PhD thesis

EXTRACELLULAR MATRIX PROTEINS AND EPITHELIAL CELL PLASTICITY IN PROGRESSION OF BREAST AND PROSTATE CANCER

by

Gvantsa Kharaishvili, MD

Supervisor: Jan Bouchal, PhD

Laboratory of Molecular Pathology and Department of Clinical and Molecular Pathology, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University

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DECLARATION

I hereby declare that I have written the thesis and performed all the work myself unless otherwise specified below.

The PhD thesis includes results which have been obtained in co-operation with the following colleagues: i) in vitro experiments using breast cancer cell lines (RNA isolation, expression analysis, collagen fibrillogenesis and invasion assays) were performed by Mgr. Dana Simkova. ii) experiments with prostate cancer cell lines (androgen depletion studies, SA-β-gal cytochemistry, western blot analysis, flow cytometry, confocal microscopy) were carried out by Mgr. Zuzana Pernicova and Mgr. Karel Soucek, PhD from the Department of Cytokinetics, Institute of Biophysics, Brno. Contributions to the research by others are explicitly acknowledged in the thesis.

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1. INTRODUCTION

1.1 Importance of extracellular matrix and tissue microenvironment in carcinogenesis

Tumor progression is in part recognized as a product of an evolving crosstalk between different cell types within the tumor and its surrounding supportive tissue, or tumor stroma (Liotta and Kohn, 2001). Invasive tumor cells interact with their microenvironment and remodel it into supportive milieu for tumor growth and progression. The altered environment is recognizable in the light microscope as desmoplasia and is used for assessment of invasion (Koperek et al., 2011). Tumor cells communicate bidirectionally with the surrounding microenvironment (diverse extracellular matrix components and growth factors) from which genetic cell programming signals are supplied that control cell survival, growth, differentiation, and invasion.

The importance of microenvironment in tumor progression is documented by model systems. It has been shown on animal xenograft that injection of purified malignant epithelial cells results in formation of histologically complex tumors, with 80% of the cells being stromal cells (Elenbaas and Weinberg, 2001). Tumor stromal cells are distinct from their normal counterparts (Allinen et al., 2004), and their modified characteristics lead to active formation of the tumor microenvironment (Zumsteg and Christofori, 2009). Influence of microenvironment on tumor formation, growth, invasiveness and metastatic potential was shown in many pathologic conditions, such as chronic inflammation leading to stomach cancer, caused by *H. pylori* infection and hepatitis C infection of the liver, leading to chronic inflammation, proceeding to liver cirrhosis and cancer (Coussens and Werb, 2002). Furthermore, injection of non transformed mammary epithelial cells into irradiated mammary stromal fat pads resulted in increased tumor growth when compared to those injected into contralateral, non irradiated mammary fat pads (Barcellos-Hoff and Ravani, 2000). Irradiated stromal cells altered microenvironment and resulted in tumor promotion. Moreover, malignant cells exist within normal tissues but are restrained by normal milieu signals. Similar patterns of mutations are found in tumor tissue and its adjacent normal epithelial tissue (Bissell et al., 1999; Deng etal., 1996). The authors concluded that the mutation that initiates the carcinoma occurs in the epithelium, but events that promote tumor progression involve the stroma. In some cases, the trigger for neoplastic

progression is speculated to come from signals within the stromal microenvironment (Egeblad et al., 2005).

1.1.1. Extracellular matrix: its composition and molecular profile

Extracellular matrix (ECM) is a proteinaceous component of the stroma. It is a complex three-dimensional network of macromolecular protein fibers as well as non-fibrous proteoglycans. The ECM provides architectural structure, strength and contextual information for cellular growth, communication, differentiation, survival, adhesion and migration. Proteins that provide ECM structure are: glycosaminoglycans and proteoglycans (form a hydrated gel-like substance, resists compressive forces and allow rapid diffusion) and fibrous proteins and collagens (stengthens the matrix and give resilience). They represent insoluble factors of the matrix (Reviewed in Erler and Weaver, 2009). Structural proteins are synthesized by fibroblasts and other cells embedded in connective tissue. Group of ECM molecules called ,,matricellular proteins" (e.g. thrombo-spondin-1 and -2, SPARC, tenascin-C and osteopontin) do not function as structural elements but modulate cell-matrix interactions and cell functions (reviewed in Järveläinen et al., 2009). ECM is in a dynamic state and undergoes turnover and remodeling in conjunction with signals and is enhanced during inflammation, wound repair and tumor invasion. Key enzymes which remodel ECM are matrix metalloproteinases (MMPs) and urokinase-type plasminogen activators (uPAs). They degrade components of the basement membrane as well as proteins and proteoglycans of connective tissue and liberate latent growth factors from their storage sites in the extracellular matrix. Factors that are activated in this fashion are for example fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), and transforming growth factors (TGFβs) (Schulz, 2005). As structural and metabolic alterations of ECM can lead to the development or progression of disease, its molecules can serve as important targets for pharmacotherapy (Figure 2).

1.1.1.1. Collagens. Collagens play structural roles and contribute to mechanical properties, organization, and shape of tissues. They interact with cells via several receptor families and regulate their proliferation, migration, and differentiation. Some collagens have a restricted tissue distribution and hence specific biological functions (Ricard-Blum, 2011). Collagens are trimeric molecules composed of three polypeptide α chains, which contain the sequence repeat that allow

the formation of a triple helix. Each member of the collagen family contains at least one triple-helical domain, which is located in the extracellular matrix, and most collagens are able to form supramolecular aggregates. Besides triple-helical domains, collagens contain non triple-helical domains, used as building blocks by other extracellular matrix proteins and are thus modular proteins. At present, 28 types of collagens are classified as fibrillar collagens, unconventional collagens including collagen VII, network-forming collagens (VI, VIII and X), fibril-associated collagens with interrupted triple helix (IX, XII, XIV, XVI and XIX), basement membrane collagens, transmembrane collagens and multiplexins (Heino, 2007; Ricard-Blum and Ruggiero, 2005).

1.1.1.2. Proteoglycans. Next to collagens, proteoglycans (PGs) constitute a major class of extracellular matrix/cell surface components known to be involved in primary physiological and pathological phenomena; and due to the altered transcription/translation patterns that these PGs exhibit, they have been identified as potential diagnostic/prognostic and therapeutic targets in diverse disease states. PGs are widely expressed throughout embryonic and adult life of invertebrates, underscoring the highly evolutionary conserved nature of these macromolecules and their multiple biological roles. Based upon its direct involvement in cell–cell and cell–ECM interactions, this gene family has been strongly implicated in the regulation of cell movement. However, how PGs actually affect this process is only partially understood and in some instances controversial. Assignment of diverse roles of PGs in promoting, or inhibiting, cell movement seems to be dictated by the biological system in which the function of the PG has been investigated (Cattaruzza and Perris, 2005). The proteoglycan superfamily now contains more than 30 molecules. They are involved in maintaining the transparency of the cornea, the tensile strength of the skin and tendon, the viscoelasticity of blood vessels, the compressive properties of cartilage, and the mineralized matrix of bones. In addition, PGs play key roles as storage deposits for growth factors and cytokines and are able to alter the biology of these factors (Iozzo and Murdoch, 1997).

The basic proteoglycan unit consists of a "core protein" with one or more covalently attached glycosaminoglycan (GAG) chain(s). Proteoglycans can be categorised depending upon the nature of their glycosaminoglycan chains and/or by size (kDa). Four major classes of PGs exist: i) chondroitin sulfate/dermatan sulfate PGs (decorin, biglycan, versican); heparan sulfate/

chondroitin sulfate PGs (testican, perlecan); ii) chondroitin sulfate (neurocan, aggrecan); iii) keratan sulfate (fibromodulin, lumican). Among them, decorin, biglycan, testican, fibromodulin, lumican are small proteoglycans, and versican, perlecan, neurocan and aggrecan are large proteoglycans.

The small leucine-rich repeat proteoglycans (SLRPs) form a group of structurally and functionally related molecules and were originally grouped on the basis of their relatively small protein core (36-42 kDa), compared with the larger aggregating proteoglycans such as aggrecan and versican, and on their unique structural organization composed of tandem leucine-rich repeats (LRRs) (Iozzo and Murdoch, 1996; Iozzo, 1997). The small leucine-rich proteoglycan family encompass five discrete classes, grouped by common structural and functional properties (Figure 1). Some of these gene products are not classical proteoglycans, whereas others have new and unique features. Despite being structural proteins, SLRPs constitute a network of signal regulation: being mostly extracellular, they are upstream of multiple signaling cascades. They affect intracellular phosphorylation, a major conduit of information for cellular responses, and modulate distinct pathways, including those driven by bone morphogenetic protein/transforming growth factor β superfamily members, receptor tyrosine kinases such as ErbB family members and the insulin-like growth factor I receptor, and Toll-like receptors.

Class I includes decorin, biglycan, and asporin. Their N termini have a typical cluster of Cys residues that form two disulfide bonds. Decorin and biglycan contain one or two chondroitin/dermatan sulfate side chains, while asporin lacks Ser-Gly dipeptide and flanking amino acids required for glycanation and thus it is not a classical proteoglycan. However, asporin contains a stretch of Asp residues which is also found in class II (osteoadherin), class III (epiphycan), and class V (podocan) members, located in either the N- or C-terminal region (further details on asporin are provided in chapter **1.7.4**).

Class II members are fibromodulin, lumican, keratan sulfate and polylactosamine and contain clusters of Tyr sulfate residues at their N termini that could contribute to the polyanionic nature of SLRPs. Class III have three members characterized by a relatively low number of LRRs and a some of them exist as glycoproteins in tissues. Class IV is composed of related chondroadherin and nyctalopin (Bech-Hansen et al., 2000; Pusch et al., 2000) and of a new member called tsukushi. Both tsukushi and nyctalopin have 11 homologous LRRs flanked by an N-terminal Cys-rich region. Tsukushi shares functional properties with class I SLRPs (Chen et

al., 2004; Moreno et al., 2005) as it is a bone morphogenetic protein (BMP) inhibitor that forms a ternary complex with BMP and chordin (Ohta et al., 2004). Class V contains two genes, podocan and a highly homologous podocan-like protein 1. Although these proteins have a different C-terminal Cys-rich cluster, they have 20 LRRs with homology to class I and II molecules. Moreover, podocan binds collagen I and inhibits cell growth via induction of p21 (Shimizu-Hirota et al., 2004), both functional properties shared by other SLRP members (Schaeffer et al., 2008).

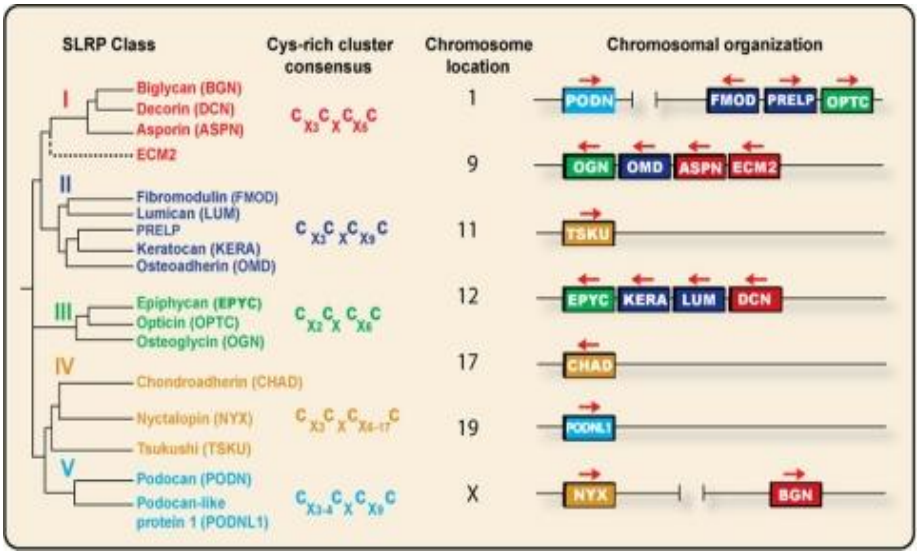


Figure 1. Phylogenetic analysis and chromosomal organization of various human SLRP classes. The color-coded dendrogram (*left*) shows the presence of five distinct families of SLRPs and related LRR proteins. The consensus for the N-terminal Cys-rich cluster is also shown. The chromosomal arrangement of the various SLRP genes is shown in a telomeric orientation (*right*). Transcriptional direction is shown by the *arrows* above the color-coded boxes. The horizontal distance between genes is not to scale. (Source: Schaefer et al. 2008).

1.1.2. Cell-cell interactions

Epithelial cells adhere to each other and interact with each other through several types of contacts. Morphologically distinct and functionally important contacts include adherens junctions, gap junctions, tight junctions (occluding junctions) and desmosomes (please see below). Loss of polarity through altered cell-cell or cell-basal membrane interactions gives rise to tumor phenotype and vice versa (Zhan et al., 2008). Unity of the basement membrane

distinguishes non-invasive state from invasive, and its gatekeeper function is essential as a determinant of cancer progression (Muschler and Streuli, 2010). The mechanisms that control transition from non-invasive to invasive state are still uncertain. However, it is known that leading factor is tumor cell itself, with increased synthesis of matrix degrading proteins and altered signaling program.

1.1.2.1. Adherence junctions are arranged in a belt-like configuration (belt desmosomes) between adjacent epithelial cells and are intracellularly connected to actin filaments. The proteins actually mediating these homotypic interactions are cadherins, of which E-cadherin is a classical representative. Interaction between E-cadherin molecules is Ca2+ - dependent. On the cytoplasmic surface of the cell membrane, E-cadherin is linked to actin filaments by α-catenin and β-catenin. Mutation or down-regulation of E-cadherin is frequent event in cancers and often occurs during cancer progression. In some invasive tumors, it is replaced by other cadherins, e.g. N-cadherin. This event is named as ,,cadherin switch".

1.1.2.2. Gap junctions are communication channels that connect cells of the same type. They allow the passage of small molecules and Ca2+ and K+. Gap junctions are formed by connexins which are frequently inactivated during tumor progression (Schulz, 2005).

1.1.2.3. Tight junctions, formed by occludins (e.g. zonula occludens-1), claudin family proteins (claudin 1-22) and the IgG-like family of junctional adhesion molecules (JAMs), seal epithelium and define the apical and lateral membrane compartments of epithelial cells (Niessen, 2007). Normal distribution of cadherins, integrins and actin cytoskeleton provides "top–bottom" apico-basal polarity of epithelial cells and limits the movement of epithelial cells to the two dimensional space of the epithelial plane.

1.1.2.4. Desmosomes are the most common type of intercellular junction in vertebrate epithelial cells. They are characterized by 2 forms of interaction with other cellular structures. First, they form membrane anchorage sites for intermediate-size filaments, which are seen as electron-dense plaques evident beneath the plasma membrane. Second, a specific membrane core domain interacts with a corresponding domain of the plasma membrane of an adjacent cell, apparently

mediating intercellular adhesion in a stable way. The desmosome intermediate filament complex is thought to impart tensile strength and resilience to the epithelium. Desmosomal proteins can be divided into 2 groups based on whether they fractionate with the urea-insoluble 'core' or the urea-soluble 'plaque' components. Desmoglein is, for example, a protein of the core. The main proteins of the plaque comprise the desmoplakins and plakoglobin.

1.1.3. Cell–matrix interactions

1.1.3.1. Integrins. Integrins are main receptors involved in cell-matrix contacts. They contain transmembrane subunits α and β, large extracellular domain, and intracellular domain which interacts with cytoskeleton proteins. Subunits form 24 integrins. Integrins provide transmission of chemical and mechanical signals, which results in rearrangement of the cell cytoskeleton and activation of pathways that control cell survival and motility, angiogenesis, differentiation and apoptosis. In normal cells, destruction of integrin-dependent contacts between cell-ECM results in special type of apoptosis – anoikis. The ability of cell to survive without contact with a substrate is a feature of tumor cells. Mesenchymal cells are elongated and motile in a three-dimensional space defining a front–back polarity with a leading edge enriched in integrins and matrix metalloproteinases.

Integrin expression changes significantly during carcinogenesis and different tumors express different integrins. Integrin α6β4 in cooperation with epidermal growth factor receptor (EGFR) are expressed mostly in breast carcinoma (Soung and Chung, 2011), while integrin αVβ3 in cooperation with platelet-derived growth factor (PDGF) and EGFR are expressed in glioblastomas and melanomas (Desgrosellier and Cheresh, 2010; Zhang et al., 2011).

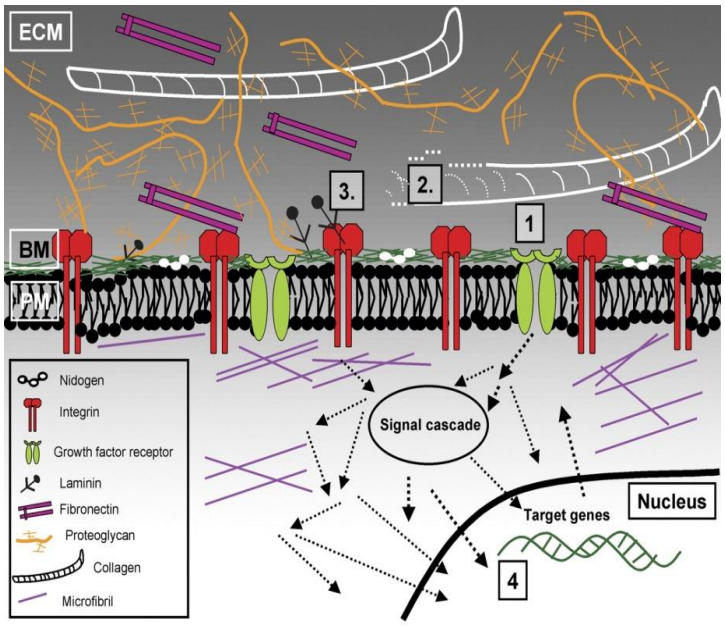


Figure 2. Schematic illustration of the ECM and potential targets in pharmacotherapy. 1, targeting the synthesis of the ECM by blocking of specific growth factors such as TGF-β, their receptor molecules, or intracellular signal transduction. 2, controlling of the degradation of ECM by interfering with enzymes involved in ECM remodelling (e.g., MMPs, ADAMTS, cathepsins) and/or their inhibitors. 3, interfering with the ECM signaling pathways (e.g., via integrins) either by blocking the ECM and integrin interactions or subsequent signal transduction. 4, influencing the transcription of specific ECM molecules [e.g., by triplex-forming oligonucleotides (TFOs)]. BM, basement membrane; PM, plasma membrane. (Source Järveläinen et al., 2009).

1.1.3.2. Matrix metalloproteinases (MMPs). There are 187 members of MMPs which are encoded in human genome and 28 members are secreted MMPs. They are able to degrade every protein in ECM and basement membranes and display preference for certain substrates. All MMPs have similar structures, with Zn2+ ion for catalytic activity, and they are synthesized as "zymogens". Several MMPs are membrane-type which contribute to precise localization of protease activity, as this is required at the edge of migrating cell. Several MMPs - collagenase, gelatinase, matrilysin degrades collagen, gelatin and fibronectin, respectively. Stromelysin degrades structural proteins and proteoglycans. MMP activity is regulated by tissue inhibitors of MMPs (TIMPs 1-4) which are produced by more cells than MMPs itself (Zucker et al., 2002). MMPs are directly implicated in embryonic growth and tissue morphogenesis that require disruption of ECM barriers for microenvironment remodeling and cell migration; and contribute

to the formation of a complex micro-environment for tumor development and progression through activation of growth factors, suppression of tumor cell apoptosis, destruction of chemokine gradients developed by host immune responce, or release angiogenic factors (Folgueras et al., 2004). For example, MMP-11 (human stromelysin-3, hST-3) favoured release of insulin-like growth factor 1 that are bound to specific binding proteins (IGFBPs) (Manes et al., 1997). MMP-9 can proteolytically activate TGF-β and promote tumor invasion and angiogenesis (Yu and Stamenkovic, 2000). Several pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or TGF-β are induced/activated by MMPs, trigger angiogenic switch during carcinogenesis and facilitate vascular remodeling and neovascularization at distant sites. In some circumstances, MMPs play opposite role in tumor progression (Folgueras et al., 2004).

1.1.3.3. Urokinase plasminogen activator (uPA) clefts plasminogen and converts it into plasmin. uPA acts on cell surface, interacts with its receptor (uPAR) which is in dynamic interaction with specific integrins**.** Conversely, its inhibitor **(**PAI-1) prevents cleavage of plasminogen and excludes plasmin from cell environment. Another inhibitor α-antiplasmin limits plasmin activity in the extracellular environment. Plasmin is known to digest fibrin and by clefting activates other ECM proteins: pro-MMPs and pro-uPA.

In general, MMP activity is increased and enhanced in cancer, while TIMPs are downregulated. Enhanced expression of MMPs and uPA/plasmin are associated with a worse outcome, i.e. primary tumors with increased expression are more likely to metastasize. However, in some cancers the relationship is inverse and depends where MMPs and TIMPs are expressed (Zucker et al., 2002). For example, increased expression of TIMP-1 may indicate a worse prognosis in breast cancer. In many carcinomas, the increased expression of MMPs is found in the tumor stroma. While tumor cells largely stain negative for many MMP proteins, the stromal cells (fibroblasts, macrophages, monocytes) express full range of active proteases. In this situation, the expression of TIMPs may actually protect the carcinoma cells (Schulz, 2005).

1.1.3.4. Chemokines are chemotactic cytokines that cause the direct migration of leukocytes, and are induced by inflammatory cytokines, growth factors and pathogenic stimuli (Balkwill, 2004). A family of chemotactic proteins is divided into C, CC, CXC and CXC3C chemokines,

depending on the number and spacing of conserved cysteine residues in the amino-terminal part of the protein. Unlike their normal counterparts, cancer cells express chemokine receptors at their surface. Chemokine network and their receptors influence development of primary tumors and metastasis. Chemokine signaling results in the transcription of target genes that are involved in invasion, motility, cell-matrix interactions and cell survival (Locati et al., 2002). Cytokines and chemokines are essential for epithelial-mesenchymal transition (EMT), tumor formation and late events in epithelial cancers (Waerner et al., 2006). A variety of pro-inflammatory cytokines, which display EMT-inducing abilities *in vitro*, have been reported to act as mitogens and motogens for epithelial cells and their mesenchymal derivatives (Thiery, 2002). Waerner et al. (2006) showed that during human and mouse mammary tumorigenesis, ILEI (secreted interleukin-related protein), a cytokine-like protein is produced by inflammatory cells, favouring epithelial plasticity, EMT, migration, and autocrine production of ILEI during tumor cell progression/dedifferentiation. Recent studies demonstrate also chemoattractant chemokine C-C motif ligand 2 (CCL2) in correlation with poor prognosis in breast, cervical and bladder cancer (Pollard, 2004; Leek and Harris, 2002). Furthermore, injection of mice with cancer cells secreting chemokine (C-C motif) ligand 5 (CCL5, RANTES), resulted in tumors with metastatic potential, in contrast to injection of breast cancer cells alone (Karnoub et al., 2007).

1.2. Cell types and microenvironmental factors affecting tumor development and progression

1.2.1. Different populations of cancer cells within tumor

Cancer cells alter their adjacent stroma and form a permissive and supportive environment for tumor progression. They influence not only stromal fibroblasts, but endothelial and immune cells. Cancer cells release stroma-modulating growth factors such as fibroblast growth factor, members of the VEGF family, PDGF, EGFR ligands, interleukins, colony-stimulating factors, transforming growth factor β and others (Mueller and Fusenig, 2004). These factors act in a paracrine manner, disrupt normal tissue homeostasis resulting in stromal reactions such as angiogenesis and inflammatory response (Baeriswyl and Christofori, 2009; Coussens and Werb, 2002).

1.2.1.1. Fibroblasts in tumor progression. Carcinoma associated fibroblasts (CAF) are believed to influence tumor behavior and outcome, and thus understanding their biology is of importance to the overall understanding of cancer. CAFs are large, spindle-shaped mesenchymal cells that share characteristics with smooth muscle cells and fibroblasts (Mueller and Fusenig, 2004). They constitute a significant component of the stroma and represent the cells responsible for the change of extracellular matrix composition to one with increased amounts of collagens (desmoplastic response) (Elenbaas and Weinberg, 2001). CAFs itself are likely to derive from resident fibroblasts and marrow-derived mesenchymal precursor cells, whereas their generation through epithelial-mesenchymal transition of tumor cells is more controversial (Mueller and Fusenig, 2004; Kalluri and Zeisberg, 2006; Karnoub et al., 2007). CAFs are phenotypically and functionally distinct from their normal counterparts and are identified immunocyto/histo-chemically based on a combination of different markers such as α-smooth muscle actin (α-SMA), vimentin, desmin and fibroblast activation protein (FAP) (Garin-Chesa et al., 1990). Some of these differences are reversible, whereas others persist when the fibroblasts are removed from the vicinity of carcinoma cells. Their gene expression differences are due to epigenetic and genetic alterations (Hu et al., 2005; Patocs et al., 2007).

CAFs promote tumor progression in several ways. Recent breast cancer gene expression profile of the stromal compartment has revealed significantly different gene sets than normal mammary stroma, with increased cytokines, ECM molecules and proteases (Singer et al., 2008; Casey et al., 2008). Secreted ECM components such as tenascin reveal pro-migratory activity (De

Wever et al., 2004). TGF-β induces HGF expression by fibroblast but also induces the transition of fibroblasts to myofibroblasts by increasing alpha smooth muscle actin and tenascin C expression (Untergasser et al., 2005). Gene expression changes, reported by Rajski et al., (2010), which were induced by IGF-I in human breast fibroblasts contained several soluble factors, such as periostin (POSTN), which is involved in bone metastasis and angiogenesis (Sasaki et al., 2003; Shao et al., 2004), Tenascin, which enhances tumor cell proliferation (Ruiz et al., 2004), as well as LOXL1, a member of lysyl oxidase family. LOXL1 similar to LOXL2, may act in the vicinity of epithelial cells during tissue remodelling. LOXL2 has previously been reported to be involved in invasiveness (Akiri et al., 2003) and specifically expressed by fibroblasts in tumor tissue (Chang et al., 2004). The presence of these factors indicates that the IGF-I activated stroma enhances proliferation and the metastatic potential of the cancer cells. Further, stromal fibroblasts have an impact on tumor stroma composition by expression of different metalloproteinases, for example, MMP-13 which is expressed by CAF-like cells in human breast cancer (Nielsen et al., 2001). *In vivo*, breast cancer cells can stimulate fibroblasts to secrete MMP-13 (Uria et al., 1997). MMP-13 acts on the proteins building the ECM and modulates signaling pathways from the ECM and changes the bioavailability of growth factors. Other metalloproteinases such as MMP-1 and MMP-3 participate directly in ECM degradation (Sato et al., 2004).

Unlike their normal counterparts, carcinoma cells express receptors for chemokines. Breast cancers tend to express the chemokine (C-X-C motif) receptor 4 (CXCR4) for chemokine (C-X-C motif) ligand 12 (CXCL12). CXCL12/SDF-1 and CXCR4, a G-protein-coupled receptor, have prominent roles in the crosstalk between tumor cells and stromal cells in the progression of breast cancer. Using a coimplantation tumor xenograft model, CAFs extracted from human breast carcinomas secrete stromal cell-derived factor 1 (SDF-1) and promote angiogenesis by recruiting endothelial progenitor cells (EPCs) into carcinomas, an effect mediated in part by SDF-1. CAF-secreted SDF-1 also stimulates tumor growth directly, acting through the cognate receptor, CXCR4, which is expressed by carcinoma cells (Orimo and Weinberg, 2006). CXCL12 has an impact on angiogenesis as it is involved in recruitment of endothelial cell progenitors to the growing tumor. Fibroblast secreted protein-1 (FSP1) is secreted by both fibroblasts and cancer cells making the environment more favorable for tumor progression by regulating angiogenesis and inflammation and promoting metastasis (Grum-Schwensen et al., 2005).

In breast carcinomas, mutations of PTEN and TP53 have been reported to occur in either carcinoma, or stromal cells in a mutually exclusive fashion. Such findings indicate establishment of vicious circle, in which mutations in the carcinoma drive alterations in the stroma that again promote the progression of carcinoma (Kurose et al., 2002; Schulz, 2005).

1.2.1.2. Endothelial cells in tumor progression. Tumor angiogenesis is an important factor in the proliferation, metastasis, and drug sensitivity of human neoplasms. Tumor neovasculature consists of endothelial cells and pericytes. Primary tumors without vasculature are small and dormant, while growth of the tumor mass creates hypoxic conditions in the center of tumor that induces expression of vascular endothelial growth factor-A and subsequently, tumor vascularization (Ferrara, 2005). Newly formed tumor vessels or capillaries have leaky and weak basement membranes; thus, tumor cells can penetrate through them more easily than through mature vessels (Fukumura and Jain, 2008). CAFs are suggested to be an important source for growth factors and cytokines recruiting endothelial cells. Tumor-endothelial cell interaction during hematogenous dissemination, and the following interaction with endothelium and subendothelial matrix constitute the most crucial factors in determining the organ preference of metastasis. Endothelial cells are involved in the establishment of the cancer stem cell niche and metastatic spread of tumor cells into distant organs (Li and Neaves, 2006). Angiogenesis in malignant tumors, as measured by microvessel density, has been reported to correlate with clinicopathological factors or survival in breast, ovarian, esophageal, gastric, colorectal, and prostate cancers, malignant melanoma, and non-small-cell lung carcinoma (Hansen et al., 1998; Schoell et al., 1997; Tanigawa et al., 1997; Takahashi et al., 1997). Tumor-endothelial cell interaction differ between breast cancer subtypes (Buess et al., 2009). Cell surface adhesion molecules (i.e., integrins, cadherins, immunoglobulins and selectins) and many other molecules, mediate these interactions (Honn and Tang, 1992). CD34, CD31, and factor-VIII-related antigen are commonly used as tumor endothelial cell markers, and microvessel density is determined based on staining of blood vessels with these markers. However, the markers identify not only newly formed small tumor blood vessels but also pre-existing large blood vessel (Ishiwata et al., 2011). Nestin has recently received attention as a marker of newly formed endothelial cells (Teranishi et al., 2007) and seems promising marker in evaluation of neoangiogenesis of different tumors. Targeting tumor angiogenesis seems to be an effective therapy. However, the benefits are

of short-term and are followed by a restoration of angiogenesis and more aggressive behavior. Taken together, tumor micro-environment and its complexity are responsible for this pathological angiogenic switch.

1.2.1.3. Other cell types in tumor progression. Besides endothelial cells and fibroblasts, the tumor microenvironment also harbors innate and adaptive immune cells. It is a complex and highly dynamic system that should concomitantly work to eradicate a tumor. However, once a system is deformed, immunity becomes a benefit for the tumor, provide very important cue to its development and progression (Chouaib et al., 2010).

Tumor-promoting effect of chronic inflammation has been reported many times (Castellsagué et al., 2002; Nelson et al., 2002). However, how tumors promote inflammation and engage inflammatory cells in this process, is still being intensively studied. Immune cells, particularly macrophages and neutrophils are sources of chemokines, growth factors and proteases. Chemokines are produced not only by activated macrophages, but stromal, and even cancer cells themselves, e.g., CXCL12 activates CXCR4 on the surface of immune cells, but also of hematopoetic and endothelial precursors. The receptor is also expressed on some cancer cells. So, CXCL12 has several consequences: i) Attraction of immune cells leads to tissue destruction favoring invasion and metastasis; ii) promotes growth and survival of cancer cells expressing the CXCR4 receptor; iii) promotes the recruitment of precursor cells for vasculogenesis; iv) activation of CXCR4 leads to strongly increased production of tumor necrosis factor alpha (TNFα) which itself exhibit other effects. Inflammatory cytokines, overexpressed by tumor cells recruit monocytes (macrophages), lymphocytes and neutrophils to tumor stroma, where they release VEGF, HGF, metalloproteinase 2 and interleukin 8 (IL-8) which affect endothelial cells and contribute to tumor progression (Zumsteg and Christofori, 2009). Recent studies demonstrate that colony stimulating factor 1 (CSF 1) which represents main growth and differentiation factor for mononuclear phagocytes (macrophages) is overexpressed in breast, ovarian and prostate cancers (Lin et al., 2010). Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) contribute to progression of various cancers through recruitment of monocytes, macrophages and neutrophils into the tumor vicinity. These factors also induce angiogenesis through the recruitment of endothelial progenitors to the tumor tissue (Reviewed in Mueller and Fusenig, 2004).

1.2.2. Physical and chemical characteristics of the tumor microenvironment

1.2.2.1. Matrix topology and stiffness. ECM topology can provide important regulation of cell motility through physical cues that geometrically constrain adhesion sites to guide directional migration (Petrie et al., 2009). Directional migration process can now be studied in native *in vivo* experiments using imaging techniques. For example, analysis of breast cancer metastasis reveals metastatic tumor cells and macrophages that migrated rapidly along collagen fibers (Sidani et al., 2006). Highly metastatic tumor cells migrated preferentially along fibers. Reticular orientation of the collagen matrix surrounding mammary glands may anchor and/or restrain cells (reviewed in Petrie et al., 2009). Dense fibrous collagen that is characteristic of breast cancer stroma forms radial patterns extending away from tumors. Thus, non-linear matrix reduces invasion, while linear structure promote. Tumor cells remodel the matrix into these parallel fibers in order to migrate.

ECM topology can influence intracellular signaling to promote directional cell migration. Integrin receptors and the physical arrangement of adhesions could trigger orientation of the cytoskeleton to favour directional cell migration. Matrix orientation can also stabilize leading edge protrusions to promote directionally persistent migration in which specific signaling pathways are involved (Petrie et al., 2001). In case of single cell migration, subcellular formation of leading edge protrusions (functional zones) occurs and in addition, the engagement with the substrate and rear-end retraction leads to translocation of the cell body (Khalil and Friedl, 2010). In collective migration, these functions are coordinated within individual and between neighboring cells via their cell-cell junctions (Friedl and Wolf, 2010). The most important intracellular regulatory elements of this system are proteins of the family of small GTPases (Rac, Rho, Cdc42) (Hall, 1998; Nobes and Hall, 1999; Etienne-Manneville and Hall, 2002). Rac is responsible for actin polymerization at the cell edge, regulating formation of lamellopodia (thin wide pseudopodia) and nascent focal complexes. Rho is responsible for formation actomyosin bundles (stress fibers), their tension, and maturation of focal contacts. Cdc42 is a master regulator of cell polarization responsible for generation of filopodia (narrow processes) (Rottner et al., 1999; Ridley, 2001). On the other hand, matrix concentration and post-translational modifications such as cross-linking and glycosylation affect its mechanical properties, including visco-elasticity or stiffness (Reviewed in Erler and Weaver, 2009). Tumors are stiffer than their normal adjacent tissue. Increased matrix stiffness is observed in fibrotic lungs, in scar tissue, irradiated and aged

tissue. Mammary epithelial growth and morphogenesis is regulated by matrix stiffness. Of two different mesenchymal tissues that are involved in mammary gland development, fibroblastic mesenchyme induces embryonic or adult mammary epithelial cells and form atypical ductal branching with hyperplasic ducts, while the fat pad induces epithelial cell elongation and branching (Sakakura et al., 1982). The latter is induced by laminin and protoheparan sulphate. Epithelial cell modifications are modulated through transmembrane receptors such as integrins (collagen, laminin, and fibronectin receptors), dystroglycan (laminin-1 receptor), discoidin domain receptor 1 tyrosine kinase (collagen receptor), and syndecans (co-receptors for heparan sulfate proteoglycans and fibronectin, laminin, collagen and growth factors) (Fata et al., 2004). Increased collagen deposition can also alter the biophysical properties of the ECM augmenting extracellular tension. This elevation in tension has been shown to perturb mammary epithelial cell differentiation (Kass et al., 2007). Biophysical properties of the ECM can regulate cell shape and basement membrane-dependent mammary epithelial cell (MEC) morphogenesis (acini formation). For example, Paszek et al. (2005) used two- and three-dimensional natural and synthetic laminin-rich matrices and observed that substrate compliance regulated cell shape (rounding), mammary tissue morphogenesis, and endogenous basement membrane assembly (Paszek et al., 2005).

1.2.2.2. Interstitial fluid pressure (IFP). Another feature of the pathophysiology of tumor microenvironment is elevated interstitial fluid pressure ranging from 10 to 100 mmHg (Nathanson and Nelson, 1994; Milosevic et al., 2001) while IFP of normal tissue is around zero (Fukumura and Jain, 2007). It is thought that the tumor vasculature is the driving force in increasing tumor IFP (Lunt et al., 2008; Jain et al., 2007). In contrast to normal vasculature which is characterized by dichotomous branching, tumor vasculature is unorganized and has trifurcations and branches with uneven diameters. Large inter-endothelial junctions, increased numbers of fenestrations, vesicles and vesico-vacuolar channels, and a lack of normal basement membrane are often found in tumor vessels (Winkler et al., 2004). Due to ultrastructural alterations in the tumor vessel wall, vascular permeability in solid tumors is generally higher than that in various normal tissues (Fukumura and Jain, 2007), tumors either lack in lymphatic vessels, or the intra-tumoral vessels are non-functional (Padera et al., 2002; Leu et al., 2000) and as a result, excess fluid accumulates in the interstitium, extending the elastic ECM and elevating IFP.

Using the model of IFP regulation Heldin et al. (2004) showed that fibroblasts actively regulate the tension applied to the ECM through integrins which enable them to exert or modify tension on the collagen fibre network, thereby modulating the elasticity of the ECM in response to hyaluronan and proteoglycan expansion (Heldin et al., 2004).

Interstitial fluid pressure may have important clinical implications with regard to cancer therapy. Roh et al. (1991) reported an inverse relationship between tumor IFP and tissue oxygenation and hypothesized that IFP may aid in predicting the efficacy of radiation therapy. Elevated tumor IFP can also act as a barrier to delivery of therapeutic agents, thereby reducing their efficacy. Multiple studies have demonstrated improved uptake of chemotherapeutic drugs following a reduction in tumor IFP (Pietras et al., 2002; Vlahovic et al., 2007).

1.2.2.3. Hypoxia and pH. Hypoxia is a next characteristic of abnormal tumor microenvironment that is intrinsically linked to the formation of neovasculature (please see the chapter ***1.2.1.2.*** on neoangiogenesis) and is clinically associated with metastasis and poor patient outcome (Lunt et al., 2009; Hockel and Vaupel, 2001). Diffusion-limited hypoxia is a consequence of tumor cells that are distant from the vascular supply. Such cells are exposed to prolonged or chronic hypoxia and tumor cells are viable in such environment for hours or few days (Franko and Sutherland, 1978, Durand and Raleigh, 1998). Acute hypoxia is due to fluctuations in blood flow and might also play an important role in solid tumors (Brown, 1979, Sutherland and Franko, 1980).
Hypoxia induces oncogene expression, enhances DNA mutation rate, and selects for cells with increased apoptotic thresholds (Lunt et al., 2009; Erler et al., 2004). Hypoxia drives tumor progression through increased matrix deposition, cross-linking and remodeling, enhances collagen turnover and its fibril deposition (Horino et al., 2002). Secreted protein lysyl oxidase (LOX) is overexpressed by hypoxic human tumor cells (Denko et al., 2003). The well described inducers of EMT, Snail, Slug and Twist are themselves induced by hypoxia. Hypoxia may also affect stem cells (Keith and Simon, 2007), and studies focusing on this particular subpopulation of cells in tumors would be relevant to the metastatic process.

Low extracellular pH is another consequence of the abnormal metabolism in the tumor and supportive factor for its progression. Products of anaerobic glycolysis - lactic acid and carbonic acid (a product of CO2 and H2O by carbonic anhydrase), are the known sources of H+ ions in tumors (Helmlinger et al., 2002; Pouyssegur et al., 2006). The imbalance between

increased production of H+ ions and their reduced removal lower extracellular pH in tumors. The mean pH profiles also decrease in tumors with increasing distance from nearest blood vessels. Low extracellular pH causes stress-induced alteration of gene expression, including the upregulation of VEGF and IL-8 in tumor cells *in vitro* (Xu et al., 2002). Coordinated study of pH, pO2, and VEGF expression *in vivo* (Fukumura et al., 2001) indicated that in low pH or oxygenated regions, tissue pH, but not pO2, regulates VEGF promoter activity. Conversely, in hypoxic or neutral pH regions, tissue pO2 and not pH regulates VEGF expression (Fukumura et al., 2001). Tissue pO2 and pH appeared to regulate VEGF transcription in tumors independently. These data suggest that these key microenvironmental parameters in solid tumors regulate angiogenic factors in a complementary manner.

1.3. Signaling pathways affecting normal development, tissue homeostasis and tumor microenvironment

1.3.1. TGF-β signaling pathway

Transforming growth factor (TGF-β) superfamily of growth factors consists of more than 35 structurally related secreted polypeptides, including TGF-βs, activins and BMPs (reviewed in Leivonen and Kahari, 2007) with diverse roles in regulation of cell proliferation, differentiation, migration and extracellular matrix deposition (Massague et al., 2000). Mammalian organisms contain three distinct isoforms termed TGF-β1, TGF-β2 and TGF-β3, which function similarly *in vitro,* but give rise to more than 30 distinct phenotypes upon their genetic deletion in mice (Chang et al., 2002).

TGF-β signaling begins with ligand binding to its receptor (Figure 3). The human genome encodes seven type I receptors [i.e. ALK (activin receptor-like kinase 1–7)] and five type°II receptors [i.e. TβRII (TGF-β type II receptor), ActR-II (activin type°II receptor), ActR-IIB, BMPR-II (BMP type II receptor) and AMHR-II (MIS type II receptor)]. Type III receptor is an accessory receptor required for TGF- β1 assistance. Type I and II receptors contain an N-terminal extracellular ligand-binding domain, transmembrane and cytoplasmic serine/threonine kinase domains. Upon ligand binding, the type II receptor kinase activates the type I receptor kinase through phosphorylation in the glycine-serine motif of the type I receptor. This results in activation of type I receptor kinase domain and propagation of downstream signaling through phosphorylation of R-Smads (receptor-activated Smads) in the canonical Smad pathway

or signaling via mitogen-activated protein kinase (MAPK) pathway. Activated TGF-β – receptor complex phosphorylates receptor-activated Smads (R-Smads) in their C-terminus. Activated R-Smads associate with a common-mediator Smad (Co-Smad), and formed complex enters the nucleus, where Smads bind to DNA or associate with transcriptional coactivators or corepressors. Inhibitory Smads (I-Smads) whose expression is induced by TGF-β, can inhibit the phosphorylation of R-Smads. I-Smads also recruit Smurf E3-ubiquitin ligases to the receptor complex, thus directing TGF-β receptors to degradation (Taylor et al., 2010; Leivonen and Kahari, 2007).

Besides its ability to activate canonical Smad2/3-dependent pathways, TGF-β also regulates numerous ,,non-canonical" effector systems, including i) small GTP-binding proteins (Ras, Rho, Rac1); ii) phosphoinositide-3-kinase (PI3K), AKT, and mTOR; iii) MAP kinases and iv) NF-κB and Cox-2 (Zavadil et al., 2001; Neil et al., 2008).

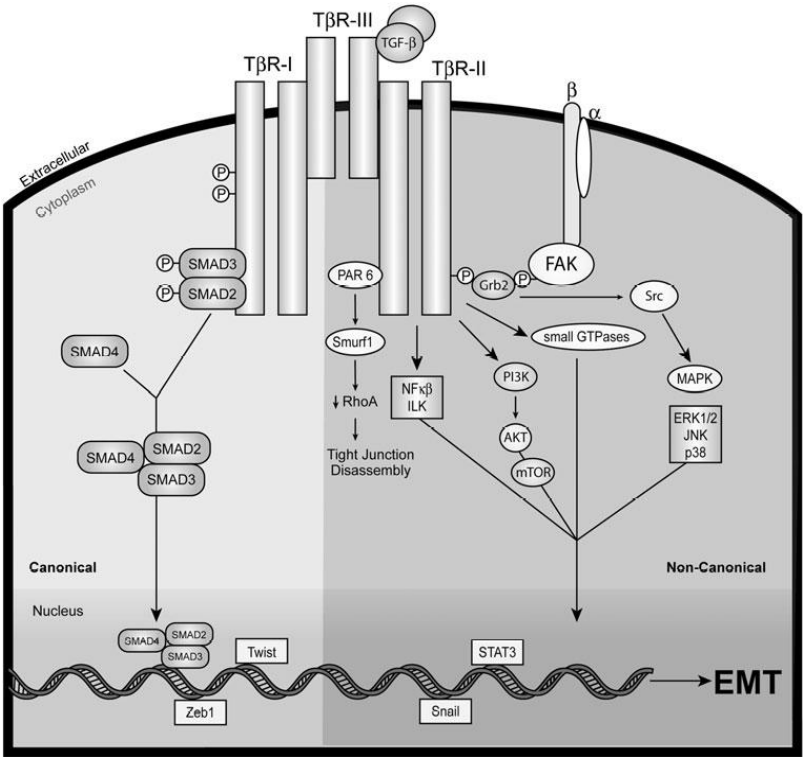


Figure 3. Schematic depicting the canonical and noncanonical TGF-β signaling systems coupled to EMT in mammary epithelial cells. (Source: Taylor et al., 2010).

TGF-β-activated MAPK pathways include the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), the c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2), and the four p38 isoforms (α, β, γ and δ) (Leivonen et al., 2006).

TGF-βs are known to be tumor suppressive (Markowitz and Roberts, 1996), but paradoxically, increasing evidence shows that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. In normal epithelial cells and in early tumor stages, TGFβ functions as an antiproliferative factor inhibiting cell growth by induction of apoptosis and cell cycle arrest. Loss of the antiproliferative responsiveness to TGF-β is often considered as a leading step in cancer progression (Hanahan and Weinberg, 2000). Study of a mouse skin model of chemical carcinogenesis showed that targeted expression of TGF-β1 in suprabasal keratinocytes appears to have dual effects. It suppresses the formation of benign skin tumors, but once tumors develop, it enhances their progression to a highly invasive spindle cell phenotype (Dumont and Arteaga, 2000). TGF-β upregulation in many types of tumors correlates with disease progression (Cohen, 2003). Tumor-derived TGF-β affects different cells in tumors and remodels its microenvironment to support tumor growth, invasion and metastasis (Dumont and Arteaga, 2000).

TGF-βs can promote tumorigenesis by modulating EMT as they are involved in the critical processes of downregulation of cellular adhesion molecules, elevated expression of metalloproteases, increased motility and angiogenesis. TGFβ stimulates disassembly of tight junctions. Par6, a key component of epithelial polarity complexes, regulate tight junction assembly. TGFβ -ligand binding enables type II TGFβ receptor kinase, which is associated with occludins at tight junctions, to phosphorylate Par6. Phosphorylated Par6 allows it to recruit Smurf1 which in turn leads to ubiquitination and degradation of RhoA, a small GTPase family member responsible for stress fiber formation and for the maintenance of apico-basal polarity and junctional stability (Reviewed in Zavadil and Bottinger, 2005).

Although mutations in various components of the TGF-β signaling pathway have been observed in some carcinomas, complete abrogation of TGF-β signaling is not a general event in malignancies as some tumors can exhibit increased invasiveness in response to exogenous TGF-β (Pardali and Moustakas, 2007). In a recent study, loss or low levels of the type II TGF-β receptor correlated with high tumor grade, but 60% of *in situ* and invasive breast carcinomas retained robust levels of TβRII expression by immunohistochemistry (Gobbi et al., 2000). As well,

although Smad4 is frequently inactivated in pancreatic cancers, the Smad genes, which encode proteins that transduce TGF-β signals, are rarely mutated in most human carcinomas. This suggests that after cells lose their sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling promote tumor progression.

TGF-β is also one of breast cancer bone metastasis-relevant factor. The bone stroma produces TGF-β that promotes tumor growth in bone (Onishi et al., 2010). TGF-β-relevance in this process has been documented earlier in mouse models where TGF-β promote bone metastasis mediated by secreted factors such as parathyroid hormone-related peptide, interleukin-11 and connective tissue growth factor (CTGF) (Yin et al., 1999; Kang et al., 2003). TGF-β upregulates expression of Hedgehog signaling molecule Gli2, which in turn increases Parathyroid hormone-related protein (PTHrP) secretion. When Gli signaling in MDA-MB-231metastatic breast cancer cells was blocked with a Gli2-repressor gene (Gli2-rep), endogenous and TGF-β-stimulated PTHrP mRNA expression was reduced. Mice inoculated with Gli2-Rep-expressing cells exhibited a decrease in osteolysis, suggesting that Gli2 inhibition blocked TGF-β propagation of a vicious osteolytic cycle in MDA-MB-231 model of bone metastasis. Gli2 was required for TGF-β to stimulate PTHrP expression and blocking Hh-independent Gli2 activity inhibited tumor-induced bone destruction (Johnson et al., 2011).

1.3.1.1. Cripto-1 signaling. Cripto-1 is a signaling protein member of the epidermal growth factor (EGF)-CFC protein family that plays an important role during early embryonic development (Bianco et al., 2004). Cripto-1 is overexpressed in a variety of human carcinomas and has a potential role in EMT (Strizzi et al., 2004). *In vitro* and *in vivo* studies have demonstrated that Cripto-1 overexpression in mouse mammary epithelial cells can enhance migration, branching morphogenesis and development of mammary hyperplasias and adenocarcinomas in mice (Wechselberger et al., 2001). Cripto-1 functions as a co-receptor for the TGF-β–related protein Nodal, activating the Alk4-Smad dependent pathway (Bianco et al., 2002) but can also bind to glypican-1 and activate the tyrosine kinase c-Src pathway in a Nodal-independent manner. The activation of the tyrosine kinase c-Src is required for cell proliferation and migration of mammary epithelial cells (Bianco et al., 2003).

1.3.2. Wnt signaling pathway

The Wnt (wingless type) signaling pathway is crucial for cell fate decisions, stem cell renewal, regulation of cell proliferation and differentiation. Deregulated Wnt signaling is also implicated in a number of hereditary and degenerative diseases and cancer.

Wnt signaling is currently known to include two major pathways: 1) the canonical or Wnt/β-catenin pathway (Figure 4), and 2) the non-canonical pathways which do not involve β-catenin stabilization. There is also a pathway which controls the orientation of mitotic spindles in *Drosophila* and *Caenorhabditis elegans* but this has not yet been found in vertebrates (Jones and Jomary, 2002).

1.3.2.1. Canonical Wnt signaling pathway. The canonical Wnt pathway regulates cellular responses through β-catenin. In the absence of Wnt ligand binding (Wnt signal), β-catenin is phosphorylated by casein kinase 1γ (CK1γ) and a multicomponent destruction complex [containing scaffolding proteins GSK3β (glycogen synthase kinase 3 beta), AXIN and adenomatosis polyposis coli (APC) protein]. Phosphorylated β-catenin is recognized by the E3 ubiquitin ligase β-TRCP (β-transducin repeat-containing protein) and targeted to rapid degradation in the cytoplasm through the ubiquitin proteasome pathway. Low nuclear levels of β-catenin are maintained by nuclear exporters APC and AXIN which shuttle β-catenin from nucleus back to the cytoplasm (Moon and Miller, 1997). Without the AXIN-based scaffold, β-catenin escapes capture, phosphorylation and ubiquitination (Yardy and Brewster, 2005; Tanner et al., 2009).

Wnt signaling is initiated at the plasma membrane where Wnt ligands form ternary complexes with their respective frizzled receptors and transmembrane coreceptors LRP5 and LRP6. Co-factors such as R-spondin and Wise also take part in Wnt-receptor complex activity. Signaling proceeds through the protein Dishevelled that is recruited to the plasma membrane, interacts with frizzled receptors and polymerizes with other Dishevelled molecules (Bilic et al., 2007; Schwarz-Romond et al., 2007). Phosphorylation of the cytoplasmic tail of LRP5 or LRP6 and the formation of the Dishevelled polymer serve as mediators for the translocation of AXIN to the plasma membrane and inactivation of the destruction complex. Thus β-catenin gradually accumulates in cytoplasm and enters the nucleus where it forms a complex with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. Binding of β-catenin to transcription factors transactivates downstream target genes, such as c-myc, cyclin D1,

urokinase-type plasminogen activator, MMP-7, CD44, survivin, endothelin-1, Cox-2 and -9, versican, periostin, fibronectin, the androgen receptor gene and others. These influence cell cycle regulation, invasion and metastasis (He et al., 1998; Shtutman et al., 1999; Carlson et al., 2003; Rahmani et al., 2005; Haertel-Wiesmann et al., 2000; ten Berge et al., 2008). Aberrant β-catenin signaling is thought to be a very early step in colon cancer development but its nuclear accumulation is only observed in colon cancer cells that already invaded into the stroma, e.g. at the more advanced stage of cancer progression (Gavert and Ben-Ze'ev, 2007). Similarly, β-catenin delocalization from the membrane to the cytoplasm is frequently observed at early stages of breast carcinomas, but its nuclear localization is associated with invasiveness (Uchino et al., 2010).

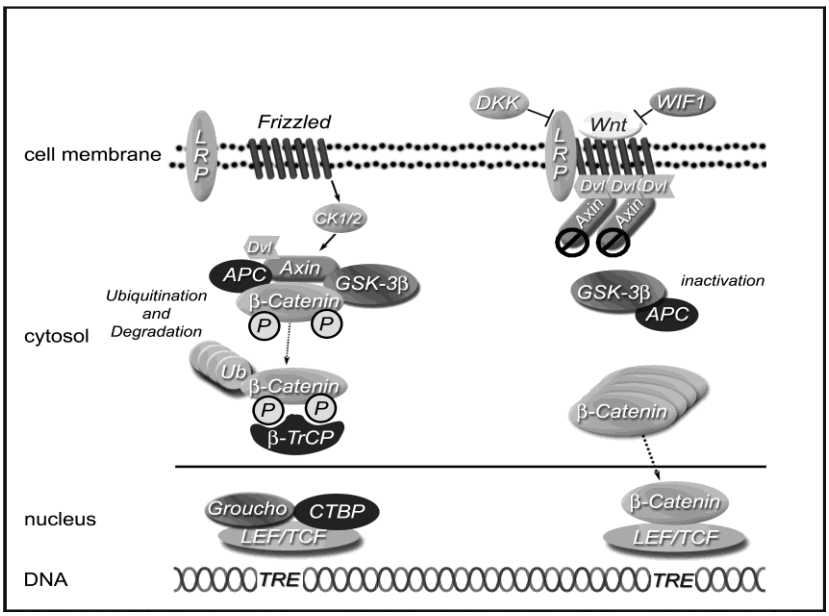


Figure 4. Canonical Wnt signaling pathway. See text for explanation. **(Source: Kharaishvili et al., 2011).**

β-catenin-TCF activity can be modified by mammalian mitogen activated protein (MAP) kinase pathway components: 1) transforming growth factor β-activated kinase (TAK1) and 2) NEMO-like kinase (NLK) (Meneghini et ak., 2008). TAK1 activates NLK and the latter phosphorylates members of the TCF family. Phosphorylation alters the DNA-binding properties of the β-catenin–TCF complex and in this way blocks Wnt target gene activation. Input from the MAP kinase pathway can thus negatively regulate the Wnt pathway in mammalian cells. Besides

TCF/LEF, Wnt signaling can modify other transcription factors, e. g. β-catenin acts as a binary switch to simultaneously activate expression of nuclear factor kappa B (NFκB).

1.3.2.2. Non-canonical (β-catenin-independent) Wnt signaling pathway. Wnts such as Wnt4, Wnt5a, and Wnt11 do not liberate β-catenin but signal noncanonically. The best characterized of these ,,noncanonical'' pathways are the Wnt/Ca2+ pathway (Kuhl et al., 2001), and the planar polarity pathway *(*McEwen and Peifer, 2000). Other noncanonical pathways include Wnt/Jnk and Wnt/ Rho signaling (Veeman et al., 2003).

Vertebrate noncanonical Wnt signaling requires frizzled receptors. Wnt ligand binding to frizzled can increase levels of intracellular calcium and activate two Ca2+-sensitive kinases: 1) calcium/calmodulin-dependent protein kinase II (CAMK2) and 2) protein kinase C (PKC). G-proteins and *Drosophila* dishevelled (dsh) are also involved in signal transduction by Wnt/Ca2+ pathway and signaling specificity may be achieved via co-receptors, such as Knypek and Ror2 (Turashvili et al., 2006). This pathway has been implicated in cell movement processes required for embryonic patterning (Slusarski et al., 1997; Sledahl et al., 1999; Ahumada et al., 2002). It also functions in promoting ventral cell fate and antagonizing dorsal cell fate during early *Xenopus* development, in regulating gastrulation movements or heart and muscle development (Pandur et al., 2002).

Noncanonical Wnt signaling pathways can overlap with each other. Wnt5a and Wnt11 that are involved in PCP signaling can also activate calcium signaling (Heisenberg et al., 2000). Some PCP proteins, including *flamingo* (CELSR2), become localized to both the proximal and distal sides of the cell. Others, however, including *frizzled*, *dishevelled* and Rho, become localized specifically to the distal side, whereas *prickle homolog 1* (PRICKLE1) and *strabismus* (STBMS1) become localized to the proximal side. The function of all of these proteins is required to ensure both correct segregation into proximal and distal domains and the subsequent development of correct planar polarity (Veeman et al., 2003). In vertebrates, this pathway requires Wnt ligands, such as *silberblick* (Wnt11 precursor) and *pipe tail* (Wnt5b), whereas no Wnt ligand is known to be involved in *Drosophila* PCP signaling. Sometimes Wnt-receptor interaction requires recruitment of additional co-factors. For example, secreted collagen glycoprotein, CTHRC1 can promote the formation of a Wnt-frizzled-Ror2 complex, leading to activation of the PCP pathway (van Amerogen and Nusse, 2009; Yamamoto et al., 2008).

The Wnt-frizzled interaction may also be enhanced by proteoglycans, such as protein Dally in *Drosophila*, or inhibited by secreted proteins including *dickkopf 1* (DKK1), *cerberus* (CER1) and SFRPs (secreted frizzled-related proteins). In some instances, noncanonical Wnt pathway can inhibit canonical Wnt signaling. One example is competition for Dishevelled molecules, that are shared between the two pathways (Veeman et al., 2003). Another example involves the Wnt5a-induced transcriptional upregulation of Siah2 which can stimulate β-catenin degradation (Topol et al., 2003).

Similar to TGF-β, Wnt signaling pathway is also implicated in EMT and epithelial plasticity during development and cancer. Wnt/β-catenin signaling pathway plays a pivotal role either in gastric cancer formation or in tumor invasion and dissemination (Cheng et al., 2004). Adenomatous polyposis, a human colon cancer is associated with truncating mutations in APC gene (Groden et al., 1991). Axin1 and Axin2 loss-of –function mutations have also been found in rare cases of colorectal cancer (Liu et al., 2000; Sato et al., 2000).

Cells with β-catenin activation lose their polarity and disrupt cell-cell contacts and EMT morphologically (Mariadason et al., 2001; Naishiro et al., 2001). Immunohistochemical studies demonstrate alternations of the actin cytoskeleton in these cells, indicating that nuclear β-catenin accumulation is functionally related to EMT in budding tumor cells at the tumor-host interface. Li and Zhou (2011) showed that β-catenin and Akt pathways were activated in Twist-overexpressing cells and activation of β-catenin correlated with the expression of stem cells marker CD44. Numerous mammary carcinoma models show that the Wnt/β-catenin pathway is capable of inducing epithelial plasticity (Kim et al., 2002; Yook et al., 2006). Nuclear β-catenin correlates clinically with poor prognosis in breast cancer patients (Prasad et al., 2009; Logullo et al., 2010).

Canonical and noncanonical Wnt signaling pathways regulate the expression of structural proteins and MMPs (Schambony et al., 2004) to modify the surrounding matrix. Targets of canonical Wnt among others are periostin, versican and fibronectin, a mesenchyme-specific genes that are upregulated in beast and other cancers. Extracellular matrix, rich in those proteins possesses anti-adhesive properties and has the ability to modulate proliferation and the migration of different cell types (Sasaki et al., 2003; Zheng et al., 2004; Nikitovic et al., 2006; Wight, 2008). PCP pathway is involved in cell motility. CTHRC1 acts as a co-factor of PCP pathway

and may thus enhance process of cell motility (van Amerogen and Nusse, 2009; Yamamoto et al., 2008).

One of the prominent regulatory proteins which mediate cross-talk between integrin-based cell-matrix adhesion and Wnt signaling, is the integrin-linked kinase (ILK) that regulates the translocation of β-catenin to the nucleus independent of altering the expression level; It up-regulates Lef-1 expression and down-regulates GSK-3β activity, resulting in stabilized cytoplasmic/nuclear β-catenin. GSK3β, along with E-cadherin and Slug play important role in EMT transition via Wnt/β-catenin signaling (Prasad et al., 2009). GSK3β resides at the junction of PI3K/AKT and Wnt/β-catenin/TCF survival pathways, thereby serving critical roles in cellular metabolism, growth and proliferation (Kim and Kimmel, 2000; Katoh, 2007).

1.3.3. Hedgehog signaling pathway

Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH) represent mammalian Hedgehog family ligands, consisting of N-terminal signal peptide, Hedgehog core domain, and C-terminal processing domain. Patched family members (PTCH) are Hedgehog receptors, while Cdon homolog (CDON), Boc homolog (BOC) and growth arrest-specific 1 (GAS1) are Hedgehog coreceptors. Receptors are distantly related to Dispatch family members. Patched 1 and 2 (PTCH1 and PTCH2) do not directly transduce Hedgehog signals to the intracellular signaling cascade, but indirectly through Smoothened seven-transmembrane-type receptor. In the absence of Hedgehog signaling, GLI family zinc finger 1 (GLI1) is transcriptionally repressed, GLI2 is phosphorylated by GSK3 and CK1 for the FBXW11-mediated degradation, and GLI3 is processed to a cleaved repressor. In the presence of Hedgehog signaling, Smoothened is relieved from Patched-mediated suppression to induce MAP3K10 activation and suppressor of fused homolog (SUFU) inactivation. GLI activators then bind to the GACCACCCA motif for the transcriptional upregulation of target genes (Figure 5). Hedgehog target genes are involved in HH signaling cascade itself, cell cycle regulation, cell fate determination and stem cell signaling. Representative targets are: GLI1, PTCH1, CCND1 and 2, SFRP1, runt-related transcription factor (RUNX2), etc. (Katoh and Katoh, 2008).

Hedgehog signaling regulate embryogenesis, adult tissue homeostasis, and carcinogenesis. Constitutive overexpression of the HH pathway is observed in a number of cancers. For example, gain-of-function mutations underlie the nevoid basal cell carcinoma

syndrome in which affected individuals have an increased risc of developing medulloblastomas and basal cell carcinomas of the skin. PTCH mutations are found in sporadic medulloblastomas, while GLI1 overexpression is noted in various central nervous system tumors. Enhanced HH signalind is described in small cell lung cancer and esophageal, gastric and pancreatic cancers (Miller et al., 2005).

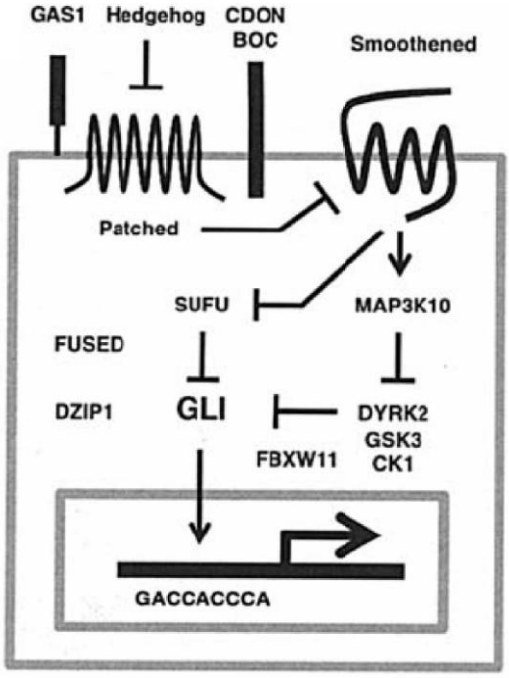


Figure 5. Schematic representation of Hedgehog signaling cascade. See text for explanation. (Source Katoh and Katoh, 2008).

1.3.4. Notch signaling pathway

Notch signaling is one of key pathways implicated in self-renewal of stem cells, cell-fate determination of progenitor cells and terminal differentiation of proliferating cells. Notch-ligand binding induces the cleavage of Notch receptor (1-4) by metalloproteinase and γ-secretase to release Notch intracellular domain (NICD). Canonical Notch signaling to CSL-NICD-Mastermind complex inhibits the differentiation of stem cells or progenitor (transit-ampifying) cells, while non-canonical Notch signaling to CSL-NICD-Deltex complex promotes the differentiation of progenitor cells (Katoh and Katoh, 2007). Notch signaling transcriptionally activate HES1-7, HEY1 and HEY2, and HEYL genes, and also to the NF-κB-NICD complex for the augmentation of NF-κB signaling. Notch signaling is aberrantly activated due to chromosomal translocation of NOTCH1 in acute lymphoblastic leukemia (Ellisen et al., 1991),

amplification and overexpression of NOTCH2 in medulloblastoma (Fan et al., 2004), chromosomal translocation of NOTCH3 in lung cancer (Dang et al., 2000), amplification and overexpression of NOTCH3 in ovarian cancer (Park et al., 2006), and upregulation of JAG1/NOTCH1 or down-regulation of NUMB in breast cancer (Reedijk et al., 2005; Pece et al., 2004). These facts indicate that Notch signaling is oncogenic in a variety of human tumors. On the other hand, Notch signaling has anti-oncogenic role in squamous cell carcinoma of skin and cervical uterus and in basal cell carcinoma of skin (Radtke and Raj, 2003; Proweller et al., 2006), partially due to the interference with canonical Wnt signaling. Notch signaling activation also leads to transcriptional activation of NF-κB target genes, such as IFN-γ through direct association between NICD and NF-κB.

1.3.5. NF-κB signaling pathway

The transcription factor NF-κB which was first demonstrated as a key player in controlling both innate and adaptive immunity, is presently considered as a key molecule controlling the apoptosis acting mainly to prevent cell death. The ability of NF-κB to suppress apoptosis is one of the main NF-κB functions determining its proinflammatory activity and implicating this transcription factor in the pathogenesis of inflammatory diseases, oncogenesis and cancer therapy resistance (Torchinsky and Toder, 2004).

The inhibitor of κB (IκB) kinase (IKK) complex is composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ. In response to stimuli such as tumour-necrosis factor-α (TNF-α), CD40 ligand (CD40L), interleukin-1 (IL-1) or lipopolysaccharide (LPS), the IKKβ subunit is activated, and phosphorylates the IκB proteins (bound to the NF-κB heterodimers) at two conserved serines. This phosphorylation event triggers the ubiquitin-dependent degradation of IκB by the 26S proteasome, resulting in the nuclear translocation of RELA–p50 (or c-REL–p50) heterodimers and transcriptional activation of target genes. In response to other stimuli, such as the TNF family members lymphotoxin β (LTβ) and BAFF, IKKα is activated to induce the phosphorylation of p100 (bound to RELB) at two serine residues at its carboxyl terminus. This phosphorylation event triggers the ubiquitin-dependent degradation of the carboxy-terminal half of p100, releasing its amino-terminal half, the p52 polypeptide, which together with its heterodimer partner, RELB, translocates to the nucleus to activate transcription. The genes that are induced in response to NF-κB activation can be divided into four

functional classes: genes which products are involved in negative-feedback control of NF-κB activity; genes which products serve various immunoregulatory functions; genes which products inhibit caspase activation and apoptosis; and genes that promote cell proliferation (Karin et al., 2002). NF-κB suppress the expression of epithelial specific genes E-cadherin and desmoplakin and induces mesenchymal vimentin in breast cancer cells (Chua et al., 2007).

1.4. Epithelial-mesenchymal transition

1.4.1. Description and types of EMT

Epithelial mesenchymal transition (EMT) is a complex manifestation of epithelial plasiticity (Thiery, 2002). EMT is a basic biologic process that allows a polarized epithelial cells, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes changes in cytoskeleton and cell shape, enhanced migratory potential, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components (Kalluri 2003). EMT was first recognized as distinct cell differentiation process in the late 70's, and has received increasing attention, as it not only occurs in normal development but is also an integral component of various pathological conditions. An increasing understanding of the signaling and transcription processes that mediate EMT is starting to provide a framework of the underlying molecular mechanisms. Conversely, mesenchymal to epithelial transition (MET) also occurs and may account for the reversal of cells that have undergone EMT back to the epithelial phenotype, illustrating the potential of EMT to be a transient and reversible process. Finally, it has become increasingly apparent that endothelial cells, similarly to epithelial cells, can lose endothelial characteristics and acquire mesenchymal properties. EMTs are encountered in three distinct settings with different functional consequences.

1.4.1.1. Type 1 EMT. This type of EMT occurs as early as gastrulation, when ectodermal cells give rise to mesoderm. Type 1 EMT is associated with implantation, embryo formation, and occurs at distinct sites and stages in organogenesis and as a result diverse cell types are generated that share mesenchymal phenotypes and biomarkers. Developmental EMT typically proceeds stepwise under the tight control of morphogenic signals and generally correlates with a cell fate change (Reviewed in Micalizzi et al., 2010). Type 1 EMT does not cause fibrosis and

uncontrolled systemic invasion by high-grade epithelial cancer cells; rather, it generates cells with a mesenchymal phenotype to create new tissues (Kalluri, 2009).

1.4.1.2. Type 2 EMT. Second type EMT is associated with wound healing, tissue regeneration and organ fibrosis. The program begins as part of a repair-associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury (Kalluri, 2009). In contrast to type 1 EMT, these types are associated with inflammation and cease when it is attenuated.

1.4.1.3. Type 3 EMT. Type 3 EMT occurs in neoplastic cells that have previously undergone genetic and epigenetic changes affecting oncogenes and tumor suppressor genes. Carcinoma cells undergoing this type of EMT may invade and metastasize and thus favour cancer progression. In cancer, features of EMT have been observed for example in breast (Micalizzi et al., 2010), prostate (Sethi et al., 2010), ovarian (Vergara et al., 2010) or colon (Bates et al., 2007) cancers. According to hypothesis that developmental programs are reactivated during tumorigenesis and contribute to tumor progression, numerous EMT regulators in development are also inappropriately expressed in human cancer and associated with features of EMT, although with less order and coordination than observed in developmental EMT (Gavert and Ben-zeev, 2008).

1.4.2. Cellular and tissue morphology characterizing EMT

Polarized epithelial cells embedded in organized stratified or single cell layers convert into single fibroblastoid cells capable of locomotion (Zavadil and Bottinger, 2005) (Figure 6). Core events of EMT include reduction of cell–cell adherence via the transcriptional repression and delocalization of cadherins (adherens junctions), occludin and claudin (tight junctions), and desmoplakin (desmosomes). The E-cadherin supporting molecule β-catenin is frequently lost from the cell membrane and translocates to the nucleus to participate in further EMT events (Klymkowsky, 2005; Stemmer et al. 2008). Circumferential F-actin fibres of the cytoskeleton are replaced by a network of stress fibers, at the tips of which ECM adhesion molecules (including integrins, paxillin, focal adhesion kinase) localize (Vignjevic et al. 2007). These changes potentially allow cells to separate, lose the apico-basal polarity typical of epithelial cells, acquire front-back polarity (Hay, 1995) and gain a more variable, elongated, fibroblast-like cell shape

(develop filopodia and lamellopodia) which facilitate cell movement. Motile fibroblastoid cells activate molecular programs capable of simultaneous degradation and *de novo* synthesis of ECM (Zavadil and Bottinger, 2005).

Tumor cells having acquired mesenchymal-like phenotype have shown increased invasive and metastatic ability, resistance to chemo- and endocrine therapy, resistance to radiation-induced DNA damage, increased interaction with stromal inflammatory mechanisms, and increased cell survival (Buck et al., 2007; Kurrey et al., 2009).

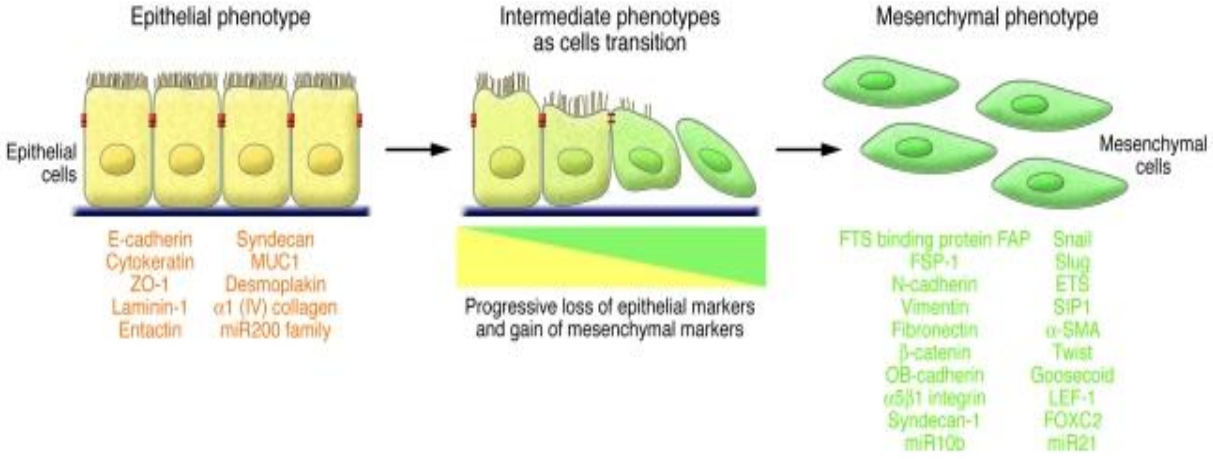


Figure 6. **Epithelial mesenchymal transition**. An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component–secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Detection of cells expressing both sets of markers makes it impossible to identify all mesenchymal cells that originate from the epithelia via EMT, as many mesenchymal cells likely shed all epithelial markers once a transition is completed. ZO-1, zona occludens 1; MUC1, mucin 1, cell surface associated; miR200, microRNA 200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C2. (Source: Kalluri and Weinberg, 2009).

Expression of epithelial intermediate filaments containing cytokeratins and other epithelial genes (β4 integrin, and ZO-1) is typically reduced and the equivalent mesenchymal filament protein vimentin together with N-cadherin and α-SMA, increased. Matrix

metalloproteases such as MMP-1, -2, -3, -7, and -14 are frequently upregulated, potentially enabling cells to detach from each other (via E-cadherin cleavage) and to penetrate the basement membrane. This phenotypic conversion requires the molecular reprogramming of epithelium with new biochemical instructions.

1.4.3. EMT induction and mechanism

EMT is induced by cytokines associated with proteolytic digestion of basement membranes upon which epithelia reside. EMT can be induced by several oncogenes, including RASV12 (Janda et al., 2002), ErbB2 (Jenndahl et al., 2005) and TRKB (Kupferman et al., 2010, Smit et al., 2009). They activate multiple effectors including the PI3 and MAP kinases, Wnt, Notch and NF-κB pathways, all of which are involved in EMT regulation (Smit and Pepper, 2010).

A group of transcription factors, including Twist, Snail 1, Snail 2, δEF1 (ZEB 1), SIP-1 (ZEB 2) and E12/E47, have been shown to induce EMT. However, it is unknown whether these transcription factors function independently or coordinately to activate the EMT program. It is evident that hierarchy exists in the expression of these transcriptional factors, with Snai1 being expressed at the onset of EMT whereas Snai2, Zeb1 and Twist are induced later to maintain the migratory phenotype. Their role in EMT will be described later.

Locally expressed growth factors TGF-β, EGF, IGF-II, FGF-2 and HGF facilitate EMT by binding epithelial receptors with ligand-inducible intrinsic kinase activity. HGF, also known as scatter factor (SF), the ligand of the c-met receptor was first validated as EMT inducer in 1985 (Stoker and Perryman, 1985). Cancer cells also start to produce proteolytic enzymes. They remodel ECM and the basement membrane and prepare pro-migratory and pro-invasive environment.

1.4.4. EMT proteome and genome; molecular switch

EMT proteome reflects a fundamental change in proteins gained, maintained or lost with the conversion of epiblasts to primary mesenchyme, secondary epithelium to fibroblasts, or in the transition of tumor epithelia to metastatic cells (Figure 7). Most information about EMT proteome is inferred from proteins found in epithelia than in fibroblasts or metastatic cancer cells.

Proteins gained or maintained during EMT are: Snail, Slug, Twist, Scratch, SIP1, E47, Ets, FTS binding protein, RhoB, FSP1 (S100A4), TGF-beta, FGF-1,-2,-8, MMP-2, MMP-9, vimentin, αSMA, fibronectin, collagen type 1, collagen type III, thrombospondin, PAI-1, etc.
Proteins that are attenuated during EMT are: E-cadherin, β-catenin, desmoplakin, Muc-1, ZO-1, syndecan-1, cytokeratin 18 (Reviewed in Kalluri, 2003).

1.4.4.1. Twist. Transcription factor Twist is a major regulator of embryonic morphogenesis and regulator of EMT in invasive and metastatic carcinomas (Yang et al., 2004). Twist, together with Snail 1, upregulates Zeb1 and leads to E-cadherin downregulation (Dave et al., 2011). Ectopic expression of Twist in MDCK (canine kidney cells) cells caused transcriptional repression of E-cadherin, α-, β- and γ-catenins, and induction of mesenchymal markers vimentin, fibronectin, smooth muscle actin and N-cadherin (Yang et al., 2004).
Twits can induce EMT in Hela and MCF7 cells, and this was accompanied by increased stem cell-like properties with the upregulation of ALDH1 and CD44 (Li and Zhou, 2011). Twist mRNA expression significantly correlates with tumor depth and advanced tumor stage in gastric cancer (Otsuki et al., 2011). *In vivo* studies suggested that elevated expression of Twist might be responsible for breast cancer lung metastases (Yang et al., 2004).

1.4.4.2. Snail family. Snails are essential for induction of EMT. Snail1 is required for mesoderm and neural crest formation during embryonic development and has recently been implicated in the EMT associated with tumour progression. It induced EMT in pancreatic cancer cells (Panc-1) and coexpressed with vimentin in these cells, where E-cadherin was not detectable. Snail1 further induced and enhanced tumor growth, invasion and distant metastasis *in vivo* (Nishioka et al., 2010). Snail1) expression was also associated with tumor dedifferentiation in breast cancer. For example, Snail1 was detected in 47% of IDCs . Most of the grade 3 tumours and more than a half of grade 2 tumors expressed Snail1, but it was not found in any of the grade 1 IDCs and *in situ* carcinoma (Blanco et al., 2002).

Snail 2 (Slug) knockdown completely blocks the ability of Twist1 to suppress E-cadherin transcription (Casas et al., 2011). Twist1 binds to an evolutionarily conserved E-box on the proximate Snail 2 promoter to induce its transcription. Snail 2 induction is essential for Twist

1-induced cell invasion and distant metastasis in mice. In human breast tumors, the expression of Twist 1 and Snail 2 is highly correlated.

Gene expression analysis of CD44+/CD24- breast cells compared to CD44-/CD24+ cells revealed increased expression of 32 EMT associated genes including SLUG, ZEB-1, ZEB-2, periostin, Hedgehog signaling associated gene Gli-2, and the metastasis-associated gene SATB-1 (Bhat-Nakshatri et al., 2010). Transgenic overexpression studies showed that only SLUG had the capacity to alter the phenotype of CD44-/CD24+ MCF-10A cells to induce a subpopulation of CD44+/CD24- cells. However, transgenic overexpression of SLUG in the luminal type breast cancer cell line MCF-7, generated cells with a CD44+/CD24+ phenotype, suggesting that basal cell types but not luminal cell types are susceptible to EMT associated acquisition of CD44+/CD24- phenotype. Additionally, only specific EMT associated genes (ST-2, N-cadherin, ETV5, FHL-1, Wnt5B, FOXC2, SATB-1, SLUG, and Gli-2) induced a CD44+/CD24- phenotype in MCF-10A cells. For example, overexpression of the NF-κB subunit of p65 upregulated expression of ZEB-1 and ZEB-2 genes (Chua et al., 2007), which resulted an increase in CD44+/CD24+ cells but not in CD44+/CD24- MCF-10A cells (Bhat-Nakshatri et al., 2010).

Another member of Snail family, Snail3 have been identified by Katoh and Katoh, (2003). Human SNAI3 (Snail3) mRNA was expressed in skin melanotic melanoma, lung epidermoid carcinoma, and germ cell tumor. Authors suggested that because SNAG zinc-finger proteins are transcriptional repressors implicated in carcinogenesis and embryogenesis, SNAI3 gene might be a potent target of pharmacogenomics in the field of oncology and regenerative medicine (Katoh and Katoh, (2003). There is not much experimental data of Snail3 function. In situ hybridization analysis showed that mRNAs coding for SNAIL1, SNAIL2, or SNAIL3 were localized predominantly to ductal epithelial invaginations or budding structures in developing sheep pancreas (Cole et al., 2009).

1.4.4.3. Zeb1 and Zeb 2. The Zeb family of transcription factors consists of two members: Zeb1 (also known as TCF8 and δEF1) and Zeb2 (ZFXH1B and SIP1) (Vandewalle et al., 2009). The members of this family interact with the DNA through the simultaneous binding of the two zinc-finger domains to E-boxes (Vandewalle et al., 2009). Both proteins are strong repressors of CDH1 (E-cadherin gene). Although they are not as potent as Snail in the induction of EMT or in the repression of CDH1 in *in vitro* assays (Vega et al., 2004), their silencing, especially that of

Zeb1, has a higher impact on CDH1 expression than Snail (Eger A et al., 2005, Vandewalle et al., 2009). Inhibition of Zeb1 and 2 by the miR200 family restores E-cadherin protein expression (Kajita M et al., 2004). Snail1 and Slug activate of Zeb family members, TCF3, TCF4, Twist, Goosecoid and FOXC2 which all may be important in maintaining the EMT phenotype (Hugo et al., 2010).

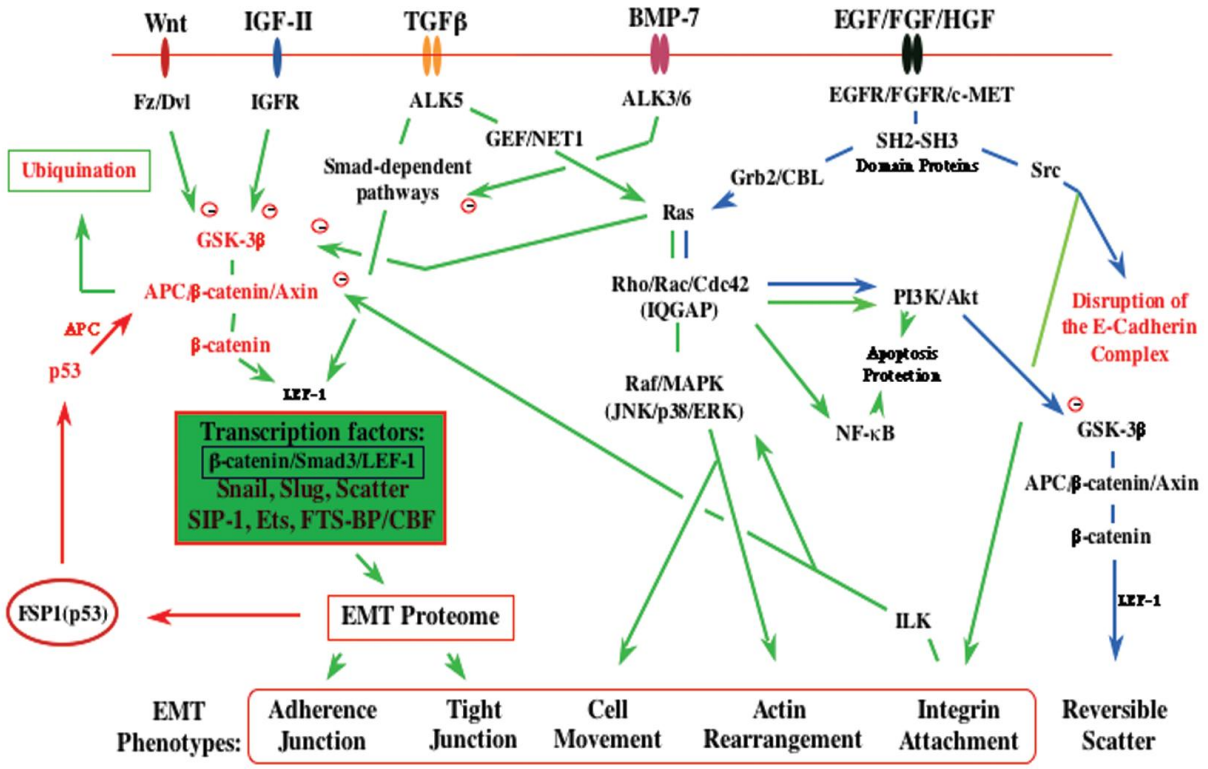


Figure 7. Epithelial plasticity can lead to classical EMT (loss of cell-cell and cell-substratum attachments, new actin rearrangements, and gain of mobility) or reversible scatter, which looks like EMT but is not enduring and can revert. These events are regulated by ligand-inducible intrinsic kinase receptors on the cell surface, which modulate small GTPases, Smads, PI3Ks, MAP kinases, and the availability of β-catenin to coactivate LEF in the nucleus. Free levels of β-catenin are regulated by E-cadherin or APC/β-catenin/Axin complexes, the latter of which shuttle β-catenin between ubiquination or utilization in adherens junctions. Activation of nuclear transcription provides new transcriptional regulators (Snail, SIP1, Ets, and FTS-BP/CarG box binding factor) of the EMT proteome. The variability of receptors, kinases, and the emergence of combined preferences for signaling pathways determine the plasticity unique to each epithelium. (Source Kalluri and Neilson, 2003).

1.4.4.4. MicroRNAs. Noncoding microRNAs are also components of the cellular signaling that regulate the EMT (Zavadil et al., 2007). For example, microRNA 200 (miR200) and miR205 inhibit the repressors of E-cadherin, ZEB1 and ZEB2, and help in maintaining the epithelial cell phenotype (Gregory et al., 2008). In breast carcinoma, a loss of miR200 correlates with increased expression of vimentin and a decrease in the levels of E-cadherin in cancer cells (Gregory et al., 2008). Acting in the opposite direction, miR21 is upregulated in many cancers and facilitates TGF-β–induced EMT (Riviewed in Kalluri, 2003). The miR200-mediated down-regulation of WAVE3 (an actin cytoskeleton remodeling and metastasis promoter protein) results in a significant reduction in the invasive phenotype of breast (MDA-MB-231) and prostate (LNCaP) cancer cells (Sossey-Alaoui et al., 2009). Inhibition of WAVE3 expression with either overexpression of miR200b or siRNA to WAVE3 both resulted in a change of cell phenotype from mesenchymal to epithelial one, indicating that WAVE3 might be implicated in this cellular process, however, WAVE3 might not be directly involved in the regulatory pathway of ZEB1/ZEB2/E-cadherin (Sossey-Alaoui et al., 2009).

1.5. Breast Cancer

1.5.1. Mammary gland development

The mammary gland development mostly occurs postnatally and is only completed in adulthood. Some aspects of mammary epithelial cell differentiation even require the completion of a full-term pregnancy, lactation, and involution cycle. In mammary gland development, three distinct stages: embryonic stage of rudimentary gland development, a pubertal stage of ductal elongation and branching, and a pregnancy stage of alveolar differentiation and tertiary ductal branching are crucial (Watson and Khaled, 2008). Mammary gland is a modified sweat gland derived from the ectoderm. Its development begins during mid-gestation when ectodermal cell migration is induced by the mesenchyme. Subsequently, mammary lines are formed and placodes develop along these lines. The placodes invaginate into the underlying subdermal stroma forming the mammary bud. Along embryogenesis, the mammary bud begins to invade into the surrounding adipose tissue producing a rudimentary mammary gland that is arrested at this stage until puberty.

During puberty, branching morphogenesis, coupled with ductal elongation, fills the mammary fat pad and arborizes the ductal tree, preparing the mammary gland for pregnancy associated alveolar development and lactation. As puberty progresses, the rudimentary gland begins to elongate with the formation of terminal end buds (TEB) (Watson and Khaled, 2008). The TEB is a multi-layered epithelial structure at the forefront of ductal development which gives rise to the bilayered mammary ducts that consist of luminal epithelial and myoepithelial cells, and also is the predominant site of branching during puberty (Sternlicht et al., 2008). The TEB bifurcates under the regulation of a number of extracellular cues known to regulate epithelial plasticity and induce EMT, including epidermal growth factor, hepatocyte growth factor/scatter factor, and the activity of proteases such as the matrix metalloproteinases (Fata et al., 2003). Unlike other branching organs, the elongation of the ducts depends on proliferation within the TEB instead of the protrusive activity of the cap cells (Friedl and Gilmour, 2009).

Two different mesenchymal cells are involved in mammary gland development during embryogenesis: the fibroblastic cells which surround the epithelial rudiment (the fibroblastic mesenchyme) and the fat pad cells (the fat pad mesenchyme). The fibroblastic mesenchyme induces formation of atypical ductal branching, while the fat pad induces epithelial cell elongation and branching (Sakakura et al., 1982). ECM of fibroblastic mesenchyme and fat pad are of different composition which explains these changes. During pregnancy, alveolar epithelial cells proliferate and differentiate in response to lactogenic stimuli – hormones and growth factors to produce milk. Alveolar morphogenesis at this stage is dependent on ECM message, that are interpreted by the mammary epithelial cells through β1 integrin signaling (Kass et al., 2007). The association of β1 integrins with their heterodimeric α subunits attach the cell to the basement membrane (through laminin and collagen IV binding) and surrounding stroma (through binding to collagen I or fibronectin). In the mammary gland, both α5β1 (fibronectin receptor) and α2β1 (collagen 1 and laminin receptor) integrin expression levels are regulated by ovarian hormones and are implicated in transduction of hormonal signals that drive growth and differentiation of the mammary gland during pregnancy (Bussard and Smith, 2011).

EMT regulators including Snail/Slug, Twist, Six1, and Cripto, along with developmental signaling pathways including TGF-β and Wnt/β-catenin induce epithelial plasticity during mammary gland development. It is clear that the combination of biochemical signals along with the physical constraints of the developing mammary gland directs branching and importance of

integrins in mammary morphogenesis and epithelial plasticity is evident. Cells of the TEB exhibit a different complement of integrins and extracellular matrix receptors compared to quiescent mammary epithelium, since numerous integrin knockout mice, including α2, α3 and β1, exhibit decreased branching (Fata et al., 2003). Gene knockout studies in mice where particular integrin subunit has been deleted indicate that integrin heterodimers containing α3, α6, or β4 integrin subunits are not required for mammary branching morphogenesis (Klinowska et al., 2001), while α2 integrin knockouts exhibit diminished branching (Chen et al., 2002). Cap cells do not exhibit protrusions characteristic of an invasive phenotype and do not lose their cell-cell adhesions, however these cells still exhibit signs of epithelial plasticity. Such signs include, among others, the loss of apico-basal polarity, as determined by the lack of specific localization of β-catenin to the basolateral domain and atypical protein kinase C-ζ to the apical domain (Ewald et al., 2008).
Molecular mechanisms of branching in the mammary gland are still being elucidated. Gene expression analysis in mammary epithelial MCF-10A cells cultivated on matrigel revealed substantial amounts of Snail 2 and E-cadherin mRNA, but very little Snail1 mRNA (Debnath et al., 2003). Similar result by Côme and colleagues suggests that Snail 2 has an active role during the lobuloalveolar phase (Come et al., 2006). Additionally, microarray analysis of gene expression in the TEB identified that Snail and Twist significantly increased in the TEB compared to the mature ducts (Kouros-Mehr and Werb, 2006). In addition, cells of the TEB secrete extracellular proteases, including MMP-3, which further activate the EMT inducing Msx2/Cripto-1 pathway (di Bari et al., 2009, Wechselberger et al., 2001). MMP-3 activity leads to a transient EMT that may be necessary for the invasion of the mammary gland ductal tree in the fat pad coincident with a thinning of the basement membrane (Fata et al., 2004).

The existence of epithelial plasticity during branching morphogenesis has been confirmed in organotypic cultures where mammary epithelial cells treated with EGF or HGF begin to branch (Nelson et al., 2006). Cells at the sites of branching activate the promoter of the mesenchymal marker, vimentin, and express MMP-3, an inducer of EMT (Nelson et al., 2006). These and other results suggest that at the site of branching, mammary epithelial cells display a significant alteration of their interaction with the extracellular matrix. Overall, while mammary epithelial cells of the TEB do not exhibit the hallmarks of a complete EMT, these cells acquire multiple features of epithelial plasticity.

1.5.2. Normal mammary gland

The mammary gland is composed of two tissue compartments separated by a basement membrane: the epithelium, which includes the ducts and lobules, and the stroma, which consists of the connective tissue that constitutes the mammary fat pad. In the nonlactating adult breast, the stroma occupies that majority of the tissue, where the proportions of fibrous and adipose tissue vary with age. Within the stroma, breast tissue consists of a complex network of lobules and mammary ducts, and fat. Breast lobules, comprised of groups of alveoli (also called acini or terminal ductules), are spherical-shaped, glandular structures that produce milk. Within the alveoli, a single layer of luminal epithelial cells that surround the inner lumen can be found. Adjacent to the luminal cells exists a layer of myoepithelial cells responsible for basement membrane protein deposition as well as providing contractile motion during milk secretion. During pregnancy, epithelial cells of the alveoli proliferate extensively, leading to an increase in the number of alveolar units, and therefore increase in the size and number of lobules. The milk that is produced by breast lobules is then drained by a branching network of ducts that carry milk from the lobules to the nipples during lactation. Ducts that drain individual alveoli lead to progressively larger ducts, which connect with the nipple. At the nipple, the ducts expand to form lactiferous sinuses. The sinuses then terminate into cone-shaped ampullae immediately below the surface of the nipple (Bussard and Smith, 2011).

1.5.3. Breast precursor lesions

Numerous breast proliferative lesions are associated with increased risc of breast cancer development and are called as risc indicators, either in the ipsilateral or in the contralateral breast. Some of these indicators are clonal, undergo neoplastic proliferation and have histological, immunohistochemical and molecular similarities with those of matched with invasive breast cancer, either synchronous, or metachronous. Lesions that fulfil these criteria are considered as breast cancer precursors (Lopez-Garcia et al., 2010).

1.5.3.1. Benign proliferative and non-proliferative lesions

Radial scar (RS) is a benign lesion characterized by obliterated ducts and „infiltrating" tubules, lined by epithelial and myoepithelial cells, surrounded by contracted ducts and lobules exhibiting variable epithelial hyperplasia, duct ectasia, adenosis and papilomatosis (Rosen, 2001).

Some evidence exist about association of of RS with breast cancer development, however, majority of them are tubular and lobular carcinomas of low histological grade (Manfrin et al., 2008).

Apocrine metaplasia is usual and incidental finding associated with wide spectrum of benign lesions and at the periphery of *in situ* and invasive carcinomas. Apocrine cells are characterized by eosinophylic, granular cytoplasm, large and vesicular nucleoli (Rosen, 2001). It is currently accepted that the presence of apocrine lesions is not associated with the risc of breast cancer development (Lopez-Garcia et al., 2010).

Hyperplasia of usual type (HUT) is an intraductal lesion characterized by proliferation of a mixed population of cells towards the lumen of ducts, leading to the formation of secondary lumens often peripherially distributed and streaming of the central proliferating cells (Rosen, 2001). HUT has been shown to be associated with low risc of breast cancer development and is considered as non-obligate precursor of atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS) (Aubele et al., 2000; Jones et al., 2003). In contrast, genetic data suggests that majority of HUT represents risc indicators rather than breast cancer precursor (Simpson et al., 2005).

1.5.3.2. Low-grade precursor lesions

Columnar cell lesions (CCL) are characterized by distended acini lined by tightly packed columnar epithelial cells with apical snouts, displaying variety of cytological and/or architectural atypia, ranging from columnar cell chage to flat epithelial atypia (FEA) (Lopez-Garcia et al., 2010). The coexistance of CCL with ADH, DCIS and LN in the same breast and similar IHC profile (ER and PR expression, low MIB1/Ki67 indexes, lack of Her2 and basal cytokeratin expression) give evidence that they are first morphologically identifieble precursor of low grade breast cancers (Pinder and Reis-Filho, 2006).

Atypical lobular hyperplasia/classic lobular carcinoma *in situ* (ALH/LCIS). LCIS is composed of monomorphic population of small and loosely discohesive cells that expand the terminal duct lobular units (TDLUs) with or without pagetoid involvement of terminal ducts, where ALH is morphologically similar but less developed lesion and Haagensen et al. (1978) suggested term of lobular neoplasia (LN). LN is characterized by expression of ER and PR and negativity of Her2 and basal markers (Lopez-Garcia et al., 2010). ALH and LCIS have been

accepted as risk indicators and non-obligate precursors of breast cancer development with higher risc in the ipsilateral breast (Page et al., 2003).

Atypical ductal hyperplasia and low grade ductal carcinoma *in situ* (ADH/DCIS). Low grade DCIS is characterized by a proliferation of mononorphic cells with uniform-sized nuclei and rare mitotic figures growing in arcades, micropapillae, cribriform and solid patterns. ADH share some but not all morphological features of low grade DCIS, and the risk of breast cancer development is higher in the latter. Immunophenotypically, both lesions are ER and PR positive, and Her2 negative. ADH is recognized as both risc indicator and non-obligate precursor of low-grade DCIS and invasive breast cancer (Lopez-Garcia et al., 2010).

1.5.3.3. High-grade precursor lesions

Microglandular adenosis (MGA) is an uncommon entity and is characterized by a haphazard proliferation of homogenous small and rounded glands lined by a single layer of epithelial cells around lumen containing secretions and/or calcifications. It has all ER, PR, Her2 negative (triple negative) phenotype and invasive carcinomas associated with MGA are often of high histological grade and frequently express basal markers. This lesion is the non-obligate precursor of invasive breast cancer (Lopez-Garcia et al., 2010).

Pleomorphic lobular carcinoma in situ (PLCIS) exhibits cytological and architectural features of classical LCIS and high grade DCIS. It is composed of large and discohesive cells with granular cytoplasm and intracytoplasmic vacuoles, show marked nuclear atypia and pleomorphism. Immunophenotypically express low levels of ER and PR, lack of E-cadherin expression and occasional overexpression and amplification of Her2. PLCIS can also be found as an isolated lesion. It is a non-obligate precursor of ILC (Eusebi et al., 1992; Lopez-Garcia et al., 2010).

High-grade DCIS is populated of atypical cells with marked nuclear pleomorphism, arranged in various architectural patterns such as micropapillary, solid and cribriform. Immunophenotype of DCIS is more heterogenous than of low-grade DCIS. DCIS has tendency to progress in invasive cancer but not always, however, diagnosis of high-grade DCIS is associated with significantly higher risc of invasive breast cancer development than low-grade DCIS (Lopez-Garcia et al., 2010).

1.5.4. Breast cancer histological classification

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths worldwide (Jemal et al., 2011). Breast cancer is a heterogenous disease with a wide array of histologic appearances and with variable prognosis depending upon the developmental stage of the breast tissue at diagnosis.

Despite all breast carcinomas arise from cells in the terminal duct lobular unit (TDLU), the use of terms lobular and ductal to describe *in situ* and invasive carcinomas still exists. According to current convention term lobular refers to carcinomas of a special type and ductal is used more generally for adenocarcinomas that have no other designation.

1.5.4.1. Invasive carcinoma, No Special Type (Invasive Ductal Carcinoma). The diagnosis of ductal or NST carcinoma is based on the absence of histologic patterns and cytologic features, the constellation of which characterize special type carcinomas (Lawton, 2009). 70-80% of invasive carcinomas are of no special type. Since many of the tumors do not form ducts, all infiltrating ductal carcinomas (IDC) are characterized by the formation of tubules, or ducts that infiltrate throughout the supporting breast parenchyma. Common gross appearance is that of sclerotic, stellate mass, however some are well-circomscribed. NST carcinoma is most commonly graded using the Nottingham system based on tubule formation, nuclear grade and mitotic rate. NST is a prognostically heterogenous group of carcinomas that needs to be stratified by use of morphologic-prognostic factors.

1.5.4.2. Invasive lobular carcinoma. Invasive lobular carcinoma (ILC) is the second most common type of invasive breast carcinoma and accounts for 8-14 % of all breast cancers. According to recent epidemiological data, its incidence is increasing in postmenopausal woman (Lawton, 2009). Microscopically, ILC is composed of small uniform cells invading the supporting breast parenchyma in a linear, single-file arrangement or concentrically around benign ducts in a target-like fashion. ILC is a low grade tumor with little or no nuclear atypia and a low mitotic rate. Unlike ductal carcinomas, ILC has ill-defined margins and does not form microcalcifications, making it difficult to detect on screening mammography and ultrasound, however, it is detectable as palpable mass or mammographic abnormality. Infiltrating lobular

carcinomas have unique patterns of metastasis and spread compared with IDC. ILC have an increased frequency of multicentricity, multifocality and bilaterality and a predilection for metastasizing to the peritoneum, gastro-intestinal system, gynaecologic organs and bone marrow. ILC have a better prognosis than ductal carcinoma NST. ILC is more likely to be hormone receptor positive and are frequently Her2-negative. Loss of E-cadherin expression is a frequent event in ILC and is used as a tool for differentiation from ductal carcinoma. ILC has several variants/subtypes that differ in histological growth pattern and cytologic atypia. The most common is classic ILC that preferentially display a luminal phenotype. Solid variant accout 10 percent of ILC and alveolar variant 4-5 percent of ILC. The pleomorphic variant of ILCs (PLCs) is characterized by atypical cells with pleomorphic nuclei and is reported to have an aggressive clinical behavior. By expression profiling, classic ILCs are of luminal, whereas PLCs may be of luminal or Her2 molecular subtypes (Lawton, 2009).

Other special type carcinomas such as tubular/cribriform carcinoma, mucinouc (colloid) carcinoma, medullary, papillary and metaplastic carcinomas ranges 10-12% of all invasive carcinomas. Compared with ductal carcinoma NST, tubular carcinoma, papillary carcinoma, and medullary carcinoma have a better prognosis while micropapillary and metaplastic carcinomas have a worse prognosis (Tavassoli, 1999; Rosen, 2001).

1.5.5. Breast cancer molecular classification

Recent gene expression profiling studies based on measuring the relative quantities of mRNA for every gene have confirmed that there are many types of cancers but most carcinomas are grouped in several major clusters that have important biologic and clinical characteristics. Breast cancer subtypes suggested first by Sorlie et al. (2001 and 2003) was divided into five major molecular subtypes: luminal A, luminal B, basal, normal breast-like and ErbB2-overexpressing subtypes associated with distinct clinical outcomes and providing more accurate information about prognosis or response to specific therapies (see below). Three broad classes of breast tumors drawn from receptor status are commonly used in the clinics such as luminal, triple negative and Her2 positive subtypes (Foulkes et al., 2010). Later, this initial classification was reproduced because of biological heterogeneity within classes. Recent studies based on immunohistochemical validation of biomarkers and also large-scale unsupervised classification methods have refined breast cancer stratification across subclasses. For example, Blows et al.

(2010) has defined five subtypes (luminal 1, luminal 2, non-luminal Her2+, 5 negative phenotype and core basal phenotype based on immunohistochemical expression of five markers (ER, PR, Her2, EGFR and/or CK5/6) and evaluated their prognostic significance (Figure 8). Existance of quintuple negative breast cancer along triple negative and basal-like has also been shown by Choi et al. (2010) where this subgroup showed worse prognosis. Guedj et al. (2011) presented a classification of breast cancer into six molecular subgroups (LumA, NormL, LumB, LumC, mApo, BasL, explanations in the legend to figure 9), which differ upon gene expression, genomic profiles, differentiation level and clinical features. Gene expression differences strongly suggested that they outlined distinct biological entities, reflecting initiating mutations and/or cell-of-origin. Specific sets of signaling pathways were associated to each subgroup (Table 1). The distribution of the six subgroups was determined by the combination of the expression of three large gene clusters organized around the (i) estrogen receptor, (ii) androgen receptor and (iii) cell cycle regulator genes.

Sorlie subtypes:

Luminal A (ER+, PR+, Her2-) is the largest group. Its gene signature is built by genes that are under the control of ER, they are well or moderately differentiated, slow growing, most of them occur in postmenopausal woman and respond well to hormonal treatments. Only a small number respond to chemotherapy.

Luminal B (ER+, PR+, Her2+) are of higher grade, have a higher proliferative rate, are more likely to have lymph node metastases and may respond to chemotherapy. They are also referred as triple-positive cancers. Luminal B patients have worse prognosis than luminal A. It is also thought that they are endocrine therapy refractory (Brenton et al., 2005). There is a suggestion that other approaches beyond endocrine therapy may be effective. The anti-VEGF antibody bevacizumab was recently shown to improve survival in metastatic breast cancer when combined with paclitaxel (Brenton et al., 2005).

Normal breast-like (ER-, PR-, Her2-) cancers are well-differentiated, characterised by the similarity of their gene expression pattern to normal breast.

Basal-like (triple negative) subtype (ER-, PR-, Her2-, CK5/6+, CK17+). Histologically they are medullary carcinomas, metaplastic carcinomas and carcinomas with central fibrotic focus. Women with BRCA1 mutations are often basal-like type. These cancers are generally high grade and have high proliferation rate. They are characterized by aggressive course, frequent

metastasis to viscera and the brain, and have poor prognosis. However, approximately 20% of patients respond completely to chemotherapy.

HER2 positive subtype (ER-, PR-, Her2+). In most of HER2/neu positive cancers, overexpression is due to amplification of the segment of DNA on 17q21 locus that includes the HER2/neu gene. These cancers are usually poorly differentiated, have a high proliferation rate and are associated with a high frequency of brain metastasis. Therapy with monoclonal antibodies in combination with chemotherapy are highly effective.

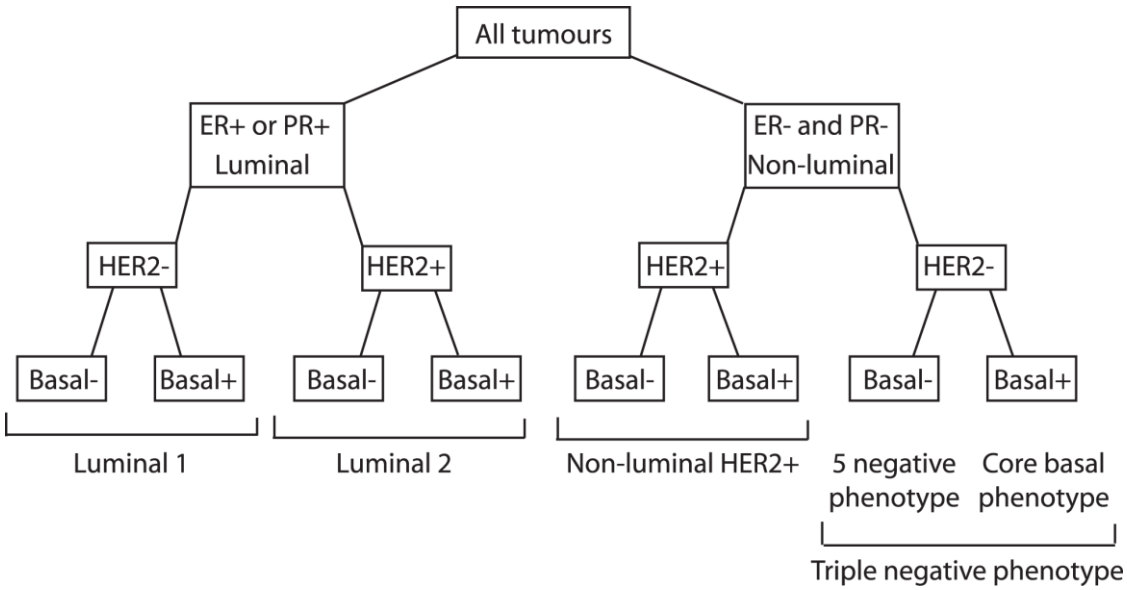


Figure 8. Classification of breast cancer subtypes according to IHC marker profile. (Source Blows et al., 2010).

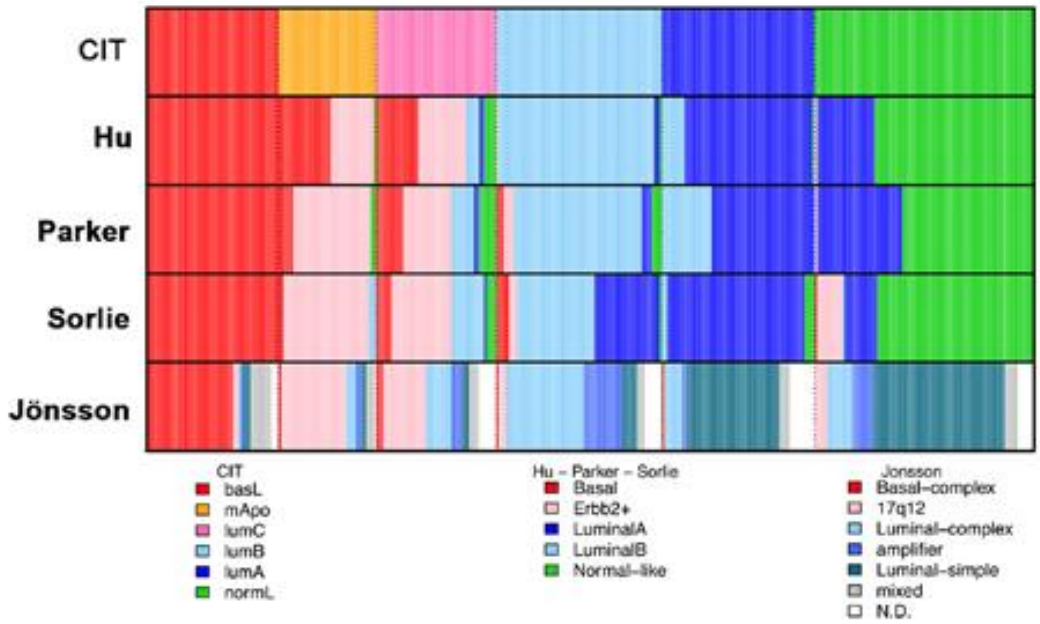


Figure 9. Comparison of different molecular classifiers. LumA–luminal A, NormL–normal-like, LumB–luminal B, LumC–luminal C, mApo–molecular-apocrine, BasL– basal-like. CIT *(Cartes d'Identité des Tumeurs program, Paris)* (Source Guedj et al., 2011).

1.5.6. Genomic profile of Breast Carcinoma

The use of comparative genomic hybridization, loss of heterozygosity (LOH), cytogenetics, and fluorescence in situ hybridization (FISH) techniques identified recurrent genomic imbalances in breast carcinoma. The most common genomic aberrations identified are gains along chromosomes 1q, 8q, 17q, 20q, and 11q and losses along 8p, 13q, 16q, 18q, and 11q (Reis-Filho, 2005; Shackney, 2003; Simpson, 2005). Many of these chromosomal segments harbor known proto-oncogenes and/or tumor suppressor genes such as BRCA1, BRCA2, HER2-neu, C-MYC, and Cyclin D1. Genomic features are in correlation with clinicopathologic parameters such as tumor stage, grade, hormonal status, histologic subtype and disease recurrence. Strong association is found with histologic grade (Yoder et al., 2007).

Table 1. Molecular subgroups show differential activation of major signaling pathways. (Source: Guedj et al., 2011).

Category	Pathways	BasL	mApo	LumC	LumB	LumA	NormL
Cell communication	Adherens junction		↑	↓		↓	
	Focal adhesion				↓		↑
Motility	Cell motility			↑	↑		↑
	Regulation of actin cytoskeleton			↑			
Cell growth and death	Apoptosis					↑	
	Cell cycle	↑			↑		↓
	p53 signaling pathway	↓			↓		↑
Replication and repair	Base excision repair				↑		↓
	DNA replication	↑			↑		↓
	Mismatch repair	↑					↓
	Nucleotide excision repair				↑		
Lipid metabolism	Androgen and estrogen metabolism	↓					
	Fatty acid metabolism	↓	↓				
Endocrine system	GnRH signaling pathway		↑				
	Insulin signaling pathway	↓	↓		↓	↑	
	Renin-angiotensin system					↑	
Signal transduction	Androgen receptor signaling	↓	↑			↑	
	Calcium signaing pathway		↑			↓	
	ErbB signaing pathway		↑	↑		↓	
	Estrogen receptor signaling	↓	↓	↑	↑	↑	↑
	mTOR signaling pathway						↑
	Phosphatidylinositol signaling		↓				
	PTEN cell cycle arrest and apoptosis	↓					
	TGF-β signaling						↑
	Wnt signaling					↓	
Immune system	Antigen processing and presentation			↑			
	B cell receptor signaling				↓		
	Hematopoetic cell lineage			↑			
	Natural killer cell mediated cytotoxicity			↑			
	T cell receptor signaling			↑			
	Toll-like receptor signaling			↑			

↑ and ↓ show up- and downregulation, respectively. Empty areas show no clear direction.

1.5.7. Breast cancer stem cells

According to current knowledge, malignant epithelial cell population contains a subset of cells with stem cell properties and their differentiated progenies. There are many reasons of malignant transformation and many cells are regarded to be cancer cell's ancestors. Particular accent is on somatic stem cells. They are long living and therefore hypothesized to be able to accumulate multiple mutations since they are exposed to damaging agents or an unfavorable environment for a long time. These cells are termed as cancer stem cell (CSC) or tumor initiating cells (TIC) (Reya et al., 2001). The concept of cancer stem cells, first proven in acute myeloid leukemia (Bonnet and Dick, 1997), is nowadays widely confirmed for number of tumors, such as glioblastomas, medulloblastomas, breast or prostate cancer (Ponti et al., 2005; Shipitsin et al., 2007; Charafe-Jauffret et al., 2009; Goldstein et al., 2010). There are many markers that are suspected to define cancer stem cell including different combinations of CD24, CD29, epithelial specific antigen (ESA), CD44, CD49f, CD133 and stem cell antigen one (Sca1) (reviewed in Klonisch et al., 2008).

Breast cancer is the first human carcinoma for which a putative cancer stem cell subpopulation has been isolated (Al-Hajj and Clarke, 2004). Researchers used four cell surface markers (adhesion molecules CD44 and CD24, a breast/ovarian cancer specific marker B38.1, and ESA) and isolated a cell population characterized by high CD44 expression and low or undetectable levels of CD24 ($Lin^{-}ESA^{+}CD44^{+}CD24^{-/low}$ cells) (Al-Hajj and Clarke, 2004; Creighton et al., 2010). This rare breast cancer initiating cells (BrCa-IC) is the only cell type capable of establishing human breast cancer after transplant into NOD/SCID mice as well as ability to reestablish tumor heterogeneity after transplantation. The percentage of tumor initiating cells (TICs) varies from tumor to tumor and between molecular subtypes. Recently, along known luminal, triple negative and Her2, a new, claudin-low subtype has been identified (Herschkowitz et al., 2007). This group represents less than 5% of breast cancers and are generally triple negative. However, they uniquely express low levels of tight and adherens junction genes Claudin 3 and E-cadherin. Claudin-low tumors also highly express markers associated with EMT, such as vimentin and Twist. TICs gene expression signature was also enriched for $CD44^{+}CD24^{-/low}$ cells (Creighton et al., 2010).

During the process of tumor metastasis, which is often enabled by EMT (Thiery, 2003), disseminated cancer cells, similar to stem cells, require self-renewal ability, in order to generate

macroscopic metastases. Thereby, the EMT may also enhance a self-renewal capability to disseminating cancer cells. Mani et al. (2008) induced an EMT in non-tumorigenic, immortalized human mammary epithelial cells (HMLEs) by ectopic expression of transcription factors Twist or Snail. The resulting cells acquired fibroblast-like appearances with down-regulated epithelial, and upregulated mesenchymal markers. They sorted cells using flow cytometry based on the expression CD44 and CD24 whose expression in the $CD44^{high}/CD24^{low}$ configuration is associated with both human breast CSCs and normal mammary epithelial stem cells. Most of the mesenchymal-like cells generated by these EMTs acquired a $CD44^{high}/CD24^{low}$ expression pattern - precisely the antigenic phenotype that has been ascribed to neoplastic mammary stem cells. HMLEs that underwent an EMT formed >30-fold more mammospheres than did HMLEs infected with the corresponding control vector.

1.6. Prostate cancer

1.6.1. Classification

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males, accounting for 14% (903,500) of the total new cancer cases and 6% (258,400) of the total cancer deaths in males worldwide (Jemal et al., 2011). Despite advances in prevention, early detection and improvements in therapy, its cure remains elusive.
Prostate gland consists of two major cellular types, the stroma and the epithelium. The stromal component is comprised of smooth muscle cells, fibroblasts and endothelial cells. Epithelial compartment consists of five types of cells: basal epithelium, secretory epithelium, transit amplifying cells, neuroendocrine cells and stem cells (Humprey, 2003).

Several classification of prostate cancer exists. WHO (Mostofi) grading system is based on light microscope evaluation of differentiation (the ability of carcinoma cells to form glands) and the degree of nuclear anaplasia. Slight anaplasia is evaluated as G1, moderate variation in differentiation and moderate anaplasia is seen in G2 and marked variation in differentiation and marked anaplasia are present in G3 (Mostofi, 1975).

The most commonly used system of classifying histologic characteristics of prostate cancer is the Gleason score, which is based entirely on the histologic patterns in haematoxylin-eosin stained sections. Grades are based on the extent to which the epithelium assumes a normal glandular structure. Grade 1 indicates a near-normal pattern, and grade 5 indicates the absence of

any glandular pattern (from less malignant to more malignant). Scoring based on the two patterns (primary pattern is a predominant in tumor areas and the second is the most common) in an attempt to factor in the considerable heterogeneity within cases of prostate cancer. The sum of these two grades is referred to as the Gleason score (Gleason, 1992).

Clinical and pathological TNM staging is based on the information about tumor size and location, lymph node invasion and presence of distant metastases. TNM staging is an important prognostic factor of prostate carcinoma.

1.6.2. Prostate development

Prostate development, growth and function is androgen dependent, however, other steroid receptors, such as estrogen receptors (ER) and retinoid receptors (RARs and RXRs), also contribute to prostate morphogenesis and differentiation (Cunha et al., 2004). Prostate development begins prenatally and lasts until the end of puberty. At 10 weeks of gestation, androgens produced by the fetal testes induce morphogenesis of the endodermal prostatic buds which grow into the surrounding urogenital sinus mesenchyme (UGM), lengthen and arborize to form a complex ductal network. Prostatic buds arise from different parts of the endodermal urogenital sinus and form various prostatic lobes whose ductal branching patterns are unique for each prostatic lobe. The immature prostatic acini and ducts are lined with multiple layers of immature cells that express cytokeratins (Wernert et al., 1987). During prenatal development, the UGM expresses high levels of androgen receptor (AR) which are initially undetectable in the epithelium of the developing male urogenital tract but are expressed postnatally, several days before the epithelium initiates production of tissue-specific secretory proteins (Cunha et al., 2004). The pubertal period is marked by androgen-driven increase in gland size, further branching and differentiation of immature prostatic epithelium into the adult-type (outer cuboidal basal and inner cylindrical secretory cells). Mature luminal cells constitute the exocrine part of the prostate and secrete PSA (prostate specific antigen) and PAP (prostate acid phosphatase) while AR appears in the secretory cell layer. In the adult prostate, androgens act on stromal and secretory epithelial cells, except for basal cells. These are relatively undifferentiated, express low or undetectable levels of AR and are androgen-independent.

In the developing prostate model, Wnt signaling regulates prostatic epithelial branching morphogenesis, luminal epithelial cell differentiation and proliferation of prostate epithelial

progenitor cells. Wnt5a mRNA is expressed at the distal tips and along the centro-distal periductal UGM during branching morphogenesis and is focally upregulated as buds emerge from anterior, dorsolateral and ventral UGS regions. Abnormal UGS morphology, bud patterns and decrease in prostatic bud number have been observed in Wnt5a null recombinant fetuses (Allgeier et al., 2008). During prostate growth, Wnt5a may be secreted from the mesenchyme and act as an inhibitor of UGE growth as addition of ectopic Wnt5a to wild-type UGS in culture inhibits growth of the ventral prostate. Wnt5a can also modulate canonical Wnt signaling through downregulation of Wif1 in neonatal rat prostate (Huang et al., 2009).

Better understanding of the complex regulation of prostate development has been provided by tissue recombination techniques using TGF-β type II receptor conditional knockout mouse with loss of TGF-β responsivity in the mesenchymal compartment ($Tgfbr2^{KO}$) (Li et al., 2009) . UGM from $Tgfbr2^{KO}$ or control mice was recombined with wild-type adult mice bladder urothelial cells. The resulting urothelium associated with $Tgfbr2^{+}$ UGM was instructively differentiated into prostatic epithelium, as expected. In contrast, the urothelium associated with $Tgfbr2^{KO}$ UGM permissively maintained the phenotype of bladder epithelial cells. Microarray analysis of UGM tissues suggested down-regulation of multiple Wnt ligands and up-regulation of the Wnt antagonist, Wif-1, in the $Tgfbr2^{KO}$ UGM compared to $Tgfbr2^{+}$ UGM. Furthermore, Wif-1 lentivirus overexpression in the wild-type UGM resulted in the inhibition of prostate epithelium induction. These results suggested that paracrine Wnt signaling mediates the role of TGF-β in the inductive effects of the UGM on the adjacent epithelium (Li et al., 2009).

1.6.3. Prostate cancer stem cells

Human prostate stem cells (PSCs) reside within the basal cell compartment of the gland (English et al., 1987). This idea is supported by an experiment where mice null for the basal marker p63 are born without prostate or mammary gland (Mills and Bradley, 2002). Several other molecular markers of the prostate stem cells have recently been proposed, including α2β1 integrin, CD133 and Sca-1 (Stem cell antigen 1). Human $\alpha2\beta1^{hi}/CD133^{+}$ enriched cells, established and maintained prostate epithelium when transplanted into an immunodeficient mouse, although entire functional prostate did not develop (Richardson et al., 2004). More recently, Lawson et al. (2007 and 2010) enriched murine prostate epithelial stem cells with $Lin(CD45/CD31/Ter119)^{-}Sca\text{-}1^{+}CD49f1^{+}$ cell surface profile by FACS (fluorescence-activated

cell sorting). These cells showed low levels of luminal cell markers (NKX3.1, cytokeratins 8 and 18) but expressed high levels of CK5, CK14 and p63 and possessed basal-like phenotype. The cells were also further used for testing their oncogenic potential following genetic manipulation.

Human prostate cancer has a markedly luminal phenotype (Lawson and Witte, 2007) . Wang et al. (2009) showed by genetic lineage-marking that rare luminal cells (CARNs, castration-resistant Nkx3-1 expressing cells) are bipotential and can self-renew in vivo. Single-cell transplantation assays also showed that CARNs can reconstitute prostate ducts in renal grafts. Further, inducible deletion of the PTEN tumor suppressor in CARNs resulted in rapid carcinoma formation after androgen-mediated prostate regeneration (Wang et al., 2009). On the other hand, Lawson et al.(2010), found basal/stem cells more efficient targets of oncogenic transformation than luminal cells. FGF10 paracrine stimulation of basal but not luminal cells resulted in multifocal adenocarcinoma. Similarly, overexpression of AKT1 or ERG1, the most frequent partner for chromosomal translocations with transmembrane protease, serine 2 (TMPRSS2) in prostate cancer, in the basal/stem cells resulted in PIN lesions. Importantly, large tumors were generated from basal but not luminal cells upon combined activation of AKT1 and AR (Lawson et al., 2010; Huang and Witte, 2010). These studies pose the question, if prostate cancers indeed originate from different cell types (e.g., the basal or luminal compartment), the resulting tumors might have different genetic profiles, biologic behavior and therapeutic responses. Self-renewing prostaspheres, formed by PCa cell lines expressing proliferation, differentiation and stem cell-associated markers CD44, ABCG2 and CD133 treated with Wnt inhibitors revealed reduction in their size and self-renewal. In contrast, addition of Wnt3a caused increased prostasphere size and self-renewal associated with increased nuclear β-catenin, CK18, CD133 and CD44 expression (Bisson and Prowse, 2009). LNCaP and C4-2B prostate cancer cell lines expressing androgen receptor were treated by its antagonist bicalutamide which led to reduced prostasphere size and expression of PSA but did not inhibit prostasphere formation. These effects are concordant with the androgen-independent self-renewal of cells with stem cell characteristics and androgen-dependent proliferation of transit amplifying cells

1.6.4. Androgen independent prostate cancer (AR in prostate cancer initiation and progression)

Prostate cancer is very heterogeneous in its etiology and progression. Androgens serve as a most important ligands for prostate cancer, stimulating growth via the androgen receptor pathway that includes the androgen receptor, the 5-alpha-reductase gene and the various members of the cytochrome p450 family (Tindal and Mohler, 2009).

Androgen deprivation therapy (ADT) through medical or surgical castration and/or blockade of the androgen receptor with anti-androgens is an important treatment for advanced stage prostate cancer that results in a decrease in tumor volume and decline in serum PSA in the majority of patients (Cronauer et al., 2003). However, during these treatments almost all tumors relapse to a androgen-independent prostate cancer (AIPC), which is currently incurable and fatal. The mechanisms that lead from androgen-sensitive to androgen-unresponsive tumor cell growth have been partly elucidated. Progress toward resistance is seen mostly in the presence of functional AR. Amplification or duplication of the AR gene has also been associated with the transition from hormone sensitive to hormone refractory stage (Edwards et al., 2003). Activation of mutated AR by glucocorticoids, adrenal androgens, dihydrotestesterone metabolites, estrogens, progestagenic steroids and antiandrogens also represents am mechanism by which tumor growth is facilitated (Culig et al., 2003). Furthermore, androgen-independent transactivation of the AR by peptide growth factors such as EGF, and IGF-I, keratinocyte growth factor (KGF) (Culig et al., 1994) and cytokines such as IL-6 (Malinowska et al., 2009) and IL-8 (Seaton et al., 2008) has been discovered as microenvironmental factors in development of AIPC.

1.6.5. Neuroendocrine differentiation of prostate cancer cells

Tumor microenvironment may be influenced by various factors released by adjacent tumor cells or normal tissue. Important role in this influence may have neuroendocrine (NE) cells that arise in prostate tumor as well as normal prostate cells. The process of occurrence of neuroendocrine cells is named as neuroendocrine differentiation. The origin of NE cells in prostate remains a subject of controversy. Bonkhoff (1998) postulate that NE cells share a common origin with other epithelial cell types from pluripotent stem cells. Aumuller et al. (1999) propose that NE cells originate from the neural crest during embryogenesis. Existence of those two functionally distinct populations was earlier suggested by Cohen et al (1990) who postulated

that peripherial NE cells are derived from pluripotent stem cells and periurethral NE cells originate from the neural crest.

Neuroendocrine cells are hybrid epithelial/neural endocrine cells which release several biologically active peptides such as serotonin, chromogranin A and others (see below) that appear as useful markers for IHC detection of NE cells (Bostwick et al., 2002). These molecules are also released by neural cells. NE cells resemble neurons by their morphology as possess irregular dendritic processes extending between adjacent epithelial cells. Among them the "open" type NE cells resembles an open flask-shaped form with long slender luminal extensions, and "closed" type NE cells lack these extensions (Daneshmand et al., 2005). Both cell types have granule – a typical sign of endocrine cells. They play important function in storing and secretion of endogenic substances.

Function of NE cells has intensively been studied. NE cells likely affect cellular growth and differentiation and exocrine secretions of the prostate through the local release of various neurosecretory products. This was shown in both benign and malignant prostatic tissue (Daneshmand et al., 2005).

Prostate cells express the androgen receptor and are androgen dependent while NE cells lack AR (Huang et al., 2006) and do not respond to hormonal therapy of prostate carcinoma. NE (trans)differentiation of prostate cancer epithelial cells may also serve as one possible mechanism for the development of AIPC: NE-like cells are shown to be emerged following ADT in response to changes in the hormonal and growth factor milieu of the microenvironment (De La Taille et al., 2001; Nelson et al., 2007).

Normal NE cells express cytokeratin 5, a basal cell marker (Schalken and van Leenders, 2003) while cancer NE cells have characteristics of luminal cells such as cytokeratin 18 and prostate acid phosphatase expression (Vashchenko and Abrahamsson, 2005; Huang et al., 2006). Further, NE-like malignant cells express anti-apoptotic protein bcl-2 (Xue et al., 1997) and α-methylacyl-CoA racemase (AMACR) (Huang et al., 2006). Multivariate analysis of NE differentiation and malignant cell proliferation based on Ki-67 in radical prostatectomy specimens found NED as a second most significant predictor of biochemical progression, after Gleason score (May et al., 2007). They also observed a significantly higher Ki-67 reactivity in chromogranin A positive group in comparison to negative one.

1.6.5.1. Markers of neuroendocrine differentiation of prostate carcinoma

Chromogranin A (CgA) is the first identified member of granine family of neuroendocrine secretory proteins. High expression of CgA was observed in tumor of neuroendocrine origin such as neuroblastomas and small cell lung cancer (Nobels et al., 1997) as well as non-neuroendocrine diseases, for example, in renal failure (O'Connor et al., 1989). Its serum level was found to be in positive association with neuroendocrine activity of prostate cancer cells (Ranno et al., 2006). Elevated CgA was frequently observed in patients with prostate carcinoma who do not respond to hormonal therapy (Berruti et al., 2005) and its elevated expression indicated poor prognosis.

Neuron-specific enolase (NSE, γ-enolase) is one of the five izoenzymes of 2-phospho-D-glycerat hydrolase (enolase). Enolase participates in the process of glycolysis and is present in all organisms. Enzyme enolase is a homodimer or heterodymer composed of three subunits. α subunit is found in all tissues, β subunit in muscles while γ subunit is expressed in neurons and normal and cancerous neuroendocrine cells (Rider and Taylor, 1975).

NSE is frequently used as a marker of neuroendocrine differentiation together with CgA, however, its specificity in serum is usually lower that of CgA (Nobels et al., 1997). Measurement of NSE is important in patients with neuroblastoma, lung cancer or prostate carcinoma. NSE was associated with poor prognosis in patients with metastatic prostate cancer who underwent hormonal therapy (Kamiya et al., 2003).

Tubulin β III. Tubulins, major components of the cellular cytoskeleton, form microtubules that are critical for cellular structure, migration, cell division, and intracellular trafficking. There are at least three types of tubulins: alpha, beta, and gamma. Class III β-tubulin is a microtubule element expressed exclusively in neurons, and is a popular marker specific for neurons in tissue.

N-cadherin is a member of cadherin family important in cell migration process. N-cadherin is expressed in different tissues during development. In adults, it is expressed in neural and endothelial tissues, in retina, osteoblasts, myocytes, oocytes, sperm and Sertoli cells. High expression of N-cadherin was observed in advanced stage of prostate cancer when in normal prostate it was not detected (Tomita et al., 2000). Underexpression of E-cadherin and N-cadherin overexpression was linked with prostate cancer progression and epithelial-mesenchymal transition (Tran et al., 1999).

Despite above described markers, gastrin-releasin peptide, somatostatin, nauropeptide Y, cholecystokinin, amino acid decarboxilase, histidine decarboxilase, tryptophan hydroxylase, serotonin receptors, calcitonin are also released by neuroendocrine cells and their expression could be associated with neuroendocrine differentiation.

1.6.6. Senescence and epithelial plasticity in prostate cancer

Senescence is a general phenomenon that limits the lifespan of cells and prevents unlimited cell proliferation (Caino et al., 2009). It can be triggered by short or malfunctioning telomeres (replicative senescence), but also prematurely, by a variety of stress signals, including unscheduled oncogenic signaling (Smit and Peeper, 2010) or deregulated expression of tumor suppressors (Chen et al., 2005). Senescent cells differ from their pre-senescent counterparts in three ways: i) they arrest growth and cannot be stimulated to reenter the cell cycle by physiological mitogens; ii) they become resistant to apoptotic cell death; iii) they acquire altered differentiated functions (Campisi, 2000).

Senescence relies on the activation of gene networks often comprising p53-$p21^{Cip1/Waf}$, $p19^{Arf}$–p53, and $p^{16INK4a}$-RB pathways (Caino et al., 2009), that may interact with one another or act independently to arrest cell proliferation (Campisi, 2000; Prieur and Peeper, 2008). Other hallmarks of sensecence include increased activity of lysosomal β-galactosidase (SA-β-GAL), chromatin remodeling and induction of DNA damage (Adams, 2009). In many settings, senescence is associated with the secretion of dozens of cytokines, comprising the Senescence-Messaging Secretome (SMS), denoting its communicative role (Kuilman and Peeper, 2009).

Several lines of evidence suggest that senescence evolved to protect higher eukaryotes, particularly mammals, from developing cancer (Shay and Roninson, 2004). However, recent findings have shown that the accrual of senescent cells may provide a permissive environment in epithelial tumors (Sprenger et al., 2010). Once a cell enters senescence, its transcriptome is altered such that genes associated with inflammation, angiogenesis and immune cell recruitment/activation are secreted locally (Sprenger et al., 2010). This unique secretome is termed as senescence associated secretory phenotype (SASP) (Coppe et al., 2010) and such cells have paracrine effects on nonsenescent neighbouring cells (Campisi, 2000).

Senescent cells also alter structural proteins of the extracellular matrix such as collagens and laminins as well as their regulatind enzymes (MMPs, TIMPs).

Factors regulating epithelial-mesenchymal transition appear to play dominant role as well in oncogene-induced senescence (Figure 10). Twist reverts p53-induced cell cycle arrest and suppresses mouse *Arf*, a gene that is highly induced during cellular and oncogene-induced senescence (Kamijo et al., 1997; Zindy et al., 2003). Similarly, in human prostate epithelial cells, Twist bypasses cellular senescence in a $p14^{ARF}$-dependent fashion (Kwok et al., 2007). Twist also prevents the upregulation of $p21^{CIP1}$ and p53 upon genotoxic stress in cell lines (Vichalkovski et al., 2010). Vice versa, senescence regulators also influence EMT. For example, the viral oncoprotein SV40 LT, which simultaneously downregulates cell cycle regulators p53 and RB, suppresses E-cadherin to induce a mesenchymal-like morphology (Martel et al., 1997). Further, when EMT is induced by TGFβ/TNFα in MCF10A cells, RB is downregulated (Batsche et al., 1998). Another factor associated with senescence is $p21^{CIP1}$ which is inhibited when Ras^{V12} induces EMT in MCF10A cells (Liu et al., 2009).

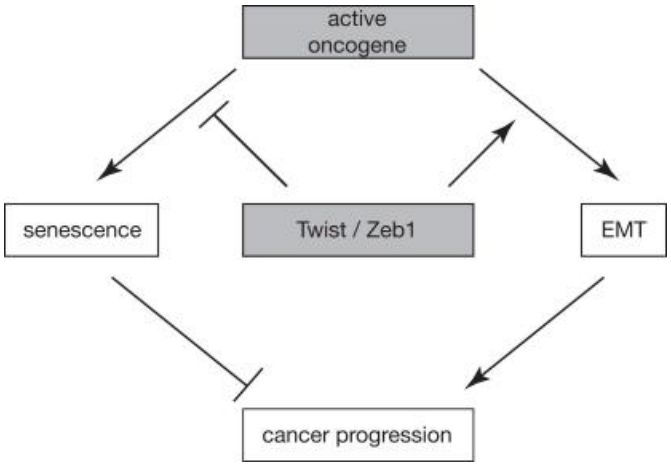


Figure 10. Schematic link between EMT and senescence in cancer progression. (Source Smit and Peeper, 2010).

1.7. Selected proteins

1.7.1. Collagen triple helix repeat containing 1

We and others have identified collagen triple helix containing 1 (CTHRC1) by expression profiling as breast cancer related protein (Tang et al. 2006; Turashvili et al., 2007). Tang et al. (2006) primarily studied melanomas, but they found expression of CTHRC1 also in breast cancer. CTHRC1 was absent in non-invasive stages of melanoma but greatly incresed in samples of primary invasive melanomas and further upregulated in metastatic melanomas. We found its upregulation in breast cancer, in particular in ILC, using laser capture microdissection and full genome profiling (Turashvili et al., 2007).

CTHRC1 is a highly conserved molecule found among vertebrates and there are no other family members. Mammalian CTHRC1 gene was first found in balloon-injured rat arteries where it is expressed by fibroblasts of the remodeling adventitia and by smooth muscle cells of the neointima (Pyagay et al. 2005). CTHRC1 is involved in vascular remodeling by limiting collagen matrix deposition and promoting cell migration (Pyagay et al. 2005). Its expression has also been described in a number of embryonal and postnatal tissues, including dermal sebaceous glands around hair follicles, esophageal epithelial cells and their surrounding matrix (Durmus et al., 2006). CTHRC1 was increased in response to TGF-β and bone morphogenetic protein 4 (BMP4) and contributed to enhanced migratory activity of fibroblasts and smooth muscle cells in injured artheries (Pyagay et al. 2005). In this context, melanoma cells were also reported to produce TGF-β and BMP4 and expression of both directly correlated with the advanced depth of tumor invasion (Rothhammer et al. 2005). Sequence analysis of the CTHRC1 promoter region reveals a binding site of SMAD, which is responsive to both TGF-β and BMP4. On the other hand, CTHRC1 inhibits transforming growth factor β in neointimal lesion formation (LeClair and Lindner, 2007). Further, overexpressing cells migrated faster than control cells in a scratch wound assay (LeClair and Lindner, 2007). CTHRC1 can also stabilize frizzled receptor-ligand complex on the cell surface and activate the Wnt/PCP pathway (Yamamoto et al., 2008). CTHRC1, together with TIMP3, cystatin C (CST3) was expressed both in myoepithelial cells and myofibroblasts in DCIS and invasive breast carcinomas (Allinen et al., 2004).

1.7.2. Periostin

Periostin, originally isolated as an osteoblast-specific factor 2, is a disulfide-linked secretory protein. It is a unique ECM protein found in collagen-rich connective tissues and is highly expressed in the embryonic periosteum, cardiac valves, placenta, periodontal ligaments and in many adult tissues. Its deposition was augmented by an increase in mechanical pressure and it acts as a critical regulator of bone and tooth formation and maintenance. Periostin was re-expressed after myocardial, vascular and skeletal muscle injuries and bone fracture (reviewed in Ruan, 2009). Shao et al. (2004) described periostin as a mesenchyme-specific gene whose level was undetectable in normal breast tissues and cell lines. However, it was overexpressed in breast cancer. Acquired expression of periostin by human breast cancers led to a significant enhancement in tumor progression and angiogenesis. Periostin levels were also elevated in breast cancer patients with bone metastasis (Sasaki et al., 2003). Periostin mRNA and protein were found to be dramatically increased in breast cancer tissues and their lymph node metastases, and this increase correlated with TNM stage of breast cancer (Zhang et al., 2009). Periostin was also involved in the development of a number of tumors including lung, colon, pancreatic and ovarian cancers and thymoma. For example, 42% of patients with non small cell lung cancer showed high periostin positivity and this expression was associated with tumor size, disease stage, metastasis and lymph node invasion (Takanami et al., 2008). In another study, associaion between periostin stromal and/or epithelial expression and density of both circulatory and lymphatic microvessels was revealed. Periostin association with disease relapse and poor survival has also been reported (Sasaki et al., 2001). In pancreatic cancer, periostin was predominantly expressed by stromal rather than cancer cells, while the latter stimulated stromal cells to secrete periostin (Kanno et al., 2008; Erkan et al., 2007). Periostin promoted cell survival and invasion in colon (Bao et al., 2004) and cell motility in ovarian (Gillan et al., 2002) cancers. In thymoma and neuroblastoma it correlated with tumor progression (Sasaki et al., 2001b and Sasaki et al., 2002).

1.7.3. Versican

Versican, also known as chondroitin sulfate proteoglycan 2 (CSPG2), is a member of the large aggregating chondroitin sulfate proteoglycan family. It exists in at least four different isoforms (V1-4) created by alternative splicing of mRNA from a single gene (Wight and Merrilees, 2004). Versican is one of the main components of the extracellular matrix that

regulates hygroscopic properties and creates a loose and hydrated matrix which is necessary to support key events in development and disease. Versican binds to other ECM components such as hyaluronan, type I collagen, tenascin-R, fibronectin, chemokines and cell surface proteins (CD44, integrin β1, EGFR, and P-selectin glycoprotein ligand-1) (reviewed in Rahmani et al. 2006). The versican-rich extracellular matrix possesses anti-adhesive properties and has the ability to modulate proliferation and the migration of different cell types, including osteosarcoma cells, astrocytoma cells, smooth muscle cells and various types of fibroblasts (Zheng et al., 2004; Nikitovic et al., 2006; Wight, 2008). Versican has been described in a number of tumors, most frequently in melanomas and breast cancer (Rahmani etal., 2006). There was a close relationship between cell differentiation degree and melanoma cell differentiation where differentiated cells did not express any versican isoform and undifferentiated cell lines produced V0 and V1 isoforms (Touab et al., 2002). In primary pancreatic cancers, a high amount of CSPG2/versican was detected in the desmoplastic stroma while cancer cells were immunonegative (Mauri et al. 2005). To demonstrate that CSPG2/versican is released by cancer cells *in vivo*, the authors set up an experimental model consisting in Suit-2 cells resuspended in an amorphous matrix (Matrigel), xenografted in the nu/nu mice, and allowed to proliferate for one week. The formalin-fixed paraffin-embedded implant was then immunostained with an anti-human versican antibody and the analysis clearly demonstrated that secretion does occur in cancer cells. Accumulation of anti-adhesive versican was reported in prostate cancer (Cross et al. 2005) and its enhanced expression was also found in our microdissected breast cancer cells (Turashvili et al., 2007).

1.7.4. Asporin

Asporin, also known as periodontal ligament-associated protein 1 (PLAP1) was primarily identified by three research groups in 2001 (Henry et al. from Houston, Lorenzo et al. from Lund/London/Tampa and Yamada et al. from Osaka). The name asporin reflects the unique aspartate stretch at the N terminus and 70% similarity to decorin (Henry et al., 2001). Asporin mRNA was expressed primarily in the skeleton (perichondrium /periosteum of cartilage/bone) and other specialized connective tissues (tendon, sclera of the eye, the connective tissue sheath surrounding muscle and dermis). Hybridization of asporin cDNA probe to dot blot of mRNAs from human fetal and adult tissues showed the highest expression in aorta and uterus. Besides others, moderate expression of asporin was found in mammary gland and low expression in

prostate (Henry et al. 2001). Lorenzo et al. (2001) reported association with osteoarthritis, which has later been extensively studied by others (Kizawa et al. (2005); Gruber et al., (2009); Sakao et al., (2009); Van Der Kraan et al., (2010)). Asporin blocks chondrogenesis and inhibits TGF-β1-induced expression of matrix genes and the resulting chondrocyte phenotypes (Nakajima et al., 2007). Small interfering RNA-mediated knockdown of asporin increases the expression of cartilage marker genes and TGF-β1; in turn, TGF-β1 stimulates asporin expression in articular cartilage cells, suggesting that asporin and TGF-β1 form a regulatory feedback loop. Asporin inhibits TGF-β/Smad signaling upstream of TGF-β type I receptor activation *in vivo* by co-localizing with TGF-β1 on the cell surface and blocking its interaction with the TGF-β type II receptor (Nakajima et al., 2007). Increased TGF-β production is the hallmark of a number of fibrotic diseases that are characterized by abundant accumulation of extracellular matrix components. Asporin, like decorin can bind collagen at the same site, but in contrast to decorin and biglycan, it drives collagen biomineralization (Kalamajski et al., 2009). In this sense, ASPN is involved in the regulation of the initial calcium deposition in the predentin layer and plays an important role in tooth mineralization (Lee et al. 2010).

Our laboratory has identified asporin as a novel cancer-related protein in invasive breast cancer (Turashvili et al. 2007). We have studied microdissected cells of both normal and cancer origin by whole genome Affymetrix U133 Plus 2.0 Arrays. Asporin was found to be upregulated in cancer, in particular in lobular carcinomas. Asporin was also reported among upregulated genes after aromatase inhibitor treatment in primary breast cancer (Mackay et al. 2007). Importantly, asporin can be found as a cancer related gene also in other microarray articles, however, the authors neither discussed nor focused on the asporin in detail (please see Discussion). Only recently, asporin was specifically reported in pancreas and prostate cancer by two additional groups (Turtoi et al. 2011; Orr et al. 2011). ASPN expression was significantly higher in ECM of pancreatic adenocarcinoma , than inflammatory or normal pancreatic tissue; In prostate cancer, asporin was expressed in CAFs and in small subset of epithelium.

1.7.5. Wnt5a

Wnt5a is a member of nontransforming subclass of Wnts, which also includes Wnt4 and Wnt11 and all they may trigger intracellular Ca^{2+} release to activate Ca^{2+}-sensitive enzymes in a G-protein-dependent manner (Slusarski *et al.*, 1997; Kuhl *et al.*, 2000). This results in various

cellular effects and inhibition of the canonical Wnt signaling pathway. Wnt5a is implicated in cell polarity, adhesion, and motility (Moon et al., 1993, Pandur et al., 2002). It binds the ROR-2 receptor activating JNK and the cytoskeleton as well as inhibiting β-catenin/TCF -dependent transcription. Wnt5a reveals its inhibitory effect on β-catenin/TCF-dependent transcription through Shia-1 (McDonald and Silver, 2009). Wnt5a signals noncanonically through frizzled receptors and do not liberate β-catenin. However, in the presence of Frizzled 4 and LRP5, Wnt5a can activate β-catenin/TCF-dependent transcription. Wnt5a can also activate PKA, which in turn can inhibit GSKβ to promote β-catenin/TCF-dependent transcription. Wnt5a is also identified as a downstream target for TGFβ in mammary gland development (Roarty and Serra, 2007) and consequential loss of TGFβ and Wnt5a redirect tumor phenotype so that they resemble tumors induced by activation of Wnt/β-catenin (Roarty et al., 2009). There are controversies about Wnt5a function: it has been shown to behave as a putative oncogene and also as a tumor suppressor gene in different cancers (Leris et al., 2005; Da Forno et al., 2008). In several cellular models including hematopoietic tissue, breast and uroepithelial cancers, it inhibits tumor cell proliferation (Olson et al., 1998; Liang et al., 2003; Dejmek et al., 2005). Moreover, Wnt5a has been identified as a good prognostic marker in breast cancer patients (Dejmek et al., 2005). This probably depends on its role within a multi-step pathway and in the variety of ways in which its production can be stimulated or attenuated. According to which frizzled receptor is present, Wnt5a may activate distinct pathways. Therefore, the observed function of Wnt5a is entirely dependent upon its context, hence the controversy about its function in tumorigenesis.

1.7.6. Nestin

Nestin is an intermediate filament (IF) protein that was originally described in 1990 as a neuronal stem cell/progenitor cell marker during central nervous system (CNS) development (Lendahl et al., 1990). Other IF protein members, for example, keratin in epithelial cells, vimentin in mesenchymal cells, desmin in muscular cells, neurofilament in neuronal cells, and glial fibrillar acidic protein in glial cells are expressed in specific cell types.

Nestin is expressed in dividing cells during the early stages of development in the central and peripheral nervous system as well as myogenic and other tissues. During differentiation, nestin is downregulated and replaced by tissue-specific IF proteins, and therefore, is widely used

as a neuronal stem cell marker. Nestin is also expressed in immature or progenitor cells in non-neuronal cells in normal tissues (Ishiwata et al., 2011).

High levels of nestin expression have been detected in oligodendroglial lineage cells, ependymocytes, Sertoli cells, enteric glia, hair follicle cells, podocytes of renal glomeruli, pancreatic stellate cells, pericytes, islets, optic nerve, and odontoblasts (Ishiwata et al., 2011). In adult organisms, nestin-expressing cells are restricted to defined locations, where they function as a cellular reserve that is capable of proliferation, differentiation, and migration after reactivation (Wiese et al., 2004).

In pathological conditions, nestin is expressed in repair processes in the central nervous system, liver, muscle and infarcted myocardium. Increased nestin expression has been reported in various tumors, including central nervous system tumors, pancreatic cancer, gastrointestinal stromal tumors, prostate cancer, breast cancer, malignant melanoma, dermatofibrosarcoma protuberances, and thyroid tumors and was associated with poor prognosis. Recently, nestin has also received attention as a cancer stem cell marker in brain tumors, uterine and cervical cancers, prostate cancer, bladder cancer, head and neck cancers, ovarian cancer, testicular cancer, pancreatic cancer, and malignant rhabdoid tumors. In the tumor tissues, proliferating vascular endothelial cells also highly express nestin, and nestin is therefore closely correlated with tumor angiogenesis (Ishiwata et al., 2011).

2. AIMS OF THE STUDY

Extracellular matrix provides structural and mechanical support to cells and tissues, and also has an important role in the regulation of gene expression, cell division, survival, shape, and movement. Interactions during which the tumor creates a microenvironment favourable for proliferation, for the recruitment of new blood vessels, and for the stimulation of the production of proteases that can degrade adjacent tissues, increase the likelihood of tumour development and invasion.Tumour development can be viewed as a process where cells are constantly subjected to mutations and these multiple alterations occur in epithelial cells. However, there is also growing evidence that changes in the microenvironment of cancer cells can promote their proliferation. This specialized stroma has an abundance of inflammatory cells and activated fibroblasts both expressing extracellular matrix components and growth factors that support survival and proliferation of tumour cells in a paracrine fashion. Growing evidence points towards a key role of the extracellular matrix in epithelial plasticity, modulation of tumor environment and hence tumor progression. Breast and prostate cancers are most frequently diagnosed cancers in woman and man, respectively. Understanding of the mechanisms of their development and progression is of crucial importance. Therefore, aims of the presented dissertation were:

1. To study collagen triple helix repeat containing 1 protein, periostin and versican expression in breast cancer and evaluate their prognostic significance in cancer progression.

2. To evaluate importance of nestin, Wnt5a and asporin in modulation of breast tumor environment, their significance in neoangiogenesis, importance for invasive growth and tumor progression.

3. To investigate whether androgen depletion induces senescence and neuroendocrine differentiation and modulate tissue environment in prostate cancer.

3. PATIENTS, MATERIAL AND METHODS

3.1. Patients

3.1.1. Breast cancer

Archival tissue samples of 173 invasive breast cancer, 27 lymph node metastases, 36 precursor lesions and 31 normal tissues (adjacent to a tumor, see chapter 3.1.2 for details), 7 autopsy cases of breast cancer bone metastases were obtained from patients between years 1996 and 2008. The study was approved by the Ethics Committee of the Faculty of Medicine and Dentistry, Palacky University. The patient set was categorized into WHO histological subtypes [invasive ductal (IDC), lobular (ILC) and other (see chapter 3.1.2 for details)]. We have previously found higher levels of ASPN and CTHRC1 mRNA in ILC than IDC (Turashvili et al., 2007), therefore, ILC cases were specifically selected to reach sufficient sample size. Luminal, Her2 and triple negative surrogates of molecular subtypes were defined according to expression of ER, PR and Her2 (triple negative set was previously generated for another project). Patient median age was 60.5 (range 30-86 years). The study was censored on 8th October 2010 with median follow-up 54 months (range 2-159 months). Other characteristics of patients and tumors are summarized in Table 2 and Table 3 (distribution of histotypes into IHC subtypes).

3.1.2. Histological characterization of precursor breast lesions and special type carcinomas

In the precursor group we included both atypical hyperplasia and carcinoma in situ (tumor adjacent; Lopez-Garcia et al. 2010). There were 3 cases of atypical ductal hyperplasia and 4 cases of atypical lobular hyperplasia. Carcinoma in situ group consisted of 17 cases of DCIS, 1 case of DCIS matched with ductal intraepithelial neoplasia grade 3, and 11 cases of LCIS.

Special type carcinomas were as follows. Eight cases of medullary carcinoma, 4 cases of invasive micropapillary carcinoma, 2 cases of invasive papillary carcinoma, tubular carcinoma and medullary carcinoma matched with invasive papillary carcinoma, and one case of secretory carcinoma, tubulolobular carcinoma, adenoid cystic carcinoma and secretory carcinoma matched with adenoid cystic carcinoma.

Table 2. Patients and tumor characteristics.

Characteristics	Subgroups	N	%
Histology	IDC	97	56.3%
	ILC	53	30.8%
	other subtype	22	12.9%
Grade	G1	13	7.5%
	G2	79	45.7%
	G3	81	46.8%
Molecular subtype	Her2	22	12.7%
	luminal	58	33.5%
	triple negative	93	53.8%
Nodal status	negative	103	59.5%
	positive	59	34.1%
	not specified	11	6.4%
Menopausal status	postmenopausal	96	55.5%
	premenopausal	15	8.7%
	not specified	62	35.8%
Stage	I	44	25.4%
	II	92	53.2%
	III	13	7.5%
	IV	3	1.7%
	not specified	21	12.1%
Distant metastases	none	106	61.3%
	bone	21	12.1%
	other organs	27	15.6%
	not specified	19	11.0%

Table 3. Distribution of histotypes into subtypes

Histology	Subtype	Frequency	%	Valid %
IDC	Her2	16	16.5	16.5
	luminal	14	15.5	15.5
	triple negative	67	68.0	68.0
	Total	97	100.0	100.0
ILC	Her2	3	5.7	5.7
	luminal	42	79.2	79.2
	triple negative	8	15.1	15.1
	Total	53	100.0	100.0
OTHER	Her2	3	13.6	13.6
	luminal	1	9.1	9.1
	triple negative	18	77.3	77.3
	Total	22	100.0	100.0

3.1.3. Prostate cancer

Formalin-fixed, paraffin-embedded prostate tumor samples were obtained between 1998 and 2003 years (Table 4). This small patients set was generated in order to verify in-vitro results on effects of androgen deprivation therapy (ADT). Samples were either fine-needle biopsies (before neoadjuvant ADT) or prostatectomies (after neoadjuvant ADT). Untreated control prostate-ctomies were randomly selected from another project.

Table 4. Characteristics of prostate cancer patients

Patient No	Age at dg	PSA at dg	Neoadjuvant therapy	Tissue	pT	pN	GS
1	55	83.5	HFM	both	pT3b	pN0	5+3
2	68	108.4	BIC	both	pT3b	pN1	3+4
3	54	60	HFM,LHRH	both	pT4	pN0	3+3
4	60	9.7	BIC, HFM,LHRH	both	pT2b	pN0	3+4
5	64	52	HFM	after	pT3a	pN1	4+5
6	58	29.1	HFM	after	pT2b	pN0	3+4
7	50	47.2	HFM	after	pT3b	pN1	4+5
8	54	24.39	HFM, CPA	after	pT3b	pN0	4+5
9	66	8.2	HFM	after	pT2b	pN0	3+5
10	63	5.7	BIC, HFM	after	pT2b	pN0	3+4
11	59	8.5	none	before	pT2b	pNx	3+4
12	66	7.07	none	before	pT2b	pN0	4+4
13	69	8.22	none	before	pT3a	pN0	2+2
14	63	9.8	none	before	pT2c	pNx	3+4
15	66	32	none	before	pT2b	pN0	3+2
16	66	8.29	none	before	pT2b	pN0	4+5

Tissue, type of tissue available with respect to the neoadjuvant therapy (before, after, both); BIC, bicalutamide; CPA, cyproterone acetate; dg, diagnosis; GS, Gleason score, HFM, hydroxyflutamide; LHRH, luteinizing-hormone-releasing hormone analog; pNx, no lymphadenectomy; pT, pathologic T stage

3.2. Analysis of formalin-fixed paraffin-embedded tissues

3.2.1. Immunohistochemistry

Four-micrometer-thick sections were dewaxed in xylene, hydrated through graded series of alcohol (96%, 80% and 70%) and rinsed in deionized water. Detailed information on the antigen retrieval procedures, antibodies and their dilutions is provided in Table 5. After antigen retrieval, slides were rinsed in tap and deionized water. For blocking endogenous peroxidase activity, slides were treated with 0.3% hydrogen peroxide solution for 15 min. Sections were washed in deionized water for 5 min, then twice in 0.05M Tris buffer (pH 7.4-7.6), once in Tris buffer with 0.5% Tween solution and incubated with primary antibody for 1 hour at room temperature in a humid chamber. Slides were washed twice in Tris buffer, once in Tris buffer with 0.5% Tween solution, and incubated with secondary antibody (Dual Link, Dako) for 1 hour. After the last washing step in Tris buffer, slides were incubated in substrate solution (DAB), counterstained in haematoxylin, dehydrated through alcohols and xylene and mounted.

Specimens were assessed semiquantitatively by histoscore [percentage positivity of epithelial cells or of area of interest (non-epithelial region for stroma evaluation)] in 10% increments multiplied by staining intensity (categorized as 0-absent, 1-weak, 2-moderate and 3-strong), resulting in histoscore (from 0-minimum to 300-maximum). Non-representative, damaged or non-evaluable cases were regarded as missing values. Score 0 was regarded as negative, where scores ranging 10-90 were regarded as low, 100-190 as moderate and 200-300 as high positivity. In addition, moderate and high positivities were regarded as high positivity when appropriate. For each experiment, an additional negative control was included in which primary antibody was replaced by non-immune serum.

Neoangiogenesis was assessed as a number of nestin positive newly formed vessels. Degrees of tissue fibrosis (percent of fibrotic transformation in tumor area) and inflammation (existance of inflammatory infiltrate categorized as 1-weak, 2-moderate and 3-high) was also evaluated on hematoxylin-eosin-stained slides.

3.2.2. Validation of antibodies

CTHRC1

Melanoma cells are recommended as a positive control for CTHRC1 rabbit polyclonal antibody (Abcam, Ab54181). The image of strongly positive invasive melanoma, provided by Abcam (http://www.abcam.com/CTHRC1-antibody-ab54181.html), is identical with Figure 3 from Tang et al. (2006), who generated antibody against C-terminus of the protein. We used invasive melanoma tissues and normal skin as positive and negative controls, respectively. The same antibody has recently been used for CTHRC1 staining in dermatofibrosarcoma protuberans and dermatofibroma (Wang et al. 2010).

Periostin

Periostin rabbit polyclonal antibody (Biovendor, RD181045050) has previously been used for breast cancer samples, e.g. Grigoriadis et al. (2006) and Puglisi et al. (2008). Like these authors, we also found positivity of breast cancer cells and negative or faint staining of normal breast tissue.

Versican

Versican mouse monoclonal antibody (DSHB, 12C5) was used by Erdelyi et al. (2003) and Castronovo et al. (2007). The first article reported precise co-localization with versican clone 2B1 (Seikagaku Co., Tokyo, Japan) in canine mammary tumors. The authors of the second article validated clone 12C5 using human cancer and benign breast tissues as well as various normal tissues. Both clones should be negative in cytoplasm of normal and benign epithelium, which is consistent with our observations for the clone 12C5. Apart from the whole patient set, several other samples were used for confirmation of strong ECM positivity in invasive areas of breast cancer.

Asporin

Our in-house rabbit polyclonal antibody (immunization by 16aa peptide within the eighth LRR domain of asporin; produced by Dr. Vojtesek, Masaryk Memorial Institute, Brno) was validated on uterus tissue as a positive control. Uterus was found as a tissue with the highest expression of asporin at Gene Expression Omnibus database (GDS1096). Until 2010, Abcam

antibody (#58741, distributed also by GenWay) was the only commercial antibody with published results (Gruber et al. 2009, Lee et al. 2010; specification of antibodies in the recent articles Turtoi et al. 2011 and Orr et al. 2011 is not clear). We also used Abnova antibody (#H00054829-D01P) which is raised against the whole recombinant protein. All antibodies showed similar staining pattern in the uterus tissue (endometrial glandular cells**,** Figure 11). The strongest staining of the lobular cancer was observed with our in-house antibody. Abcam antibody peptide is overlapping with our one, however, it contains an asparagine which may be abundantly glycosylated in cancer (Brooks, 2009) and subsequently Abcam antibody epitopes may be masked.

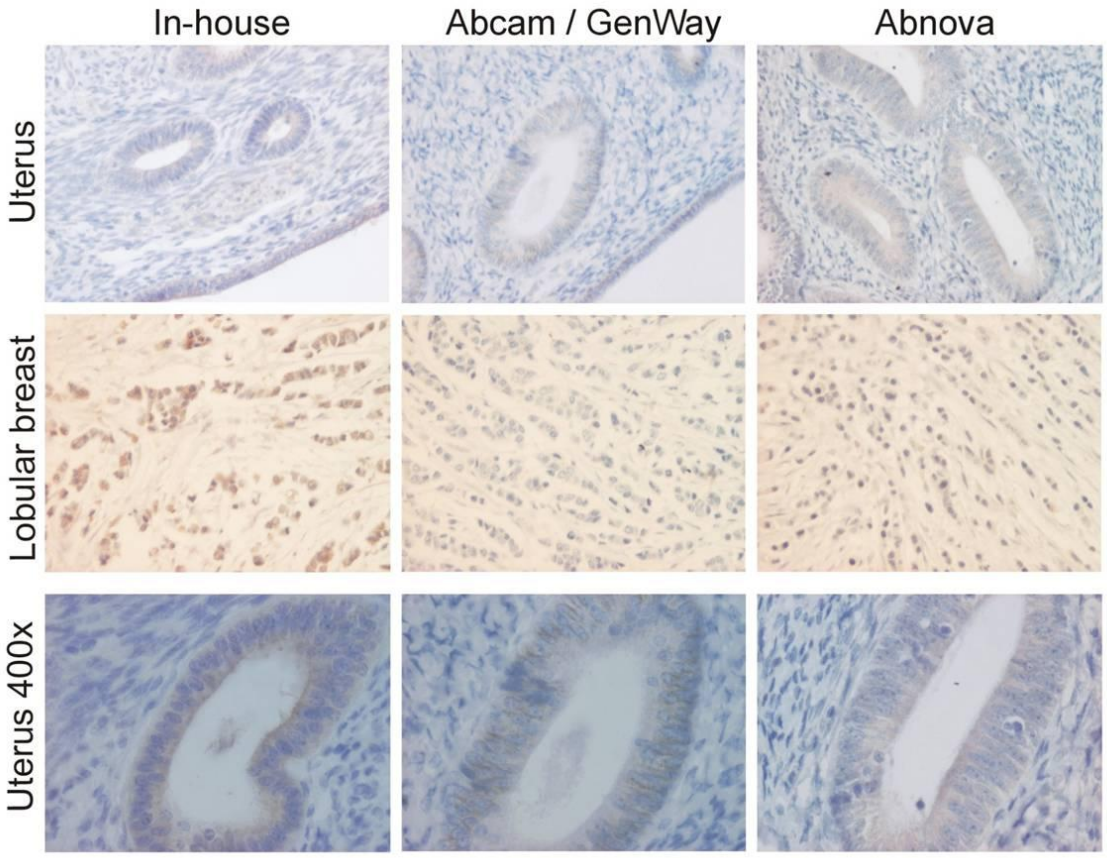


Figure 11. Validation of asporin antibodies.

Table 5. Antibody and method specification

Antibody	Species	Clone	Antigen retrieval method	Dilution	Supplier
Primary antibodies used for breast samples					
Asporin	rabbit	polyclonal	MW, sodium citrate, pH 6.0, 12 min	1:200	in-house
CTHRC1	rabbit	polyclonal	WB, EDTA, pH 9.0, 20 min	1:400	Abcam, Ab54181
Versican	mouse	12C5	No antigen retrival	1:50	DSHB
Periostin	rabbit	polyclonal	No antigen retrival	1:1500	Biovendor, RD181045050
β-catenin	mouse	E-5	MW, sodium citrate, pH 6.0, 12 min	1:20	Santa Cruz
E-cadherin	mouse	NCH-38	MW, sodium citrate, pH 6.0, 12 min	1:50	Dako
Nestin	mouse	10C2	MW, sodium citrate, pH 6.0, 12 min	1:100	Chemicon
Wnt5a	rabbit	polyclonal	MW, sodium citrate, pH 6.0, 12 min	1:100	Abcam, Ab72583
ER	mouse	1D5	MW, sodium citrate, pH 6.0, 12 min	1:20	Dako
PR	mouse	PgR636	MW, sodium citrate, pH 6.0, 12 min	1:100	Dako
c-erbB-2	rabbit	polyclonal	MW, sodium citrate, pH 6.0, 12 min	1:5000	Dako, A0485
Primary antibodies used for prostate samples					
NSE(γ-enolase)	mouse	BBS/NC/VI-H14	MW, sodium citrate, pH 6.0, 12 min	prediluted	AbD serotec
Chromogranin A	mouse	LK2H10	No antigen retrival	prediluted	AbD serotec
Vimentin*	mouse	V9	MW, sodium citrate, pH 6.0, 12 min	prediluted	Dako
Secondary antibody					
Dual Link (Anti- mouse and anti-rabbit)					Dako

DSHB (Developmental Studies Hybridoma Bank, WB (water bath), MW (microwave); *used for both breast and prostate

3.2.3. Dual immunofluorescence and confocal microscopy

Prostate cancer FFPE sections were dewaxed in xylene twice for 10 min each, hydrated through graded series of alcohol (96%, 80% and 70%) and rinsed in deionized water. Microwave antigen retrival was done with Dako buffer High pH, at 98°C for 20 min. Slides were cooled, rinsed in deionized water and treated with 0.5% Sudan Black in 70% ethanol solution for 5 min, rinsed in tap water for 3 min and treated with Image iT^{TM} FX signal enhancer (200 µl for each slide) for 30 min in a humid chamber. Sections were washed in PBS+Tween buffer 4X for 5 min and incubated with primary antibody (mouse monoclonal anti-NSE, clone BBS/NC/VI-H14, AbD serotec, prediluted; and rabbit polyclonal anti-vimentin antibody, clone C-20, dilution 1:200, Santa Cruz) overnight at 4^0 C in a humid chamber. Slides were washed in PBS+Tween buffer 4X for 5 min and and incubated with secondary antibodies (Alexa-Fluor-488; 1:200 and Alexa-Fluor-594; 1:200) for 30 min in the dark. After the next washing step, nuclei were visualized by counterstaining with DAPI (4', 6-diamidino-2-phenylindole, AppliChem). Slides were washed again in PBS+Tween buffer 4X for 5 min, mounted in fluorescein mounting medium Mowiol 4-88/DABCO (Calbiochem) and viewed on a Olympus DP 71 fluorescence microscope (Palacky University, Olomouc) and LSM Leica SP5 confocal microscope (confocal microscopy was performed by Dr. Karel Souček, Institute of Biophysics, Brno).

3.2.4. Statistical analysis

Comparisons between different groups were determined by Mann-Whitney U or Wilcoxon Signed Ranks tests, while correlations between quantitative variables (histoscores) were assessed by the Spearman's rho test. Pearson's X^2 was used to check associations between qualitative variables (contingency tables) and for the assessement of the strength of such associations, Phi (r_φ) and Cramer's V were used. Degree of association was assessed according to Cohen's scale. For survival and Cox regression analysis, relapse-free survival was defined as the time from initial surgery to the date of clinically documented local or distant recurrence (event) or to the time of last clinical examination (censored value). Kaplan-Meier survival and Cox regression analyses were done for CTHRC1 and periostin as prognostic factors of bone metastasis. Ordinal regression analysis (logit model) was performed to define Wnt5a as a factor for patient having lower grade tumor; and logistic regression analysis was done to define odds of cancer-related death in relation to Wnt5a expression. For all regression analyses, the Wald chi-

square test was used to assess hypotheses and the performance of models was checked by (likelihood) X^2. For logistic and ordinal regression analyses additional model fit tests were performed such as Deviance and Pearson Goodness-of-Fit $X^2$and Hosmer & Lemeshow X^2 (for the model fit, P should be less than 0.05). P-value less than .05 was considered as statistically significant. All tests were performed using SPSS version 15.0 (SPSS, Inc., ***Chicago***, IL) statistical analysis software.

3.3. Molecular biology methods

3.3.1. Cell lines

All cells were grown in a humidified incubator (37°C, 5% CO2). Breast cancer cell lines HS578T and MDA MB 231 were obtained from European Collection of Cell Cultures (ECACC). Both cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM), containing 10% foetal bovine serum (FBS), L-glutamine and antibiotics (Invitrogen). Medium for HS578T was enriched by 10 µg/ml bovine insulin (Invitrogen). Prostate cancer LNCaP cells (obtained from DSMZ) were cultivated in phenol red-free RPMI 1640 media (Invitrogen) supplemented with NaHCO3, penicillin/streptomycin, and 5% fetal bovine serum (PAA). Prostate cancer LAPC-4 cells (kind gift from Prof. Zoran Culig, University of Innsbruck) were cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with NaHCO3, penicillin/streptomycin, 10% FBS and 1 nM R1881 (PerkinElmer). For androgen depletion studies, LNCaP cells were cultivated in 5% dextran/charcoal-stripped FBS (CS) and LAPC-4 cells were cultivated in IMDM with 10% CS.

3.3.2. RNA isolation, qRT-PCR and expression analysis

Total RNA was isolated by the GenElute™ Mammalian Total RNA Miniprep Kit(Sigma-Aldrich), quantified by Nanodrop, pretreated with DNAse I (Invitrogen) and reverse transcribed with SuperScript® III Reverse Transcriptase (Invitrogen). The real-time PCR reactions were performed with Light Cycler® 480 Probes Master Mix for 50 cycles of denaturation, annealing and extension (95°C-60°C-72°C each for 20 seconds) on LightCycler® 480, Roche. Relative quantification was carried out according to the ΔC_t method using a reference gene ($\Delta C_t = C_{t\ ASPN} - C_{tTBP}$). Primers and probes were as follows: ASPN specific and UPL, TBP-hex.

Expression of asporin in both tissues and cell lines was also queried in databases Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo), ArrayExpress (http://www.ebi.ac.uk/arrayexpress) and Oncomine (www.oncomine.org).

3.3.3. Collagen *in vitro* fibrillogenesis and invasion assay

Pepsin-extracted acid-solubilized collagen (Purecol, Nutacon) was neutralized by 0.1 M NaOH and diluted in 150 mM NaCl buffered with 20 mM Hepes (pH 7.4). The collagen solution (200 ug/ml) was incubated with or without recombinant asporin (kind gift from professor Åke Oldberg, Department of Experimental Medical Science, University of Lund) at 37°C for 8 hours (inducing collagen fibrillogenesis). Starved cells were seeded on the collagen pre-coated transwells (pre-coating by bovine serum albumine was used as a control) while FBS was used as a chemoattractant in bottom wells. Cell impedance was monitored every 10 minutes for 48 hours (xCELLigence system, Roche).

3.3.4. Flow cytometry and senescence assessment

Trypsinized cells were fixed in 2% PFA at 4.C, permeabilized and incubated with appropriate antibodies (vimentin (V6389), serotonin (S5545), histamine (H7403), and IGFBP3 (HPA013357, all from Sigma) diluted in PBS with 300 mg/ml digitonin. Afterwards, the cells were washed and incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Far red LIVE/DEAD Cell Stain Kit (Invitrogen) was used for the exclusion of dead cells. Stained cells were analyzed on a FACSCalibur or FACS Aria II Sorp (Becton Dickinson) cell sorter, and flow cytometry data analyzed using FlowJo software (TreeStar). Senescence-associated β-galactosidase (SA-β-gal) cytochemistry was performed at pH 6.0 using X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Pierce) as previously described (Dimri et al., 1995), and photographed using an Olympus IX-71 microscope.

4. RESULTS

4.1. Collagen triple helix repeat containing 1 protein, periostin and versican in primary and metastatic breast cancer

CTHRC1 was predominantly positive in cancer cell cytoplasm. Lower expression was also observed in adjacent stroma (stromal cells and extracellular matrix with dominance of the latter) (Figure 12a-c). Periostin was either absent or low positive in cancer cells while being highly positive in stroma of almost all cases (Figure 12d-f). Versican was also less expressed in cancer cells than in stroma compartment (Figure 12g-i). The above mentioned predominant localizations refer to the results below, if not otherwise specified.

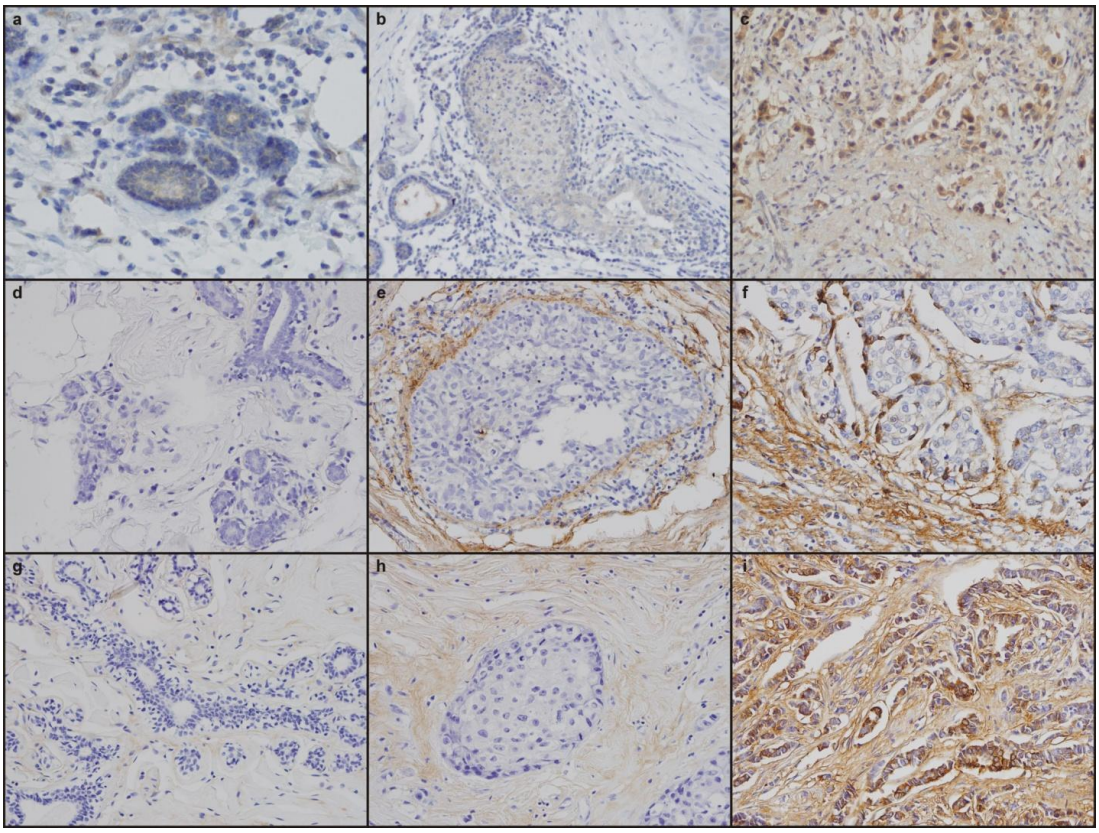


Figure 12. CTHRC1, periostin and versican immunohistochemical expression. a, b. Low to moderate CTHRC1 immunostaining of normal tissue and atypical ductal hyperplasia and **c.** strong CTHRC1 immunostaining of breast cancer epithelium and faint positivity of stroma. **d.** Negative (normal tissue), **e.** low positive (ductal carcinoma in situ) and **f.** strong periostin staining of breast cancer stroma and negative cancer cells. **g.** Negative (normal tissue), **h.** low positive (atypical lobular hyperplasia) and **i**. strong versican staining of breast cancer stroma with moderate positivity of cancer cells.

Expressions of CTHRC1, versican and periostin were significantly higher in breast cancer than in normal tissue and precursor lesions (both $p<0.001$, Figures 12 and 13a). Further, CTHRC1 and versican were overexpressed (both $p<0.001$) in invasive lobular carcinoma compared to invasive ductal ones, while periostin showed opposite trend ($p=0.012$, Figure 13b). Versican was also overexpressed in luminal subtype compared to both Her2 and triple negative cases (both $p<0.01$, Figure 13c). On the other hand, periostin was underexpressed in Her2 subtype compared to triple negative cases ($p=0.018$, Figure 13c). Importantly, its epithelial expression was higher in patients with positive lymph nodes compared to negative ones ($p=0.04$), while it was also expressed in lymph node metastases themselves.

There was no difference in epithelial expression of CTHRC1 between molecular subtypes but its stromal expression was significantly higher in triple negative cases than luminal ones ($p=0.013$, Figure 13c). The stromal expression of CTHRC1 was higher in patients with bone metastasis than in non-metastasizing cases ($p=0.04$), while, there were no significant differences with respect to other distant metastases. Our study cohort

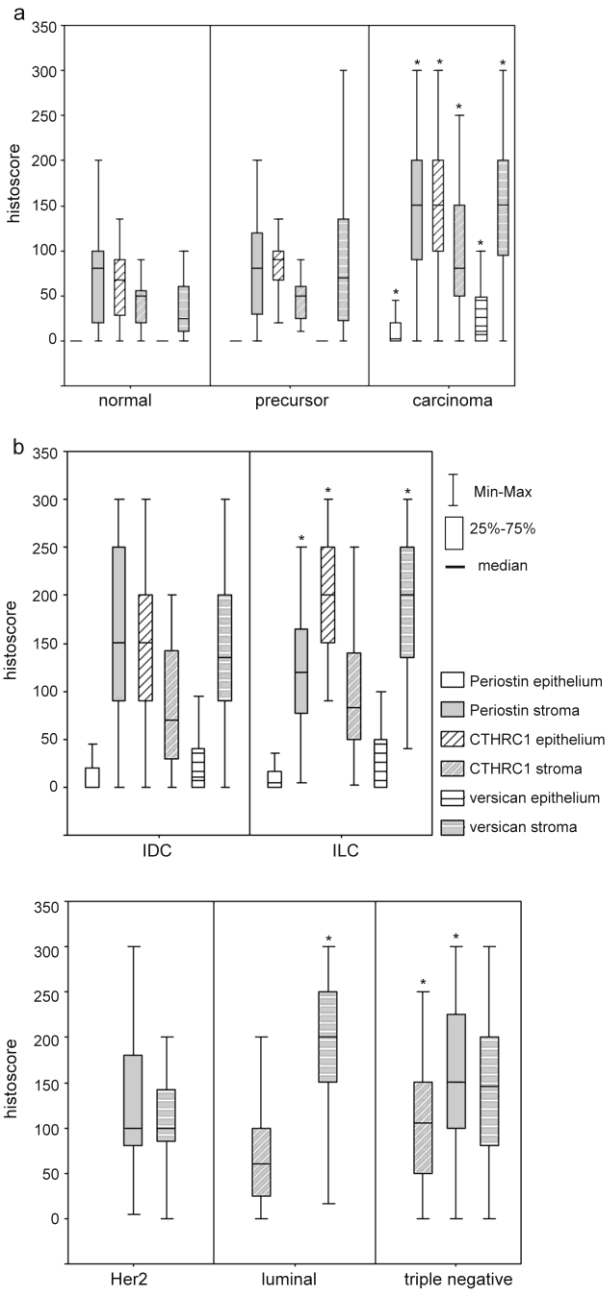


Figure 13. Differential expression of CTHRC1, versican and periostin. a. in normal tissue, precursor lesions and cancer. **b.** in invasive ductal and lobular carcinomas. Significant differences are marked with asterisk. **c.** Expression of CTHRC1, versican and periostin in immunohistochemical subtypes. Only significant differences are displayed.

contained a higher proportion of triple negative tumors, however, they did not metastasise to bone more often than other IHC subtypes (p=0.819).

In univariate Cox regression analysis, neither CTHRC1 nor periostin stromal expressions alone showed statistically significant relationship with bone metastasis (p=0.182 and p=0.053, respectively). However, the Cox regression analysis revealed that the risk of bone metastases increased with increase in CTHRC1 stromal expression in patients with high periostin stromal expression [HR=1.007, p=0.024; 95% CI (1.001-1.012)] (Figure 14a-c). The same patients had shorter time to relapse (bone metastasis) than patients with other combinations of CTHRC1 and periostin stromal expressions (p=0.006, Figure 14d).

Finally, the correlations for the studied proteins were calculated for either the whole patient set or in subgroups (Table 6). Besides these, membrane-associated β-catenin and E-cadherin were positively correlated (r=0.287, p=0.02), as expected. We have frequently observed partial loss of membrane-bound β-catenin and its accumulation in cytoplasm but nuclear β-catenin was found in only 2% of all patients. Neither membrane nor cytoplasmic β-catenin were associated with versican which is a target of the Wnt/β-catenin signaling pathway.

Table 6. Significant associations (Spearman's rank correlations) between CTHRC1, periostin and versican and other variables.

Variable	Variable	Group [a]	r_s	p-value
CTHRC1 epithelial	CTHRC1 stromal	IDC and ILC	0.45 and 0.51	both <0.001
CTHRC1 epithelial	E-cadherin	luminal	-0.413	0.014
CTHRC1 epithelial	membranous β-catenin	luminal	-0.564	<0.001
CTHRC1 epithelial	versican epithelial	IDC	0.477	0.045
CTHRC1 epithelial and stromal	membranous β-catenin	G3	-0.4 and -0.46	0.012 and 0.002
CTHRC1 stromal	ER and PR	IDC	-0.35 and -0.34	both 0.001
CTHRC1 stromal	tumor grade	Other hist. subtype	0.467	0.04
CTHRC1 stromal	versican stromal	IDC	-0.35	0.003
periostin epithelial	versican epithelial and stromal	whole set	0.35 and 0.28	0.003 and 0.016
periostin stromal	versican epithelial and stromal	whole set	0.42 and 0.44	both <0.001
versican stromal	Tissue fibrosis	G3, advanced stage	0.52 and 0.55	0.012 and 0.008
versican stromal	patients age	patients with relapse	0.424	0.006

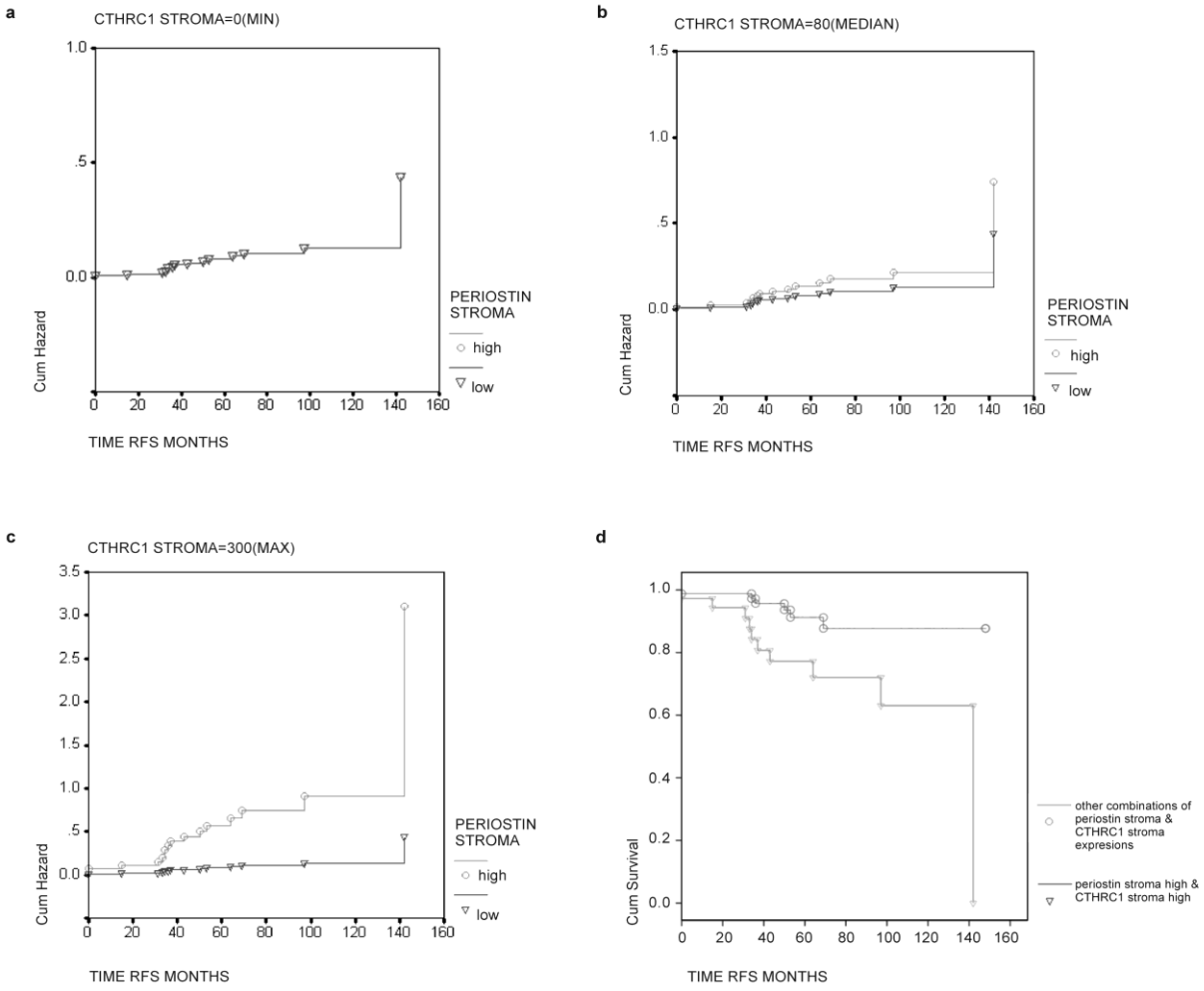


Figure 14. Cox regression hazard function. a. When CTHRC1 stroma histoscore is 0, hazard of bone metastasis between periostin high and low groups are same. **b.** When CTHRC1 stroma histoscore is 80 (median) hazard is higher in periostin high group in comparison to low group; **c.** when CTHRC1 stroma histoscore is 300 (max) difference between hazard of high group in comparison to low group is the highest (HR=1.007; p=0.024). Periostin stroma low group was used as a reference variable. **d.** Kaplan-Meier survival plot showing prevalence in time to relapse (bone metastasis) of CTHRC1 and periostin stroma high group vs other combinations of CTHRC1 and periostin expression. RFS, relapse-free survival.

4.2. Importance of nestin, Wnt5a and asporin in breast cancer

4.2.1. Importance of nestin in neoangiogenesis and triple-negative breast cancer

Nestin positivity was found in 32.9% of carcinomas with the highest expression in other histotypes (Figure 16b, p=0.044). Both CTHRC1 stromal (p=0.013, please see above) and nestin epithelial expression (Figure 16c, p<0.001) were higher in the triple negative subtype than in other subtypes. We found strong association between nestin expression in cancer cells and CTHRC1 stromal expression in advanced stage patients (r=0.614; p=0.007). Further, nestin was highly expressed by endothelium (in 75.7%) of all cases (Figure 15c). In this sense, neoangiogenesis was more evident in triple negative than in luminal or Her2 subtypes (p=0.036). Nestin expression was also associated with vimentin expression in breast cancer cells (r=0.491; p<0.001). Besides stromal positivity, vimentin was expressed in 38% of all cases in breast cancer

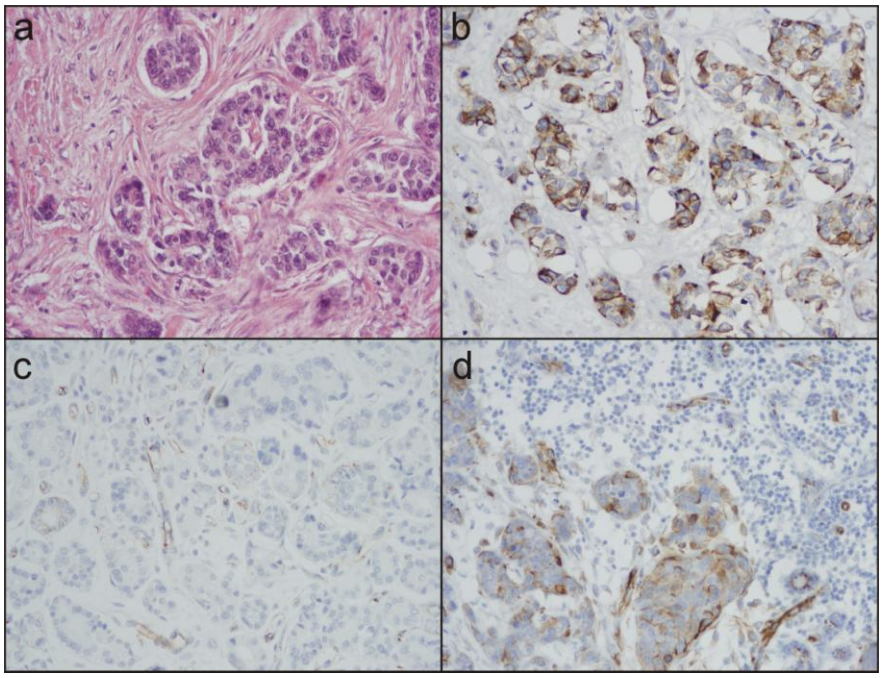
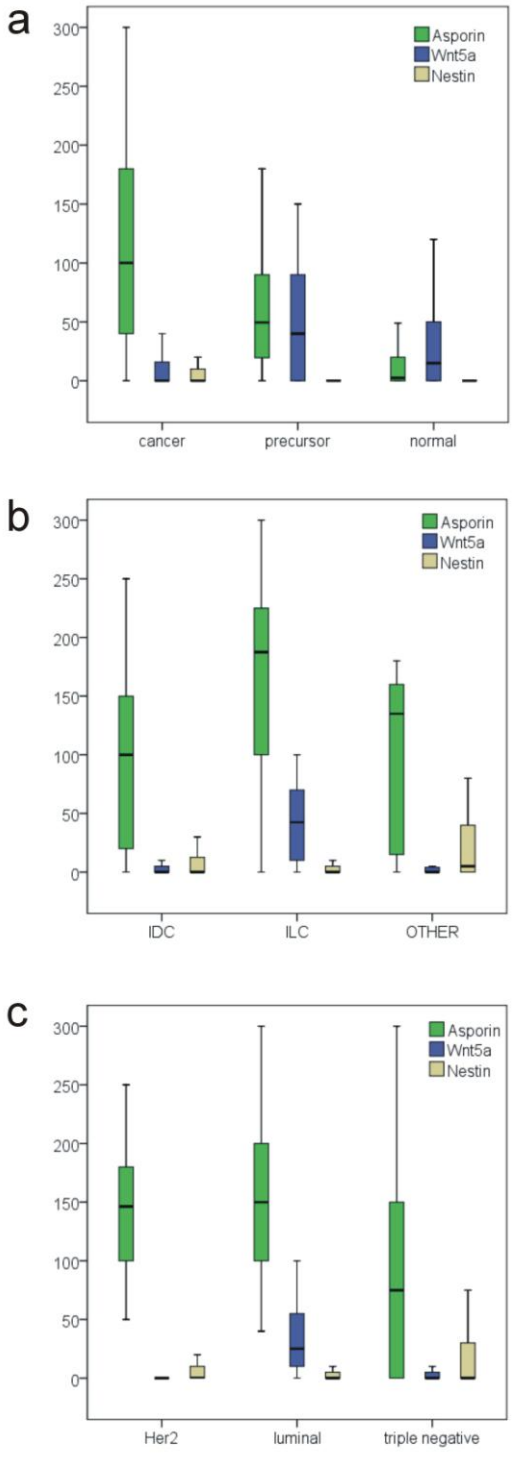


Figure 15. Nestin expression in breast cancer. a, b. Hematoxylin-eosin and nestin-positive slides from the same patient (invasive ductal carcinoma). **c.** Nestin positive newly formed endothelium; **d.** Nestin positive cancer epithelium and endothelium.

epithelium. Both nestin positivity and vimentin expression showed positive association with degree of inflammation in the whole set (r_φ=0.317 and 0.369; both p<0.001), in triple negative (r_φ=0.422 and 0.521; both p<0.001) and high grade patients (r_φ=0.374 and 0.311; p=0.017 and p<0.001). We also observed higher nestin positivity in patients with lymph node metastases and high periostin stromal expression (p=0.031).



4.2.2. Wnt5a as a positive prognostic factor in breast cancer

We also aimed to investigate the role of Wnt5a molecule in breast cancer progression. Our results show that Wnt5a protein expression was lost in 51.5% of breast cancer patients, while positive samples ranged between histoscore 1-100 (full histoscore range is usually 1-300). Its expression was higher in normal (p=0.042), and precursor (p=0.001) breast samples in comparison to the breast cancer group (Figure 16a). Wnt5a positivity was significantly higher in ILC, than in IDC (p<0.001) (Figure16b and 18a,b). Luminal subtype showed higher expression of Wnt5a than Her2 and triple negative cases (both p<0.001). Wnt5a expression was also higher in patients who did not relapse in comparison to patients with distant metastases (p=0.026).

Figure 16. Expression of asporin, Wnt5a and nestin in **a.** breast cancer, precursor lesions and normal breast tissues; **b**. different histotypes **c.** different IHC-based subtypes. Box and whisker plots display median, 25%-75% percentiles and minimal/maximal values.

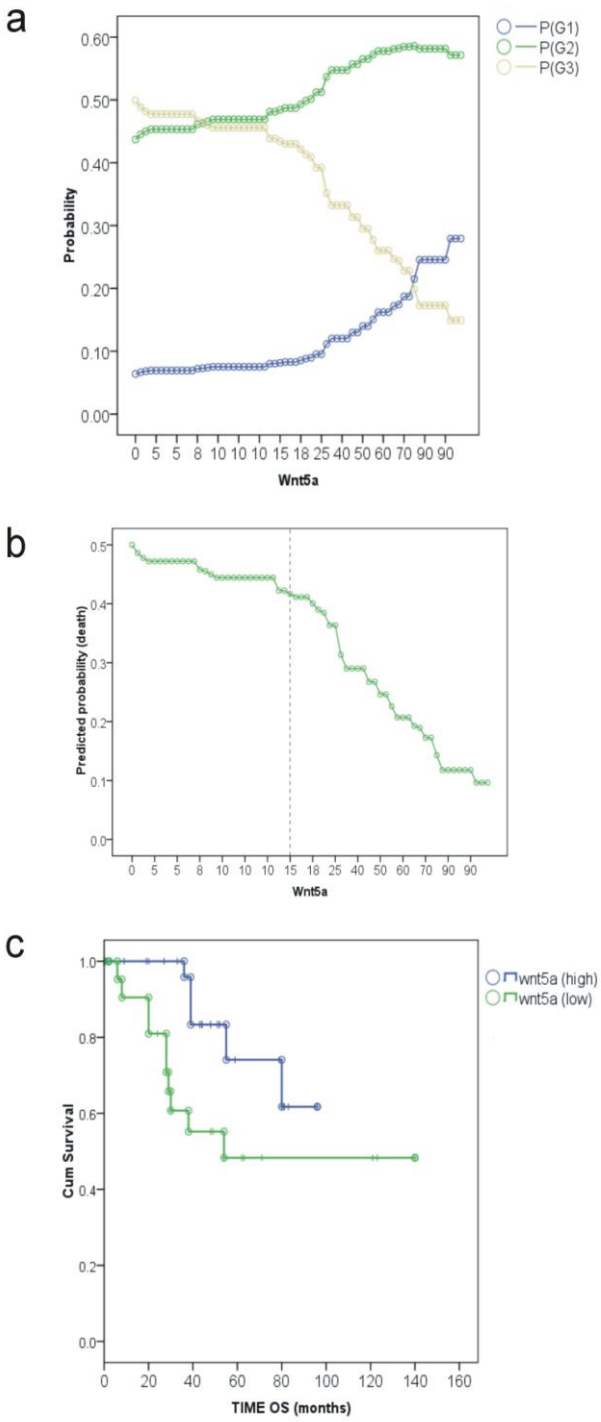


Figure 17. **Association of Wnt5a expression with tumor grade, risk of death and overall survival. a**. Probabilities of having lower tumor grade (G1-3) in relation to Wnt5a expression (each patient displayed in all three lines); **b**. Probability of cancer-related death in relation to Wnt5a expression; **c.** Kaplan-Meier survival plot showing better overall survival (OS) of Wnt5a high group (histoscore ≥15) in comparison to Wnt5a low group (histoscore<15).

Ordinal regression analysis revealed that odds of being patients in lower grade is increasing by 17.2 % with every increment of 10 in Wnt5a histoscore [(Figure 17a), 95% CI 4.7%-29.6% (p=0.007; OR=0.983)]. Similarly, increase in Wnt5a expression by each 10 histoscore decreases odds of cancer-related death by 22% [(Figure 17b, 95% CI 8% - 36% (p=0.003; OR=0.978)]. We have observed shorter overall survival in patients with low Wnt5a expression in comparison to group with high Wnt5a expression (Figure 17c, p=0.033).

Wnt5a was found to be positively correlated with estrogen and progesterone receptors (r=0.499 and 0.461 respectively; both p<0.001). Similarly, positive correlation with ER and PR was observed in patients with negative lymph node status (r=0.507 and r=0.474, respectively; both p<0.001).

In our breast cancer set, E-cadherin was completely lost in 31.8 % of all cases, among them in 51.9% of lobular carcinomas, 22.7% of ductal carcinomas and 44% of other histotypes.

Membrane-associated β-catenin and E-cadherin showed positive correlation in whole set ($r=0.314$; $p<0.001$), in particular in Her2 and luminal ($r=0.650$, $p=0.002$; $r=0.651$, $p<0.001$), but not in triple negative subtypes. Underexpression of Wnt5a was associated with low levels of membrane-associated β-catenin ($r=0.327$ and $p=0.048$) in ILC; and Wnt5a was significantly higher in E-cadherin-positive ILC patients in comparison to E-cadherin-negative ones (Mann-Whitney test, $p=0.048$ [1-tailed]). We also found positive association between Wnt5a and asporin expression ($r=0.326$; $p<0.001$).

4.2.3. Asporin protein expression in normal breast tissue, precursor breast lesions and breast carcinoma

We have studied asporin protein expression in breast cancer samples, normal tissue and precursor lesions, as well as autopsy specimens of breast cancer bone metastases. Levels of asporin were significantly higher in breast cancer samples compared to normal tissues and precursor lesions ($p<0.001$; Figure 16a), and as expected, higher in invasive lobular carcinomas than ductal ones ($p<0.001$; Figure 16b and 18c,d).

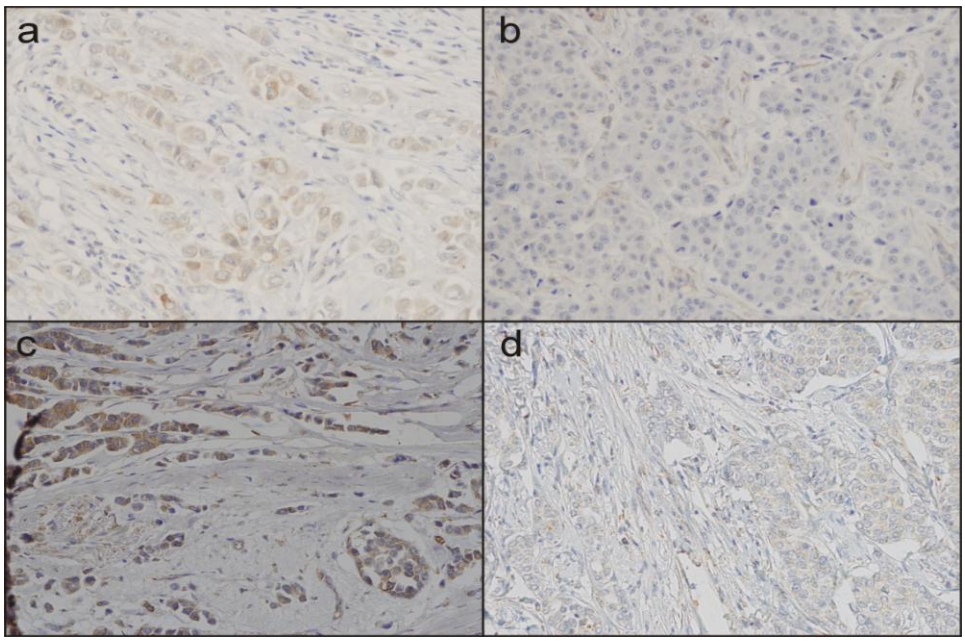


Figure 18. Expression of Wnt5a and asporin in invasive lobular and ductal carcinomas. a. Faint Wnt5a positivity in ILC and **b.** negativity in IDC. Asporin expression in **c.** invasive lobular and **d.** ductal carcinomas. Note high asporin positivity of cancer epithelium and lower positivity of adjacent stroma in ILC; both epithelium and stroma are faint positive in IDC.

Asporin was less expressed in triple negative patients ($p<0.001$; Figure 16c) but it positively correlated with tumor grade within this subtype ($r_s=0.4$; $p<0.001$) but not in luminal and Her2 subtypes. Asporin was also highly expressed in all breast cancer bone metastases. However, its expression in primary tumor was not associated with metastatic potential.

4.2.4. Asporin expression in breast cancer cell lines and its importance for invasive growth

The expression of asporin was tested by RT-PCR in selected breast cancer cell lines, however, we did´t find any breast cancer cell line overexpressing asporin (please refer also to Figure 20). We have also searched microarray databases for any positive cell line which pointed to Hs578T cells with slightly higher signals than other breast cancer lines (please refer to GSE15026 at http://www.ncbi.nlm.nih.gov/gds). The expression of asporin in this cell line was confirmed by RT-PCR and mechanistic experiments are still on-going (e.g. aspn modulation by shRNA or TGF-β). Because of the lack of asporin-positive cell lines (we have identified Hs578T cells later in the project), we have used recombinant asporin (kind gift of prof. Oldberg, University of Lund) for in-vitro experiments. MDA-MB-231 cells migrated faster through collagen I with recombinant asporin as documented by real-time cell analysis xCELLigence system (Figure 19).

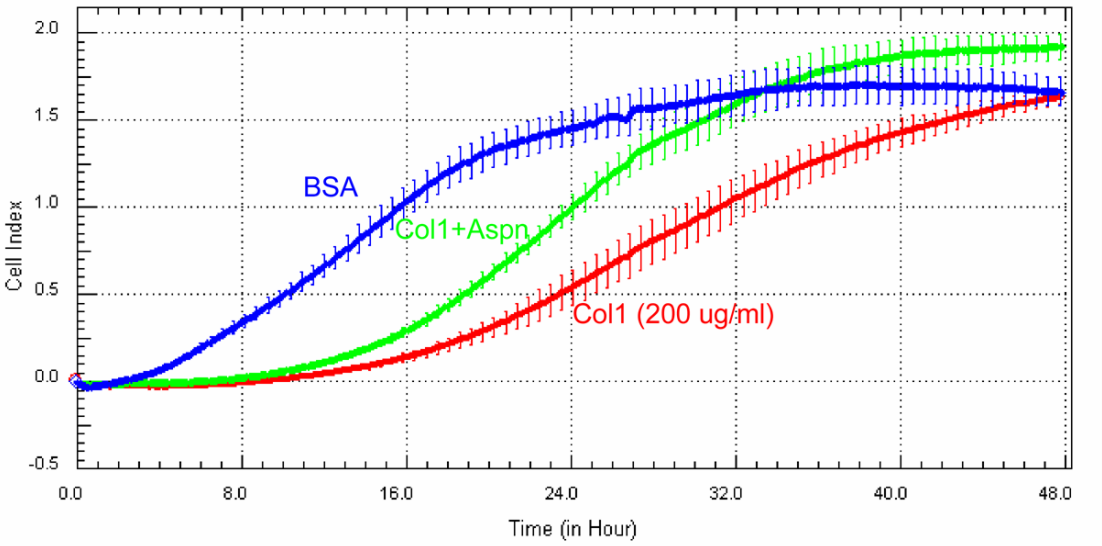


Figure 19. Migration of MDA-MB-231 cells through collagen 1 with asporin using real-time cell analysis xCELLigence systém. Upper transwells were coated by collagen with or without recombinant asporin (BSA was used as a control). Starved cells (for 8 hours) were seeded on the collagen while 10% FBS was used as a chemoattractant in bottom wells. Asporin enhanced invasion of the cancer cells through collagen 1 matrix**.** (performed by Dana Simkova).

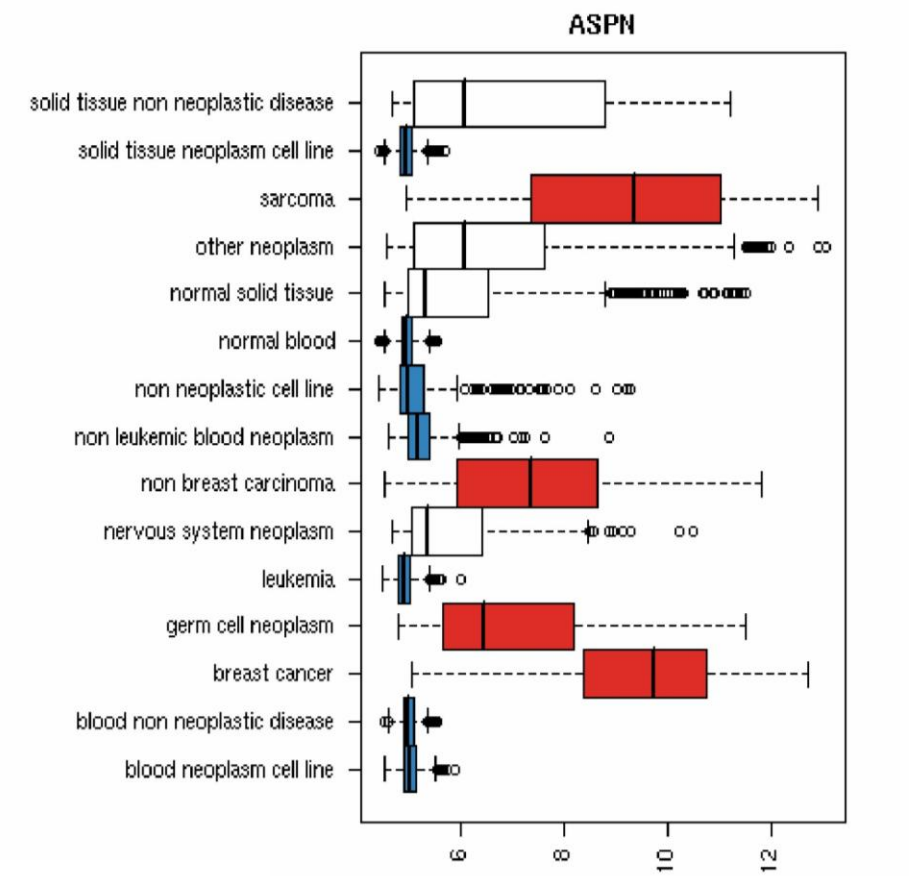


Figure 20. **Asporin expression in the Global Map of Human Gene Expression.** Lukk et al. (2010) constructed the map by integrating microarray data from 5372 human samples representing 369 different tissue types, disease states and cell lines (http://www.ebi.ac.uk/gxa/array/U133A). Asporin overexpression is displayed in red while underexpression in blue.

4.3. Modulation of tissue environment by senescent cells in prostate cancer

4.3.1. Androgen depletion induces senescence associated secretory phenotype and neuro-endocrine differentiation in prostate cancer cells

One possible mechanism for the development of androgen-independent prostate cancer is modulation of the tissue microenvironment by neuroendocrine (NE)-like cancer cells, which emerge following androgen-deprivation therapy (ADT).

Long term androgen depletion induces cell cycle arrest without apoptosis in LNCaP and LAPC-4 prostate cancer cells. In many cell types, irreversible cell cycle arrest correlates with senescence. Using a cytochemical reaction, many LNCaP cells were strongly positive for SA-β-gal (senescence associated β-galactosidase) after 16 days growth without androgens (Figure 21). Quantification of SA-β-gal reactivity using a fluorescent substrate showed that androgen depletion led to a statistically significant increase in SA-β-gal activity in both LNCaP and LAPC-4 cells compared to control cells cultivated in the presence of androgens. Senescent phenotype of prostate cancer cells following ADT was also documented by other markers (decreased telomerase activity and senescence-associated secretory phenotype).

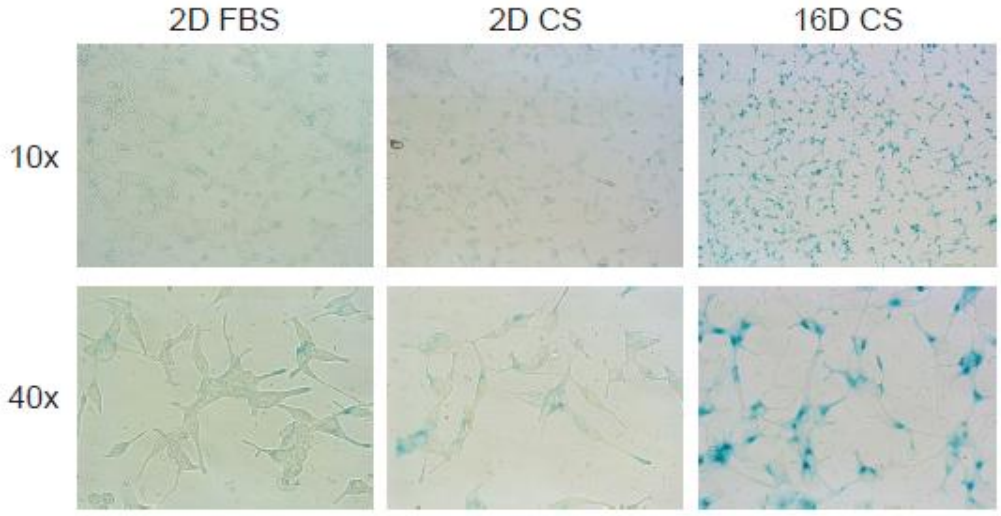


Figure 21. Induction of senescence by androgen deprivation. Cytochemical detection of SA-β-gal activity in LNCaP cells cultivated in presence (FBS) or absence (CS) of androgens for 2 or 16 days (2D, 16D) (performed by Z. Pernicova).

NED, a frequent finding following ADT of human prostate cancer patients (Nelson et al., 2007), was also observed in our *in vitro* model system, based on increased levels of widely used the NED markers γ-enolase and tubulin β-III. To determine if functional NE-like cells were present in our cultures, we used flow cytometry to analyze intracellular levels of serotonin and histamine. Cells cultivated for 8 days under androgen-depleted conditions expressed higher intracellular levels of both these NE markers compared with cells cultivated in the presence of androgens.

In summary, these data show that long term androgen depletion in our *in vitro* model induced irreversible senescence, and that this was associated with increased expression of senescence associated secretory factors and markers of NED. Further details are available in the original article Pernicova et al. 2011, as an attachment of the thesis.

4.3.2. Androgen depletion induces vimentin and neuroendocrine markers in prostate cancer cell lines and tissues

To further investigate the phenotype of prostate cancer cells undergoing senescence and NED in response to androgen depletion we analyzed the expression of cytokeratins and vimentin, which are markers of epithelial cells and mesenchymal cells, respectively. Using a pan-cytokeratin antibody, we found that androgen depletion upregulated the expression of several cytokeratins. Surprisingly, we also found that vimentin was also strongly induced by androgen depletion that was confirmed using flow cytometry (Figure 22a) and confocal microscopy (Figure 22b). Vimentin expression in epithelial cells may indicate an epithelial-mesenchymal transition (EMT) (Zeisberg and Neilson, 2009). To examine whether EMT occurred in our cultures, we examined expression of N-cadherin and E-cadherin, which are up-regulated and down-regulated, respectively, during EMT (Lee et al., 2006). N-cadherin was not expressed by LNCaP cells either before or after androgen depletion, and expression of the epithelial marker E-cadherin was not significantly down-regulated following androgen withdrawal. These data indicate that the up-regulation of vimentin following androgen depletion is unlikely to be due to EMT. Since vimentin is also expressed in senescent fibroblasts (Nishio et al., 2001), it is possible that induction of vimentin in our model is indicative of senescence.

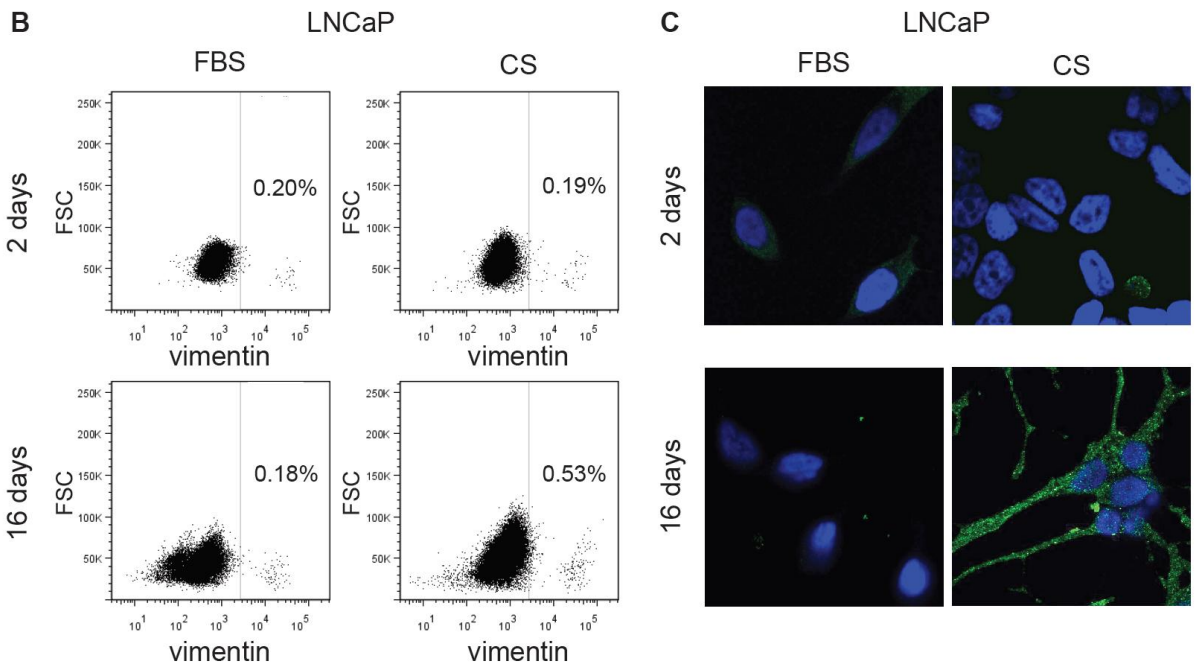


Figure 22. Androgen depletion induces expression of vimentin in LNCaP cells. a. Flow cytometric analysis showing the percentage of cells expressing high levels of vimentin. Similar results were obtained from three independent experiments. **b.** Immunocytochemical detection of vimentin in cultured cells. (Performed by Z. Pernicova).

We wanted to know if induction of vimentin occurred in human prostate tumors following ADT and examined expression of vimentin, as well as the NED markers chromogranin A and γ-enolase, in samples of human prostate cancers collected pre- and/or post-neoadjuvant ADT (Table 4). Prior to ADT, expression of vimentin, chromogranin A and γ-enolase in epithelial cells of the prostate tumor samples were either undetectable or at low levels (Figure. 23a,b; pre, epi). Following ADT, expression of all three markers was significantly increased in epithelial cells in samples from the same individuals (Figure. 23a, post), and their scores were significantly higher (Figure. 23b, epi). In contrast, vimentin expression in stromal cells of the prostate was unaffected by ADT. Using dual color immunostaining and confocal microscopy, we found that vimentin was colocalized with γ-enolase, indicating that NE-like cells in human tumors express vimentin following ADT (Figure 24). These results show that induction of senescence and NED are associated with expression of vimentin in epithelial prostate cancer cells.

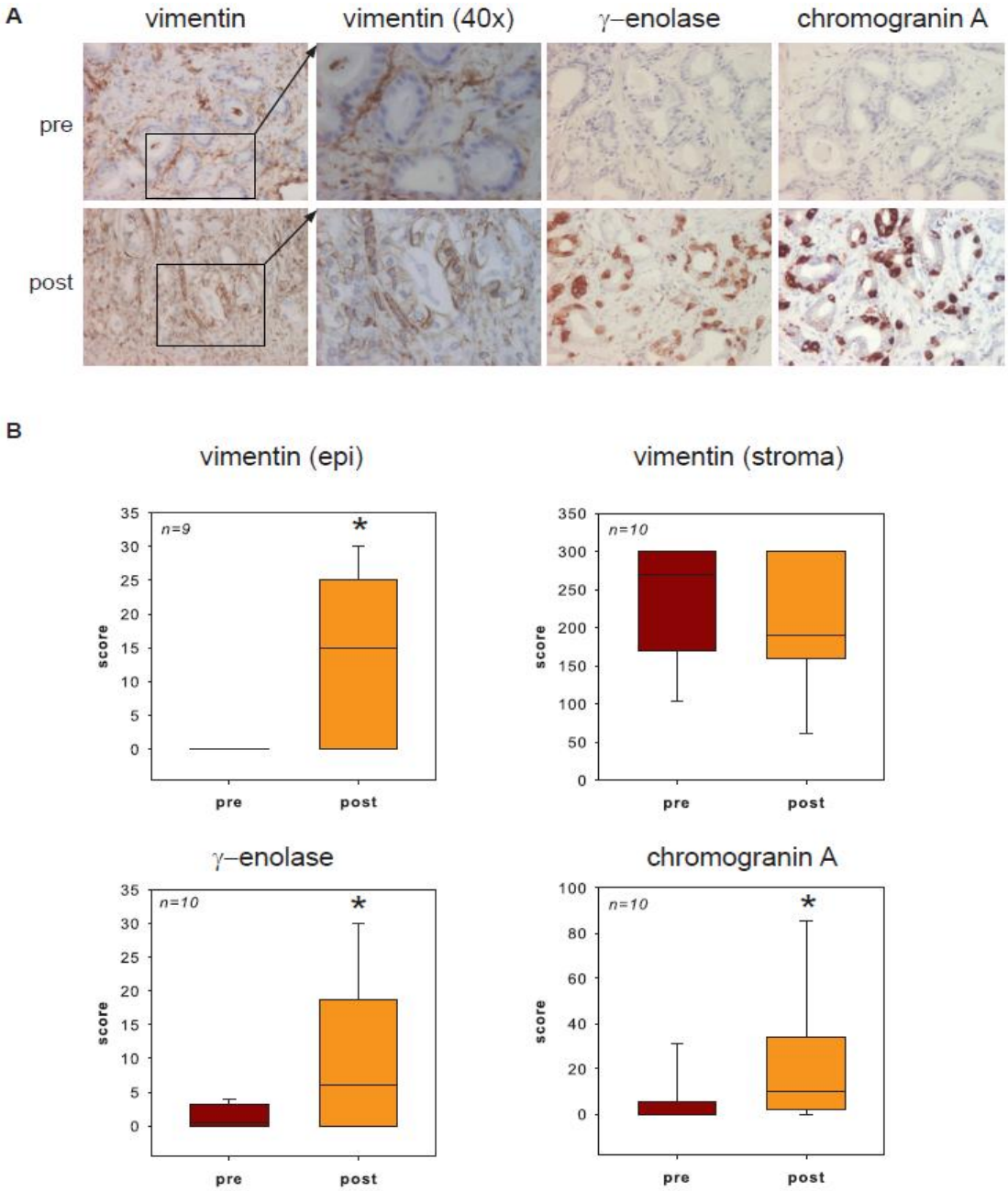


Figure 23. Up-regulation of vimentin and NED markers in human prostate cancer epithelial cells following neoadjuvant ADT. a. Immunohistochemical detection of γ-enolase, chromogranin A, and vimentin. Sample pairs are from the same individual pre- and post-neoadjuvant ADT. Expression of vimentin in epithelial and stromal cells is easily distinguishable at 40. magnification (inset). **b.** Quantification of γ-enolase, chromogranin A, and vimentin expression in patient tumor samples.

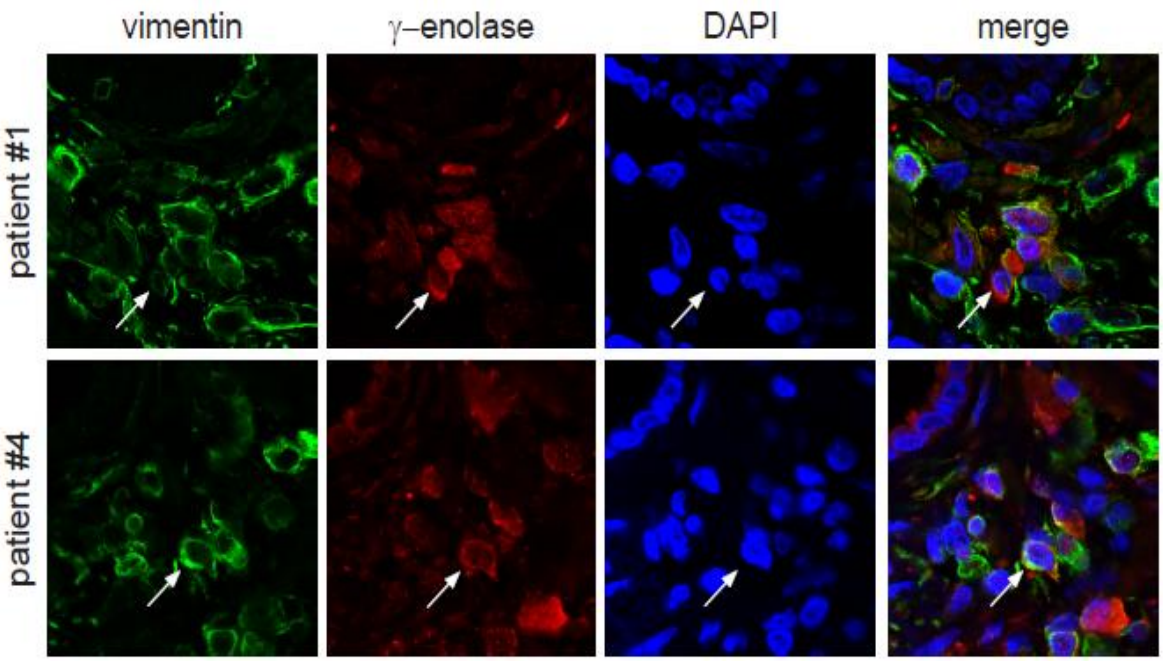


Figure 24. Immunofluorescent staining for vimentin and γ-enolase in prostate cancer samples following ADT. White arrows indicate epithelial cells co-expressing both markers.

5. DISCUSSION

Recent data in breast and other cancers suggest that modulation of tissue environment, epithelial plasticity and its manifestation such as epithelial-mesenchymal transition are potential mechanisms of tumor initiation and progression. In humans, early breast lesions are often present, however only a limited number of them progress towards malignancy. The high numbers of lesions not progressing into invasive carcinomas suggests that other mechanisms leading to malignancy must be involved including a "tumor prone" microenvironment (Hu et al., 2008). Animal tumor models have shown that fibroblasts overexpressing hepatocyte growth factor (HGF) or transforming growth factor beta (TGF-β) are able to induce formation of tumors at diverse sites including stomach and prostate (Bhowmick et al., 2004; Kuperwasser et al., 2004). These data suggest that microenvironment is crucial in the earlier steps of breast tumorigenesis. Later, towards progression to invasiveness and metastases, cancer cells become more autonomous and less affected by paracrine, negative feedback mechanisms that normally prevent outgrowth, excessive migration, dissemination and organ colonization. Alterations in the expression of ECM-related genes have frequently been identified in gene expression signatures related to poor prognosis and metastasis in breast cancers (Ramaswamy et al., 2003; Feng et al., 2007; Calvo et al., 2008; Ma et al., 2009). ECM homeostasis is maintained in the normal stroma by a tight balance between ECM synthesis, organization, cross-linking, and degradation. In the presence of tumor cells, ECM homeostasis is disrupted by the tumor cells themselves, by stromal components such as fibroblasts, macrophages, and leucocytes and by their interaction with tumor (Tlsty and Coussens, 2006; Jodele et al., 2006). Hence, induction of ECM remodeling and modulation of tumor microenvironment by these stromal components may lead to a permissive „soil" that enables tumor cells to escape from dormancy, invade and metastasize (Barkan et al., 2011).

5.1. CTHRC1, periostin, versican in primary and metastatic breast cancer

Using immunohistochemistry analysis we showed that collagen triple helix repeat containing 1 protein relates to breast cancer and is differentially expressed in normal tissue, precursor lesions and cancer. The first observations of CTHRC1 transcript in breast cancer were reported by microarray and in situ hybridization (West et al., 2005; Tang et al., 2006; Turashvili

et al., 2007). West et al (2005) described CTHRC1 and periostin as connective tissue markers which are expressed in breast cancer stroma and enhance tumor progression, while we have previously identified CTHRC1 as differentially expressed between ILC and IDC by laser microdissection and full-genome expression analysis (Turashvili et al., 2007). Tang et al. thoroughly examined CTHRC1 protein in melanomas with a brief transription survey for other solid cancers including breast (Tang et al., 2006). They didn't detect CTHRC1 in non-invasive stages of melanoma but it greatly enhanced in primary invasive melanomas and further upregulated in metastatic melanomas. Functional experiment showed that inhibition of CTHRC1 expression resulted in decreased cell migration *in vitro* (Tang et al., 2006). More recently, Tano et al. (2010) found that CTHRC1 knockdown affects cell motility in lung cancer, too. Like Tang et al. (2006) we found immunohistochemical positivity of CTHRC1 predominantly in cancer cells with lower expression in surrounding stroma. Consistent with our previous data, CTHRC1 protein was upregulated in ILC compared to IDC. In the luminal subtype, CTHRC1 positivity in cancer cells correlated negatively with membrane β-catenin and E-cadherin, the proteins that are frequently altered in ILC. CTHRC1 stromal expression was significantly higher in triple negative patients and negatively correlated with estrogen and progesterone receptor status. These results suggest that CTHRC1 may be significant for tumor development and more aggressive behavior of breast cancer cells. In this sense, Wang et al. (2010) has recently reported high levels of CTHRC1 in dermatofibrosarcoma protuberans which is a locally aggressive spindle cell neoplasm that frequently recurs and metastasizes.

Periostin and versican are other proteins affecting the tumor microenvironment. Like others (Nara et al., 1997; Soikkeli et al., 2010), we observed their peri-lobular and mesh-like staining patterns with high positivity in peripherial areas of infiltrating carcinomas while expression was either absent or less intense in normal tissues and precursor lesions. Periostin stable overexpression in tumorigenic but not metastatic 293T cells, was found to induce a fibroblast-like transformation characterized by increased expression of specific proteins such as vimentin, EGFR and MMP-9 (Yan and Shao, 2006). Further, the acquired expression of periostin in breast cancer cells upregulated vascular endothelial growth factor receptors in endothelial cells and promoted tumor growth and angiogenesis *in vivo* (Shao et al., 2004).

We observed that periostin expression was higher in patients with positive lymph nodes than in patients with negative lymph nodes and, in addition, periostin was also expressed in their

lymph node metastases. Similarly, Soikkeli et al. (2010) reported simultaneous upregulation of POSTN, CSPG2, FN1 and COL-1 genes in melanoma lymph node metastases. These proteins can form intricate fibrillar networks around tumor cell nests in melanoma and breast cancer metastases. Enhanced expression of periostin and other mesenchymal genes was also found in breast cancer patients and, importantly, periostin positivity was associated with their poor outcome (Grigoriadis et al., 2006). Periostin was also expressed by circulating endothelial cells (Grigoriadis et al., 2006) and elevated in the serum (Contie et al., 2011), both in cancer patients with metastatic disease.

We also found significant increase in CTHRC1 stromal expression in patients with bone metastasis compared to non-metastatic ones. The significance of this observation was further tested by Cox regression analysis which revealed that the risk of bone metastases increased with increase in CTHRC1 stromal expression in patients with high periostin stromal expression. The patients with high CTHRC1 and periostin stromal expression had also shorter time to relapse (bone metastasis) than patients with other combinations of CTHRC1 and periostin stromal expressions. Bone mass regulation and the balance between osteoblastic bone formation and osteoclastic bone resorption are mediated by multiple pathways including the parathyroid hormone, TGF-β and BMPs (Smirnov et al., 2006; Barginear et al., 2009). In this sense, BMP2-induced CTHRC1 is required for the maintenance of bone homeostasis (Theriault, 2010) and CTHRC1 overexpression in primary breast tumor stroma may contribute to osteotropic cancer cell invasion to the bone. Our study cohort contained high proportion of triple negative tumors with generally poor prognosis. Nevertheless, they were reported to develop metastases more often to visceral organs, but not to bone, when compared with non-triple-negative tumors (Liedtke et al., 2008; Dent et al., 2009). CTHRC1 stromal expression was indeed significantly higher in our triple negative patient group. However, as triple negative breast cancer tends to develop metastases mostly to visceral organs (Dent et al., 2009; Liedtke et al., 2008), besides this, our metastatic triple negative patients had distant metastases to other organs in 57.1% and to bone in 42.9%. We have also checked association between triple negativity and bone metastasis on our triple negative patients and Fisher's exact test revealed no association between them. Of course, our observation needs validation both on larger cohorts and by functional experiments.

5.2. Importance of nestin, Wnt5a and asporin in breast cancer

Nestin is an intermediate filament (IF) protein whose expression has been confirmed in stem/progenitor cells of the dermis, hair follicles, intestine, pancreas, bone marrow as well as in neural, muscle and other tissues (Mokry et al., 2004). Its expression is downregulated in the course of differentiation and subsequently replaced by another type of IF (Cattaneo and McKay, 1990; Sjoberg et al., 1994). Nestin expression has also been detected in the endothelium (Sugawara et al., 2002), and nestin expression was noted in newly formed human blood vessels (Kolar et al., 2007).

We found nestin positivity in 32.9% of carcinomas with the highest expression in other histotypes composed of papillary, micropapillary, medullary, secretory, tubular, tubulolobular and adenoid cystic carcinomas. We have confirmed previous observation that nestin positivity in cancer cells was associated with triple negativity/basal phenotype which might also indicate higher proportion of cancer stem cells in this subtype (Kolar et al., 2007). Further, nestin was highly expressed in endothelium of neovessels (in 75.7% of all cases) with the most profound neoangiogenesis in triple negative subtype. One of the mechanisms by which tumor environment controls tumor growth, invasion and metastasis is vascularization process. Tumor vascularization is required for the transition of epithelial cells into a mesenchymal state (Rouhi et al., 2010). In this sense, nestin expression was associated with vimentin expression in breast cancer cells that supports previous observation where nestin was associated with mesenchymal phenotype in highly metastatic prostate cancer cells (Kleeberger et al., 2007). We also observed higher nestin positivity in patients with lymph node metastases and high periostin stromal expression. Both nestin and periostin were associated with the potential of metastasis to lymph nodes in breast (Grigoriadis et al., 2006; Liu et al., 2010) and non small cell lung cancer (Sasaki et al., 2001; Chen et al., 2010). Finally, we have observed positive association between nestin positivity and degree of inflammation both in triple negative and high grade groups. Inflammation can induce lymphangiogenesis (Flister et al., 2010) which has been associated with high nestin expression (Chen et al. (2010) and poor outcome of breast carcinoma (Gu et al., 2008).

In summary, nestin, as a candidate marker for a cancer stem cell phenotype, showed association with mesenchymal markers vimentin, periostin, inflamation and neoangiogenesis which all can contribute to progression of advanced breast cancers, in particular of the triple negative subtype.

We also aimed to evaluate the expression of Wnt5a protein in breast cancer, normal tissues and precursor breast lesions, its relationship with E-cadherin and β-catenin adhesion molecules, and whether Wnt5a expression participates in breast cancer progression. According to our results, Wnt5a protein is lost in approximately one half of our breast cancer patients and its expression was higher in normal tissues and precursor breast lesions. This is in line with previous observations where Wnt5a is frequently lost in breast cancer and associated with an increased risc of relapse (Jonsson et al., 2002). Its effect on cell migration and invasion properties has been studied by Hansen et al. (2009) who showed that Wnt5a induces a cAMP response leading to Thr-34 phosphorylation of DARPP-32 and a subsequent downstream activation of CREB resulting in inhibition of breast cancer cell migration (Hansen et al., 2009). Similarly, majority of studies indicate that Wnt5a has tumor suppressing effect and is also decreased in colorectal cancer, leukaemias and neuroblastomas (reviewed in McDonald et al., 2009). Wnt5a overexpression or treatment of FTC-133 thyroid carcinoma cells with recombinant Wnt5a protein, decreased their proliferation, migration and invasiveness (Kremenevskaja et al., 2005). However, some investigators show its oncogenic effect in melanoma (Da Forno et al, 2008), breast cancer cells (Fernandez-Cobo et al, 2007), gastric (Kurayoshi et al, 2006), pancreatic (Ripka et al, 2007), prostate (Wang et al, 2007) and non-small-cell lung cancer (Huang et al, 2005). Fernandez-Cobo et al. (2007) studied Wnt5a mRNA expression by qPCR and found its overexpression in primary cells from metastatic patients, but not in established breast cancer cell lines. This needs to be further elucidated also with respect to potential difference in Wnt5a protein and mRNA expression as it was observed by Dejmek et al. (2005).

In our results, Wnt5a was found in higher levels in luminal subtype and positively correlated with estrogen and progesterone receptors in moderate and high grade patients. This is in support to results by Ford et al., (2009), however, their article has been retracted (due to other reasons which are not related to the above mentioned finding). Wnt5a was overexpressed in ILC, while its underexpression was associated with low levels of membrane-associated β-catenin in ILC. Wnt5a was significantly higher in membrane-associated β-catenin-positive ILC patients in comparison to negative ones, and similarly, Wnt5a was significantly higher in E-cadherin-positive ILC patients in comparison to E-cadherin-negative ones. Association between β-catenin and E-cadherin is regulated through phosphorylation of a serine-rich stretch of E-cadherin that comprises the β-catenin binding domain and by phosphorylation of the latter (Stappert and

Kemler, 1994). Wnt5a, through CKIα signaling can increase formation of β-catenin/E-cadherin complex (Medreck et al., 2009). These effects of Wnt5a are also in relation with the membrane-associated β-catenin translocation and c-myc oncogene suppression and are mediated through an increase in intracellular Ca^{2+} release, which via CaMKII pathways promotes β-catenin phosphor-rylation (Kremenevskaja et al., 2005).

We found higher Wnt5a expression in patients who did not relapse in comparison to patients whose disease had progressed to distant organs. Higher levels of Wnt5a associated also with lower stage of the disease in non-relapsing and lymph node negative patients. This supports the notion that Wnt5a suppresses cell migration and invasion (Hansen et al., 2009). In this regard we did ordinal and logistic regression analysis and revealed that odds of being patients in lower grade is 17.2 % when Wnt5a expression increases in each 10 score. Moreover, increase in Wnt5a expression by each 10 score decreases odds of cancer-related death in 22%. Survival analysis showed better survival in patients with Wnt5a high expression in comparison to Wnt5a low expression.

In summary, our results indicate decrease of Wnt5a expression along with breast cancer progression. Wnt5a might an independent factor determining decreased invasiveness and cancer-related death in breast cancer patients.

ECM evolution in higher animals includes the collagen-associated small leucine-rich proteoglycans (SLRPs). These proteins are not only differentially expressed in normal tissues, but also during matrix remodeling and pathological conditions (Kalamajski et al., 2009). Yamada and co-workers (2001) identified asporin in periodontal ligament which is characterized by high turnover and remodeling with rapid synthesis and breakdown of matrix components, most notably the collagenous meshwork (most active genes were COL1A2, COL1A3 and COL3A1) that stretches out between the cementum and bone. Importantly, the extremely high turnover and remodeling rate of matrix proteins (including collagens) is also associated with invasive cancer (Weigelt et al. 2005). The increased synthesis of the asporin frequently alters deposition of calcium phosphate (Lorenzo et al. 2001). Asporin also blocks chondrogenesis and inhibits TGF-β1-induced expression of matrix genes. Small interfering RNA-mediated knockdown of asporin increases the expression of cartilage marker genes and TGF-β1; in turn, TGF-β1 stimulates

asporin expression in articular cartilage cells, suggesting that asporin and TGF-β1 form a regulatory feedback loop (Kalamajski etal., 2009).

Asporin as a novel cancer-related gene was first reported by our laboratory (Turashvili et al., 2007) using laser microdissection and whole genome Affymetrix U133 Plus 2.0 Arrays. Asporin was found to be upregulated among normal and cancer samples and among invasive ductal and lobular carcinomas. In detail, ASPN was 22.1 and 23.3-fold upregulated in invasive lobular carcinoma in comparison to normal lobular and ductal cells, respectively; and 3.9-fold upregulated in invasive lobular carcinoma versus invasive ductal carcinoma (Turashvili et al., 2007).

Our current immunohistochemistry analysis confirmed that asporin was overexpressed in ILC in comparison to IDC. Asporin was also overexpressed in breast cancer tissue in comparison to precursor lesions and normal tissue that supports its role in breast cancer progression. Using expression profiling, Klein et al. (2009) described asporin among 22 breast cancer bone metastasis specific genes (Klein et al., 2009). In this sense, we observed high positivity of all 7 bone breast cancer metastasis samples from autopsies. However, asporin expression in primary tumor was not associated with the metastatic potential. With respect to bone metastasis, another protein involved in mineralization process, bone sialoprotein, has been reported as elevated in breast and prostate cancers (reviewed in Bellahcene et al. 2008). Gordon et al. (2009) have recently shown that bone sialoprotein stimulates focal adhesion-related signaling pathways and promotes migration and survival of these cancers.

Asporin was less expressed in triple negative subtype , nevertheless it was associated with tumor grade within this subtype. On the other hand, we observed better survival with higher asporin expression, and association with ER, PR and Wnt5a within the whole patients set. These data suggest that asporin may serve as a marker of tumor aggressiveness in a subtype-dependent manner. There are also controversial data on another SLRPs, lumican and decorin regarding the prognosis of breast and other cancers (Troup et al., 2003; Matsuda et al., 2008). In this sense, prognostic importance of asporin needs further study on larger cohorts and functional experiments.

For our immunohistochemistry analysis, we used in-house rabbit polyclonal antibody (immunization by 16aa peptide within the eighth LRR domain of asporin; produced by Dr. Vojtesek, Masaryk Memorial Institute, Brno) that was validated on uterus tissue as a positive

control. Uterus was found as a tissue with the highest expression of asporin at Gene Expression Omnibus database. Until 2010, Abcam antibody (#58741, distributed also by GenWay) was the only commercial antibody with published results (Gruber et al. 2009, Lee et al. 2010). We also used Abnova antibody (#H00054829-D01P) which is raised against the whole recombinant protein. All antibodies showed similar staining pattern in the uterus tissue. The strongest staining of the lobular cancer was observed with our in-house antibody. Abcam antibody peptide is overlapping with our one, however, it contains an asparagine which may be abundantly glycosylated in cancer (Brooks, 2009) and subsequently Abcam antibody epitopes may be masked. Immunohistochemical study of asporin expression was also carried out in two recent articles on pancreatic and prostate cancer by Turtoi et al. (2011) and Orr et al. (2011), however, specification of antibodies they used is not clear (and authors have not replied to our query yet).
Asporin binds to collagen (high-affinity site is located in the C-terminal part of the protein) and induces calcium mineralization (through the variable domain that contains a polyaspartic tail). As asporin is an important modulator of tissue environment (Kalamajski et al., 2009), we tested its importance for cancer cell migration and invasion in-vitro. MDA-MB-231 cells indeed migrated faster through collagen I with recombinant asporin. Asporin is known to inhibit collagen fibrillogenesis and therefore collagen matrix may be loosened which supports movement of cells through the matrix. On the other hand, importance of matrix stiffness as invasion-promoting physical mechanism has been recently reported (Levental et al. 2009). Another issue to be dissected is the primary source of the asporin expression. Ma et al., (2009) compared expression profiles of stroma and epithelium of breast carcinoma and asporin is among genes upregulated in the stroma. We haven´t found any breast cancer cell line overexpressing asporin, except Hs578T, which is described as myofibroblast-like. We have also observed asporin expression in normal gingival and skin fibroblasts (data not shown, fibroblast kindly provided by Dr. Galandakova, Department of Medical Chemistry and Biochemistry, Olomouc).

In summary, asporin may affect extracellular matrix structure and promote invasive growth of breast cancer cells. However, further investigation of the role of asporin is needed both by functional experiments and large cohort studies.

5.3. Modulation of tissue environment by senescent cells in prostate cancer

Senescence, a permanent cell cycle arrest coupled with resistance to apoptosis and high metabolic activity, is a potent defense against tumorigenesis. However it is now becoming clear that cells with a senescence associated secretory phenotype may actually promote tumor progression through their secretion of factors that can significantly modulate the tissue microenvironment (Krtolica et al., 2001; Sprenger et al., 2008; Coppe et al., 2010a). Using a panel of markers to identify sensescent cells, including SA-β-gal activity and markers described in original article by Z. Pernicova such as telomerase activity, and formation of HP1β foci, we have found that androgen depletion induced irreversible senescence in prostate cancer cells *in vitro*. We also found that expression of both cathepsin B and IGFBP3, two markers of SASP (Coppe et al., 2010b; Untergasser et al., 2002), was significantly increased following androgen depletion, and confirmed that androgen depletion promoted NED of prostate cancer cells (Yuan et al., 2006). This is the first demonstration that androgen depletion leads to senescence and NED of prostate cancer cells. Interestingly, senescent and NE-like cells are associated with high metabolic activity and the potential to influence the behavior of non-senescent neighboring cells.

To further characterize the phenotype of prostate cancer cells following androgen depletion, we examined markers of epithelial and mesenchymal cells. Surprisingly, ADT increased expression of the epithelial marker cytokeratin and the mesenchymal marker vimentin (Kalluri, 2009). Similar findings were observed in tumor samples from prostate cancer patients following ADT, and are the first demonstration that androgen depletion up-regulates vimentin in prostate cancer epithelial cells. Moreover, vimentin was co-expressed with γ-enolase, indicating that vimentin was expressed by NE-like cells. Although vimentin is a well-known marker of EMT (Zeisberg, 2009), it is also expressed by senescent fibroblasts (Nishio et al., 2006). Since the expression of N-cadherin and E-cadherin were both unaffected by androgen depletion, the NE-like cells in our cultures were unlikely to be undergoing an EMT. Instead, the robust expression of vimentin following androgen depletion may be associated with the induction of senescence in prostate cancer epithelial cells.

In summary, our findings demonstrate a novel linkage between the inhibition of androgen receptor activity and the formation of secretory, senescent cells in prostate tumors. These observations suggest that modulation of the prostate tumor environment following androgen depletion is a major contributory factor in the development of androgen-independent prostate

cancer, especially since several components of the SASP secretome are capable of trans-activating ARs under androgen-depleted conditions. We suggest that in prostate cancer patients undergoing ADT, paracrine factors released by senescent cells override the requirement for androgen ligand and promote the clonal expansion of androgen-independent cells, leading to failure of ADT and progression of the disease to androgen-independence.

6. SUMMARY

Tumor cells communicate bidirectionally with the surrounding microenvironment (diverse extracellular matrix components and growth factors) from which genetic cell programming signals are supplied that control cell survival, growth, differentiation, and invasion. Breast and prostate cancers are most frequently diagnosed cancers in woman and man, respectively. Understanding of the mechanisms of their development and progression is of crucial importance.

The initial objective of the thesis was to investigate the role of CTHRC1, periostin, versican and asporin in the progression of breast cancer. We also wanted to characterize breast tumor environment and studied Wnt5a protein as a signaling molecule and its role in breast cancer. Further we studied nestin as a marker of stem cells and neoangiogenesis. In parallel, we wanted to know if androgen deprivation therapy could induce neuroendocrine differentiation and senescence in prostate cancer cells and whether modulation of the prostate tumor environment following androgen depletion contributed to the development of androgen-independent prostate cancer.

Throughout the work, the following materials were used: breast and prostate cancer tissues, normal and precursor breast samples, lymph node tissue from metastatic breast cancer patients and autopsy specimens obtained from patients with breast cancer bone metastases as well as breast and prostate cancer cell lines such as MDA-MB-231, Hs578T, LNCaP and LAPC-4. We carried out immunohistochemistry using antibodies after validation steps, dual immunofluorescence, qRT-PCR, collagen in vitro fibrillogenesis and invasion assay, flow cytometry and senescence assessment.

When studying CTHRC1, periostin and versican, we have found that CTHRC1 protein was predominantly expressed in breast cancer cell cytoplasm and to a lesser extent, in stroma (stromal cells and extracellular matrix with dominance of the latter). All three proteins were significantly highly expressed in breast cancer tissue. CTHRC1 and versican were overexpressed in invasive lobular cancer and periostin in invasive ductal cancer. Periostin epithelial expression was higher in patients with positive lymph nodes, while it was also expressed in lymph node metastases themselves. CTHRC1 stromal expression was higher in triple negative subtype and similarly, it was higher in patients with bone metastases than in non-metastasizing cases. Cox regression

analysis revealed that the risk of bone metastases increased with increase in CTHRC1 stromal expression in patients with high periostin stromal expression. The same patients had shorter time to relapse (bone metastasis) than patients with other combinations of CTHRC1 and periostin stromal expressions.

In parallel, we were interested if asporin is expressed in breast and other cancers as we have shown previously by microarray analysis. Immunohistochemistry analysis confirmed that asporin is overexpressed in invasive lobular cancer in comparison to invasive ductal cancer. Asporin was also overexpressed in breast cancer tissue in comparison to precursor lesions and normal tissue that supports its role in breast cancer progression. Asporin was less expressed in triple negative patients but it positively correlated with tumor grade within this subtype. Asporin was also highly expressed in all breast cancer bone metastases. However, its expression in primary tumor was not associated with metastatic potential. Functional experiments are still on-going in order to clarify asporin role in tumor microenvironment and progression.

We have also studied nestin and Wnt5a at the same cohort of breast cancer patients. Nestin, as a candidate marker for a cancer stem cell phenotype, showed association with mesenchymal markers vimentin, periostin, inflamation and neoangiogenesis which all can contribute to progression of advanced breast cancers, in particular of the triple negative subtype. Wnt5a expression was higher in patients who did not relapse in comparison to patients with progression to distant organs. Using logistic and ordinal regression analyses we also showed that Wnt5a is an independent factor determining decreased invasiveness and cancer-related death in breast cancer patients.

In a joint project we complemented results of Dr. Souček´s group who demonstrated that long term androgen depletion (ADT) induces neuroendocrine differentiation and senescence-associated secretory phenotype of LNCaP and LAPC-4 prostate cancer cells. We have confirmed that ADT induced neuroendocrine differentiation and vimentin expression in prostate cancer samples. We suggest that in prostate cancer patients undergoing ADT, paracrine factors released by senescent cells override the requirement for androgen ligand and promote the clonal expansion of androgen-independent cells, leading to failure of ADT and progression of the disease to androgen-independence.

7. SOUHRN

Nádorové buňky vzájemně komunikují s okolním mikroprostředím (rozličné komponenty extracelulární matrix a růstové faktory), které může ovlivňovat genetické naprogramování buněk, a modulovat tak jejich životaschopnost, růst, diferenciaci nebo invazivní potenciál. Karcinomy prsu a prostaty jsou nejčastěji diagnostikované nádory u žen a mužů a pochopení mechanismu jejich karcinogeneze a progrese má zásadní význam.

Primárním cílem disertace bylo zkoumat význam CTHRC1, periostinu, versicanu a asporinu v progresi karcinomu prsu. Dále jsme chtěli blíže charakterizovat nádorové mikroprostředí a studovali jsme Wnt5a jako signální molekulu a její roli v nádorech prsu. Rovněž jsme studovali nestin jako marker neoangiogeneze a kmenových buněk. Paralelně jsme testovali hypotézu, zda androgenní ablace může indukovat neuroendokrinní diferenciaci a senescenci v buňkách odvozených od karcinomu prostaty a zda modulace nádorového mikroprostředí po androgenní ablaci přispívá k rozvoji androgen-independentního karcinomu prostaty.

V práci byl použit následující materiál: normální tkáň prsní žlázy, tkáň nádorů prsu i jejich prekurzorových lézí, metastázy v lymfatických uzlinách, nekroptické vzorky pacientek s kostními metastázemi a malý soubor tkání karcinomu prostaty před a po androgen deprivační terapii. In vitro experimenty byly prováděny s buněčnými liniemi odvozenými od karcinomu prsu (MDA-MB-231 a Hs578T) a prostaty (LNCaP a LAPC-4). Byly použity následující metody: imunohistochemie včetně optimalizace a validace protilátek, dvojitá imunofluorescence, qRT-PCR, kolagenová fibrilogeneze in vitro, invazivní testy, průtoková cytometrie a hodnocení senescence.

V rámci studia CTHRC1, periostinu a versicanu jsme zjistili, že CTHRC1 protein byl převážně exprimován v cytoplazmě nádorových buněk a v menší míře ve stromatu (stromální buňky a zejména extracelulární matrix). Všechny tři proteiny byly výrazně exprimovány v nádorové tkáni. Hladiny CTHRC1 a versicanu byly vyšší v invazivních lobulárních karcinomech zatímco hladina periostinu byla vyšší v invazivních duktálních karcinomech. Epiteliální exprese periostinu byla zvýšená u pacientů s pozitivními lymfatickými uzlinami, přičemž uzliny samotné vykazovaly pozitivní barvení periostinu. Stromální exprese CTHRC1 byla vyšší v triple-negativních nádorech a zároveň byla vyšší u pacientek s kostními metastázemi. Cox regresní analýza ukázala, že riziko kostních metastáz roste s intenzitou stromální exprese CTHRC1 u

pacientek s vysokou hladinou periostinu. Tyto pacientky měly kratší dobu do relapsu (kostní metastázy) oproti pacientkám s jinými kombinacemi exprese CTHRC1 a periostinu.

Další část dizertace navazuje na naši dřívější identifikaci asporinu jako nádorového proteinu. Imunohistochemická analýza potvrdila, že asporin je více exprimován u invazivních lobulárních než u duktálních karcinomů. Hladina asporinu byla zvýšená v nádorech ve srovnání k normální tkáni i prekurzorovým lézím, což indikuje jeho roli v nádorové progresi. Jeho hladina byla nižší v triple-negativních nádorech, nicméně v rámci tohoto subtypu pozitivně korelovala se stupněm malignity. Asporin byl vysoce exprimován ve všech kostních metastázách, ale jeho exprese v primárních nádorech nebyla asociována s metastatickým potenciálem. Mechanistické experimenty dále pokračují s cílem lépe objasnit funkci tohoto proteinu v nádorovém mikroprostředí a progresi.

Na stejném souboru pacientek jsme rovněž studovali nestin a Wnt5a. Nestin, potenciální marker nádorových kmenových buněk, pozitivně koreloval s vimentinem, periostinem, zánětem a neoangiogenezí, přičemž všechny tyto parametry mohou přispívat k progresi nádorů prsu, především triple-negativního subtypu. Wnt5a exprese byla vyšší u pacientek bez relapsu oproti pacientkám s distálními metastázemi. Obecná a logistická regresní analýza ukázala, že Wnt5a je nezávislý faktor určující nižší invazivitu a smrt v souvislosti nádorovým onemocněním.

Ve společném projektu se skupinou Dr. Součka jsme doplnili pomocí pacientských vzorků jejich zjištění, že dlouhodobá androgenová ablace indukuje neuroendokrinní diferenciaci a sekreční fenotyp asociovaný se senescencí u LNCaP a LAPC-4 prostatických linií. Potvrdili jsme, že androgenová ablace indukuje neuroendokrinní diferenciaci a expresi vimentinu ve vzorcích karcinomu prostaty. U pacientů podstupujících androgenovou ablaci mohou parakrinní faktory, které jsou uvolňovány senescentními buňkami, nahradit stimulaci nádorových buněk androgeny a podpořit klonální expanzi androgen-independentních buněk, což přispívá k selhání androgen deprivační terapie a progresi k refraktornímu karcinomu prostaty.

8. ABBREVIATIONS

ADH	atypical ductal hyperplasia
ADT	androgen deprivation therapy
AIPC	andogen independent prostate cancer
ALH	atypical lobular hyperplasia
APC	adenomatous polyposis coli
AR	androgen receptor
α-SMA	alpha smooth muscle actin
AXIN	axis inhibitor
BMP	bone morphogenetic protein
CAF	carcinoma associated fibroblasts
CAMK2	calcium/calmodulin-dependent protein kinase II
CCL	columnar cell lesions
CgA	chromogranin A
CK1γ	casein kinase 1γ
CTHRC1	collagen triple helix repeat containing 1
CTNNB1	catenin (cadherin-associated protein), beta 1
CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
DCIS	ductal carcinoma *in situ*
DKK1	dickkopf homolog 1 (Xenopus laevis)
dsh	Drosophila dishevelled
DVL	dishevelled, dsh homolog 1 (Drosophila)
E12/E47 E2A	immunoglobulin enhancer binding factors E12/E47
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ErbB2 v-erb-b2	erythroblastic leukemia viral oncogene homolog 2*
ESC	embryonic stem cells
FGF	fibroblast growth factor
FOXC2	forkhead box C2
GLI1 GLI	family zinc finger 1
GSK3β	glycogen synthase kinase 3 beta
HES1 – 7	hairy and enhancer of split 1 - 7 *
HEY1 and HEY2	hairy/enhancer-of-split related with YRPW motif 1and 2*
HEYL	hairy/enhancer-of-split related with YRPW motif-like*
HGF	hepatocyte growth factor
HUT	hyperplasia of usual type
IFP	interstitial fluid pressure
IKK	inhibitor of κB (IκB) kinase
IL	interleukin
TRCP	human homolog of slmb (Drosophila), also known as BTRC (beta-transducin repeat containing)
TRKB	neurotrophic tyrosine kinase, receptor, type 2*
LBD	ligand binding domain
LCIS	lobular carcinoma *in situ*
LRP5 and LRP6	low-density lipoprotein receptor-related protein 5 and 6
LRR	leucine-rich repeat
MAPK	mitogen activated protein kinase
MGA	microglandular adenosis

MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
NE	neuroendocrine
NLK	NEMO-like kinase
NUMB	numb homolog (Drosophila)*
NSE	neuron-specific enolase, γ-enolase
PAP	prostate acid phosphatase
PCP	planar cell polarity
PDGF	platelet-derived growth factor
PG	proteoglycan
PI3	peptidase inhibitor 3, skin-derived
PIN	prostate intraepithelial neoplasia
PKC	protein kinase C
PLCIS	pleomorphic lobular carcinoma in situ
POSTN	periostin
PSA	prostate specific antigen
PSCs	prostate stem cells
RS	radial scar
PTHrP	parathyroid hormone-related protein
RT-qPCR	quantitative reverse-transcriptase polymerase chain reaction
SA-β-GAL	senescence-associated beta-galactosidase
SASP	senescence associated secretory phenotype
SDF-1	stromal cell-derived factor 1
SFRP1	secreted frizzled-related protein 1
SFRP2	secreted frizzled-related protein 2
SLRP	small leucine-rich repeat proteoglycan
Snail 1 and 2	snali homolog 1 and 2*
TAK1	transforming growth factor β-activated kinase 1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGFβ	transforming growth factor
TIMP	tissue inhibitors of MMP
TNFα	tumor necrosis factor alpha
UGM	urogenital sinus mesenchyme
UGS	urogenital sinus
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
WIF-1	Wnt inhibitory factor-1
Wnt	wingless type
ZEB 1 and 2	zinc finger E-box binding homeobox 1 and 2
ZO-1	zona occludens 1

**abbreviations are explained only here*

9. PUBLICATIONS

Publications related to the thesis:

1. G. Kharaishvili, M. Cizkova, K. Bouchalova, G. Mgebrishvili, Z. Kolar, J. Bouchal. Collagen triple helix repeat containing 1 protein, periostin and versican in primary and metastatic breast cancer: an immunohistochemical study. J Clin Pathol. 2011 Jul 8. [Epub ahead of print].

2. Z. Pernicová, E. Slabáková, **G. Kharaishvili**, J. Bouchal, M. Král, Z. Kunická, M. Machala, A. Kozubík, K. Souček. Androgen depletion induces senescence in prostate cancer cells through down-regulation of Skp2. Neoplasia. 2011;13(6):526-36.

3. G. Kharaishvili, D.Simkova, E. Makharoblidze, K.Trtkova, Z. Kolar, J. Bouchal. Wnt signaling in prostate development and carcinogenesis. Biomed Pap Med Fac Univ Palacky Olomouc Czech Republic 2011;155(1):11-8.

Other publications:

1. G. Kharaishvili, A. Gogelia. Changes of blood paramagnetic centres of animals irradiated with low-intensity laser. **J. Georgian Medical News**.No 4(133).April, 2006.

2. G. Kharaishvili, M. Dzamashvili, M. phirosmanashvili, C. Getsadze. Effects of Submaximal dozes of Low-intensity Laser Therapy on bone marrow of white laboratory mice. Tbilisi State Medical University. Collection of scientific works. Vol.XXXIX. 2003. pp.399-401. (In Georgian).

3. G. Kharaishvili, A. Gogelia. Comparative analysis of cytogenetic effects of red (0,63 mkm) and infrared (0,85 mkm) Low-intensity Lasers. J. Annals of Biomedical Research and education. Vol.4, Issue 2. April-June 2004. pp.100-101.(In English).

4. G. Kharaishvili, M. Dzamashvili, M. phirosmanashvili, C. Getsadze. Mutagenic effects of submaximal dozes of low-intensity infrared lasers on laboratory mice bone marrow chromosomes. Tbilisi State Medical University. Collection of scientific works. Vol. XL. 2004. pp.468-470. (In Georgian).

5. G. Kharaishvili, A. Gogelia, M. Dzamashvili, M. phirosmanashvili, C. Getsadze. Cytogenetic and EPR-spectroscopic changes induced by therapeutic dozes Of low intensity laser. Tbilisi State Medical University. Collection of scientific works. 2005.

Presentations:

1. Kharaishvili G, Cizkova M, Bouchalova K, Sedlakova E, Kolar Z, Bouchal J. Asporin modulates tissue microenvironment, invasive growth and bone metastasis of breast cancer. 8th International Symposium & Workshop on Molecular Pathology and Histo(cyto)chemistry and 97th Seminar of the Czech division of the International Academy of Pathology, 2011, Olomouc, Czech Republic.

2. Kharaishvili G, Cizkova M, Bouchalova K, Sedlakova E, Mgebrishvili G, Kolar Z, Bouchal J. Collagen triple helix repeat containing 1 protein in primary and metastatic breast cancer. 8th International Symposium & Workshop on Molecular Pathology and Histo(cyto)chemistry and 97th Seminar of the Czech division of the International Academy of Pathology, 2011, Olomouc, Czech Republic.

3. Kharaishvili, G. Cizkova, M. Sedlakova, E. Kolar, Z. Bouchal, J. Immunohistochemical analysis of collagen triple helix containing 1 protein in breast cancer patiens. In VI. Days of diagnostic, predictive and experimental oncology. Onkologie, 2010, s. 29. (vol. 4, Suppl.A., ISSN: 1803-5922), Olomouc, Czech Republic.

4. G. Kharaishvili, G. Mgebrishvili, Z. Kolar, J. Bouchal. Wnt5a as an independent factor determining decreased invasiveness and cancer-related death in breast cancer patients. This lecture will be presented at „Analytická cytometrie VI“. 2011, Prague, Czech Republic.

5. Bouchal J, Simkova D, **Kharaishvili G**: Asporin Is Associated with Invasive Growth and Bone Metastasis of Breast Cancer. Cancer Res 70 (24Suppl): 326-327 (2010) - 33rd San Antonio Breast Cancer Symposium, San Antonio, Texas, USA, 8.-12.12.2010.

6. Simkova D, **Kharaishvili G**, Cizkova M, Bouchalova K, Kolar Z, Bouchal J. Proteoglycan asporin a jeho vliv na invazivni rust karcinomu prsu. XXXV. Brnenske onkologicke dny a XXV. Konference pro nelekarske zdravotnicke pracovniky. Edukacni sbornik. 21-23. dubna 2011.

10. REFERENCES

1. Adams PD. Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Mol Cell* 2009; 36: 2-14.
2. Akiri G, Sabo E, Dafni H, Vadasz Z, Kartvelishvily Y, Gan N, Kessler O, Cohen T, Resnick M, Neeman M, Neufeld G. Lysyl oxidase-related protein-1 promotes tumor fibrosis and tumor progression in vivo. *Cancer Res* 2003; 63: 1657-66.
3. Al-Hajj M, Clarke MF. Self-renewal and solid tumor stem cells. *Oncogene* 2004; 23: 7274-82.
4. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell* 2004; 6: 17-32.
5. Aubele MM, Cummings MC, Mattis AE, Zitzelsberger HF, Walch AK, Kremer M, Hofler H, Werner M. Accumulation of chromosomal imbalances from intraductal proliferative lesions to adjacent in situ and invasive ductal breast cancer. *Diagn Mol Pathol* 2000; 9: 14-9.
6. Aumuller G, Leonhardt M, Janssen M, Konrad L, Bjartell A, Abrahamsson PA. Neurogenic origin of human prostate endocrine cells. *Urology* 1999; 53: 1041-8.
7. Baeriswyl V, Christofori G. The angiogenic switch in carcinogenesis. *Semin Cancer Biol* 2009; 19: 329-37.
8. Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer* 2004; 4: 540-50.
9. Bao S, Ouyang G, Bai X, Huang Z, Ma C, Liu M, Shao R, Anderson RM, Rich JN, Wang XF. Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway. *Cancer Cell* 2004; 5: 329-39.
10. Barcellos-Hoff MH, Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer Res* 2000; 60: 1254-60.
11. Barginear M, Clotfelter A, Poznak CV. Markers of bone metabolism in women receiving aromatase inhibitors for early-stage breast cancer. *Clin Breast Cancer*.2009;9:72-6.
12. Barkan D, Green JE, Chambers AF. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. *Eur J Cancer*. 2010; 46: 1181-8.
13. Bates RC, Pursell BM, Mercurio AM. Epithelial-mesenchymal transition and colorectal cancer: gaining insights into tumor progression using LIM 1863 cells. *Cells Tissues Organs* 2007; 185: 29-39.
14. Batsche E, Muchardt C, Behrens J, Hurst HC, Cremisi C. RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol Cell Biol* 1998; 18: 3647-58.
15. Bech-Hansen NT, Naylor MJ, Maybaum TA, Sparkes RL, Koop B, Birch DG, Bergen AA, Prinsen CF, Polomeno RC, Gal A, Drack AV, Musarella MA, Jacobson SG, Young RS, Weleber RG. Mutations in NYX, encoding the leucine-rich proteoglycan nyctalopin, cause X-linked complete congenital stationary night blindness. *Nat Genet* 2000; 26: 319-23.
16. Bellahcène A, Castronovo V, Ogbureke KU, Fisher LW, Fedarko NS. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nat Rev Cancer*. 2008; 8: 212-26.
17. Berruti A, Mosca A, Tucci M, Terrone C, Torta M, Tarabuzzi R, Russo L, Cracco C, Bollito E, Scarpa RM, Angeli A, Dogliotti L. Independent prognostic role of circulating chromogranin A in prostate cancer patients with hormone-refractory disease. *Endocr Relat Cancer*. 2005; 12: 109-17.
18. Bhat-Nakshatri P, Appaiah H, Ballas C, Pick-Franke P, Goulet R Jr, Badve S, Srour EF, Nakshatri H. SLUG/SNAI2 and tumor necrosis factor generate breast cells with CD44+/CD24 phenotype. *BMC Cancer* 2010; 10: 411.

19. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL. TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science*. 2004; 30: 848-51.

20. Bianco C, Adkins HB, Wechselberger C, Seno M, Normanno N, De Luca A, Sun Y,Khan N, Kenney N, Ebert A, Williams KP, Sanicola M, Salomon DS. Cripto-1 activates nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial Cells. *Mol Cell Biol*. 2002; 22: 2586-97.

21. Bianco C, Normanno N, Salomon DS, Ciardiello F. Role of the cripto (EGF-CFC)family in embryogenesis and cancer. *Growth Factors*. 2004; 22: 133-9.

22. Bianco C, Strizzi L, Rehman A, Normanno N, Wechselberger C, Sun Y, Khan N,Hirota M, Adkins H, Williams K, Margolis RU, Sanicola M, Salomon DS. A Nodal- and ALK4-independent signaling pathway activated by Cripto-1 through Glypican-1 and c-Src. *Cancer Res*. 2003; 63: 1192-7.

23. Bissell MJ, Weaver VM, Lelievre SA, Wang F, Petersen OW, Schmeichel KL. Tissue structure, nuclear organization, and gene expression in normal and malignant breast. *Cancer Res* 1999; 59: 1757-1763s-discussion1763s-1764s.

24. Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, Nieto MA. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 2002; 21: 3241-6.

25. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, Wesseling J, Cheang MC, Gelmon K, Nielsen TO, Blomqvist C, Heikkilä P, Heikkinen T, Nevanlinna H,Akslen LA, Bégin LR, Foulkes WD, Couch FJ, Wang X, Cafourek V, Olson JE,Baglietto L, Giles GG, Severi G, McLean CA, Southey MC, Rakha E, Green AR, Ellis IO, Sherman ME, Lissowska J, Anderson WF, Cox A, Cross SS, Reed MW, Provenzano E,Dawson SJ, Dunning AM, Humphreys M, Easton DF, García-Closas M, Caldas C, Pharoah PD, Huntsman D. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. *PLoS Med*. 2010; 7:e1000279.

26. Bonkhoff H. Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. *Prostate Suppl* 1998; 8: 18-22.

27. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; 3: 730-7.

28. Bostwick DG, Qian J, Pacelli A, Zincke H, Blute M, Bergstralh EJ, Slezak JM, Cheng L. Neuroendocrine expression in node positive prostate cancer: correlation with systemic progression and patient survival. *J Urol* 2002; 168: 1204-11.

29. Brenton JD, Carey LA, Ahmed AA, Caldas C. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J Clin Oncol*. 2005; 23:7350-60.

30. Brooks SA. Strategies for analysis of the glycosylation of proteins: current status and future perspectives. *Mol Biotechnol.* 2009; 43: 76-88.

31. Buck E, Eyzaguirre A, Barr S, Thompson S, Sennello R, Young D, Iwata KK, Gibson NW, Cagnoni P, Haley JD. Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Mol Cancer Ther* 2007; 6: 532-41.

32. Bussard KM, Smith GH. The mammary gland microenvironment directs progenitor cell fate in vivo. *Int J Cell Biol.* 2011;2011:451676.

33. Caino MC, Meshki J, Kazanietz MG. Hallmarks for senescence in carcinogenesis: novel signaling players. *Apoptosis* 2009; 14: 392-408.

34. Calvo A, Catena R, Noble MS, et al. Identification of VEGF-regulated genes associated with increased lung metastatic potential: functional involvement of tenascin-C in tumor growth and lung metastasis. *Oncogene*. 2008; 27:5373–84.

35. Campisi J. Cancer, aging and cellular senescence. *In Vivo* 2000; 14: 183-8.

36. Casey T, Bond J, Tighe S, Hunter T, Lintault L, Patel O, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, Weaver D, Muss H, Plaut K. Molecular signatures suggest a major role for stromal cells in development of invasive breast cancer. *Breast Cancer Res Treat* 2009; 114: 47-62.

37. Castellsague X, Bosch FX, Munoz N. Environmental co-factors in HPV carcinogenesis. *Virus Res* 2002; 89: 191-9.

38. Castronovo V, Kischel P, Guillonneau F, de Leval L, Defechereux T, De Pauw E, Neri D, Waltregny D. Identification of specific reachable molecular targets in human breast cancer using a versatile ex vivo proteomic method. *Proteomics* 2007; 7: 1188-96.

39. Cattaneo E, McKay R. Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature*. 1990; 347:762-5.

40. Cattaruzza S, Perris R. Proteoglycan control of cell movement during wound healing and cancer spreading. *Matrix Biol*. 2005; 24: 400-17.

41. Cohen MM Jr. TGF beta/Smad signaling system and its pathologic correlates. *Am J Med Genet A* 2003; 116A: 1-10.

42. Cohen RJ, Glezerson G, Haffejee Z, Afrika D. Prostatic carcinoma: histological and immunohistological factors affecting prognosis. *Br J Urol* 1990; 66: 405-10.

43. Cole L, Anderson M, Antin PB, Limesand SW. One process for pancreatic beta-cell coalescence into islets involves an epithelial-mesenchymal transition. *J Endocrinol* 2009; 203: 19-31.

44. Come C, Magnino F, Bibeau F, De Santa Barbara P, Becker KF, Theillet C, Savagner P. Snail and slug play distinct roles during breast carcinoma progression. *Clin Cancer Res* 2006; 12: 5395-402.

45. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010; 5: 99-118.

46. Coppé JP, Patil CK, Rodier F, Krtolica A, Beauséjour CM, Parrinello S, Hodgson JG, Chin K, Desprez PY, Campisi J. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PloS One*. 2010; 5:e9188.

47. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420: 860-7.

48. Creighton CJ, Chang JC, Rosen JM. Epithelial-mesenchymal transition (EMT) in tumor-initiating cells and its clinical implications in breast cancer. *J Mammary Gland Biol Neoplasia* 2010; 15: 253-60.

49. Cronauer MV, Schulz WA, Burchardt T, Anastasiadis AG, de la Taille A, Ackermann R, Burchardt M. The androgen receptor in hormone-refractory prostate cancer: relevance of different mechanisms of androgen receptor signaling (Review). *Int J Oncol* 2003; 23: 1095-102.

50. Cross NA, Chandrasekharan S, Jokonya N, Fowles A, Hamdy FC, Buttle DJ, Eaton CL. The expression and regulation of ADAMTS-1, -4, -5, -9, and -15, and TIMP-3 by TGFbeta1 in prostate cells: relevance to the accumulation of versican. *Prostate*. 2005; 63: 269-75.

51. Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. *Cancer Res* 1994; 54: 5474-8.

52. Culig Z, Klocker H, Bartsch G, Steiner H, Hobisch A. Androgen receptors in prostate cancer. *J Urol* 2003; 170: 1363-9.

53. Cunha GR, Tuohimaa P, Visakorpi T. Steroids and prostate cancer. *J Steroid Biochem Mol Biol*. 2004; 92: 219-20.

54. Da Forno PD, Pringle JH, Hutchinson P, Osborn J, Huang Q, Potter L, Hancox RA, Fletcher A, Saldanha GS. WNT5A expression increases during melanoma progression and correlates with outcome. *Clin Cancer Res*. 2008; 14: 5825-32.

55. Daneshmand S, Dorff TB, Quek ML, Cai J, Pike MC, Nichols PW, Pinski J. Ethnic differences in neuroendocrine cell expression in normal human prostatic tissue. *Urology* 2005; 65: 1008-12.

56. Dang TP, Gazdar AF, Virmani AK, Sepetavec T, Hande KR, Minna JD, Roberts JR, Carbone DP. Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. *J Natl Cancer Inst* 2000; 92: 1355-7.

57. De La Taille A, Vacherot F, Salomon L, Druel C, Gil Diez De Medina S, Abbou C, Buttyan R, Chopin D. Hormone-refractory prostate cancer: a multi-step and multi-event process. *Prostate Cancer Prostatic Dis* 2001; 4: 204-212.

58. De Wever O, Nguyen QD, Van Hoorde L, Bracke M, Bruyneel E, Gespach C, Mareel M. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* 2004; 18: 1016-8.

59. Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 2003; 30: 256-68.

60. Dejmek J, Leandersson K, Manjer J, Bjartell A, Emdin SO, Vogel WF, Landberg G, Andersson T. Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clin Cancer Res* 2005; 11: 520-8.

61. Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science* 1996; 274: 2057-9.

62. Denko NC, Fontana LA, Hudson KM, Sutphin PD, Raychaudhuri S, Altman R, Giaccia AJ. Investigating hypoxic tumor physiology through gene expression patterns.*Oncogene*. 2003; 22: 5907-14.

63. Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA. Pattern of metastatic spread in triple-negative breast cancer. *Breast Cancer Res Treat*. 2009; 115: 423-8.

64. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 2010; 10: 9-22.

65. di Bari MG, Ginsburg E, Plant J, Strizzi L, Salomon DS, Vonderhaar BK. Msx2 induces epithelial-mesenchymal transition in mouse mammary epithelial cells through upregulation of Cripto-1. *J Cell Physiol* 2009; 219: 659-66.

66. Dumont N, Arteaga CL. Transforming growth factor-beta and breast cancer: Tumor promoting effects of transforming growth factor-beta. *Breast Cancer Res* 2000; 2: 125-32.

67. Durand RE, Raleigh JA. Identification of nonproliferating but viable hypoxic tumor cells in vivo. *Cancer Res* 1998; 58: 3547-50.

68. Durmus T, LeClair RJ, Park KS, Terzic A, Yoon JK, Lindner V. Expression analysis of the novel gene collagen triple helix repeat containing-1 (Cthrc1). *Gene Expr Patterns* 2006; 6: 935-40.

69. Edwards J, Krishna NS, Grigor KM, Bartlett JM. Androgen receptor gene amplification and protein expression in hormone refractory prostate cancer. *Br J Cancer* 2003; 89: 552-6.

70. Eger A, Aigner K, Sonderegger S, Dampier B, Oehler S, Schreiber M, Berx G, Cano A, Beug H, Foisner R. DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* 2005; 24: 2375-85.

71. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp Cell Res*. 2001;264:169-84.

72. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 1991; 66: 649-61.

73. Erdelyi I, Nieskens DH, Van Dijk JE, Vass L, Nederbragt H. Immunohistochemical evaluation of versican, in relation to chondroitin sulphate, in canine mammary tumours. *Histol Histopathol* 2003; 18: 1067-80.

74. Erkan M, Kleeff J, Gorbachevski A, Reiser C, Mitkus T, Esposito I, Giese T, Buchler MW, Giese NA, Friess H. Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity. *Gastroenterology* 2007; 132: 1447-64.

75. Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C, Miller C, Demonacos C, Stratford IJ, Dive C. Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. *Mol Cell Biol* 2004; 24: 2875-89.

76. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002; 420: 629-35.

77. Eusebi V, Magalhaes F, Azzopardi JG. Pleomorphic lobular carcinoma of the breast: an aggressive tumor showing apocrine differentiation. *Hum Pathol* 1992; 23: 655-62.

78. Ewald AJ, Brenot A, Duong M, Chan BS, Werb Z. Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Dev Cell* 2008; 14: 570-81.

79. Fan X, Mikolaenko I, Elhassan I, Ni X, Wang Y, Ball D, Brat DJ, Perry A, Eberhart CG. Notch1 and notch2 have opposite effects on embryonal brain tumor growth. *Cancer Res* 2004; 64: 7787-93.

80. Fata JE, Werb Z, Bissell MJ. Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast Cancer Res* 2004; 6: 1-11.

81. Feng Y, Sun B, Li X, Zhang L, Niu Y, Xiao C, Ning L, Fang Z, Wang Y, Zhang L, Cheng J, Zhang W, Hao X. Differentially expressed genes between primary cancer and paired lymph node metastases predict clinical outcome of node-positive breast cancer patients. *Breast Cancer Res Treat*. 2007;103:319-29.

82. Fernandez-Cobo M, Zammarchi F, Mandeli J, Holland JF, Pogo BG. Expression of Wnt5A and Wnt10B in non immortalized breast cancer cells. *Oncol Rep*. 2007; 17: 903-7.

83. Flister MJ, Wilber A, Hall KL, Iwata C, Miyazono K, Nisato RE, Pepper MS, Zawieja DC, Ran S. Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NF-kappaB and Prox1. *Blood* 2010; 115: 418-29.

84. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int J Dev Biol* 2004; 48: 411-24.

85. Ford CE, Ekström EJ, Andersson T. Wnt-5a signaling restores tamoxifen sensitivity in estrogen receptor-negative breast cancer cells. Proc Natl Acad SciU S A. 2009;106(10):3919-24. Retraction in: Ford CE, Ekström EJ, Anderson T. *Proc Natl Acad Sci U S A*. 2010; 107: 22360.

86. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med* 2010; 363: 1938-48.

87. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 2009; 10: 445-57.

88. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. *J Cell Biol* 2010; 188: 11-9.

89. Fukumura D, Jain RK. Tumor microenvironment abnormalities: causes, consequences, and strategies to normalize. *J Cell Biochem* 2007; 101: 937-49.

90. Garin-Chesa P, Old LJ, Rettig WJ. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci U S A*. 1990:7235-9.

91. Gavert N, Ben-Ze'ev A. Epithelial-mesenchymal transition and the invasive potential of tumors. *Trends Mol Med* 2008; 14: 199-209.

92. Gillan L, Matei D, Fishman DA, Gerbin CS, Karlan BY, Chang DD. Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. *Cancer Res* 2002; 62: 5358-64.

93. Gleason DF. Histologic grading of prostate cancer: a perspective. *Hum Pathol* 1992; 23: 273-9.

94. Gobbi H, Arteaga CL, Jensen RA, Simpson JF, Dupont WD, Olson SJ, Schuyler PA, Plummer WD Jr, Page DL. Loss of expression of transforming growth factor beta type II receptor correlates with high tumour grade in human breast in-situ and invasive carcinomas. *Histopathology* 2000; 36: 168-77.

95. Goldstein AS, Huang J, Guo C, Garraway IP, Witte ON. Identification of a cell of origin for human prostate cancer. *Science* 2010; 329: 568-71.

96. Gordon JA, Sodek J, Hunter GK, Goldberg HA. Bone sialoprotein stimulates focal adhesion-related signaling pathways: role in migration and survival of breast and prostate cancer cells. *J Cell Biochem*. 2009; 107: 1118-28.

97. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008; 10: 593-601.

98. Gregory PA, Bracken CP, Bert AG, Goodall GJ. MicroRNAs as regulators of epithelial-mesenchymal transition. *Cell Cycle* 2008; 7: 3112-8.

99. Grigoriadis A, Mackay A, Reis-Filho JS, Steele D, Iseli C, Stevenson BJ,Jongeneel CV, Valgeirsson H, Fenwick K, Iravani M, Leao M, Simpson AJ, Strausberg RL, Jat PS, Ashworth A, Neville AM, O'Hare MJ. Establishment of the epithelial-specific transcriptome of normal and malignant human breast cells based on MPSS and array expression data. *Breast Cancer Res*.2006; 8:R56.

100. Gruber HE, Ingram JA, Hoelscher GL, Zinchenko N, Hanley EN Jr, Sun Y. Asporin, a susceptibility gene in osteoarthritis, is expressed at higher levels in the more degenerate human intervertebral disc. *Arthritis Res Ther* 2009; 11: R47.

101. Gu Y, Qi X, Guo S. Lymphangiogenesis induced by VEGF-C and VEGF-D promotes metastasis and a poor outcome in breast carcinoma: a retrospective study of 61 cases. *Clin Exp Metastasis* 2008; 25: 717-25.

102. Guedj M, Marisa L, de Reynies A, Orsetti B, Schiappa R, Bibeau F, Macgrogan G, Lerebours F, Finetti P, Longy M, Bertheau P, Bertrand F, Bonnet F, Martin AL, Feugeas JP, Bieche I, Lehmann-Che J, Lidereau R, Birnbaum D, Bertucci F, de The H, Theillet C. A refined molecular taxonomy of breast cancer. *Oncogene* 2011.

103. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000: 57-70.

104. Hansen C, Howlin J, Tengholm A, Dyachok O, Vogel WF, Nairn AC, Greengard P, Andersson T. Wnt-5a-induced phosphorylation of DARPP-32 inhibits breast cancer cell migration in a CREB-dependent manner. *J Biol Chem* 2009; 284: 27533-43.

105. Hansen S, Grabau DA, Rose C, Bak M, Sorensen FB. Angiogenesis in breast cancer: a comparative study of the observer variability of methods for determining microvessel density. *Lab Invest* 1998; 78: 1563-73.

106. Heino J. The collagen family members as cell adhesion proteins. *Bioessays* 2007; 29: 1001-10.

107. Heldin CH, Rubin K, Pietras K, Ostman A. High interstitial fluid pressure - an obstacle in cancer therapy. *Nat Rev Cancer* 2004; 4: 806-13.

108. Helmlinger G, Sckell A, Dellian M, Forbes NS, Jain RK. Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism. *Clin Cancer Res* 2002; 8: 1284-91.

109. Henry SP, Takanosu M, Boyd TC, Mayne PM, Eberspaecher H, Zhou W, de Crombrugghe B, Hook M, Mayne R. Expression pattern and gene characterization of asporin. a newly discovered member of the leucine-rich repeat protein family. *J Biol Chem* 2001; 276: 12212-21.

110. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, Backlund MG, Yin Y, Khramtsov AI, Bastein R, Quackenbush J, Glazer RI, Brown PH, Green JE, Kopelovich L, Furth PA, Palazzo JP, Olopade OI, Bernard PS, Churchill GA, Van Dyke T, Perou CM. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol* 2007; 8: R76.

111. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001; 93: 266-76.

112. Honn KV, Tang DG. Adhesion molecules and tumor cell interaction with endothelium and subendothelial matrix. *Cancer Metastasis Rev* 1992; 11: 353-75.

113. Horino Y, Takahashi S, Miura T, Takahashi Y. Prolonged hypoxia accelerates the posttranscriptional process of collagen synthesis in cultured fibroblasts. *Life Sci* 2002; 71: 3031-45.

114. Hu M, Yao J, Cai L, Bachman KE, van den Brule F, Velculescu V, Polyak K. Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet* 2005; 37: 899-905.

115. Huang CL, Liu D, Nakano J, Ishikawa S, Kontani K, Yokomise H, Ueno M. Wnt5a expression is associated with the tumor proliferation and the stromal vascular endothelial growth factor--an expression in non-small-cell lung cancer. J Clin Oncol. 2005;23:8765-73.

116. Huang J, Yao JL, di Sant'Agnese PA, Yang Q, Bourne PA, Na Y. Immunohistochemical characterization of neuroendocrine cells in prostate cancer. *Prostate* 2006; 66: 1399-406.

117. Humphrey PA. Prostate pathology. Chicago: ASCP Press; 2003.

118. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 2002; 23: 787-823.

119. Chang HY, Sneddon JB, Alizadeh AA, Sood R, West RB, Montgomery K, Chi JT, van de Rijn M, Botstein D, Brown PO. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol* 2004; 2: E7.

120. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res* 2009; 69: 1302-13.

121. Chen J, Diacovo TG, Grenache DG, Santoro SA, Zutter MM. The alpha(2) integrin subunit-deficient mouse: a multifaceted phenotype including defects of branching morphogenesis and hemostasis. *Am J Pathol* 2002; 161: 337-44.

122. Chen XD, Fisher LW, Robey PG, Young MF. The small leucine-rich proteoglycan biglycan modulates BMP-4-induced osteoblast differentiation. *FASEB J* 2004; 18: 948-58.

123. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 2005; 436: 725-30.

124. Chen Z, Wang T, Luo H, Lai Y, Yang X, Li F, Lei Y, Su C, Zhang X, Lahn BT, Xiang AP. Expression of nestin in lymph node metastasis and lymphangiogenesis in non-small cell lung cancer patients. *Hum Pathol* 2010; 41: 737-44.

125. Choi YL, Oh E, Park S, Kim Y, Park YH, Song K, Cho EY, Hong YC, Choi JS, Lee JE, Kim JH, Nam SJ, Im YH, Yang JH, Shin YK. Triple-negative, basal-like, and quintuple-negative breast cancers: better prediction model for survival. *BMC Cancer*. 2010;10:507.

126. Chouaib S, Kieda C, Benlalam H, Noman MZ, Mami-Chouaib F, Ruegg C. Endothelial cells as key determinants of the tumor microenvironment: interaction with tumor cells, extracellular matrix and immune killer cells. *Crit Rev Immunol* 2010; 30: 529-45.

127. Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* 2007; 26: 711-24.

128. Iozzo RV, Murdoch AD. Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. *FASEB J* 1996; 10: 598-614.

129. Iozzo RV. The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. *Crit Rev Biochem Mol Biol* 1997; 32: 141-74.

130. Ishiwata T, Matsuda Y, Naito Z. Nestin in gastrointestinal and other cancers: effects on cells and tumor angiogenesis. *World J Gastroenterol* 2011; 17: 409-18.

131. Jain RK, Tong RT, Munn LL. Effect of vascular normalization by antiangiogenic therapy on interstitial hypertension, peritumor edema, and lymphatic metastasis: insights from a mathematical model. *Cancer Res* 2007; 67: 2729-35.

132. Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H,Grünert S. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J Cell Biol*. 2002; 156: 299-313.

133. Jarvelainen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacol Rev*. 2009; 61: 198-223.

134. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.

135. Jenndahl LE, Isakson P, Baeckstrom D. c-erbB2-induced epithelial-mesenchymal transition in mammary epithelial cells is suppressed by cell-cell contact and initiated prior to E-cadherin downregulation. *Int J Oncol* 2005; 27: 439-48.

136. Jodele S, Blavier L, Yoon JM, et al. Modifying the soil to affect the seed: role of stromal-derived matrix metalloproteinases in cancer progression. *Cancer Metastasis Rev*. 2006; 25: 35–43.

137. Johnson RW, Nguyen MP, Padalecki SS, Grubbs BG, Merkel AR, Oyajobi BO, Matrisian LM, Mundy GR, Sterling JA. TGF-beta promotion of Gli2-induced expression of parathyroid hormone-related protein, an important osteolytic factor in bone metastasis, is independent of canonical Hedgehog signaling. *Cancer Res* 2011; 71: 822-31.

138. Jones C, Merrett S, Thomas VA, Barker TH, Lakhani SR. Comparative genomic hybridization analysis of bilateral hyperplasia of usual type of the breast. *J Pathol* 2003; 199: 152-6.

139. Jönsson M, Dejmek J, Bendahl PO, Andersson T. Loss of Wnt-5a protein is associated with early relapse in invasive ductal breast carcinomas. Cancer Res. 2002; 62: 409-16.

140. Kajita M, McClinic KN, Wade PA. Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress. *Mol Cell Biol* 2004; 24: 7559-66.

141. Kalamajski S, Aspberg A, Lindblom K, Heinegård D, Oldberg A. Asporin competes with decorin for collagen binding, binds calcium and promotes osteoblast collagen mineralization. *Biochem J*. 2009; 423: 53-9.

142. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003; 112: 1776-84.

143. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest*. 2009; 119: 1420-8. doi: 10.1172/JCI39104.

144. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006; 6: 392-401.

145. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* 2009; 119: 1417-9.

146. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, Grosveld G, Sherr CJ. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997; 91: 649-59.

147. Kamiya N, Akakura K, Suzuki H, Isshiki S, Komiya A, Ueda T, Ito H.Pretreatment serum level of neuron specific enolase (NSE) as a prognostic factor in metastatic prostate cancer patients treated with endocrine therapy. *Eur Urol*. 2003; 44: 309-14.

148. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003; 3: 537-49.

149. Kanno A, Satoh K, Masamune A, Hirota M, Kimura K, Umino J, Hamada S, Satoh A, Egawa S, Motoi F, Unno M, Shimosegawa T. Periostin, secreted from stromal cells, has biphasic effect on cell migration and correlates with the epithelial to mesenchymal transition of human pancreatic cancer cells. *Int J Cancer* 2008; 122: 2707-18.

150. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002; 2: 301-10.

151. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; 449: 557-63.

152. Kass L, Erler JT, Dembo M, Weaver VM. Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis. *Int J Biochem Cell Biol* 2007; 39: 1987-94.

153. Katoh M, Katoh M. Identification and characterization of human SNAIL3 (SNAI3) gene in silico. *Int J Mol Med.* 2003;11:383-8.

154. Katoh M, Katoh M. Notch signaling in gastrointestinal tract (review). *Int J Oncol* 2007; 30: 247-51.

155. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. *Cell* 2007; 129: 465-72.

156. Khalil AA, Friedl P. Determinants of leader cells in collective cell migration. *Integr Biol (Camb)* 2010; 2: 568-74.

157. Kizawa H, Kou I, Iida A, Sudo A, Miyamoto Y, Fukuda A, Mabuchi A, Kotani A, Kawakami A, Yamamoto S, Uchida A, Nakamura K, Notoya K, Nakamura Y, Ikegawa S. An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. *Nat Genet* 2005; 37: 138-44.

158. Kleeberger W, Bova GS, Nielsen ME, Herawi M, Chuang AY, Epstein JI, Berman DM. Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. *Cancer Res*. 2007; 67: 9199-206.

159. Klein A, Olendrowitz C, Schmutzler R, Hampl J, Schlag PM, Maass N, Arnold N, Wessel R, Ramser J, Meindl A, Scherneck S, Seitz S. Identification of brain- and bone-specific breast cancer metastasis genes. *Cancer Lett*. 2009; 276: 212-20.

160. Klinowska TC, Alexander CM, Georges-Labouesse E, Van der Neut R, Kreidberg JA, Jones CJ, Sonnenberg A, Streuli CH. Epithelial development and differentiation in the mammary gland is not dependent on alpha 3 or alpha 6 integrin subunits. *Dev Biol* 2001; 233: 449-67.

161. Klonisch T, Wiechec E, Hombach-Klonisch S, Ande SR, Wesselborg S, Schulze-Osthoff K, Los M. Cancer stem cell markers in common cancers - therapeutic implications. *Trends Mol Med* 2008; 14: 450-60.

162. Kolar Z, Ehrmann J Jr, Turashvili G, Bouchal J, Mokry J. A novel myoepithelial/progenitor cell marker in the breast? Virchows Arch. 2007; 450: 607-9.

163. Koperek O, Asari R, Niederle B, Kaserer K. Desmoplastic stromal reaction in papillary thyroid microcarcinoma. *Histopathology*. 2011; 58: 919-24.

164. Kremenevskaja N, von Wasielewski R, Rao AS, Schöfl C, Andersson T, Brabant G. Wnt-5a has tumor suppressor activity in thyroid carcinoma. *Oncogene*. 2005; 24: 2144-54.

165. Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A*. 2001; 98: 12072-7.

166. Kühl M, Sheldahl LC, Park M, Miller JR, Moon RT. The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet*. 2000;16:279-83.

167. Kuilman T, Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer* 2009; 9: 81-94.

168. Kuperwasser C, Dessain S, Bierbaum BE, Garnet D, Sperandio K, Gauvin GP, Naber SP, Weinberg RA, Rosenblatt M. A mouse model of human breast cancer metastasis to human bone. *Cancer Res* 2005; 65: 6130-8.

169. Kupferman ME, Jiffar T, El-Naggar A, Yilmaz T, Zhou G, Xie T, Feng L, Wang J, Holsinger FC, Yu D, Myers JN. TrkB induces EMT and has a key role in invasion of head and neck squamous cell carcinoma. *Oncogene* 2010; 29: 2047-59.

170. Kwok WK, Ling MT, Yuen HF, Wong YC, Wang X. Role of p14ARF in TWIST-mediated senescence in prostate epithelial cells. *Carcinogenesis* 2007; 28: 2467-75.

171. Lawton T. Breast. Cambridge illustrated surgical pathology. Cambridge university press, 2009.

172. LeClair R, Lindner V. The role of collagen triple helix repeat containing 1 in injured arteries, collagen expression, and transforming growth factor beta signaling. *Trends Cardiovasc Med* 2007; 17: 202-5.

173. Lee EH, Park HJ, Jeong JH, Kim YJ, Cha DW, Kwon DK, Lee SH, Cho JY. The role of asporin in mineralization of human dental pulp stem cells. *J Cell Physiol* 2010.

174. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006; 172: 973-81.

175. Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia* 2002; 7: 177-89.

176. Leivonen SK, Ala-Aho R, Koli K, Grenman R, Peltonen J, Kahari VM. Activation of Smad signaling enhances collagenase-3 (MMP-13) expression and invasion of head and neck squamous carcinoma cells. *Oncogene* 2006; 25: 2588-600.

177. Leivonen SK, Kahari VM. Transforming growth factor-beta signaling in cancer invasion and metastasis. *Int J Cancer* 2007; 121: 2119-24.

178. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990; 60: 585-95.

179. Leris AC, Roberts TR, Jiang WG, Newbold RF, Mokbel K. WNT5A expression in human breast cancer. Anticancer Res. 2005; 25: 731-4.

180. Leu AJ, Berk DA, Lymboussaki A, Alitalo K, Jain RK. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res* 2000; 60: 4324-7.

181. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell.* 2009; 139: 891-906.

182. Li J, Zhou BP. Activation of beta-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. *BMC Cancer* 2011; 11: 49.

183. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. *Cancer Res* 2006; 66: 4553-7.

184. Liang H, Chen Q, Coles AH, Anderson SJ, Pihan G, Bradley A, Gerstein R, Jurecic R, Jones SN. Wnt5a inhibits B cell proliferation and functions as a tumorsuppressor in hematopoietic tissue. *Cancer Cell*. 2003; 4: 349-60.

185. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M, Cristofanilli M, Hortobagyi GN, Pusztai L. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol*. 2008; 26: 1275-81.

186. Lin CW, Shen SC, Ko CH, Lin HY, Chen YC. Reciprocal activation of macrophages and breast carcinoma cells by nitric oxide and colony-stimulating factor-1. *Carcinogenesis* 2010; 31: 2039-48.

187. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001; 411: 375-9.

188. Liu C, Chen B, Zhu J, Zhang R, Yao F, Jin F, Xu H, Lu P. Clinical implications for nestin protein expression in breast cancer. *Cancer Sci* 2010; 101: 815-9.

189. Liu M, Casimiro MC, Wang C, Shirley LA, Jiao X, Katiyar S, Ju X, Li Z, Yu Z, Zhou J, Johnson M, Fortina P, Hyslop T, Windle JJ, Pestell RG. p21CIP1 attenuates Ras- and c-Myc-dependent breast tumor epithelial mesenchymal transition and cancer stem cell-like gene expression in vivo. *Proc Natl Acad Sci U S A* 2009; 106: 19035-9.

190. Locati M, Deuschle U, Massardi ML, Martinez FO, Sironi M, Sozzani S, Bartfai T, Mantovani A. Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. *J Immunol* 2002; 168: 3557-62.

191. Lopez-Garcia MA, Geyer FC, Lacroix-Triki M, Marchio C, Reis-Filho JS. Breast cancer precursors revisited: molecular features and progression pathways. *Histopathology* 2010; 57: 171-92.

192. Lorenzo P, Aspberg A, Onnerfjord P, Bayliss MT, Neame PJ, Heinegard D. Identification and characterization of asporin. a novel member of the leucine-rich repeat protein family closely related to decorin and biglycan. *J Biol Chem* 2001; 276: 12201-11.

193. Lukk M, Kapushesky M, Nikkilä J, Parkinson H, Goncalves A, Huber W, Ukkonen E,Brazma A. A global map of human gene expression. *Nat Biotechnol*. 2010; 28: 322-4.

194. Lunt SJ, Chaudary N, Hill RP. The tumor microenvironment and metastatic disease. *Clin Exp Metastasis* 2009; 26: 19-34.

195. Lunt SJ, Kalliomaki TM, Brown A, Yang VX, Milosevic M, Hill RP. Interstitial fluid pressure, vascularity and metastasis in ectopic, orthotopic and spontaneous tumours. *BMC Cancer* 2008; 8: 2.

196. Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC. Gene expression profiling of the tumor microenvironment during breast cancer progression. *Breast Cancer Res*. 2009; 11: R7.

197. Malinowska K, Neuwirt H, Cavarretta IT, Bektic J, Steiner H, Dietrich H, Moser PL, Fuchs D, Hobisch A, Culig Z. Interleukin-6 stimulation of growth of prostate cancer in vitro and in vivo through activation of the androgen receptor. *Endocr Relat Cancer* 2009; 16: 155-69.

198. Manes S, Mira E, Barbacid MM, Cipres A, Fernandez-Resa P, Buesa JM, Merida I, Aracil M, Marquez G, Martinez-A C. Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3. *J Biol Chem* 1997; 272: 25706-12.

199. Manfrin E, Remo A, Falsirollo F, Reghellin D, Bonetti F. Risk of neoplastic transformation in asymptomatic radial scar. Analysis of 117 cases. *Breast Cancer Res Treat* 2008; 107: 371-7.

200. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; 133: 704-15.

201. Markowitz SD, Roberts AB. Tumor suppressor activity of the TGF-beta pathway in human cancers. *Cytokine Growth Factor Rev* 1996; 7: 93-102.

202. Martel C, Harper F, Cereghini S, Noe V, Mareel M, Cremisi C. Inactivation of retinoblastoma family proteins by SV40 T antigen results in creation of a hepatocyte growth factor/scatter factor autocrine loop associated with an epithelial-fibroblastoid conversion and invasiveness. *Cell Growth Differ* 1997; 8: 165-78.

203. Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 2000; 103: 295-309.

204. Matsuda Y, Yamamoto T, Kudo M, Kawahara K, Kawamoto M, Nakajima Y, Koizumi K, Nakazawa N, Ishiwata T, Naito Z. Expression and roles of lumican in lung adenocarcinoma and squamous cell carcinoma. *Int J Oncol* 2008; 33: 1177-85.

205. Mauri P, Scarpa A, Nascimbeni AC, Benazzi L, Parmagnani E, Mafficini A, Della Peruta M, Bassi C, Miyazaki K, Sorio C. Identification of proteins released by pancreatic cancer cells by multidimensional protein identification technology: a strategy for identification of novel cancer markers. *FASEB J*. 2005; 19: 1125-7.

206. May M, Siegsmund M, Hammermann F, Loy V, Gunia S. Prognostic significance of proliferation activity and neuroendocrine differentiation to predict treatment failure after radical prostatectomy. *Scand J Urol Nephrol* 2007; 41: 375-81.

207. McDonald SL, Silver A. The opposing roles of Wnt-5a in cancer. *Br J Cancer*. 2009; 101: 209-14.

208. Medrek C, Landberg G, Andersson T, Leandersson K. Wnt-5a-CKI{alpha} signaling promotes {beta}-catenin/E-cadherin complex formation and intercellular adhesion in human breast epithelial cells. *J Biol Chem*. 2009; 284: 10968-79.

209. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia* 2010; 15: 117-34.

210. Miller SJ, Lavker RM, Sun TT. Interpreting epithelial cancer biology in thecontext of stem cells: tumor properties and therapeutic implications. *Biochim Biophys Acta*. 2005; 1756: 25-52.

211. Milosevic M, Fyles A, Hedley D, Pintilie M, Levin W, Manchul L, Hill R. Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements. *Cancer Res* 2001; 61: 6400-5.

212. Mokrý J, Cízková D, Filip S, Ehrmann J, Osterreicher J, Kolár Z, English D. Nestin expression by newly formed human blood vessels. *Stem Cells Dev*. 2004; 13: 658-64.

213. Moon RT, Campbell RM, Christian JL, McGrew LL, Shih J, Fraser S. Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of Xenopus laevis. *Development*. 1993; 119: 97-111.

214. Moreno M, Munoz R, Aroca F, Labarca M, Brandan E, Larrain J. Biglycan is a new extracellular component of the Chordin-BMP4 signaling pathway. *EMBO J* 2005; 24: 1397-405.

215. Mostofi FK. Grading of prostatic carcinoma. *Cancer Chemother Rep*. 1975; 59: 111-7.

216. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* 2004; 4: 839-49.

217. Muschler J, Streuli CH. Cell-matrix interactions in mammary gland development and breast cancer. *Cold Spring Harb Perspect Biol*. 2010; 2:a003202.

218. Nakajima M, Kizawa H, Saitoh M, Kou I, Miyazono K, Ikegawa S. Mechanisms for asporin function and regulation in articular cartilage. *J Biol Chem* 2007; 282: 32185-92.

219. Nara Y, Kato Y, Torii Y, Tsuji Y, Nakagaki S, Goto S, Isobe H, Nakashima N, Takeuchi J. Immunohistochemical localization of extracellular matrix components in human breast tumours with special reference to PG-M/versican. *Histochem J* 1997; 29: 21-30.

220. Nathanson SD, Nelson L. Interstitial fluid pressure in breast cancer, benign breast conditions, and breast parenchyma. *Ann Surg Oncol* 1994; 1: 333-8.

221. Neil JR, Johnson KM, Nemenoff RA, Schiemann WP. Cox-2 inactivates Smad signaling and enhances EMT stimulated by TGF-beta through a PGE2-dependent mechanisms. *Carcinogenesis* 2008; 29: 2227-35.

222. Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science* 2006; 314: 298-300.

223. Nelson EC, Cambio AJ, Yang JC, Ok JH, Lara PN Jr, Evans CP. Clinical implications of neuroendocrine differentiation in prostate cancer. *Prostate Cancer Prostatic Dis* 2007; 10: 6-14.

224. Nelson WG, DeWeese TL, DeMarzo AM. The diet, prostate inflammation, and the development of prostate cancer. *Cancer Metastasis Rev* 2002; 21: 3-16.

225. Nielsen BS, Rank F, Lopez JM, Balbin M, Vizoso F, Lund LR, Dano K, Lopez-Otin C. Collagenase-3 expression in breast myofibroblasts as a molecular marker of transition of ductal carcinoma in situ lesions to invasive ductal carcinomas. *Cancer Res* 2001; 61: 7091-100.

226. Niessen CM. Tight junctions/adherens junctions: basic structure and function. *J Invest Dermatol* 2007; 127: 2525-32.

227. Nikitovic D, Zafiropoulos A, Katonis P, Tsatsakis A, Theocharis AD, Karamanos NK, Tzanakakis GN. Transforming growth factor-beta as a key molecule triggering the expression of versican isoforms v0 and v1, hyaluronan synthase-2 and synthesis of hyaluronan in malignant osteosarcoma cells. *IUBMB Life* 2006; 58: 47-53.

228. Nishio K, Inoue A, Qiao S, Kondo H, Mimura A. Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts. *Histochem Cell Biol* 2001; 116: 321-7.

229. Nishioka R, Itoh S, Gui T, Gai Z, Oikawa K, Kawai M, Tani M, Yamaue H, Muragaki Y. SNAIL induces epithelial-to-mesenchymal transition in a human pancreatic cancer cell line (BxPC3) and promotes distant metastasis and invasiveness in vivo. *Exp Mol Pathol* 2010; 89: 149-57.

230. Nobels FR, Kwekkeboom DJ, Coopmans W, Schoenmakers CH, Lindemans J, De Herder WW, Krenning EP, Bouillon R, Lamberts SW. Chromogranin A as serum marker for neuroendocrine neoplasia: comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones. *J Clin Endocrinol Metab*. 1997; 82: 2622-8.

231. O'Connor DT, Pandlan MR, Carlton E, Cervenka JH, Hslao RJ. Rapid radioimmunoassay of circulating chromogranin A: in vitro stability, exploration of the neuroendocrine character of neoplasia, and assessment of the effects of organ failure. *Clin Chem*. 1989; 35: 1631-7.

232. Ohta K, Lupo G, Kuriyama S, Keynes R, Holt CE, Harris WA, Tanaka H, Ohnuma S. Tsukushi functions as an organizer inducer by inhibition of BMP activity in cooperation with chordin. *Dev Cell* 2004; 7: 347-58.

233. Olson DJ, Gibo DM. Antisense wnt-5a mimics wnt-1-mediated C57MG mammary epithelial cell transformation. *Exp Cell Res*. 1998; 241: 134-41.

234. Onishi T, Hayashi N, Theriault RL, Hortobagyi GN, Ueno NT. Future directions of bone-targeted therapy for metastatic breast cancer. *Nat Rev Clin Oncol* 2010; 7: 641-51.

235. Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell Cycle* 2006; 5: 1597-601.

236. Orr B, Riddick AC, Stewart GD, Anderson RA, Franco OE, Hayward SW, Thomson AA.Identification of stromally expressed molecules in the prostate by tag-profiling of cancer-associated fibroblasts, normal fibroblasts and fetal prostate.*Oncogene*. 2011. doi: 10.1038/onc.2011.312.

237. Otsuki S, Inokuchi M, Enjoji M, Ishikawa T, Takagi Y, Kato K, Yamada H, Kojima K, Sugihara K. Vimentin expression is associated with decreased survival in gastric cancer. *Oncol Rep* 2011; 25: 1235-42.

238. Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK. Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002; 296: 1883-6.

239. Page DL, Schuyler PA, Dupont WD, Jensen RA, Plummer WD Jr, Simpson JF. Atypical lobular hyperplasia as a unilateral predictor of breast cancer risk: a retrospective cohort study. *Lancet* 2003; 361: 125-9.

240. Pardali K, Moustakas A. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta* 2007; 1775: 21-62.

241. Park JT, Li M, Nakayama K, Mao TL, Davidson B, Zhang Z, Kurman RJ, Eberhart CG, Shih IeM, Wang TL. Notch3 gene amplification in ovarian cancer. *Cancer Res* 2006; 66: 6312-8.

242. Paszek MJ, Weaver VM. The tension mounts: mechanics meets morphogenesis and malignancy. *J Mammary Gland Biol Neoplasia* 2004; 9: 325-42.

243. Patocs A, Zhang L, Xu Y, Weber F, Caldes T, Mutter GL, Platzer P, Eng C. Breast-cancer stromal cells with TP53 mutations and nodal metastases. *N Engl J Med* 2007; 357: 2543-51.

244. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, Zurrida S, Maisonneuve P, Viale G, Di Fiore PP. Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J Cell Biol* 2004; 167: 215-21.

245. Petrie RJ, Doyle AD, Yamada KM. Random versus directionally persistent cell migration. *Nat Rev Mol Cell Biol* 2009; 10: 538-49.

246. Pietras K, Rubin K, Sjoblom T, Buchdunger E, Sjoquist M, Heldin CH, Ostman A. Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy. *Cancer Res* 2002; 62: 5476-84.

247. Pinder SE, Reis-Filho JS. Non-operative breast pathology: columnar cell lesions. *J Clin Pathol* 2007; 60: 1307-12.

248. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004; 4: 71-8.

249. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005; 65: 5506-11.

250. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 2006; 441: 437-43.

251. Proweller A, Tu L, Lepore JJ, Cheng L, Lu MM, Seykora J, Millar SE, Pear WS, Parmacek MS. Impaired notch signaling promotes de novo squamous cell carcinoma formation. *Cancer Res* 2006; 66: 7438-44.

252. Puglisi F, Puppin C, Pegolo E, Andreetta C, Pascoletti G, D'Aurizio F, Pandolfi M, Fasola G, Piga A, Damante G, Di Loreto C. Expression of periostin in human breast cancer. *J Clin Pathol* 2008; 61: 494-8.

253. Pusch CM, Zeitz C, Brandau O, Pesch K, Achatz H, Feil S, Scharfe C, Maurer J, Jacobi FK, Pinckers A, Andreasson S, Hardcastle A, Wissinger B, Berger W, Meindl A. The complete form of X-linked congenital stationary night blindness is caused by mutations in a gene encoding a leucine-rich repeat protein. *Nat Genet* 2000; 26: 324-7.

254. Pyagay P, Heroult M, Wang Q, Lehnert W, Belden J, Liaw L, Friesel RE, Lindner V. Collagen triple helix repeat containing 1, a novel secreted protein in injured and diseased arteries, inhibits collagen expression and promotes cell migration. *Circ Res* 2005; 96: 261-8.

255. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* 2003; 3: 756-67.

256. Ramaswamy S, Perou CM. DNA microarrays in breast cancer: the promise of personalised medicine. *Lancet*. 2003; 361: 1576-7.

257. Ranno S, Motta M, Rampello E, Risino C, Bennati E, Malaguarnera M. The chromogranin-A (CgA) in prostate cancer. *Arch Gerontol Geriatr*. 2006; 43: 117-26.

258. Reedijk M, Odorcic S, Chang L, Zhang H, Miller N, McCready DR, Lockwood G, Egan SE. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res* 2005; 65: 8530-7.

259. Reis-Filho JS, Simpson PT, Gale T, Lakhani SR. The molecular genetics of breast cancer: the contribution of comparative genomic hybridization. *Pathol Res Pract* 2005; 201: 713-25.

260. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; 414: 105-11.

261. Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol Biol (Paris)* 2005; 53: 430-42.

262. Ricard-Blum S. The collagen family. *Cold Spring Harb Perspect Biol* 2011; 3: a004978.

263. Ridley AJ. Rho family proteins: coordinating cell responses. *Trends Cell Biol* 2001; 11: 471-7.

264. Roarty K, Baxley SE, Crowley MR, Frost AR, Serra R. Loss of TGF-beta or Wnt5a results in an increase in Wnt/beta-catenin activity and redirects mammary tumour phenotype. *Breast Cancer Res*. 2009; 11: R19.

265. Roarty K, Serra R. Wnt5a is required for proper mammary gland development and TGF-beta-mediated inhibition of ductal growth. *Development*. 2007;134:3929-39.

266. Roh HD, Boucher Y, Kalnicki S, Buchsbaum R, Bloomer WD, Jain RK. Interstitial hypertension in carcinoma of uterine cervix in patients: possible correlation with tumor oxygenation and radiation response. *Cancer Res* 1991; 51: 6695-8.

267. Rosen PP. Rosen's Breast Pathology. 3rd ed. Philadelphia, PA: Lippincott-Raven; 2008.

268. Rothhammer T, Poser I, Soncin F, Bataille F, Moser M and Bosserhoff AK: Bone morphogenic proteins are overexpressed in malignant melanoma and promote cell invasion and migration. *Cancer Res* 65: 448-456, 2005.

269. Rottner K, Hall A, Small JV. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol* 1999; 9: 640-8.

270. Rouhi P, Lee SL, Cao Z, Hedlund EM, Jensen LD, Cao Y. Pathological angiogenesis facilitates tumor cell dissemination and metastasis. *Cell Cycle*. 2010; 9: 913-7.

271. Ruiz C, Huang W, Hegi ME, Lange K, Hamou MF, Fluri E, Oakeley EJ, Chiquet-Ehrismann R, Orend G. Growth promoting signaling by tenascin-C [corrected]. *Cancer Res* 2004; 64: 7377-85.

272. Sakakura T, Sakagami Y, Nishizuka Y. Dual origin of mesenchymal tissues participating in mouse mammary gland embryogenesis. *Dev Biol* 1982; 91: 202-7.

273. Sakao K, Takahashi KA, Arai Y, Saito M, Honjyo K, Hiraoka N, Kishida T, Mazda O, Imanishi J, Kubo T. Asporin and transforming growth factor-beta gene expression in osteoblasts from subchondral bone and osteophytes in osteoarthritis. *J Orthop Sci* 2009; 14: 738-47.

274. Sasaki H, Dai M, Auclair D, Fukai I, Kiriyama M, Yamakawa Y, Fujii Y, Chen LB. Serum level of the periostin, a homologue of an insect cell adhesion molecule, as a prognostic marker in nonsmall cell lung carcinomas. *Cancer* 2001; 92: 843-8.

275. Sasaki H, Sato Y, Kondo S, Fukai I, Kiriyama M, Yamakawa Y, Fuji Y. Expression of the periostin mRNA level in neuroblastoma. *J Pediatr Surg* 2002; 37: 1293-7.

276. Sasaki H, Yu CY, Dai M, Tam C, Loda M, Auclair D, Chen LB, Elias A. Elevated serum periostin levels in patients with bone metastases from breast but not lung cancer. *Breast Cancer Res Treat* 2003; 77: 245-52.

277. Sato T, Sakai T, Noguchi Y, Takita M, Hirakawa S, Ito A. Tumor-stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. *Gynecol Oncol* 2004; 92: 47-56.

278. Seaton A, Scullin P, Maxwell PJ, Wilson C, Pettigrew J, Gallagher R, O'Sullivan JM, Johnston PG, Waugh DJ. Interleukin-8 signaling promotes androgen-independent proliferation of prostate cancer cells via induction of androgen receptor expression and activation. *Carcinogenesis* 2008; 29: 1148-56.

279. Sethi S, Macoska J, Chen W, Sarkar FH. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am J Transl Res* 2010; 3: 90-9.

280. Shackney SE, Silverman JF. Molecular evolutionary patterns in breast cancer. *Adv Anat Pathol* 2003; 10: 278-90.

281. Shao R, Bao S, Bai X, Blanchette C, Anderson RM, Dang T, Gishizky ML, Marks JR, Wang XF. Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. *Mol Cell Biol* 2004; 24: 3992-4003.

282. Shay JW, Roninson IB. Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene*. 2004; 23: 2919-33.

283. Shimizu-Hirota R, Sasamura H, Kuroda M, Kobayashi E, Saruta T. Functional characterization of podocan, a member of a new class in the small leucine-rich repeat protein family. *FEBS Lett* 2004; 563: 69-74.

284. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K. Molecular definition of breast tumor heterogeneity. *Cancer Cell* 2007; 11: 259-73.

285. Schaefer L, Iozzo RV. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. *J Biol Chem* 2008; 283: 21305-9.

286. Schalken JA, van Leenders G. Cellular and molecular biology of the prostate: stem cell biology. *Urology* 2003; 62: 11-20.

287. Schoell WM, Pieber D, Reich O, Lahousen M, Janicek M, Guecer F, Winter R. Tumor angiogenesis as a prognostic factor in ovarian carcinoma: quantification of endothelial immunoreactivity by image analysis. *Cancer* 1997; 80: 2257-62.

288. Schulz WA. Molecular biology of human cancers. Springer Science + Business Media, Inc. 2005.

289. Sidani M, Wyckoff J, Xue C, Segall JE, Condeelis J. Probing the microenvironment of mammary tumors using multiphoton microscopy. *J Mammary Gland Biol Neoplasia* 2006; 11: 151-63.

290. Simpson PT, Reis-Filho JS, Gale T, Lakhani SR. Molecular evolution of breast cancer. *J Pathol* 2005; 205: 248-54.

291. Singer CF, Gschwantler-Kaulich D, Fink-Retter A, Haas C, Hudelist G, Czerwenka K, Kubista E. Differential gene expression profile in breast cancer-derived stromal fibroblasts. *Breast Cancer Res Treat* 2008; 110: 273-81.

292. Sjöberg G, Edström L, Lendahl U, Sejersen T. Myofibers from Duchenne/Becker muscular dystrophy and myositis express the intermediate filament nestin. *J Neuropathol Exp Neurol*. 1994; 53: 416-23.

293. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res*. 2006; 66: 2918-22.

294. Smit MA, Geiger TR, Song JY, Gitelman I, Peeper DS. A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis. *Mol Cell Biol* 2009; 29: 3722-37.

295. Smit MA, Peeper DS. Epithelial-mesenchymal transition and senescence: two cancer-related processes are crossing paths. *Aging (Albany NY*). 2010; 2: 735-41.

296. Soikkeli J, Podlasz P, Yin M, Nummela P, Jahkola T, Virolainen S, Krogerus L, Heikkilä P, von Smitten K, Saksela O, Hölttä E. Metastatic outgrowth encompasses COL-I, FN1, and POSTN up-regulation and assembly to fibrillar networks regulating cell adhesion, migration, and growth. *Am J Pathol*. 2010; 177: 387-403.

297. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001; 98: 10869-74.

298. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S,Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lønning PE, Brown PO,Børresen-Dale AL, Botstein D. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*. 2003; 100: 8418-23.

299. Sossey-Alaoui K, Bialkowska K, Plow EF. The miR200 family of microRNAs regulates WAVE3-dependent cancer cell invasion. *J Biol Chem* 2009; 284: 33019-29.

300. Soung YH, Chung J. Curcumin Inhibition of the Functional Interaction between Integrin {alpha}6{beta}4 and the Epidermal Growth Factor Receptor. *Mol Cancer Ther* 2011; 10: 883-91.

301. Sprenger CC, Drivdahl RH, Woodke LB, Eyman D, Reed MJ, Carter WG, Plymate SR. Senescence-induced alterations of laminin chain expression modulate tumorigenicity of prostate cancer cells. *Neoplasia*. 2008; 10: 1350-61.

302. Sprenger CC, Plymate SR, Reed MJ. Aging-related alterations in the extracellular matrix modulate the microenvironment and influence tumor progression. *Int J Cancer*. 2010; 127: 2739-48. doi: 10.1002/ijc.25615.

303. Stappert J, Kemler R. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes Commun*. 1994; 2: 319-27.

304. Sternlicht MD, Sunnarborg SW. The ADAM17-amphiregulin-EGFR axis in mammary development and cancer. *J Mammary Gland Biol Neoplasia*. 2000; 13: 181-94.

305. Stoker M, Perryman M. An epithelial scatter factor released by embryo fibroblasts. *J Cell Sci* 1985; 77: 209-23.

306. Strizzi L, Bianco C, Normanno N, Seno M, Wechselberger C, Wallace-Jones B,Khan NI, Hirota M, Sun Y, Sanicola M, Salomon DS. Epithelial mesenchymal transition is a characteristic of hyperplasias and tumors in mammary gland from MMTV-Cripto-1 transgenic mice. *J Cell Physiol*. 2004; 201: 266-76.

307. Sugawara K, Kurihara H, Negishi M, Saito N, Nakazato Y, Sasaki T, Takeuchi T. Nestin as a marker for proliferative endothelium in gliomas. *Lab Invest*. 2002; 82: 345-51.

308. Sutherland RM, Franko AJ. On the nature of the radiobiologically hypoxic fraction in tumors. Int J Radiat *Oncol Biol Phys*. 1980; 6: 117-20.

309. Takahashi Y, Tucker SL, Kitadai Y, Koura AN, Bucana CD, Cleary KR, Ellis LM. Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Arch Surg* 1997; 132: 541-6.

310. Takanami I, Abiko T, Koizumi S. Expression of periostin in patients with non-small cell lung cancer: correlation with angiogenesis and lymphangiogenesis. *Int J Biol Markers* 2008; 23:182-6.

311. Tang Y, Kesavan P, Nakada MT, Yan L. Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. *Mol Cancer Res* 2004; 2:73-80.

312. Tanigawa N, Amaya H, Matsumura M, Shimomatsuya T. Association of tumour vasculature with tumour progression and overall survival of patients with non-early gastric carcinomas. *Br J Cancer* 1997; 75: 566-71.

313. Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y, Ijiri K, Akimitsu N. MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing theexpression of motility-related genes. *FEBS Lett*. 2010; 584: 4575-80.

314. Tavassoli F. Pathology of the breast. 2nd ed. Stamford, conn: Appleton & Lange. 1999.

315. Taylor MA, Parvani JG, Schiemann WP. The pathophysiology of epithelial-mesenchymal transition induced by transforming growth factor-beta in normal and malignant mammary epithelial cells. *J Mammary Gland Biol Neoplasia*.2010; 15:169-90.

316. Teranishi N, Naito Z, Ishiwata T, Tanaka N, Furukawa K, Seya T, Shinji S, Tajiri T. Identification of neovasculature using nestin in colorectal cancer. *Int J Oncol* 2007; 30: 593-603.

317. Theriault R. Bone-directed therapy and breast cancer: Bisphosphonates, Monoclonal Antibody, and Radionuclides. In: Harris J, Lippman ME, Osborne CK, Morrow M (eds) Diseases of the Breast. Lippincott Williams & Wilkins, Philadelphia, 2010.

318. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003; 15: 740-6.

319. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; 2: 442-54.

320. Tindal D and Mohler J. Androgen action in prostate cancer. Springer Dordrecht Heidelberg London New Yourk 2009.

321. Tindal D and Mohler J. Androgen action in prostate cancer. Springer Dordrecht Heidelberg London New Yourk 2009.

322. Tlsty TD, Coussens LM. Tumor stroma and regulation of cancer development. *Annu Rev Pathol*.2006; 1: 119–50.

323. Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, Schalken JA. Cadherin switching in human prostate cancer progression. Cancer Res. 2000; 60: 3650-4.

324. Torchinsky A, Toder V. To die or not to die: the function of the transcription factor NF-kappaB in embryos exposed to stress. *Am J Reprod Immunol* 2004; 51: 138-43.

325. Touab M, Villena J, Barranco C, Arumi-Uria M, Bassols A. Versican is differentially expressed in human melanoma and may play a role in tumor development. *Am J Pathol* 2002; 160: 549-57.

326. Tran NL, Nagle RB, Cress AE, Heimark RL. N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion withStromal cells. Am J Pathol. 1999; 155: 787-98.

327. Troup S, Njue C, Kliewer EV, Parisien M, Roskelley C, Chakravarti S, Roughley PJ, Murphy LC, Watson PH. Reduced expression of the small leucine-rich proteoglycans, lumican, and decorin is associated with poor outcome in node-negative invasive breast cancer. *Clin Cancer Res* 2003; 9: 207-14.

328. Turashvili G, Bouchal J, Baumforth K, Wei W, Dziechciarkova M, Ehrmann J, Klein J, Fridman E, Skarda J, Srovnal J, Hajduch M, Murray P, Kolar Z .Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis. *BMC Cancer* 2007; 27;7:55.

329. Turtoi A, Musmeci D, Wang Y, Dumont B, Somja J, Bevilacqua G, De Pauw E,Delvenne P, Castronovo V. Identification of Novel Accessible Proteins Bearing Diagnostic and Therapeutic Potential in Human Pancreatic Ductal Adenocarcinoma. *J Proteome Res*. 2011 Jul 29.

330. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech Ageing Dev* 2005; 126: 59-69.

331. Untergasser G, Koch HB, Menssen A, Hermeking H. Characterization of epithelial senescence by serial analysis of gene expression: identification of genes potentially involved in prostate cancer. *Cancer Res*. 2002; 62: 6255-62.

332. Uria JA, Stahle-Backdahl M, Seiki M, Fueyo A, Lopez-Otin C. Regulation of collagenase-3 expression in human breast carcinomas is mediated by stromal-epithelial cell interactions. *Cancer Res* 1997; 57: 4882-8.

333. van der Kraan PM, Blaney Davidson EN, van den Berg WB. A role for age-related changes in TGFbeta signaling in aberrant chondrocyte differentiation and osteoarthritis. *Arthritis Res Ther* 2010; 12: 201.

334. Vandewalle C, Van Roy F, Berx G. The role of the ZEB family of transcription factors in development and disease. *Cell Mol Life Sci* 2009; 66: 773-87.

335. Vashchenko N, Abrahamsson PA. Neuroendocrine differentiation in prostate cancer: implications for new treatment modalities. *Eur Urol* 2005; 47: 147-55.

336. Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA. Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev* 2004; 18: 1131-43.

337. Vergara D, Merlot B, Lucot JP, Collinet P, Vinatier D, Fournier I, Salzet M. Epithelial-mesenchymal transition in ovarian cancer. *Cancer Lett* 2010; 291: 59-66.

338. Vignjevic D, Schoumacher M, Gavert N, Janssen KP, Jih G, Lae M, Louvard D, Ben-Ze'ev A, Robine S. Fascin, a novel target of beta-catenin-TCF signaling, is expressed at the invasive front of human colon cancer. *Cancer Res* 2007; 67: 6844-53.

339. Vichalkovski A, Gresko E, Hess D, Restuccia DF, Hemmings BA. PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage. *Oncogene* 2010; 29: 3554-65.

340. Vlahovic G, Ponce AM, Rabbani Z, Salahuddin FK, Zgonjanin L, Spasojevic I, Vujaskovic Z, Dewhirst MW. Treatment with imatinib improves drug delivery and efficacy in NSCLC xenografts. *Br J Cancer* 2007; 97: 735-40.

341. Waerner T, Alacakaptan M, Tamir I, Oberauer R, Gal A, Brabletz T, Schreiber M, Jechlinger M, Beug H. ILEI: a cytokine essential for EMT, tumor formation, and late events in metastasis in epithelial cells. *Cancer Cell* 2006; 10: 227-39.

342. Wang L, Xiang YN, Zhang YH, Tu YT, Chen HX. Collagen triple helix repeat containing-1 in the differential diagnosis of dermatofibrosarcoma protuberans and dermatofibroma. *Br J Dermatol*. 2011;164:135-40.

343. Watson CJ, Khaled WT. Mammary development in the embryo and adult: a journey of morphogenesis and commitment. *Development* 2008; 135: 995-1003.

344. Wechselberger C, Ebert AD, Bianco C, Khan NI, Sun Y, Wallace-Jones B, Montesano R, Salomon DS. Cripto-1 enhances migration and branching morphogenesis of mouse mammary epithelial cells. *Exp Cell Res* 2001; 266: 95-105.

345. West RB, Nuyten DS, Subramanian S, et al. Determination of stromal signatures in breast carcinoma. *PLoS Biol* 2005;3:e187

346. Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, Boheler KR, Wobus AM. Nestin expression--a property of multi-lineage progenitor cells? *Cell Mol Life Sci* 2004; 61: 2510-22.

347. Wight TN. Arterial remodeling in vascular disease: a key role for hyaluronan and versican. *Front Biosci* 2008;13:4933-7.

348. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res*. 2004;94:1158-67.

349. Winkler F, Kozin SV, Tong RT, Chae SS, Booth MF, Garkavtsev I, Xu L, Hicklin DJ, Fukumura D, di Tomaso E, Munn LL, Jain RK. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* 2004; 6: 553-63.

350. Xu L, Fukumura D, Jain RK. Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway: mechanism of low pH-induced VEGF. *J Biol Chem* 2002; 277: 11368-74.

351. Xue Y, Verhofstad A, Lange W, Smedts F, Debruyne F, de la Rosette J, Schalken J. Prostatic neuroendocrine cells have a unique keratin expression pattern and do not express Bcl-2: cell kinetic features of neuroendocrine cells in the human prostate. *Am J Pathol* 1997; 151: 1759-65.

352. Yamada S, Murakami S, Matoba R, Ozawa Y, Yokokoji T, Nakahira Y, Ikezawa K, Takayama S, Matsubara K, Okada H. Expression profile of active genes in human periodontal ligament and isolation of PLAP-1, a novel SLRP family gene. *Gene* 2001; 275: 279-86.

353. Yamamoto S, Nishimura O, Misaki K, Nishita M, Minami Y, Yonemura S, Tarui H, Sasaki H (2008) Cthrc1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex. *Dev Cell* 15:23-36.

354. Yan W, Shao R. Transduction of a mesenchyme-specific gene periostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation. *J Biol Chem*. 2006; 281: 19700-8.

355. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004; 117: 927-39.

356. Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA. TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* 1999; 103: 197-206.

357. Yoder BJ, Wilkinson EJ, Massoll NA. Molecular and morphologic distinctions between infiltrating ductal and lobular carcinoma of the breast. *Breast J* 2007; 13: 172-9.

358. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; 14: 163-76.

359. Yuan TC, Veeramani S, Lin FF, Kondrikou D, Zelivianski S, Igawa T, Karan D, Batra SK, Lin MF. Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells. *Endocr Relat Cancer*. 2006;13:151-67.

360. Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, Kneitz S, Piek E, Bottinger EP. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A* 2001; 98: 6686-91.

361. Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* 2005; 24: 5764-74.

362. Zavadil J, Narasimhan M, Blumenberg M, Schneider RJ. Transforming growth factor-beta and microRNA:mRNA regulatory networks in epithelial plasticity. *Cells Tissues Organs* 2007; 185: 157-61.

363. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* 2009; 119: 1429-37.

364. Zhan L, Rosenberg A, Bergami KC, Yu M, Xuan Z, Jaffe AB, Allred C, Muthuswamy SK. Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell.* 2008; 135: 865-78.

365. Zhang Y, Yang M, Ji Q, Fan D, Peng H, Yang C, Xiong D, Zhou Y. Anoikis induction and metastasis suppression by a new integrin alphavbeta3 inhibitor in human melanoma cell line M21. *Invest New Drugs* 2011; 29: 666-73.

366. Zhang Y, Zhang G, Li J, Tao Q, Tang W. The expression analysis of periostin in human breast cancer. *J Surg Res.* 2010; 160: 102-6.

367. Zheng PS, Wen J, Ang LC, Sheng W, Viloria-Petit A, Wang Y, Wu Y, Kerbel RS, Yang BB Versican/PG-M G3 domain promotes tumor growth and angiogenesis. *FASEB J* 2004; 18: 754-6.

368. Zindy F, Williams RT, Baudino TA, Rehg JE, Skapek SX, Cleveland JL, Roussel MF, Sherr CJ. Arf tumor suppressor promoter monitors latent oncogenic signals in vivo. *Proc Natl Acad Sci U S A* 2003; 100: 15930-5.

369. Zucker S, Hymowitz M, Conner CE, DiYanni EA, Cao J. Rapid trafficking of membrane type 1-matrix metalloproteinase to the cell surface regulates progelatinase a activation. *Lab Invest*. 2002 ; 1673-84.

370. Zumsteg A, Christofori G. Corrupt policemen: inflammatory cells promote tumor angiogenesis. *Curr Opin Oncol* 2009; 21: 60-70.