View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Helsingin yliopiston digitaalinen arkisto

Fibronectin-integrin interaction promotes fibroblast activation (nemosis) and crosstalk with tumor cells

PERTTELI SALMENPERÄ

Department of Virology
Haartman Institute

Helsinki Graduate Program in Biotechnology and Molecular Biology

Research Programs Unit
Infection Biology Research Program

University of Helsinki
Finland

Academic dissertation

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Auditorium XII at the University of Helsinki Main Building, Unioninkatu 34, on 27th April 2012, at 12 noon

Helsinki 2012

Supervisor

Professor Emeritus Antti Vaheri
Department of Virology
Haartman Institute
University of Helsinki
Finland

Reviewers

Docent Eeva-Liisa Eskelinen
Department of Biosciences
University of Helsinki
Finland

Professor Jorma Keski-Oja
Department of Pathology
Haartman Institute
University of Helsinki
Finland

Opponent

Professor Marja Jäättelä
Institute of Cancer Biology
Danish Cancer Society
Copenhagen
Denmark

ISBN 978-952-10-7927-6 (Paperback)
ISBN 978-952-10-7928-3 (PDF, http://ethesis.helsinki.fi)
Helsinki University Print
Helsinki 2012

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals (I-IV) in the text.

I. Salmenperä P, Kankuri E, Bizik J, Sirén V, Virtanen I, Takahashi S, Leiss M, Fässler R and Vaheri A. Formation and activation of fibroblast spheroids depend on fibronectin-integrin interaction. *Experimental Cell Research* 314(19):3444-3452, 2008

II. Salmenperä P, Karhemo PR, Räsänen K, Zhao F, Laakkonen P, and Vaheri A. Fibroblast activation by spheroid formation involves a secretory phenotype, autophagy and features of senescence. *Submitted*

III. Sirén V, Salmenperä P, Kankuri E, Bizik J, Sorsa T, Tervahartiala T and Vaheri A. Cell-cell contact activation of fibroblasts increases the expression of matrix metalloproteinases. *Annals of Medicine* 38(3):212-220, 2006

IV. Räsänen K, Salmenperä P, Baumann M, Virtanen I and Vaheri A. Nemosis of fibroblasts is inhibited by benign HaCaT keratinocytes but promoted by malignant HaCaT cells. *Molecular Oncology* 2(4):340-348, 2008

These original articles have been reprinted with the permission of their copyright holders. IV has previously been included in the Ph.D. thesis of Kati Räsänen.

ABBREVIATIONS

3-MeA	3-Methyladenine
ATG	Autophagy-related
Baf A1	Bafilomycin A1
CAFs	Cancer associated fibroblasts
Cav-1	caveolin-1
CDK	Cyclin-dependent kinase
CHO	Chinese hamster ovary cells
CK7	cytokeratin-7
CMA	Chaperone-mediated autophagy
COX-2	Cyclo-oxygenase-2
CSC	Cancer stem cells
ECM	Extracellular matrix
EDA	Extra domain A
EDB	Extra domain B
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein
Erk	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FN (pFN/cFN)	Fibronectin (plasma fibronectin/cellular fibronectin)
G0, G1, G2	Gap 0, Gap 1, Gap 2
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1α
Hsc70	Heat shock cognate 70 kDa protein
IHC	Immunohistochemistry
ISH	In situ hybridization
KGF	Keratinocyte growth factor
LAMP-2A	Lysosome-associated membrane protein type 2A
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
LC3	Microtubule-associated protein light chain
MEK1/2	Mitogen-activated protein kinase kinase 1 and 2
MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloproteinase
MVBs	Multivesicular bodies
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
NSAIDs	Non-steroidal anti-inflammatory drugs
OIS	Oncogene-induced senescence
PCNA	Proliferating cell nuclear antigen
PE	Phosphatidyl ethanolamine
PGs	Prostaglandins
PI3K	Phosphoinositide-3-kinase
PK	Pyruvate kinase
RB	Retinoblastoma
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SA-β-gal	Senescence-associated β-galactosidase
α-SMA	α-smooth muscle actin

SMS	Senescence-messaging secretome
TEM	Transmission electron microscopy
TORC1	TOR complex 1
tPA	Tissue-type plasminogen activator
ULK	unc-51-like kinase
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor

ABSTRACT

Cancer is a complex disease. It is a multistep process where genetic changes lead to cellular transformation and uncontrolled proliferation. However, cancer is not only a disease of these transformed cells, since tumor stroma and microenvironment synchronously evolve and become activated together with these genetic changes. The interactions between different cell types in tumor microenvironment are mediated by soluble factors, such as cytokines, chemokines, growth factors and proteases. They modulate cell proliferation, activation and differentiation, as well as the composition of the extracellular matrix in tumor and its microenvironment.

Nemosis is an in vitro model of fibroblast activation, which is initiated by forcing fibroblast to cluster together instead of providing solid support for attachment. This results in a multicellular spheroid that upregulates soluble paracrine molecules known to be important mediators of tumor microenvironment. Furthermore, nemotic fibroblasts affect cancer cell proliferation, invasion and differentiation through these soluble factors. In addition to direct effects on cancer cells they stimulate angiogenesis and the chemotaxis of leukocytes.

This thesis study shows that fibroblast spheroid formation depends on the interaction between fibronectin (FN) with its integrin receptors, more accurately $\alpha 5$ and $\beta 1$ integrin subunits, whereas fibroblasts activation in spheroids was mediated by the interaction of FN with $\alpha 5$, αV and $\beta 1$ integrin subunits. The activation was mediated by the binding of integrins to the RGD -motif in FN molecules and the synergy site that is known to stimulate RGD-motif binding to integrins enhanced it. Unexpectedly, FN-matrix assembly was not essential for the activation of fibroblasts in spheroids although it had an effect on spheroid formation. FN deposit to matrix is an acknowledged consequence of integrin binding to fibronectin.

Nemosis was accompanied by a dramatic change in gene expression. The change could be roughly categorized in three classes; the upregulation of secreted molecules and downregulation of cell cycle and cytoskeleton. Nemosis was associated with a quiescent withdrawal from the cell cycle, as the cells downregulated cyclin D and upregulated p27, the driver and the inhibitor of the

cell cycle, respectively. Furthermore, nemotic fibroblasts resumed to the cell cycle when taken out from the spheroid, indicating reversible cell cycle arrest, a known characteristic of quiescence.

Fibroblast activation by spheroid formation was accompanied by stress-related changes in the cellular ultrastructure, such as dilated endoplasmic reticulum, increased lipofuscin and degenerated organelles. Hence, nemosis is a cellular stress response. This observation was in agreement with the induction of autophagy in fibroblasts spheroids. Autophagy is a well-known stress response that helps cell survival under stress conditions. Furthermore, nemosis resembled another cellular stress condition, the cellular senescence. They both had a similar secretory phenotype, expressed senescence-associated β-galactosidase and lipofuscin, and there was a cell cycle arrest in both. However, there were also features to distinguish nemosis from senescence, such as nemosis being a reversible phenotype, and cell cycle inhibitors that regulate senescence being downregulated in nemosis.

Nemosis attenuated tumor growth in vivo in a mouse xenograft model. The attenuation was associated with the expression of senescence-associated β-galactosidase and the expression of the p14ARF cell-cycle inhibitor in human RT3 malignant keratinocytes. This suggests that nemosis causes cellular senescence in the RT3 keratinocytes in vivo. In addition to the senescence response, nemosis was found to increase the cytokeratin-7 (CK7) mediated differentiation of RT3 cells in xenografts.

It is becoming obvious that cancer is not just a disease of uncontrolled proliferation of cancer cells, but a disease where normal stromal fibroblasts actively participate in its progression. The current work reveals new mechanistic insights of fibroblast activation and concludes that nemosis can be a useful model to study the activation of fibroblasts and interactions between fibroblasts and cancer cells.

1. REVIEW OF LITERATURE

1.1 Cancer

Cancer is a leading cause of death and accounted for 7.6 million deaths worldwide in 2008, according to World Health Organizations GLOBOCAN 2008 –project [1]. Cancer is more a term for a vast group of different diseases than just one specific disease. It is a disease where malignant cells proliferate and grow uncontrollably. Cancers are classified according to their cellular origin. Cancers originating from epithelial tissues, carcinomas, are responsible for ~80% of cancer-related deaths in the western world. Non-epithelial cancers can be classified in three different classes according their embryonic origin. Sarcomas are derived from mesenchymal cells, like fibroblasts, adipocytes, osteoblasts and myocytes. The second group of non-epithelial cancers originate from hematopoietic (blood-forming) cells. Leukemias and lymphomas are two major subtypes of these cancers. The last major group of non-epithelial cancers is derived from various components of the central and peripheral nervous system. These cancers (gliomas, glioblastomas, neuroblastomas, schwannomas and medulloblastomas) are rare, but very deadly. Some cancers, such as melanoma that originates from melanocytes, cannot be grouped in any of the above-mentioned major classifications [2].

Cancer progression is a multistage process, where a normal cell transforms from a pre-cancerous lesion to a malignant tumor. These changes are driven by mutations in the cellular genome, which are the result of interaction of genetic and environmental factors. Hanahan and Weinberg stated six hallmarks of cancer (sustaining proliferative signaling, evading growth suppression, activation of invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death), two emerging hallmarks (deregulated cellular energetics, avoiding immune destruction) and two enabling characteristics (genome instability and mutation, tumor-promoting inflammation) (Table 1) [3]. The cells acquire these capabilities during a multistep process that is required for the progression from a benign to malignant cell and the formation of a tumor. In addition to these malignant cells, tumors consist also of a variety of untransformed stromal cells, such as fibroblasts, pericytes, and endothelial

cells, and a variety of immune cells. Hence, cancer is a heterogeneous disease consisting of multiple cell types. The interactions between these cell types generate a tumor-prone microenvironment [4, 5].

Table 1. Hallmarks of cancer according to Weinberg and Hanahan [3] (VEGF=vascular endothelial growth factor)

Hallmarks of cancer	Main reasons
Sustaining proliferative signaling	Deregulated growth factor signaling
Evading growth suppression	Deregulated cell cycle inhibitors and antiproliferative signaling
Activation of invasion and metastasis	Epithelial-mesenchymal-transition
Enabling replicative immortality	Induction of telomerase
Inducing angiogenesis	Secretion of angiogenic factors, such as VEGF
Resisting cell death	Evading apoptosis, necrosis and autophagic cell death, but increasing survival promoting autophagy
Emerging hallmarks	
Deregulated cellular energetics	Aerobic glycolysis (Warburg effect)
Avoiding immune destruction	Immunoediting
Enabling characteristics	
Genome instability and mutation	Random mutations and chromosomal rearrangements
Tumor-promoting inflammation	Inflammatory cells supply bioactive molecules and reactive oxygen species

1.2 Fibroblasts

Fibroblasts are the most common cell type in connective tissue. They represent a heterogeneous mesenchymal cell type, characterized by flat spindle-shaped morphology [6]. They lack endothelial, epithelial and hematopoietic cell markers, such as CD34, cytokeratin and CD45, respectively. Their main function is to maintain proper extracellular matrix (ECM) structures. Hence, they produce high amounts of different ECM components, such as fibronectin (FN), laminin, collagen and proteoglycans (Figure 1). ECM has many functions: It provides support and anchorage for cells, leading to correct tissue architecture; it also affects intercellular communication and stores a wide range of growth factors [7, 8].

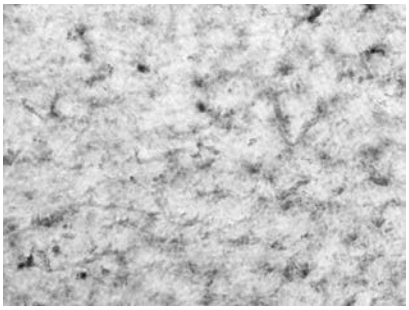

Figure 1. Phase-contrast image of ECM produced by confluent fibroblasts

In addition to maintaining proper ECM structure, fibroblasts can serve as sentinel cells. They initiate and modulate inflammatory processes by secreting various cytokines and chemokines, as well as releasing growth factors from the ECM by secreting proteases [9-12]. In normal healthy tissue, fibroblasts maintain in a less active state, but in pathological conditions, such as inflammation, wound healing or cancer, fibroblasts become activated [5]. Activated fibroblasts express α-smooth muscle actin (α-SMA) and the extra domain A (EDA) containing splice variant of FN, which are used as markers of activated fibroblasts, or so-called myofibroblasts [13]. Interestingly, the activated fibroblasts can make direct cell-cell contacts through gap junctions and form a body-wide network [14-16].

1.2.1 Fibroblasts in tumor microenvironment

Activated fibroblasts in tumors are often called cancer-associated fibroblasts (CAFs). They are the most abundant stromal cell type in cancer and in some cancers stromal cells can even create most of the tumor mass [17]. CAFs are a highly heterogeneous population and they, like myofibroblasts, have different cellular origins. The main progenitor of CAFs are local resident fibroblasts, but they can also originate from smooth-muscle cells, pericytes and bone marrow-derived mesenchymal stem cells, or as a result of epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EnMT) [18-21]. Activated fibroblasts share many consistent features regardless of their origin and tissue type. To describe this analogy between stroma in cancer microenvironment and wound healing, Dvorak used the famous quotation of tumors: “wounds that do not heal” [22].

The exact mechanism of how resident fibroblasts become activated to CAFs is under intense investigation. Several hypotheses have been postulated; acute phase and stress response, fibroblasts senescence, interaction with cancer cells and somatic mutations in fibroblasts [23]. Maffini et al. showed that cancer

progression in response to the carcinogen N-nitrosomethylurea depends on stress-related changes in stromal cells and not on the direct effects of carcinogens on epithelial cells [24]. Age correlates with chronic increase in tissue inflammation [25, 26], as well as cancer incidence [27]. In addition to these assumptions, senescent fibroblasts secrete various inflammatory cytokines to maintain an inflammatory state [28]. In addition, senescent fibroblasts have gene expression profiles similar to CAFs, which has led to the hypothesis that they might be a source of CAFs [23]. Several experiments have shown that the co-culture of fibroblasts with cancer cells leads to the myofibroblast differentiation of fibroblasts and growth promotion of cancer cells [29-32]. Mutations in tumor suppressor genes, such as PTEN and p53, in stromal fibroblasts also activate fibroblasts and promote tumor growth [33-35].

CAFs have both direct and indirect effects on cancer cells. Hence, the variety of growth factors and cytokines they produce exert their paracrine effects directly on cancer cells as well as other cell types in the tumor, which further stimulates or inhibits tumor progression [12]. They also produce proteases, such as matrix metalloproteinases (MMPs) and plasminogen activators that modulate the ECM structure, as well as release and activate growth factors embedded in it [36-38].

It is well appreciated that CAFs support and promote tumor growth. In addition, several studies have shown that CAFs can even cause epithelial cell transformation and initiate tumor growth [39]. In contrast to CAFs, normal fibroblasts can convert malignant epithelia to benign lesions [12, 39]. It remains unclear how normal fibroblasts limit cancer progression, but it has been suggested that fibroblasts as a source of immuno-modulatory cytokines, such as IL-6, provoke immune defense against cancer cells [12]. In addition, Mina Bissell's laboratory has found that ECM structure affects cancer cells' malignant phenotype and vice versa, proper ECM can convert malignant cells to benign, and thus limit cancer progression [40-42].

1.2.2 Cells in tumor microenvironment

In addition to cancer cells and fibroblasts, tumors contain other cell types, which all can affect tumor growth. Hence, tumors must be seen as complex organs where the complicated interplay of various cells affects the outcome.

Cancer cells themselves are considered to be a heterogeneous population and according to one theory all cancer cells in tumors originate from cancer stem cells (CSCs). CSCs are tumorigenic cells that express markers of stem cells and have the ability of self-renewal [43]. Although CSCs cause cellular heterogeneity within tumors, most heterogeneity is generated by the stromal compartment. Interactions between tumor-stromal cells have a fundamental role in tumor initiation and progression. This interaction leads to co-evolution of cancer cells and their microenvironment. It has been postulated that co-evolution can develop in two ways: transformed epithelia causes activation and changes in microenvironment, or stromal changes occur first leading to subsequent transformation of epithelial cells [44].

Tumor growth, as well as normal organ growth, depends on the formation of novel blood and lymphatic vessels, angiogenesis and lymphangiogenesis, respectively. Blood vessels are assembled from endothelial cells that form the interlining of the vessels, and pericytes and smooth muscle cells, which give structural support for the vessels. Endothelial cells in already-formed vessels retain their ability to become activated and divide in response to angiogenic factors, such as VEGF and FGF, released by cancer cells to initiate angiogenesis [45].

Tumor microenvironment is very inflammatory (wound that never heals), hence it is not surprising that the cells of the immune system have a complex impact on tumor growth [46]. Leukocytes can have either antagonistic or tumor-promoting effects. It is clear that cancer cells must avoid immune destruction to fully develop, as stated by Hanahan and Weinberg as an emerging hallmark [3]. This immune destruction, also known as immunosurveillance, is mainly mediated by cytotoxic T cells and natural killer cells [47], whereas macrophages, mast cells, neutrophils, and B and T lymphocytes promote tumor growth by secreting growth factors, cytokines, chemokines and proteases to increase angiogenesis, cancer cell proliferation and invasion [46, 48]. Moreover, they further amplify the inflammatory state of tumors, which causes an increase in the production of reactive oxygen species (ROS) that leads to additional genomic instability in cancer cells [46].

1.2.3 Fibronectin

FN is a ubiquitous glycoprotein that is found in ECM and body fluids. FN protein is secreted from cells as a dimer and assembled to a multimeric matrix in an integrin-mediated process. Two types of FN are found, soluble plasma FN (pFN) and insoluble cellular FN (cFN). Hepatocytes synthesize pFN and it is a major component of the plasma (300 µg/ml). Several cell types are able to produce cFN, but it is primarily produced by fibroblasts [49-52]. It is synthesized as a soluble form and is then assembled into ECM [53]. FN is a product of a single gene and it contains three types of repeating units (type I, II and III), but in humans alternative splicing can generate 20 different isoforms. Splicing can occur in three different splice sites celled EDA, EDB and V (variable) (Figure 2.). EDA and EDB can be either completely excluded or included and are present in cFN and very rarely in pFN. V domain can be alternatively spliced in five different regions [51].

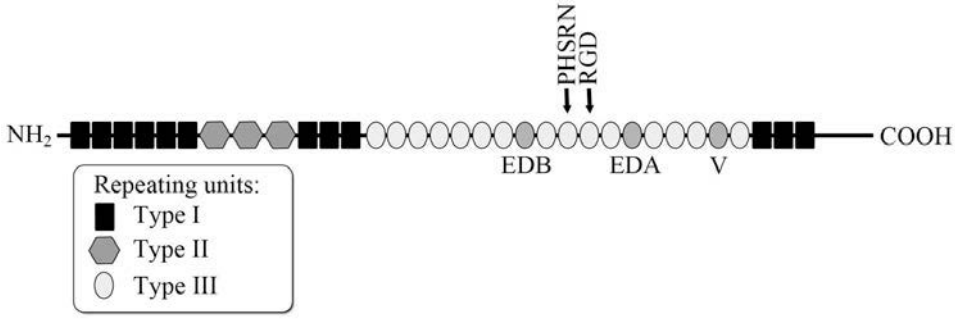

Figure 2. The structure of fibronectin

FN mediates cell adhesion to ECM and plays an important role in cell migration, differentiation and growth. FN deposition to ECM is a complex, tightly regulated, cell mediated process, which is initiated by soluble FN binding to integrins. This causes FN-bound integrins to cluster, bringing FN molecules in close proximity to interact with one another [54]. It is not completely known how final insoluble matrix is formed, but it seems that dimeric FN is stretched to uncover cryptic bindings sites along the FN molecule [53] and interaction with fibrillin is needed to make large insoluble matrix [55].

1.2.4 Integrins as fibronectin receptors

Integrins are cell-surface-adhesion receptors that are responsible mainly for cell-ECM adhesion [56, 57], although they can also mediate cell-cell adhesion [58-60]. There are at least 24 distinct integrin heterodimers in humans, which arise form the noncovalent association between one of each 18 α– and 8 β-subunits. Distinct integrins bind to distinct molecules in ECM, giving cell specific adherence and migration on different matrixes [57]. Besides their mechanical role in cell adhesion, integrins transmit signals from ECM into cells (outside-in signaling). These signals determine whether cells differentiate, survive or migrate in response to their environment. In addition to outside-in signaling, integrins also signal inside-out (termed also as activation) and regulate the affinity of integrins to their extracellular ligand [61].

Most cell types in the body harbor one or several of these receptors and thereby can adhere to fibronectin, an abundant molecule in tissues, making fibronectin-integrin interaction important in a variety of different biological processes. At least a dozen integrins can bind FN (Table 2) [51, 62]. α5β1 integrin is probably the most important receptor for FN in several cell types. It was the first receptor to be identified for FN [63], and differs from other FN-binding integrins by being specialized only to FN [64].

Table 2. FN-binding integrins [51, 62].

Receptor	Recognition sequence in FN
α2β1	
α3β1	RGD
α4β1	CS-1 peptide, CS-5 peptide, IDAPS
α5β1	RGD
α8β1	RGD
αVβ1	RGD
αVβ3	RGD
αIIbβ3	RGD
αVβ5	RGD
αVβ6	RGD
α4β7	CS-1 peptide
αVβ8	

1.3 Autophagy

Autophagy is an evolutionarily conserved pathway that exerts protein and organelle degradation. It has an essential role in development, cell survival, homeostasis and immune response. Autophagy is a “self-eating” process where cytosolic cargo is delivered to lysosomes for catabolic and energy-generating degradation of the engulfed material. In addition to the energy-generating role of autophagy, constitutive autophagy has a housekeeping role in the clearance of damaged organelles and misfolded or aggregated proteins to maintain cellular homeostasis [65]. Furthermore, various cellular stress conditions, such as starvation, hypoxia, unfolded protein response and infection, induce autophagic activity [66].

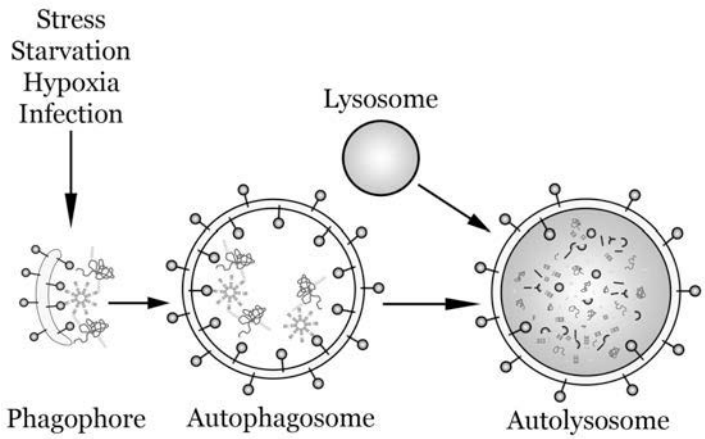

Figure 3. The main events in macroautophagy. In response to stress cytosolic material is sequestered to autophagosome. Autophagosomes fuse with lysosomes for degradation of the cargo [65].

The delivery of material to lysosomes can occur in three different ways, macroautophagy, microautophagy or chaperone-mediated autophagy (CMA). They differ from each other by the way they target cytosolic cargo to lysosomes. In macroautophagy, hereafter called autophagy (as in most literature), cytosolic material is first sequestered by double-membrane structures, phagophores, which are then sealed to form double-membrane vesicles, autophagosomes. Autophagosomes fuse with lysosomes to form autolysosomes for degradation of the cargo carried by autophagosomes (Figure 3) [65]. In microautophagy, endosomes and lysosomes sequester cytosolic material directly without initial

autophagosome formation [67]. CMA can degrade only proteins, not organelles, which contain a peptide sequence that is recognized by the heat shock cognate 70 kDa protein (Hsc70). Hsc70 with co-chaperons forms a complex that targets substrate protein to the lysosomal receptor lysosome-associated membrane protein type 2A (LAMP-2A) in the lysosomal membrane. Target proteins are unfolded and translocated to lysosomes for degradation [68]. In addition to non-selective bulk degradation of cytosolic material, organelles, proteins and intracellular parasites can be selectively targeted to autophagic degradation. Autophagy can also be named to describe the destruction of the target organelle, for instance mitophagy targets selectively mitochondria for degradation (Table 3) [69].

Table 3. Types of selective autophagy

Type of autophagy	Target organelle	Reference
Mitophagy	Mitochondria	[70]
Ribophagy	Ribosomes	[71]
Aggrephagy	Protein aggregates	[72]
Pexophagy	Peroxisomes	[73]
Lipophagy	Lipids	[74]
Reticulophagy	Endoplasmic reticulum	[75]

1.3.1 Induction and progression of autophagy

Autophagic flux is a multistep process that consists of five steps, nucleation, elongation, completion, fusion and degradation (Figure 3). Autophagosome is formed and cytosolic cargo is sequestered to it during the first three steps. During fusion and degradation, autophagosome fuses with lysosome and the cargo is degraded by lysosomal hydrolases. Highly conserved *atg* (autophagy-related) genes control both basal and stimulus-induced autophagy. Induced autophagy is, in most cases, associated with inhibition of mammalian target of rapamycin (mTOR), a master regulator of cell growth and autophagy [76]. It forms and functions as a catalytic subunit of TOR complex 1 (TORC1) together with Raptor, Deptor, mLST8 and PRAS40 subunits [77]. The activity of mTOR is controlled through different signaling routes, such as class I phosphoinositide-3-kinase (PI3K), Akt/protein kinase B and AMP-activated protein kinase, in response to cellular and environmental energy resources as

well as nutrient, growth factor and stress levels [77]. When nutrient levels are high and cells are in a stress-free environment, mTOR remains activated and inhibits autophagy by phosphorylating the unc-51-like kinase (ULK) 1 or 2 (mammalian homologs for Atg1), which form a complex with Atg13 and FIP200 [78]. When the activity of mTOR is inhibited, ULK1/2 is dephosphorylated together with Atg13, and becomes able to phosphorylate FIP200, which triggers initial autophagosome formation (Figure 4) [78]. Recently it was shown that AMPK can directly activate ULK1 through phosphorylation, in addition to its ability to inhibit the activity of mTOR [79].

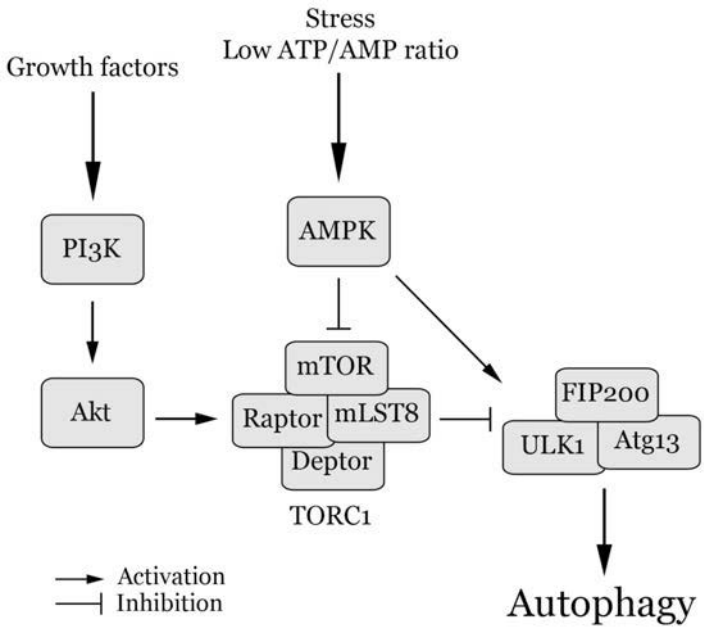

Figure 4. The overview of mTOR pathway that regulate autophagy. AMPK = AMP-activated protein kinase. For other abbreviations see text.

Beclin-1 together with class III PI3K, also called Vps34, drives the nucleation of autophagosomes. The binding of Vps34 with Beclin-1 promotes its catalytic activity to generate phosphatidyl inositol 3-phosphate, a phospholipid that is required for autophagosome formation [80]. The most widely used autophagic inhibitor, 3-methyladenine (3-MeA), targets class III PI3K and thus blocks the early steps of autophagosome formation [81].

There are two important ubiquitin-like conjugation systems that are required for the autophagic process, Atg12-Atg5 and microtubule-associated protein light chain (LC3, Atg8)-phosphatidyl ethanolamine (PE). Atg12-Atg5 conjugate is a ubiquitin ligase E3-like enzyme that assists the formation and elongation of the autophagosome, but dissociates when the autophagosome is complete, whereas conjugated LC3 stays in the autophagosome [82]. Upon the induction of autophagy, LC3 is cleaved by Atg4 to generate LC3-I. LC3-I is then conjugated to PE to form LC3-II, which localizes to inner and outer membranes of the developing autophagosome. This step is required for proper autophagosome formation [83]. In addition to aiding autophagosome formation, LC3 interacts with most of the known receptors and substrates of selective autophagy [69]. The receptors for selective autophagy, such as sequestosome 1 (p62/SQSTM1), interact with cargo and LC3, often through ubiquitin, to target cargo to the forming autophagosome (Figure 5) [84-86].

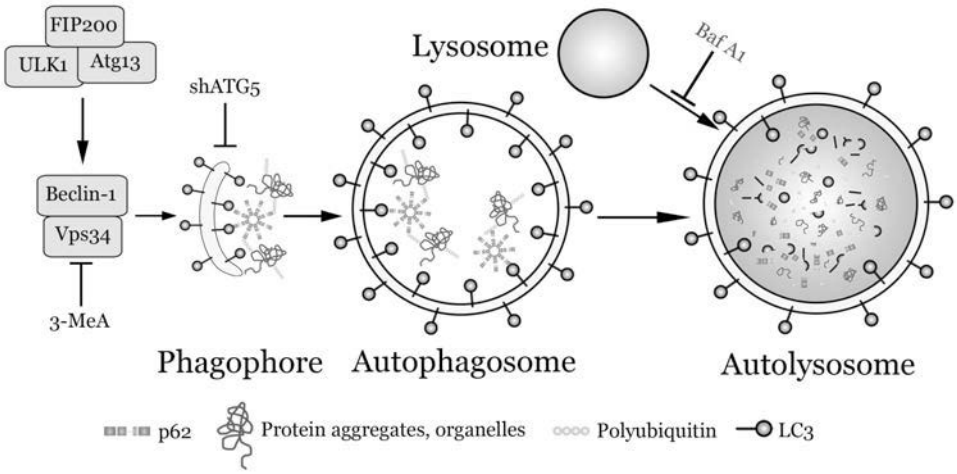

Figure 5. Molecular and cellular events during autophagy. ULK1 together with FIP200 and Atg13 regulates initial autophagosome formation. Beclin-1 binds Vsp34 and drives nucleation of forming autophagosomes. The mature autophagosome fuses with a lysosome to degrade autophagosomal cargo with lysosomal hydrolases. LC3 is required for autophagosome formation. In addition LC3 can bind p62 that binds to polyubiquitinated protein aggregates and organelles to target them into forming autophagosome. shATG5 and 3-MeA prevent initial formation of autophagosome, whereas Baf A1 inhibits fusion of autophagosome and lysosome.

After the autophagosome is formed it matures and then fuses with a lysosome to degrade its content using lysosomal hydrolases. Although this pathway is not fully understood, it is known to converge with the endocytic

pathway. It has been suggested that the autophagosomes fuse with early and late endosomes prior to fusion with lysosomes [87]. It has also been postulated that the maturation requires multivesicular bodies (MVBs), which provide a platform for sorting events in the endocytic pathway [88, 89]. The fusion events are mediated by the endocytic factors, such as Rab7 that associates with the autophagosomes and mediates the fusion of the outer membrane of the autophagosome with the lysosomal membrane in a LAMP1/LAMP2 –dependent manner to form an autolysosome [90, 91]. The detailed mechanism that mediates the autophagosome maturation and fusion with lysosomes is still partly uncharacterized, but recently it was shown that SNARE proteins that mediate vesicle fusion are required for the proper maturation of autophagosomes and their fusion with lysosomes [92, 93]. In addition, it has been suggested that also ESCRT proteins, which have been initially characterized to regulate the biogenesis of MVBs, plays an important role in the autophagosome-lysosome fusion, since impairing their function leads to the accumulation of autophagosomes [94, 95].

The final stage, the degradation of autolysosomal content, contains two steps; first a putative lipase Aut5p/Atg15p degrades the inner membrane of the autophagosome and after that the lysosomal content (acid hydrolases) is released to the autophagosome to degrade its cargo [96]. The degradation products, such as amino acids and monosaccharides, are transported out of the autolysosomes through the lysosomal membrane and recycled [97].

1.3.3 Autophagy in cancer

The role of autophagy in cancer is complex: it can be both a tumor-suppressive and tumor-promoting, depending on the type and stage of the tumor. Basal autophagy is considered to be tumor suppressive by clearing the damaged organelles and protein aggregates that are toxic to cells and cause genomic instability. This is supported by the finding that Beclin 1 gene is monoallelically deleted in 70% of ovarian, 50% of breast and 40% of prostate cancers [98]. Furthermore, overexpression of Beclin 1 in breast cancer cells leads to decreased tumorigenicity and has been related to increased autophagy [99]. Mathew et al. showed that defective autophagy caused p62 accumulation, which led to increased tumorigenesis through deregulated nuclear factor-κB (NF-κB)

signaling, elevated ROS production and DNA damage [100]. p62 is a signal modulator and adaptor protein that functions as a scaffold for several signaling proteins such as RIP, TRAF6, ERK, aPKC and caspase-8 (Figure 6) [101]. In addition to these interaction partners, p62 uses its PB1 domain for self-oligomerization to generate intracellular speckles or aggregates to form signal-organization centers [101, 102]. The levels of p62 are controlled by autophagy through LC3-interaction region (LIR) that binds to LC3 and targets p62 for degradation in autophagosomes [103]. Furthermore, through its ubiquitin-associated (UBA) domain p62 binds polyubiquitinated proteins and organelles, such as misfolded and aggregated proteins, and dysfunctional organelles, for their clearance through autophagy. By removing these dangerous cytosolic elements, p62 protects cells from oxidative and genotoxic stress. Hence, p62 is a tumor suppressor that functions as a receptor for selective autophagy [104].

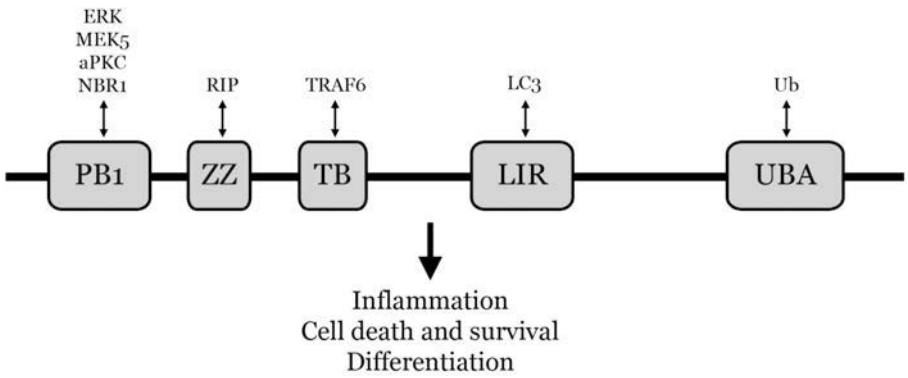

Figure 6. The structural domain organization of p62 and its interaction partners [101, 102].

In addition to autophagy's ability to prevent genotoxic stress, the induction of autophagy is required for mitotic transition to oncogene-induced senescence [105], an irreversible cell-cycle arrest that provides resistance against cellular transformation [106]. p53, a regulator of senescence, apoptosis and cell-cycle arrest, that is most commonly mutated in cancers [107], induces autophagy to protect cells from malignant transformation [108-110].

The role of autophagy in cell death is complex. Although in most cases the basal autophagy supports cell survival by removing damaged proteins and

organelles, prolonged and/or high levels of autophagy can lead to subsequent autophagic cell death, also known as type II programmed or cytosolic cell death [111], which further corroborates the role of autophagy in tumor suppression. The suppression of apoptosis induces autophagy, whereas the inhibition of autophagy upregulates apoptosis [112, 113]. Although the link between apoptosis and autophagy is not so straightforward, both are regulated by a set of common molecules and can occur in the same cell [113]. Furthermore the increased autophagy can lead to a subsequent apoptosis response [114, 115]. In addition to increased apoptosis, autophagy can lead to catalase depletion dependent (ROS mediated) necrosis in cells where caspase activity is inhibited [116]. In contrast, in the cells where apoptosis is impaired, such as in cancer cells, the inhibition of autophagy increases the necrotic form of cell death [117, 118], highlighting the role of autophagy in the resistance against both apoptosis and necrosis.

During tumor progression, the role of autophagy changes from tumor suppressive to tumor promoting. It is well-established that autophagy promotes cell survival under various stress conditions, like hypoxia and starvation. When the size of the tumor exceeds a certain limit, some areas become deprived of oxygen and/or nutrients, which leads to increased necrosis [119]. In these areas autophagy promotes tumor growth by improving cell survival during hypoxic stress as well as by providing amino acids and carbohydrates for energy source [120]. In opposition to this theory, Degenhardt et al. showed that in the apoptosis-defective cells, autophagy restricted tumorigenesis, although it promoted cell survival by preventing necrosis in the tumor. This restriction was provoked by reduced necrosis-mediated infiltration of the tumor growth supporting inflammatory cells and the inflammatory state of the tumor [117]. In addition to increased survival during hypoxia and starvation, autophagy can promote the therapeutic resistance of the tumor [121]. Many chemotherapeutic treatments, such as toxic compounds, induce autophagy that in turn increases survival and counteracts cell killing. The combination of simultaneous chemotherapy and autophagy inhibition has been shown to potentiate cell killing in several studies [122].

1.3.3.1 Autophagy in tumor stroma and fibroblast activation

Michael Lisanti's group has extensively characterized a model for CAF activation called "The Autophagic Tumor Stroma Model of Cancer Cell Metabolism" [123, 124]. In this model cancer cells increase ROS production through downregulation of the scaffolding protein caveolin-1 (Cav-1) [125-127]. In addition to being a structural component of caveolae, Cav-1 inhibits a variety of signaling pathways, such as mitogen-activated protein kinases and PI3K [128, 129], whereas it is required for FN-integrin mediated signaling [130, 131]. The loss of stromal Cav-1 is a powerful independent biomarker for early tumor recurrence, metastasis and poor prognosis [132, 133].

The loss of Cav-1 expression in fibroblasts leads to myofibroblastic (α-SMA and vimentin expression) differentation [134]. Cav-1 degradation was found to depend on autophagy and moreover, Cav-1 downregulation in fibroblasts caused induction autophagic activity [135]. Cav-1 downregulation and myofibroblast differentiation was driven by the conditions that upregulate ROS production in mitochondria, such as the dysfunction of mitochondria and hypoxia [136, 137]. In addition to induced myofibroblast markers, oxidative stress was shown to drive the activation of the transcription factors hypoxia-inducible factor-1α (HIF-1α) and NF-κB [136], which increased the secretion of inflammatory mediators, such as interleukin-6 (IL-6), IL-8, IL-10, macrophage inflammatory protein-1α (MIP-1α), interferon-γ, RANTES (CCL5) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [138].

These activated fibroblasts also shift their metabolic regulation to aerobic glycolysis (The so called Warburg effect) [139]. In the Warburg effect, cancer cells produce energy using unusually high rate of glycolysis and lactate production in cytosol even in the presence of oxygen instead of oxidation of pyruvate in mitochondria like normal cells [140, 141]. Hence, Pavlides et al. called this stromal glycolysis as "Reversed Warburg effect" [139]. When fibroblasts use glycolysis to produce energy they secrete high-energy metabolites, such as lactate, ketone and pyruvate to the microenvironment [142]. These energy metabolites together with the secreted inflammatory mediators were shown to enhance the proliferation of the neighboring cancer cells [143]. Induction of Pyruvate kinase (PK) isozyme M2 drives Warburg effect

in cancer cells [144]. To further elaborate the role of Warburg effect in the stromal fibroblasts, Chiavarina et al. overexpressed PK isozymes M1 and M2 and activated aerobic glycolysis pathway (Reversed Warburg effect) for ATP generation in fibroblasts. These M1-PK or M2-PK overexpressing fibroblasts promoted tumorigenesis through different mechanisms. The M1-PK overexpression increased tumor inflammation and produced lactate for an energy source to cancer cells, whereas M2-PK overexpression led to "pseudo-starvation" response with increased autophagy in fibroblasts and increased ketone-body production as a energy source to cancer cells [145]. Furthermore, upregulation of these enzymes was found in Cav-1 negative fibroblasts in human breast cancer samples [145].

1.4 The Cell cycle

Active cell cycle results in the proliferation of cells. The cell cycle consists of four different phases: Gap 1 (G1), Synthesis (S), G2, and Mitosis (M). During G1 cell size increases and the decision whether the cells will move on to the phase S is made according to external signals, such as the presence of mitogens and nutrients. When DNA is duplicated in the S phase, the cells move on to G2 to further grow in size to prepare for cell division in mitosis (M phase). In the M-phase the cell growth and protein production are stopped as cells use their energy for division into two daughter cells [146].

The progression through the cell cycle phases is controlled accurately and specifically by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDIs) (Figure 5) [147]. Varying the concentrations of cyclin proteins through different cell-cycle phases drives the progression of the cell cycle [148, 149]. Cyclins form a complex with CDKs and activate them through phosphorylation [147]. The levels of D-type cyclins, controlled by the extracellular environment, start to increase in early G1 and they form complexes with CDK4 and CDK6 [150]. Cyclin E starts to accumulate close to G1/S transition and activates specially CDK2. Both cyclin D-CDK4/6 and cyclin E/CDK2 -complexes hyperphosphorylate retinoblastoma (RB) protein to release E2F transcription factor bound to it [151]. The released E2F can subsequently push cells to the S phase by regulating the expression of genes necessary for the S phase entry [152]. In the S phase, also cyclin A can activate CDK2 and control

the progression towards G2 when the levels of cyclin E start to decrease. During the G2/M transition cyclin A-CDK1 activity is needed. Finally, cyclin B-CDK1 complex mediates the progression and the completion of mitosis (Figure 7) [153].

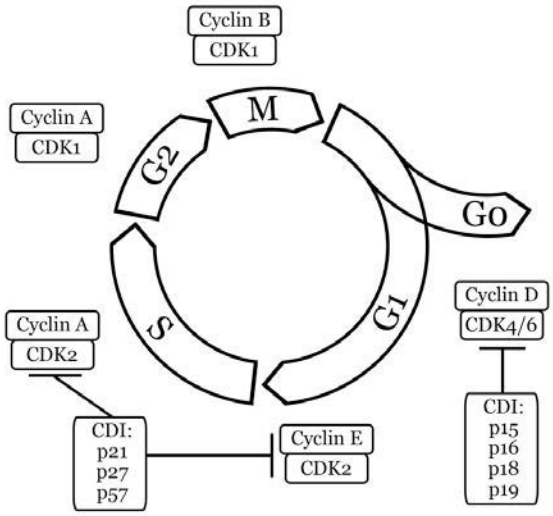

Figure 7. Cyclins and cyclin dependent kinases in different cell cycle phases [147]. CDK, cyclin-dependent kinase. CDI, cyclin-dependent kinase inhibitor.

1.4.1 Checkpoints

The progression of cell cycle and activities of cyclin-CDKs are controlled by checkpoints under normal, as well as stress conditions, such as DNA damage, and telomere dysfunction. If the preceding phase of the cell cycle is not finished or the cell has reparable or irreparable damage, the cell cycle is arrested to the checkpoint by CDIs [154, 155]. There are two families of CDIs: the INK4 family (p15, p14Arf, p16, p18) binds to CDK4 and CDK6 to prevent cyclin D activity and the Cip/Kip family (p21, p27, p57) binds and inhibits cyclin E-CDK2, cyclin A-CDK2, cyclin A-CDK1 and cyclin B-CDK1 (Figure 7) [146].

There are multiple checkpoints in different stages along the cell cycle. The best-known checkpoint, the DNA damage checkpoint, is always active even in the non-cycling cells, such as in differentiated and quiescent cells. If DNA damage is reparable, the cells return to the cell cycle after the damaged DNA is repaired. Whereas if DNA damage is irreparable, the cells withdraw permanently from the cell cycle by the induction of cellular senescence, or die

through apoptosis. The DNA-damage checkpoints occur at the G1/S and G2/M boundaries, but can occur also in the middle of the S phase. DNA damage is detected by using sensor mechanisms and the checkpoints are activated by two master kinases, ATM and ATR [156].

The G1 checkpoint, also known as a restriction point, is located at the end of G1 just before the entry into the S phase. At this point the cells make the decision whether they should divide, delay division or withdraw from the cell cycle to the resting stage G0, also called quiescence. When the cell progresses through this restriction point, the completion of the cycle becomes independent of growth factors [157].

1.4.3 Quiescence

Cellular quiescence (G0) is a common state of somatic cells where proliferation is reversibly arrested due to environmental reasons. When the cells proceed to the quiescence they exit from the cell cycle in G1 [158]. This is induced by contact inhibition, the loss of anchorage or nutrient and/or growth factor deprivation [159]. The transition to G0 is mediated by the downregulation of the cell cycle-related genes, rather than the induction of the cell cycle inhibitors [160], although CDI p27 plays an important role in the initiation and maintenance of the quiescent state. Quiescent fibroblasts are not only passively arrested from the cell cycle, but they seem to posses a controlled program where reversibility is insured, and they maintain high metabolic activity [161]. Furthermore, terminal differentiation, apoptosis and senescence states are actively suppressed [159].

1.4.4 Cellular senescence

Cellular senescence is a tumor suppression mechanism, where cells are terminally withdrawn from the cell cycle [162] in response to a variety of intracellular or extracellular stressors such as telomere erosion (replicative senescence), oxidative stress, irreparable DNA damage and oncogenic stimulation (oncogene-induced senescence; OIS) (Figure 8) [163]. Senescence is characterized by enlarged flattened cell morphology, the lack of DNA replication and positivity for senescence-associated β-galactosidase (SA-β–gal) [163, 164].

The irreversible growth arrest in senescence is controlled by the activity of major tumor-suppressor pathways: p21, p53, retinoblastoma (RB) and p16 [163]. The induction of these proteins has been used as a marker of senescent cells both in cell culture and in vivo.

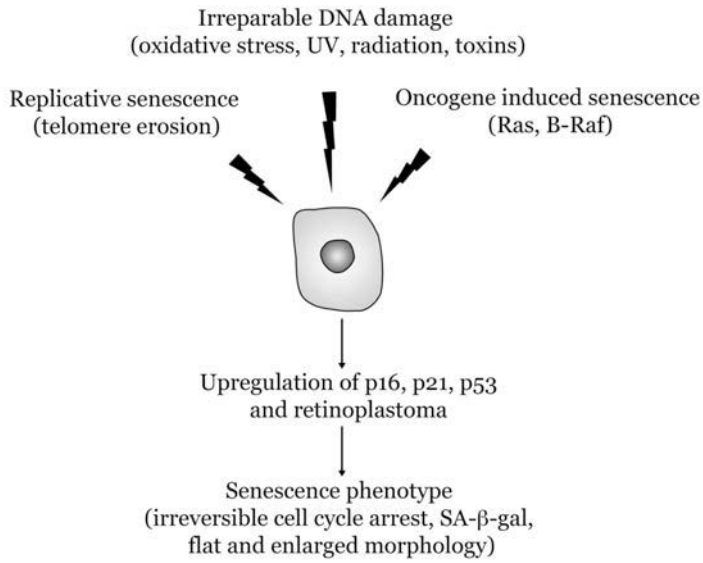

Figure 8. Cellular senescence is induced by a variety of DNA damage causing stressors. SA-β-gal, Senescence-associated-β-galactosidase.

Cellular senescence was first discovered by Leonard Hayflick in 1961, when he observed that fibroblast cells can divide 40 to 60 times [165]. This was called Hayflick's limit [166] and was found to be due to the erosion of telomeres during the cell division [167]. This led to the hypothesis that organism aging is dependent on cellular senescence, which was strengthened by the observation that during the aging senescent cells accumulate in to the tissues [164, 168, 169]. Furthermore, it was discovered recently that the clearance of the senescent cells from the tissues delays ageing-associated disorders [170].

It has been suggested that the senescent cells secrete components that mediate tissue degenerative effects [171]. In addition to the ageing-associated disorders these components also have a great impact in cancer and inflammatory conditions. Coppe et al. found that upon induction of senescence, the cells acquire a senescence-associated secretory phenotype (SASP), where they secrete various inflammation- and malignancy-associated growth factors

such as hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), cytokines and chemokines including IL-1, IL-6, GM-CSF, granulocyte colony-stimulating factor (G-CSF), IL-8, monocyte chemotactic protein-1 (MCP-1), growth-related oncogene α (GROα) and matrix metalloproteinases (MMP-1, MMP-10, MMP-14) [172]. These secreted factors may even drive the transformation of the adjacent benign epithelial cells and stimulate the growth and invasion of cancer cells [172]. SASP has also effects on other non-transformed cells, as evidenced by the increased infiltration of leukocytes, the stimulation of angiogenesis and the differentiation of various cell types. Interestingly, some of the secreted SASP factors can also potentially induce or reinforce senescence in normal cells and are part of the so-called "senescence-messaging secretome" (SMS) [173]. Several of these SMS factors activate transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) in target cells, which in turn is able to enhance senescence response and further increase the secretion of SMS factors [28, 174]. Although senescence is considered to be the first line of defense against oncogenic transformation, it seems to have intricate effects on the nearby cells.

1.5 Nemosis

Nemosis is a novel fibroblast activation model [175]. *In vitro,* nemosis is initiated by plating fibroblasts on a non-adhesive substratum causing them to spontaneously adhere together, forming multicellular spheroids [176]. This process of activation was named after Nemesis, Greek goddess of retribution and inevitable consequence [177]. The formation of fibroblast spheroid is associated with increase in expression of inflammatory and tumor associated cytokines and growth factors [177-182] as well as fibroblast activation protein (FAP) [178], a marker of activated fibroblasts [12]. Hence, nemotic fibroblast express similar genes that activated fibroblast express in wound and tumor microenvironments [5, 12].

The initial hallmark of nemosis was considered to be the induction and activity of the stress-related enzyme cyclo-oxygenase-2 (COX-2). Moreover, the modulation of COX-2 activity with non-steroidal anti-inflammatory drugs (NSAIDs) was found to inhibit its induction in nemosis, whereas addition of its

catalytic products, prostaglandins (PGs), further stimulated its induction [176]. This was quite unexpected and suggested that COX-2 has an important role in the regulation of nemotic activation. Spheroid formation is also associated with damaged cell membrane. Transmission electron microscopy (TEM) revealed that nemosis is associated with features of necrotic cell death. Furthermore, nemotic fibroblasts do not express any markers of apoptosis (morphology, activated caspase-3, Bax, Fas and Daxx) (Table 4). Nevertheless, nemosis is not a completely caspase-independent process. The stimulation of spheroids with the universal caspase inhibitor (Z-VAD-FMK) inhibited the induction of COX-2 protein and membrane damage with unknown mechanisms. Notably, in addition to the apoptosis execution, active caspase is required for the activation of inflammasome that mediates inflammatory processes. In addition to caspase proteases, the α-enolase mediated plasmin activation was found to be a major proteolytic mechanism in the nemosis-associated cell death [176].

Table 4. Different types of cell death [183, 184]

Type of cell death	Morphological feature
Apoptosis (Anoikis)	Rounding-up of the cell Blebbing of plasma membrane Nucleus condensation (Pyknosis) Nuclear fragmentation
Necrosis (Nemosis)	Swelling of cytoplasm and organelles Rupture of cytoplasm
Autophagic cell death	Massive vacuolization of the cytoplasm (autophagic vacuoles)
Mitotic catastrophe	Multinucleation and micronucleation

1.5.1 Nemosis and paracrine signaling

Nemotic fibroblasts secrete a variety of cytokines, chemokines and growth factors, which can potentially have autocrine or paracrine effects. Many of these are important modulators of the tumor microenvironment, such as the proinflammatory cytokines; IL-1β, IL-6, IL-8, IL-11 and leukemia inhibitory factor (LIF), chemokines; IL-8, MIP-1α and RANTES, and growth factors; VEGF, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) [177-182].

Nemotic fibroblasts are able to attract neutrophils and monocytic THP-1 cells in vitro. Neutrophil chemotaxis is mostly dependent on IL-8 secreted by fibroblast spheroids, whereas THP-1 chemotaxis is mediated by the activation of receptors for MIP-1α and RANTES [179]. In addition to chemotaxis, nemosis also stimulates THP-1 adhesion to endothelium and the opening of endothelial tight junctions, thus activating the extravasation of monocytes from the circulating blood to the tissue [185]. Furthermore, fibroblast spheroids cause cell cycle arrest and differentiation of monocytic THP-1 and KG-1 cells to dendritic or macrophage-like cells, which suggests their activation [181].

In addition to activation, as mentioned above, nemosis induces an angiogenic response in endothelium. In cell culture model, nemotic fibroblasts increased migration, motility and cell sprouting of HUVEC cells by secreting the angiogenic growth factors HGF and VEGF [182].

1.5.2 Nemosis and cancer

Nemosis has effects on different cell types that are important in the microenvironment of wounds and tumors. In addition to controlling the development of microenvironment, nemosis also has a direct impact on cancer cells. Many *in vitro* experiments have indicated that nemotic fibroblasts induce more migratory, invasive and proliferative phenotypes of cancer cells [177, 181, 186]. The above-mentioned effect on monocytic leukemia cell differentiation and cell cycle arrest were only applicable for c-Met (receptor for HGF) negative leukemia cells and where counteracted by the introduction of c-MET expression [181]. The cell cycle arrest and differentiation effect were suggested to result in response to molecules (IL-1β, IL-6, IL-8, IL-11, GM-CSF and LIF) secreted by nemotic fibroblasts, whereas active HGF/c-Met pathway was somehow able to counteract their effects. The differentiation of KG-1 and THP-1 cells was accompanied by increased expression of the cell surface markers CD45RA, CD11c, CD86, CD54 and CD13, indicating phenotype change to antigen-presenting cells. The differentiation was also associated with increased chemotaxis towards the nemotic fibroblasts [181].

Conditioned medium from nemotic fibroblasts stimulates HGF-dependent outgrowth and invasiveness of c-Met positive melanoma cells [177], as well as

motility and proliferation of keratinocytes, presenting different stages of skin carcinoma progression. Nemosis stimulates benign and low-grade malignant keratinocyte invasion, whereas it was unable to further stimulate the invasiveness of metastasizing keratinocytes [186]. Moreover, the two-directional interaction between cancer cells and nemotic fibroblasts is highlighted by the findings showing that conditioned medium from melanoma cells, or co-culture with squamous carcinoma cells causes fibroblasts to spontaneously form spheroid-like structures in adherent cultures [177, 178].

2. AIMS OF THE STUDY

Nemosis is a novel type of fibroblast activation that is initiated by fibroblast spheroid formation. Nemosis can regulate the differentiation of leukemia cells, invasion of melanoma cells, proliferation and migration of malignant keratinocytes, and the chemotaxis of monocytes. The aim in the current study was to elucidate the phenotype of nemosis and its effects on tumor growth.

The detailed aims were:

1. To investigate the role of FN-integrin interaction and FN matrix assembly in the formation and activation of fibroblast spheroid.
2. To determine gene expression changes in nemosis compared to adherent fibroblasts cultures.
3. To uncover the phenotype that fibroblasts undertake during spheroid activation.
4. To characterize the effects of spheroid-activated fibroblasts on tumor growth.

3. MATERIALS AND METHODS

3.1 Cell culture (I-IV)

Human foreskin dermal fibroblasts: HFSF (kindly provided by Dr. Magdalena Eisinger, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and CCD-1072sk (ATCC, Manassas, VA). Mouse embryonic fibroblasts: $FN^{-/-}$, $FN^{fl/fl}$ (control for $FN^{-/-}$), $FN^{RGE/RGE}$ and $FN^{RGD/RGD}$ (control for $FN^{RGE/REG}$). Human fibrosarcoma cell lines: HT-1080 and AT9733 were cultured at +37° C in 5% CO_2 atmosphere in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% Fetal calf serum (FCS; Invitrogen), 100 µg/ml streptomycin, and 100 U/ml penicillin. HFSF cells were used at passages 10 to 25. $FN^{-/-}$ and $FN^{RGE/RGE}$ cells were previously described in references [187, 188].

Spontaneously immortalized HaCat human keratinocyte cell line and its variants (A5, II-4 and RT3), containing H-ras oncogene (Val 12 mutation), represent different stages of tumor progression. A5 clone is classified as benign, whereas II-4 clone forms well differentiated squamous cell carcinoma. The high-grade malignant and metastasizing variant RT3 was generated by in vivo passaging of A5 clone [189]. HaCat, A5, II-4 and RT3 cells (kindly provided by Dr. Petra Boukamp and Dr. Norbert E. Fusenig, DKFZ, Heidelberg, Germany) were cultured in DMEM medium supplemented with 10% FCS (Invitrogen), 100 µg/ml streptomycin, and 100 U/ml penicillin.

Fibroblast spheroids were formed by plating 200 µl aliquots of cell suspensions ($5x10^4$ cells/ml) on agarose-coated U-bottom 96-well plates (Costar, Cambridge, MA). For LDH activity assay 100 µl cell suspension ($20x10^4$ cells/ml) was plated per well. In some experiments, inhibitors, peptides, proteins or antibodies (Table 5) were added to the cell suspension before initiation of spheroid formation. To study the effect of FN-integrin interaction on spheroid formation and activation, the experiments were done using 1% FN-depleted FCS. FCS was depleted of FN by incubating it with gelatin-Sepharose (Pharmacia, Uppsala, Sweden) overnight at +4 °C. The depleted FCS was sterile filtered through 0.2 µm filter (Millipore, Cork, Ireland). The removal of FN was confirmed by dot blotting.

Table 5. Inhibitors, peptides, proteins and antibodies used to stimulate fibroblast spheroid formation.

Compound	Concentration or dilution	Action	Company
3-MeA	5 mM	Inhibitor of autophagy	Sigma-Aldrich
Aprotinin	200 KIU/ml	Inhibitor of several proteases	Sigma-Aldrich
Anti-β1 integrin (Clone P5D2)	1:100	Blocks FN binding to β1 integrin	Santa-Cruz Biotechnology
Anti-α5 integrin (Clone B1E5)	1:10	Blocks FN binding to α5 integrin	In house [190]
Anti-αV integrin (Clone AV1)	1:10	Blocks FN binding to αV integrin	In house [191]
Anti-FN (Clone HFN 7.1)	1:10	Binds to FN	Transduction Laboratories
Bafilomycin A1	10 nM	Inhibitor of vacuolar proton bump	Tocris
CMT-3, CMT-5, CMT-308	5 μM	MMP inhibitors	CollaGenex Pharmaceuticals
Cycloheximide	10 ng/ml	Translational inhibitor	Sigma-Aldrich
pFN	50 μg/ml	Plasma fibronectin	Roche Applied Science
N-terminal 70 kDa FN fragment	100 μg/ml	Inhibits FN matrix assembly	Sigma-Aldrich
GRGDSP	5 mM	Integrin binding peptide	Bachem
GRGESP	5 mM	Control peptide for GRGDSP	Bachem
Ilomastat	5 μM	MMP inhibitor	Sigma-Aldrich
U0126	20 μM	Inhibitor of MEK	Tocris

3.1.1. Liposome-mediated siRNA transfections (I)

For siRNA experiments, human foreskin fibroblasts were cultured for one passage without antibiotics to avoid antibiotic-associated toxicity. The cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, one day before the transfection the cells were split to 50% confluence in DMEM:F-12 containing glutamine and nonessential amino acids (Invitrogen). The transfection was done in Opti-MEM with siGENOME oligonucleotides against the human FN1 gene (Gene ID: 2335) (Dharmacon, Lafayette, CO) at a concentration of 80 nM. After a 5-h incubation, the transfection medium was replaced with fresh medium (5% FN-depleted FCS) without antibiotics. 24 hours after transfection antibiotics were added to the culture medium. Three days after transfection spheroid formation was initiated. The spheroids were processed for immunoblotting after another 72 hours.

3.1.2. Lentiviral mediated shRNA (II)

293FT cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) together with pLKO.1 plasmid, expressing shRNA against ATG5 and puromycin-resistance gene (Clone: TRCN0000151963; Open Biosystem, Huntsville, AL) or non-targeting scramble shRNA (Biomedicum Genomics, Helsinki, Finland), and packaging vector pCMVΔ8.91 and pHCMV-G, which express the vesicular stomatitis virus envelope glycoprotein G. After 48 h, the lentivirus-containing supernatant was collected and filtered (Millex-HV 0.45 µm low protein binding PVDH filter) (Millipore Corporation, Bedford, MA) to exclude carryover of 293FT packaging cells. HFSF cells were transduced with filtered supernatant containing 8 µg/ml polybrene (Sigma-Aldrich) overnight. After 48 h, tranduced cells were selected using puromycin (2 µg/ml) for five days.

3.2 Functional assays

3.2.1 Soft-agar assay (II)

The soft-agar colony forming assay was done as described [178]. Briefly, adherent fibroblast cultures were plated on a 6 well plate (5 000, 50 000 or 500 000 cells/well) one day before the experiment. The cells or empty wells (control) were overlaid with 0.5% low-melting point agarose (Invitrogen, Carlsbad, CA) in DMEM:F-12 supplemented with 5% FCS and antibiotics, and were allowed to solidify at +4 °C for 30 min. In spheroid groups, the indicated amounts of spheroids were mixed in the bottom agar, which was let to solidify. RT3 keratinocytes (5 000 cells/well) were mixed with 0.3% top agarose in DMEM:F-12 supplemented with 5% FCS and antibiotics, which was added to the bottom agar and let to solidify at +4 °C for 30 min (Figure 9). After the agar had solidified, the plates were placed in a standard cell incubator and 100 µl of growth medium was added once a week to prevent the agar from drying. All groups were done in duplicate and three images per well were taken at indicated times for analyzing the number and growth of colonies. The size of the colonies was quantified using NIH ImageJ software (http://rsb.info.nih.gov/ij/) [192].

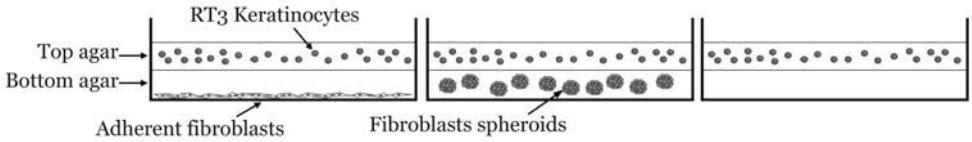

Figure 9. Soft-agar experimental setup

3.2.2 Collagen co-culture assay (III)

24-well plates were overlaid with 200 μl of ice-cold mixture containing rat tail collagen type I (80 μl; final concentration in lattice was 1.6 mg/ml) (Millipore, Cork, Ireland), 2xDMEM (100 μl) and DMEM (20 μl) and let to polymerize for 30 min in the cell culture incubator. 45 human fibroblast spheroids (5-day old) were collected and diluted to 200 μl of ice-cold 2xDMEM. After a short incubation on ice 40 μl DMEM and 160 μl of ice-cold collagen were rapidly mixed with the spheroids and the mixture was overlaid on top of the first collagen lattice. Cell-free collagen- and fibroblast-collagen lattices were prepared by mixing ice-cold 2xDMEM (with or without 450 000 fibroblasts) (300 μl), DMEM (60 μl) and collagen (240 μl), and poured on a clean 24-well plate well. The collagen lattices were permitted to polymerize for additional 45 min in the cell culture incubator, and 250 000 cells/well of HaCaT or RT3 cells were plated on top of the collagen lattice (Figure 10). After 6 days of incubation, the collagen lattices were fixed with cytoskeletal buffer with sucrose (4% paraformaldehyde, 320 mM sucrose, 10 mM MES, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA), permeabilized with 0.5% Triton X-100 in PBS and embedded in paraffin.

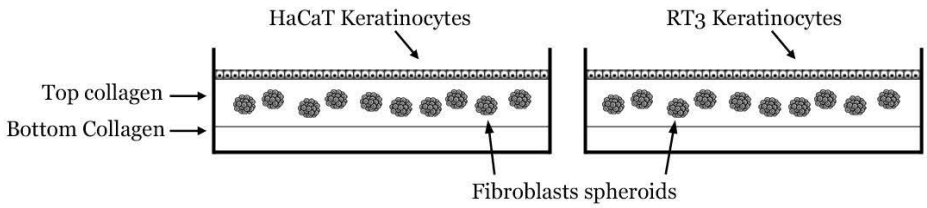

Figure 10. Collagen co-culture experimental setup

3.2.3 Tumor xenograft models (II)

In order to generate tumors, $6x10^5$ RT3 cells alone, together with $3.6x10^5$ fibroblasts or with 36 spheroids were suspended in 100 µl of 1:1 dilution of PBS and growth factor reduced Matrigel (BD Biosciences), and injected to the peritoneal cavity of female Balb/c nude mice (Scanbur, Sweden). The tumors were measured using a calibre at indicated times and volumes were calculated using the following formula: Volume = 0.5 X height X width X length. The experiments in NOD/SCID (Charles Rivers Laboratories, Margate, U. K.) mice were conducted in a similar way, except the number of implanted cells was $6x10^5$ RT3 cells with either $3.2x10^5$ fibroblasts or 32 spheroids.

3.3. Protein expression (I-IV)

3.3.1 Western blot (I-IV)

The spheroid and monolayer cultures were lysed in reducing Laemmli sample buffer and incubated at +100 °C for 5 min. Protein extracts were separated by 8 to 20% gradient SDS-PAGE and transferred to nitrocellulose membranes. Unspecific binding was blocked with 2.5% powdered non-fat milk in 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20. Immunoreactive proteins were visualized with the appropriate primary and secondary antibodies using ECL detection (Pierce, Rockford, IL). The densiometric analysis of the films was done using NIH ImageJ software.

3.3.2 Immunohistochemistry, immunofluorescence and senescence-associated β-galactosidase (I-IV)

Spheroids were collected at the indicated times, snap frozen in liquid nitrogen and embedded in OCT (Tissue Tek, Sakura Finetek Europe B.V., Zoeterwoude, NL). For tumors, the samples were collected immediately after the sacrifice and fixed with 4% paraformaldehyde solution for 1 hour, soaked in 30% PBS-sucrose overnight, embedded in OCT and frozen in the freezer. Eight-µm frozen sections were cut, fixed with freezer-cold acetone and stained with the indicated antibodies. For paraffin-embedded spheroid and tissue blocks, the samples were fixed with 4% paraformaldehyde (spheroids for 1 hour and tumor overnight),

dehydrated and embedded to paraffin blocks. Immunohistochemistry (IHC) was performed with the Ventana Discovery immunohistochemistry Slide Stainer (Ventana Medical Systems, Inc., Tucson, AZ). Paraffin-embedded 5-μm sections of spheroids were incubated with primary antibodies for 32 min each. The staining was performed with the Ventana 3,3'-diaminobenzidine tetrahydrochloride (DAB) biotin avidin detection kit.

SA-β-gal activity was detected as described in [164]. Briefly: whole spheroids, frozen sections or monolayer cultures were fixed with 2% PFA/0.2% glutaraldehyde in PBS for 5 min, washed and incubated with staining buffer (1 mg/ml X-gal, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ in 40 mM phosphate buffer pH 6) overnight. In SA-β-gal and IHC double staining, frozen sections were stained first with SA-β-gal and then stained using antibodies with Ventana Discovery immunohistochemistry Slide Stainer.

3.3.3 Enzyme-linked immunosorbent assay (ELISA) (IV)

The concentrations of VEGF and HGF in the culture media were determined using ELISA assays (R&D Systems for HGF and Orgenium Laboratories for VEGF) according to manufacturers' instructions.

3.3.4 LDH activity assay (II, III)

Media from fibroblast spheroids were collected at the indicated times and clarified by centrifugation at 5 000 x *g* for 5 min. LDH activity was measured using colorometric assays (Cayman Chemicals (II) or Roche (III)). Aliquots of 100 µl of medium were mixed with 100 µl of LDH assay buffer, incubated for 30 min and optical density was measured at 492 nm.

3.3.5 Caseinolysis assay

Proteolytic activity in fibroblast spheroid-conditioned medium was quantified using radial caseinolysis assays [193]. Aliquots of 10 µl of spheroid-conditioned medium were added to the wells of an agarose gel containing 1% casein. The gels were incubated at +37 °C for 72 h and the lysis zones were measured.

Table 6. Antibodies used in different protein analysis methods (I-IV)

Primary antibody	Host	Supplier	Method	
Pan-actin	Mm	Thermo Scientific	WB	
Akt	Rp	Cell Signaling Technology	WB	
p-Akt	Mm	Cell Signaling Technology	WB	
ATG5	Rp	Novus Biologicals	WB	
C/EBPβ	Mm	Santa-Cruz Biotechnology	WB, IHC	
CK7	Rp	Abcam	IHC	
COX-2	Rm,Rp	Thermo Scientific	WB, IHC	
Cyclin D1	Mm	Thermo Scientific	WB	
Fibronectin	Rp	Abcam	WB, IHC	
Erk	Rp	Cell Signaling Technology	WB	
p-Erk	Gp	Santa-Cruz Biotechnology	WB	
GAPDH	Rp	Santa-Cruz Biotechnology	WB	
Ki67	Rm	Thermo Scientific	IF	
LC3B	Rp	Sigma-Aldrich	WB	
MMP-1	Mm	Oncogene Research Products	WB	
MMP-9	Sp	Calbiochem	WB	
MMP-10	Mm	R&D Systems	WB	
MMP-13	Mm	Oncogene Research Products	WB	
MT1-MMP	Rp	Biogenesis Ltd	WB	
p14Arf	Mm	Abcam	IHC	
p21	Mm	DakoCytomation	IF, WB	
p27	Mm	BD Biosciences	WB	
p53	Mm	Thermo Scientific	WB	
p62	Rp	Sigma-Aldrich	WB	
p63	Rp	Santa-Cruz Biotechnology	IHC	
PCNA	Rp	Thermo Scientific	WB	
Polyubiquitin	Sp	Biomol	WB	
RB	Mm	Thermo Scientific	WB	
α-SMA	Mm	DakoCytomation	IHC	
TIMP-1	Rp	Chemicon International	WB	
β-tubulin	Mm	Sigma-Aldrich	WB	
Vimentin	Mm	Ventana Medical Systems	IHC	
Secondary antibody	**Host**	**Supplier**	**Label**	**Method**
Anti-mouse IgG	Donkey	Jackson	HRP	WB
Anti-mouse IgG	Donkey	Jackson	Biotin	IHC
Anti-mouse IgG	Goat	Molecular Probes	Alexa Fluor 488	IF
Anti-rabbit IgG	Donkey	Jackson	HRP	WB
Anti-rabbit IgG	Rabbit	Jackson	Biotin	IHC
Anti-rabbit IgG	Goat	Molecular Probes	Alexa Fluor 488	IF
Anti-Sheep IgG	Donkey	Jackson	HRP	WB
Anti-Goat IgG	Donkey	Jackson	HRP	WB

Mm: mouse monoclonal, Rm: rabbit monoclonal, Rp: rabbit polyclonal, Gp: Goat polyclonal, Sp: Sheep polyclonal, IF: immunofluorescence, IHC: immunohistochemistry, WB: Western blot, HRP: horseradish peroxidase, Jackson: Jackson ImmunoResearch Laboratories

3.4 mRNA expression (II-IV)

3.4.1 RT-qPCR (II, IV)

The samples for RT-qPCR were harvested in RNAprotect Cell Reagent and total RNA was extracted according to the manufacturer's instructions using the RNeasy Plus Mini kit (QIAGEN, Hilden, Germany). One μg of RNA from each sample was reverse-transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was performed using the DyNAmo Capillary SYBR Green Quantitative PCR kit (Finnzymes, Espoo, Finland) with a LightCycler Instrument (Roche Applied Science, Mannheim, Germany). The primers were purchased from Oligomer (Helsinki, Finland) (Table 7). The relative expression of target gene mRNA normalized to GAPDH expression was calculated using the REST-MSC software's Pair-Wise Fixed Reallocation Randomization Test.

Table 7. Primer sequences

GENE	Forward primer	Reverse primer
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
C/EBPβ	CTCTCTGCTTCTCCCTCTGC	CGTGTACACACGCGTTCAG
VEGF	TACCTCCACCATGCCAAGTG	ATGATTCTGCCCTCCTCCTTC
IL-6	AGTGAGGAACAAGCCAGAGC	GTCAGGGGTGGTTATTGCAT
HGF	CGCTGGGAGTACTGTGCAAT	CCCTGTAGCCTTCTCCTTGA
KGF	GGGACCCAAGAGATGAAGAA	TTCACTTTCCACCCCTTTGA
Groa	GAAAGCTTGCCTCAATCCTG	CTTCCTCCTCCCTTCTGGTC

3.4.2 In situ hybridization (III)

In situ hybridization (ISH) was used to determine the urokinase-type plasminogen actiovator (uPA) and the tissue-type plasminogen activator (tPA) mRNA expression and localization in fibroblast spheroids. Pst I fragment of tPA (243 bases) and uPA (240 bases) cDNA were cloned to pHUK-8 plasmid and in vitro transcribed from T7 and SP6 promoters to digoxigenin labeled riboprobes. Sense strand was used as a negative control. ISH was performed with the Ventana Discovery Slide Stainer (Ventana Medical Systems). Sections were incubated with probe overnight. The hybridized probe was detected with anti-digoxin and visualized with Ventana Blue Map kit.

3.4.3 Microarray

Total RNA was extracted from the corresponding adherent and spheroid cultures 3, 12, 24 and 36 h after seeding by standard methods using Trizol Reagent (Invitrogen) followed by purification with the RNeasy kit according to the manufacturer's instructions (QIAGEN). RNA quality was assessed by using the Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA).

Biotinylated cRNAs were prepared from 5 μg of total RNA for hybridization according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Briefly, RNA was converted to first-strand cDNA by the use of a T7-$(dT)_{24}$ primer (Invitrogen), followed by second-strand synthesis (Invitrogen). This double-stranded cDNA then served as a template for labeling the *in vitro* transcripts using biotinylated ribonucleotides (Enzo, Farmingdale, NY). Fifteen μg of each labeled cRNA was chemically fragmented and then hybridized to Affymetrix HG-U133A GeneChips under standard conditions in an Affymetrix fluidics station. To eliminate false-positive results, duplicate samples and chips were performed for both adherent and spheroid cultures at 36 hours.

3.4.3.1 Microarray data analysis

The arrays were scanned according to the manufacturer's instructions (Affymetrix). Obvious artifacts and the outliers on the scanned images were excluded from the analysis by Affymetrix® Microarray Suite 5.0 software.

The gene expression profiles of adherent and spheroid cultures were compared by means of the GeneSpring™ software version 7.2 (Agilent Technologies). The data were normalized by GC-RMA normalization and per gene: Normalize to median. The "Cross gene error model for replicates" was active. Those transcripts that were not at least 2-fold upregulated or downregulated compared to adherent cultures, whose normalized values were under 2 (as 2-fold upregulated) and over 0.5 (as 2-fold downregulated), or were not given a present tag by Affymetrix® Microarray Suite 5.0 (MAS5.0) at 36 h (either in adherent or spheroid cultures) were removed from the statistical analysis. The decision on the present tags was made based on the present and absent tags generated by MAS5.0, expecting that the transcript was present in both replicates in monolayer or spheroid samples. Significantly ($p \leq 0.05$)

upregulated or downregulated transcripts were determined by comparison of adherent and spheroid cultures at 36 h using student's t-test and the Benjamini and Hochberg False Discovery Rate as a multiple testing correction. The data are deposited at http://www.ebi.ac.uk/arrayexpress/, accession number: E-MEXP-1226.

3.5 Fluorescence-activated cell sorting (FACS) (II)

For cell cycle analysis the cells were dissociated from the spheroids or from monolayers with trypsin-EDTA and fixed with 70% ethanol at least overnight. The cells were washed once with PBS and stained 10 min with propidium iodide (10 µg/ml for 10 min at room temperature) (Invitrogen) and analyzed with FACScanner (BD Biosciences)

3.6 Transmission electron microscopy (TEM) (II)

The spheroids were collected at the indicated time points and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed with phosphate buffer and fixed again with 2% osmium tetroxide. After washing the spheroids were dehydrated with Ethanol gradient and embedded into Epoxy resin LX-112. The epon blocks were cut into ultrathin (80 nm) sections, and stained with uranyl acetate and led citrate. Electron microscopy was performed using JEOL 1400 TEM (JEOL, Tokyo Japan) at 80 kV.

3.7 Statistical analyses

SPSS software was used to calculate statistical significances.

4. RESULTS AND DISCUSSION

4.1 Fibronectin-integrin interaction is required for fibroblast spheroid formation and activation (I)

Nemotic fibroblasts have been shown to produce a variety of interesting inflammation and tumor-associated secretory factors, and the paracrine signaling mediated by these factors to other cell types is relatively well characterized *in vitro*. However, nothing is known about the molecules that mediate the formation of fibroblast spheroids or cellular processes that lead to nemotic activation. In this study, we sought to investigate the molecules that participate in the initial formation of fibroblast spheroids.

4.1.1 Effect of fibronectin on spheroid formation (I)

FN started to accumulate in fibroblast spheroids already 3 h after the initiation of spheroid formation and its amount began to decline after 24 h, indicating an induction of proteolytic mechanisms in the spheroids (Figure 1A and 1B in I). This is in agreement with activation of FN-degrading plasminogen in fibroblast spheroids [176, 194]. As expected, cycloheximide, an inhibitor of translation [195], prevented the accumulation of FN, but at the same time resulted in looser-structured spheroids. Adding exogenous pFN counteracted this effect and resulted in more compact spheroids, suggesting that FN is needed for the formation of tight spheroids (Figure 1B, 1C and 1D in I).

This observation was further tested using immortalized mouse embryonic fibroblasts where the FN gene was knocked-out ($FN^{-/-}$ cells) or its integrin binding RGD motif was mutated to RGE ($FN^{RGE/RGE}$ cells). Both of these cell lines resulted in more loosely-structured spheroids and adding pFN restored the formation of tight spheroid structures (Figure 2A and 2C in I). This confirmed that FN mediates the formation of fibroblast spheroids and suggested that the integrins mediate this interaction through the FN's integrin binding motif RGD.

To investigate whether FN protein mediated spheroid formation is necessary for nemotic activation as well, we used siRNA to deplete FN from primary human skin fibroblasts. FN siRNA -treated fibroblast spheroids expressed only a residual amount of FN protein and were unable to induce

COX-2, considered to be a marker of nemotic activation, in response to spheroid formation. Unexpectedly, adding pFN to the forming fibroblast spheroids did not restore the induction of COX-2 (Figure 2E in I). This suggests that although the addition of pFN can counteract the deletion of FN synthesis in spheroid formation, the activation of fibroblasts requires that FN be produced in the cells themselves, or alternatively spliced cFN is needed.

To further elaborate the role of the FN-RGD-motif on activation of fibroblast spheroids we used the hexapeptide (GRGDSP) and an inactive control peptide (GRGESP) to stimulate spheroids during formation. GRGDSP peptide was able to prevent spheroid formation and FN accumulation, but also augmented COX-2 induction (Figure 3D and 3E in I), considered a hallmark of nemotic activation [176], indicating that it can function as a competitive peptide for FN, and that fibroblast activation is mediated via the RGD motif.

4.1.2 FN-integrin interaction and FN matrix formation in fibroblast spheroids (I)

As shown above FN's integrin binding motif RGD plays an important role in the spheroid formation and activation. We characterized FN-binding integrins more closely using antibodies that prevent FN adhesion to a particular integrin subunit. We focused on α5, αV and β1 integrin subunits, as those were the highly expressed ones in fibroblasts according to microarray data. Antibodies against α5 and β1 integrins were able to retard spheroid formation, whereas αV integrin did not have any effect (Figure 3A and 3B in I). On the contrary, αV and β1 integrins inhibited COX-2 induction whereas α5 integrin did not have an effect (Table 7, Figure 3C in I). This might be due to the fact that the antibody we used has been shown to act as an activating ligand for α5 integrin [196].

The binding of RGD to integrin is enhanced by the synergy sequence PHSRN located between the 9th and 10th type III repeats in FN molecule (Figure 2) [197]. FN binding to integrins also activates FN matrix formation [53]. To study the effect of synergy site and FN matrix on spheroid formation, we used a FN antibody that targets FN in the same site where the synergy site is located and in addition, we also used a 70 kDa N-terminal fragment of FN, which has been shown to prevent FN matrix formation [198]. Interestingly, both treatments delayed spheroid formation, but did not affect the amount of FN in

the spheroid (Figure 2C in I). The synergy site was found to decrease the induction of COX-2 (Figure 2C in I), indicating that integrins are important in mediating nemotic activation. The 70 kDa N-terminal fragment did not inhibit COX-2 induction, but seemed to enhance its induction slightly (Table 8 and Figure 3C in I), suggesting that FN matrix assembly is not important in fibroblast nemosis, since the formation of insoluble FN matrix is prevented already at namomolar concentrations of the 70 kDa N-terminal FN fragment [199, 200]. Although 70 kDa N-terminal FN fragment prevents matrix formation it still might have a role in the activation of fibroblast spheroids, given that it can stimulate the migration of fibroblast [201], indicating that it stimulates the activation of integrins [54]. This might explain the modest induction of COX-2 in 70 kDa N-terminal FN fragment stimulated fibroblast spheroids. The absence of FN matrix formation in fibroblast spheroids may also explain the decline of FN in later time points (Figure 1A in I), since non-deposited FN is degraded in Cav-1 dependent turnover [202].

Myofibroblasts make direct cell-cell contacts through N-cadherin and OB-cadherin [203]. Whether fibroblasts make direct homotypic cell-cell contacts after initial FN-integrin adhesion in spheroids is still unclear. TEM images revealed some contacts between fibroblasts, but their number did not increase with time and they did not mature further to real cell-cell contacts (previously unpublished; Figure 11).

Table 8. The effect of peptides and integrin antibodies on spheroids

Compound	Spheroid formation	Activation (COX-2)
GRGDSP peptide	Prevents	Activates
α5 integrin Ab	Prevents	No effect
αV integrin Ab	No effect	Inhibits
β1 integrin Ab	Prevents	Inhibits
FN PHSNR site Ab	Prevents marginally	Inhibits
70 kDA FN fragment	Prevents marginally	Modest induction

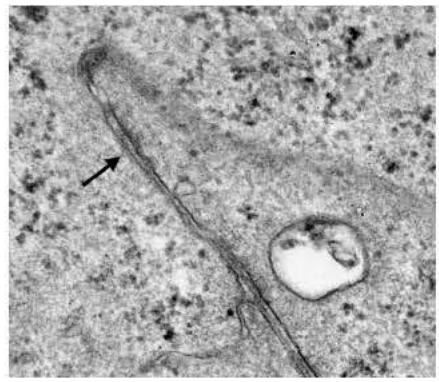

Figure 11. TEM image of 12 h old spheroid. Arrow: unmaturated cell-cell contact

These results are in agreement with other studies showing that integrin $\alpha5\beta1$ regulate FN matrix deposition and strong compaction of Chinese hamster ovary (CHO) B2 cell spheroids that where engineered to express $\alpha5\beta1$ integrin [60, 204, 205]. These studies also showed that fibronectin matrix deposition is essential to mediate strong cell-cell cohesion in spheroid, whereas in our setup inhibition of matrix deposition by 70 kDa FN fragment delayed spheroid formation only marginally. The difference might be due to the cell types used. The surface of normal fibroblasts is rich in a variety of integrins, whereas CHO B2 lacks the integrin $\alpha5$ subunit and is not able to bind FN [206], indicating that other subunits are not able to compensate FN binding in CHO B2 cells. Hence, other integrin subunits could counteract $\alpha5\beta1$ integrin mediated FN matrix deposition in normal fibroblasts.

It is well established that activated fibroblasts (myofibroblasts) produce FN EDA splice variant [12]. This is in line with our result showing that fibronectin-integrin interaction is required for the activation of fibroblast spheroids. Furthermore, adding pFN that lacks EDA domain was not able to rescue the activated phenotype of FN depleted fibroblast spheroids. In addition cell adhesion to fibronectin induces and stabilizes COX-2 protein [207, 208], as well as activates inflammatory-associated transcription factor NF-κB [209, 210].

4.2 Gene expression changes in fibroblast spheroids compared to adherent fibroblast cultures (II)

To tentatively characterize the phenotype that cells acquire in fibroblast spheroids, we performed genome-wide gene expression microarray analysis 3, 12, 24 and 36 h after the initiation of spheroid formation. The change in gene expression within time is shown in figures S1A and S1B in II. An independent duplicate analysis at the 36 h time-point revealed a massive changes in gene expression compared to standard adherent cultures. Over 16% of genes expressed in fibroblast spheroids had altered expression levels compared to adherent fibroblasts. When the list of up- and downregulated genes was categorized according to their Gene Ontologies, the change could be roughly divided into three groups: Most of the upregulated genes encoded secreted

proteins, such as cytokines, chemokines and growth factors, whereas the downregulated encoded proteins associated with cell cycle and cytoskeleton. Over 50 fold up- and downregulated genes are classified according to their biological process in Table S1 in II. The protein products of up- or downregulated genes that are significant in this thesis are shown in Table 9.

Table 9. The protein products of up- and downregulated genes in fibroblast spheroids compared to adherent fibroblast cultures

Upregulated		Downregulated	
Protein	Fold change (mRNA)	Protein	Fold change (mRNA)
COX-2	93.1	Cyclin B1	132.3
p62	12	Cyclin A2	65.8
C/EBPβ	4.1	Cyclin B2	33.8
IL-1β	211.5	Cyclin E2	8.9
IL-1α	188.3	p53	3.4
IL-6	200.3	α-actin	23.9
IL-8	231.5	β-actin	6
MCP-1	15.8	γ-actin	3.5
MIP-1α	787.9	β-tubulin	22.1
RANTES	46.8	α-tubulin	5.7
GM-CSF	106.6	PCNA	6.3
G-CSF	161.8	Ki67	24
GROα	5.8		
HGF	138.9		
LIF	82.7		
IL-11	178		
PAI-2	9		

4.3 Autophagic activity increases during early time-points (II)

To better understand the cell morphology of fibroblast spheroids, we performed TEM analysis of fibroblast spheroids as a function of time. When fibroblast spheroids aged (2-4 days) they started to show typical structures of cellular stress, such as disrupted rough endoplasmic reticulum, degenerated organelles, accumulation of lipofuscin and intermediate filaments (Figure 3A in II).

A prominent finding in TEM analysis was an increased amount of autolysosomes, lipid droplets and MVBs, suggesting either an increase in autophagic activity or reduced turnover of autolysosomes (Figure 3A in II). To confirm the increase in autophagy we used immunoblotting assays to detect

conversion of LC3B-I to LC3B-II form, a universally used marker of autophagic vacuoles. LC3B-II appeared already 1.5 h after the initiation of spheroid formation. After 12 h both forms, LC3B-I and LC3B-II started to decline, indicating active autophagy (Figure 3B in II). The expression of LC3B-II correlated with the expressions of p62 and polyubiquitinated proteins (Figure 3B in II). During induced autophagy p62 becomes incorporated to autophagosome and is degraded completely [211]. There is a marginal induction of p62 in fibroblast spheroids, peaking at 12 h after the initiation of spheroid formation, and after that its level starts to decline, although it never disappears completely (Figure 3B in II). The initial induction of p62 protein is explained by the induction of p62 mRNA (12 fold in microarray analysis) in fibroblasts spheroids. The disappearance of polyubiquitinated proteins and the decline of p62 suggest that autophagy was induced in fibroblast spheroids.

To confirm the induction of autophagy we used shRNA against ATG5, as well as two inhibitors, 3-MeA, which prevents autophagosome formation, and bafilomycin A1 (Baf A1), which prevents the lysosomal acidification, to block autophagy at different steps of the process. The formation of LC3B-II was almost completely blocked in response to ATG5 shRNA (Figure 4A and 4B in II). It was not entirely effective in preventing the degradation of LC3B-I and p62 (Figure 4A and 4B in II), suggesting that a residual amount of ATG5 is active or that the proteins are also degraded in an alternative process in fibroblast spheroids. 3-MeA inhibited the formation of LC3B-II for 3 h, but after this it was ineffective in blocking the formation of LC3B-II (Figure 4A and 4B in II). This is because the ability of 3-MeA to inhibit class III PI3K is only transient and it lasts only less than 6 h [212]. Nonetheless, 3-MeA was able to decrease the degradation of LC3B-II and p62, proposing that it is inhibiting autophagic flux in fibroblast spheroids. The lysosomal inhibitor Baf A1 blocked autophagic flux completely, as evidenced by the accumulation of LC3B-II and p62 (Figure 4A and 4B in II). Baf A1 prevents the acidification of lysosomes and other vesicles, as well as the fusion of autophagosome and lysosome [213]. The accumulation of LC3B-II and p62 in fibroblast spheroids treated with Baf A1 indicates that autophagy is induced during spheroid formation and that the increase in autophagosomes is not due to reduced turnover of autophagosomes and autolysosomes.

The induction of autophagy happens approximately at the same time as FN-integrin interaction mediates spheroid formation. This is contrary to other studies showing that disintegrated integrins signal to induce autophagy during cell detachment from ECM to provide cell survival against anoikis [214]. However, recently it was found that the plasminogen activator inhibitor-1 and -2-mediated remodeling of ECM causes autophagy dependent fibroblast activation [215]. This may explain the discrepancy in FN-integrin interaction and induction of autophagy.

The inhibition of autophagy, using ATG5 targeted shRNA, caused cell death as measured by the increased release of LDH in culture media (Figure 4C in II). This is in agreement with numerous studies showing autophagy as a survival mechanism against various stress conditions [216]. Usually the inhibition of stress induced autophagy leads to enhanced apoptic response [216]. Interestingly, shATG5-mediated cell death in fibroblast spheroids was not associated with increased cleaved-caspase-3, cleaved-caspase-8 or cleaved-PARP (the activated forms of these proteins) (unpublished data), indicating that the inhibition of ATG5 does not induce apoptosis in fibroblast spheroids. This is in agreement with the findings that the inhibition of autophagy induces necrosis in apoptosis impaired cells [117, 118], and that fibroblast spheroids seem to actively avoid apoptosis by downregulating apoptosis regulators and upregulating anti-apoptotic molecules, such as NF-κB, COX-2, PAI-2 and HGF [176, 177, 179].

4.3.1 ERK and Akt as possible regulators of autophagy in fibroblast spheroids (II)

A major negative regulator of autophagy is the mammalian target of rapamycin (mTOR), which senses environmental signals and controls cell growth and proliferation [77]. These signals are conveyed by Akt, which in turn regulates mTOR activity [217]. In fibroblast spheroids Akt was dephosphorylated, indicating inactivation, already after a few hours after the initiation of spheroid formation as shown by the immunoblot (Figure 3C in II). This suggests that the induction of autophagy might be mediated by the negative regulation of Akt and the subsequent inactivation of mTOR in fibroblast spheroids, although additional experimentation is needed to evidence its direct role. The

dephosphorylation of Akt was timely associated with the fibronectin-integrin mediated spheroid formation. This is somewhat contrary to the integrin mediated Akt activation in response to fibronectin adhesion [218], but strengthen the conclusion that FN-integrin interaction leads to unusual signal transduction.

Another well-known mediator of extracellular signals is the extracellular signal-regulated kinase (Erk). Its activity is regulated by mitogens. Erk was transiently (between 1.5-12 h) activated in fibroblast spheroids as shown by the increase of the phosphorylated form in the immunoblot (Figure 3C in II). Erk participates in the regulation of autophagy [219], but its role as a regulator of autophagy is not as straightforward as Akt's. To explore its effects on spheroid-induced autophagy we used mitogen-activated protein kinase kinase 1 and 2 (MEK1/2) inhibitor U0126 [220]. MEK1/2 is located upstream of ERK and regulates its activity through phosphorylation [221]. As expected, 20 μM U0126 efficiently blocked ERK phosphorylation (Figure S4C in II). The stimulation of spheroids with U0126 also decreased the formation of LC3B-II and degradation of LC3B-I and p62 (Figure 4A and 4B in II), suggesting that ERK might mediate the induction of autophagy in fibroblast spheroids.

ERK conveys signals of many growth factors and cytokines that negatively regulate autophagy [219]. In this study ERK inhibition resulted almost complete inhibition of LC3B-II formation. Whether this is a direct effect of ERK on the formation of autophagosome, or an indirect effect on other molecules, remains unsolved. Interestingly, ROS have been shown to mediate the induction of autophagy through ERK activation [222].

4.4 The downregulation of cytoskeleton is associated with fibroblast spheroid formation (II)

Another distinct feature from TEM analysis was a clear decrease in cytosol volume (Figure S3 in II). Furthermore, when the changes in the gene expression of fibroblast spheroids were compared to adherent fibroblast cultures using gene expression microarrays, it became clear that the genes associated with the cytoskeleton were downregulated (Figure 1A in II). Immunoblot analysis confirmed the downregulation of the cytoskeletal proteins actin and β-tubulin also at protein level (Figure 1G in II). The smaller cell size was also visible in the

phase-contrast microscopy and FACS analysis (Figure 1F and 11D in II). To study whether autophagy participated in the cell-size shrinkage, we measured how the above-mentioned autophagy inhibitors affected fibroblast size in spheroids. Forming spheroids were treated with the inhibitors (3-MeA, Baf A1, U0126, shATG5) for 48 h. The cell size was determined by taking images of cells using phase-contrast microscopy and the cell area was estimated using ImageJ –software. The cells in spheroids treated with the inhibitors were significantly larger than the control cells in untreated spheroids (Figure 4D and 4E in II), indicating that the shrinkage of cytosol was due to the degradation of cytosolic components through autophagy and the downregulation of the expression of cytoskeletal proteins.

Cytoskeleton plays an important role in the function of myofibroblasts. Fibroblasts are able to convey signals from changed ECM structure through contractile actin cytoskeleton to regulate gene expression in a process called mechanotransduction [223]. The reason why nemotic fibroblasts downregulate their cytoskeleton is still unclear, but it has been shown that cells downregulate their cytoskeleton in response to stress [100], and it has been suggested that cytoskeleton needs to be degraded to achieve efficient remodeling of cell structure when cells change their phenotype [224]. Interestingly, stressed cancer cells have been shown to shrink in autophagy-dependent manner to the state of reversible dormancy [225, 226]. Proliferation of dormant cells is arrested in quiescence state in a new microenvironment, and the dormant cells are resistant to stress and therapy induced cell death [227, 228].

4.5 Fibroblast spheroid formation relates to cell cycle arrest (II)

Microarray experiments also revealed that fibroblast spheroids are associated with the universal downregulation of genes associated with the cell cycle, suggesting a decrease in cellular proliferation (Figure 1A in II). To confirm this, we analyzed the expression of proliferation markers, such as proliferating cell nuclear antigen (PCNA) and Ki67. As expected on the basis of the microarray analysis, the expression of PCNA started to decline 24 h after the initiation of spheroid formation as shown by immunoblotting (Figure 1B in II). Ki67 expression correlated with PCNA and the only proliferating cells were found in

the outermost layer of fibroblast spheroids as show by immunofluorescence with Ki67 antibody (Figure 1C in II). FACS analysis of the DNA content of cells indicated that the cells are arrested at G1/G0 cell cycle phase (Figure 1D in II).

The most important cyclins that regulate the cell cycle progression in different phases (Cyclin A2, B1, B2 and E2) were downregulated according to the microarray analysis. Furthermore, cyclin D1 protein expression was also downregulated 24 h after the initiation of spheroid formation (Figure 1E in II). This indicates that the cells were rather withdrawn from the cell cycle than actually arrested. This observation was further strengthened by the finding that CDI p27 was induced at the same time when cyclin D1 was downregulated (Figure 1E in II). The simultaneous upregulation of p27 and downregulation of cyclin D1 are considered to be markers of cellular quiescence [229]. Cellular quiescence can be effectively induced by the loss of anchorage, contact inhibition or growth factor deprivation in fibroblasts [159].

4.6 Spheroid-activated fibroblasts acquire secretory phenotype (II-III)

Based on microarray data, the change in the gene expression profile of fibroblast spheroid vs. adherent fibroblast cultures could roughly be divided in three categories, to the previously mentioned downregulated cytoskeleton- and cell-cycle-associated genes and the upregulation of genes that encode secretory proteins (Figure 1A in II). This is in agreement with our previous studies showing the induction of various cytokines and growth factors in response to spheroid formation, as well as paracrine effects mediated by these factors on other cell types. Interestingly, this secretory phenotype was very similar to SASP, which is seen in cellular senescence [172].

4.7 Fibroblast spheroids express markers of senescence (II)

Taken together, the fibroblast in spheroids are associated with similar features as the senescent cells, such as a secretory phenotype, increased lipofuscin and decreased proliferation. To determine whether cellular senescence is induced in response to spheroid formation, we analyzed common hallmarks of senescence.

Activity of SA-β-gal was induced 24 h after the initiation of the spheroid formation (Figure 2C in II). SA-β-gal activity is considered to be a marker of cellular senescence, although increased activity is also found in confluent fibroblast cell cultures [164]. Moreover, SA-β-gal activity reflects lysosomal mass [230], which is in agreement with our TEM findings, showing increased amount of autolysosomes. Unexpectedly, cell cycle inhibitors (p53, p21 and RB) that regulate cell cycle arrest in senescence, were all downregulated in fibroblast spheroids (Figure 2D in II).

Furthermore, when the cells of 96-h-old fibroblast spheroids were dispersed using trypsin and plated as standard monolayer culture, they resumed cell proliferation as seen by the increased cell number and restoration of PCNA expression (Figure 2E in II). These cells also restored their actin expression, suggesting that the expression of cytoskeletal proteins was normalized (Figure 2E in II). In addition, the expression of the stress proteins COX-2 (Figure 2E in II) and IL-6 (data not shown) disappeared suggesting the reversal of the secretory phenotype. Quiescent cells are considered to be passive, non-active and resting, although recently it was shown that they maintain high metabolic activity [161]. Our results indicate that in addition to metabolic activity quiescent cells can also harbor a secretory phenotype.

Interestingly, cell cycle arrest and secretory phenotype seen in nemosis seem to be very similar to cellular senescence. In senescence cell cycle arrest is controlled by induction of CKI, such as p21, p53 and RB, and at the same time the expression of cyclins remains high [231], whereas in nemosis there is universal downregulation of cell cycle associated genes, indicating that fibroblasts in spheroids are withdrawn from the cell cycle to quiescence. Although nemosis relates to quiescence, secretory phenotype suggests that there is a simultaneous stress response that resembles senescence. Recently, it was shown that rapamycin, the inhibitor of mTOR, shifts p53 or p21 induced senescence to quiescence [232].

The most compelling evidence that nemosis is not associated with senescence was that the nemotic phenotype was found to be reversible. Senescence is considered always to be an irreversible cell cycle arrest, although there are some studies suggesting that senescence could also be reversible.

However, in these studies reversibility was achieved only by genetic inactivation or depleting of p53 in senescent cells where p16 expression was low [233, 234].

4.8 Nemotic fibroblasts secrete matrix metalloproteinases to modulate their environment (III)

Plasmin is known to function as a major extracellular proteolytic system in fibroblast spheroids [176]. We wanted to characterize other possible proteolytic mechanisms to better understand the role of nemosis in the tumor microenvironment. According to the microarray results of fibroblast spheroids, matrix metalloproteinases (MMPs)-1, -10 and -14 (MT1-MMP) were induced 5.8, 106 and 5.6-fold, respectively (Table I in III). Fibroblast spheroids also secreted these MMPs to their culture media as shown by immunoblotting (Figure 1A, 1B, 1C in III). MT1-MMP, which is bound to cell membranes, was also found in fibroblast spheroids (Figure 1E in III). These same MMPs that fibroblast spheroids produce are overexpressed in tumor stroma [235-237].

The inhibition of MMP activity by pooled specific MMP inhibitors (CMT-3, CMT-5 and CMT-308) decreased LDH release by 11% and when combined with the broad-spectrum proteinase inhibitor, aprotinin, the LDH release was decreased by 21%. This indicates that nemosis-induced proteases, MMPs and plasmin, mediate the membrane damage related to nemosis. Proteinase activity was confirmed by using the casein-agarose assay. In this assay, ilomastat, an inhibitor of MMPs, inhibited casein degradation caused by fibroblast spheroid, indicating that spheroid mediated proteolytic activity was mediated by MMP activity. Suggesting that MMPs may also participate in the degradation of FN (Figure 1A in I). MMP-mediated degradation of ECM leads to increased cytokine and chemokine production [238], suggesting that MMP might functions as autocrine stimulators of inflammation response seen in fibroblast spheroids. In addition to degradation, MT1-MMP regulates endocytosis of non-polymerized fibronectin [239].

Unexpectedly, we did not find any activation of uPA and tPA, in the conditioned media of the spheroids. Although in situ hybridization indicated increased uPA and tPA mRNA in fibroblast spheroids, IHC showed no increase in uPA and tPA protein levels at the different time points in fibroblast spheroids (Figure 2 in III).

Nemosis expressed MMPs, together with previously published plasmin, are important mediators in cancer progression and inflammation. They modulate ECM, activate growth factors, such as TGF-β, promote angiogenesis, and assist invasion and metastasis by the degradation of basement membranes. In addition, they play a key role in the molecular communication between tumor and stroma, by processing cytokines to influence many inflammatory pathways [240, 241]. Although the expression of MMPs in tumors was long thought to only promote cancer growth and metastasis, clinical trials with specific MMP inhibitors yielded mostly negative results [242]. Moreover, new experimental data on the protective role of MMP activity in tumor progression has started to accumulate [243, 244]. It seems that most of protective role come from MMPs that are expressed in stromal cells [243].

4.9 Benign keratinocytes inhibit, whereas malignant keratinocytes promote nemotic activation (IV)

Tumor cell derived conditioned medium causes adherent fibroblast cultures to spontaneously form spheroid structures [177, 178]. This finding led us to further characterize tumor cell mediated paracrine effect on nemotic fibroblasts. We used a cell panel of HaCaT-keratinocytes representing different stages of tumor progression from immortalized to high-grade malignant, metastasizing cells. We stimulated the forming fibroblast spheroids with conditioned medium from different HaCaT cell clones and analyzed the expression of COX-2 by immunoblotting, and HGF and VEGF growth factors by ELISA and qRT-PCR. IHC was used to evaluate markers of activated fibroblasts, α-SMA and vimentin.

Conditioned medium from benign HaCaT clones (parental HaCaT and A5) inhibited COX-2 expression, whereas medium from II-4 keratinocytes had no effect and metastasizing RT3-cell-conditioned medium enhanced COX-2 induction in fibroblast spheroids (Figure 1A and 1B in IV). This indicated that benign cells produce anti-nemotic factors whereas malignant cells produce pro-nemotic factors. Further characterization of these factors may reveal new therapeutic targets for the treatment of inflammation. To test whether these factors are able to overrule each other, we added 25% of RT3-conditioned medium to HaCaT-conditioned medium. This treatment was able to restore

COX-2 expression and vice versa, adding HaCaT conditioned medium was able to prevent the effect of RT3-conditioned medium (Figure 4A and 4B in IV).

In carcinogenesis, COX-2 is linked to tumor progression, inflammation and angiogenesis [245, 246]. Other important mediators of tumor angiogenesis are HGF and VEGF, which are also upregulated in fibroblast spheroids [177, 178, 182]. Therefore, we examined their secretion from nemotic fibroblasts in response to HaCaT clones. Spheroids secreted 60-fold more HGF and 6-fold more VEGF on protein level than adherent fibroblast cultures after 72 hour incubation (Figure 2A in IV). This was also reflected at the mRNA levels with 10- and 2-fold increased expression of HGF and VEGF mRNA, respectively (Figure 2B in IV). All HaCaT clones further stimulated the secretion of HGF and VEGF from fibroblast spheroids, but only Ras-transformed HaCaT cells (A5, II-4 and RT3) significantly increased HGF secretion (Figure 2A and 2D in IV), further strengthening the observation that malignant cells promoted nemotic response of fibroblasts.

To further characterize the effect of HaCaT and RT3 cells on fibroblast spheroids, we performed co-culture experiments. Spheroids were embedded in collagen type I and adherent keratinocyte cultures were seeded on top of the collagen lattice. HaCaT cells stimulated the outgrowth and migration of fibroblasts from spheroids more than malignant RT3 keratinocytes (Figure 5A in IV). We also observed increased degradation of collagen around fibroblast spheroids in the cultures containing RT3 cells (Figure 5D, 5F and 5H in IV), suggesting increased expression of proteases in fibroblast spheroids in response to RT3 cells. This is in agreement with the earlier observations of increased MMP production in fibroblasts in co-culture with keratinocytes [247-249]. To confirm that the malignant RT3 cells promote nemosis in co-culture assays, similar to their conditioned medium, we used IHC to stain COX-2 in fibroblast spheroids (Figure 5C and 5D in IV). In agreement with the immunoblotting results, the co-culture with RT3 keratinocytes induced strong COX-2 staining, whereas the co-cultures with HaCaT cells gave only faint staining in fibroblast spheroids. We also detected increased α-SMA and decreased vimentin staining in the fibroblasts spheroids in RT3 co-cultures compared to HaCaT co-cultures (Figure 1E, 1F, 1G and 1H in IV), indicating myofibroblastic differentiation of

nemotic fibroblasts in response to malignant RT3 keratinocytes. Thus, our results further extend previous findings that malignant cells activate fibroblasts.

4.10 Fibroblast spheroids inhibit growth of malignant keratinocytes (II)

Nemotic fibroblasts increase cancer cell motility, invasiveness and proliferation in cell culture experiments, but are also able to induce differentiation of c-Met-negative leukemia cells. Furthermore, as shown above, malignant keratinocytes (RT3) promote the nemotic response of fibroblasts. To further study the effect of this paracrine signaling on RT3 cells we measured the ability of RT3 cells to form colonies in soft-agar co-culture with fibroblast spheroids. We also used a mouse xenograft model to measure the growth of RT3 tumors *in vivo*.

4.10.1 RT3 cell growth on soft-agar is attenuated by co-culture with fibroblast spheroids (II)

To study how fibroblast spheroids impact the anchorage-independent growth of RT3 cells, we plated different amounts (6, 12, 32, 96 or 180 spheroids) of fibroblast spheroids in the bottom agar and RT3 keratinocytes in top agar of soft agar cultures. Fibroblast spheroids stimulated the growth of RT3 colonies during the first eight days. This stimulation was dependent on the number of spheroids plated in the agarose (Figure 6A in II). When the experiment progressed the higher numbers (over 32) of spheroids started to have an adverse effect on the RT3 colonies (Figure 6B and 6D in II). The colonies changed their shape from spherical to irregular and appeared as decomposed. High number of fibroblast spheroids also lost the ability to stimulate the growth of RT3 colonies as measured as an increase in their size. Fibroblast spheroids had no effect on the number of RT3 colonies (Figure 6C in II).

Fibroblast spheroids are able to stimulate the proliferation and motility of adherent RT3 cells [186]. Our results are in line with this. Small numbers of fibroblast spheroids stimulated the growth of RT3 colonies in soft agarose, whereas higher numbers had deleterious effects on RT3 cells in soft agarose. The concentration of paracrine factors secreted in nemosis may explain this difference. Cells are also known to behave differently when they are cultivated in

a 3-dimensional environment, such as soft agarose, compared to 2-dimensional standard cell culture conditions.

4.10.2 Fibroblast spheroids attenuate the growth of xenograft tumors by inducing tumor cell senescence (II)

In addition to direct effects on cancer cells, nemotic fibroblasts may have effects on other stromal cell types in tumors. To better elucidate the impact of nemotic fibroblast spheroids on the progression of tumors, we injected RT3 keratinocytes together with adherent fibroblast cultures or fibroblast spheroids to the peritoneal cavity of female Balb/c nude or NOD/SCID mice. Fibroblast spheroids significantly reduced the RT3 tumor growth compared to control RT3 tumors or RT3 tumors containing monolayer-cultivated fibroblasts. This was seen as a reduction of both tumor size and tumor weight (Figure 7A, 7B, S6A and S6B in II).

The slower tumor growth was associated with an increase in SA-β-gal staining in tumor sections (Figure 6C in II), suggesting an induction of tumor cell senescence. This observation was further strengthened by the simultaneous upregulation of p14ARF and downregulation of p63 in these same SA-β-gal positive areas (Figure 7D in II). The upregulation of p14ARF induces cellular senescence and its upregulation depends on the downregulation of p63 [250]. Remarkably, RT3 cells harbor a mutation in p53 gene enabling them to escape classical p53-mediated senescence. The induction of p14ARF can mediate senescence by both p53-dependent and independent mechanisms [251, 252].

In addition to the induction of senescence, fibroblast spheroids caused cytokeratin-7 mediated differentiation in RT3 tumors (Figure 7F in II). This is in agreement with previous publications showing close connection between cytokeratin-7 mediated differentiation and tumor senescence [253, 254]. This finding is also in line with the differentiation of KG-1 and THP-1 cells in response to nemotic fibroblasts [181]. Although nemotic fibroblasts stimulate the growth and invasion of cancer cell in vitro, our results show that the complex secretion of paracrine mediators by nemotic fibroblasts may also restrict tumor growth by induction of senescence and differentiation of cancer cells, emphasizing the complex role of tumor stroma in the progression and restriction of tumor growth [12, 39].

4.11 The summary of results

The results of this thesis indicate that when primary fibroblasts lose their solid support, they tend to cluster to a multicellular spheroid in a fibronectin-integrin dependent manner. This interaction leads to a rapid induction of stress-related autophagy, and sequential withdraw from the cell-cycle to cellular quiescence. These events lead to the secretion of tumor-associated cytokines, chemokines, growth factors and proteases, which attenuated tumor growth by inducing cellular senescence and the differentiation of malignant RT keratinocytes in a mouse xenograft models (Figure 12).

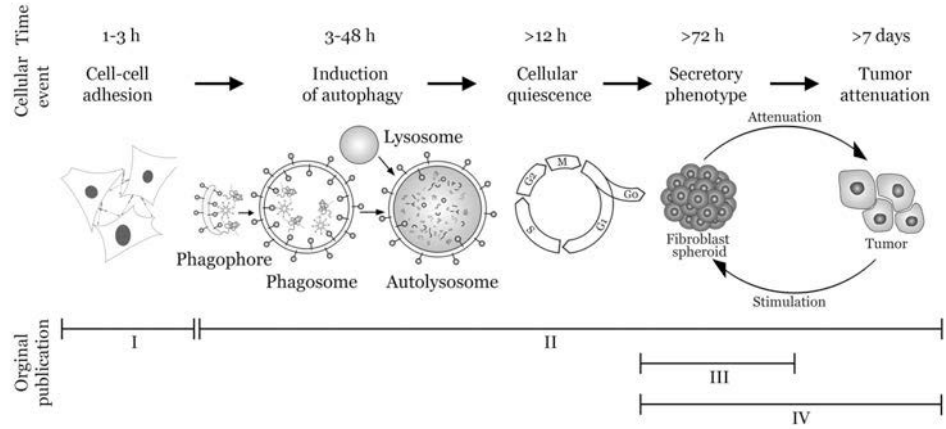

Figure 12. The schematic summary of major results.

CONCLUDING REMARKS

Fibroblast activation plays an important role in many physiological and pathophysiological conditions, such as wound healing and carcinogenesis. Regardless of the considerable impact of fibroblast activation on the progression of these conditions, very little is known of the events and mechanisms leading to this activation [12]. CAFs, and myofibroblasts, are highly heterogeneous cell populations [255]. Hence they probably can be activated by several different mechanisms. The present results show that fibroblasts can be activated solely by changing their growth environment, and that this activation is mediated by an abnormal FN-integrin interaction, indicating that fibroblasts can be activated in conditions where their normal growth properties change dramatically. Furthermore, both tumors and wound healing, the conditions where activated fibroblasts are found, express altered ECM compositions [7, 198].

Autophagy has a prominent role in the activation of CAFs in response to tumor cell stimulus [135]. This is in agreement with our finding showing that autophagy has an essential role during nemotic activation. Interestingly, in co-culture or through conditioned media, tumor cells are known to cause spontaneous clustering of fibroblasts, which resembles fibroblast spheroid formation [177, 178]. In addition, we showed that malignant keratinocytes were able to potentiate nemotic activation. These results suggest that our experimental model of fibroblast activation, nemosis, shares many features of fibroblasts activation in vivo, although more specific studies are needed to reveal the precise mechanism of nemosis and its connection to in vivo situations.

The nemotic phenotype was associated with the upregulation of secreted molecules. Interestingly, this phenotype is very similar to that seen in cellular senescence. Nemosis shares also other features related to senescence, although it lacks some of the hallmarks, indicating that nemosis is a similar, but not identical stress response to senescence. Cell cycle arrest, the downregulation of the cytoskeleton, and the induction of secretory phenotype suggest that nemotic activation is directed to paracrine modulation of other cells in the microenvironment (summarized in Figure 13 [175]).

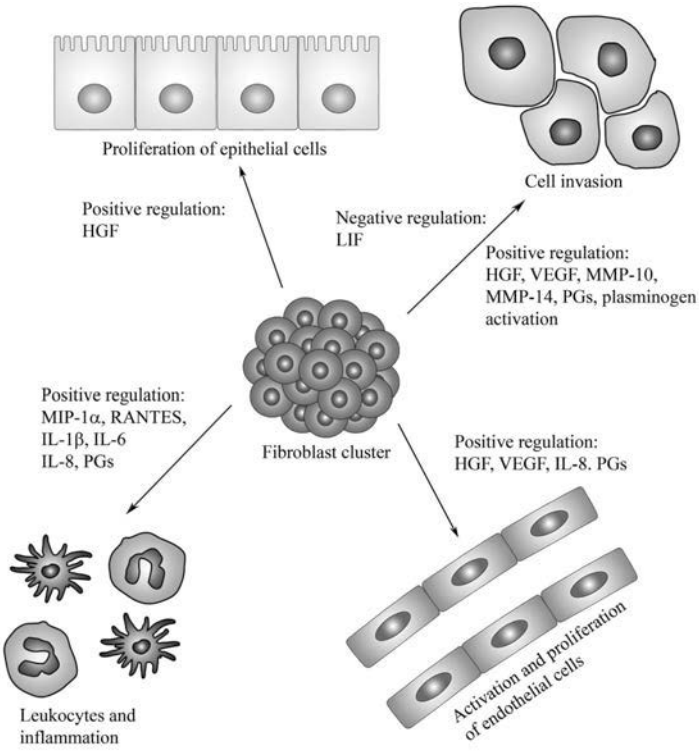

Figure 13. The paracrine effects of nemosis-associated secretory phenotype to other cell types. Reprinted with permission from ref [175].

In cell culture experiments, nemotic fibroblasts can either stimulate cancer cell proliferation or cause differentiation, which was limited to c-Met negative cells [175]. In this study, we show that nemotic fibroblasts promote tumor cell senescence in xenograft experiments. This is in agreement with earlier publications showing that SASP factors can induce senescence in a cell-autonomous or cell-non-autonomous manner [173], and that stromal activation can lead to tumor cell senescence [256].

In conclusion, our results indicate that nemosis is a powerful mechanism for fibroblast activation and a suitable model for studies on cellular events leading to activation and crosstalk between tumor cells and fibroblasts.

ACKNOWLEDGMENTS

This study was carried out at the Department of Virology, Haartman Institute, University of Helsinki. The head of department, Professor Kalle Saksela, is acknowledged for providing excellent research facilities also to a non-virologist.

I would like to thank Professor Antti Vaheri for guidance and support, as well as believing in me even times when it looked like that everything had come to a standstill. Professor Jorma Keski-Oja and Docent Eeva-Liisa Eskelinen are acknowledged for reviewing this thesis and providing valuable comments. In addition, Professor Jorma Keski-Oja together with Docent Erkki Hölttä are thanked for being members of my thesis committee. I am grateful to Riikka for the language revision of this thesis.

I owe my sincere gratitude to Irina, our expert technician, for all the help in the lab, as well as tolerating complains about scientific and unscientific topics. Kati and Anna are thanked for numerous discussions on meaning of nemosis and for good travel company to scientific meetings. Kati, without your input this thesis would still be unfinished. Esko, Vappu and Jozef are thanked for introducing me to nemosis. Esko is additionally thanked for being my first mentor.

I am grateful to Pirjo and Piia-Riitta for fruitful discussions and collaboration. All co-authors are acknowledged for their contributions in the original publications. Samu is thanked for good company at lunch breaks and vital support during these final months. Zoonosis group, thank you for adopting me and considering me as one of your own. Former PA group, Marjaleena, Oskari, and Sari are thanked for conversations and coffee meetings. Thanks to members of “maanantai sähly”, a nice way to start the week.

In addition, I would like to thank friends outside work, all the members of FC Degu, FBD, KPPK, Hurrjat, and particularly Espoo-Expos. Tomi J, Minttu, Imppu, Keiju, Tomi S, Irina, Kipa, JP, Tuke, Vilo, Sanna, Jasu, Lauri, Suvi, Päivi, Eeva, Köpi Ray and Aapo are thanked for their friendship and all the discussions unrelated to science.

My family father Markku, Ritva, brother Otso, sister Heta and her family Aapo, Frans and Eemil are thanked for support and care.

This work has been financially supported by Helsinki Graduate Program in Biotechnology and Molecular Biology, Research Foundation of the University of

Helsinki, Biomedicum Helsinki Foundation, Magnus Ehrnrooth Foundation, Finnish Cancer Organizations, and Orion-Farmos Research Foundation.

Pertteli Salmenperä, Helsinki, March, 2012

REFERENCES

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM, GLOBOCAN 2008 v1.2, cancer incidence and mortality worldwide: IARC CancerBase no. 10 [http://globocan.iarc.fr], 2011.

[2] Weinberg RA, The Biology of Cancer, Garland Science, 2007.

[3] Hanahan D, Weinberg RA, Hallmarks of cancer: The next generation, Cell. 144 (2011) 646-674.

[4] Pietras K, Östman A, Hallmarks of cancer: Interactions with the tumor stroma, Exp. Cell Res. 316 (2010) 1324-1331.

[5] Räsänen K, Vaheri A, Activation of fibroblasts in cancer stroma, Exp. Cell Res. 316 (2010) 2713-2722.

[6] Kulonen E, Pikkarainen J, Biology of Fibroblast, Academic Press, London, 1973.

[7] Boudreau N, Bissell MJ, Extracellular matrix signaling: Integration of form and function in normal and malignant cells, Curr. Opin. Cell Biol. 10 (1998) 640-646.

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

[9] Baglole CJ, Ray DM, Bernstein SH, Feldon SE, Smith TJ, Sime PJ, Phipps RP, More than structural cells, fibroblasts create and orchestrate the tumor microenvironment, Immunol. Invest. 35 (2006) 297-325.

[10] Smith RS, Smith TJ, Blieden TM, Phipps RP, Fibroblasts as sentinel cells. synthesis of chemokines and regulation of inflammation, Am. J. Pathol. 151 (1997) 317-322.

[11] Buckley CD, Why does chronic inflammation persist: An unexpected role for fibroblasts, Immunol. Lett. 138 (2011) 12-14.

[12] Kalluri R, Zeisberg M, Fibroblasts in cancer, Nat. Rev. Cancer. 6 (2006) 392-401.

[13] Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA, Myofibroblasts and mechano-regulation of connective tissue remodelling, Nat. Rev. Mol. Cell Biol. 3 (2002) 349-363.

[14] Hinz B, Gabbiani G, Cell-matrix and cell-cell contacts of myofibroblasts: Role in connective tissue remodeling, Thromb. Haemost. 90 (2003) 993-1002.

[15] Ehrlich HP, Diez T, Role for gap junctional intercellular communications in wound repair, Wound Repair Regen. 11 (2003) 481-489.

[16] Langevin HM, Cornbrooks CJ, Taatjes DJ, Fibroblasts form a body-wide cellular network, Histochem. Cell Biol. 122 (2004) 7-15.

[17] Ronnov-Jessen L, Petersen OW, Bissell MJ, Cellular changes involved in conversion of normal to malignant breast: Importance of the stromal reaction, Physiol. Rev. 76 (1996) 69-125.

[18] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G, The myofibroblast: One function, multiple origins, Am. J. Pathol. 170 (2007) 1807-1816.

[19] McAnulty RJ, Fibroblasts and myofibroblasts: Their source, function and role in disease, Int. J. Biochem. Cell Biol. 39 (2007) 666-671.

[20] Östman A, Augsten M, Cancer-associated fibroblasts and tumor growth--bystanders turning into key players, Curr. Opin. Genet. Dev. 19 (2009) 67-73.

[21] Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R, Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts, Cancer Res. 67 (2007) 10123-10128.

[22] Dvorak HF, Tumors: Wounds that do not heal. similarities between tumor stroma generation and wound healing, N. Engl. J. Med. 315 (1986) 1650-1659.

[23] Haviv I, Polyak K, Qiu W, Hu M, Campbell I, Origin of carcinoma associated fibroblasts, Cell. Cycle. 8 (2009) 589-595.

[24] Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C, The stroma as a crucial target in rat mammary gland carcinogenesis, J. Cell. Sci. 117 (2004) 1495-1502.

[25] Ben-Neriah Y, Karin M, Inflammation meets cancer, with NF-kappaB as the matchmaker, Nat. Immunol. 12 (2011) 715-723.

[26] Mantovani A, Allavena P, Sica A, Balkwill F, Cancer-related inflammation, Nature. 454 (2008) 436-444.

[27] Gerashchenko BI, At a crossroads of cancer risk and aging: The role of telomeres, Exp. Oncol. 32 (2010) 224-227.

[28] Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, Aarden LA, Mooi WJ, Peeper DS, Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network, Cell. 133 (2008) 1019-1031.

[29] Fromigue O, Louis K, Dayem M, Milanini J, Pages G, Tartare-Deckert S, Ponzio G, Hofman P, Barbry P, Auberger P, Mari B, Gene expression profiling of normal human pulmonary fibroblasts following coculture with non-small-cell lung cancer cells reveals alterations related to matrix degradation, angiogenesis, cell growth and survival, Oncogene. 22 (2003) 8487-8497.

[30] Gallagher PG, Bao Y, Prorock A, Zigrino P, Nischt R, Politi V, Mauch C, Dragulev B, Fox JW, Gene expression profiling reveals cross-talk between melanoma and fibroblasts: Implications for host-tumor interactions in metastasis, Cancer Res. 65 (2005) 4134-4146.

[31] Ma H, Liu T, Qin J, Lin B, Characterization of the interaction between fibroblasts and tumor cells on a microfluidic co-culture device, Electrophoresis. 31 (2010) 1599-1605.

[32] Mukaratirwa S, Koninkx JF, Gruys E, Nederbragt H, Mutual paracrine effects of colorectal tumour cells and stromal cells: Modulation of tumour and stromal cell differentiation and extracellular matrix component production in culture, Int. J. Exp. Pathol. 86 (2005) 219-229.

[33] Addadi Y, Moskovits N, Granot D, Lozano G, Carmi Y, Apte RN, Neeman M, Oren M, p53 status in stromal fibroblasts modulates tumor growth in an SDF1-dependent manner, Cancer Res. 70 (2010) 9650-9658.

[34] Trimboli AJ, Cantemir-Stone CZ, Li F, Wallace JA, Merchant A, Creasap N, Thompson JC, Caserta E, Wang H, Chong JL, Naidu S, Wei G, Sharma SM, Stephens JA, Fernandez SA, Gurcan MN, Weinstein MB, Barsky SH, Yee L, Rosol TJ, Stromberg PC, Robinson ML, Pepin F, Hallett M, Park M, Ostrowski MC, Leone G, Pten in stromal fibroblasts suppresses mammary epithelial tumours, Nature. 461 (2009) 1084-1091.

[35] Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou XP, Eng C, Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas, Nat. Genet. 32 (2002) 355-357.

[36] Taipale J, Saharinen J, Keski-Oja J, Extracellular matrix-associated transforming growth factor-beta: Role in cancer cell growth and invasion, Adv. Cancer Res. 75 (1998) 87-134.

[37] Noel A, Jost M, Maquoi E, Matrix metalloproteinases at cancer tumor-host interface, Semin. Cell Dev. Biol. 19 (2008) 52-60.

[38] Almholt K, Johnsen M, Stromal cell involvement in cancer, Recent Results Cancer Res. 162 (2003) 31-42.

[39] Bissell MJ, Hines WC, Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression, Nat. Med. 17 (2011) 320-329.

[40] Petersen OW, Ronnov-Jessen L, Howlett AR, Bissell MJ, Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 9064-9068.

[41] Howlett AR, Petersen OW, Steeg PS, Bissell MJ, A novel function for the nm23-H1 gene: Overexpression in human breast carcinoma cells leads to the formation of basement membrane and growth arrest, J. Natl. Cancer Inst. 86 (1994) 1838-1844.

[42] Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ, Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies, J. Cell Biol. 137 (1997) 231-245.

[43] Gupta PB, Chaffer CL, Weinberg RA, Cancer stem cells: Mirage or reality? Nat. Med. 15 (2009) 1010-1012.

[44] Polyak K, Haviv I, Campbell IG, Co-evolution of tumor cells and their microenvironment, Trends Genet. 25 (2009) 30-38.

[45] Holopainen T, Bry M, Alitalo K, Saaristo A, Perspectives on lymphangiogenesis and angiogenesis in cancer, J. Surg. Oncol. 103 (2011) 484-488.

[46] Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A, Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability, Carcinogenesis. 30 (2009) 1073-1081.

[47] Zitvogel L, Tesniere A, Kroemer G, Cancer despite immunosurveillance: Immunoselection and immunosubversion, Nat. Rev. Immunol. 6 (2006) 715-727.

[48] Qian BZ, Pollard JW, Macrophage diversity enhances tumor progression and metastasis, Cell. 141 (2010) 39-51.

[49] Hynes RO, Fibronectins, Springer-Verlag Inc, New York, 1990.

[50] Hynes RO, Yamada KM, Fibronectins: Multifunctional modular glycoproteins, J. Cell Biol. 95 (1982) 369-377.

[51] Pankov R, Yamada KM, Fibronectin at a glance, J. Cell. Sci. 115 (2002) 3861-3863.

[52] Vaheri A, Salonen EM, Vartio T, Fibronectin in formation and degradation of the pericellular matrix, Ciba found. Symp. 114 (1985) 111-126.

[53] Singh P, Carraher C, Schwarzbauer JE, Assembly of fibronectin extracellular matrix, Annu. Rev. Cell Dev. Biol. 26 (2010) 397-419.

[54] Margadant C, Monsuur HN, Norman JC, Sonnenberg A, Mechanisms of integrin activation and trafficking, Curr. Opin. Cell Biol. 23 (2011) 607-614.

[55] Ohashi T, Erickson HP, Revisiting the mystery of fibronectin multimers: The fibronectin matrix is composed of fibronectin dimers cross-linked by non-covalent bonds, Matrix Biol. 28 (2009) 170-175.

[56] Harburger DS, Calderwood DA, Integrin signalling at a glance, J. Cell. Sci. 122 (2009) 159-163.

[57] Schwartz MA, Integrins and extracellular matrix in mechanotransduction, Cold Spring Harb Perspect. Biol. 2 (2010) a005066.

[58] Kim LT, Yamada KM, Evidence that beta1 integrins in keratinocyte cell-cell junctions are not in the ligand-occupied conformation, J. Invest. Dermatol. 108 (1997) 876-880.

[59] Larjava H, Peltonen J, Akiyama SK, Yamada SS, Gralnick HR, Uitto J, Yamada KM, Novel function for beta 1 integrins in keratinocyte cell-cell interactions, J. Cell Biol. 110 (1990) 803-815.

[60] Robinson EE, Zazzali KM, Corbett SA, Foty RA, Alpha5beta1 integrin mediates strong tissue cohesion, J. Cell. Sci. 116 (2003) 377-386.

[61] Ye F, Kim C, Ginsberg MH, Molecular mechanism of inside-out integrin regulation, J. Thromb. Haemost. 9 Suppl 1 (2011) 20-25.

[62] Plow EF, Haas TA, Zhang L, Loftus J, Smith JW, Ligand binding to integrins, J. Biol. Chem. 275 (2000) 21785-21788.

[63] Pytela R, Pierschbacher MD, Ruoslahti E, Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor, Cell. 40 (1985) 191-198.

[64] Johansson S, Svineng G, Wennerberg K, Armulik A, Lohikangas L, Fibronectin-integrin interactions, Front. Biosci. 2 (1997) d126-46.

[65] Mizushima N, Komatsu M, Autophagy: Renovation of cells and tissues, Cell. 147 (2011) 728-741.

[66] Kroemer G, Marino G, Levine B, Autophagy and the integrated stress response, Mol. Cell. 40 (2010) 280-293.

[67] Li WW, Li J, Bao JK, Microautophagy: Lesser-known self-eating, Cell Mol. Life Sci. (2011) .

[68] Cuervo AM, Chaperone-mediated autophagy: Dice's 'wild' idea about lysosomal selectivity, Nat. Rev. Mol. Cell Biol. 12 (2011) 535-541.

[69] Komatsu M, Ichimura Y, Selective autophagy regulates various cellular functions, Genes Cells. 15 (2010) 923-933.

[70] Lemasters JJ, Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging, Rejuvenation Res. 8 (2005) 3-5.

[71] Kraft C, Peter M, Is the Rsp5 ubiquitin ligase involved in the regulation of ribophagy? Autophagy. 4 (2008) 838-840.

[72] Yamamoto A, Simonsen A, The elimination of accumulated and aggregated proteins: A role for aggrephagy in neurodegeneration, Neurobiol. Dis. 43 (2011) 17-28.

[73] Meijer WH, van der Klei IJ, Veenhuis M, Kiel JA, ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective cvt and pexophagy pathways also require organism-specific genes, Autophagy. 3 (2007) 106-116.

[74] Weidberg H, Shvets E, Elazar Z, Lipophagy: Selective catabolism designed for lipids, Dev. Cell. 16 (2009) 628-630.

[75] Tasdemir E, Maiuri MC, Tajeddine N, Vitale I, Criollo A, Vicencio JM, Hickman JA, Geneste O, Kroemer G, Cell cycle-dependent induction of autophagy, mitophagy and reticulophagy, Cell. Cycle. 6 (2007) 2263-2267.

[76] Jung CH, Ro SH, Cao J, Otto NM, Kim DH, mTOR regulation of autophagy, FEBS Lett. 584 (2010) 1287-1295.

[77] Sengupta S, Peterson TR, Sabatini DM, Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress, Mol. Cell. 40 (2010) 310-322.

[78] Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH, ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery, Mol. Biol. Cell. 20 (2009) 1992-2003.

[79] Kim J, Kundu M, Viollet B, Guan KL, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell Biol. 13 (2011) 132-141.

[80] Simonsen A, Tooze SA, Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes, J. Cell Biol. 186 (2009) 773-782.

[81] Seglen PO, Gordon PB, 3-methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 1889-1892.

[82] Yang Z, Klionsky DJ, Mammalian autophagy: Core molecular machinery and signaling regulation, Curr. Opin. Cell Biol. 22 (2010) 124-131.

[83] Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y, A ubiquitin-like system mediates protein lipidation, Nature. 408 (2000) 488-492.

[84] Noda NN, Kumeta H, Nakatogawa H, Satoo K, Adachi W, Ishii J, Fujioka Y, Ohsumi Y, Inagaki F, Structural basis of target recognition by Atg8/LC3 during selective autophagy, Genes Cells. 13 (2008) 1211-1218.

[85] Ichimura Y, Kumanomidou T, Sou YS, Mizushima T, Ezaki J, Ueno T, Kominami E, Yamane T, Tanaka K, Komatsu M, Structural basis for sorting mechanism of p62 in selective autophagy, J. Biol. Chem. 283 (2008) 22847-22857.

[86] Ichimura Y, Kominami E, Tanaka K, Komatsu M, Selective turnover of p62/A170/SQSTM1 by autophagy, Autophagy. 4 (2008) 1063-1066.

[87] Tong J, Yan X, Yu L, The late stage of autophagy: Cellular events and molecular regulation, Protein Cell. 1 (2010) 907-915.

[88] Fader CM, Colombo MI, Autophagy and multivesicular bodies: Two closely related partners, Cell Death Differ. 16 (2009) 70-78.

[89] Fader CM, Sanchez D, Furlan M, Colombo MI, Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells, Traffic. 9 (2008) 230-250.

[90] Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL, Role for Rab7 in maturation of late autophagic vacuoles, J. Cell. Sci. 117 (2004) 4837-4848.

[91] Huynh KK, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S, LAMP proteins are required for fusion of lysosomes with phagosomes, EMBO J. 26 (2007) 313-324.

[92] Moreau K, Ravikumar B, Renna M, Puri C, Rubinsztein DC, Autophagosome precursor maturation requires homotypic fusion, Cell. 146 (2011) 303-317.

[93] Nair U, Jotwani A, Geng J, Gammoh N, Richerson D, Yen WL, Griffith J, Nag S, Wang K, Moss T, Baba M, McNew JA, Jiang X, Reggiori F, Melia TJ, Klionsky DJ, SNARE proteins are required for macroautophagy, Cell. 146 (2011) 290-302.

[94] Tamai K, Tanaka N, Nara A, Yamamoto A, Nakagawa I, Yoshimori T, Ueno Y, Shimosegawa T, Sugamura K, Role of hrs in maturation of autophagosomes in mammalian cells, Biochem. Biophys. Res. Commun. 360 (2007) 721-727.

[95] Metcalf D, Isaacs AM, The role of ESCRT proteins in fusion events involving lysosomes, endosomes and autophagosomes, Biochem. Soc. Trans. 38 (2010) 1469-1473.

[96] Epple UD, Suriapranata I, Eskelinen EL, Thumm M, Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole, J. Bacteriol. 183 (2001) 5942-5955.

[97] Lloyd JB, Metabolite efflux and influx across the lysosome membrane, Subcell. Biochem. 27 (1996) 361-386.

[98] Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B, Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21, Genomics. 59 (1999) 59-65.

[99] Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B, Induction of autophagy and inhibition of tumorigenesis by beclin 1, Nature. 402 (1999) 672-676.

[100] Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E, Autophagy suppresses tumorigenesis through elimination of p62, Cell. 137 (2009) 1062-1075.

[101] Ichimura Y, Komatsu M, Selective degradation of p62 by autophagy, Semin. Immunopathol. 32 (2010) 431-436.

[102] Moscat J, Diaz-Meco MT, P62 at the crossroads of autophagy, apoptosis, and cancer, Cell. 137 (2009) 1001-1004.

[103] Ichimura Y, Kominami E, Tanaka K, Komatsu M, Selective turnover of p62/A170/SQSTM1 by autophagy, Autophagy. 4 (2008) 1063-1066.

[104] Dikic I, Johansen T, Kirkin V, Selective autophagy in cancer development and therapy, Cancer Res. 70 (2010) 3431-3434.

[105] Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavare S, Arakawa S, Shimizu S, Watt FM, Narita M, Autophagy mediates the mitotic senescence transition, Genes Dev. 23 (2009) 798-803.

[106] Campisi J, Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors, Cell. 120 (2005) 513-522.

[107] Muller PA, Vousden KH, Norman JC, P53 and its mutants in tumor cell migration and invasion, J. Cell Biol. 192 (2011) 209-218.

[108] Feng Z, Zhang H, Levine AJ, Jin S, The coordinate regulation of the p53 and mTOR pathways in cells, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 8204-8209.

[109] Crighton D, Wilkinson S, Ryan KM, DRAM links autophagy to p53 and programmed cell death, Autophagy. 3 (2007) 72-74.

[110] Gao W, Shen Z, Shang L, Wang X, Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death, Cell Death Differ. 18 (2011) 1598-1607.

[111] Denton D, Nicolson S, Kumar S, Cell death by autophagy: Facts and apparent artefacts, Cell Death Differ. 19 (2012) 87-95.

[112] Platini F, Perez-Tomas R, Ambrosio S, Tessitore L, Understanding autophagy in cell death control, Curr. Pharm. Des. 16 (2010) 101-113.

[113] Maiuri MC, Zalckvar E, Kimchi A, Kroemer G, Self-eating and self-killing: Crosstalk between autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 8 (2007) 741-752.

[114] Scott RC, Juhasz G, Neufeld TP, Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death, Curr. Biol. 17 (2007) 1-11.

[115] Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, Varbanov M, Codogno P, Biard-Piechaczyk M, Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4, J. Clin. Invest. 116 (2006) 2161-2172.

[116] Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M, Autophagic programmed cell death by selective catalase degradation, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 4952-4957.

[117] Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E, Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis, Cancer. Cell. 10 (2006) 51-64.

[118] White E, Autophagic cell death unraveled: Pharmacological inhibition of apoptosis and autophagy enables necrosis, Autophagy. 4 (2008) 399-401.

[119] Proskuryakov SY, Gabai VL, Mechanisms of tumor cell necrosis, Curr. Pharm. Des. 16 (2010) 56-68.

[120] Pouyssegur J, Dayan F, Mazure NM, Hypoxia signalling in cancer and approaches to enforce tumour regression, Nature. 441 (2006) 437-443.

[121] Rzymski T, Milani M, Singleton DC, Harris AL, Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia, Cell. Cycle. 8 (2009) 3838-3847.

[122] Eskelinen EL, The dual role of autophagy in cancer, Curr. Opin. Pharmacol. 11 (2011) 294-300.

[123] Pavlides S, Tsirigos A, Migneco G, Whitaker-Menezes D, Chiavarina B, Flomenberg N, Frank PG, Casimiro MC, Wang C, Pestell RG, Martinez-Outschoorn UE, Howell A, Sotgia F, Lisanti MP, The autophagic tumor stroma model of cancer: Role of oxidative stress and ketone production in fueling tumor cell metabolism, Cell. Cycle. 9 (2010) 3485-3505.

[124] Martinez-Outschoorn UE, Whitaker-Menezes D, Pavlides S, Chiavarina B, Bonuccelli G, Casey T, Tsirigos A, Migneco G, Witkiewicz A, Balliet R, Mercier I, Wang C, Flomenberg N, Howell A, Lin Z, Caro J, Pestell RG, Sotgia F, Lisanti MP, The autophagic tumor stroma model of cancer or "battery-operated tumor growth": A simple solution to the autophagy paradox, Cell. Cycle. 9 (2010) 4297-4306.

[125] Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, Migneco G, Witkiewicz AK, Martinez-Cantarin MP, Flomenberg N, Howell A, Pestell RG, Lisanti MP, Sotgia F,

Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors, Cell. Cycle. 9 (2010) 2423-2433.

[126] Pavlides S, Tsirigos A, Vera I, Flomenberg N, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG, Martinez-Outschoorn UE, Sotgia F, Lisanti MP, Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the "reverse warburg effect": A transcriptional informatics analysis with validation, Cell. Cycle. 9 (2010) 2201-2219.

[127] Pavlides S, Tsirigos A, Vera I, Flomenberg N, Frank PG, Casimiro MC, Wang C, Pestell RG, Martinez-Outschoorn UE, Howell A, Sotgia F, Lisanti MP, Transcriptional evidence for the "reverse warburg effect" in human breast cancer tumor stroma and metastasis: Similarities with oxidative stress, inflammation, alzheimer's disease, and "neuron-glia metabolic coupling", Aging (Albany NY). 2 (2010) 185-199.

[128] Goetz JG, Lajoie P, Wiseman SM, Nabi IR, Caveolin-1 in tumor progression: The good, the bad and the ugly, Cancer Metastasis Rev. 27 (2008) 715-735.

[129] Krajewska WM, Maslowska I, Caveolins: Structure and function in signal transduction, Cell. Mol. Biol. Lett. 9 (2004) 195-220.

[130] Park JH, Ryu JM, Han HJ, Involvement of caveolin-1 in fibronectin-induced mouse embryonic stem cell proliferation: Role of FAK, RhoA, PI3K/Akt, and ERK 1/2 pathways, J. Cell. Physiol. 226 (2011) 267-275.

[131] Del Pozo MA, Schwartz MA, Rac, membrane heterogeneity, caveolin and regulation of growth by integrins, Trends Cell Biol. 17 (2007) 246-250.

[132] Witkiewicz AK, Dasgupta A, Sammons S, Er O, Potoczek MB, Guiles F, Sotgia F, Brody JR, Mitchell EP, Lisanti MP, Loss of stromal caveolin-1 expression predicts poor clinical outcome in triple negative and basal-like breast cancers, Cancer. Biol. Ther. 10 (2010) 135-143.

[133] Witkiewicz AK, Dasgupta A, Sotgia F, Mercier I, Pestell RG, Sabel M, Kleer CG, Brody JR, Lisanti MP, An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers, Am. J. Pathol. 174 (2009) 2023-2034.

[134] Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, Migneco G, Witkiewicz AK, Martinez-Cantarin MP, Flomenberg N, Howell A, Pestell RG, Lisanti MP, Sotgia F, Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors, Cell. Cycle. 9 (2010) 2423-2433.

[135] Lisanti MP, Martinez-Outschoorn UE, Chiavarina B, Pavlides S, Whitaker-Menezes D, Tsirigos A, Witkiewicz A, Lin Z, Balliet R, Howell A, Sotgia F, Understanding the "lethal" drivers of tumor-stroma co-evolution: Emerging role(s) for hypoxia, oxidative stress and autophagy/mitophagy in the tumor micro-environment, Cancer. Biol. Ther. 10 (2010) 537-542.

[136] Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, Zhou J, Wang C, Pavlides S, Martinez-Cantarin MP, Capozza F, Witkiewicz AK, Flomenberg N, Howell A, Pestell RG, Caro J, Lisanti MP, Sotgia F, Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFkappaB activation in the tumor stromal microenvironment, Cell. Cycle. 9 (2010) 3515-3533.

[137] Balliet RM, Capparelli C, Guido C, Pestell TG, Martinez-Outschoorn UE, Lin Z, Whitaker-Menezes D, Chiavarina B, Pestell RG, Howell A, Sotgia F, Lisanti MP, Mitochondrial oxidative stress in cancer-associated fibroblasts drives lactate production, promoting breast cancer tumor growth: Understanding the aging and cancer connection, Cell. Cycle. 10 (2011) 4065-4073.

[138] Martinez-Outschoorn UE, Whitaker-Menezes D, Lin Z, Flomenberg N, Howell A, Pestell RG, Lisanti MP, Sotgia F, Cytokine production and inflammation drive autophagy in the tumor microenvironment: Role of stromal caveolin-1 as a key regulator, Cell. Cycle. 10 (2011) 1784-1793.

[139] Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG, Martinez-Outschoorn UE, Sotgia F, Lisanti MP, The reverse warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma, Cell. Cycle. 8 (2009) 3984-4001.

[140] Warburg O, Wind F, Negelein E, The metabolism of tumors in the body, J. Gen. Physiol. 8 (1927) 519-530.

[141] Bayley JP, Devilee P, The warburg effect in 2012, Curr. Opin. Oncol. 24 (2012) 62-67.

[142] Pavlides S, Tsirigos A, Migneco G, Whitaker-Menezes D, Chiavarina B, Flomenberg N, Frank PG, Casimiro MC, Wang C, Pestell RG, Martinez-Outschoorn UE, Howell A, Sotgia F, Lisanti MP, The autophagic tumor stroma model of cancer: Role of oxidative stress and ketone production in fueling tumor cell metabolism, Cell. Cycle. 9 (2010) 3485-3505.

[143] Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG, Tanowitz HB, Sotgia F, Lisanti MP, Stromal-epithelial metabolic coupling in cancer: Integrating autophagy and metabolism in the tumor microenvironment, Int. J. Biochem. Cell Biol. 43 (2011) 1045-1051.

[144] Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL, Cantley LC, The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth, Nature. 452 (2008) 230-233.

[145] Chiavarina B, Whitaker-Menezes D, Martinez-Outschoorn UE, Witkiewicz AK, Birbe RC, Howell A, Pestell RG, Smith J, Daniel R, Sotgia F, Lisanti MP, Pyruvate kinase expression (PKM1 and PKM2) in cancer-associated fibroblasts drives stromal nutrient production and tumor growth, Cancer. Biol. Ther. 12 (2011) .

[146] Williams GH, Stoeber K, The cell cycle and cancer, J. Pathol. 226 (2012) 352-364.

[147] Satyanarayana A, Kaldis P, Mammalian cell-cycle regulation: Several cdks, numerous cyclins and diverse compensatory mechanisms, Oncogene. 28 (2009) 2925-2939.

[148] Arooz T, Yam CH, Siu WY, Lau A, Li KK, Poon RY, On the concentrations of cyclins and cyclin-dependent kinases in extracts of cultured human cells, Biochemistry. 39 (2000) 9494-9501.

[149] Obeyesekere MN, Tucker SL, Zimmerman SO, Mathematical models for the cellular concentrations of cyclin and MPF, Biochem. Biophys. Res. Commun. 184 (1992) 782-789.

[150] Massague J, G1 cell-cycle control and cancer, Nature. 432 (2004) 298-306.

[151] Keenan SM, Lents NH, Baldassare JJ, Expression of cyclin E renders cyclin D-CDK4 dispensable for inactivation of the retinoblastoma tumor suppressor protein, activation of E2F, and G1-S phase progression, J. Biol. Chem. 279 (2004) 5387-5396.

[152] Sun A, Bagella L, Tutton S, Romano G, Giordano A, From G0 to S phase: A view of the roles played by the retinoblastoma (rb) family members in the rb-E2F pathway, J. Cell. Biochem. 102 (2007) 1400-1404.

[153] Nigg EA, Mitotic kinases as regulators of cell division and its checkpoints, Nat. Rev. Mol. Cell Biol. 2 (2001) 21-32.

[154] Kastan MB, Bartek J, Cell-cycle checkpoints and cancer, Nature. 432 (2004) 316-323.

[155] Khodjakov A, Rieder CL, The nature of cell-cycle checkpoints: Facts and fallacies, J. Biol. 8 (2009) 88.

[156] Bartek J, Lukas J, DNA damage checkpoints: From initiation to recovery or adaptation, Curr. Opin. Cell Biol. 19 (2007) 238-245.

[157] Foster DA, Yellen P, Xu L, Saqcena M, Regulation of G1 cell cycle progression: Distinguishing the restriction point from a nutrient-sensing cell growth checkpoint(s), Genes Cancer. 1 (2010) 1124-1131.

[158] Coller HA, Cell biology. The essence of quiescence, Science. 334 (2011) 1074-1075.

[159] Coller HA, Sang L, Roberts JM, A new description of cellular quiescence, PLoS Biol. 4 (2006) e83.

[160] Srivastava S, Mishra RK, Dhawan J, Regulation of cellular chromatin state: Insights from quiescence and differentiation, Organogenesis. 6 (2010) 37-47.

[161] Lemons JM, Feng XJ, Bennett BD, Legesse-Miller A, Johnson EL, Raitman I, Pollina EA, Rabitz HA, Rabinowitz JD, Coller HA, Quiescent fibroblasts exhibit high metabolic activity, PLoS Biol. 8 (2010) e1000514.

[162] Rodier F, Campisi J, Four faces of cellular senescence, J. Cell Biol. (2011) .

[163] Campisi J, d'Adda di Fagagna F, Cellular senescence: When bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (2007) 729-740.

[164] Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9363-9367.

[165] Hayflick L, Moorhead PS, The serial cultivation of human diploid cell strains, Exp. Cell Res. 25 (1961) 585-621.

[166] Hayflick L, The limited in vitro lifetime of human diploid cell strains, Exp. Cell Res. 37 (1965) 614-636.

[167] Harley CB, Futcher AB, Greider CW, Telomeres shorten during ageing of human fibroblasts, Nature. 345 (1990) 458-460.

[168] Collado M, Blasco MA, Serrano M, Cellular senescence in cancer and aging, Cell. 130 (2007) 223-233.

[169] Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM, Cellular senescence in aging primates, Science. 311 (2006) 1257.

[170] Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM, Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders, Nature. 479 (2011) 232-236.

[171] Campisi J, Andersen JK, Kapahi P, Melov S, Cellular senescence: A link between cancer and age-related degenerative disease? Semin. Cancer Biol. 21 (2011) 354-359.

[172] Coppe JP, Desprez PY, Krtolica A, Campisi J, The senescence-associated secretory phenotype: The dark side of tumor suppression, Annu. Rev. Pathol. 5 (2010) 99-118.

[173] Kuilman T, Peeper DS, Senescence-messaging secretome: SMS-ing cellular stress, Nat. Rev. Cancer. 9 (2009) 81-94.

[174] Sebastian T, Malik R, Thomas S, Sage J, Johnson PF, C/EBPbeta cooperates with RB:E2F to implement ras(V12)-induced cellular senescence, EMBO J. 24 (2005) 3301-3312.

[175] Vaheri A, Enzerink A, Räsänen K, Salmenperä P, Nemosis, a novel way of fibroblast activation, in inflammation and cancer, Exp. Cell Res. 315 (2009) 1633-1638.

[176] Bizik J, Kankuri E, Ristimäki A, Taieb A, Vapaatalo H, Lubitz W, Vaheri A, Cell-cell contacts trigger programmed necrosis and induce cyclooxygenase-2 expression, Cell Death Differ. 11 (2004) 183-195.

[177] Kankuri E, Cholujova D, Comajova M, Vaheri A, Bizik J, Induction of hepatocyte growth factor/scatter factor by fibroblast clustering directly promotes tumor cell invasiveness, Cancer Res. 65 (2005) 9914-9922.

[178] Räsänen K, Virtanen I, Salmenperä P, Grenman R, Vaheri A, Differences in the nemosis response of normal and cancer-associated fibroblasts from patients with oral squamous cell carcinoma, PLoS One. 4 (2009) e6879.

[179] Enzerink A, Salmenperä P, Kankuri E, Vaheri A, Clustering of fibroblasts induces proinflammatory chemokine secretion promoting leukocyte migration, Mol. Immunol. 46 (2009) 1787-1795.

[180] Peura M, Bizik J, Salmenperä P, Noro A, Korhonen M, Patila T, Vento A, Vaheri A, Alitalo R, Vuola J, Harjula A, Kankuri E, Bone marrow mesenchymal stem cells undergo nemosis and induce keratinocyte wound healing utilizing the HGF/c-Met/PI3K pathway, Wound Repair Regen. 17 (2009) 569-577.

[181] Kankuri E, Babusikova O, Hlubinova K, Salmenperä P, Boccaccio C, Lubitz W, Harjula A, Bizik J, Fibroblast nemosis arrests growth and induces differentiation of human leukemia cells, Int. J. Cancer. 122 (2008) 1243-1252.

[182] Enzerink A, Rantanen V, Vaheri A, Fibroblast nemosis induces angiogenic responses of endothelial cells, Exp. Cell Res. 316 (2010) 826-835.

[183] Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G, Nomenclature Committee on Cell Death 2009, Classification of cell death: Recommendations of the nomenclature committee on cell death 2009, Cell Death Differ. 16 (2009) 3-11.

[184] Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G, Molecular definitions of cell death subroutines: Recommendations of the nomenclature committee on cell death 2012, Cell Death Differ. 19 (2012) 107-120.

[185] Enzerink A, Vaheri A, Fibroblast nemosis promotes proinflammatory properties of endothelial cells by modulating cell-cell junctions and leukocytes by augmenting adhesion, SUBMITTED. (2012) .

[186] Räsänen K, Vaheri A, Proliferation and motility of HaCaT keratinocyte derivatives is enhanced by fibroblast nemosis, Exp. Cell Res. 316 (2010) 1739-1747.

[187] Fontana L, Chen Y, Prijatelj P, Sakai T, Fassler R, Sakai LY, Rifkin DB, Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1, FASEB J. 19 (2005) 1798-1808.

[188] Takahashi S, Leiss M, Moser M, Ohashi T, Kitao T, Heckmann D, Pfeifer A, Kessler H, Takagi J, Erickson HP, Fassler R, The RGD motif in fibronectin is essential for development but dispensable for fibril assembly, J. Cell Biol. 178 (2007) 167-178.

[189] Fusenig NE, Boukamp P, Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes, Mol. Carcinog. 23 (1998) 144-158.

[190] Hall DE, Reichardt LF, Crowley E, Holley B, Moezzi H, Sonnenberg A, Damsky CH, The alpha 1/beta 1 and alpha 6/beta 1 integrin heterodimers mediate cell attachment to distinct sites on laminin, J. Cell Biol. 110 (1990) 2175-2184.

[191] Carlson TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO, Direct cell adhesion to the angiopoietins mediated by integrins, J. Biol. Chem. 276 (2001) 26516-26525.

[192] Abramoff MD, Magelhaes PJ, Ram SJ, Image processing with ImageJ, Biophotonics Int. 11 (2004) 36-42.

[193] Saksela O, Radial caseinolysis in agarose: A simple method for detection of plasminogen activator in the presence of inhibitory substances and serum, Anal. Biochem. 111 (1981) 276-282.

[194] Ehrlich MI, Krushell JS, Blumenstock FA, Kaplan JE, Depression of phagocytosis by plasmin degradation products of plasma fibronectin, J. Lab. Clin. Med. 98 (1981) 263-271.

[195] Baliga BS, Pronczuk AW, Munro HN, Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver, J. Biol. Chem. 244 (1969) 4480-4489.

[196] Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH, Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression, J. Cell Biol. 109 (1989) 877-889.

[197] Aota S, Nomizu M, Yamada KM, The short amino acid sequence pro-his-ser-arg-asn in human fibronectin enhances cell-adhesive function, J. Biol. Chem. 269 (1994) 24756-24761.

[198] Wierzbicka-Patynowski I, Schwarzbauer JE, The ins and outs of fibronectin matrix assembly, J. Cell. Sci. 116 (2003) 3269-3276.

[199] McKeown-Longo PJ, Mosher DF, Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts, J. Cell Biol. 100 (1985) 364-374.

[200] Sottile J, Mosher DF, N-terminal type I modules required for fibronectin binding to fibroblasts and to fibronectin's III1 module, Biochem. J. 323 (Pt 1) (1997) 51-60.

[201] Maurer LM, Annis DS, Mosher DF, IGD motifs, which are required for migration stimulatory activity of fibronectin type I modules, do not mediate binding in matrix assembly, PLoS One. 7 (2012) e30615.

[202] Sottile J, Chandler J, Fibronectin matrix turnover occurs through a caveolin-1-dependent process, Mol. Biol. Cell. 16 (2005) 757-768.

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

[204] Robinson EE, Foty RA, Corbett SA, Fibronectin matrix assembly regulates alpha5beta1-mediated cell cohesion, Mol. Biol. Cell. 15 (2004) 973-981.

[205] Caicedo-Carvajal CE, Shinbrot T, Foty RA, Alpha5beta1 integrin-fibronectin interactions specify liquid to solid phase transition of 3D cellular aggregates, PLoS One. 5 (2010) e11830.

[206] Zhang Z, Morla AO, Vuori K, Bauer JS, Juliano RL, Ruoslahti E, The alpha v beta 1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin, J. Cell Biol. 122 (1993) 235-242.

[207] Han S, Sidell N, Roser-Page S, Roman J, Fibronectin stimulates human lung carcinoma cell growth by inducing cyclooxygenase-2 (COX-2) expression, Int. J. Cancer. 111 (2004) 322-331.

[208] Zaric J, Ruegg C, Integrin-mediated adhesion and soluble ligand binding stabilize COX-2 protein levels in endothelial cells by inducing expression and preventing degradation, J. Biol. Chem. 280 (2005) 1077-1085.

[209] You R, Zheng M, McKeown-Longo PJ, The first type III repeat in fibronectin activates an inflammatory pathway in dermal fibroblasts, J. Biol. Chem. 285 (2010) 36255-36259.

[210] Kolachala VL, Bajaj R, Wang L, Yan Y, Ritzenthaler JD, Gewirtz AT, Roman J, Merlin D, Sitaraman SV, Epithelial-derived fibronectin expression, signaling, and function in intestinal inflammation, J. Biol. Chem. 282 (2007) 32965-32973.

[211] Johansen T, Lamark T, Selective autophagy mediated by autophagic adapter proteins, Autophagy. 7 (2011) 279-296.

[212] Wu YT, Tan HL, Shui G, Bauvy C, Huang Q, Wenk MR, Ong CN, Codogno P, Shen HM, Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase, J. Biol. Chem. 285 (2010) 10850-10861.

[213] Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y, Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells, Cell Struct. Funct. 23 (1998) 33-42.

[214] Lock R, Debnath J, Extracellular matrix regulation of autophagy, Curr. Opin. Cell Biol. 20 (2008) 583-588.

[215] Castello-Cros R, Bonuccelli G, Molchansky A, Capozza F, Witkiewicz AK, Birbe RC, Howell A, Pestell RG, Whitaker-Menezes D, Sotgia F, Lisanti MP, Matrix remodeling stimulates stromal autophagy, "fueling" cancer cell mitochondrial metabolism and metastasis, Cell. Cycle. 10 (2011) 2021-2034.

[216] Kroemer G, Marino G, Levine B, Autophagy and the integrated stress response, Mol. Cell. 40 (2010) 280-293.

[217] Memmott RM, Dennis PA, Akt-dependent and -independent mechanisms of mTOR regulation in cancer, Cell. Signal. 21 (2009) 656-664.

[218] Danen EH, Yamada KM, Fibronectin, integrins, and growth control, J. Cell. Physiol. 189 (2001) 1-13.

[219] Corcelle EA, Puustinen P, Jäättelä M, Apoptosis and autophagy: Targeting autophagy signalling in cancer cells -'trick or treats'? FEBS J. 276 (2009) 6084-6096.

[220] Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM, Identification of a novel inhibitor of mitogen-activated protein kinase kinase, J. Biol. Chem. 273 (1998) 18623-18632.

[221] Kyriakis JM, Avruch J, Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation, Physiol. Rev. 81 (2001) 807-869.

[222] Wong CH, Iskandar KB, Yadav SK, Hirpara JL, Loh T, Pervaiz S, Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation, PLoS One. 5 (2010) e9996.

[223] Chiquet M, Gelman L, Lutz R, Maier S, From mechanotransduction to extracellular matrix gene expression in fibroblasts, Biochim. Biophys. Acta. 1793 (2009) 911-920.

[224] White E, Lowe SW, Eating to exit: Autophagy-enabled senescence revealed, Genes Dev. 23 (2009) 784-787.

[225] White E, DiPaola RS, The double-edged sword of autophagy modulation in cancer, Clin. Cancer Res. 15 (2009) 5308-5316.

[226] Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, Kondo S, Kondo Y, Yu Y, Mills GB, Liao WS, Bast RC,Jr, The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells, J. Clin. Invest. 118 (2008) 3917-3929.

[227] Essers MA, Trumpp A, Targeting leukemic stem cells by breaking their dormancy, Mol. Oncol. 4 (2010) 443-450.

[228] Aguirre-Ghiso JA, Models, mechanisms and clinical evidence for cancer dormancy, Nat. Rev. Cancer. 7 (2007) 834-846.

[229] Blomen VA, Boonstra J, Cell fate determination during G1 phase progression, Cell Mol. Life Sci. 64 (2007) 3084-3104.

[230] Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, Kleijer WJ, DiMaio D, Hwang ES, Senescence-associated beta-galactosidase is lysosomal beta-galactosidase, Aging Cell. 5 (2006) 187-195.

[231] Blagosklonny MV, Cell cycle arrest is not senescence, Aging (Albany NY). 3 (2011) 94-101.

[232] Leontieva OV, Demidenko ZN, Gudkov AV, Blagosklonny MV, Elimination of proliferating cells unmasks the shift from senescence to quiescence caused by rapamycin, PLoS One. 6 (2011) e26126.

[233] Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J, Reversal of human cellular senescence: Roles of the p53 and p16 pathways, EMBO J. 22 (2003) 4212-4222.

[234] Dirac AM, Bernards R, Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53, J. Biol. Chem. 278 (2003) 11731-11734.

[235] Sobral LM, Zecchin KG, Nascimento de Aquino S, Lopes MA, Graner E, Coletta RD, Isolation and characterization of myofibroblast cell lines from oral squamous cell carcinoma, Oncol. Rep. 25 (2011) 1013-1020.

[236] Suomela S, Koljonen V, Skoog T, Kukko H, Bohling T, Saarialho-Kere U, Expression of MMP-10, MMP-21, MMP-26, and MMP-28 in merkel cell carcinoma, Virchows Arch. 455 (2009) 495-503.

[237] Sugiyama N, Varjosalo M, Meller P, Lohi J, Hyytiainen M, Kilpinen S, Kallioniemi O, Ingvarsen S, Engelholm LH, Taipale J, Alitalo K, Keski-Oja J, Lehti K, Fibroblast growth factor receptor 4 regulates tumor invasion by coupling fibroblast growth factor signaling to extracellular matrix degradation, Cancer Res. 70 (2010) 7851-7861.

[238] Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG, The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction, J. Mol. Cell. Cardiol. 48 (2010) 504-511.

[239] Shi F, Sottile J, MT1-MMP regulates the turnover and endocytosis of extracellular matrix fibronectin, J. Cell. Sci. 124 (2011) 4039-4050.

[240] Van Lint P, Libert C, Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation, J. Leukoc. Biol. 82 (2007) 1375-1381.

[241] Kessenbrock K, Plaks V, Werb Z, Matrix metalloproteinases: Regulators of the tumor microenvironment, Cell. 141 (2010) 52-67.

[242] Tu G, Xu W, Huang H, Li S, Progress in the development of matrix metalloproteinase inhibitors, Curr. Med. Chem. 15 (2008) 1388-1395.

[243] Martin MD, Matrisian LM, The other side of MMPs: Protective roles in tumor progression, Cancer Metastasis Rev. 26 (2007) 717-724.

[244] Decock J, Thirkettle S, Wagstaff L, Edwards DR, Matrix metalloproteinases: Protective roles in cancer, J. Cell. Mol. Med. 15 (2011) 1254-1265.

[245] Muller-Decker K, Furstenberger G, The cyclooxygenase-2-mediated prostaglandin signaling is causally related to epithelial carcinogenesis, Mol. Carcinog. 46 (2007) 705-710.

[246] Wang W, Bergh A, Damber JE, Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer, Clin. Cancer Res. 11 (2005) 3250-3256.

[247] Laeeq S, Faust R, Modeling the cholesteatoma microenvironment: Coculture of HaCaT keratinocytes with WS1 fibroblasts induces MMP-2 activation, invasive phenotype, and proteolysis of the extracellular matrix, Laryngoscope. 117 (2007) 313-318.

[248] Bachmeier BE, Boukamp P, Lichtinghagen R, Fusenig NE, Fink E, Matrix metalloproteinases-2,-3,-7,-9 and-10, but not MMP-11, are differentially expressed in normal, benign tumorigenic and malignant human keratinocyte cell lines, Biol. Chem. 381 (2000) 497-507.

[249] Moon SE, Dame MK, Remick DR, Elder JT, Varani J, Induction of matrix metalloproteinase-1 (MMP-1) during epidermal invasion of the stroma in human skin organ culture: Keratinocyte stimulation of fibroblast MMP-1 production, Br. J. Cancer. 85 (2001) 1600-1605.

[250] Ha L, Ponnamperuma RM, Jay S, Ricci MS, Weinberg WC, Dysregulated DeltaNp63alpha inhibits expression of Ink4a/arf, blocks senescence, and promotes malignant conversion of keratinocytes, PLoS One. 6 (2011) e21877.

[251] Keyes WM, Wu Y, Vogel H, Guo X, Lowe SW, Mills AA, p63 deficiency activates a program of cellular senescence and leads to accelerated aging, Genes Dev. 19 (2005) 1986-1999.

[252] Ha L, Ichikawa T, Anver M, Dickins R, Lowe S, Sharpless NE, Krimpenfort P, Depinho RA, Bennett DC, Sviderskaya EV, Merlino G, ARF functions as a melanoma tumor suppressor by inducing p53-independent senescence, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 10968-10973.

[253] Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW, Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas, Nature. 445 (2007) 656-660.

[254] Ikeda H, Sasaki M, Sato Y, Harada K, Zen Y, Mitsui T, Nakanuma Y, Bile ductular cell reaction with senescent hepatocytes in chronic viral hepatitis is lost during hepatocarcinogenesis, Pathol. Int. 59 (2009) 471-478.

[255] Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO, Diversity, topographic differentiation, and positional memory in human fibroblasts, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12877-12882.

[256] Reimann M, Schmitt CA, Lee S, Non-cell-autonomous tumor suppression: Oncogene-provoked apoptosis promotes tumor cell senescence via stromal crosstalk, J. Mol. Med. (Berl). 89 (2011) 869-875.

ORGINAL PUBLICATIONS