibition of aggregation, disturbance of epithelial morphology and induction of invasion (Mbalaviele *et al.*, 1995; Willems *et al.*, 1995; Noë *et al.*, 1999, Makagiansar *et al.*, 2001). Moreover, sE-CAD, released following cleavage by MMP's, has been shown to inhibit aggregation and induce invasion of invasion-competent cells (Noë *et al.*, 2001). In the article presented here we show that a plasmin-derived E-cadherin ectodomain is capable of disrupting cell aggregation and induction of invasion into collagen type I.

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II.7. Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion

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Plasmin Produces an E-Cadherin Fragment That Stimulates Cancer Cell Invasion

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Matrix metalloproteases from the cell surface cleave an 80 kDa E-cadherin fragment (sE-CAD) that induces invasion of cancer cells into collagen type I and inhibits cellular aggregation. Conditioned media from MDCKts.*src*Cl2 cells at 40 °C and 35 °C, PCm.src5 and COLO-16 cells at 37 °C contained spontaneously released sE-CAD; these 48 h old conditioned media were capable of inhibiting E-cadherin functions in a paracrine way. Here we show direct cleavage of the extracellular domain of E-cadherin by the serine protease plasmin. sE-CAD released by plasmin inhibits E-cadherin functions as evidenced by induction of invasion into collagen type I and inhibition of cellular aggregation. This functional inhibition by sE-CAD was reversed by aprotinin or by immunoadsorption on protein Sepharose 4 fast flow beads with antibodies against the extracellular part of E-cadherin. Our results demonstrate that plasmin produces extracellular E-cadherin fragments which regulate E-cadherin function in cells containing an intact E-cadherin/catenin complex.

Key words: E-cadherin / Ectodomain shedding / Invasion / Plasmin / Proteolysis.

Introduction

Proteolytic degradation of the extracellular matrix is crucial for cancer invasion. One of the proteolytic systems implicated in cancer invasion is the plasminogen activation system generating the serine protease plasmin (Andreasen *et al.,* 1997; Bugge *et al.,* 1998; Reuning *et al.,* 1998; Wang, 2001). During invasion, the urokinase-type plasminogen activator (uPA) is released as an inactive proenzyme, pro-uPA, that binds to its cell surface receptor uPAR. Coincident binding of pro-uPA and plasminogen to uPAR enhances plasminogen activation, a phenomenon that is also controlled by two inhibitors, PAI-1 and PAI-2 (Mignatti and Rifkin, 1996). The widely accepted promotion of invasiveness by proteases is due to the breakdown of the extracellular matrix (ECM), facilitating the passage of cancer cells through the basement membrane and through the stroma. Plasmin, the end product of the plasminogen activation system, can degrade directly most extracellular matrix proteins and, moreover, promotes matrix degradation through activation of some pro-MMPs (pro-matrix metalloproteases). We recently described another mode of action: some MMPs cleave sE-CADs (soluble E-cadherin fragment), and such fragments stimulate invasion (Noë *et al.*, 2001).

E-cadherin is a calcium-dependent cell-cell adhesion and signaling molecule that is often inactivated during carcinoma progression. E-cadherin forms together with its intracellular associated proteins, termed catenins, a powerful invasion-suppressor complex that is regulated at different levels (Van Aken *et al.,* 2001). Proteolytic cleavage, resulting in ectodomain shedding, has been observed for cell surface molecules such as growth factor receptors and their ligands and adhesion molecules including E-cadherin, N-cadherin and VE-cadherin (Pyke *et al.,* 1991, 1993; Paradies *et al.,* 1993; Rabbani *et al.,* 1995; Lochter *et al.,* 1997; Werb et *al.*, 1997; Herren *et al.,* 1998). E-cadherin ectodomain shedding was first suggested by the detection of a soluble 80 kDa fragment in the medium of the human breast cancer cell line MCF-7. This E-cadherin fragment caused scattering of epithelial cells in culture, an effect that could be neutralized by antibodies against E-cadherin (Wheelock *et al.,* 1987). E-cadherin cleavage and release of the 80 kDa sE-CAD also occurs during uterine invasion in pregnant mice and can be induced by treatment with oestradiol (Mbalaviele *et al.,* 1995). sE-CAD activity was mimicked by synthetic E-cadherin peptides containing the histidine-alanine-valine amino acid sequence (Noë *et al.,* 1999). This sequence is conserved in the family of classical cadherins and is crucial for homophilic interactions.

Since plasmin is also involved in ectodomain shedding of the cell adhesion molecules L1, N-CAM and of the cytokine TNF-α (Murdoch *et al.,* 1999; Nayeem *et al.,* 1999), we wanted to examine whether or not plasmin cleaves the E-cadherin ectodomain and, if so, whether the plasmin-generated sE-CAD stimulates invasion of cancer cells into collagen type I.

[a] These authors contributed equally to this work.

Here we show that plasmin can directly cleave E-cadherin and that the resulting ectodomain fragments are capable of inhibiting E-cadherin functions in canine kidney MDCKts.*src*Cl2 cells as tested by the fast aggregation and collagen type I invasion assays. This observation provides a novel mechanism by which plasmin may induce tumor cell invasion.

Results

Plasmin Cleavage of E-Cadherin on Cells

Treatment of MDCKts.*src*Cl2 at 40 °C and 35 °C, PCm.src5 and COLO-16 cells at 37 °C with 1 µg/ml plasmin for 2 h resulted in the release of an 80 kDa E-cadherin fragment (Figure 1, lane 2) as detected by immunoprecipitation with DECMA-1 or HECD-1 and immunostaining of Western blots with HECD-1 or SEC-11 antibody and compared with untreated cells (lane 1). This activity of plasmin was blocked by the serine protease inhibitor aprotinin at 10 µg/ml (lane 3). The cells also spontaneously released sE-CAD, as is evident from the analysis of 48 h old conditioned medium (lane 5). sE-CAD could be removed from the conditioned medium by repeated immunoadsorption (lane 4).

Plasmin Cleavage of E-Cadherin Immunoadsorbed on Sepharose Beads

sE-CAD production by plasmin treatment could be due to direct cleavage of E-cadherin or to the activation of a common enzymatic cascade, since plasmin may activate other proteases some of which cleave E-cadherin (Noë *et al.,* 1999). To distinguish between these two possibilities, we purified canine E-cadherin from MDCKts.*src*Cl2 cells by immunoprecipitation on protein A Sepharose 4 fast flow beads with an antibody against the cytoplasmic domain of cadherins. Immobilized E-cadherin was treated with different concentrations of plasmin or with trypsin in the presence of Ca^{2+}. Cleavage of E-cadherin was apparent by decreasing amounts of full length E-cadherin retained on the beads (Figure 2A) and by the appearance of lower molecular weight bands in the supernatant (Figure 2B). Plasmin treatment of immobilized canine E-cadherin results in the release of different E-cadherin fragments in the supernatant, depending upon the plasmin concentration. Treatment with 0.01 µg/ml plasmin results in an E-cadherin fragment of about 99 kDa (lane 3). This fragment gradually disappears when higher doses of plasmin are added, probably due to further proteolytic degradation. Finally, two E-cadherin fragments of 80 kDa and 60 kDa are detected when human E-cadherin is treated with plasmin or trypsin (lane 5 and 6).

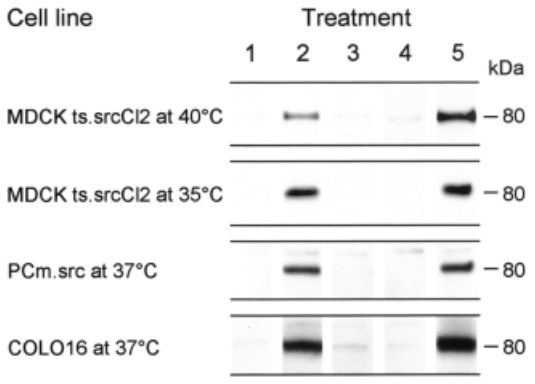

Fig. 1 Effect of Plasmin on the Release of Soluble E-Cadherin Fragments (sE-CAD) from Cells Cultured on Solid Substrate. Conditioned media were harvested from cells that were left untreated for 2 h (1) or 48 h (5) or were treated for 2 h with 1 µg/ml plasmin without (2) or with 10 µg/ml aprotinin (3). Precipitated proteins were separated by SDS-PAGE, blotted, immunostained with SEC-11 (for canine cells) or HECD-1 antibodies (for human cells), and detected with the ECL reagent. Lane 4 shows medium prepared with 1 µg/ml plasmin and sE-CAD immunodepletion.

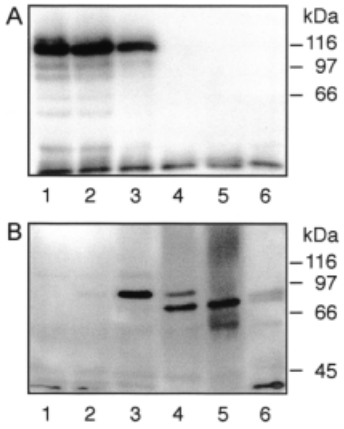

Fig. 2 Direct Cleavage of Canine E-Cadherin by Plasmin. E-cadherin was immunoprecipitated from lysates of MDCKts.*src*Cl2 cells with a polyclonal pan-cadherin antibody on Sepharose beads. The beads were incubated at 37 °C in 50 µl PBS only (l), with 1 ng (2), 10 ng (3), 100 ng (4) or 1 µg (5) plasmin or with 0.025% trypsin (6) in the presence of 0.04 mM Ca^{2+}. Beads (panel A) and supernatant (panel B) were separated, proteins were resolved by SDS-PAGE, blotted and immunostained with the anti-E-cadherin antibody recognizing the cytoplasmic tail (panel A), or DECMA-1 recognizing the extracellular part of E-cadherin (panel B).

Stimulation of MDCKts.*src*Cl2 Invasion into Collagen Type I by Plasmin-Released sE-CAD

Control MDCKts.*src*Cl2 cells do not invade into collagen type I at 40 °C. Conditioned medium from MDCKts.*src*-Cl2 cells at 40 °C and 35 °C, PCm.src5 and COLO-16 cells at 37 °C treated with plasmin for 2 h induced collagen type I invasion in a paracrine way, whereas culture medium incubated for 2 h with untreated cells or with aprotinin in the presence of plasmin for 2 h had no effect (Figure 3). The invasion index observed with conditioned medium from 2 h plasmin treated cells was similar to the invasion index seen with the 48 h conditioned medium containing constitutively shed sE-CAD, illustrating the strong effect of protease activity. To ensure that the in-

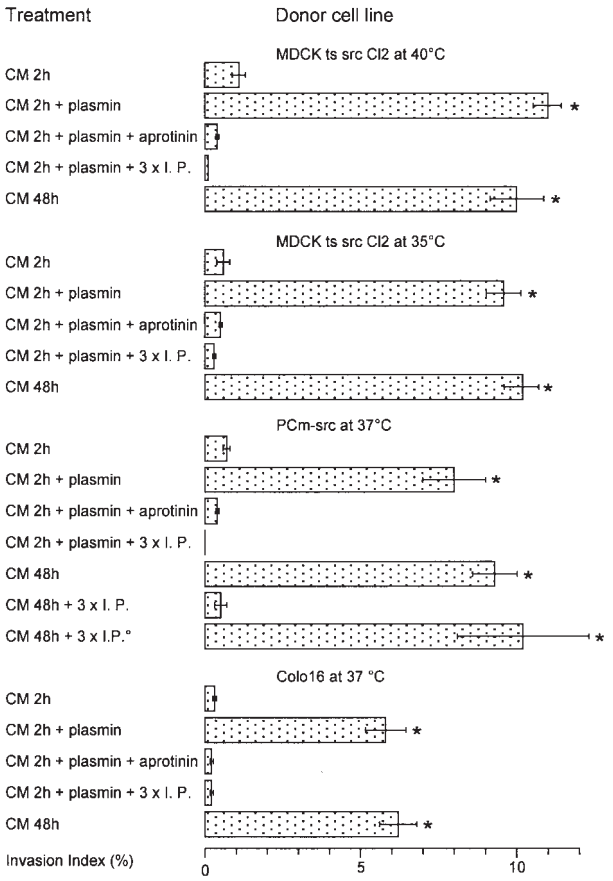

Fig. 3 Induction of Invasion of MDCKts.*src*Cl2 Cells into Collagen Type I by Plasmin-Released sE-CAD.
Cells were seeded on top of a collagen gel and incubated at 40 °C for 24 h with or without conditioned medium that was obtained from the indicated donor cell lines. Donor cells were incubated for 2 h either without or with 1 µg/ml plasmin or with 1 µg/ml plasmin plus 10 µg/ml aprotinin. As a control for the sE-CAD-specificity of stimulation of invasion, sE-CAD was immunodepleted from the conditioned media treated with 1 µg/ml plasmin for 2 h by immunoprecipitating three times with an antibody against the extracellular part of E-cadherin (3 × I.P.). As a positive control for spontaneously released sE-CAD, we used conditioned media of 48 h untreated donor cells. A mouse IgG1 antibody (3 × I.P.°) was used as a control for HECD-1 specificity. *Invasion of treated cells that are significantly different (Student's *t*-test) from untreated cells.

creased invasion was due to the presence of sE-CAD and not a direct result of the added protease acting on the collagen gel, sE-CAD was selectively removed. Immunodepletion of sE-CAD from the conditioned media completely abrogated the invasion-inducing activity of the media, thus indicating that this phenomenon was not due to the presence of the added plasmin. Moreover, addition of plasmin to unconditioned medium did not induce invasion (data not shown).

Inhibition of MDCKts.*src*Cl2 Aggregation by Plasmin-Released sE-CAD

Because the E-cadherin/catenin complex is responsible for epithelial cell-cell adhesion, we tested the effect of the

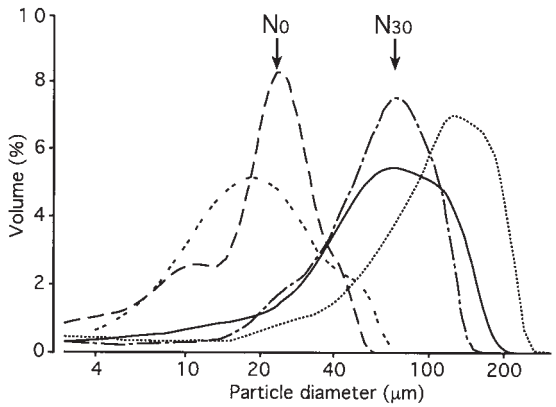

Fig. 4 Inhibition of Cellular Aggregation by sE-CAD Released by Plasmin.
Cells were detached under E-cadherin saving conditions. Control MDCKts.*src*Cl2 cells were incubated in aggregation buffer and allowed to aggregate for 30 minutes. Arrows indicate peaks of MDCKts.*src*Cl2 cells at 40 °C, as mearured by a particle size counter either after 0 (N0) or 30 (N30) minutes. Aggregation was inhibited by sE-CAD containing conditioned medium after 2 h plasmin treatment (- - -) and 48 h conditioned medium (-- --); aggregation was not inhibited by 2 h conditioned medium (——), by 2 h conditioned medium treated with plasmin + aprotinin (—· —) and by immunodepletion of sE-CAD in plasmin-derived 2 h conditioned medium (·····).

sE-CAD released by plasmin on this function. Aggregation of MDCKts.*src*Cl2 cells at 40 °C was abundant for cells left untreated (arrows indicate peak values at N0' and N30'), or treated with 2 h conditioned medium or with 2 h conditioned medium produced with plasmin in the presence of aprotinin. The 48 h conditioned medium from donor cells or 2 h conditioned medium from cells treated with plasmin significantly inhibited aggregation. When sE-CAD was immunodepleted from the conditioned medium inhibition of aggregation did no longer occur (Figure 4). We could also demonstrate that donor MDCKts.*src*Cl2 cells after treatment with plasmin or trypsin become non-aggregating (data not shown).

Discussion

Plasmin is an invasion promoter as evidenced by *in vitro* experiments and by observations of animal and human tumors *in vivo*; it acts through breakdown of the extracellular membrane, providing space for invading cancer cells and through stimulation of migration (Andreasen *et al.,* 1997). Our present experiments suggest one more mechanism, namely enzymatic release of invasion-stimulating fragments from the extracellular part of E-cadherin, called sE-CAD, on the surface of cancer cells.

Our test tube experiments with immunoadsorbed E-cadherin demonstrate that plasmin may produce sE-CAD through direct enzymatic digestion of the protein. In cell culture or *in vivo*, however, plasmin might also cause cleavage of sE-CAD in a proteolytic cascade, *e. g.*

through activation of pro-MMPs (pro matrix metalloproteases), such as pro-MMP-3 or pro-MMP-9 (Werb *et al.,* 1997; Ramos-DeSimone *et al.,* 1999). We know from previous experiments that MMP-3 (stromelysin-1) as well as MMP-7 (matrilysin) release invasion-stimulating sE-CAD from epithelial cancer cells in culture (Noë *et al.,* 2001). In the present experiments, we demonstrated the invasion-stimulating capacity of plasmin-generated sE-CAD in a paracrine system: the MDCKts.*src*Cl2 acceptor cells stimulated to invade by sE-CAD had an intact E-cadherin/catenin complex or restored it during the assay. They were exposed to conditioned medium not only from the same type of cultures but also from cultures of cells of different types and different species. Since the E-cadherin/catenin complex is a powerful invasion-suppressor (Bracke *et al.,* 1996) whose downregulation leads to invasion in multiple *in vitro* and *in vivo* situations, one might expect that plasmin would also induce invasion of the cells from which the extracellular part of E-cadherin has been removed. We were, however, unable to demonstrate a direct effect of plasmin on invasion. Neither did the spontaneously released sE-CAD from 1- and 2-day old collagen type I invasion cultures stimulate invasion in an autocrine manner. One possible explanation might be that the minimum concentration of sE-CAD necessary to stimulate invasion is not reached by the release during the invasion assay because of dilution in the culture medium. The concentration of sE-CAD is 50 times lower in an experiment without conditioned medium than with conditioned medium as measured by ELISA. In conditioned medium of PCm.src5, the minimum concentration of sE-CAD that stimulated invasion of MDCKts.*src*Cl2 at 40 °C was between 600 and 300ng/ml (our unpublished results). Release of the 80 kDa sE-CAD can be inhibited by overexpression of the tissue inhibitor of MMP-2 and stimulated by phorbol-12-myristate-13-acetate (Noë *et al.,* 1999). Interestingly, ectodomain shedding of E-cadherin results in a loss of function in the fast aggregation assay. In long-term experiments, such as the slow aggregation assay and the collagen type I invasion assay, we find recovery of E-cadherin and results that are difficult to interprete (our unpublished results).

Since sE-CAD is also found in human cancers, our present *in vitro* experiments may also be relevant for invasion *in vivo* (Katayama *et al.,* 1994; Banks *et al.,* 1995; Griffiths *et al.*, 1996; Gofuku *et al.,* 1998). Here, tumor microecosystems may generate higher amounts of sE-CAD through proteases from host cells (De Wever and Mareel, 2002). Furthermore, there may be contributions from cells undergoing apoptosis and producing MMPs in concert with caspases acting on β-catenin and plakoglobin (Herren *et al.,* 1998; Steinhusen *et al.,* 2001).

Although our data reveal the invasion-promoting effect of the released sE-CAD, it is possible that the remnant transmembrane fragment may have a similar function. There is evidence in the literature for a function of such a fragment of receptor tyrosine kinases, where removal of the ectodomain results in constitutive activation of the kinase activity present in the remaining fragment (Rodrigues and Park, 1994; Cabrera *et al.,* 1996). It has been reported that trypsin-mediated removal of the E-cadherin ectodomain induces tyrosine phosphorylation of ß-catenin (Takahashi *et al.,* 1997), a protein that associates with the E-cadherin cytoplasmic tail, forming part of the adherens complex. Mutant cadherins containing the transmembrane domain and the cytoplasmic tail, thus mimicking the 40 kDa remnant fragment, exert dominant-negative effects when expressed in *Xenopus* embryos (Kintner, 1992), in cultured epithelial cells (Fujimori *et al.,* 1993), and in mouse intestinal epithelia (Hermiston and Gordon, 1995a, b). Such fragments have also been shown to directly block ß-catenin signaling (Crawford *et al.,* 1999; Orsulic *et al.,* 1999). E-cadherin cleavage may therefore promote invasion through both an sE-CAD fragment acting in a paracrine manner and a 40 kDa remnant fragment that acts in an autocrine manner.

How sE-CAD stimulates invasion is not yet clear (Willems *et al.,* 1995; Noë *et al.,* 2001). The present aggregation assay suggests that sE-CAD inhibits the homophilic cell-cell adhesion function of the E-cadherin/catenin complex, which is in line with stimulation of invasion. Both aggregation and lack of invasion require an intact E-cadherin/catenin complex as evidenced by neutralizing antibodies against E-cadherin (Boterberg *et al.,* 2000; Bracke *et al.,* 2000). The E-cadherin-specificity of the loss of function caused by sE-CAD is suggested by increased tyrosine phosphorylation of ß-catenin. Experiments with dominant negative mutants and with pharmacological inhibitors point to invasion-promoter or invasion-suppressor pathways implicating Rho GTPases, PI3 kinase and trimeric G proteins (our unpublished results in collaboration with C. Gespach, Paris, France).

The immunodepletion of sE-CAD from conditioned media, associated with loss of activity, proves that sE-CAD is responsible for inhibition of aggregation and stimulation of invasion by these conditioned media. Here, sE-CAD might mimic an intact E-cadherin molecule anchored to the cell surface and thus disturb homophilic interactions responsible for cell-cell adhesion. The presence of multiple molecular networks around cell surface receptors (Mareel *et al.,* 1997) does, however, suggest that this simple physical disruption of cell-cell contacts is not solely responsible and that other signal transduction pathways, either stimulated directly by sE-CAD interactions with E-cadherin or indirectly by the disruption of cell-cell contacts, contribute to the stimulation of invasion.

Materials and Methods

Cell Culture

Cells expressing E-cadherin were used as donor cells to monitor the release of sE-CAD into their conditioned medium and to test its influence on E-cadherin functions in acceptor cells. MDCK-ts.*src*Cl2 (Behrens *et al.,* 1993) are Madin-Darby canine kidney (MDCK) cells transformed with a temperature sensitive *src* oncogene. They were used as donor cells at the non-permissive tem-

perature for $p60^{v\text{-}src}$ activity (40 °C), when their E-cadherin/catenin complex is functional and at the *src* permissive temperature (35 °C), when the E-cadherin/catenin complex is deficient as evidenced by loss of aggregation and gain of invasion. The following cells were also used as donor cells. Parental PC/AA/C1 cells are derived from a colonic adenoma in a patient with familial adenomatous polyposis (FAP); they are non-tumorigenic in nude mice. PCm.src5 cells are PC/AA/C1 cells transfected with an activated *c-src* oncogene, which becomes susceptible to invasiveness upon addition of HGF (Empereur *et al.,* 1997). MDCKts.*src*Cl2 and PCm.src5 cells are maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Ghent, Belgium), supplemented with 10% (w/v) fetal bovine serum (FBS, Life Technologies) and 0.05% (v/v) L-glutamine. COLO-16 cells are derived from a human skin squamous carcinoma; they are cultured in RPMI-1640 supplemented with 10% (v/v) FBS and 0.05% (w/v) L-glutamine (Moore *et al.,* 1975). The MDCKts.*src*Cl2 cells were used as acceptor cells at the non-permissive temperature for $p60^{v\text{-}src}$ activity (40 °C).

Antibodies and Chemicals

DECMA-1 is a rat monoclonal antibody, raised against the murine embryonal cell line PCC7 Aza RI, recognizing the extracellular domain of murine and canine E-cadherin (Sigma, St. Louis, USA). HECD-1 is a murine monoclonal antibody recognizing the extracellular domain of human E-cadherin (Takara, San Diego, USA). A rabbit polyclonal pan-cadherin antibody was raised against a synthetic peptide corresponding to a conserved sequence in the cytoplasmic tail of classical cadherins. The immune serum recognizes bands corresponding with E-cadherin, N-cadherin and P-cadherin on Western blots. We also used the mouse monoclonal anti-E-cadherin, recognizing the cytoplasmic tail of E-cadherin (Transduction Laboratories, Lexington, USA). The monoclonal antibody SEC-11 was raised against a synthetic peptide corresponding to amino acids 9–21 (CPENEKGPFPKNL) of mature human E-cadherin, a sequence shared with only dog E-cadherin and implied in calcium binding. Briefly, female BALB/c mice were immunized with one subcutaneous and two intraperitoneal booster injections of the peptide conjugated to keyhole limpet hemocyanin. Spleen cells were harvested after 7 weeks and fused with NS0 mouse myeloma cells according to the protocol of Brown and Ling (1988). Hybridomas were screened using ELISA for specific recognition of the synthetic peptide. After testing positive hybridomas for recognition of E-cadherin by Western blotting, limiting dilution was performed to obtain stable clones. SEC-11 (IgG1) was found to recognize human and dog, but not mouse or rat 120 kDa E-cadherin and its 80 kDa extracellular part (sE-CAD) by Western blotting.

For incubation with cells, 1 µg/ml plasmin or 0.025% trypsin were dissolved in serum-free medium. To test the effect on purified E-cadherin, 1 ng, 10 ng, 100 ng or 1 µg plasmin were dissolved in 50 µl PBS and 0.025% trypsin was diluted in PBS containing 0.04 mM $CaCl_2$. The serine protease inhibitor aprotinin (ICN, Costa Mesa, USA) was used at a concentration of 10 µg/ml. Immunodetection was achieved with HECD-1 for the human cell-lines and with SEC-11 for the canine cells.

Immunoprecipitation and Western Blotting

Cells were lysed with PBS containing 1% NP-40, 1% Triton-X 100 and the following protease inhibitors (all from ICN): phenylmethylsulfonyl fluoride (1.72 mM), leupeptin (21 mM), aprotinin (10 µg/ml). Equal amounts of protein (1 mg) or conditioned media from equal amounts of cells were incubated with an antibody for 3 h at 4 °C followed by protein A Sepharose 4 fast flow beads or protein G Sepharose 4 fast flow beads (Pharmacia LKB, Uppsala, Sweden) for 1 h. Precipitated proteins were dissolved in sample buffer, boiled in the presence of 5% 2-mercaptoethanol, separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, USA). For immunostaining, blots were quenched with 5% non-fat dry milk in PBS containing 0.5% Tween 20, the membranes were incubated with primary antibody, followed by 3 times washing for 5 minutes and incubation with horseradish peroxidase conjugated secondary antibodies. Proteins were stained using ECL reagent (Amersham Life Science, Buckinghamshire, UK) as substrate.

Conditioned Medium

Conditioned medium of donor cells was obtained as follows. Seventy-five cm^2 monolayers of 75% confluency of MDCKts.*src*Cl2 at 40 °C or 35 °C, PCm.src5 or COLO-16 donor cells at 37 °C were washed 3 times with serum-free DMEM (Life Technologies) and incubated for 2 h at 37 °C with serum-free DMEM to which 1 µg/ml plasmin was added or not. The medium was harvested, centrifuged at 2000 *g* for 15 min, 5-fold concentrated in centriprep tubes YM-30 (Amicon, Millipore Corp., Bedford, USA) and filtered through a 0.22 µm filter. To remove sE-CAD, 1 ml of conditioned medium was incubated at 4 °C for 3 h with 2 µg DECMA-1 for canine kidney cells and 10 µg HECD-1 for human cells, followed by protein A Sepharose 4 fast flow beads or protein G Sepharose 4 fast flow beads for 1 h. This procedure was repeated a second and a third time to remove all sE-CAD. As a control for specificity of the HECD-1 precipitation, we used an isotype mouse IgG1 antibody. Finally the conditioned media were filtered through a 0.22 µm filter and diluted 1:2 before use in the collagen type I assay.

Proteolytic Cleavage of E-Cadherin

Direct cleavage of E-cadherin was examined by the treatment of pan-cadherin immunoprecipitated E-cadherin on beads. After 3 washes with PBS, the beads carrying E-cadherin were incubated for 30 min at 37 °C in 50 µl PBS containing plasmin or trypsin. Supernatant and beads were separately dissolved in sample buffer, boiled in the presence of 5% 2-mercaptoethanol, separated by 7.5% SDS-PAGE, transferred onto Immobilon-P membranes (Millipore Corp.) and immunostained.

Collagen Invasion Assay

Type I collagen (Upstate Biotechnology, Lake Placid, USA) was dissolved in DMEM containing bicarbonate buffer and neutralized with 1 M NaOH. 1.25 ml aliquots were poured into a 6 well-plate and incubated for at least two hours at 37 °C for gelation. 2×10^5 cells were seeded on top of the collagen gel with or without conditioned medium. After 24 h of incubation, the number of cells invading into the gel was counted (Bracke *et al*., 2000). The invasion index was calculated as the number of cells inside the gel over the total number of cells.

Aggregation Assay

Cell-cell adhesion was quantified in an aggregation assay as described earlier (Boterberg *et al*., 2000). Briefly, incubation with collagenase A in presence of 0.04 mM Ca^{2+} (Boehringer Mannheim, Mannheim, Germany), followed by trypsin in presence of 0.04 mM Ca^{2+} (Sigma, St-Louis, MS) and neutralization with soy bean trypsin inhibitor released cells from the substrate with preservation of E-cadherin. The cells were allowed to aggregate on a Gyrotory shaker (New Brunswick Scientific, New Brunswick, USA) at 80 rpm for 30 min in an aggregation buffer containing 1.25 mM Ca^{2+}, 0.1 mg DNase/ml, 10 mM Hepes and

0.1% (w/v) BSA and equilibrated at physiological pH and osmolarity. Cell aggregation was measured with a LS particle size analyser (LS 200, Coulter Electronics, Miami, USA) after 0 min and 30 min of aggregation. The relative volume in function of the particle size was used as an index of aggregation.

Statistics

All experiments were performed at least twice. Student's *t*-test was used for statistical analysis of the collagen invasion assay and Kolmogorov-Smirnov statistics to analyze the differences between the cumulative distribution curves obtained in the fast aggregation assay.

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Discussion and Perspectives

The experiments discussed in this thesis provide novel insights into several systems that may play a role in carcinogenesis. Tumorigenesis mostly results from the accumulation of genetic events, in a first phase leading to the establishment of a primary tumor whose growth has to be sustained. Activation of particular pro-survival cellular pathways is likely to be crucial in this phase. In a second phase, the tumor cells may leave the primary site and invade the surrounding tissue, which may eventually result in metastasis. Impaired function of cell adhesion molecules may facilitate this process. Although many cancer types go through this multi-step process of progression, recently more attention is being given to the so-called 'metastatic signature' of cancer cells, a profile which may distinguish in an early stage tumors that are likely to progress aggressively from more benign tumors.

Our identification of two new growth factor systems, the heregulin (HRG)-human epidermal growth factor receptor (HER) system (**Section I.3**) and the follistatin-activin-activin receptor system (**Section I.4**), in human melanoma cell lines has added these systems to the ever expanding list of growth factors that may play a role in carcinogenesis, more particularly melanomagenesis. Both systems may contribute to the tight balance that regulates senescence and proliferation of melanocytes *in vivo*. Disruption of this balance, either towards increased proliferation or towards decreased inhibition, may represent a first step towards melanoma. Considering the tight interplay that exists between tumors and the surrounding micro-environment, examination of these systems in experimental models *ex vivo* or *in vivo* may provide additional valuable information regarding this mutual interaction. The use of retro- or lentivirally transduced cell lines, either leading to overexpression or gene silencing, may be very helpful in this approach. Furthermore, these experimental systems also allow the evaluation of invasion and metastasis in a physiologically relevant model. Since neither of both growth factor systems has been described in melanoma before, it will be essential to examine whether they occur in melanoma patients as well. If so, they may represent interesting targets for directed therapies. This is particularly true for the heregulin-HER system, for which several therapies exist, including the use of receptor-blocking antibodies and small tyrosine kinase inhibitors.

For many years, the E-cadherin/catenin system is known to function as a gatekeeper in cancer. The studies described here have extended the knowledge about the mechanisms on how the anti-invasive function of E-cadherin can be modulated. Our finding that the anti-invasive activity of a melanoma conditioned medium can be attributed, at least in part, to the stimulation of the E-cadherin/catenin complex by HRG present in this medium (**Section II.2**), extends the wide array of functions attributed to this growth factor. Although HRG has been implicated in the malignant behavior of cells in many studies, it has a well-known differentiating function as well (**Section I.2**). This is reflected in its role in the mammary gland *in vivo,* which exists of a combination of maintenance of proliferation and, at the same time, inducing differentiation. In future studies, it may be interesting to determine what signaling pathways underlie the pro-aggregating and anti-invasive effects of HRG we observed.

Tumor cells have developed a wide array of mechanisms by which they can interfere with E-cadherin functionality. These include genomic, transcriptional and post-transcriptional events. The latter include the proteolytic cleavage of E-cadherin, releasing the E-cadherin ectodomain (sE-CAD). In our study we focused on the effects of sE-CAD, generated following the exposure of E-cadherin positive cells to the serine protease plasmin (**Section II.7**.). We found that sE-CAD can interfere with aggregation and induced invasion of E-cadherin positive cells *in vitro*. However, the way by which the sE-CAD signal is received by the cell, how it is transduced and what underlies the altered phenotype of the cells, remains elusive. As an alternative approach, it may be worthwhile to look at the cells that have produced the sE-CAD fragment. In these cells, the remaining transmembrane-intracellular part may be subjected to further processing, releasing the intracellular tail, and possibly influencing signaling pathways as has been described for the corresponding N-cadherin cytoplasmic tail.

In contrast to the wealth of publications on E-cadherin, relatively little is known about the role P-cadherin may play in cancer. The two manuscripts on P-cadherin in this thesis describe new, opposite roles for this protein in melanoma and breast cancer. Loss of P-cadherin frequently occurs during melanoma progression. We found that d*e novo* expression of P-cadherin in melanoma cell lines leads to a more epithelioid morphology, coinciding with a reduction of invasion and promotion of cell-cell adhesion (**Section II.4**). Analysis of P-cadherin mutants suggested that these effects were dependent on the 'classical' function of this cadherin, the support of cell-cell adhesion. Another scenario accounts for breast cancer, where a correlation exists between P-cadherin expression, estrogen receptor negativity, and poor survival. In our study, we could provide a direct link between a lack of estrogen receptor signaling and

P-cadherin expression. Moreover, in contrast to melanoma, where P-cadherin has essentially an inhibitory effect on invasion, invasion of breast cancer cells is promoted by P-cadherin (**Section II.5**.). Further investigation revealed that the juxtamembrane domain was required for this effect, suggesting that this particular domain, although only differing in some amino acids from the corresponding domain in E-cadherin, actively provides the cells with a pro-invasive signal. The mechanisms by which P-cadherin drives cells towards invasion remain to be elucidated. Possible mechanisms include differences in binding partners or binding characteristics of these partners. Domain-swapping experiments, in which the corresponding domains of E- and P-cadherin are exchanged, approaches destined to map interaction partners (yeast-two-hybrid, mass spectrometry), as well as experiments using dominant negative and constitutively active constructs of signal transduction molecules or pharmacological inhibitors may provide an answer to this question. In addition, although P-cadherin transgenic mice, in which P-cadherin expression is driven by a murine mammary tumor virus (MMTV) promoter, do not spontaneously form tumors (Radice *et al.*, 2002), crossing these mice with other transgenic mice may provide a valuable *in vivo* model to evaluate our findings. As evidenced by the lack of P-cadherin positive tumors upon crossing these mice with mice expressing the *neu* oncogene under the same promoter, the choice of the partner transgene may be crucial to identify tumors in which P-cadherin expression is present. Having in mind that in healthy breast tissue, P-cadherin expression is confined to the myoepithelial cells, good candidates may be transgenic mice in which components of the β-catenin pathway are expressed under the MMTV promoter, since, unlike *neu* transgenic mice, the tumors of these mice display both epithelial and myoepithelial characteristics (Li *et al.*, 2003).

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Notes