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GROWTH FACTOR EFFECTS ON CELL SURVIVAL IN THREE-DIMENSIONAL CULTURES

TUOMO ALANKO

DEPARTMENT OF VIROLOGY
HAARTMAN INSTITUTE

AND

DEPARTMENT OF DERMATOLOGY

UNIVERSITY OF HELSINKI
FINLAND

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SUPERVISOR:

Docent Olli Saksela
Department of Dermatology
Helsinki University Central Hospital
Helsinki, Finland

REVIEWED BY:

Docent Juha Partanen
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

AND

Docent Ulpu Saarialho-Kere
Department of Dermatology
Helsinki University Central Hospital
Helsinki, Finland

OPPONENT:

Docent Veli-Matti Kähäri
Turku Centre for Biotechnology
University of Turku
Turku, Finland

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Alanko, T., Tienari, J., Lehtonen, E., and Saksela, O.: Development of FGF-dependency in human embryonic carcinoma cells after retinoic acid-induced differentiation. *Developmental Biology* 161:141-153, 1994.

II Alanko, T., Tienari, J., Lehtonen, E., and Saksela, O.: FGF-2 inhibits apoptosis in human teratocarcinoma cells during differentiation on collagen substratum. *Experimental Cell Research* 228:306-312, 1996.

III Alanko, T., Rosenberg, M., and Saksela, O.: FGF expression allows nevus cells to survive in three-dimensional collagen gel under conditions that induce apoptosis in normal human melanocytes. *Journal of Investigative Dermatology* 113:111-116, 1999.

IV Alanko, T. and Saksela, O.: Transforming growth factor β1 induces apoptosis in normal melanocytes but not in nevus cells in type I collagen. *Journal of Investigative Dermatology* 115:286-291, 2000.

V Alanko, T. and Saksela, O.: Melanocyte apoptosis after transforming growth factor β induced oxidative stress. Submitted.

ABBREVIATIONS

5-BrdU	5-bromo-2'-deoxyuridine
AIF	apoptosis inducing factor
BM	basement membrane
CAM	cell adhesion molecule
Cdk	cyclin dependent kinase
CdkI	Cdk inhibitor
CHX	cycloheximide
DD	death domain
EC	embryonal carcinoma
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FACIT	fibril associated collagens with interrupted triple helix
FGF	fibroblast growth factor
FGF-1	fibroblast growth factor-1, former acidic FGF (aFGF)
FGF-2	fibroblast growth factor-2, former basic FGF (bFGF)
FGF-4	fibroblast growth factor-4, former Kaposi FGF (K-FGF)
FGF-7	fibroblast growth factor-7, former keratinocyte growth factor (KGF)
FGFR	FGF receptor
fgfr	FGF receptor gene
GM-CSF	granulocyte-macrophage colony stimulating factor
Hsp	heat shock protein
HSPG	heparan sulfate proteoglycan
IAP	inhibitor of apoptosis protein
IFN-α	interferon α
IL	interleukin
LAP	latency associated peptide
LTPB	latent TGF-β binding protein
MAPK	mitogen activated protein kinase
PDGF	platelet derived growth factor
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
VEGF	vascular endothelial growth factor

SUMMARY

The aim of this study was to develop a three-dimensional cell culture model to study the growth factor dependency of a human teratocarcinoma cell line Tera-2 and human skin pigment cells, and their responses to extracellular matrix (ECM) mediated stimuli.

Malignant Tera-2 cells originally derived from a human germ cell tumor have a remarkable ability to differentiate in response to retinoic acid (RA) stimulation. The resulting cells are phenotypically benign neurons. Tera-2 served as a model of differentiation-induced changes in cell behavior during development.

Human skin melanocytes form a loose network of pigment producing cells in the basal epidermis where they are exclusively found in contact with the basal layer of keratinocytes and the basement membrane. Pigment cell nevus represents a local, benign proliferation of melanocytes. Nevus cells, like malignant melanoma cells, are in many cases able to grow in the dermis. The differential *in vivo* localization of these cell types was the reason to use them as a model to study changes in growth factor and ECM requirements during various stages of malignant progression.

Type I collagen gel was used as a three-dimensional cell culture lattice. The growth factors of interest were either mixed into the gel or added as a single spot to form a concentration gradient. The cells were observed by microscopy and analyzed by immunohistochemistry, Western blotting, Northern blotting, or flow cytometry.

Undifferentiated Tera-2 cells grew in collagen as tight clusters. FGF-2 induced scattering of the cells or, when in gradient, directional migration towards the FGF-2 source. After RA induced differentiation, Tera-2 cells growing in collagen gel started to undergo apoptosis. FGF-2 and FGF-2 containing ECM preparations promoted the survival but not migration of differentiated cells. Tera-2 cells express two members of the FGF family, FGF-2 and FGF-4. The expression of both FGFs was turned off during differentiation in collagen, whereas on the plastic surface of a tissue culture dish, the expression of FGF-4 only became undetectable.

The results suggest that signaling through FGF receptors is vital for Tera-2 cells. Differentiation in collagen results in complete extinction of their autocrine stimulatory FGF loops. *In vivo*, such differentiation-induced growth factor dependency would result in apoptotic death of the cells that fail to find adequate FGF stimulation.

Epidermal melanocytes are dependent on FGF-mediated paracrine signaling from neighboring keratinocytes. In vitro, unstimulated melanocytes rapidly entered apoptosis when cultured in collagen gel. Addition of FGF-2 strongly promoted their survival. Dermal nevus cells survived without exogenous FGF stimulation. Nevus cells were found to produce fairly large amounts of FGF-2 themselves, while normal melanocytes were almost completely negative. The survival of nevus cells in collagen was repressed by protamine, an inhibitor of FGF-2 mediated effects.

TGF-β1 dramatically accelerated the apoptosis of melanocytes in collagen. Neutralizing TGF-β1 antibodies not only inhibited the effect of exogenous TGF-β1 but also improved the survival of melanocytes even in the absence of exogenous growth factors. This suggests a new role for TGF-β as a mediator of melanocyte apoptosis. FGF-2 was found to counteract the effects of TGF-β. Melanocytes, nevus cells, and melanoma cells were found to produce and secrete TGF-β in cell culture. For nevus cells TGF-β acted as a growth inhibitor but not inducer of apoptosis. Melanoma cells were totally resistant to TGF-β effects.

The TGF-β induced apoptosis of melanocytes in collagen was found to be associated with downregulation of the antiapoptotic protein Bcl-2 and an early increase in intracellular oxidative stress. Antioxidants N-acetylcysteine and ascorbic acid effectively decreased the oxidative stress and inhibited apoptosis. FGF-2 both increased Bcl-2 and decreased the oxidative stress. Caspase inhibitors inhibited melanocyte apoptosis in collagen without effects on Bcl-2 or radical levels.

The results suggest that the balance between inhibitory (TGF-β) and stimulatory (FGF-2) growth factors has the potential to regulate the growth, localization, and survival of melanocytes also *in vivo*. Autocrine or paracrine TGF-β stimulation induces apoptotic death of normal melanocytes in a collagen environment in the absence of sufficient FGF-2 stimulation. FGF-2 expression and resistance to TGF-β mediated apoptosis allow nevus cells and tumorigenic melanoma cells to survive and grow in the dermis that consists largely of type I and III collagens.

INTRODUCTION

Research in cell biology explores fundamental processes that not only underlie the behavior of a single cell or cell type but also form the basis for functions of tissues and whole organisms. The aim of biological research is to learn to understand normal biology. However, in many cases it is possible to learn things about what is normal by studying pathological phenomena. Cancer in its many appearances has been the favored subject of interest for thousands of scientists and for decades of time.

Cancer is characterized by a disturbance in cellular growth control. Tissue growth can be abnormal because of faults in cellular signaling cascades normally leading to quiescence (growth arrest), differentiation, or apoptosis. Apoptosis, the suicide of aged, damaged or surplus cells, is important during normal development and maintenance of healthy organs. Abnormalities of apoptosis have been described in many serious pathologic conditions ranging from cancer to AIDS.

Normal growth is tightly regulated by extracellular cues that trigger complex intracellular programs. Each cell has contact with neighboring cells and with the extracellular matrix. Much of the paracrine signaling between neighboring cells is mediated by growth factor action on their respective cell surface receptors. In many cases these growth factor signals are mediated by the extracellular matrix which can guide and restrict growth factor diffusion and in some cases act as a reservoir of signaling molecules.

The aim of this study was to explore changes in growth requirements and survival of two cell types: Tera-2, a malignant cell line that can, when properly stimulated, differentiate *in vitro* into benign cells, and melanocytes, among which both benign and malignant cells of the melanocyte lineage were studied. To achieve a controllable system where even subtle changes would be easily detectable, a model was developed that utilizes type I collagen as an artificial matrix. This model makes it feasible to compare the growth requirements of cells under conventional and three-dimensional culture conditions.

REVIEW OF THE LITERATURE

Extracellular matrix

Functions of the ECM

The extracellular matrix (ECM) is a highly organized meshwork of molecules. The structural components include collagens, noncollagenous glycoproteins, and proteoglycans. Like the cement in a brick wall the ECM provides cells with a scaffold that they can attach to. It also acts as a barrier that insulates tissues, impedes cell migration and regulates diffusion and the spreading of physiological and non-physiological stimuli. Besides these seemingly passive functions it serves as a dynamic cellular microenvironment that can mediate information to and from the cells and store and protect essential regulatory factors produced by the cells. ECM is actively produced and molded by the cells. The interplay and signalling between cells and ECM is continuous. The broad spectrum of functions makes it understandable that mutations affecting ECM components and their normal function can cause a wide range of pathologic conditions in any organ of the body (for review see Bruckner-Tuderman and Bruckner, 1998; Aumailley and Gayraud, 1998).

The fine composition of the ECM is under tight regulation and is different in every specific tissue. Two broad categories of ECM can be distinguished with differing main structural components, the basement membranes (BM) and the interstitial connective tissue.

Structure of the ECM

The basement membrane

BMs are specialized, sheet-like extracellular matrices. They are secreted by epithelial, endothelial, and many mesenchymal cells and serve to separate these from the underlying cells and matrix structures. In different organs BM can have very specialized additional functions ranging from regulation of gene expression to acting as a molecular sieve. The main components of BM are type IV collagen, laminin, entactin/nidogen, and heparan sulfate proteoglycans (HSPGs) (**Table 1**; for review see Yurchenco and Schittny, 1990).

Type IV collagen molecules are heterotrimers of α(IV) chains. Six genetically different chains (α1(IV) to α6(IV)) have been cloned but only the combination α1(IV)2α2(IV) has been

Table 1
Main structural components of the ECM

Basement membrane	Interstitial connective tissue
Type IV collagen	Fibril-forming collagens (types I, II, III, V, XI)
Laminins	
Proteoglycans	Non-fibrillar collagens (types VI, IX, XII, XVI, XIX)
Entactin	
Type VII collagen	Elastin
Fibulins	Fibrillins
	Fibronectin
	Vitronectin
	Chondronectin
	SPARC
	Thrombospondin
	Tenascin
	Proteoglycans

purified (Brown and Timpl, 1995). The chains form a triple-helical structure with several interruptions that make it comparatively flexible. Two molecules can interact and bind each other through the C-terminal globular noncollagenous domain (NC1) and four molecules can tetramerize by lateral association of the N-terminal 7S-termini. In addition, certain regions of the triple helical parts of the molecule can interact and form a supercoiled structure. A self-assembly process leads to the formation of a scaffold that is stabilized by disulfide bonding and covalent cross-linking (Yurchenco and Schittny, 1990).

Laminin (mostly laminin 1) provides another polymeric network in BM. Laminin is a flexible cruciform glycoprotein (carbohydrate content 12-15%) that consists of three polypeptides, the larger (c. 400 kDa) A (α) chain and the two smaller (c. 200 kDa) chains designated B1 (β) and B2 (γ). The terminal globular domains of the arms of laminin molecule are involved in the attachment of several molecules to dimers and oligomers. The self-assembly of laminin network is calcium-dependent. The three-dimensional scaffold is an important component of all BMs. Several cell types interact with laminin that promotes their attachment, spreading and differentiation (see Aumailley and Rousselle, 1999; Timpl, 1989 for review, Panayotou et al., 1989). Laminin apparently interacts with type IV collagen network through a bridging molecule, entactin/nidogen.

Entactin is a dumbbell-shaped glycoprotein with two terminal globules and a connecting rod-like domain. It binds to the central region of laminin with the COOH-terminal globule and to type IV collagen apparently with the same domain (Timpl, 1989; Paulsson et al., 1987).

Proteoglycans are present in all BM structures (see Iozzo, 1998 for review). The three most important mammalian BM proteoglycans are perlecan, agrin, and bamacan. As highly charged macromolecules, they are probably responsible for the charge-dependent molecular sieving functions of BM for example in the glomeruli. Perlecan, that carries primarily HSPG side chains, is a prominent component of the endothelial BMs and it may play a key role in early

vasculogenesis (Handler et al., 1997). Several heparin-binding growth factors, including fibroblast growth factor 2 (FGF-2, basic FGF, bFGF) and vascular endothelial growth factor (VEGF), are involved in the process of vasculogenesis. Perlecan has the potential to store these growth factors and regulate their diffusion and activity. Agrin is a HSPG primarily found on the synaptic BM (Tsen et al., 1995; Iozzo, 1998). Bamacan, a chondroitin sulfate proteoglycan, is found in nearly all of the BMs investigated (McCarthy and Couchman, 1990; McCarthy et al., 1994). Its functions are poorly known but from developmental studies it has been inferred that bamacan may inhibit, while perlecan seems to enhance, branching morphogenesis (Iozzo, 1998).

It is not known exactly how BM is connected to the underlying interstitial connective tissue. Considerable progress has been made during the past few years by studying hereditary blistering skin diseases caused by mutations in the gene for *type VII collagen* (see Bruckner-Tuderman et al., 1999 for review). Anchoring fibrils composed of type VII collagen extend from the BM to foci of BM components (anchoring plaques) deep in the stroma. On the ventral surface of BM anchoring fibrils interact with specialized cellular junctional complexes called hemidesmosomes through another minor BM component, *laminin 5*. Hemidesmosomes serve to attach epithelial cells to the underlying BM in complex epithelia such as the skin. Unlike other laminins, laminin 5 does not bind nidogen and it lacks the self-association domain needed to form an independent network (see Borradori and Sonnenberg, 1999 for review). *Fibulins*, a group of small glycoproteins rich in epidermal growth factor (EGF)-like repeats, are also thought to be involved in connecting BM networks to the fibrils of the interstitium (Aumailley and Gayraud, 1998).

The information needed to form such complicated structures as a BM seems, rather surprisingly, to be intrinsic to the molecules (Yurchenco, 1994). As mentioned above, both type IV collagen and laminin are able to self-assemble into a three-dimensional network. Grant et al. (1989) have also shown that structures resembling a BM can be formed by co-incubation of laminin, type IV collagen, and HSPG at 35°C.

The interstitial connective tissue

The interstitial connective tissue surrounds and is formed by connective tissue cells (**Table 1**). It is the major structure determining the mechanical strength and elasticity of tissues like bone, cartilage, tendons, and dermis. As could be predicted from the extremely varying qualities

represented by these tissue types, there are marked differences in the composition and molecular architecture of their matrices.

Collagens are the most abundant structural components of the intersitial matrix. *The fibril-forming collagens* (types I, II, III, V, and XI) are synthesized as procollagens with large globular N- and C-terminal propeptides. The enzymatical cleavage of these leads to the formation of mature collagen (van der Rest and Garrone, 1992). During fibrillogenesis the collagen molecules are assembled longitudinally to thick fibrils. Type II collagen fibrils are typical for cartilage. Type I collagen is the major structural protein of most other tissues including skin, bone and tendon. Varying amounts of type XI collagen is found in association to type II fibrils and types III and V are found within type I fibrils (Henkel and Glanville, 1982; Mendler et al., 1989). These minor fibrillar collagens are thought to be involved in regulating fibril morphogenesis. Collagen fibrils account for the ability of tissues to resist stretching. In tendon, where the load is longitudinal, the fibrils align longitudinally. In the skin, on the other hand, the fibers form a seemingly random meshwork but they get aligned during loading.

Non-fibrillar collagens form a rather heterogenous group. Fibril associated collagens with interrupted triple helix (FACIT, types IX, XII, XIV, XVI, and XIX) are found on the surface of collagen fibrils possibly facilitating interactions with proteoglycans through their large extending noncollagenous domains (Brown et al., 1993). Type VI collagen forms a polymeric microfibrillar network. Glycoasaminoglycan interactions probably connect the type VI collagen network to other polymers of the ECM but its biological roles are still not fully known (Bidanset et al., 1992; Burg et al., 1996; Kielty et al., 1992).

Elastin and fibrillins form an important part of the connective tissue matrix. Together they form the elastic fibers that confer elasticity to tissues. Elastin, a hydrophobic, extensively cross-linked protein, forms the amorphous part of elastic fibers. It has the property of rubber-like recoil. Fibrillin microfibrils that form the core of elastic fibers probably have a role in the assembly of elastin (Mecham and Davis, 1994). Fibrillins form a family of proteins that consist almost entirely of EGF-like motifs. Several other small proteins are thought to be involved in the assembly of the elastin/microfibril system (Brown-Augsburger et al., 1996; Cleary and Gibson, 1996).

Another ECM meshwork is provided by *fibronectin.* Fibronectin is a large (c. 250 kD) noncollagenous glycoprotein that forms disulfide linked dimers. Alternative splicing gives rise to at least 20 variants in man. Fibronectin also forms fibrils but, in contrast to collagen self-assembly, the polymerization is cell driven. Fibronectin has several binding regions that

interact with cells, heparin, fibrin, and collagens (Hynes, 1990). Mouse embryos defective in both fibronectin alleles die *in utero* (George et al., 1993).

Other glycoproteins of the ECM that display adhesive properties include *vitronectin, chondronectin, SPARC, thrombospondin and tenascin* (Yamada, 1991).

Proteoglycans form the amorphous mass of interstitial extracellular matrices. They provide the glue between various networks and, as highly hydrophilic molecules, retain water that is important for maintaining normal tissue volume. Several classes with different sizes and protein cores are present in the ECM (see Wight et al., 1991; Iozzo, 1998 for review).

The group of *small leucine-rich proteoglycans* (protein core of 36-42 kD) includes decorin, biglycan, fibromodulin, lumican, and epiphycan. The protein core of decorin or fibromodulin binds to fibrillar collagen. The protein core has also been reported to interact with type VI collagen, fibronectin, and thrombospondin (Bidanset et al., 1992; Winnemoller et al., 1991; Winnemoller et al., 1992).

The *hyalectans* are proteoglycans with a large protein core (100-370 kD). The protein core has several functional domains including an N-terminal domain that binds hyaluronan, a central domain that carries the glycosaminoglycan side chains, and a C-terminal region that binds lectins. Of these, versican has a widespread distribution, while aggregan is mostly expressed in cartilage, and neurocan and brevican are found almost exclusively in neural tissue (Iozzo, 1998).

Cell-matrix and cell-cell interactions

Cell adhesion is fundamental to formation and maintenance of tissue structure and function. Changes in adhesive interactions are found early in conjunction with malignant transformation and metastasis (Takeichi, 1993; Yap, 1998). The environment can regulate cellular functions in several ways. Receptor molecules for ECM proteins or cellular counter-receptors can mediate both mechanical stress and ligand specific interactions leading to activation of specific intracellular signal pathways (outside-in signalling). *Vice versa*, intracellular events can lead to changes in the expression and affinity state of cell surface adhesion receptors (inside-out signalling) (see Aplin et al., 1998 for review). Additionally, neighboring cells secrete and ECM harbors a multitude of growth factors and other signalling molecules.

The most studied family of adhesion receptors, *integrins*, are transmembrane heterodimeric glycoproteins. The family consists of at least 16 α subunits and at least 8 β subunits which can

form more than 20 different integrins with distinct ligand binding specificities (Hynes, 1992; Rosales et al., 1995). For example, adhesion to type I collagen is mediated by β1 integrins, principally α1β1, α2β1, and α3β1 (Tuckwell and Humphries, 1993), while denatured collagen is a ligand for αvβ3 (Davis, 1992). Aggregation of integrin molecules by their natural ligands or monoclonal antibodies induces intracellular accumulation of a multitude of associated proteins and activation of the JNK, ERK, and MEK pathways (Miyamoto et al., 1995a; Miyamoto et al., 1995b; Galbraith and Sheetz, 1998). Further, tension applied to the cell through integrins initiates other signals. Mechanical tension has been reported to induce shifting of ribosomes and mRNA into focal adhesions, complexes of aggregated integrins and associated proteins (Chicurel et al., 1998).

Integrins undergo a set of dynamic conformational changes after ligand binding (Loftus and Liddington, 1997). According to a widely accepted model, the cytoplasmic domain of the α subunit is inhibitory to the activating β cytoplasmic domain. Ligand binding releases the inhibition possibly by allowing the domains to separate (Burridge and Chrzanowska-Wodnicka, 1996; Hughes et al., 1996). Intracellular factors have been shown to modulate the affinity of integrin receptors to their ligands. Different Ras-related small GTPases and their downstream effectors can have either positive or negative effects on ligand binding (Zhang et al., 1996; Hughes et al., 1997).

Cadherins form another important family of cell adhesion receptors. They are transmembrane proteins with a homologous extracellular domain consisting of multiple repeats of a cadherin motif (Suzuki, 1996). The “classic” cadherin subfamily includes N-, P- and E-cadherins along with a dozen other members (Takeichi, 1995). “Classic” cadherins mediate cell-cell adhesion by homotypic interactions in specific sites called adherence junctions. At these sites the cadherins of neighboring cells form a zipper-like structure. Another subgroup of cadherins is involved in intercellular interactions that take place in desmosomes (Cowin and Burke, 1996). The intracellular domains of cadherins interact with a group of proteins called catenins. Catenins mediate interactions of adherens junctions with the actin cytoskeleton (Cowin and Burke, 1996). Cadherins are critical for maintenance of normal tissue architecture. Several malignancies are associated with loss of normal cadherin expression or function (Risinger et al., 1994; Oda et al., 1994; Yoshiura et al., 1995; Berx et al., 1996). Considerably less is known about the role of cadherins in signal transduction.

The immunoglobulin superfamily of cell adhesion molecules (Ig-CAMs) is a diverse group of proteins that contain one or more Ig folds. A typical example is NCAM, which contains five Ig

folds in its extracellular part. NCAM functions as a homotypic adhesion receptor in the nervous system (Edelman and Crossin, 1991). Several other Ig-CAMs are present in developing and mature nervous tissue and are thought to be involved in axon guidance and formation and maintenance of neural connections (Baldwin et al., 1996). Ig-CAMs also have a critical role in the immune system. They mediate several aspects of immune response from antigen recognition and presentation to leukocyte trafficking (Dustin and Springer, 1991; Rosales and Juliano, 1995; Springer, 1995).

Selectins form a small group of adhesion receptors. They mediate heterotypic cell-cell interactions like the adherence of leukocytes and platelets (L-selectin and P-selectin) to endothelium (E-selectin) during inflammation (Lasky, 1995; Rosen and Bertozzi, 1994; Tedder et al., 1995).

ECM resident growth factors

Several growth factors bind to ECM either directly or via specific binding proteins. The largest group of ECM associated growth factors binds to *HSPG* or heparin. Heparan sulfates are highly heterogeneous and their growth factor binding ability seems to be affected by remarkably small critical modifications. For example, FGF-2 binding to HSPG is promoted by 2-O-sulfation of the uronic acid (Faham et al., 1996; Maccarana et al., 1993), whereas N- or 6-O-sulfation of glucosamine inhibits binding (Ernst et al., 1995). Other members of the FGF family also bind to HSPG but the critical residues are not conserved suggesting that they may bind to different HSPG substructures (Faham et al., 1996). Treatment of tissue preparations with heparin, heparanases or proteases releases FGF-2 from the matrix in a biologically active form (Flaumenhaft and Rifkin, 1992). HSPG protects FGF from proteolytic degradation and inactivation by heat and extremes of pH (Saksela et al., 1988). Cell surface HSPGs also bind to FGF receptor molecules with high affinity and act as modulators of FGF-receptor interactions. This modulation can be either stimulatory or inhibitory (Kan et al., 1999; Plotnikov et al., 2000).

Other growth factors that interact with HSPG include the hepatocyte growth factor family (Mizuno et al., 1994), platelet derived growth factor (PDGF) (Raines and Ross, 1992), VEGF (Park et al., 1993), heparin-binding EGF-like growth factor (Raab and Klagsbrun, 1997), and several hematopoietic cytokines, like interleukin 3 (IL-3) and granulocyte macrophage growth factor (GM-CSF) (Roberts et al., 1988). TGF−β can bind to HSPG via an accessory protein (Bützow et al., 1993).

Growth factors can associate with other ECM components besides HSPG. TGF−βs are secreted as latent, inactive complexes. During secretion, TGF−β is cleaved from its propeptide LAP (latency associated peptide) but LAP remains associated with TGF−β by noncovalent interactions (Gentry et al., 1988; Sha et al., 1989; Dubois et al., 1995). This complex interacts with a group of proteins called *latent TGF−β binding proteins* (LTBP). LTBPs are highly homologous to fibrillins, which are major constituents of the connective tissue microfibrils. They mediate the association of latent TGF−β with the matrix (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Taipale et al., 1994). TGF−β can be released and activated by proteolysis (Taipale et al., 1992; Taipale et al., 1995). Active TGF−β also has interactions with ECM components like the protein core of small leucine-rich proteoglycans (Hildebrand et al., 1994).

PDGF-AB and -BB bind to *SPARC*. The expression of SPARC is especially high in tissues undergoing remodeling or repair (Porter et al., 1995). The binding of complexed PDGF to its receptors is inhibited but the complex seems to dissociate in the mildly acidic conditions created during inflammation and tissue damage.

Insulin-like growth factors associate with a group of binding proteins (*IGFBPs*) some of which are components of the connective tissue matrix (Jones et al., 1993). Also, tumor necrosis factor α (TNF−α) has been shown to have interactions with *fibronectin* (Alon et al., 1994).

Matrix association can serve several functions. It allows generation of rapid and highly localized signals in situations like tissue damage and microbial invasion. The low affinity binding sites of the matrix enable formation of growth factor gradients in tissues. This facilitates for example the directional migration of certain cell populations during development. Also, growth factors of the ECM can have important regulatory feedback signal functions during tissue degradation and remodeling (Taipale and Keski-Oja, 1997).

Regulation of cell growth and survival

Cell multiplication

Multicellular organisms are composed of millions of cells. They all are progeny of one cell, the fertilized oocyte, which during development form highly specialized subpopulations. In most cases cells loose multipotency during the process of differentiation and are not able to switch into another cell type anymore. Certain cells, like neurones, almost completely stop proliferating while several other types undergo continuous divisions throughout the life of the

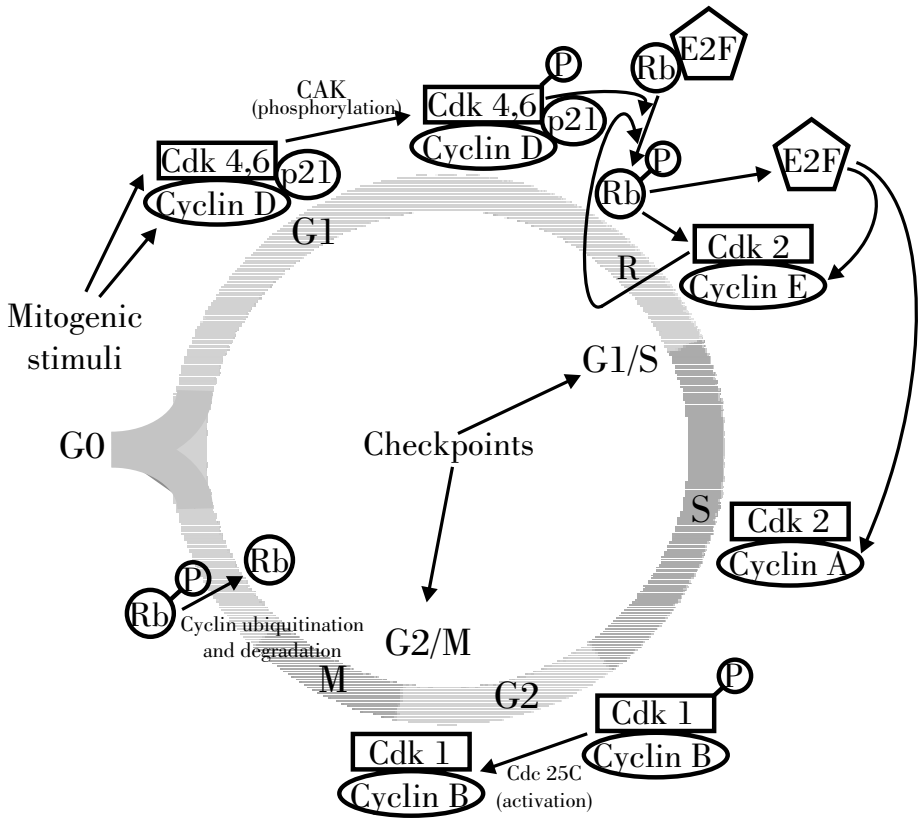

Figure 1. The cell cycle. A simplistic diagram depicting changes in the activity of cyclin-Cdk complexes and pRb during cell cycle progression. D-type cyclins are the key regulators of G1 progression. They are induced as a response to mitogenic stimulation and are rapidly degraded when mitogens are withdrawn. The cyclins assemble with the catalytic partners, Cdk 4 and Cdk 6. Cdk Inhibitors (CKI) p21Cip1 and p27Kip1 act as assembly factors. The cyclin D-Cdk-CKI complex is active and serves to sequester CKIs that would otherwise inhibit other cyclin-Cdk complexes. Assembled complexes enter the cell nucleus and are phosphorylated by a Cdk-activating kinase (CAK). The most important function of active cyclin D-Cdk is to phosphorylate Rb. Rb phosphorylation releases transcription factor E2F, which leads to transcription of several genes necessary for DNA synthesis, including cyclins E and A. Cyclin E-Cdk 2 becomes active and completes the phosphorylation of Rb on several additional sites. The cell is then triggered to enter the S-phase and start DNA synthesis. Cyclin A- and B-dependent Cdks maintain Rb phosphorylation until the end of mitosis. Cyclin A-Cdk 2 is essential for progression through S-phase and again for the G2/M transition. Cyclin B-Cdk 1 complexes accumulate in an inactive state during S and G2 phases. At the end of G2, Cdc25C dephosphorylates Cdk 1, which triggers entry to mitosis. Cyclins A and B are degraded during mitosis, Rb becomes dephosphorylated, and another cycle can begin. Checkpoints indicate critical steps of the cell cycle where disturbances (ex.g. DNA damage) can trigger arrest. Based on Hunter and Pines, 1994; King et al., 1994; Sherr, 1994; Sherr and Roberts, 1999.

organism. Cells like endothelial cells of the dermal capillaries proliferate only slowly but increase their proliferation rate for example during the process of wound healing.

The sequence of events during division of mammalian cells is described as the cell cycle (**Fig. 1**). Resting cells outside the cycle are in G0-phase. When properly stimulated they enter the cell cycle that consists of four phases. In G1-phase the cell is under the influence of extracellular signals that control its progression to the next phase. When the cell proceeds through the restriction point (R), it is committed to go through the rest of the cell cycle. The cell also prepares itself for DNA synthesis by synthesizing the necessary components. In S-phase the cellular DNA is doubled by copying the whole genome. In G2-phase the cell prepares for mitosis, the M-phase. After mitosis it returns to G1 and another decision has to be made whether to halt or to continue. The duration of a complete cycle is different in each cell type (Pardee, 1989; Sherr, 1996; Hamel and Hanley-Hyde, 1997).

The cell cycle machinery is regulated by sequential activation of cyclin-cyclin dependent kinase (Cdk) complexes. Formation of the complexes and regulation of their activity is highly specific. External and internal cues regulate the activation and inactivation of specific cyclin-Cdk complexes (reviewed in Pines, 1995). These, in turn, have essential roles in the transition of the cells from one cell cycle state to the next one. For instance, the regulation of D-type cyclins is important in transition through the restriction point, and their activity depends on the presence of certain growth factors (reviewed in Sherr, 1994). The signal routes leading from the extracellular growth factors to the cell cycle proteins are very complex and only partially known.

Fibroblast growth factors (FGFs) form a large group of at least 17 structurally related and predominantly growth stimulatory factors (see Szebenyi and Fallon, 1999 for review). The prototype member of the group, FGF-2, is found ubiquitously in mammalian tissues from where it can be quickly mobilized when necessary. FGF signaling is mediated by specific high affinity cell surface receptors coded by a family of at least 4 distinct *fgfr* genes (Coutts and Gallagher, 1995). Alternatively spliced transcripts of *fgfr* genes extend the complexity of the FGFR protein family. Each variant has a distinctive specificity profile (**Table 2**; Ornitz et al., 1996, Plotnikov et al., 2000). The binding to FGFRs is also affected by cell surface HSPGs (Vlodavsky et al., 1996). HSPGs bind to the self-associated oligomers of the FGFR ectodomain to form a complex that will bind FGF (Kan et al., 1996, Wang et al., 1997). Cell type specific HSPGs modulate the ligand specificity of the FGFR complex, and modify the ability of the four FGFRs to bind the large repertoire of FGFs (Kan et al., 1999). FGF binding leads to receptor transphosphorylation

Table 2
Specificity of FGFR-FGF interactions

FGFR variant	FGF-1	FGF-2	FGF-3	FGF-4	FGF-5	FGF-6	FGF-7	FGF-8	FGF-9
FGFR1									
B variant	+++	++	+	+	-	-	-	-	-
C variant	+++	+++	-	+++	++	++	-	-	+
FGFR2									
B variant	+++	-	++	+	-	-	+++	-	-
C variant	+++	++	-	+++	+	++	-	+	+++
FGFR3									
B variant	+++	-	-	-	-	-	-	-	++
C variant	+++	+++	-	+++	+	-	-	++	+++
FGFR4									
C variant	+++	+++	-	+++	-	+++	-	+++	+++

Note. Mitogenic activities of FGF paralogs were measured in BaF3 cells expressing various FGFR variants. FGF-1 showed a maximal response in these assays and the activities of other FGFs were expressed as a percentage of FGF-1 activity: -, <10%; +, 10-40%; ++, 40-75%; +++, 75-100%. Based on Ornitz et al., 1996; Szebenyi and Fallon, 1999.

in the intracellular tyrosine kinase domain (Neilson and Friesel, 1996). The phosphorylation steps are essential for mediating many FGF functions (Itoh et al., 1996; McFarlane et al., 1996; Saffell et al., 1997). However, it has been convincingly shown that FGFRs also mediate internalization of the FGF ligand. Several FGFs contain a nuclear localization signal sequence and for example FGF-2 has been reported to translocate to the nucleus in G1 (Kilkenny and Hill, 1996). Thus, FGFs may have functions that involve signalling pathways distinct from those activated at the cell surface.

Several signaling pathways lead down from FGFRs (**Fig. 2**). It seems that differences in downstream signaling exist between the different receptor molecules (Kanai et al., 1997; Reichman-Fried and Shilo, 1995; Vainikka et al., 1996). The signaling pathway that is thought to mediate the proliferation effects of FGFs is described here in short. When activated, FGFR1 phosphorylates FRS2 (FGFR substrate) docking molecules (Kouhara et al., 1997). The phosphorylated FRS2 binds directly to the Grb2-Sos complex that mediates interactions with Ras. Membrane associated Ras recruits Raf-1 that functions as a mitogen activated protein kinase kinase kinase (MAPKKK). This leads to sequential activation of MAPKK (MEK), and MAPK (Ferrell, 1996). The Ras-MAPK pathway regulates cyclin D expression and links FGF stimulation to the cell cycle machinery (Sherr and Roberts, 1999; Aktas et al., 1997). *In vivo,* the set of activation steps is very complex and several parallel MAPK pathways probably exist. FGFRs also activate other pathways leading to stimulation of PLC-γ, PKC, IP3, and Ca2+ release (see Klint and Claesson-Welsh, 1999 for review).

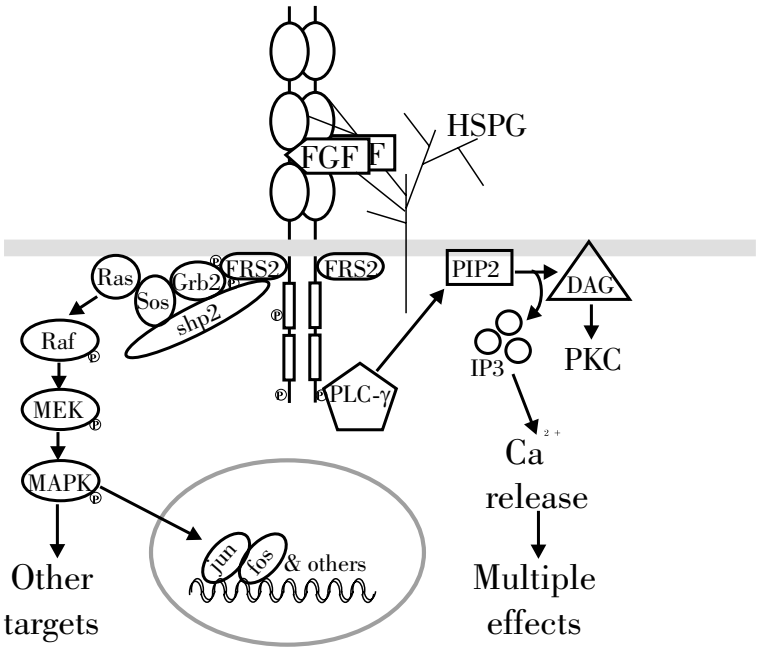

Figure 2. FGF receptor signaling. FGF binds to its receptor (FGFR) facilitated by heparan sulfate proteoglycan (HSPG) interactions. Several signaling pathways lead down from the activated FGFR. Some of the interactions involved in mitogen activated protein kinase (MAPK) and phospholipase C-γ (PLC-γ) pathways are shown. Ligand binding leads to transphosphorylation and activation of FGFR. The adaptor protein FGF receptor substrate 2 (FRS2) becomes tyrosine phosphorylated at several sites. Both the Grb2-Sos complex and shp2 adaptor protein are needed for sustained activation of the MAPK pathway. FGFR activation also leads to binding and tyrosine phosphorylation of PLC-γ. Activated PLC-γ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 generation leads to release of Ca2+ from intracellular stores and DAG accumulation leads to activation of protein kinase C (PKC) family members. Based on Klint and Claesson-Welsh, 1999; Szebenyi and Fallon, 1999; Carpenter and Ji, 1999; Garrington and Johnson, 1999.

FGF regulates the expression of a multitude of genes. They include immediate early response genes, delayed early response genes, homeobox genes, patterning genes, growth factors and their receptors, proteases, protease activators and inhibitors, matrix proteins etc. (see Szebenyi and Fallon, 1999 for review). The regulated genes are often cell-type specific.

TGF–βs comprise a family of multifunctional polypeptides that regulate the growth of epithelial, endothelial, and hematopoietic cells (see Massagué, 1996; Massagué, 1998; Bonewald, 1999 for review). Three different subtypes, TGF–β1, TGF–β2, and TGF–β3 with overlapping biologic functions have been described in mammals. In epithelial and endothelial cells they cause growth arrest to the G1-phase of the cell cycle. As explained above, TGF–β activity is regulated by its association to LAP (latency associated peptide). The activation of secreted TGF-β is the critical step for expression of its biological effects. Active TGF–β can bind to specific cell surface receptor molecules (**Fig. 3**). It is thought first to associate with a cell surface binding factor betaglycan (López-Casillas et al., 1991; López-Casillas et al., 1993; López-Casillas et al., 1994). Betaglycan presents TGF–β to type II TGF–β receptor, a constitutively active serine/threonine protein kinase, and a complex is formed between these two. The complex is bound by type I receptor that becomes phosphorylated. The phosphorylated type I TGF–β receptor is activated and propagates the signal further (Wrana et

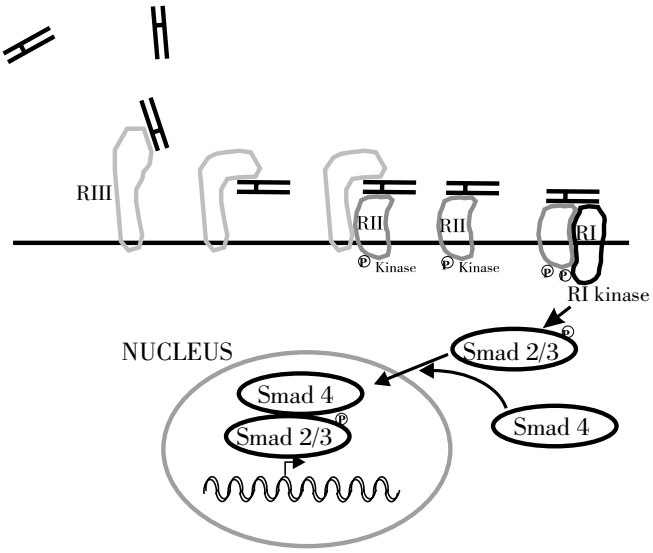

Figure 3. TGF-β receptor signaling. TGF-β binds to type II receptor (RII) either directly or facilitated by its association to betaglycan (RIII). The complex recruits type I receptor (RI) by means of the bound TGF-β. The constitutively active RII serine/threonine kinase phosphorylates RI, which leads to signal transduction. A downstream Smad pathway is thought to mediate at least part of TGF-β effects. Based on Lin and Lodish, 1993; Wrana et al., 1992; Wrana et al., 1994; Zhang and Derynck, 1999.

al., 1992). The intracellular downstream signalling is at least partly mediated by Smad proteins. The Smad pathway leads to the nucleus where the involved complexes regulate the expression of target genes (Heldin et al., 1997). Several mechanisms for TGF−β-mediated cell cycle arrest have been suggested. TGF−β can downregulate the expression of c-myc proto-oncogene and suppress the phosphorylation and thus the inactivation of the retinoblastoma (Rb) tumor suppressor protein (Laiho et al., 1990). Rb binds several transcription factors and represses their activity (Wang et al., 1994). In various studies TGF−β has also been shown to suppress the activity of cyclin-dependent kinases Cdc2 (Cdk1) (Howe et al., 1991), Cdk2 (Koff et al., 1993) and Cdk4 (Ewen et al., 1993). The function of these kinases is important for cell cycle progression. TGF−β can also induce the expression of cyclin dependent kinase inhibitors p15Ink4B (Hannon and Beach, 1994), p21Cip1 (Li et al., 1995), and p27Kip1 (Polyak et al., 1994).

Modes and regulation of cell death

Many cells and tissues of a multicellular organism are under constant wear and tear. In particular, the epithelial cells of skin and intestine are exposed to mechanical and chemical irritation and, in the case of skin, also radiation. As a consequence, the epithelial cells have to be replaced frequently. Dead or old cells are shed from the surface and division of basal cells produces new ones. Since epithelia are often exposed to genotoxic stimuli, this process is an important defence against accumulation of genetic mutations that could eventually lead to development of cancer. Elimination of cells is also necessary during development of an organism, when cells with definite functions during embryogenesis are not needed in the mature

organism. In some cases dead cells serve a specific function (e.g. cornified epithelium) (Ellis et al., 1991, Fesus et al., 1991).

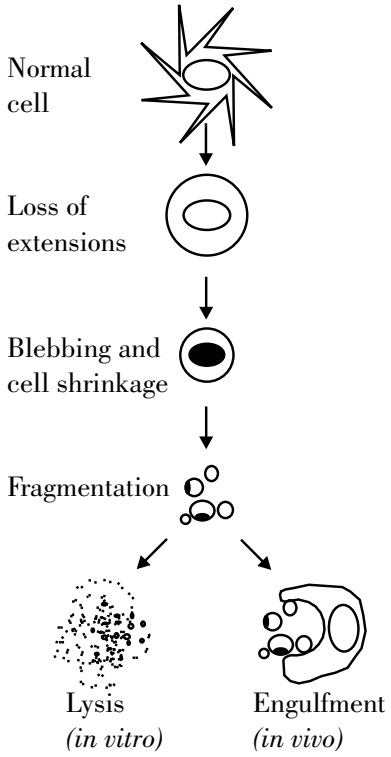

Figure 4. The morphological changes during apoptotic death. A characteristic pattern of morphological changes can be detected in most cases of apoptosis. They include 1) loss of extensions and cell rounding, 2) blebbing of the cell membrane and shrinkage of the nucleus and cytosol, 3) segmentation of the nucleus and cytosol, and 4) lysis that is very similar to necrosis. *In vivo*, the remnants of apoptotic cells are normally phagocytozed rapidly and lysis does not take place. Based on Kroemer et al., 1995; Willingham, 1999.

Cell death was recognized as a normal part of development and homeostasis about fifty years ago (Glücksmann, 1950). The pathologic form of cell death, necrosis, occurs in response to a variety of conditions, including hyperthermia, hypoxia, ischemia, complement attack, metabolic poisons, and direct cell trauma (Schwartzman and Cidlowski, 1993) usually leading to destruction of the plasma membrane or the biochemical supports of its integrity.

It gradually became evident that there is a distinct form of naturally occurring cell death that uses pathways and mechanisms quite different from necrosis. This type of cell death deletes cells that have become useless or harmful to the organism. It followed a certain defined pattern of events (a program) that seemed to be genetically coded in the cells and hence the concept of programmed cell death arose. Subsequently, the terminal phase of programmed cell death was termed apoptosis, an ancient Greek word for the falling off of leaves from trees (Kerr et al., 1972). Apoptotic cells show distinctive morphologic features (**Fig. 4**). The earliest changes include loss of cell junctions and plasma membrane extensions. The cytoplasm and nuclear chromatin condense. The DNA starts to degrade producing oligonucleosomal fragments. Later, the cell breaks into membrane-bound pouches called apoptotic bodies. The apoptotic bodies are phagocytozed by nearby cells that can be members of the mononuclear-phagocyte system or just normal neighboring cells (Arends et al., 1990; Walker et al., 1988). The membrane integrity of apoptotic bodies and their rapid phagocytosis prevent the generation of an inflammatory reaction. Lack of inflammation is one of the hallmarks of apoptosis *in vivo* since it facilitates cell elimination without injury to the surrounding tissue (Wyllie et al., 1980). It is also one of the greatest obstacles of accurately

quantitating apoptosis *in vivo*. In tissues apoptosis mostly appears asyncronously and as the cells are phagocytozed quickly afterwards only a few can be detected at each time although apoptosis can be quite abundant in the tissue (Willingham, 1999).

The mechanisms of apoptosis have been studied extensively. The nematode *Caenorhabditis elegans* has provided an excellent model system (Ellis et al., 1991). The genes controlling programmed cell death in *C. elegans* are known in detail. As research has moved over to more complex organisms like mammalians, it has become clear that the same gene families exist but, while they consist of a few genes in *C. elegans*, they are extremely complex in mammalians.

Apoptosis is the most common form of cell death. The apoptotic stimuli include a variety of both physiological death signals and pathological factors. The stimuli are cell type specific so that for example the same growth factor that is needed for survival of a given cell type induces death in another. The initial steps during the start of the death program are tightly regulated and complex but the final mechanism seems to be common.

Different death signals converge into two main pathways of apoptosis initiation. One pathway involves the so-called death receptors. Death receptors are a subfamily of transmembrane receptors with two to four cysteine rich extracellular domains and a conserved cytoplasmic sequence called death domain (DD). The prototype death receptor tumor necrosis factor receptor (TNFR) binds its ligand TNF−α (see Bazzoni and Beutler, 1996 for review). Almost all cells carry TNFR and are therefore potential targets for its effects. It seems, however, that triggering of apoptosis by TNF-α is in most cells suppressed by inhibitory proteins. Since certain oncogenes activated during tumorigenesis render cells sensitive to TNF−α or other death ligands like TRAIL (TNF related apoptosis inducing ligand), these factors could potentially be used to kill tumor cells selectively. Intensive research is going on to find ways to benefit from this remarkable feature. Upon ligation, the DD domain of death receptors binds a cytoplasmic adaptor protein called FADD (Fas associated death domain) by DD-DD interactions. FADD recruits caspase 8 by homotypic interactions through another conserved domain called DED (death effector domain). Caspase 8 is activated by autoproteolysis and initiates a cascade leading to activation of downstream effector caspases (cysteinyl aspartate specific proteases), specific cellular proteinases involved in execution of apoptosis (Chinnaiyan et al., 1996; Los et al., 1999; Wehrli et al., 2000; Griffith and Lynch, 1998).

Another apoptosis pathway involves the participation of mitochondria. Several mechanisms can lead to dysfunction and leakiness of mitochondria. A variety of apoptotic stimuli mediate their effects through Bcl-2 family of proteins (see Gross et al., 1999 for review). Bcl-2 family

includes both pro- as well as anti-apoptotic molecules. The expression of such molecules can be under regulation of a specific survival factor or apoptotic factor, respectively. Following a death signal, the pro-apoptotic members undergo changes that enable them to target and integrate into the mitochondrial outer membrane (Hsu et al., 1997; Goping et al., 1998). This leads, among other things, to release of mitochondrial cytochrome c to the cytoplasm (Desangher et al., 1999). Together with dATP cytochorome c activates the adaptor protein Apaf1 that, in turn, can bind and activate the zymogen form of caspase 9 (Li et al., 1997; Los et al., 1999). Mitochondria can be damaged by several other mechanisms besides the action of pro-apoptotic Bcl-2 family members. For example, radiation and changes in certain homeostatic enzyme activities can lead to accumulation of intracellular reactive oxygen species. Oxygen radicals as such damage several types of biological membranes and cause mitochondrial leakiness as well as dysfunction of other cellular organelles (see Clutton, 1997 for review).

Both of these pathways lead to activation of caspases. Although there are indications of caspase-independent apoptosis, they are considered by many to be the essential components of most, if not all apoptotic pathways. Caspases comprise a family of more than a dozen members that can promote apoptosis (Los et al., 1999). As a result of activation they cleave several cellular targets (see Wolf and Green, 1999 for review). The first caspase targets include pro- and anti-apoptotic proteins (Cohen, 1997; Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). This leads to signal amplification and inhibitor inactivation. Caspases also cleave structural proteins of the nucleus and cytoskeleton which probably promotes packaging of the cell to apoptotic bodies and engulfment by phagocytes (Cohen, 1997; Nicholson and Thornberry, 1997; Cryns and Yuan, 1998; Gohring et al., 1997). A third group of caspase substrates include homeostatic proteins like enzymes and factors necessary for protein and nucleic acid synthesis (Cohen, 1997; Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). Caspases seem to act on the final apoptotic pathway and inhibition of their activity by specific peptide inhibitors can in many cases effectively stop the progression of apoptosis and the cells survive. This further emphasizes their central role in the execution of apoptotic cell death.

Oxidative stress has been implicated in apoptosis, although there still is controversy regarding its importance. Normally, a fine balance exists between cellular oxidants and their defence mechanisms. During normal aerobic conditions many redox reactions produce highly reactive intermediates. These reactive oxygen species (ROS) can also have beneficial roles in the cell and are mostly neutralized by specialized cellular enzymes and antioxidants. If ROS are produced in excess or the neutralization mechanisms are hampered the cell suffers from oxidative stress. In extreme cases this leads to severe damage and necrosis (Duvall and Wyllie,

1986). Less severe oxidative overload causes lipid peroxidation, loss of calcium homeostasis and alterations of metabolic pathways (Slater, 1987). Increasing evidence shows that in certain situations these changes mediate apoptotic death of the cell. ROS have been suggested to lead to apoptosis in response to several stimuli including ionizing radiation, certain chemicals and chemotherapy agents, and in some cases serum or growth factor deprivation (see Anderson et al., 1999 for review).

Cancer-dysregulation of cell growth and survival

Mature, normal tissues are characterized by a homeostatic balance between cellular proliferation and cell death. Imbalance leads to development of disease. Malignant cells most often have disturbances in both of these processes leading to uncontrolled multiplication and growth. On the other hand, excessive cell death can result from acquired or genetic conditions that lead to accumulation of signals that induce apoptosis (see Thompson, 1995 for review). In AIDS, the HIV infection leads to depletion of CD4+ T cells by apoptosis (Ameisen and Capron, 1991; Groux et al., 1992; Gougeon and Montagnier, 1993). Several neurodegenerative disorders are characterized by the gradual loss of a subset of neurons by apoptosis (Isacson, 1993; Heintz, 1993). These include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular dystrophy and various forms of cerebellar degeneration.

The apoptotic pathways of cancer cells are not fully unfunctional and many treatments are targeted to inducing apoptosis in malignant cells. These include both radiation treatments and most of the chemotherapeutic drugs. The cumulation of genetic lesions can ultimately lead to development of cancer cells that are resistant to these therapies as well as motile and invasive enough to metastasize. The importance of apoptosis in defence against malignancies is reflected in the several aberrations of the apoptotic pathways found in cancer cell lines and tumors *in vivo* (see Jäättelä, 1999; Lyons and Clarke, 1997 for review).

The expression of *c-Myc* proto-oncogene is elevated in many human tumors (Spencer and Groudine, 1991). Over-expression studies have shown that c-Myc alone can drive cells into continuous proliferation even in the absence of normal mitogenic stimulation (Eilers et al., 1989; Evan et al., 1992). However, when c-Myc over-expressing cells are cultured in low serum, their numbers stabilize despite of frequent mitoses (Evan et al., 1992; Askew et al., 1991). The discrepancy is explained by a dramatic increase in apoptosis. Thus, it seems that a genetic lesion causing overproduction of c-Myc is not sufficient for cell transformation.

Together with other mutations causing changes in apoptotic pathways, it has been proven to cause direct tumorigenesis (Amundadottir et al., 1996; Santoni-Rugiu et al., 1996). Additionally, although apoptosis takes place and limits the rate of growth of c-Myc over-expressing tissues, increased cell turnover means that the probability of subsequent mutations arises and places selective pressure upon mutations that block apoptosis (Lyons and Clarke, 1997).

Bcl-2 was initially identified as highly overexpressed protein in follicular lymphoma (Tsujimoto et al., 1984). In the chromosomal translocation t(14;18) the *bcl-2* gene is translocated into the immunoglobulin heavy chain locus. It was soon found out that, unlike other oncogenes known at that time, it did not stimulate proliferation but increased cell survival by inhibiting apoptosis (Vaux et al., 1988). Recently, several transgenic Bcl-2 overexpressing mice strains have been engineered. These mice are prone to develop lymphomas but only after a long latency period (McDonnell et al., 1989; McDonnell and Korsmeyer, 1991). In analogy to c-Myc, Bcl-2 alone doesn't seem to be able to cause tumorigenesis. It leads to impaired apoptosis, though, and facilitates subsequent mutations that induce cellular proliferation. Rather unexpectedly, in at least human breast carcinoma (Leek et al., 1994; Joensuu et al., 1994; Silvestrini et al., 1994; Hurlimann et al., 1995) and colon carcinoma (Watson et al., 1996; Sinicrope et al., 1995; Ofner et al., 1995; Baretton et al., 1996) high Bcl-2 expression has been shown to correlate with improved disease-free and overall survival. The controversy is at least partly explained by the ratio between anti- and proapoptotic members of the Bcl-2 family. In breast and gastrointestinal cancers Bax expression or Bax/Bcl-2 ratio correlate with longer survival and better response to therapy (Krajewski et al., 1995; Ogura et al., 1999). Another explanation is that Bcl-2 is also an inhibitor of cell cycle progression (Huang et al., 1997). It probably rescues cancer cells carrying otherwise lethal DNA damage but it also slows down their cycling until accumulating mutations provide other ways to protect the cells against apoptosis. After this the selection pressure favors cells that have lost Bcl-2 expression.

Loss of function of tumor suppressor gene *p53* is frequently found in association with almost all human tumors (Hollstein et al., 1991; Beroud and Soussi, 1998). p53 regulates both cell cycle and apoptosis. DNA strand breaks following for example UV radiation induce p53 accumulation (Liu et al., 1994). p53 activates the transcription of several genes involved in DNA repair, growth arrest, and apoptosis. If the damage is too extensive to be repaired, the cell dies. If p53 activity is lost the cells carrying damaged DNA survive and the numbers of mutation bearing cells increase (Yuan et al., 1995). Genetically engineered p53 -/- mice develop lymphomas and sarcomas soon after birth. Also heterozygous p53 +/- mice are prone to develop

tumors but only after a longer latency (Purdie et al., 1994; Jacks et al., 1995). Also the +/- mice are very susceptible to tumors and double strand chromosomal breaks after exposure to ionizing radiation (Lee et al., 1994). p53 is important for protection against UV induced skin cancer (Ziegler et al., 1994). Sunburn keratinocytes enter apoptosis p53 dependently. Cells with unfunctional p53 acquire lesions in their genome and are predisposed to malignancy. In melanoma cells, other mechanisms besides p53 pathway have been shown to control UV responses (Haapajärvi et al., 1999).

Another response to cellular stress is mediated by a group of specialized stress proteins called *heat shock proteins* (Hsps). Hsps were first identified on the basis of their synthesis after exposure of cells to elevated temperatures (Landry et al., 1982). Induced endogenous expression or overexpression by gene transfer confers resistance to heat stress (Li et al., 1991; Landry et al., 1989). Hsps function as chaperones and probably mediate their effects by inhibiting heat induced denaturation and aggregation of cellular proteins (Nollen et al., 1999). Hsp70 and Hsp27 have been shown to protect cells from several other apoptotic stimuli besides heat (Jäättelä et al., 1992; Simon et al., 1995; Bellman et al., 1996; Mosser et al., 1997; Jäättelä et al., 1998). The exact mechanisms are not known. Hsp70 has been shown to be able to inhibit apoptosis even after the activation of effector caspases, a step that has previously been considered “the point of no return” (Jäättelä et al., 1998). Hsp27 seems to protect cells from ROS both by increasing glutathione levels and by neutralizing their effects by its chaperone activity (Préville et al., 1999). Several human tumors have been reported to express Hsp70 and some also Hsp27 (see Jäättelä, 1999 for review). With some exceptions, they are typically associated with poor prognosis.

Recently, a group of *Inhibitor of Apoptosis Proteins* (IAP) has been found originally from baculovirus infected cells (see LaCasse et al., 1998 for review; Crook et al., 1993; Birnbaum et al., 1994). Most of the IAPs seems to mediate their effects by inhibiting the functions of activated effector caspases (Roy et al., 1997; Deveraux et al., 1998). The cancer-associated member of the family, survivin, is expressed in normal tissues only fetally and in almost all cancer cell lines and primary carcinomas studied so far (Ambrosini et al., 1997). Its prognostic value remains to be investigated but there are indications that it favors the cycling of cells with an aberrant genome (Li et al., 1998).

Embryonal carcinoma - a model for cancer and for cell differentiation

The origin of embryonal carcinoma cells

Teratoma is a germ cell tumor that often consists of an array of more or less organized tissues. The tumor mass contains hair, skin, myocardium, or endocrine tissues, sometimes even a completely formed organ like the eye (Sergi et al., 1999). The malignant form of teratoma is called teratocarcinoma. Teratocarcinomas include nests of undifferentiated stem cells, embryonal carcinoma (EC) cells, that have at least partly retained the ability to differentiate into various tissues. EC cells have been shown in several aspects to be similar to the embryonal stem (ES) cells of the inner cell mass and primitive ectoderm (Solter and Damjanov, 1979; Martin, 1980). It could be shown in a mouse model that when pluripotent EC cells were placed into a normal blastocyst, it developed into a chimeric mouse with normal and EC cell derived tissues (Brinster, 1974; Papaioannou et al., 1975). It has also been shown that murine primordial germ cells transform spontaneously into EC-like cells when cultured *in vitro* under suitable conditions (Matsui et al., 1992). Most EC cell lines probably represent a degenerate form of ES cells and have limited capability to differentiate.

The germ cell tumors are caused by an unknown mutation in the primordial germ cells during early development. The abnormal cells can remain dormant for long periods of time until they start proliferating, without entering meiosis like normal germ cells. They form a teratoma or a carcinoma *in situ* that can develop into a seminoma or transform into EC cells.

Tera-2 cell line

The most widely used pluripotent human EC cell line is Tera-2 and its clonal derivatives. Tera-2 was originally derived from a lung metastasis of a testicular teratocarcinoma (Fogh and Tremple, 1975). Later, another subline called NTera-2 was derived by passaging Tera-2 through athymic mouse (Andrews et al., 1984) in which it causes tumors.

Retinoic acid treatment induces the differentiation of Tera-2 cells into neurons and other cell types yet to be specified. Simultaneously, the cells lose their tumorigenic potential (Dmitrovsky et al., 1990). The commitment to differentiate takes place within a few days of exposure to retinoic acid (Andrews, 1998). At least NTera-2 can be induced to differentiate in distinct, non-neural directions by hexamethylene bisacetamide (Andrews et al., 1990) and by bone morphogenetic protein-7 (Andrews et al., 1994). The neurons differentiated from Tera-2 cells

are functional cells that, when implanted to the brain of immunodeficient mice, differentiate and adjust to the site of implantation (Kleppner et al., 1995; Miyazono et al., 1995; Miyazono et al., 1996). In a recent study, NTera-2 derived neurons were implanted into the brain of rats with unilateral striatal lesions, a model of Huntington's disease. Compared to control animals, the rats showed improvement in several aspects of their deficits suggesting that the transplanted neurons could augment the function of damaged brain tissue (Hurlbert et al., 1999).

The mechanisms that regulate Tera-2 differentiation are poorly known. It has been shown that different combinations of retinoic acid receptors and retinoid X receptors direct differentiation into mesenchymal vs. neural cells (Moasser et al., 1994; Moasser et al., 1995; Spinella et al., 1998). The expression of several growth factors and their receptors is altered during differentiation. For example, the expression of epidermal growth factor receptor and its ligands transforming growth factor α (TGF-α) and CRIPTO are downregulated during differentiation (Dmitrovsky et al., 1990; Miller et al., 1990). Forced expression of TGF-α in differentiated cells restores their tumorigenic potential without affecting the expression of differentiation markers (Baselga et al., 1993). This proposes a function for an EGFR dependent autocrine loop in facilitating Tera-2 tumorigenicity. The presence of several other autocrine loops in undifferentiated cells is probable.

Melanocyte-nevus-melanoma

Origin and functions of pigment cells of the skin

Skin is the largest organ of the body. Among its many functions is to act as an active as well as passive protective barrier against harmful factors of the environment. The basic structure of the skin consists of two separate layers, the dermis and the epidermis. The dermis is composed of connective tissue cells and nourishing blood vessels and capillaries along with several types of other adnexal structures. The epidermis, separated from the dermis by a BM, is structurally highly organized. Keratinocytes are the principal cell type of the epidermis and form the cornified epithelium of the skin. Basal keratinocytes are proliferating cells that continuously produce new cells to replace those shed from the outer surface. During differentiation the keratinocytes lose their nuclei and ability to proliferate. When moved towards the surface of the skin they transform into scale-like keratinous remnants that provide both chemical and physical resistance. The thickness of the epidermis differs greatly in various parts of the body as a function of the mechanical load it is exposed to.

Melanocytes are located within the basal layer of the epidermis. Unlike keratinocytes, they are neural crest-derived cells that migrate to the epidermis as melanoblasts from the neural tube during fetal development (see Goding and Fisher, 1997 for review). The main function of melanocytes is to produce melanin pigment. One melanocyte transfers melanin particles, melanosomes, to several surrounding keratinocytes through its dendrite-like extensions to form a so-called epidermal melanin unit. The color of melanin dictates the color of the skin, while there are no marked racial differences in the total amount of melanocytes. Melanin has an important photoprotective function. It absorbs UV light and radicals generated by interaction of UV photons with membrane lipids and other cellular chromophores (Pathak, 1995; Riley, 1997). In keratinocytes, melanin is distributed in protective caps around and on top of their nuclei (Kobayashi et al., 1998). The significance of melanin can be clearly seen in amelanotic conditions, such as vitiligo, where the demelanized areas of skin are extremely sensitive to UV-induced sunburn (Gniadecka et al., 1996). Melanocytes respond to UV exposure by enhanced melanin synthesis and transport. Tanning after sun exposure is the result of increased amount of melanin in keratinocytes, not increased amount of melanocytes (Jimbow et al., 1993; Gilchrest et al., 1998). Melanocytes are remarkably quiescent and dividing cells are rarely seen in normal skin.

The localization of melanocytes in the skin is tightly regulated. In cell culture, melanocytes need either keratinocyte "feeder cells" or substancial growth factor stimulation to survive and proliferate. Basal keratinocytes seem to support melanocytes also *in vivo* by producing several paracrine growth factors. FGF-2 has been shown to be among these factors (Halaban et al., 1987). Disturbance of the strict dependence of melanocytes on basal keratinocytes is a general feature of melanocyte neoplasms.

Development and significance of nevi

Moles (benign nevus, common nevus, pigment nevus) are small (mostly less than 6 mm in diameter), local areas of melanocyte proliferation. Moles owe their darkish color to melanin pigment that is often found in large quantities inside nevus cells. Besides the proliferative activity, nevus cells differ from normal melanocytes by their localization. While normal melanocytes are only found intermingled in the basal layer of keratinocytes, nevus cells are located to the vicinity of the BM (junctional nevus), deeper in the dermis (dermal nevus) or both (compound nevus). Nevus cells seem to be less dependent on the supportive growth factors secreted by keratinocytes.

There is no known function for common nevi. Not very much is known about the causative factors leading to their development. A twin study revealed a strong correlation in the number of nevi among monozygotic twins but not among dizygotic twins suggesting a role for inheritance (Easton et al., 1991). Exposure to sun (ultraviolet radiation) in childhood has also been shown to correlate with subsequent development of nevi in the exposed areas (Pavlotsky et al., 1997; Kelly et al., 1994; Luther et al., 1996). It has been suggested that this is the consequence of UV-induced subtle mutations. Other, possibly immune mediated mechanisms are suggested by a study showing inverse association of nevi with sun exposure in immunosuppressed patients (Bouwes Bavinck et al., 1996).

While nevus, as such, is a benign lesion, it seems to have a role in the development of more malignant melanocyte neoplasms. Interestingly, the relatives of melanoma patients are at elevated risk of developing dysplastic features in their nevi. The finding of dysplasia in a patients nevus is an important risk factor for melanoma development. Dysplasia has also been partly attributed to the same genetic markers as melanoma (see Greene, 1997 for review). The risk of melanoma rises with the number of moles. In a large study the risk of melanoma in subjects with more than 50 common nevi was 4.2 times higher compared to subjects with 0-4

nevi while in subjects with more than 4 dysplastic nevi it was 23.7 times higher (Bataille et al., 1996).

Malignant melanoma - clinical stages vs. molecular changes

Melanoma is one of the most common cancers in young adults. The frequency of melanoma has increased by a factor of about 15 during the past 60 years (Glass and Hoover, 1989; Koh et al., 1995). According to the Finnish Cancer Registry, the age-adjusted incidence of melanoma in the Finnish male population has risen from 2.8 cases per 100.000 person-years in 1965-1969 to 7.8 cases in 1995-1997. In 1997 melanoma of the skin was the 12th most common primary cancer diagnosed in the male and 8th most common in the female population (Finnish Cancer Registry, 2000). Although melanoma mortality seems, at least in some populations, to have reached a plateau (Iscovich et al., 1995; Giles et al., 1996), possibly because of enhanced early detection, the incidence still seems to be growing (Armstrong and Kricker, 1994).

Both environmental and genetic factors predispose to the development of melanoma. UV radiation is clearly the most important environmental factor. The incidence of melanoma is highest in countries with subtropical or tropical climate and light-skinned population. In the USA, the incidence among Afro-Americans is only 1/10 of that among caucasians (Parkin et al., 1992). Melanomas have been experimentally induced by UV in certain animals and in human skin grafted onto immunologically tolerant mice (see Satyamoorthy et al., 1999 for review). Epidemiological studies have revealed that daily (for example occupational) substantial sun exposure is less harmful than intense intermittent exposure (Bentham and Aase, 1996; Holman et al., 1986; Nelemans et al., 1993). The risk is especially high in subjects with exposures that induce sunburn. A history of five or more severe sunburns during adolescence causes doubling of melanoma risk (Weinstock, 1996). In contrast to melanoma, other forms of skin cancer, including basal and squamous cell carcinoma, are associated with cumulative sun exposure. This discrepancy could be explained by the differential effects of UV on the apoptosis of melanocytes and keratinocytes *in vivo*. After strong sun exposure, a large number of "dyskeratotic" or apoptotic keratinocytes can be detected in the skin (Young, 1987). In contrast, melanocytes seem to resist UV-induced apoptosis. This should be considered as rational since melanocytes are needed to prepare the skin for subsequent exposures. Still, in pure cell cultures *in vitro*, melanocytes undergo apoptosis after physiologic UV exposures (Zhai et al., 1996). The greater *in vivo* resistance of melanocytes to apoptosis is partly explained by the fact that they are far less likely to be actively cycling during UV-induced damage, and cells are most vulnerable

to radiation effects during S phase of the cell cycle (DNA synthesis) (Danno and Horio, 1987). Also, in response to UV radiation, keratinocytes have been shown to secrete growth factors, such as NGF, that act as survival factors for melanocytes (Zhai et al., 1996).

Resistance to apoptosis after sunburn facilitates pigmentation of the skin and better protection against further exposures. On the other hand, it can be speculated that melanocytes are susceptible to accumulation of genetic lesions that are incompletely repaired by cellular DNA repair mechanisms and should lead to deletion of the damaged cell.

Melanoma has long been known to occur familially (Cawley, 1952). The proportion of familial cases is estimated to be 5-10%. Familial melanoma follows an autosomal dominant inheritance pattern with penetrance of less than 80%. The tools for studying genes predisposing to a certain disease have been developed during the past 20 years. The first successful linkage analyses with melanoma-prone families showed moderately strong evidence for linkage between melanoma and the short arm of chromosome 1 (Greene et al., 1983). Further analysis led to more accurate localization to 1p36 (Bale et al., 1989; Goldstein et al., 1993). This finding could not be duplicated by later studies, however, and a candidate gene in this region has not been identified. More recently, a second site on chromosome 9p was identified on the basis of frequent abnormalities in that area in melanoma cell lines. Multiple linkage analysis of subjects in 11 melanoma families further narrowed the locus to 9p21 (Cannon-Albright et al., 1992; Cannon-Albright et al., 1994). Later, a candidate gene was identified from that area coding for a Cdk inhibitor protein p16INK4a (Kamb et al., 1994) and germline mutations of this gene were found in 33 of 36 patients with melanoma from 9 different families in the same study material (Hussussian et al., 1994). Others have confirmed the finding of p16INK4a germline mutations in about half of the families studied. Although a mutation in a CdkI is compelling as a predisposing factor for melanoma progression, it is clear that p16INK4a mutations alone cannot explain familial melanoma and further work is needed to identify other hereditary factors. Molecular abnormalities are frequently found in sporadic melanoma, as well. The most frequent currently known gene alterations are listed in **Table 3**.

Table 3
Gene abnormalities in sporadic melanoma

Gene	Mechanisms	Percent
p16	Absent or mutated gene	5-20
N-ras	Overexpression or mutation	5-15
p53	Mutated or absent gene	2-7
β-catenin	Overexpression or mutation	15
PTEN	Mutated	25
myc	Overexpression	25
Other		<1
PKC-α	Mutated	
c-myb	Mutated	
CDK-4	Mutated	
EWS-AFT-1	Translocation	
NF-1	Mutated	

Based on Meier et al., 1998.

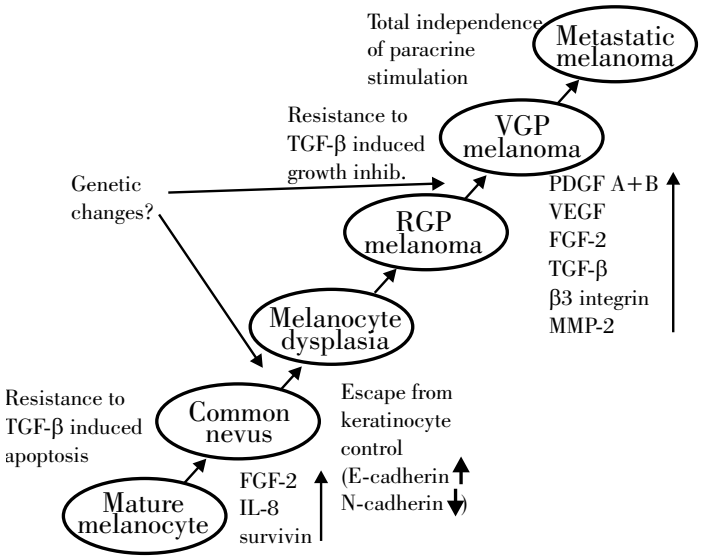

Figure 5. Melanoma development. Melanoma development can be divided into steps. According to the present view, most but not all melanomas are associated to benign nevi. The expression of certain autocrine growth factors (e.g. FGF-2) is upregulated in nevus cells, possibly causing the resistance to TGF-β-induced apoptosis in collagen gel and dermis. Nevus cells have recently been shown also to express a member of the family of inhibitor of apoptosis proteins (IAP) called survivin (Grossman et al., 1999). The cadherin profile of nevus cells is different from normal melanocytes, which is thought to enable growth independently of keratinocyte support. Dysplasia, the first sign of malignant progression in nevus cells, is thought to be the result of as yet undefined genetic changes. Radial growth phase (RGP, *in situ*) melanoma cells are poorly tumorigenic and not capable of metastasis. The differences between dysplastic and RGP melanoma cells are often minor. On the contrary, progression to vertical growth phase (VGP) seems to be associated with further genetic changes and upregulation of several growth factors. The forced expression of β3 integrin alone permits conversion from RGP to VGP melanoma *in vitro* (Hsu et al., 1998). Invasive melanoma cells also express several matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) (Hoffmann et al., 2000; Airola et al., 1999). Aggressively growing, metastatic melanoma cells produce a multitude of growth factor receptors and ligands and are evidently independent of support from neighboring cells. Adapted from Meier et al., 1998.

Clinically and histopathologically melanoma development and progression can be divided into five steps (**Fig. 5**; Meier et al.,1998). Histological evidence for nevic origin of melanoma can be found in about 35% of cases. It is possible that some melanomas arise directly from melanocytes but this is difficult to show conclusively. The first step, from melanocytes to nevus, has been discussed above. The second step, development of dysplasia in nevi is the first one with some evidence of (so far poorly specified) genetic changes (Greene, 1997). The relative independence of nevus cells from keratinocyte control has been recently explained by a gradual shift in cadherin expression (Hsu et al., 1996). E-cadherin has been proposed to mediate the phenotypic control of keratinocytes over melanocytes. Keratinocytes and normal melanocytes express almost exclusively E-cadherin, while it's expression in nevus cells is downregulated and N-cadherin upregulated. Melanoma cells express almost exclusively N-cadherin, which is thought to facilitate interactions of melanoma cells with N-cadherin-expressing fibroblasts and endothelial cells.

The shift from dysplastic nevus to *in situ* radially spreading melanoma is gradual (Meier et al., 1998). Although the former is considered to be premalignant and the latter malignant, there is little difference in the cells' behavior for example in cell culture. In contrast, the shift from

radial growth phase to vertically growing melanoma is critical and probably preceded by further genetic changes since the growth characteristics of these cells are quite different (Satyamoorthy et al., 1997). Cells from vertically growing melanoma are almost independent of exogenous growth factor stimulation, they can grow anchorage-independently in soft agar and cause tumors in nude mice. Rather surprisingly, vertical growth, invasiveness and tumorigenicity could be experimentally induced in radially growing melanoma cells by forced expression of β3 integrin (Hsu et al., 1998). The genetic instability of melanoma cells favors selection of poorly differentiated, highly invasive and metastatic phenotypes with very modest growth requirements. Metastatic melanoma cells express several growth factors and cytokines with both autocrine and paracrine stimulatory functions (Meier et al., 1998).

AIMS OF THE PRESENT STUDY

Research in cell biology is for the most part based on studies utilizing cultured cells. Although the actual environment of cells *in vivo* is a three-dimensional network, they are still cultured and observed in two-dimensional cultures, where they are surrounded by a thin layer of matrix produced by the cells themselves. The current work was undertaken to study the behavior of cells in a controllable, three-dimensional matrix. The goals were:

1) To develop a controllable, three-dimensional cell culture system where the cells can be easily observed in the presence of even or graded concentrations of various growth factors and chemicals.

2) To use this system to study the effects of growth factors on migration and phenotype of cells undergoing differentiation.

3) To explore differences in the behavior of normal melanocytes, nevus cells, and melanoma cells grown in a three-dimensional environment, which bears some resemblance with the dermal compartment of the skin.

MATERIALS AND METHODS

Cell lines

Cell line	Description	Source	Used in
Tera-2 (clone 13)	human embryonic carcinoma	Dr. C.F. Graham	**I, II**
BCE	bovine capillary endothelial	bovine adrenal cortex	**I**
U-251 MG	human glioma	ATCC	**I**
Melanocytes (Mela)	human epidermal melanocytes	human skin	**III, IV, V**
Nevus cells	human dermal nevus cells	human dermal or junctional pigment cell nevus	**III, IV**
ML793 (WML793)	human VGP melanoma	Wistar Institute	**IV**
G-361	human metastatic melanoma	ATCC	**IV**
Mv1Lu	mink lung epithelial cells	ATCC	**IV**

Cell culture (**I-V**)

Tera-2 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Differentiation was induced by plating the cells on gelatin-coated tissue culture plates (2 x 10^3 cells/cm2) and adding 20 μM retinoic acid (RA) to the medium the following day. The cells were typically used after at least 10-d differentiation.

BCE cells were isolated from adrenal cortex according to published procedures (Folkman et al., 1979). Initial isolates were grown in MEM alpha medium (Gibco/BRL) containing 10% newborn calf serum (NCS) and endothelial cell growth supplement (Sigma). After cloning, the cells were grown in the absence of growth supplement. The clones stained positively for factor VIII-related antigen (α-FVIII-Ag, Dako).

U-251 MG cells were maintained in MEM + 10% NCS. In co-culture experiments the cells were γ-irradiated (6000 rad) to prevent cell proliferation and allowed to recover for 1 d before use.

Human skin melanocyte cultures were established from normal adult human skin. Most of the dermis was removed by a scalpel in visual control. The remaining layers were treated with dispase (Collaborative Biomedical Products) for 20 min to enzymatically separate the epidermis. The epidermis was then treated with trypsin-EDTA for 15-30 min. Detached cells were cultivated in a selective F-12 medium (Gibco/BRL) containing 10% FCS, 1 μg/ml cholera toxin (CT, Sigma), 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), and 3 ng/ml human recombinant FGF-2 (a gift from Dr. Andreas Sommer). In 50% confluence the plates were treated with Geneticin (100 ug/ml, Sigma) for 7-10 d to kill remaining contaminant cells.

Nevus cell cultures were established from excised intradermal or compound-type nevi. The epidermis was mechanically removed to the depth of the dermal papillae. The superficial dermis was mechanically minced and treated with collagenase (type IX, Sigma) to produce a cell suspension. The cells were plated and treated as described above for melanocytes.

Both melanocyte and nevus cell cultures stained 100% positive for S-100 (antibodies from Dako) and GD3 ganglioside (antibodies from Biogenesis). The cells were used between

passages 4-15. Before each experiment the cells were kept in F-12 + 10% FCS without supplements for 24-48 h.

ML793 and G-361 melanoma cell lines were cultured in RPMI-1640 medium containing 10% FBS.

Mv1Lu cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Construction of three-dimensional collagen lattices (**I-V**)

The collagen gels were prepared by mixing type I collagen stock solution (3-5 mg/ml) with an equal volume of 2X medium. The gel was allowed to polymerize for 30 min at 37°C (5% CO_2 atmosphere). Cells were seeded on top of this layer. They attached tightly in 30 minutes. A second layer of collagen was cast and solidified. Complete culture medium was added on top of the collagen sandwich to provide the cells with sufficient nutrients during prolonged experiments.

When desired, growth factors, cytokines and antibodies were either included in collagen layers or added to the medium on top from which most substances readily spread to the collagen layers by passive diffusion.

For migration assays (**I**), the cells were allowed to attach inside a plastic ring inserted on top of the first collagen layer. After 30 min the ring was removed and unattached cells were rinsed away. A second layer of collagen was cast as usual. The culture medium layer was solidified by 0.75% low-melting-point agarose (FMC Bioproducts). To establish a growth factor gradient, a well (diameter 3 mm) was punched through all the layers on both sides of the cell spot (distance 4 mm), and the growth factor was introduced into one of the wells. The second well served as a vehicle control. Photomicrographs of the migrating cells were taken through an Olympus CK-2 inverted microscope.

Preparation of ECM isolates (**I**)

BCE and glioma cells were maintained in the presence of 5% Dextran T40 as confluent cultures for 5-7 d. The matrices were prepared by extracting cells with 0.5% Triton X-100 and 20 mM ammonium hydroxide in PBS (Ishai-Michaeli et al., 1990). The resulting matrix was rinsed extensively with the extraction buffer and PBS.

HSPGs were purified from the matrix preparations by dissolving the matrix in 4 M guanidine in 50 mM Na-acetate, pH 6.0. The buffer was changed to 8 M urea in 50 mM Na-acetate, pH 6.0, using a G-10 Sepharose salt exchange column (Pharmacia) and the proteoglycans were bound to DEAE-dextran (DE-52, Whatman Biosystems). The HSPGs were eluted between 0.25 and 0.5 M NaCl in 0.1 M PO_4, pH 7.4 (Yanagishita et al., 1987; Saksela et al., 1988).

Northern blotting (**I-III**)

Poly A+ mRNA was isolated according to published procedures (Glisin et al., 1974; Schwab et al., 1983). Equal amounts, determined by OD_{260}, were electrophoresed on 0.8% agarose gels and blotted onto nylon membranes (Pall).

The cDNA fragments used as probes were labeled with [α-32P]-dCTP (Amersham) using a random priming kit (Pharmacia Biotech). The probes included: FGF-2 (**I-III**) (Sommer et al., 1987), FGF-4 (**I-II**) (Delli-Bovi et al., 1987), Rb (**II**) (provided by Dr. Yuen Kai Fung), FGFR-1 and FGFR-2 (**III**) (Dionne et al., 1990), and FGFR-3 and FGFR-4 (**III**) (Partanen et al., 1991).

Signal quantitation was accomplished by densitometric scanning of exposed autoradiography film (**I-II**) or by a bio-imaging analyzer (Fuji BAS-1500; **III**).

Immunohistochemistry (**I**)

The cells and collagen lattices were fixed in 3.5% PFA at 4°C for 1 h. After washing with PBS the specimens were permeabilized with 0.5% Triton X-100, rewashed, and exposed to primary antibodies followed by FITC-conjugated secondary antibodies for 4-6 h each. The stained cells were photographed with an Olympus BH-2 fluorescence microscope.

Western blotting (**II, III, IV**)

FGF-2 sample preparation

Cells were lyzed with 1% Triton X-100/2 M NaCl in PBS in the presence of proteinase inhibitors (aprotinin, AEBSF, Pepstatin A). Equal amounts of protein were diluted 1:10 with distilled, deionized water and incubated on a mixer for several h with Heparin-Sepharose particles (Pharmacia Biotech). The heparin-bound FGF-2 was released by either eluting with 2 M NaCl followed by TCA precipitation (**II**) or by resuspending the particles in reducing Laemmli sample buffer and boiling (**III**).

TGF-β sample preparation

Cells were lyzed with 0.5% NP-40 in 120 mM NaCl, 25 mM Tris, pH 8, in the presence of proteinase inhibitors (aprotinin, AEBSF, Pepstatin A). Equal amounts of protein were mixed with 2X reducing Laemmli sample buffer and boiled.

Conditioned media were collected and concentrated 10X with Centricon 10 (Amicon). A 50-µl aliquot was mixed with 2X reducing Laemmli sample buffer.

Electrophoresis and antigen detection

The samples were electrophoresed in 12% SDS-PAGE gel followed by electroblotting onto a nitrocellulose filter (Schleicher & Schuell). After primary antibodies, biotinylated species-specific secondary antibodies followed by streptavidin-HRP were used. HRP was visualized with enhanced chemiluminescence (Amersham). Monoclonal antibodies against neurofilament polypeptides (**I-II**) were kindly provided by Dr. Ismo Virtanen (Virtanen et al., 1985). FGF-2 antibodies (**I-III**) were produced in rabbits as described in **I**. Rabbit polyclonal TGF-β1 antibodies (**IV**) were a kind gift from Dr. Jorma Keski-Oja (Taipale et al., 1992).

Staining of apoptotic cells (**II-V**)

Hoechst staining

The collagen gel cultures were fixed with 3-3.5% paraformaldehyde for 30 min at room temperature or for 12 h in +4EC. The gels were then exposed to 1 μg/ml bisbenzimide (Hoechst 33342; Sigma) for 10-30 min. After thorough rinsing with PBS, the gels were inspected and photographed with a fluorescence microscope under UV illumination. Condensed and fragmented nuclei were counted as apoptotic.

TUNEL staining

Apoptotic DNA cleavage was detected in situ by a modification of the original TUNEL method (Gavrieli et al., 1992). The collagen gels were either fixed with 3.7% formaldehyde in PBS for at least 16 h or fixed with 3.5% paraformaldehyde for 12 h and treated with 2% H_2O_2 for 20 min. They were covered with a labeling mixture containing dATP, terminal deoxinucleotidyl transferase (Promega) and either digoxigenin- (Boehringer Mannheim) or fluorescein-labeled (New England Nucleotides) dUTP. Digoxigenin-labeled nucleotides were detected by anti-digoxigenin-POD conjugate (Boehringer Mannheim) and DAB substrate. Fluorescein-labeled nucleotides were detected using a fluorescence microscope equipped with the appropriate filters.

DNA fragmentation analysis (**II, III**)

To analyze low-molecular-weight fragments of genomic DNA the cells were lyzed with 150 mM NaCl, 25 mM EDTA, 0.2% SDS. The lysate was treated with proteinase K (Boehringer Mannheim), extracted with phenol-chloroform and treated with RNAse A (Sigma). DNA was precipitated with ethanol and loaded onto a 1.8% agarose gel with markers. After electrophoresis DNA was visualized by staining with ethidium bromide.

To analyze high-molecular-weight fragments the cells were lyzed with 1% SDS and loaded onto a 1% agarose gel. The electrophoresis was performed in a clamped homogenous field apparatus (CHEF-DR-II; Bio-Rad). The gel was stained with ethidium bromide.

Cell proliferation assays (**III, IV**)

Cell proliferation was measured either using CellTiter 96 AQ kit (Promega) according to manufacturers instructions (**III**) or by 5-BrdU incorporation and immunostaining (**IV**). In latter, the cells were seeded on glass coverslips and grown overnight. The factors of interest were added to the growth medium for the following 48 h. 250 μM 5-BrdU (Sigma) was added 4 h (melanoma cells) or 48 h (melanocytes) before the end of the incubation. The cells were fixed with ice-cold methanol and permeabilized by a brief treatment with 1.5 N HCl. The cells were stained with monoclonal antibodies to BrdU (Amersham) and a secondary, rhodamine-conjugated antibody (Jackson). The nuclei were briefly stained with Hoechst as above.

Mv1Lu growth inhibition test (**IV**)

The growth-inhibitory activity secreted by cultured cells was analyzed in Mv1Lu cell assay (Laiho et al., 1990). The cells were plated on 24-well plates 24 h prior to the experiment. Conditioned media were collected from the experimental cultures and either used as such or heat-treated (90°C, 1 min) to activate latent forms of TGF-β. Mv1Lu medium was replaced by the conditioned medium of interest. After 18 h 5-BrdU was added for the last 2 h. The cells were fixed with 3.5% paraformaldehyde, permeabilized with 0.5% NP-40 and 1.5 N HCl, and the incorporation of 5-BrdU was measured essentially as described above. The growth inhibitory activity was identified as TGF-β by using neutralizing chicken egg yolk anti-TGF-β1 IgY.

Analysis of intracellular ROS level (**V**)

The oxidative stress of cultured cells was analyzed using fluorescent, oxidation sensitive markers, dichlorofluorescin diacetate (DCFH-DA; Sigma) and dihydroethidium (DHE; Molecular Probes). The markers were added to the culture medium for the last 4 h of incubation with desired supplements. Cells were either trypsinized from plates or released from collagen gel by collagenase treatment. Intracellular production of highly fuorescent oxidation products dichlorofluorescein (DCF) and ethidium was measured by a FACS analysator using FL1 and FL2 filter sets, respectively. 10.000 events were recorded per sample. Cell debris was gated out based on forward scatter.

Analysis of Bcl-2 protein expression by FACS (**V**)

Bcl-2 expression was analyzed by FACS analysis. Cells were either trypsinized from plates or released from collagen gel by collagenase treatment. After washing the cells were fixed with 1% PFA at room temperature for 5 min, centrifuged and permeabilized with ice-cold 70% methanol for 15 min. The cells were resuspended in PBS + 1% serum and incubated with monoclonal anti-Bcl-2 (Santa Cruz Biotechnology) for 30 min in +4°C. The secondary antibody used was species specific FITC-conjugated Fab. Thorough washing was followed by FACS analysis of the samples using FL1 filter set. 10.000 events were recorded per sample. Cell debris was gated out based on forward scatter.

RESULTS AND DISCUSSION

A model to study the migration and phenotype of cells *in vitro* in a three-dimensional lattice (**I-V**)

The three-dimensional cell culture model used throughout this thesis was originally developed in our laboratory to study angiogenesis in a true *in vitro* assay as an attempt to avoid some of the problems arising in the conventional cell culture system. The angiogenic potential of growth factors and other substances can also be determined by often laborous *in vivo* assays, for example by applying biologically active materials on the chorioallantoid membrane of a chicken embryo or in the cornea of a rabbit. Several extracellular matrix preparations were tested during development of the assay (Alanko and Saksela, unpublished results). Collagen gels had previously been widely used mainly to study fibroblasts in a three-dimensional environment (Elsdale and Bard, 1972; Schor, 1980; Tomasek et al., 1982; Bell et al., 1979; Mauch et al., 1988) and collagen was commercially available in large quantities. Type I collagen gel also had the benefit of being almost completely transparent. It allowed diffusion of growth factors and development of a concentration gradient. The model has since been used in several studies to quantitate the potential of growth factor preparations to trigger cell migration and/or angiogenesis (Joukov et al., 1996; Joukov et al., 1997; Joukov et al., 1998).

Type I collagen gel is a protein matrix that lacks several components of natural intersitial connective tissue. It still clearly differs from soft agar and related growth environments that prohibit cell attachment. Firstly, type I collagen is a naturally occurring glycoprotein polymer and although it is not the sole constituent of any ECM, it is one of the major components of some. Secondly, during gelling collagen renatures and takes on a conformation that resembles naturally occurring collagen networks. In contrast, denatured collagen, or gelatin, that has widely been used to coat cell culture surfaces, forms a thin film of mostly denatured collagen. Thirdly, collagen gel favors cell attachment. All cell types tested attach to collagen quickly. After 30 minutes practically all viable cells have attached. Malignant but also several benign, terminally differentiated cell types remain attached and viable for very long periods of time.

More advanced three-dimensional cell culture environments have been designed lately. For example, the structure of skin can be reconstructed *in vitro* by layering keratinocyte-melanocyte cocultures (epidermis) on top of collagen embedded fibroblasts (dermis) (Chen et al., 1995; Meier et al., 2000; Satyamoorthy et al., 1999). In this kind of organotypic cultures, the cells

appear surprisingly similar to their *in vivo* counterparts. On the other hand, the presence of several cell types hampers the use of many biochemical methods to analyze one single subpopulation such as the melanocytes.

Differentiation of Tera-2 cells is accompanied by development of growth factor dependency

Undifferentiated Tera-2 cells respond to FGF-2 by enhanced proteolysis and migration towards higher concentration (I)

Tera-2 cells represent malignant neural crest-derived cells capable of differentiation to neurons in response to all-trans retinoic acid (RA) treatment (Thompson et al., 1984). Undifferentiated Tera-2 cells proliferate rapidly in cell culture and are tumorigenic in nude mice. FGF-2 does not markedly affect their proliferation rate but it induces a change in their phenotype towards proteolytically active, migratory cells (Tienari et al., 1991).

To study the migration of Tera-2 cells in response to various growth factors the cells were grown between two layers of collagen gel in a sharply demarcated area in the middle of a cell culture dish and the growth factors were applied to the gel outside this area to form a concentration gradient (**I**).

Undifferentiated Tera-2 cells grew in collagen as tight cell clusters. A gradient of FGF-2 induced directional migration towards higher concentrations, whereas addition of FGF-2 to the gel caused scattering of the cells through random migration. Several other growth factors were tested for their ability to induce migration of Tera-2 cells. Only FGF-1 and, to a somewhat lesser extent, EGF were effective (**I**).

The ability of FGF-2 to induce migration of Tera-2 cells can be partly explained by their enhanced proteolysis (Tienari et al., 1991). It is also possible that FGF-2 substitutes a paracrine, matrix-bound growth factor required by the cells, such as FGF-4 or FGF-2 itself, and thus allows growth independently of contacts to matrix and the neighboring cells.

Differentiation of Tera-2 cells inhibits their migration and renders them susceptible to apoptosis in three-dimensional collagen gel (I, II)

Retinoic acid induces neuronal differentiation of Tera-2 cells. Differentiation causes a dramatic change in the appearance of Tera-2 cells. They gradually start expressing neuronal marker molecules and develop neuron-like extensions. Differentiated cells express specific proteinase inhibitors in their pericellular structures. FGF-2 enhances their overall proteolytic potential only slightly (Tienari et al., 1991).

When the differentiated cells were embedded in collagen gel the cells rapidly started to die. Cell death was effectively counteracted by addition of FGF-2. A gradient of growth factor did not induce migration of the differentiated cells but they survived in the areas with sufficiently high concentration of FGF-2. Of the other growth factors tested, only FGF-1 and FGF-4 (**II**) could induce Tera-2 survival. EGF, that had an effect on the migration of undifferentiated Tera-2 cells, could not promote the survival of differentiated cells (**I**).

As a control experiment, the differentiated cells were first let to attach onto the surface of a plain culture dish and then covered with collagen gel. The cells did not die when in contact with the bottom of the dish, which implies that their death is not mediated by soluble factors possibly contaminating the collagen preparation.

After release *in vitro* and probably also *in vivo*, FGF-2 is quickly bound to HSPGs of the ECM (Saksela et al., 1988; Flaumenhaft and Rifkin, 1992). To find out whether Tera-2 cells could benefit from matrix bound FGF-2, the HSPG rich matrix of glioma cells and bovine capillary endothelial cells was isolated. Both cell types synthesize FGF-2. When the cells were induced to differentiate inside the collagen gel, they remained alive only in areas of the isolated matrix preparations. Also addition of purified HSPG from glioma or capillary endothelial cells known to contain FGF-2 mediated survival of the cells. Their survival was inhibited by neutralizing FGF-2 antibodies (**I**).

Close observation of the death of Tera-2 cells in collagen gel revealed morphological resemblance to apoptotic cell death. The cells first attached to collagen and sent several dendrite-like extensions to their environment. Within a few days the cells lost these extensions and rounded up with extensive blebbing of the plasma membrane. A DNA stain (Hoechst 33342) revealed chromatin condensation and, later, fragmentation of the nucleus. To confirm the apoptotic nature of cell death, the cells were stained by the TUNEL method. TUNEL staining revealed DNA strand breaks in 75-80% of the cells after 4 days in collagen gel.

Addition of FGF-2 (20 ng/ml) slowed down the rate of apoptosis by about 50%. Although FGF-4 also had some protective effect, it was less potent than FGF-2 (**II**).

Tera-2 cells lose the expression of endogenous FGF-2 during differentiation in three-dimensional collagen gel

It is known from previous work by others (Miller et al., 1990) that the expression of several growth factors changes during differentiation of Tera-2 cells by RA. To study whether a change in autocrine or paracrine FGF-2 stimulation could explain the death of differentiated cells in collagen, their FGF-2 expression was investigated using Western blotting enhanced by a heparin-Sepharose affinity based concentration step (see Materials and methods). It was found that in aconventional cell culture RA treatment alone does not cause marked downregulation of FGF-2 expression. However, the regulation of FGF-2 expression was different in collagen gel. Complete disappearance of endogenous FGF-2 protein was seen in three-dimensional collagen following RA-induced differentiation. The downregulation of FGF-2 protein expression took place gradually 48-72 h after transfer of the cells into collagen (**II**).

Northern blotting analysis of FGF-2 mRNA expression was performed to confirm the results obtained by Western blotting. In accordance to protein determinations, the RA treated cells cultured in collagen gel for 2 days almost completely lost the expression of FGF-2 mRNA (**II**). The mRNA expression of another member of the FGF family known to be expressed by Tera-2 cells, namely FGF-4 (K-FGF), was also analyzed. The results were similar to FGF-2 with the exception that RA seems to efficiently downregulate FGF-4 mRNA expression also in the absence of collagen (**II**).

FGF-2 is an important neurotrophic factor (Amaya et al., 1991; Anderson, 1993; Morrison et al., 1986; Slack et al., 1987; Westermann et al., 1990). Some FGFs have been demonstrated to have a role during development and differentiation of the nervous network. FGF-2 is also expressed in the mature brain. It is known to have a supportive role and be involved in regeneration after injury (Kuzis et al., 1999; Pataky et al., 2000). Development of FGF dependency in Tera-2 cells following RA induced differentiation has similarities to the events taking place *in vivo* during differentiation and migration of neural progenitor cells. A gradient of neurotrophic factors is thought to guide the migration. Most of the cells are misguided or only serve a temporary role during the process of development. These cells probably undergo apoptosis triggered either by loss of trophic factors or by some other defined cellular program (Bronner-Fraser, 1993). The

cells that reach areas where the survival factors are available remain alive and ultimately form the functioning central and peripheral nervous systems.

The apoptosis of differentiated Tera-2 cells in collagen seems to be triggered by loss of a vital autocrine growth factor loop. The mechanisms that mediate different regulation of FGF-2 expression in collagen compared to conventional two-dimensional culture, are not fully known. Alterations in the surrounding ECM are known to have profound effects on cell phenotype and growth, and even to induce apoptosis (Ruoslahti and Reed, 1994). Adhesion to a substrate creates signals that are mediated inside the cell by adhesion receptors like the integrins. The finding that apoptosis is not induced in cells that have adhered to a conventional cell culture plate even when they are covered with a layer of collagen gel indicates that the contact to collagen is not deleterious to the cells as such. Rather, a collagen environment might not facilitate stimulation of, as yet unidentified, necessary integrins. One of the functions of those vital integrins might be the regulation of FGF-2 expression.

Melanocytes are susceptible to apoptosis in collagen gel in the absence of FGF-2 stimulation

Unstimulated melanocytes undergo apoptosis in collagen gel

Normal melanocytes form an almost nonproliferating cell population in the basal epidermis. They are dependent on supportive factors produced by the surrounding keratinocytes (Halaban, 1996). In cell culture melanocytes require strong growth factor stimulation to proliferate. When left without stimulation they stop dividing and only slowly start to deteriorate after several days in culture (**III**). The localization of melanocytes is strictly regulated and normal melanocytes are never found in the dermis. On the contrary, nevus cells and malignant melanocytes have the ability to invade the dermis. To observe the behavior of melanocytes in an environment that mimicks the dermal extracellular matrix more closely than the conventional cell cultures, they were cultured in collagen gel. Melanocytes have been reported to express integrins that mediate attachment to type I collagen, namely $\alpha 2\beta 1$ and $\alpha 3\beta 1$ (Hara et al., 1994).

When normal melanocytes were seeded in three-dimensional collagen they quickly attached and spread their dendrite-like extensions into the gel. In the absence of added growth factors, the first cells started showing signs of deterioration in a few hours after seeding and then gradually lost their extensions and rounded up. In 3 days about 35% of the cells showed morphological

signs of apoptosis. Hoechst staining revealed variable degrees of chromatin condensation and nuclear fragmentation. TUNEL staining revealed DNA breaks in the same cells confirming the apoptotic nature of cell death. Addition of laminin and/or fibronectin to the collagen gel did not affect the cell death rate (**III**).

The results suggest that the extracellular matrix has an important role in the regulation of melanocyte survival. The mechanism that causes apoptotic death of cells in collagen *in vitro* has the potential of serving as an effective mechanism to restrict survival of melanocytes in the dermis *in vivo* and facilitate their strict localization into the epidermal compartment.

Fibroblast growth factor-2 acts as a survival factor for melanocytes in collagen gel

When melanocytes were seeded into three-dimensional collagen and cultured in the presence of FGF-2, the cells attached like control cells. They, however, showed considerably less signs of apoptotic death and most of the cells survived for extensive periods of time. Hoechst and TUNEL staining revealed few apoptotic cells after 5 days in collagen (**III**).

FGF-2 is a natural growth factor for melanocytes. It is one of the factors involved in paracrine stimulation of melanocytes by basal keratinocytes (Halaban et al., 1988). It has also been previously shown that although normal melanocytes express very little or no FGF-2, malignant melanocytes and nevus cells very commonly produce high levels of endogenous FGF-2 (Scott et al., 1991). The same cell types are also capable of living without support from keratinocytes in the dermis.

Nevus cells survive in three-dimensional collagen without exogenous FGF-2

Nevus cells are phenotypically benign melanocytic cells that have distinct growth characteristics *in vivo*. Unlike normal melanocytes that grow intermingled between keratinocytes and seem to be territorial in the sense that they are never found in clusters, nevus cells form nests in the epidermis and dermis. Mitotic activity is more often detected among nevus cells than among normal melanocytes *in situ* (Rieger et al., 1993).

Nevus cell cultures were established from the dermal part of human compound or dermal nevi. When grown in collagen gel, nevus cells survived without exogenous supportive growth factors (**III**). The FGF-2 expression of normal melanocytes and nevus cells was analyzed by Northern and Western blotting techniques. The expression level in melanocytes was very low. Nevus

cells showed modest expression of FGF-2 on mRNA level but relatively high amounts of FGF-2 protein. The expression of FGFR-1 was also confirmed by mRNA analysis in both cell types (**III**).

To demonstrate the possible autocrine or paracrine role for FGF-2 in nevus cells, they were cultured in three-dimensional collagen gel in the presence of protamine, a rather unspecific but effective inhibitor of FGF-2 receptor interactions. Protamine treated nevus cells became susceptible to apoptosis in collagen. The effect of protamine could be overcome by high concentrations of exogenous FGF-2. Also, protamine as equivalent or higher concentrations was not toxic to nevus cells or melanocytes in conventional cell culture.

Endogenous expression of FGF-2 has been connected to increased cell survival in several cell types, both benign and malignant (see Szebenyi and Fallon, 1999 for review). In the case of nevus cells it seems to have the potential to enable their growth outside the epidermis, without contact to keratinocytes.

Transforming growth factor β and fibroblast growth factor-2 have opposite effects on melanocyte apoptosis in collagen gel

Transforming growth factor β inhibits the growth of melanocytes and nevus cells

Transforming growth factor βs (TGF−β) comprise a family of principally inhibitory growth factors. The roles of TGF−β differ in various parts of the skin. In the dermis it enhances matrix deposition and has an important role during wound healing (Roberts et al., 1986; Massagué, 1990). It acts as a growth inhibitor for epidermal keratinocytes both *in vivo* and *in vitro*. Lack of TGF−β receptor activation in the skin of transgenic mice has been shown to lead to increased incidence of carcinomas (Wang et al., 1997; Wang et al., 1999). TGF−β has been shown to induce apoptosis in several cell types (Yanagihara and Tsumuraya, 1992; Moulton, 1994; Rotello et al., 1991; Oberhammer et al., 1992; Fabregat et al., 1996; Fukuda et al., 1993).

To investigate the effect of TGF-β on melanocyte growth and apoptosis the cells were cultured in conventional cell culture in the presence of TGF−β1. Unstimulated melanocytes are quiescent as such. TGF−β had no stimulatory effect on them, nor did it induce accelerated cell death. When TGF−β1 was introduced to melanocytes that had been stimulated to proliferate by strong PKC and cAMP activators, it effectively inhibited their replication again without

affecting the rate of cell death (**IV**). These results were in line with previous studies that have suggested a role for TGF−β as an inhibitor of melanocyte growth (Rodeck et al., 1994).

Transforming growth factor β accelerates the apoptosis of melanocytes in collagen

Melanocytes that are grown in three-dimensional collagen gradually undergo apoptosis. When TGF−β1 was added to collagen cultures, it caused significant acceleration of apoptosis. In 2 days about 90% of melanocytes had died. TGF−β effect could be effectively reversed by specific TGF−β1 antibodies. The effect was dose-dependent and detectable already at physiological concentrations (**IV**). FGF-2, that promotes the survival of melanocytes in collagen gel, counteracted also the TGF-β effect. FGF-2 was not able to totally prevent apoptosis but the proportion of apoptotic cells after 2 days was lowered by more than 50%.

As a control, the effect of two growth inhibitory factors with apoptotic potential, namely TNF-α and IFN-α was tested. These factors were unable to affect the rate of melanocyte apoptosis in collagen (**IV**).

Endogenous TGF−β produced by melanocytes is involved in their apoptosis in collagen

Apoptosis in the presence of exogenous TGF−β1 takes place faster but is otherwise morphologically very similar to the "background" apoptosis of normal melanocytes in collagen lacking proper stimulation by FGF-2 (**III**, **IV**). To explore the possibility that endogenously expressed TGF−β is involved in the apoptosis, neutralizing TGF−β1 antibodies were added to collagen gel. The apoptotic rate of normal melanocytes decreased in the presence of antibodies. However, the maximal inhibition of apoptosis did not exceed 50% (**IV**). The limited effect could result from relatively slow diffusion of antibody molecules in collagen that hampers their ability to neutralize newly synthesized and activated TGF−β1. It is also possible that other TGF−β subtypes are present and the subtype 1-specific antibody does not recognize them.

To investigate whether TGF−β is present in the collagen cultures as an impurity or whether it is endogenously produced by melanocytes, Western blot analysis of the collagen preparation, cell lysates and conditioned media was performed. A relatively high expression of TGF−β1 protein was detected in melanocyte lysates. Ten times concentrated conditioned medium and the undiluted collagen preparation were completely negative (**IV**).

Although the sensitivity of the Western blotting assay allowed detection of 0.1 ng recombinant TGF−β1 per lane, the theoretically lowest detectable concentration in conditioned medium was about 0.2 ng/ml. A mink lung epithelial cell (Mv1Lu) growth inhibition assay was used for increased sensitivity (see Materials and methods). The serum free conditioned medium of melanocytes was shown to contain growth inhibitory activity that was only detectable after heat treatment of the samples. The activity was partly inhibited by TGF-β1 antibodies, which identifies it as latent TGF-β1 (**IV**). TGF-β is secreted from most cells in latent form. The partial inhibition suggests the presence of other subtypes in the conditioned media besides TGF−β1.

As a strong inhibitor of melanocyte growth, TGF−β probably has regulatory functions in the skin. It has been shown to be present in the dermis and epidermis (Dagnino et al., 1993; Taipale et al., 1994; Karonen et al., 1997, Raghunath et al., 1998). These results point to an endogenous suicide mechanism that melanocytes carry with them and that gets activated whenever the cells end up outside their normal environment.

Nevus cells and melanoma cells are insensitive to the pro-apoptotic effect of TGF−β

Nevus cells and vertically growing melanoma cells have broken the natural barrier formed by the BM and can be found on its dermal side. As mentioned earlier, mitotic activity can be detected in nevi, although less commonly than in melanomas. In aggreement with that, unlike normal melanocytes, nevus cells proliferated in cell culture even in the absence of strong stimulants and growth factors but were further stimulated by them. In either case, TGF−β1 effectively inhibited their growth. However, TGF−β1 added to the cultures did not affect the survival of nevus cells in three-dimensional collagen gel. Nevus cell lysates and conditioned media were investigated for TGF−β1-like activity by Western blotting and Mv1Lu growth inhibition assay, respectively. The observed levels were comparable to those detected in normal melanocytes (**IV**).

Previous studies (Filmus and Kerbel, 1993; Krasagakis et al., 1999; Rodeck et al., 1994) have suggested that melanomas gain resistance to TGF−β effects during malignant progression. TGF−β did not inhibit the rapid proliferation of the two melanoma cell lines tested. When melanoma cells were cultured in three-dimensional collagen, their apoptosis was not increased and addition of TGF−β was ineffective in inducing apoptosis. Mv1Lu growth inhibition assay indicated that melanoma cells secrete large amounts of latent TGF−β into their medium (**IV**). This has also been previously described in the literature. Melanoma cell secreted TGF−β has

been suggested to have a role in regulating the synthesis of connective tissue stroma of the tumor. Also, TGF−β has immunosuppressive effects that could be very beneficial in protecting malignant cells from the host immune system (Ruffini et al., 1993).

The evidence presented above confirms that FGF-2 is a strong supportive factor for melanocytes. It promotes their survival in collagen that is an unphysiological environment for normal melanocytes. *In vivo*, melanocytes need contact to keratinocytes and the BM. By an unknown mechanism, FGF-2 can overcome this requirement. Nevus cells, which are stimulated by an autocrine FGF-2 loop, are able to grow in the dermis without keratinocyte and BM contacts. TGF-β is inhibitory to melanocyte and nevus cell growth. Endogenous TGF-β seems to mediate the apoptosis of melanocytes in collagen. Also nevus cells and melanoma cells produce and probably activate TGF-β but are not susceptible to induction of apoptosis because of the autocrine FGF-2 stimulation. TGF-β is present in conventional cell culture and, *in vivo*, in the epidermis. Supportive factors from epidermal keratinocytes explain melanocyte survival *in vivo* but it is not clear what protects them from TGF-β induced apoptosis in conventional culture. The contact to cell culture plate (or to BM *in vivo*) might generate integrin mediated signals that counteract the TGF-β effect.

Transforming growth factor β-induced apoptosis of melanocytes involves downregulation of Bcl-2 and production of reactive oxygen species

TGF−β-induced apoptosis of melanocytes in collagen gel is associated with an increase in intracellular oxidative activity and can be inhibited by antioxidants

Intracellular accumulation of reactive oxygen species (ROS) has been shown to take place in several cases of TGF−β induced apoptosis (Islam et al., 1997; Kayanoki et al., 1994; Lafon et al., 1996; Sanchez et al., 1996). To study whether ROS have a role in melanocyte apoptosis, the level of oxidative stress was monitored using a fluorescent marker, dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is cell permeable and non-fluorescent in its reduced state but becomes less permeable and strongly fluorescent dichlorofuorescein (DCF) once hydrolyzed by intracellular esterases and oxidized by intracellular radicals (Bass et al., 1982; LeBel et al., 1992).

It was found that TGF−β triggered apoptosis of melanocytes in collagen is accompanied by upregulation of intracellular oxidative potential in an early phase (**V**). The effect was detectable

already 4 hours after the transfer of cells to collagen (endogenous TGF−β stimulation; **IV**) and it was stronger if exogenous TGF−β was added to the gel. The early detection of ROS virtually rules out the possibility that ROS production is merely a secondary phenomenon that accompanies cell lysis. Interestingly, no enhancement of ROS generation in response to TGF−β treatment could be detected when the cells were cultured on cell culture plates suggesting that collagen environment is a prerequisite for this effect (**V**).

To determine whether ROS have a causative role in melanocyte apoptosis, the cells were cultured in collagen gel in the presence of NAC or ascorbic acid. NAC is a glutathione precursor as well as a radical scavenger, while ascorbic acid can act as an intracellular radical scavenger and is needed during "recycling" of oxidized vitamin E. As quantitated by TUNEL staining and nuclear morphology, the antioxidants were effective in inhibiting TGF−β induced apoptosis of melanocytes (**V**). FACS analysis of DCFH-DA treated cells revealed that the antioxidants downregulated the intracellular levels of ROS (**V**). A time-course experiment showed that NAC could be added 4 hours after TGF−β and it still had the same rescue effect, while when added 8 hours after TGF-β NAC could only rescue about half of the cells (unpublished data). This suggests that after 4-8 h of TGF-β induced ROS activity the cells start passing the "point of not return" possibly represented by activation of the terminal caspases.

TGF−β and FGF-2 have opposite effects on ROS generation and Bcl-2 expression of melanocytes

FGF-2 acts as a survival factor for melanocytes in collagen gel and it can effectively inhibit TGF−β induced apoptosis (**IV**). To study whether FGF-2 also could inhibit TGF−β induced accumulation of intracellular ROS, their generation was measured utilizing DCFH-DA. FGF-2 treatment diminished ROS generation in collagen in the absence and presence of added exogenous TGF-β. However, while antioxidants NAC and ascorbic acid reduce the measurable ROS activity also in cells growing on conventional cell culture plates, FGF-2 had little effect on them (**V**). These results suggest an indirect mode of action for FGF-2 as an inhibitor of ROS production rather than a radical scavenger.

The induction of apoptosis by TGF−β has in some cases been attributed to regulation of the expression of the Bcl-2 family of proteins. Downregulation of the antiapoptotic Bcl-2 and upregulation of some proapoptotic family members has been described (Salzman et al., 1998). On the other hand, also FGF-2 is known in many cases to regulate the Bcl-2-related proteins.

Recent reports have suggested an important role for Bcl-2 in regulating ROS production during apoptosis (Cai and Jones, 1998).

To study the level of Bcl-2 protein in melanocytes, FACS analysis of fixed, antibody stained cells was performed. Bcl-2 protein was less abundant in cells that grow in collagen than in control cells. Exogenous TGF−β caused further downregulation of Bcl-2 protein. FGF-2 upregulated the level of Bcl-2 in melanocytes grown in collagen gel and it almost completely counteracted the downregulation observed as a result of TGF−β addition (**V**).

Caspase inhibitors slow down melanocyte apoptosis in collagen without affecting ROS generation or Bcl-2 expression

Activation of a specific set of intracellular proteases, caspases, is considered one of the most uniform phenomena of apoptotic cell death (see Los et al., 1999; Wolf and Green, 1999 for recent review). To study the involvement of caspases in TGF−β induced death of melanocytes, a set of inhibitor peptides was used. TUNEL staining showed that two of the inhibitors, Z-VAD-FMK and Z-DEVD-FMK, were able to inhibit the apoptosis of melanocytes in collagen gel (**V**). Both are known to inhibit some of the executioner caspases that are necessary for completing the apoptotic sequence (Los et al., 1999). In contrast, no effect on cell death was seen by the third inhibitor used, Ac-AAVALLPAVLLALLAP-YVAD-CHO, that specifically inhibits caspases 1 and 4. These caspases are involved in processing of certain cytokines and in the regulation of the immune response. However, apoptosis is almost unaffected in caspase 1 knockout mice, which suggests that it is dispensable for at least some forms of apoptosis (Li et al., 1995; Kuida et al., 1995).

The effect of caspase inhibitors on ROS accumulation and Bcl-2 expression in the presence of TGF-β was determined to further clarify the role of caspases in melanocyte apoptosis. The inhibitors had no effect on either indicating that caspase activation takes place downstream of Bcl-2 regulation and ROS production or is not related to them (**V**).

TGF−β cannot induce apoptosis or ROS production in the presence of the translation inhibitor cycloheximide

To find out whether CHX inhibits melanocyte apoptosis, the cells were cultured in collagen gel in the presence of TGF−β and CHX. Apoptosis was quantitated by TUNEL staining. Although

most cells rounded up and lost their typical morphology they remained viable and showed no DNA strand breaks (**V**).

FACS analysis of DCFH-DA treated cells was carried out in the presence of CHX to study whether its antiapoptotic effect is attributable to inhibition of ROS generation. Decreased production of oxidized, fluorescent derivatives indicated that the oxidative potential of melanocytes is depressed by CHX treatment (**V**). This suggests that CHX inhibits the apoptotic cascade very early, upstream of the production of ROS.

Cycloheximide (CHX) can inhibit TGF−β-induced apoptosis in several cell systems (Yanagihara and Tsumaraya, 1992; Weller et al., 1994; Sanchez et al., 1997). Since CHX is an inhibitor of protein synthesis, it has been proposed that the synthesis of an as yet unknown protein is needed.

Concluding remarks

Apoptosis is induced in differentiated Tera-2 cells and normal melanocytes when grown in collagen. In Tera-2 cells transfer to collagen causes downregulation of endogenous FGF-2 expression. Integrin mediated signals not generated in collagen gel might be needed for sustained FGF-2 expression and autostimulation. Melanocytes do not normally produce significant amounts of FGF-2. Nevus cells that, according to the results presented in this thesis, have acquired an autocrine FGF-2 loop, have the ability to survive in collagen and, *in vivo*, in the dermis. A recent study that employed gene transfection to overexpress FGF-2 confirmed that FGF-2 expression induces nevus-like phenotype in melanocytes. When injected to the dermis of human skin grafted to mice, the FGF-2 expressing transfectants survived much like nevus cells, while control melanocytes showed rapid cell death (Nesbit et al., 1999). Analogously, forced overexpression of FGF-2 in nontumorigenic radial growth phase melanoma cells was shown to enable them to invade to the dermis in a skin reconstruct and to cause tumors in SCID mice (Meier et al., 2000). Inhibition of FGF-2 or its receptor with antisense oligonucleotides has been shown to inhibit melanoma growth both by slowing down the proliferation of melanoma cells and by restricting tumor induced angiogenesis (Becker et al., 1992; Wang and Becker, 1997). These results verify the significance of FGF-2 as a growth and survival factor during both normal and abnormal growth of melanocytes.

Endogenous TGF-β mediates the apoptosis of melanocytes in collagen gel. The subcellular mechanisms of TGF-β induced apoptosis remain partly undiscovered. Melanocytes respond to TGF-β stimulation differently in different growth environments. When the cells are in contact with a conventional cell culture plate, TGF-β inhibits their growth but does not accelerate their apoptosis. Only when the cells are grown inside a three-dimensional collagen gel can TGF-β induce their apoptosis. According to a recent study, TGF-β increases glioma cell expression of α2 integrin that, together with α1, is one of the most important integrin receptors for collagen (Miyake et al., 2000). This may suggest that α2 integrin mediated stimulation triggered by collagen and enforced by TGF-β is responsible for melanocyte apoptosis. However, this is not supported by the facts that melanocyte apoptosis in collagen is not affected by antibodies to integrin β1, the counterpart of α2 in collagen receptors, or cytochalacin D (Alanko and Saksela, unpublished data) that disrupts the cells' cytoskeleton and blocks most of the signaling cascades downstream of integrins.

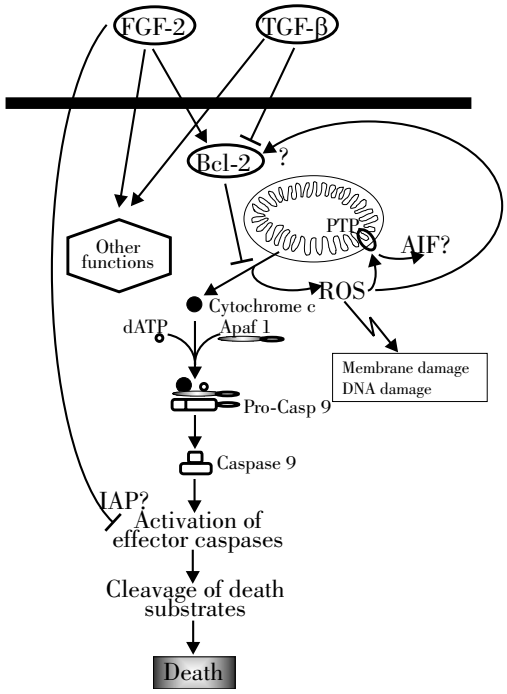

Figure 6. A hypothetical model of TGF-β induced apoptosis. TGF-β downregulates Bcl-2 that is a universal inhibitor of apoptosis. Loss of Bcl-2 leads to an inbalance between pro- and antiapoptotic Bcl-2 family members. Cytochrome c released from mitochondria associates with cytosolic Apaf 1 and dATP unmasking the Apaf 1 caspase recruitment domain (CARD). Procaspase 9 binds to the complex through CARD/CARD interaction and is activated. Cytochrome c loss from the mitochondria leads to production of ROS. ROS can induce apoptosis by damaging vital structures. DNA damage induces p53 dependent apoptosis. ROS induced opening of the mitochondrial transition pore (MPT) could lead to release of the apoptosis inducing factor (AIF). There is no evidence that ROS can directly activate caspases. FGF-2 effectively inhibits TGF-β-induced apoptosis by upregulating Bcl-2 and possibly by other mechanisms such as the induction of cellular antioxidants and inhibition of caspase activation. Based on **V**; Cai and Jones, 1998; Gross et al., 1999; Los et al., 1999.

The results presented in this thesis indicate that TGF-β induced apoptosis is accompanied by early upregulation of intracellular oxidative stress. Although considered merely a secondary phenomenon by some investigators, increasing evidence points to a central role for oxidative stress in apoptotic death. A hypothetical model describing possible mechanisms of FGF-2 and TGF-β action has been developed based on this thesis and recent publications (**Fig. 6**). According to this model, TGF-β acts primarily by downregulating the expression of Bcl-2, which is thought to have a pivotal role in deciding whether a cell will live or die (Gross et al., 1999). Bcl-2 maintains the

integrity of the outer mitochondrial membrane. Its downregulation leads to leakage of cytochrome c from mitochondria through the action of proapoptotic Bcl-2 family members. Together with cytosolic Apaf 1 and dATP, cytochrome c activates caspase 9, which initiates the caspase cascade (Los et al., 1999). Cytochrome c is an integral part of the mitochondrial electron transport chain. Its loss from the intermembrane space of mitochondria leads to generation of superoxide and increased oxidative stress (Cai and Jones, 1998).

The functions of ROS inside the cell are largely unknown. They are known to cause damage to biological membranes and DNA. ROS may also trigger the opening of mitochondrial permeability transition pore, which has been reported to augment mitochondrial dysfunction and release proteins, including the apoptosis inducing factor to the cytosol (Hirsch et al., 1997; Susin et al., 1996; Zoratti and Szabo, 1995). The mechanism, by which antioxidants block apoptosis, is currently unknown. ROS are not known to be able to activate caspases. It is clear that several links are still lacking from this model.

The model suggests that FGF-2 acts by upregulating Bcl-2 expression. FGF-2 probably has additional functions that inhibit apoptosis. It is tempting to speculate that FGF-2 regulates the expression of IAP. VEGF, that functions as a survival factor for vascular endothelial cells in a similar fashion as FGF-2, has been shown to induce the expression of IAPs survivin and XIAP in them (Tran et al., 1999). IAP can inhibit apoptosis even after activation of the effector caspases. A recent study reported that survivin is expressed by human nevus and melanoma cells but not by normal melanocytes, which could make it a potential target for novel anticancer therapies (Grossman et al., 1999).

PERSPECTIVE

This study reports the development of a novel method to monitor migration, growth, and apoptosis of cells in three-dimensional culture. The results provide new insights into the biology of the developing nervous system and skin. Differentiation of Tera-2 cells into neuron-like cells was found to be accompanied by a change in their ability to migrate through three-dimensional ECM, in their apoptotic potential, and requirement for stimulating growth factors. It is still not known what the actual targets and mechanisms of FGF-2 action *in vivo* are. Since apoptosis depends on the surrounding ECM and it has been previously shown that FGF-2 can stimulate the cell surface proteolysis, a change in interactions with matrix through integrins or other specialized molecules might be involved. FGF-2 has been used experimentally to stimulate the regeneration and inhibit apoptosis of axotomized neurons *in vivo*.

Differences in growth requirements between human skin melanocytes, dermal nevus cells, and melanoma cells were studied. Collagen gel was used as a model of dermis, a natural environment for nevus cells and metastatic melanoma cells, but not for normal skin melanocytes. FGF-2 and TGF−β were found to act as antagonists as they regulate the apoptosis of normal melanocytes. Endogenous TGF−β expression was found to be capable of inducing apoptosis of melanocytes in collagen. Nevus cells have strong endogenous FGF-2 stimulation and, thus, were protected from apoptosis in collagen gel. They were sensitive to growth inhibition but not apoptosis mediated by TGF−β. Melanoma cells, on the other hand, were totally unresponsive to TGF−β. They also exhibited strong FGF-2 expression but in their case FGF-2 probably acts less as a survival factor and more as a stroma stimulating and angiogenic factor, since most melanomas express a multitude of growth stimulatory mutations. The role of FGF-2 in melanoma development, growth and metastasis is a subject that has gained much interest. Several recent publications have confirmed the results presented in this thesis (Nesbit et al., 1999; Meier et al., 2000).

The nature of TGF−β-induced apoptosis was further characterized. It was found that TGF−β stimulation is accompanied by an upregulated level of intracellular oxidative stress in melanocytes. ROS level was downregulated by FGF-2, antioxidants, and CHX, all inhibitors of melanocyte apoptosis. The fact that antioxidants could inhibit melanocyte apoptosis suggests that ROS are needed for apoptosis. On the other hand, caspase inhibitors inhibited apoptosis without affecting ROS level, showing that elevated ROS production is not sufficient for apoptosis. It seems that ROS act upstream of caspases and perhaps are even able to activate the

caspase cascade, but the necessary cofactors are not known and the subject requires further investigation.

TGF−β and FGF-2 had antagonistic effect on Bcl-2 expression also. Bcl-2 has been suggested to act as a regulator of intracellular ROS production. The results presented here are consistent with this theory but are not conclusive. Further clarification could be obtained by transfection experiments using an inducible Bcl-2 expression vector construct and by studying the integrity of the mitochondrial membranes utilizing suitable fluorescent markers. The effect of TGF−β and FGF-2 on the expression of cellular antioxidants like superoxide dismutase, catalase and glutathione peroxidase should also be studied.

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REFERENCES

Airola, K., Karonen, T., Vaalamo, M., Lehti, K., Lohi, J., Kariniemi, A.-L., Keski-Oja, J., and Saarialho-Kere, U. (1999). Expression of collagenases-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas. Br J Cancer *80*, 733-743.

Aktas, H., Cai, H., and Cooper, G. (1997). Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the cdk inhibitor p27Kip1. Mol Cell Biol *17*, 3850-3857.

Alon, R., Cahalon, L., Hershkoviz, R., Elbaz, D., Reizis, B., Wallach, D., Akiyama, S., Yamada, K., and Lider, O. (1994). TNF-alpha binds to the N-terminal domain of fibronectin and augments the beta1-integrin-mediated adhesion of CD4+ T lymphocytes to the glycoprotein. J Immunol *152*, 1304-1313.

Amaya, E., Musci, T. J., and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. Cell *66*, 257-270.

Ambrosini, G., Adida, C., and Altieri, D. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nature Med *3*, 917-921.

Ameisen, J., and Capron, A. (1991). Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol Today *12*, 102-105.

Amundadottir, L., Nass, S., Berchem, G., Johnson, M., and Dickson, R. (1996). Cooperation of TGF alpha and c-Myc in mouse mammary tumourigenesis: coordinated stimulation of growth and suppression of apoptosis. Oncogene *13*, 757-765.

Anderson, D. J. (1993). Cell fate determination in the peripheral nervous system: the sympathoadrenal progenitor. J Neurobiol *24*, 185-198.

Anderson, K., Seed, T., Ou, D., and Harris, J. (1999). Free radicals and reactive oxygen species in programmed cell death. Med Hypotheses *52*, 451-463.

Andrews, P. (1998). Teratocarcinomas and human embryology: pluripotent human EC cell lines. APMIS *106*, 158-167.

Andrews, P., Damjanov, I., Berends, J., Kumpf, S., Zappavingna, V., Mavilio, F., and Sampath, K. (1994). Inhibition of proliferation and induction of differentiation of pluripotent human embryonal carcinoma cells by osteogenic protein-1 (or bone morphogenetic protein-7). Lab Invest *71*, 243-251.

Andrews, P., Damjanov, I., Simon, D., Banting, G., Carlin, C., Dracopoli, N., and Fogh, J. (1984). Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2: differentiation in vivo and in vitro. Lab Invest *50*, 147-162.

Andrews, P., Nudelman, E., Hakomori, S.-I., and Fenderson, B. (1990). Different patterns of glycolipid antigens are expressed following differentiation of TERA-2 human embryonal carcinoma cells induced by retinoic adic, hexamethylene bisacetamide (HMBA) or bromodeoxyuridine (BUdR). Differentiation *43*, 131-138.

Aplin, A., Howe, A., Alahari, S., and Juliano, R. (1998). Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. Pharmacol Rev *50*, 197-263.

Arends, M., Morris, R., and Wyllie, A. (1990). Apoptosis. The role of the endonuclease. Am J Pathol *136*, 593-608.

Armstrong, B., and Kricker, A. (1994). Cutaneous melanoma: trends in cancer incidence and mortality. Cancer Surv *19*, 219-240.

Askew, D., Ashmun, R., Simmons, B., and Cleveland, J. (1991). Constitutive c-Myc expression in IL-3-dependent myeloid cell line suppresses cycle arrest and accelerates apoptosis. Oncogene *6*, 1915-1922.

Aumailley, M., and Gayraud, B. (1998). Structure and biological activity of the extracellular matrix. J Mol Med *76*, 253-265.

Aumailley, M., and Rousselle, P. (1999). Laminins of the dermo-epidermal junction. Matrix Biol *18*, 19-28.

Baldwin, T., Fazeli, M., Doherty, P., and Walsh, F. (1996). Elucidation of the molecular actions of NCAM and structurally related cell adhesion molecules. J Cell Biochem *61*, 502-513.

Bale, S., Dracopoli, N., Tucker, M., Clark Jr, W., Fraser, M., Stanger, B., Green, P., Donis-Keller, H., Housman, D., and Greene, M. (1989). Mapping the gene for hereditary cutaneous malignant melanoma-dysplastic nevus to chromosome 1p. N Engl J Med *320*, 1367-1372.

Baretton, G., Diebold, J., Christoforis, G., Vogt, M., Muller, C., Dopfer, K., Schneiderbanger, K., Schmidt, M., and Lohrs, U. (1996). Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of carcinogenesis and prognostic significance. Cancer *77*, 255-264.

Baselga, J., Maerz, W., Moy, D., Miller Jr, W., Castro, L., Reuter, V., Yao, T., Masui, H., and Dmitrovsky, E. (1993). Over-expression of transforming growth factor alpha antagonizes the anti-tumorigenic but not the differentiation actions of retinoic acid in a human teratocarcinoma cell. Oncogene *8*, 3257-3263.

Bass, D., Parce, J., Dechatelet, L., Szejda, P., Seeds, M., and Thomas, M. (1982). Flow cytometry studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol *130*, 1910-1917.

Bataille, V., Newton-Bishop, J., Sasieni, P., Swerdlow, A., Pinney, E., Griffiths, K., and Cuzick, J. (1996). Risk of cutaneous melanoma in relation to the numbers, types and sites of naevi: a case-control study. Br J Cancer *73*, 1605-1611.

Bazzoni, F., and Beutler, B. (1996). The tumor necrosis factor ligand and receptor families. N Engl J Med *334*, 1717-1725.

Becker, D., Lee, P., Rodeck, U., and Herlyn, M. (1992). Inhibition of the fibroblast growth factor receptor 1 (FGFR-1) gene in human melanocytes and malignant melanomas leads to inhibition of proliferation and signs indicative of differentiation. Oncogene *7*, 2303-2313.

Bell, E., Ivarsson, B., and Merrill, C. (1979). Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci USA *76*, 1274-1278.

Bellman, K., Jäättelä, M., Wissing, D., Burkart, V., and Kolb, H. (1996). Heat shock protein Hsp70 overexpression confers resistance against nitric oxide. FEBS Lett *391*, 185-188.

Bentham, G., and Aase, A. (1996). Incidence of malignant melanoma of the skin in Norway, 1955-1989: associations with ultraviolet radiation, income and holidays abroad. Int J Epidemiol *25*, 1132-1138.

Beroud, C., and Soussi, T. (1998). p53 gene mutation: software and database. Nucleic Acids Res *26*, 202-207.

Berx, G., Cleton-Jansen, A., Strumane, K., de Leeuw, W., Nollet, F., van Roy, F., and Cornelisse, C. (1996). E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene *13*, 1919-1925.

Bidanset, D., Guidry, C., Rosenberg, L., Choi, H., Timpl, R., and Höök, M. (1992). Binding of the proteoglycan decorin to collagen type VI. J Biol Chem *267*, 5250-5256.

Birnbaum, M., Clem, R., and Miller, L. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. J Virol *68*, 2521-2528.

Bonewald, L. (1999). Regulation and regulatory activities of transforming growth factor beta. Crit Rev Eukar Gene Express *9*, 33-44.

Borradori, L., and Sonnenberg, A. (1999). Structure and function of hemidesmosomes: more than simple adhesion complexes. J Invest Dermatol *112*, 411-418.

Bouwes Bavinck, J., Crijns, M., Vermeer, B., van der Woude, F., Claas, F., Pfister, H., Green, A., and Bergman, W. (1996). Chronic sun exposure and age are inversely associated with nevi in adult renal transplant recipients. J Invest Dermatol *106*, 1036-1041.

Brinster, R. (1974). The effect of cells transferred into the mouse blastocyst on subsequent development. J Exp Med *140*, 1049-1056.

Bronner-Fraser, M. (1993). Neural crest cell migration in the developing embryo. Trends Cell Biol *3*, 392-397.

Brown, J., Mann, K., Wiedeman, H., and Timpl, R. (1993). Structure and binding properties of collagen type XIV isolated from human placenta. J Cell Biol *120*, 557-567.

Brown, J., and Timpl, R. (1995). The collagen superfamily. Int Arch Allergy Immunol *107*, 484-490.

Brown-Augsburger, P., Broekelmann, T., Rosenbloom, J., and Mecham, R. (1996). Functional domain on elastin and microfibril-associated glycoprotein involved in elastic fiber assembly. Biochem J *318*, 149-155.

Bruckner-Tuderman, L., and Bruckner, P. (1998). Genetic diseases of the extracellular matrix: more than just connective tissue disorders. J Mol Med *76*, 226-237.

Bruckner-Tuderman, L., Hopfner, B., and Hammami-Hauasli, N. (1999). Biology of anchoring fibrils: lessons from dystrophic epidermolysis bullosa. Matrix Biol *118*, 43-54.

Burg, M., Tillet, E., Timpl, R., and Stallcup, W. (1996). Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. J Biol Chem *271*, 26110-26116.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signalling. Annu Rev Cell Dev Biol *12*, 463-519.

Bützow, R., Fukushima, D., Twardzik, D., and Ruoslahti, E. (1993). A 60-kD protein mediates the binding of transforming growth factor-beta to cell surface and extracellular matrix proteoglycan. J Cell Biol *122*, 721-727.

Cai, J., and Jones, D. (1998). Superoxide in apoptosis: mitochondrial generation triggered by cytochrome c loss. J Biol Chem *273*, 11401-11404.

Cannon-Albright, L., Goldgar, D., Meyer, L., Lewis, C., Anderson, D., Fountain, J., Hegi, M., Wiseman, R., Petty, E., Bale, A., and co-workers (1992). Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-p22. Science *258*, 1148-1152.

Cannon-Albright, L., Goldgar, D., Neuhausen, S., Gruis, N., Anderson, D., Lewis, C., Jost, M., Tran, T., Nyguen, K., Kamb, A., and co-workers (1994). Localization of the 9p melanoma susceptibility locus (MLM) to a 2-cM region between D9S736 and D9S171. Genomics *23*, 265-268.

Carpenter, G., and Ji, Q. (1999). Phospholipase C-gamma as a signal-transducing element. Exp Cell Res *253*, 15-24.

Cawley, E. (1952). Genetic aspects of malignant melanoma. Arch Dermatol Forsch *65*, 440-450.

Chen, C.-S., Lavker, R., Rodeck, U., Risse, B., and Jensen, P. (1995). Use of a serum-free epidermal culture model to show deleterious effects of epidermal growth factor on morphogenesis and differentiation. J Invest Dermatol *104*, 107-112.

Chicurel, M., Singer, R., Meyer, C., and Ingber, D. (1998). Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. Nature *392*, 730-733.

Chinnaiyan, A., Tepper, C., Seldin, M., O'Rourke, K., Kischkel, F., Hellbardt, S., Krammer, P., Peter, M., and Dixit, V. (1996). FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem *271*, 4961-4965.

Cleary, E., and Gibson, M. (1996). Elastic tissue, elastin and elastin associated microfibrils. In Extracellular matrix, W. Comper, ed. (Amsterdam: Harwood), pp. 95-140.

Clutton, S. (1997). The importance of oxidative stress in apoptosis. Br Med Bull *53*, 662-668.

Cohen, G. (1997). Caspases: the executioners of apoptosis. Biochem J *326*, 1-16.

Coutts, J., and Gallagher, J. (1995). Receptors for fibroblast growth factors. Immunol Cell Biol *73*, 584-589.

Cowin, P., and Burke, B. (1996). Cytoskeleton-membrane interactions. Curr Opin Cell Biol *8*, 56-65.

Crook, N., Clem, R., and Miller, L. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol *67*, 2168-2174.

Cryns, V., and Yuan, J. (1998). Proteases to die for. Genes Dev *12*, 1551-1570.

Dagnino, L., Pietenpol, J., Yang, E., and Moses, H. (1993). Transforming growth factor regulation of keratinocyte growth. Recent Res Cancer Res *128*, 15-29.

Danno, K., and Horio, T. (1987). Sunburn cell: factors involved in its formation. Photochem Photobiol *45*, 683-690.

Davis, G. (1992). Affinity of integrins for damaged extracellular matrix: αvβ3 binds to denatured collagen type I through RGD sites. Biochem Biophys Res Comm *182*, 1025-1031.

Delli-Bovi, P., Curatola, A., Kern, F., Greco, A., Ittman, M., and Basilico, C. (1987). An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell *50*, 729-737.

Desangher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol *144*, 891-901.

Deveraux, Q., Roy, N., Stennicke, H., Van Arsdale, T., Zhou, Q., Srinivasula, S., Alnemri, E., Salvesen, G., and Reed, J. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J *17*, 2215-2223.

Dionne, C., Crumley, G., Bellot, F., Kaplow, J., Searfoss, G., Ruta, M., Burgess, W., Jaye, M., and Schlessinger, J. (1990). Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J *9*, 2685-2692.

Dmitrovsky, E., Moy, D., Miller Jr, W., Li, A., and Masui, H. (1990). Retinoic acid causes a decline in TGF-alpha expression, cloning efficiency, and tumorigenicity in a human embryonal cancer cell line. Oncogene Res *5*, 233-239.

Dubois, C., Laprise, M., Blanchette, F., Gentry, L., and Leduc, R. (1995). Processing of transforming growth factor-beta1 precursor by human furin convertase. J Biol Chem *270*, 10618-10624.

Dustin, M., and Springer, T. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. Annu Rev Immunol *8*, 27-66.

Duvall, E., and Wyllie, A. (1986). Life and death of the cell. Immunol Today *7*, 115-119.

Easton, D., Cox, G., Macdonald, A., and Ponder, B. (1991). Genetic susceptibility to naevi-a twin study. Br J Cancer *64*, 1164-1167.

Edelman, G., and Crossin, K. (1991). Cell adhesion molecules: implications for a molecular histology. Annu Rev Biochem *60*, 155-190.

Eilers, M., Picard, D., Yamamoto, K., and Bishop, M. (1989). Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature *340*, 66-68.

Ellis, R., Yuan, J., and Horwitz, H. (1991). Mechanisms and functions of cell death. Annu Rev Cell Biol *7*, 663-698.

Elsdale, T., and Bard, J. (1972). Collagen substrata for studies on cell behavior. J Cell Biol *54*, 626-637.

Ernst, S., Langer, R., Cooney, C., and Sasisekharan, R. (1995). Enzymatic degradation of glycosaminoglycans. Crit Rev Biochem Mol Biol *30*, 387-444.

Evan, G., Wyllie, A., Gilbert, C., Littlewood, T., Land, H., Brooks, M., Waters, C., Penn, L., and Hancock, D. (1992). Induction of apoptosis in fibroblasts by c-Myc protein. Cell *63*, 119-125.

Ewen, M., Sluss, H., Whitehouse, L., and Livingston, D. (1993). TGF-beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. Cell *74*, 1009-1020.

Fabregat, I., Sánchez, A., Alvarez, A., Nakamura, T., and Benito, M. (1996). Epidermal growth factor, but not hepatocyte growth factor, suppresses the apoptosis induced by transforming growth factor-beta in fetal hepatocytes in primary cultures. FEBS Lett *384*, 14-18.

Faham, S., Hileman, R., Fromm, J., Linhardt, R., and Rees, D. (1996). Heparin structure and interactions with basic fibroblast growth factor. Science *271*, 1116-1120.

Ferrell, J. J. (1996). MAP kinases in mitogenesis and development. Curr Topics Dev Biol *33*, 1-60.

Fesus, L., Davies, P., and Piacentini, M. (1991). Apoptosis: molecular mechanisms in programmed cell death. Eur J Cell Biol *56*, 170-177.

Filmus, J., and Kerbel, R. (1993). Development of resistance mechanisms to the growth-inhibitory effects of transforming growth factor-beta during tumor progression. Curr Opin Oncol *5*, 123-129.

Finnish Cancer Registry (2000). Cancer incidence in Finland in 1996 and 1997. Cancer Society of Finland Publication *61*.

Flaumenhaft, R., and Rifkin, D. (1992). The extracellular regulation of growth factor action. Mol Biol Cell *3*, 1057-1065.

Fogh, J., and Trempe, G. (1975). New human tumor cell lines. In Human tumor cells in vitro, J. Fogh, ed. (New York: Plenum Press), pp. 115-159.

Folkman, J., Haudenschild, C., and Zetter, B. (1979). Long-term culture of capillary endothelial cells. Proc Natl Acad Sci USA *76*, 5217-5721.

Fukuda, K., Kojiro, M., and Chiu, J.-F. (1993). Induction of apoptosis by transforming growth factor-beta1 in the rat hepatoma cell line McA-RH7777: a possible association with tissue transglutaminase expression. Hepatology *18*, 945-953.

Galbraith, C., and Sheetz, M. (1998). Forces on adhesive contacts affect cell function. Curr Opin in Cell Biol *10*, 566-571.

Garrington, T., and Johnson, G. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol *11*, 211-218.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol *119*, 493-501.

Gentry, L., Lioubin, M., Purchio, A., and Marquardt, H. (1988). Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. Mol Cell Biol *8*, 4162-4168.

George, E., Georges-Labouesse, E., Patel-King, R., Rayburn, H., and Hynes, R. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development *119*, 1079-1091.

Gilchrest, B., Park, H.-Y., Eller, M., and Yaar, M. (1998). The photobiology of the tanning responce. In The pigmentary system: physiology and pathophysiology, J. Nordlund, R. Boissy, V. Hearing, R. King and J.-P. Ortonne, eds. (New York: Oxford University Press), pp. 359-372.

Giles, G., Armstrong, B., Burton, R., Staples, M., and Thursfield, V. (1996). Has mortality from melanoma stopped rising in Australia? Analysis of trends between 1931 and 1994. Br Med J *312*, 1121-1125.

Glass, A., and Hoover, R. (1989). The emerging epidemic of melanoma and squamous cell skin cancer. JAMA *262*, 2097-2100.

Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry *13*, 2633-2637.

Glücksmann, A. (1950). Cell deaths in normal vertebrate ontogeny. Biol Rev Cambridge Philos Soc *26*, 59-86.

Gniadecka, M., Wulf, H., Mortensen, N., and Poulsen, T. (1996). Photoprotection in vitiligo and normal skin: a quantitative assessment of the role of stratum corneum, viable epidermis and pigmentation. Acta Dermatol Venereol *76*, 429-432.

Goding, C., and Fisher, D. (1997). Regulation of melanocyte differentiation and growth. Cell Growth Differ *8*, 935-940.

Gohring, F., Schwab, B., Nicotera, P., Leist, M., and Fackelmayer, F. (1997). The novel SAR-binding domain of scaffold attachment factor A (SAF-A) is a target in apoptotic nuclear breakdown. EMBO J *16*, 7361-7371.

Goldstein, A., Dracopoli, N., Ho, E., Fraser, M., Kearns, K., Bale, S., McBride, O., Clark, W. J., and Tucker, M. (1993). Further evidence for a locus for cutaneous malignant melanoma-dysplastic nevus (CMM/DN) on chromosome 1p, and evidence for genetic heterogeneity. Am J Hum Genet *52*, 537-550.

Goping, I., Gross, A., Lavoie, J., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S., and Shore, G. (1998). Regulated targeting of BAX to mitochondria. J Biol Chem *274*, 2225-2233.

Gougeon, M.-L., and Montagnier, L. (1993). Apoptosis in AIDS. Science *260*, 1269-1270.

Grant, D., Leblond, C., Kleinman, H., Inoue, S., and Hassell, J. (1989). The incubation of laminin, collagen IV, and heparan sulfate proteoglycan at 35°C yields basement membrane-like structures. J Cell Biol *108*, 1567-1574.

Greene, M. (1997). Genetics of cutaneous melanoma and nevi. Mayo Clin Proc *72*, 467-474.

Greene, M., Goldin, L., Clark Jr, W., Lovrien, E., Kraemer, K., Tucker, M., Elder, D., Fraser, M., and Rowe, S. (1983). Familial cutaneous malignant melanoma: autosomal dominant trait possibly linked to the Rh locus. Proc Natl Acad Sci USA *80*, 6071-6075.

Griffith, T., and Lynch, D. (1998). TRAIL: a molecule with multiple receptors and control mechanisms. Curr Opin Immunol *10*, 559-563.

Gross, A., McDonnell, J., and Korsmeyer, S. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*, 1899-1911.

Grossman, D., McNiff, J., Fengzhi, L., and Altieri, D. (1999). Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol *113*, 1076-1081.

Groux, H., Torpier, G., Monte, D., Mouton, Y., Capron, A., and JC, A. (1992). Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med *175*, 331-340.

Haapajärvi, T., Pitkänen, K., and Laiho, M. (1999). Human melanoma cell line UV responses show independency of p53 function. Cell Growth Differ *10*, 163-171.

Halaban, R. (1996). Growth factors and melanomas. Seminars Oncol *23*, 673-681.

Halaban, R., Ghosh, S., and Baird, A. (1987). bFGF is the putative natural growth factor for human melanocytes. In Vitro Cell Dev Biol *23*, 47-51.

Halaban, R., Langdon, R., Birchall, N., Cuono, C., Baird, A., Scott, G., Moellman, G., and McGuire, J. (1988). Paracrine stimulation of melanocytes by keratinocytes through basic fibroblast growth factor. Ann NY Acad Sci *548*, 180-190.

Hamel, P., and Hanley-Hyde, J. (1997). G1 cyclins and control of the cell division cycle in normal and transformed cells. Cancer Invest *15*, 143-152.

Handler, M., Yurchenco, P., and Iozzo, R. (1997). Developmental expression of perlecan during murine embryogenesis. Dev Dyn *210*, 130-145.

Hannon, G., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta -induced cell cycle arrest. Nature *371*, 257-261.

Hara, M., Yaar, M., Tang, A., Eller, M., Reenstra, W., and Gilchrest, B. (1994). Role of integrins in melanocyte attachment and dendricity. J Cell Sci *107*, 2739-2748.

Heintz, N. (1993). Cell death and the cell cycle: a relationship between transformation and neurodegeneration? Trends Biochem Sci *18*, 157-159.

Heldin, C., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.

Henkel, W., and Glanville, R. (1982). Covalent crosslinking between molecules of type I and type III collagen. The involvement of the N-terminal, nonhelical regions of the alpha1(I) and alpha1(III) chains in the formation of intermolecular crosslinks. Eur J Biochem *122*, 205-213.

Hildebrand, A., Romaris, M., Rasmussen, L., Heinegård, D., Twardzik, D., Border, W., and Ruoslahti, E. (1994). Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J *302*, 527-534.

Hirsch, T., Marzo, I., and Kroemer, G. (1997). Role of the mitochondrial transition pore in apoptosis. Biosci Rep *17*, 67-76.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). p53 mutations in human cancers. Science *253*, 49-53.

Holman, C., Armstrong, B., and Heenan, P. (1986). Relationship of cutaneous malignant melanoma to individual sunlight-exposure habits. J Natl Cancer Inst *76*, 403-414.

Howe, P., Draetta, G., and Leof, E. (1991). Transforming growth factor-beta1 inhibition of p34cdc2 phosphorylation and histone H1 kinase activity is associated with G1/S-phase growth arrest. Mol Cell Biol *11*, 1185-1194.

Hsu, M.-Y., Shih, D.-T., Meier, F., Van Belle, P., Hsu, J.-Y., Elder, D., Buck, C., and Herlyn, M. (1998). Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma. Am J Pathol *153*, 1435-1442.

Hsu, M.-Y., Wheelock, M., Johnson, K., and Herlyn, M. (1996). Shifts in cadherin profiles between human normal keratinocytes and melanomas. J Invest Dermatol Symp Proc *1*, 188-194.

Hsu, Y., Wolter, K., and Youle, R. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci *94*, 3668-3672.

Huang, D., O'Reilly, L., Strasser, A., and Cory, S. (1997). The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. EMBO J *16*, 4628-4638.

Hughes, P., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J., and Shattil, S. (1996). Breaking the integrin hinge: a defined structural constraint regulates integrin signaling. J Biol Chem *271*, 6571-6574.

Hughes, P., Renshaw, M., Pfaff, M., Forsyth, J., Keivens, V., Schwartz, M., and Ginsberg, M. (1997). Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. Cell *88*, 521-530.

Hunter, T., and Pines, J. (1994). Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell *79*, 573-582.

Hurlbert, M., Gianani, R., Hutt, C., Freed, C., and Kaddis, F. (1999). Neural transplantation of hNT neurons for Huntington's disease. Cell Transplantation *8*, 143-151.

Hurlimann, J., Larrinaga, B., and Vala, D. (1995). bcl-2 protein in invasive ductal breast carcinomas. Virchows Arch *426*, 163-168.

Hussussian, C., Struewing, J., Goldstein, A., Higgins, P., Ally, D., Sheahan, M., Clark, W. J., Tucker, M., and Dracopoli, N. (1994). Germline p16 mutations in familial melanoma. Nature Genet *8*, 15-21.

Hynes, R. (1990). Fibronectins (Berlin Heidelberg New York: Springer).

Hynes, R. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Iozzo, R. (1998). Matrix proteoglycans: from molecular design to cellular function. Annu Rev Biochem *67*, 609-52.

Isacson, O. (1993). On neuronal health. Trends Neurosci *16*, 306-308.

Iscovich, J., Andreev, H., and Steinitz, R. (1995). Incidence of cutaneous malignant melanoma in Israel, 1960-1989. Public Health Rev *23*, 1-24.

Ishai-Michaeli, R., Eldor, A., and Vlodavsky, I. (1990). Heparanase activity expressed by platelets, neutrophils and lymphoma cells releases active fibroblast growth factor from extracellular matrix. Cell Regul *1*, 833-842.

Islam, K., Kayanoki, Y., Kaneto, H., Suzuki, K., Asahi, M., Fujii, J., and Taniguchi, N. (1997). TGF-b1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase. Free Radic Biol Med *22*, 1007-1017.

Itoh, N., Mima, T., and Mikawa, T. (1996). Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. Development *122*, 291-300.

Jacks, T., Remington, L., Williams, B., Schmitt, E., Halachmi, S., Bronson, R., and Weinberg, R. (1994). Tumour spectrum analysis in p53 mutant mice. Curr Biol *4*, 1-7.

Jimbow, K., Quevado, W., Fitzpatrick, T., and Szabo, G. (1993). Biology of melanocytes. In Dermatology in general medicine, T. Fitzpatrick, A. Eisen, K. Wolff, I. Freedberg and K. Austen, eds. (New York: McGraw-Hill), pp. 261-289.

Joensuu, H., Pylkkanen, L., and Toikkanen, S. (1994). Bcl-2 protein expression and long-term survival in breast cancer. Am J Pathol *145*, 1191-1198.

Jones, J., Gockerman, A., Busby, W., Camacho-Hubner, C., and Clemmons, D. (1993). Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol *121*, 679-687.

Joukov, V., Kumar, V., Sorsa, T., Arighi, E., Weich, H., Saksela, O., and Alitalo, K. (1998). A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. J Biol Chem *273*, 6599-6602.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Kalkkinen, N., and Alitalo, K. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA *93*, 2576-2581.

Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N., and Alitalo, K. (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J *16*, 3898-3911.

Jäättelä, M. (1999). Escaping cell death: survival proteins in cancer. Exp Cell Res *248*, 30-43.

Jäättelä, M., Wissing, D., Bauer, P., and Li, G. (1992). Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. EMBO J *11*, 3507-3512.

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T., and Egeblad, M. (1998). Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J *17*, 6124-6134.

Kamb, A., Gruis, N., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S., Stockert, E., Day, R. r., Johnson, B., and Skolnick, M. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science *264*, 436-440.

Kan, M., Wang, F., To, B., Gabriel, J., and McKeehan, W. (1996). Divalent cations and heparin/heparan sulfate cooperate to control assembly and activity of the fibroblast growth factor receptor complex. J Biol Chem *271*, 26143-26148.

Kan, M., Xiaochong, W., Wang, F., and McKeehan, W. (1999). Specifity for fibroblast growth factors determined by heparan sulfate in a binary complex with the receptor kinase. J Biol Chem *274*, 15947-15952.

Kanai, M., Goke, M., Tsunekawa, S., and Podolsky, D. (1997). Signal transduction pathway of human fibroblast growth factor receptor 3. Identification of a novel 66-kDa phosphoprotein. J Biol Chem *272*, 6621-6628.

Kanzaki, T., Olofsson, A., Moren, A., Wernstedt, C., Hellman, U., Miyazono, K., Claesson-Welsh, L., and Heldin, C.-H. (1990). TGF-beta1 binding protein: a component of the large latent complex of TGF-beta1 with multiple repeat sequences. Cell *61*, 1051-1061.

Karonen, T., Jeskanen, L., and Keski-Oja, J. (1997). Transforming growth factor beta 1 and its latent form binding protein-1 associate with elastic fibres in human dermis: accumulation in actinic damage and absence in anetoderma. Br J Dermatol *137*, 51-58.

Kayanoki, Y., Fujii, J., Suzuki, K., Kawata, S., Matsuzawa, Y., and Taniguchi, N. (1994). Suppression of antioxidative enzyme expression by transforming growth factor-b1 in rat hepatocytes. J Biol Chem *269*, 15488-15492.

Kelly, J., Rivers, J., MacLennan, R., Harrison, S., Lewis, A., and Tate, B. (1994). Sunlight: a major factor associated with the development of melanocytic nevi in Australian schoolchildren. J Am Acad Dermatol *40*, 40-48.

Kerr, J., Wyllie, A., and Currie, A. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Kielty, C., Whittaker, S., Grant, M., and Shuttlewoorth, C. (1992). Type VI collagen microfibrils: evidence for a structural association with hyaluronan. J Cell Biol *118*, 979-990.

Kilkenny, D., and Hill, D. (1996). Perinuclear localization of an intracellular binding protein related to the fibroblast growth factor (FGF) receptor 1 is temporally associated with the nuclear trafficking of FGF-2 in proliferating epiphyseal growth plate chondrocytes. Endocrinology *137*, 5078-5089.

King, R., Jackson, P., and Kirschner, M. (1994). Mitosis in transition. Cell *79*, 563-571.

Kleppner, S., Robinson, K., Trojanowski, J., and Lee, V. (1995). Transplanted human neurons derived from a teratocarcinoma cell line (NTERA2) mature, integrate, and survive for over 1 year in the nude mouse brain. J Comp Neurol *357*, 618-632.

Klint, P., and Claesson-Welsh, L. (1999). Signal transduction by fibroblast growth factor receptors. Front Biosci *4*, d165-177.

Kobayashi, N., Nakagawa, A., Muramatsu, T., Yamashina, Y., Shirai, T., Hashimoto, M., Ishigaki, Y., Ohnishi, T., and Mori, T. (1998). Supranuclear melanin caps reduce ultraviolet induced DNA photoproducts in human epidermis. J Invest Dermatol *110*, 806-810.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J., and Massagué, J. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-beta. Science *260*, 536-539.

Koh, H., Geller, A., Miller, D., Davis, B., and Lew, R. (1995). Skin cancer: prevention and control. In Cancer prevention and control, P. Greenwald, B. Kramer and W. DL, eds. (New York: Marcel Dekker), pp. 611-640.

Kouhara, K., Hadari, Y., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., Lax, I., and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell *89*, 693-702.

Krajewski, S., Blomqvist, C., Franssila, K., Krajewska, M., Wasenius, V., Niskanen, E., Nordling, S., and Reed, J. (1995). Reduced expression of proapoptotic gene BAX is associated with poor responce rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res *55*, 4471-4478.

Krasagakis, K., Krüger-Krasagakes, S., Fimmel, S., Eberle, J., Thölke, D., Ohe von der, M., Mansmann, U., and Orfanos, C. (1999). Desensitization of melanoma cells to autocrine TGF-beta isoforms. J Cell Physiol *178*, 179-187.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J., and Mignotte, B. (1995). The biochemistry of programmed cell death. FASEB J *9*, 1277-1287.

Kuida, K., Lippke, J., Ku, G., Harding, M., Livingston, D., Su, M., and Flavell, R. (1995). Altered cytokine export and apoptosis in mice deficient in interleukin-1b converting enzyme. Science *267*, 2000-2003.

Kuzis, K., Coffin, J., and Eckenstein, F. (1999). Time course and age dependence of motor neuron death following facial nerve crush injury: role of fibroblast growth factor. Exp Neurol *157*, 77-87.

LaCasse, E., Baird, S., Korneluk, R., and MacKenzie, A. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene *17*, 3247-3259.

Lafon, C., Mathieu, C., Guerrin, M., Pierre, O., Vidal, S., and Valette, A. (1996). Transforming growth factor b1-induced apoptosis in human ovarian carcinoma cells: protection by the antioxidant N-acetylcysteine and Bcl-2. Cell Growth Differ *7*, 1095-1104.

Laiho, M., DeCaprio, J., Ludlow, J., Livingston, D., and Massagué, J. (1990). Growth inhibition by TGF-beta linked to suppression of retinoblastoma protein phosphorylation. Cell *62*, 175-185.

Landry, J., Bernie, R., Chértien, P., Nicole, L., Tanguay, R., and Marceau, N. (1982). Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res *42*, 2457-2461.

Landry, J., Crétien, P., Lambert, H., and Hickey, E. (1989). Heat shock resistance cenferred by expression of the human HSP27 gene in rodent cells. J Cell Biol *109*, 7-15.

Lasky, L. (1995). Selectin-carbohydrate interactions and the initiation of the inflammatory response. Annu Rev Biochem *64*, 113-139.

LeBel, C., Ishiropoulos, H., and Bondy, S. (1992). Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol *5*, 227-231.

Lee, J., Abrahamson, J., Kandel, R., Donehower, L., and Bernstein, A. (1994). Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene *9*, 3731-3736.

Leek, R., Kaklamanis, L., Pezzella, F., Gatter, K., and Harris, A. (1994). bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor-negative tumours and in situ cancer. Br J Cancer *69*, 135-139.

Li, C.-Y., Suardet, L., and Little, J. (1995). Potential role of WAF/Cip1/p21 as a mediator of TGF-beta cytoinhibitory effect. J Biol Chem *270*, 4971-4974.

Li, F., Ambrosini, G., Chu, E., Plescia, J., Tognin, S., Marchisio, P., and Altieri, D. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. Nature *396*, 580-584.

Li, G., Li, L., Liu, Y.-K., Mak, J., Chen, L., and Lee, W. (1991). Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc Natl Acad Sci USA *88*, 1681-1685.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., and Salfeld, J. (1995). Mice deficient in IL-1b-converting enzyme are defective in production of mature IL-1b and resistant to endotoxic shock. Cell *80*, 401-411.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S., Ahmad, M., Alnemri, E., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Lin, H., and Lodish, H. (1993). Receptors for the TGF-beta superfamily: multiple polypeptides and serine/threonine kinases. Trends Cell Biol *3*, 14-19.

Liu, M., Dhanwada, K., Birt, D., Hecht, S., and Pelling, J. (1994). Increase in p53 protein half-life in mouse keratinocytes following UV-B irradiation. Carcinogenesis *15*, 1089-1092.

Loftus, J., and Liddington, R. (1997). New insights into integrin-ligand interaction. J Clin Invest *99*, 2302-2306.

López-Casillas, F., Cheifetz, S., Doody, J., Andres, J., Lane, W., and Massagué, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell *67*, 785-795.

López-Casillas, F., Payne, H., Andres, J., and Massagué, J. (1994). Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol *124*, 557-568.

López-Casillas, F., Wrana, J., and Massagué, J. (1993). Betaglycan presents ligand to the TGF-beta1 signaling receptor. Cell *73*, 1435-1444.

Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999). The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. Immunity *10*, 629-639.

Luther, H., Altmeyer, P., Garbe, C., Ellwanger, U., Jahn, S., Hoffmann, K., and Segerling, M. (1996). Increase of melanocytic nevus counts in children during 5 years of follow-up and analysis of associated factors. Acta Dermatol *132*, 1473-1478.

Lyons, S., and Clarke, A. (1997). Apoptosis and carcinogenesis. Br Med Bull *52*, 554-569.

Maccarana, M., Casu, B., and Lindahl, U. (1993). Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor. J Biol Chem *268*, 23898-23905.

Martin, G. (1980). Teratocarcinomas and mammalian embryogenesis. Science *209*, 768-775.

Massagué, J. (1990). The transforming growth factor-beta family. Annu Rev Cell Biol *6*, 597-641.

Massagué, J. (1996). TGF-beta signaling: receptors, transducers, and Mad proteins. Cell *85*, 947-950.

Massagué, J. (1998). TGF-beta signal transduction. Annu Rev Biochem *67*, 753-791.

Matsui, Y., Zsebo, K., and Hogan, B. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841-847.

Mauch, C., Hatamochi, A., Scharffetter, K., and Krieg, T. (1988). Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. Exp Cell Res *178*, 493-503.

McCarthy, K., Abrahamson, D., Bynum, K., St. John, P., and Couchman, J. (1994). Basement membrane-specific chondroitin sulfate proteoglycan is abnormally associated with the glomerular capillary basement membrane of diabetic rats. J Histochem Cytochem *42*, 473-484.

McCarthy, K., and Couchman, J. (1990). Basement membrane chondroitin sulfate proteoglycans: localization in adult rat tissues. J Histochem Cytochem *38*, 1479-1486.

McDonnell, T., Deane, N., Platt, F., Nunez, G., Jaeger, U., and McKearn, J. (1989). Bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell *57*, 79-88.

McDonnell, T., and Korsmeyer, S. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature *349*, 254-256.

McFarlane, S., Cornel, E., Amaya, E., and Holt, C. (1996). Inhibition of FGF receptor activity in retinal ganglion cell axons causes errors in target recognition. Neuron *17*, 245-254.

Mecham, R., and Davis, E. (1994). Elastic fiber structure and assembly. In Extracellular Matrix Assembly and Structure, P. Yurchenco, D. Birk and R. Mecham, eds. (San Diego, CA: Academic Press), pp. 281-314.

Meier, F., Nesbit, M., Hsu, M.-Y., Martin, B., Van Belle, P., Elder, D., Schaumburg-Lever, G., Garbe, C., Walz, T., Donatien, P., Crombleholme, T., and Herlyn, M. (2000). Human melanoma progression in skin reconstructs: biological significance of bFGF. Am J Pathol *156*, 193-200.

Meier, F., Satyamoorthy, K., Nesbit, M., Hsu, M.-Y., Schittek, B., Garbe, C., and Herlyn, M. (1998). Molecular events in melanoma development and progression. Frontiers Biosci *3*, 1005-1010.

Mendler, M., Eich-Bender, S., Vaughan, L., Winterhalter, K., and Bruckner, P. (1989). Cartilage contain mixed fibrils of collagen type II, IX and XI. J Cell Biol *108*, 191-197.

Miller, W. J., Moy, D., Li, A., Grippo, J., and Dmitrovsky, E. (1990). Retinoic acid induces down-regulation of several growth factors and proto-oncogenes in a human embryonal cancer cell line. Oncogene *5*, 511-517.

Miyake, K., Kimura, S., Nakanishi, M., Hisada, A., Hasegawa, M., Nagao, S., and Abe, Y. (2000). Transforming growth factor-beta1 stimulates contraction of human glioblastoma cell-mediated collagen lattice through enhanced alpha2 integrin expression. J Neuropathol Exp Neurol *59*, 18-28.

Miyamoto, S., Akiyama, S., and Yamada, K. (1995a). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science *267*, 883-885.

Miyamoto, S., Teramoto, H., Coso, O., Gutkind, J., Burbelo, P., Akiyama, S., and Yamada, K. (1995b). Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J Cell Biol *131*, 791-805.

Miyazono, K., Hellman, U., Wernstedt, C., and Heldin, C.-H. (1988). Latent high molecular weight complex of transforming growth factor-beta1. Purification from human platelets and structural characterization. J Biol Chem *263*, 6407-6415.

Miyazono, M., Lee, V., and Trojanowski, J. (1995). Proliferation, cell death, and neuronal differentiation in transplanted human embryonal carcinoma (NTERA2) cells depend on the graft site in nude and severe combined immunodeficient mice. Lab Invest *73*, 273-283.

Miyazono, M., Nowell, P., Finan, J., Lee, V., and Trojanowski, J. (1996). Long-term integration and neuronal differentiation of human embryonal carcinoma cells (NTERA2) transplanted into the caudoputamen of nude mice. J Comp Neurol *376*, 603-613.

Mizuno, K., Inoue, H., Hagiya, M., Shimizu, S., Nose, T., Shimohigashi, Y., and Nakamura, T. (1994). Hairpin loop and second kringle domain are essential sites for heparin binding and biological activity of hepatocyte growth factor. J Biol Chem *269*, 1131-1136.

Moasser, M., DeBlasio, A., and Dmitrovsky, E. (1994). Response and resistance to retinoic acid are mediated through the retinoic acid nuclear receptorgamma in human teratocarcinomas. Oncogene *9*, 833-840.

Moasser, M., Reuter, V., and Dmitrovsky, E. (1995). Overexpression of the retinoic acid receptor gamma directly induces terminal differentiation of human embryonal carcinoma cells. Oncogene *10*, 1537-1543.

Morrison, R. S., Sharma, A., De Vellis, J., and Bradshaw, R. A. (1986). Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. Proc Natl Acad Sci USA *83*, 7537-7541.

Mosser, D., Caron, A., Bourget, L., Denis-Larose, C., and Massie, B. (1997). Role of human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol Cell Biol *17*, 5317-5327.

Moulton, B. (1994). Transforming growth factor-beta stimulates endometrial stromal apoptosis in vitro. Endocrinology *134*, 1055-1060.

Neilson, K., and Friesel, R. (1996). Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. J Biol Chem *271*, 25049-25057.

Nelemans, P., Groenendal, H., Kiemeney, L., Rampen, F., Ruiter, D., and Verbeek, A. (1993). Effect of intermittent exposure to sunlight on melanoma risk among indoor workers and sun-sensitive individuals. Environ Health Perspect *101*, 252-255.

Nesbit, M., Nesbit, H., Bennett, J., Andl, T., Hsu, M.-Y., Dejesus, E., McBrian, M., Gupta, A., Eck, S., and Herlyn, M. (1999). Basic fibroblast growth factor induces a transformed phenotype in normal human melanocytes. Oncogene *18*, 6469-6476.

Nicholson, D., and Thornberry, N. (1997). Caspases: killer proteases. Trends Biochem Sci *22*, 299-306.

Nollen, E., Brunsting, J., Roelofsen, H., Weber, L., and Kampinga, H. (1999). In vivo chaperone activity of heat shock protein 70 and thermotolerance. Mol Cell Biol *19*, 2069-2079.

Oberhammer, F., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A., Burch, W., and Schulte-Hermann, R. (1992). Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta1. Proc Natl Acad Sci USA *89*, 5408-5412.

Oda, T., Kanai, Y., Oyama, T., Yoshiura, K., Shimoyama, Y., Birchmeier, W., Sugimura, T., and Hirohashi, S. (1994). E-cadherin gene mutations in human gastric carcinoma cell lines. Proc Natl Acad Sci USA *91*, 1858-1862.

Ofner, D., Riehemann, K., Maier, H., Riedmann, B., Nehoda, H., Totsch, M., Bocker, W., Jasani, B., and Schmid, K. (1995). Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumour stage and patient survival. Br J Cancer *72*, 981-985.

Ogura, E., Senzaki, H., Yamamoto, D., Yoshida, R., Takada, H., Hioki, K., and Tsubura, A. (1999). Prognostic significance of Bcl-2, Bcl-xL/S, Bax and Bak expressions in colorectal carcinomas. Oncol Rep *6*, 365-369.

Ornitz, D., Xu, J., Colvin, J., McEwen, D., MacArthur, C., Coulier, F., Gao, G., and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. J Biol Chem *271*, 15292-15297.

Panayotou, G., End, P., Aumailley, M., Timpl, R., and Engel, J. (1989). Domains of laminin with growth-factor activity. Cell *56*, 93-101.

Papaioannou, V., McBurney, M., Gardner, R., and Evans, M. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. Nature *258*, 70-73.

Pardee, A. (1989). G1 events and regulation of cell proliferation. Science *246*, 603-608.

Park, J., Keller, G., and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell *4*, 1317-1326.

Parkin, D., Muir, C., Whelan, S., Gao, Y., Ferlay, J., and Powell, J. (1992). Cancer incidence in five continents (Lyon, France: International Agency for Research on Cancer).

Partanen, J., Makela, T., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L., and Alitalo, K. (1991). FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. EMBO J *10*, 1347-1354.

Pataky, D., Borisoff, J., Fernandes, K., Tetzlaff, W., and Steeves, J. (2000). Fibroblast growth factor treatment produces differential effects on survival and neurite outgrowth from identified bulbospinal neurons in vitro. Exp Neurol *163*, 357-372.

Pathak, M. (1995). Functions of melanin and protection by melanin. In Melanin: its role in human photoprotection, L. Zeise, M. Chedekel and T. Fitzpatrick, eds. (Overland Park, KA: Valdenmar Publishing), pp. 125-134.

Paulsson, M., Yurchenco, P., Ruben, G., Engel, J., and Timpl, R. (1987). Structure of low density heparan sulfate proteoglycan isolated from a mouse tumor basement membrane. J Mol Biol *197*, 297-313.

Pavlotsky, F., Azizi, E., Gurvich, R., Lusky, A., Barell, V., Weiner, M., and Iscovich, J. (1997). Prevalence of melanocytic nevi and freckles in young Israeli males: correletion with melanoma incidence in Jewish migrants: demographic and host factors. Am J Epidemiol *146*, 78-86.

Pines, J. (1995). Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J *308*, 697-711.

Plotnikov, A., Hubbard, S., Schlessinger, J., and Mohammadi, M. (2000). Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specifity. Cell *101*, 413-424.

Polyak, K., Kato, J., Solomon, M., Sherr, C., Massagué, J., Roberts, J., and Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev *8*, 9-22.

Porter, P., Sage, E., Lane, T., Funk, S., and Gown, A. (1995). Distribution of SPARC in normal and neoplastic human tissue. J Histochem Cytochem *43*, 791-800.

Préville, X., Salvemini, P., Giraud, S., Chaufour, S., Paul, C., Stepien, G., Ursini, M., and Arrigo, A. (1999). Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. Exp Cell Res *247*, 61-78.

Purdie, C., Harrison, D., Peter, A., Dobbie, L., White, S., Howie, S., Salter, D., Bird, C., Wyllie, A., Hooper, M., and co-workers (1994). Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. Oncogene *9*, 603-609.

Raab, G., and Klagsbrun, M. (1997). Heparin-binding EGF-like growth factor. Biochim Biophys Acta *9*, F179-199.

Raghunath, M., Unsold, C., Kubitscheck, U., Bruckner-Tuderman, U., Peters, R., and Meuli, M. (1998). The cutaneous microfibrillar apparatus contains latent transforming growth factor-beta binding protein-1 (LTBP-1) and is a repository for latent TGF-beta 1. J Invest Dermatol *111*, 559-564.

Raines, E., and Ross, R. (1992). Compartmentalization of PDGF on extracellular binding sites dependent on exon-6-encoded sequences. J Cell Biol *116*, 533-543.

Reichman-Fried, M., and Shilo, B. (1995). Breathless, a Drosophila FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. Mech Dev *52*, 265-273.

Rest van der, M., and Garrone, R. (1992). The collagen family of proteins. FASEB J *5*, 2814-2823.

Rieger, E., Hofmann-Wellenhof, R., Soyer, H., Kofier, R., Cerroni, L., Smolle, J., and Kerl, H. (1993). Comparison of proliferative activity as assessed by proliferating cell nuclear antigen (PCNA) and Ki-67 monoclonal antibodies in melanocytic lesions. A quantitative immunohistochemical study. J Cutan Pathol *29*, 229-236.

Riley, P. (1997). Melanin. Int J Biochem Cell Biol *29*, 1235-1239.

Risinger, J., Berchuck, A., Kohler, M., and Boyd, J. (1994). Mutations of the E-cadherin gene in human gynecologic cancers. Nat Genet *7*, 98-102.

Roberts, A., Sporn, M., Assoian, R., Smith, J., Roche, N., Wakefield, L., Heine, U., Liotta, L., Falanga, V., Kehrl, J., and Fauci, A. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA *83*, 4167-4171.

Roberts, R., Gallagher, J., Spooncer, E., Allen, T., Bloomfield, F., and Dexter, T. (1988). Heparan sulphate bound growth factors: a mechanism for stromal cell midiated haematopoiesis. Nature *332*, 376-378.

Rodeck, U., Bossler, A., Graeven, U., Fox, F., Nowell, P., Knabbe, C., and Kari, C. (1994). Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. Cancer Res *54*, 575-581.

Rosales, C., and Juliano, R. (1995). Signal transduction by cell adhesion receptors in leukocytes. J Leukocyte Biol *57*, 189-198.

Rosales, C., O'Brien, V., Kornberg, L., and Juliano, R. (1995). Signal transduction by cell adhesion receptors. Biochim Biophys Acta *1242*, 77-98.

Rosen, S., and Bertozzi, C. (1994). Selectins and their ligands. Curr Opin Cell Biol *6*, 663-673.

Rotello, R., Liebermann, R., Purchio, A., and Gerchenson, L. (1991). Coordinated regulation of apoptosis and cell proliferation by transforming growth factor beta1 in cultured uterine epithelial cells. Proc Natl Acad Sci USA *88*, 3412-3415.

Roy, N., Deveraux, Q., Takahashi, R., Salvesen, G., and Reed, J. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J *16*, 6914-6925.

Ruffini, P., Rivoltini, L., Silvani, A., Boiardi, A., and Parmiani, G. (1993). Factors, including transforming growth factor beta, released in the glioblastoma residual cavity, impair activity of adherent lymphokine-activated killer cells. Cancer Immunol Immunother *36*, 409-416.

Ruoslahti, E., and Reed, J. (1994). Anchorage dependence, integrins, and apoptosis. Cell *77*, 477-478.

Saffell, J., Williams, E., Mason, I., Walsh, F., and Doherty, P. (1997). Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. Neuron *18*, 231-242.

Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D. (1988). Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol *107*, 743-751.

Saltzman, A., Munro, R., Searfoss, G., Franks, C., Jaye, M., and Ivashchenko, Y. (1998). Transforming growth factor-b-mediated apoptosis in the Ramos B-lymphoma cell line is accompanied by caspase activation and Bcl-XL downregulation. Exp Cell Res *242*, 244-254.

Sanchez, A., Alvarez, A., Benito, M., and Fabregat, I. (1996). Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures. J Biol Chem *271*, 7416-7422.

Sanchez, A., Alvarez, A., Benito, M., and Fabregat, I. (1997). Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor b in fetal rat hepatocytes in primary culture. Hepatology *26*, 935-943.

Santoni-Rugiu, E., Nagy, P., Jensen, M., Factor, V., and Thorgeirsson, S. (1996). Evolution of neoplastic development in the liver of transgenic mice co-expressing c-Myc and transforming growth factor-alpha. Am J Pathol *149*, 407-428.

Satyamoorthy, K., Dejesus, E., Linnenbach, A., Kraj, B., Kornreich, D., Rendle, S., Elder, D., and Herlyn, M. (1997). Melanoma cell lines from different stages of progression and their biological and molecular analyses. Melanoma Res *7*, 35-42??

Satyamoorthy, K., Meier, F., Hsu, M., Berking, C., and Herlyn, M. (1999). Human xenografts, human skin and skin reconstructs for studies in melanoma development and progression. Cancer Metastasis Rev *18*, 401-405.

Schor, S. (1980). Cell proliferation and migration on collagen substrata in vitro. J Cell Sci *41*, 159-175.

Schwab, M., Alitalo, K., Varmus, H., Bishop, J., and George, D. (1983). A cellular oncogene (c-Ki-ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature *303*, 497-501.

Schwartzman, R., and Cidlowski, J. (1993). Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine Rev *14*, 133-151.

Scott, G., Stoler, M., Sarkar, S., and Halaban, R. (1991). Localization of basic fibroblast growth factor mRNA in melanocytic lesions by in situ hybridization. J Invest Dermatol *96*, 318-322.

Sergi, C., Ehemann, V., Beedgen, B., Linderkamp, O., and Otto, H. (1999). Huge fetal sacrococcygeal teratoma with a completely formed eye and intratumoral DNA ploidy heterogeneity. Pediatr Dev Pathol *2*, 50-57.

Sha, X., Brunner, A., Purchio, A., and Gentry, L. (1989). Transforming growth factor-beta1: importance of glycosylation and acidic proteases for processing and secretion. Mol Endocrinol *3*, 1090-1098.

Sherr, C. (1994). G1 phase progression: cycling on cue. Cell *79*, 551-555.

Sherr, C. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Sherr, C., and Roberts, J. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.

Silvestrini, R., Veneroni, S., Daidone, M., Benini, E., Boracchi, P., Mezzetti, M., Di Fronzo, G., Rilke, F., and Veronesi, U. (1994). The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-degative breast cancer patients. J Natl Cancer Inst *86*, 499-504.

Simon, M., Krone, C., Schwarz, A., Luger, T., Jäättelä, M., and Schwarz, T. (1995). Heat shock protein 70 overexpression affects the response to ultraviolet light in murine fibroblasts. Evidence for increased cell viability and suppression of cytokine release. J Clin Invest *95*, 926-933.

Sinicrope, F., Hart, J., Michelassi, F., and Lee, J. (1995). Prognostic value of bcl-2 oncoprotein expression in stage II colon carcinoma. Clin Cancer Res *1*, 1103-1110.

Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. (1987). Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature *326*, 197-200.

Slater, T. (1987). Free radicals fact or fiction? Br J Cancer *55*, 5-10.

Solter, D., and Damjanov, I. (1979). Teratocarcinoma and the expression of oncodevelopmental genes. Cancer Res *18*, 277-332.

Sommer, A., Brewer, M., Thompson, R., Moscatelli, D., Presta, M., and Rifkin, D. (1987). A form of human basic fibroblast growth factor with an extended amino terminus. Biochem Biophys Res Comm *144*, 543-550.

Spencer, C., and Groudine, M. (1991). Control of c-Myc regulation in normal and neoplastic cells. Adv Cancer Res *56*, 1-48.

Spinella, M., Kitareewan, S., Mellado, B., Sekula, D., Khoo, K., and Dmitrovsky, E. (1998). Specific retinoid receptors cooperate to signal growth suppression and maturation of human embryonal carcinoma cells. Oncogene *16*, 3471-3480.

Springer, T. (1995). Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol *57*, 827-872.

Susin, S., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an antiapoptotic protease. J Exp Med *184*, 1331-1341.

Suzuki, S. (1996). Structural and functional diversity of cadherin superfamily. J Cell Biochem *61*, 531-542.

Szebenyi, G., and Fallon, J. (1999). Fibroblast growth factors as multifunctional signaling factors. Int Rev Cytol *185*, 45-106.

Taipale, J., and Keski-Oja, J. (1997). Growth factors in the extracellular matrix. FASEB J *11*, 51-59.

Taipale, J., Koli, K., and Keski-Oja, J. (1992). Release of transforming growth factor-beta1 from the pericellular matrix of cultured fibroblast and fibrosarcoma cells by plasmin and thrombin. J Biol Chem *268*, 25378-25384.

Taipale, J., Lohi, J., Saarinen, J., Kovanen, P., and Keski-Oja, J. (1995). Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta1 from the extracellular matrix of human epithelial and endothelial cells. J Biol Chem *270*, 4689-4696.

Taipale, J., Miyazono, K., Heldin, C.-H., and Keski-Oja, J. (1994). Latent transforming growth factor-beta1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol *124*, 171-181.

Takeichi, M. (1993). Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol *5*, 806-811.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. Curr Opin Cell Biol *7*, 619-627.

Tedder, T., Steeber, D., Chen, A., and Engel, P. (1995). The selectins: vascular adhesion molecules. FASEB J *9*, 866-873.

Thompson, C. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456-1462.

Thompson, S., Stern, P., Webb, M., Walsh, F., Engström, W., Evans, P., Shi, W.-K., Hopkins, B., and Graham, C. (1984). Cloned human teratoma cells differentiate into neuron-like cells and other cell types in retinoic adic. J Cell Sci *72*, 37-64.

Tienari, J., Alanko, T., Lehtonen, E., and Saksela, O. (1991). The expression and localization of urokinase-type plasminogen activator and its type 1 inhibitor are regulated by retinoic acid and fibroblast growth factor in human teratocarcinoma cells. Cell Regul *2*, 285-297.

Timpl, R. (1989). Structure and biological activity of basement membrane proteins. Eur J Biochem *180*, 487-502.

Tomasek, J., Hay, E., and Fujiwara, K. (1982). Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts: distribution of actin, alpha-actinin, and myosin. Dev Biol *92*, 107-122.

Tran, J., Rak, J., Sheehan, C., Saibil, S., LaCasse, E., Korneluk, R., and Kerbel, R. (1999). Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Comm *264*, 781-788.

Tsen, G., Halfter, W., Kroger, S., and Cole, G. (1995). Agrin is a heparan sulfate proteoglycan. J Biol Chem *270*, 3392-3399.

Tsuji, T., Okada, F., Yamaguchi, K., and Nakamura, T. (1990). Molecular cloning of the large subunit of transforming growth factor type beta masking protein and expression of the mRNA in various rat tissues. Proc Natl Acad Sci USA *87*, 8835-8839.

Tsujimoto, Y., Finger, L., Yunis, J., Nowell, P., and Croce, C. (1984). Coning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science *226*, 1097-1099.

Tuckwell, D., and Humphries, M. (1993). Molecular and cellular biology of integrins. Crit Rev Oncol Hematol *15*, 149-171.

Vainikka, S., Joukov, V., Klint, P., and Alitalo, K. (1996). Association of a 85-kDa serine kinase with activated fibroblast growth factor receptor-4. J Biol Chem *271*, 1270-1273.

Walker, N., Harmon, B., Gobé, G., and Kerr, J. (1988). Patterns of cell death. Methods Achiev Exp Pathol *13*, 18-54.

Wang, J., Knudsen, E., and Welch, P. (1994). The retinoblastoma tumor suppressor protein. Adv Cancer Res *64*, 25-85.

Wang, X.-J., Greenhalgh, D., Bickenbach, J., Jiang, A., Bundman, D., Krieg, T., Derynck, R., and Roop, D. (1997). Expression of a dominant-negative type II transforming growth factor beta (TGF-beta) receptor in the epidermis of transgenic mice blocks TGF-beta-mediated growth inhibition. Proc Natl Acad Sci USA *94*, 2386-2391.

Wang, X.-J., Liefer, K., Tsai, S., O'Malley, B., and Roop, D. (1999). Development of gene-switch transgenic mice that inducibly express transforming growth factor beta1 in the epidermis. Proc Natl Acad Sci USA *96*, 8483-8488.

Wang, Y., and Becker, D. (1997). Antisense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth. Nature Med *3*, 887-893.

Watson, A., Merritt, A., Jones, L., Askew, J., Anderson, E., Becciolini, A., Balzi, M., Potten, C., and Hickman, J. (1996). Evidence of reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. Br J Cancer *73*, 889-895.

Vaux, D., Cory, S., and Adams, J. (1988). Bcl-2 gene promotes haematopoietic cell survival and cooperates with c-Myc to immortalize pre-B cells. Nature *335*, 440-442.

Wehrli, P., Viard, I., Bullani, R., Tschopp, J., and French, L. (2000). Death receptors in cutaneous biology and disease. J Invest Dermatol *115*, 141-148.

Weinstock, M. (1996). Controversies in the role of sunlight in the pathogenesis of cutaneous melanoma. Photochem Photobiol *63*, 406-410.

Weller, M., Constam, D., Malipiero, U., and Fontana, A. (1994). Transforming growth factor-beta2 induces apoptosis of murine T cell colonies without downregulating bcl-2 mRNA expression. Eur J Immunol *24*, 1293-1300.

Westermann, R., Grothe, C., and Unsicker, K. (1990). Basic fibroblast growth factor (bFGF), a multifunctional growth factor for neuroectodermal cells. J Cell Sci Suppl *13*, 97-117.

Wight, T., Heinegård, D., and Hascall, V. (1991). Proteoglycans: structure and function. In Cell Biology of the Extracellular Matrix, E. Hay, ed. (New York: Plenum Press), pp. 45-78.

Willingham, M. (1999). Cytochemical methods for the detection of apoptosis. J Histochem Cytochem *47*, 1101-1109.

Winnemoller, M., Schmidt, G., and Kresse, H. (1991). Influence of decorin on fibroblast adhesion to fibronectin. Eur J Cell Biol *54*, 10-17.

Winnemoller, M., Schon, P., Visher, P., and Kresse, H. (1992). Interactions between thrombospondin and the small proteoglycan decorin: interference with cell attachment. Eur J Cell Biol *59*, 47-55.

Virtanen, I., Miettinen, M., Lehto, V.-P., Kariniemi, A.-L., and Paasivuo, R. (1985). Diagnostic application of monoclonal antibodies to intermediate filaments. Ann NY Acad Sci *455*, 635-648.

Vlodavsky, I., Miao, H., Medalion, B., Danagher, P., and Ron, D. (1996). Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor. Cancer Metastasis Rev *15*, 177-186.

Wolf, B., and Green, D. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. J Biol Chem *274*, 20049-20052.

Wrana, J., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X., and Massagué, J. (1992). TGF-beta signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003-1014.

Wrana, J., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGF-beta receptor. Nature *370*, 341-347.

Wyllie, A., Kerr, J., and Currie, A. (1980). Cell death: the significance of apoptosis. Int Rev Cytol *68*, 251-306.

Yahagishita, M., Midura, R., and Hascall, V. (1987). Proteoglycans: Isolation and purification from tissue cultures. Methods Enzymol *138*, 279-289.

Yamada, K. (1991). Fibronectin and other cell interactive glycoproteins. In Cell Biology of the Extracellular Matrix, E. Hay, ed. (New York: Plenum Press), pp. 111-146.

Yanagihara, K., and Tsumuraya, M. (1992). Transforming growth factor-beta1 induces apoptotic cell death in cultured human gastric cancinoma cells. Cancer *52*, 4042-4045.

Yap, A. (1998). The morphogenetic role of cadherin cell adhesion molecules in human cancer: a thematic review. Cancer Invest *16*, 252-261.

Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. (1995). Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci USA *92*, 7416-7419.

Young, A. (1987). The sunburn cell. Photodermatol *4*, 127-134.

Yuan, J., Yeasky, T., Havre, P., and Glazer, P. (1995). Induction of p53 in mouse cells decreases mutagenesis by UV radiation. Carcinogenesis *16*, 2295-2300.

Yurchenco, P. (1994). Assembly of laminin and type IV collagen into basement membrane networks. In Extracellular matrix assembly and structure, P. Yurchenco, D. Birk and R. Mecham, eds. (San Diego, CA: Academic Press), pp. 351-388.

Yurchenco, P., and Schittny, J. (1990). Molecular architecture of basement membranes. FASEB J *4*, 1577-1590.

Zhai, S., Yaar, M., and Gilchrest, B. (1996). Nerve growth factor rescues pigment cells from ultraviolet induced apoptosis by upregulating BCL-2 levels. Exp Cell Res *224*, 335-343.

Zhang, Y., and Derynck, R. (1999). Regulation of Smad signalling by protein associations and signalling crosstalk. Trends Cell Biol *9*, 274-279.

Zhang, Z., Vuori, K., Wang, H., Reed, J., and Ruoslahti, E. (1996). Integrin activation by R-ras. Cell *85*, 61-69.

Ziegler, A., Jonason, A., Leffell, D., Simon, J., Sharma, H., Kimmelman, J., Remington, L., Jacks, T., and Brash, D. (1994). Sunburn and p53 in the onset of skin cancer. Nature *372*, 773-776.

Zoratti, M., and Szabo, I. (1995). The mitochondrial permeability transition. Biochim Biophys Acta *1241*, 139-176.