Adhesion signaling in mammary gland development, tumorgenesis and progression

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Chapter 1

Adhesion signaling in mammary gland development and tumorigenesis

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General introduction

Adhesion signaling in mammary gland development and tumorigenesis

Breast cancer is one of the most common malignancies in women causing over 3000 deaths every year in The Netherlands. Most patients die of distant metastases that are frequently unresponsive to cancer therapy. In order to metastasize, cells need to be able to migrate and invade into the surrounding tissue, intravasate in to a blood vessel or lymphatic system, survive in circulation, extravasate and finally proliferate at a distant site. During mammary gland development, several biological processes occur in the mammary gland that also take place during breast cancer development and progression. For example many of the stromal factors involved in mammary gland development also promote or protect against breast cancer. Epithelial and stromal cells communicate via the extracellular matrix (ECM). Disruption of this interaction and respective communication can induce breast cancer. To be able to understand the process of breast cancer and metastases formation and the role of adhesion signaling in this process, it is also important to gain understanding of the normal function of the mammary gland.

In the following paragraphs factors involved in mammary gland and breast cancer development will be discussed. Improved understanding of processes involved in mammary gland biology, initiation of breast cancer and metastases formation will ultimately lead to more effective cancer therapy.

Introduction to mammary gland development

Mammary gland development is a dynamic process in which proliferation, apoptosis, differentiation and migration are essential for the formation of the highly organized branched ductal network of epithelial cells. Although the mammary gland is already present in embryos, initial development at embryonic day 10 in mice, most of the branching morphogenesis that is required for the development of the ductal tree occurs postnatally around the time of ovarian hormone release at puberty. During this time the distal ends of the mammary ducts develop into bulbous structures composed of multiple layers of epithelial cells, called the terminal end buds (TEBs, Fig. 1) (1). TEBs, highly proliferative structures that are the invading fronts of the ducts, extend into the fat pad, and branch until the fat pad is completely filled (2). The final developmental fate of the mammary gland is accomplished during pregnancy and lactation. Upon stimulation by reproductive hormones, the mammary epithelium expands and differentiates into milk-producing lobular alveoli (1, 3). Eventually, when pups no longer suckle the nipple, the secretory epithelium of the mammary gland dies by apoptosis and the mammary gland is remodeled back to the state resembling the adult mammary gland, called involution (4).

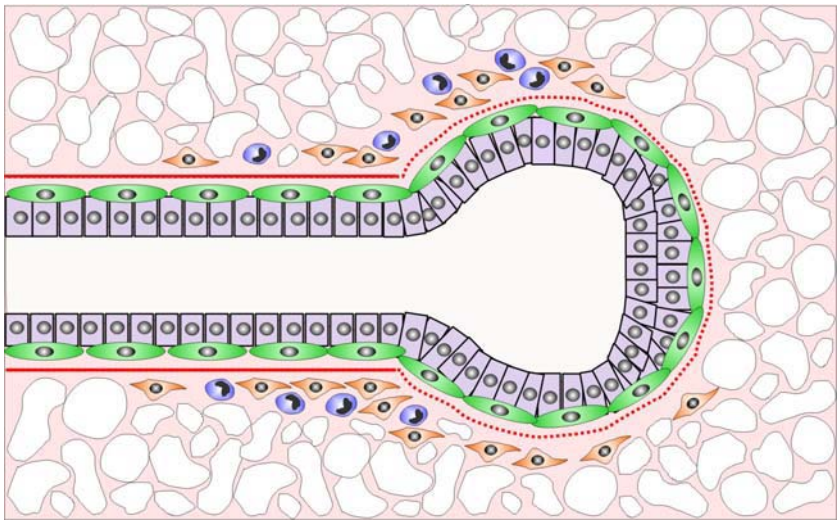

Figure 1. Schematic representation of a Terminal End Bud
Ductal structures contain a layer of luminal epithelial cells (pink) that are surrounded by a layer of myoepithelial cells (green). Terminal end buds as depicted in this diagram contain multiple layers of luminal epithelial cells. The myoepithelial cells are in contact with the laminin-rich basement membrane (red). Fibroblasts and stromal macrophages surround the ducts (orange and blue cells, respectively). The major part of the mammary gland consists of adipocytes (white).

Factors involved in mammary gland development
There are several factors, such as estrogen, progesterone and prolactin that act on stromal cells to stimulate the branching process (5). During puberty, both estrogen and EGF control ductal elongation and branching. Other growth factors such as fibroblast growth factors (FGF) (6-8), insulin growth factor (IGF) (9), neuregulin (NRG), amphiregulin and receptors ERBB2/3/4 (10-13), have also been implicated in embryonic mammary gland development and branching morphogenesis. Factors such as progesterone, prolactin and placental lactogens stimulate alveolar proliferation and differentiation during pregnancy (3). In addition to these hormones proteases are also essential in the control of the branching process. Metalloproteases are both upstream and downstream of EGF receptor signaling by which they regulate growth factor function and branching (1, 14, 15). Just recently, it was shown that collective epithelial migration and cell rearrangements drive mammary branching morphogenesis (16). Duct elongation requires proliferation, Rac and myosin light-chain kinase, whereas repolarization to a bilayer depends on Rho (16).

Mammary gland morphology and physiology

Luminal and myoepithelial cells
Quiescent mammary ducts are bilayered, one layer of luminal epithelial cells, that are surrounded by a layer of myoepithelial cells (also referred to as basal cells). Luminal and myoepithelial cells originate from the same progenitor cell. This common progenitor differentiates into a luminal progenitor and a myoepithelial progenitor cell that finally differentiates to a myoepithelial cell (17, 18). Luminal progenitors can differentiate to mature ductal, alveolar or secretory cells. Luminal epithelial cells differentiate to milk-producing cells upon stimulation with prolactin. Breast cancers mainly arise in the luminal epithelial compartment (19). This is most likely related to the dynamics of differentiation and dedifferentiation of these cells.

Myoepithelial cells function as a guardian of tissue integrity of the mammary gland by maintaining tissue polarity (20, 21). Approximately 15% of all breast cancers show basal/myoepithelial like characteristics. To distinguish luminal and myoepithelial cells several markers can be used. Luminal epithelial cells are MUC-1 positive and express cytokeratin 8 (CK8). Myoepithelial cells on the other hand are smooth muscle actin-1 (SMA-1) positive, express cytokeratin 5 and p63.

Basement membrane
Most cells are dependent on adhesion to other cells and to the extracellular matrix in order to survive. Also in the mammary gland the basement membrane has an important role as a survival factor for mammary epithelial cells. The mammary

basement membrane contains collagen IV, and laminin-1 and -11, which are cross-linked by nidogen-1 and 2 to form a gel-like structure to which mammary epithelial cells adhere (22). The adhesion of mammary epithelial cells to extracellular matrix via integrins, family of heterodimeric transmembrane glycoprotein receptors, has been shown to suppress apoptosis (23, 24). Integrins regulate cell shape and facilitate migration by providing a structural link with the actin cytoskeleton. Apart from integrins, several other receptors such as dystroglycans, syndecans and galactosyl transferases function as adhesion receptors for basement membrane proteins (25). Binding of integrins to the ECM promotes the formation of adhesion complexes at the plasma membrane. These adhesion complexes are important for cytoskeleton assembly and signaling for controlling cell behavior (26, 27). Adhesion complexes function by recruiting structural proteins, e.g. talin, vinculin, adaptor proteins, e.g. paxillin, p130Cas, and enzymes, e.g. FAK, Src, and small GTPases (reviewed in Geiger et al. (28)). The expression and deposition of extracellular matrix proteins is frequently altered in breast cancer. Reduced expression of integrins and lack of basement membrane is often observed in metastatic disease. Basement membrane isolated from Engelbreth-Holm-Swarm tumor, known as Matrigel, is thought to mimic the mammary basement membrane and is therefore often used to study cell-ECM interaction in a three-dimensional environment.

Stromal cells & microenvironment

In addition to the extracellular matrix components described in previous section, stromal cells secrete a separate set of extracellular components such as fibronectin (FN) (29). The stroma of the mammary gland is composed of a variety of cells including adipocytes, fibroblasts, and migratory leukocytes. Macrophages and eosinophils are recruited to the mammary gland simultaneous with the outgrowth of terminal end buds (TEBs). Macrophages are recruited mostly to the neck of the TEB while eosinophils are mainly located around the head of the TEB (30). Leukocyte depletion from the mammary gland was shown to result in dramatic inhibition of ductal development. No other leukocytes are found surrounding the ducts (31). Survival and proliferation of macrophages as well as macrophage behavior, morphology and motility is regulated by colony-stimulating factor-1 (CSF-1). CSF-1 has a crucial role in mammary gland development as CSF-1 null mice show clear defects in branching morphogenesis during development and pregnancy (32-35). Regulation of the CSF-1/CSF-1R system seems to be crucial for ductal outgrowth. Since the CSF-1 receptor (CSF-1R) is exclusively expressed on macrophages in the mammary gland tissue, macrophages are important stromal factors in mammary gland development. CSF-1 is essential for proper mammary gland development and elevated expression of CSF-1 in human breast cancer correlates with poor prognosis (36, 37).

Stem cells

For years researchers have been studying the regenerative capacity of the mammary gland. Fifty years ago a technique was developed to determine the regenerative capacity by transplanting small pieces of tissue into the mouse mammary fat pad cleared from endogenous epithelium (38). Later on others showed that repopulating mammary cells exist throughout the life span of an adult mouse (39). It is believed that the progenitor cells, able to differentiate into myoepithelial and luminal epithelial cells, originate from a cell from the luminal epithelial compartment. Several markers were identified that are expressed by mammary stem cells (MaSC). Expression of CD29 (β1-integrin) or CD49f (α6-integrin) and CD24 (heat-stable antigen) was found enriched in the MaSC population. A single cell from the $CD29^{hi}/CD24^{+}$ or $CD49f^{hi}/CD24^{+}$ population was found sufficient to completely reconstitute a functional mammary gland in vivo (40, 41). Integrin-β1 deficient transplanted mammary tissue failed to repopulate the cleared fat pad and deletion of β1-integrin in the basal compartment of the mammary gland was shown to affect the regenerative potential of the mammary epithelium (42, 43). This effect on stem cells can be due to loss of cell-matrix adhesion, a process that is essential in stem cell niches to establish and maintain the niche architecture (44). In addition to β1-integrin, FAK deletion was also shown to affect the stem cell niche in a breast cancer model (44, 45).

Branching morphogenesis

Factors involved in branching morphogenesis

There are two distinct mechanisms of branching morphogenesis in the pubertal mouse; bifurcation of the terminal end bud (TEB), only occurring in immature ducts and side branching, when a new branch forms from a mature duct. During TEB bifurcation, a branch point is formed through deposition of stroma at the cleft side. The duct extends into the adipose tissue, a process initiated by cap cells, without myoepithelial cells or stroma, and with a minimal amount of basement membrane at the invasive front. Factors that are involved in bifurcation of the TEB include β1 integrin, laminin-1, matrix metalloproteinase (MMPs), DDR-1, GH, insulin growth factor-1 (IGF-1), and its receptor IGF-1R (46, 47). In contrast, during side branching ducts must extend through a layer of myoepithelial cells, degrade the aligning basement membrane and invade the surrounding stroma. Factors that are involved in side branching include progesterone receptor (48), p27kip-1 (49), Wnts (50-52), heparan sulfate proteoglycans (HSPGs), NFkB, MMPs (53, 54), tissue inhibitor of metalloproteinase (TIMP-1), TGFβ, TGFβIIR (47), P-cadherin (2), C/EBPb (55), CSF-1(31, 36), Stat5a, and Stat5b.

Adhesion and cytoskeletal dynamics in mammary gland function

Cell-cell and cell-matrix adhesions are essential for the formation and maintenance of the mammary gland. Adhesion molecules control organization of groups of cells and are essential for signaling between neighboring cells and stroma. This communication is important to promote proliferation and differentiation only when the cell is in the proper microenvironment. One of the most important functions of adhesion molecules is to create and maintain the polarized structure of the mammary gland.

Cell-cell adhesion

Epithelial structures are maintained by cell-cell junctional complexes, mainly adherens junctions, desmosomes, tight junctions and gap junctions. A central component of adherens junctions are cadherins. In the mammary gland, E-cadherin is present in luminal epithelial cells and myoepithelial cells express P-cadherin (56). Blocking antibodies for E-cadherin cause disruption of the luminal epithelial cell layer, resulting in floating cells in the luminal compartment, while blocking antibodies for P-cadherin disrupted the basal layer, indicating that both E- and P-cadherin function to maintain tissue integrity. While cadherins are important to maintain the ductal integrity, tight junctions are particularly important in the alveoli of the lactating mammary gland, to ensure properly polarized secretion of milk. Tight junctions are located at the apical cell-cell junctional complexes, preventing diffusion of proteins between the apical and basolateral compartment of the cell membrane (57, 58).

Cell-matrix adhesions

As indicated, the mammary gland consists of myoepithelial and luminal epithelial cells, stromal cells and adipocytes. Ductal structures are comprised of luminal epithelial cells, cells that are located at the apical side of the duct; myoepithelial cells are located at the basolateral side of the duct and surround the luminal epithelial cells. Myoepithelial cells are in contact with the basement membrane, an interaction that is important to maintain the highly organized, polarized structure of the mammary gland. This integrin-mediated interaction with laminin-rich basement membrane supports prolactin-dependent activation of the JAK-Stat5 pathway and transcription of prolactin- and Stat5-regulated genes such as β-casein. Such integrin-mediated signaling occurs within discrete sites of close contact between the cell membrane and the extracellular matrix (ECM) termed “cell-matrix adhesions”. One important signal transducer within cell-matrix adhesions is focal adhesion kinase (FAK). Together with Src family kinases, FAK integrates signals from growth-factor receptors and integrins (Fig. 2) to control processes such as adhesion and cytoskeleton dynamics, cell migration, and proliferation and survival.

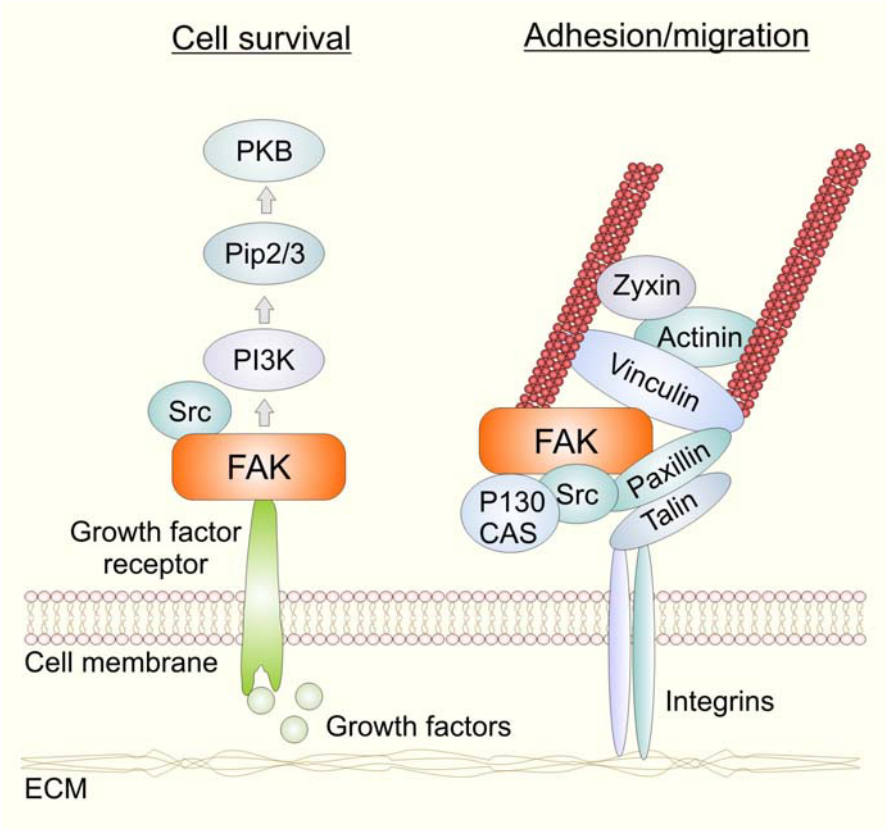

Figure 2. FAK mediated signaling
FAK mediated cell survival signaling through its interaction with growth factor receptors (left). Upon interaction with the ECM integrins cluster and subsequently recruit adaptor and signaling proteins. Integrins can directly interact with signaling proteins such as Talin that bind to adaptor proteins to recruit actin-binding proteins vinculin and paxillin, as well as regulatory proteins such as FAK to the focal contacts. These interactionsm, in combination with binding and bundling of actin, facilitates adhesion and migration (right).

Cytoskeletal dynamics & cell migration/invasion
Migration and invasion are essential processes during mammary gland development. Ductal elongation and migration into the fat pad are required for full ductal development postnatal. In order to migrate, cells need to be able to assemble and disassemble focal contacts and rearrange their actin cytoskeleton. Stress fiber assembly and contraction are predominantly induced by Rho and its downstream effector ROCK. Cell migration is a five-step model (described by Friedl & Wolf (59)). First, cells gain protrusions at the leading edge, where actin

filaments connect to adaptor proteins and push the cell membrane outwards. Actin polymerization is achieved by coupling of Arp2/3 with WASP, a complex that interacts with PIPs. PIPs are also able to bind guanine exchange factors (GEFs) that regulate the activity of small GTPases like Rac, Rho and Cdc42 (60-63). Second, cells interact with the extracellular matrix and formation of focal contacts is achieved. Upon interaction with the ECM integrins cluster and subsequently recruit adaptor and signaling proteins (64, 65). Integrins can directly interact with signaling proteins such as talin that bind to adaptor proteins to recruit actin-binding proteins (vinculin & paxillin) as well as regulatory proteins such as FAK to the focal contacts (Fig. 2) (66-68). The third step in migration is recruitment of proteases to ECM contacts. In order to migrate, cells need to be able to degrade the ECM. Cleavage of ECM components such as fibronectin, collagen and laminin is facilitated by MMPs (69-71). Next, active myosin II binds to actin filaments to generate actomyosin contraction. Rho regulates actomyosin contraction via its downstream effector ROCK (72-74). The last step is detachment of the trailing edge, a process in which focal contacts are disassembled and integrins detach from the ECM and are internalized (75-78).

During mammary gland development collective migration is essential for ductal elongation. Mammary epithelial cells migrate collectively, without formation of leading cellular extensions. One of the molecular regulators of ductal morphogenesis is small GTPase Rac that is required for ductal initiation while small GTPase Rho kinase is needed for repolarization to a bilayer. Interestingly, organization of mammary epithelium during ductal morphogenesis in cell culture, in vivo and mouse mammary hyperplasia is similar.

Introduction of deregulation in mammary gland development

Loss of cell-cell interaction

Cell-cell adhesion is crucial for survival signaling but also for maintaining mammary gland architecture. Mice deficient for adhesion molecules E-cadherin and N-cadherin are not viable. To study the effect of E-cadherin depletion in mammary gland development and tumorigenesis, Derksen *et al* made use of conditional inactivation of E-cadherin and p53 in skin and mammary gland using K14-promotor, predominantly expressed in myoepithelial cells. No abnormal ductal or alveolar development was observed in virgin, pregnant or parous mice. In addition, they showed that loss of E-cadherin alone did not predispose to cancer. However, combined deletion of E-cadherin and p53 resulted in accelerated development of invasive and metastatic carcinomas (79). Though deletion of E-cadherin did not seem to affect mammary gland development, mammary gland specific deletion of P-cadherin resulted in precocious mammary gland development and mice develop hyperplasias and dysplasias with age. Thus P-

cadherin-mediated signaling derived from cell-cell interactions most likely regulate negative growth control in the mammary gland (80).

In addition to cell adhesion molecules, several tight junction components have been shown to be directly or indirectly involved in breast cancer formation. The tight junction component ZO-1 functions as a tumor suppressor as decreased ZO-1 expression is often found in invasive breast cancer cell lines (81). ZO-2 has been shown to be down-regulated in most breast adenocarcinomas (82). Furthermore, several other tight junction components, such as Claudin-1 and 7, have been shown to be deregulated in breast cancer cell lines and breast carcinoma (83, 84). In summary, both cell-adhesion molecules as well as tight junction components are important for maintaining the integrity of the mammary gland and disruption of either one of these structures can directly or indirectly contribute to breast cancer formation.

Loss of cell-matrix interaction

β1-integrins are involved in maintenance of the integrity of mammary acini. An early study using a mouse model with expression of a dominant-negative mutant of β1-integrin showed that β1-integrin is important for processes such as proliferation, apoptosis, differentiation and for the maintenance of baso-apical polarity of mammary epithelium (85, 86). Also studies using β1-integrin specific antibodies implicated β1-integrin as an essential factor in mammary gland development (87). The importance of β1-integrin in mammary gland development was later confirmed using the Cre-lox system, in which β1-integrin was deleted specifically in luminal epithelial cells. This led to disorganized alveoli resulting from alterations in cell-basement membrane associations, impaired alveologenesis and lactation (43). Moreover, β1-integrin deleted mammary epithelial cells cultured in three-dimensional system were unable to form or maintain polarized acini (88). Deletion of β1-integrin in the basal compartment, targeting myoepithelial cells, abolished the regenerative potential of the mammary epithelium and affects mammary gland development (42). This study showed for the first time the essential role of β1-integrin in mediating mammary epithelial cell interaction with the ECM, which is needed for the maintenance of a functional stem cell population, mammary morphogenesis and segregation of the two mammary cell lineages (42). Thus β1-integrin is essential in mammary gland development and lactation. Interestingly, deletion of β1-integrin reduced FAK phosphorylation and protein levels indicating that FAK might be a central player in signal transduction required for mammary gland development (43, 88). Indeed, specific deletion of FAK in the luminal compartment of mammary ductal structures resulted in severe lobulo-alveolar hypoplasia and secretory immaturity of the mammary gland (89). However, recently others have suggested that the defects in lactational differentiation caused by β1-integrin deletion are due to

reduced integrin-linked kinase (ILK)-mediated signaling and not FAK-mediated signaling (90).

Introduction of breast cancer formation and progression

Luminal and myoepithelial cancer origin
Human breast cancers are heterogeneous in their morphology, response to therapy and clinical course. Five major subtypes of breast carcinomas can be identified (e.g. luminal A, luminal B, normal breast-like, ErbB2-positive and basal-like) (91-93). Luminal and basal-like breast cancers (BLBC) correspond to the two distinct types of epithelial cells found in the normal mammary gland, luminal epithelial and myoepithelial cells, respectively. However, although basal-like breast cancers express myoepithelial markers such as cytokeratin 5, it is not known whether these tumors develop from myoepithelial cells or that they gain myoepithelial markers in time. Luminal and basal-like breast cancers differ in their clinical course (91, 92) and response to therapeutic agents (94, 95). In particular, the triple negative, i.e. negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), basal-like subtype is characterized by a poor clinical outcome due to resistance to chemotherapy, enhanced invasiveness and formation of distant metastasis.

Factors involved in breast cancer formation/progression
Numerous factors have been described that are directly or indirectly involved in the formation of breast cancer as well as progression. As mentioned in the previous paragraph, breast cancer subtypes are characterized by the expression of a set of markers; ErbB2, progesterone receptor (PR), estrogen receptor (ER), and p53 status. These markers are used to distinguish the different breast cancer subtypes to improve the determination of patient prognosis. Triple-negative tumors (ER, PR and Her2 negative) are aggressive tumors often showing accumulation of (mutant) p53, and are frequently non-responsive to anti-cancer therapeutics (96, 97). One factor that we are particularly interested in is (mutant) p53 and its role in breast cancer formation and progression.

p53 and breast cancer
Breast cancer can develop spontaneously by mutations in tumor suppressor genes or amplification of oncogenes, but can also occur due to germline mutations in (tumor suppressor) genes resulting in familial predisposition to breast cancer and ovarian cancer. Two well studied tumor suppressor genes in which these germlines mutations occur are BRCA1 and BRCA2 (98, 99). Germline mutations in the BRCA genes account for 15-20% of women with familial history of breast cancer (100). Normally, BRCA1 binds to proteins involved in DNA damage

response and cell cycling (101). Mutations in other genes, such as phosphatase and tensin homolog (PTEN), have also been identified as a factor causing breast cancer (102).

Our focus is on the tumor suppressor gene p53 which has been implicated in diverse biological processes such as apoptosis, cell-cycle arrest, DNA repair, cellular differentiation and senescence; and has been shown to be altered in over 50% of spontaneous tumors in humans (1, 103-106). In addition to spontaneous tumors, germ-line mutations in p53 are observed in Li-Fraumeni patients which predisposes them to a wide spectrum of early-onset cancers, including breast cancer (4, 107-110). Normally, p53 is expressed in a latent form and is maintained at low levels through targeted degradation. However, in response to DNA damage or stress, p53 is activated to function as a transcription factor, resulting in a cascade of events that eventually prevents tumor development (103). P53 is the most frequently mutated gene in spontaneous breast tumors. Approximately 30% of sporadic breast tumors acquire p53 mutations, which are related to breast cancer progression and resistance to doxorubicin therapy in breast cancer patients (109, 111, 112). The majority of p53 mutations are missense substitutions (75%), other alterations such as frameshift insertions/deletions, nonsense mutations and silent mutations are less common (113). Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. In response to stress signals p53 is activated and functions to prevent tumor development. Due to inhibition or loss of wild-type p53 function tumors can ultimately develop. The focus of my thesis is on two proteins that both can interact with p53: FAK and Annexin A1 (AnxA1).

Actin remodeling/cytoskeletal dynamics/EMT

Most breast cancer patients die of distant metastases that are unresponsive to treatment. In order to metastasize, tumor cells have to acquire an invasive phenotype, a process involving a phenotypic switch from epithelial to a more mesenchymal phenotype typically referred to as EMT. During such a switch, epithelial cells lose their cell–cell interactions and cell polarity, and undergo major changes in the actin cytoskeleton network. This enables them to acquire a migratory phenotype with increased motility and the possibility to invade surrounding tissue and blood vessels (Fig. 3). Members of the transforming growth factor-β (TGF-β) super family have been implicated as major induction signals of EMT (114, 115). Activation of the TGF-β pathway induces clustering of TGF-β receptor I and II, leading to phosphorylation of receptor Smads, Smad2 and 3, after which Smad2/3 complex with Smad4. Together this complex travels to the nucleus where it is involved in transcription of genes such as Twist, leading to the switch from an epithelial to a more mesenchymal morphology (116).

In addition to TGF-β, other factors such as Notch signaling, transcriptional regulation of factors like E-cadherin, and tyrosine kinase receptors

MET, FGF, IGF, EGF and PDGF have been implicated in regulating EMT-like morphogenetic events that occur during development (reviewed in Yang & Weinberg (117)).

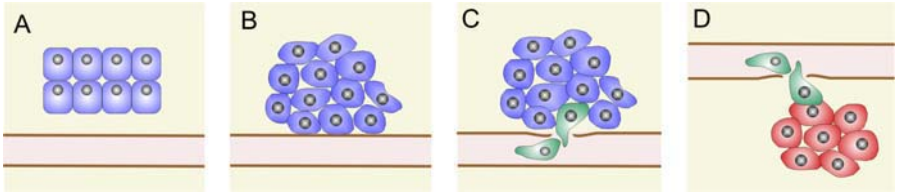

Figure 3. Key steps in metastases formation
Normally, epithelial cells have a well polarized phenotype (A). Upon stimulation/alteration, cells may proliferate uncontrollably and loose polarization (B). Next cells loose their cell-cell interactions, gain a migratory phenotype and intravasate in to a blood vessel or lymphatic system (C). Then, if the cells survive the bloodstream, cells extravasate and finally proliferate at a distant site (D).

FAK & AnxA1 in breast cancer formation
In this thesis the main focus is on the role of FAK and AnxA1 in breast cancer formation and progression. Both FAK and AnxA1 can directly or indirectly regulate cytoskeletal dynamics, thereby influencing the morphology and invasive capability of tumor cells.

FAK is located at cell-matrix adhesions where it integrates signals from extracellular cues such as growth factors, to control and regulate cell adhesion and migration. In addition, FAK regulates the turnover of the cell-matrix adhesion complexes and cytoskeletal dynamics, processes essential for migration of cells. Mammary gland development involves many processes including proliferation and migration, processes that involve FAK. However, to study the role of FAK in mammary gland development and mammary tumorigenesis FAK knockout mice cannot be used due to lethality at embryonic day 8.5. Therefore efforts have been made to develop conditional knockout models to study FAK in both mammary gland development and breast cancer. Using such models, FAK deletion was shown to cause defects in lactational differentiation during pregnancy (89). In addition to FAK, β1-integrin was shown to be involved in mammary gland development (43, 88). Interestingly, FAK levels dropped in β1-integrin-knockout mammary epithelial cells indicating that the defects observed in mammary gland development were possibly due to reduced FAK expression.

Though previous studies performed to determine the role of FAK in mammary gland development gave insight in its involvement in lactational differentiation, the MMTV-Cre-based model used does not provide information on the early steps in mammary gland development. In our study we made use of conditional deletion of FAK in both myo- and luminal epithelial cells, in

combination with transplantation of these cells in a cleared fat pad of an immunodeficient mouse. We transplanted Wt MECs in the left fat pad and *Fak* KO MECs in the right mammary fat pad. This experimental setup enabled us to study the effect of *Fak* deletion and compare that to the Wt situation in one mouse, thereby excluding variances in hormonal differentiation from mouse to mouse (Chapter 2).

Apart from their role in mammary gland development both β1-integrin and FAK have been implicated in breast cancer. β1-integrin expression is essential for the initiation of mammary tumorigenesis and for maintaining the proliferative capacity of late-stage tumor cells (118). To study the role of FAK in mammary tumorigenesis several groups have used the MMTV-PyMT model to induce mammary tumors. FAK deletion in these models reduced the incidence of MMTV-PyMT mammary tumors, suggesting an important role for FAK in breast tumor development. However, due to the strength of the PyMT or PyVT oncogene, which induces tumors within 10 weeks, the studies are unable to address the natural course of mammary tumorigenesis (45, 119-121).

Though MMTV-PyMT mouse model provides information on the role of FAK in mammary tumorigenesis it is not a clinically relevant model. One protein of which the expression is typically altered in breast cancer is the tumor suppressor protein p53. P53 inhibits transcription of *FAK* by binding to the *FAK* promoter (122, 123). However, FAK has also been shown to enter the nucleus where it binds to and causes degradation of p53 (124). We are interested in the role of FAK in breast cancer induced by alterations in p53.

In our studies described in this thesis (chapter 3 and 4) we made use of two conditional spontaneous mouse models of breast cancer to evaluate the role of FAK in mammary tumorigenesis. Both models induce mammary tumors due to its WapCre-mediated deletion or expression in the mammary gland. This enabled us to study the natural course of breast cancer formation in a clinically relevant model. The first model we describe is based on mammary gland-specific deletion of wild-type p53 and FAK. The second model we used is based on mammary gland-specific expression of p53R270H, the mouse equivalent of the human point mutation R273H, in combination with deletion of FAK. In both models deletion of FAK resulted in a decrease of mammary tumor incidence, suggesting the FAK is involved in p53-based breast cancer formation (Chapter 3 & 4).

AnxA1 & breast cancer

Morphological change is one of the key steps in tumor cell migration and invasion. Normally, epithelial cells are highly polarized and form strong cell-cell interactions. However, upon stimulations or alterations, cells rearrange their actin cytoskeleton, loose cell-cell interaction and are able to migrate. In a previous study we searched for proteins involved in scattering of cells, and found two Annexin family members, AnxA1 and AnxA2, to be differentially phosphorylated

upon Src-oncogene induced cell scattering (125). Phosphorylation of AnxA2 at tyrosine 23 increased cell scattering and protrusiveness of MDCK cells. In addition, AnxA2 phosphorylation was essential for cell spreading. The induction of cell scattering was dependent on dephosphorylation of the actin-severing protein cofilin (125). Though the study was performed in MDCK cells, a canine kidney epithelial cell line, AnxA1 and AnxA2 might generally function to regulate cell scattering. AnxA1 and AnxA2 interact with actin in a Ca^{2+}-dependent manner and regulate actin dynamics (Fig. 4) (126, 127). AnxA2 is concentrated in dynamic actin-rich protrusions of motile cells and knockdown of AnxA2 results in accumulation of stress fibers (128). AnxA2 was also implicated in the formation of actin-rich tight junctions (129) and in the regulation of cell-cell contacts through formation of complexes with Rac1 and cadherin (130). By regulating actin dynamics both AnxA1 and AnxA2 may facilitate cell migration and invasion and may thus be important in metastases formation.

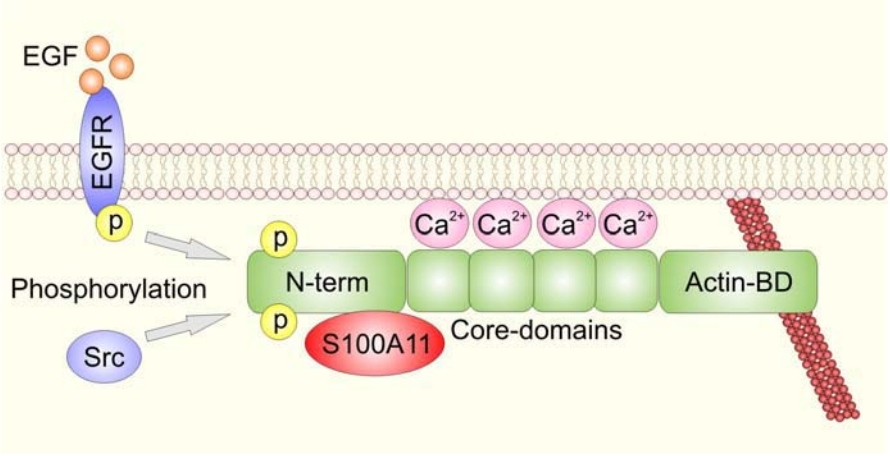

Figure 4. AnxA1 structure
AnxA1 is phosphorylated by EGFR on tyrosine 21 and was also found as one of the first proteins to be phosphorylated in response to Src-oncogene induced cell scattering. AnxA1 contains four core domains which enables AnxA1 to interact with the cell membrane in a calcium-dependent manner. In addition, it can interact with the actin-cytoskeleton through its actin-binding domain.

We aimed to evaluate the role of AnxA1 and AnxA2 (not described in this thesis) in tumor cell scattering. Indeed, high expression of AnxA1 was found in tumor cells with a scattered morphology and basal-like characteristics, while human breast cancer cells with epithelial characteristics lacked expression or had low expression of AnxA1. Basal-like breast cancer is an aggressive subtype of breast cancer that is often unresponsive to cancer therapy. In clinic, basal-like breast cancer is diagnosed based on expression of cytokeratin 5, and triple-negative (ER,

Her2, and PR) status. However, cytokeratin 5 is not exclusively expressed in triple-negative tumors, making it difficult to discriminate basal-like breast cancers from other subtypes. In chapter 5 we describe our findings on the role of AnxA1 in basal-like breast cancer formation and progression and evaluate AnxA1 as a candidate marker for basal-like breast cancer.

Concluding remarks

Mammary gland development is highly dynamic, requiring numerous processes in order to develop a functional gland. There is a clear overlap between processes involved in mammary gland development and those required for breast cancer formation/progression. Understanding the biology and physiology of mammary gland development might therefore clarify the processes involved in breast cancer formation, and identify essential proteins in this process. Migration is a process that is important during mammary gland development and is influenced by a large set of proteins, among which are adhesion receptors (e.g. integrins), cell-matrix adhesion proteins and regulators of cytoskeletal dynamics. Signaling by cell-matrix adhesion proteins plays a central role in the maintenance of tissue architecture of the mammary acini and are essential for proper expansion of alveoli during pregnancy and lactation. Deregulation of cell-matrix adhesion and cell-cell adhesion are hallmarks of breast cancer development. Though the role of some regulators of cell-matrix adhesion and cell-cell adhesions in mammary gland development and/or breast cancer have been identified, many remain unknown. Identifying additional proteins and elucidating their role in mammary gland development and breast cancer formation will contribute to the understanding of these processes and help to find ways for therapeutic intervention.

Aim and outline of this thesis

Though considerable efforts have been made to improve breast cancer diagnosis and therapy, breast cancer still causes numerous deaths every year. Therefore more research is needed to identify proteins involved in breast cancer progression and therapy (ir-)responsiveness. Moreover, new markers to improve diagnosis and therapy of specific subtypes of breast cancer are needed. The general aim of the study presented in this thesis is to evaluate the role focal adhesion kinase (FAK) and Annexin A1 (AnxA1) in mammary gland development and breast cancer formation. In **chapter 2**, we investigate the role of FAK, a cell-matrix adhesion protein, in mammary gland development. For this purpose, we setup a conditional FAK knockout mouse model and mammary epithelial cells transplantation technique. We show a crucial role for FAK in maintaining tissue architecture. In **chapter 3 and 4**, the role of FAK in breast tumor development is investigated. We evaluated the role of FAK in mammary tumorigenesis using two mouse models for spontaneous breast cancer. The first model is based on mammary gland-specific loss of p53 wild-type (chapter 3). The second model is based on mammary gland-specific expression of p53R270H, the mouse equivalent of human p53R273H hot-spot point mutation (chapter 4). In both models FAK deletion resulted in a decrease in occurrence of mammary tumors. In **chapter 5** we describe the identification of AnxA1 as one of the markers for the highly invasive basal-like breast cancer subtype and show that AnxA1 is functionally related to the progression of this breast cancer subtype. AnxA1 promotes metastasis formation by enhancing TGFβ/Smad signaling and actin reorganization, which facilitates an EMT-like switch, thereby allowing efficient cell migration and invasion of metastatic breast cancer cells. In **chapter 6** we discuss the findings described in this thesis and their implications for future research.

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