Adhesion signaling in mammary gland development, tumorgenesis and progression

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Chapter 6

Summary and Discussion

Cell-cell and cell-matrix interactions are important for cell survival, proliferation, migration and maintenance of cell polarity in the mammary gland. Loss of polarization, increased cell survival and proliferation, and subsequent filling of the ductal lumen are early steps in mammary tumor formation. Ones a tumor has formed, more transformation steps can occur, such as loss of cell-cell adhesion. Loss of cell-cell adhesion is one of the hallmarks of epithelial-to-mesenchymal transition, a process that enables cells to escape from a primary tumor and form distant metastases. The research described in this thesis highlights our recent findings on the role of focal adhesion kinase in mammary gland development and mammary tumorigenesis. In addition, we describe the identification of AnxA1 as a potential new marker for basal-like breast cancer and discuss its role in breast cancer progression. Both FAK and AnxA1 seem to control the maintenance of cell-cell interactions.

FAK in control of branching morphogenesis and mammary gland organization

Cell adhesion signaling is important for the coordinated control of mammary gland organization and structure. Mammary gland-specific deletion of integrin-β1, a cell-matrix adhesion component, has profound effects on the virgin and lactating mammary gland development. FAK is an important signal transducer within these cell-matrix adhesions, where it integrates signals from extracellular cues such as growth-factor receptors and integrins to control processes such as proliferation, cell survival, adhesion dynamics and cell migration.

Although *Fak* deletion in luminal mammary epithelial cells was shown to inhibit ductal outgrowth during pregnancy and milk production/secretion, the role of FAK in early mammary gland development was not addressed (1). In **chapter 2** of this thesis we evaluate the effect of FAK deficiency during mammary gland development. For this purpose we set-up a conditional FAK knockout mammary epithelial cell transplantation model, which allows us to study both FAK wild type and FAK deficient mammary epithelial cells (MECs) in the same mouse. *In vitro*, FAK deficient MECs spread poorly, show enhanced ROCK-mediated cytoskeletal contractility and fail to respond to receptor-mediated cytoskeletal remodeling. The impaired cytoskeletal dynamics found in FAK deficient MECs may underlie the defects observed during FAK deficient mammary gland development *in vivo*. Transplantation of *Fak* deficient MECs in a cleared mammary fat pad of immune deficient recipient mice results in development of new but dilated virgin ducts with a disrupted myo- and luminal epithelial cell multilayer, and aberrant ductal morphogenesis during pregnancy. During branching morphogenesis cytoskeletal rearrangements are required for cells to migrate, ultimately forming new ductal structures. FAK deficient organoids show impaired branching morphogenesis in three-dimensional culture, a process that can be reversed by the addition of a ROCK-inhibitor. Though addition of this inhibitor relieves the tension of FAK

deficient MECs and allows organoids to form early branches no persistent branching is observed. This suggests that cytoskeletal rearrangement during mammary gland development is a delicate process at times increasing tension within the cells but also releasing this force when required.

During initiation of cell adhesion and focal-complex formation Rac1 and Cdc42 are activated, which stimulate lamellipodia and filopodia. These processes allow membrane protrusion and cell polarization needed for the direction of movement. The subsequent assembly of tension inducing actin stress fibers and maturation of focal adhesions are controlled by RhoA and its downstream effectors such as ROCK. FAK can contribute to actin dynamics by binding to Rho protein effectors thereby influencing Rho-GTPase pathways (2, 3).

Although we could link defects in mammary gland organization and milk-secretion to impaired Rho-kinase mediated contractility, we still do not know the exact role of FAK in mammary gland development. For instance the observation that during pregnancy and lactation the number of lactating alveoli is markedly reduced can not be explained by the impaired Rho-kinase mediated contractility. However, a recent paper does give us a clue to what might happen within these mammary glands. It was proposed that FAK plays an important role in the maintenance of a mammary cancer stem/progenitor cell population during mammary tumorigenesis (4). Though the setting is completely different, we can not exclude the possibility that during mammary gland development, FAK is involved in the maintenance of mammary stem/progenitor cells. Intriguingly, basal mammary epithelial cell-ECM interactions mediated by β1 integrins were shown to be essential for the maintenance of a functional stem cell population, mammary morphogenesis and segregation of the two major mammary cell lineages (5). This suggests that loss of any important component within the cell-matrix adhesions may affect the mammary stem/progenitor cell population, and thus mammary gland development. Indeed, several cell-matrix adhesion components and downstream effectors have been implicated in the context of mammary gland development. β1-integrin-mediated adhesion to the basement membrane component laminin controls the orientation of polarization of mammary cysts via activation of Rac1 (6). β1-integrin knockout mouse models, where β1-integrin is deleted in luminal epithelial cells, have provided important data on the role of β1-integrin in mammary gland function. β1-integrin knockout mammary glands show defects in integrin-mediated adhesion to the basement membrane, mammary gland differentiation during lactation and production of milk (7-9). Interestingly, FAK levels and its activation were reduced in β1-integrin knockout cells indicating that the phenotype described in these studies could partially be caused by the reduction in FAK expression and/or activation. However, others have provided evidence that ILK, but not FAK, has a key role in lactogenesis in vivo and in the differentiation of cultured luminal epithelial cells (10). Although there are diverse views on the importance of proteins such as FAK,

ILK and β1-integrin in the context of mammary gland development, overall one could say that disruption of cell-matrix adhesions affects mammary gland development.

FAK in control of p53-mediated mammary tumorigenesis

Increased FAK protein levels and activity is frequently found in numerous human cancers, including breast cancer, and correlates with disease progression. In addition to FAK, alterations in the tumor suppressor protein p53 are found in over 50% of human breast cancers. Interestingly, p53 controls FAK proteins levels by binding and inhibiting the FAK promoter. In **chapter 3** we investigate the role of FAK in tumor formation initiated by complete deletion of p53. We hypothesize that increased protein levels of FAK are often due to loss of wild type p53 function, and that this increased FAK expression is essential for mammary tumor formation and progression. To study the role of FAK in spontaneous mammary tumor formation we generated mice that conditionally delete p53 and FAK. Complete FAK deficiency reduces mammary tumor incidence in our spontaneous mammary tumor model, but overall survival is not affected. In line with this finding, heterozygous FAK gene expression also reduces the incidence of mammary tumors. An interesting finding is that most FAK deficient tumors simultaneously showed reduced E-cadherin levels, indicating that in the absence of FAK an additional hit, in this case E-cadherin down-regulation, is required for these tumors to develop. These results are in contrast to what one would expect. FAK signaling is important for TGFβ-induced EMT, increasing mesenchymal- and invasiveness markers but also for the delocalization of membrane-bound E-cadherin (11). In addition, decreased E-cadherin and increased FAK expression are linked in metastases of laryngeal cancer (12). In the absence of FAK, cells are less invasive and motile. If there would be any effect on E-cadherin expression one would expect an up-regulation but not a down-regulation. However, in contrast to the role of FAK in TGFβ-induced EMT, FAK has an opposite function in human colon carcinoma cells. In these cells TGFβ induces FAK activation, which subsequently leads to up-regulation of E-cadherin and suppression of their malignant phenotype (13). In our spontaneous mammary tumorigenesis model FAK deficiency could force the (pre) neoplastic lesions to overcome this tumor suppression by down-regulating E-cadherin.

Thus far, direct evidence for the involvement of FAK in the regulation of E-cadherin expression is lacking. E-cadherin levels can be regulated transcriptionally or translationally, but can also be affected by delocalization. In the last couple of years research has focused on the role of miRNAs in the regulation of proteins including E-cadherin. The miRNA-200 family has been implicated to regulate the E-cadherin suppressors SIP1 and Zeb1 (14). Preliminary data suggests that at least in some FAK deficient/p53-null-induced mammary tumors SIP1 and Zeb1 are up-regulated. If up-regulation of SIP1 and Zeb1 is a

general process in FAK deficient neoplastic lesions to down-regulate E-cadherin is not known, and we are currently investigating this possibility.

In addition to down-regulation of E-cadherin other alterations may occur during the process of spontaneous mammary tumor formation. As mentioned in the previous paragraph, FAK is important for the maintenance of the mammary cancer stem/progenitor cell population. To what extent this mammary cancer stem/progenitor cell population is affected in our model is not known. Staining of the mammary tumors for mammary cancer stem/progenitor cell markers will provide additional information on the role of FAK in the maintenance of this population in our spontaneous mammary tumor model.

In human breast cancer specimens complete loss of p53 is rarely found but hot-spot p53 mutations occur frequently in human breast cancers. Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. Increased FAK expression correlates with p53 mutations in human breast cancer, but the role of FAK in p53-mutant induced mammary tumorigenesis has not been addressed. In **chapter 4** we investigate the role of FAK in p53-mutant induced mammary tumorigenesis. In our model conditional expression of p53 R270H mutant, the mouse equivalent of human hot-spot mutation R273H, is accompanied by deletion of FAK. In line with the reduction of mammary tumor incidence in p53 deficient mouse model, FAK deficiency reduces the incidence of mammary tumors in p53 mutant induced mammary tumorigenesis. Interestingly, mammary tumors that develop in FAK deficient-p53 mutant mice do not show a correlation with E-cadherin levels, indicating that expression of p53 mutant is sufficient to induce mammary tumors without down-regulating E-cadherin. In addition, p53 deficient spontaneous mammary tumors express luminal marker cytokeratin 8, while p53 mutant induced mammary tumors expressed the basal marker cytokeratin 5. Cytokeratin 5, in combination with a triple negative status (ER, HER2, and PR negative) is used in the clinic to distinguish basal-like breast cancer from other breast cancer subtypes. If the mammary tumors that develop in our p53 R270H spontaneous mammary tumorigenesis model are indeed basal-like breast cancers is not known. Additional staining to determine triple-negative status is required to determine the breast cancer subtype. Although the breast cancer subtype has yet to be determined, the incidence of p53 R270H mammary tumors is higher; the first onset is approximately 3 months earlier than $p53^{lox/lox}$ mammary tumors; and the median survival is lower in p53R270H mammary tumorigenesis. These observations suggest that p53R270H is more potent in inducing mammary tumors when compared to $p53^{lox/lox}$.

To summarize the results of chapters 3 and 4, FAK deficiency reduced the incidence of mammary tumors in both spontaneous mammary tumorigenesis studies. Therefore FAK might be a valuable target for breast cancer therapy. Indeed several studies using a FAK inhibitor have reported decreased tumor growth, again confirming the importance of FAK in tumorigenesis (15, 16).

However, when using FAK inhibitors or other ways to suppress FAK function or activity in the clinic, one must realize that suppressing FAK may trigger the tumors to down-regulate E-cadherin and thus may indirectly contribute to a phenotypic switch enabling tumor cells to escape the primary tumor and metastasize.

Regulation of metastases formation by AnxA1

Loss of polarity and disorganization of the mammary gland are often observed during the early steps of mammary tumor formation. Breast cancer progression depends, in part, on the ability of tumor cells to invade and metastasize. This metastatic spread can be initiated by a cell morphology switch, whereby cells change from a resting, epithelial- to a more migratory, mesenchymal-like phenotype, a process that is often stimulated by growth factors such as HGF and TGFβ/Smad signaling.

AnxA1, a calcium/phospholipid-binding and actin regulatory protein, is a candidate regulator of oncogene-mediated epithelial cell scattering. In **chapter 5** we investigate if AnxA1-mediated regulation of actin cytoskeletal dynamics enables cells to undergo morphological changes that are required for migration and invasion. We show that expression of AnxA1 associates with the basal-like breast cancer subtype in both breast cancer patients as well as in a panel of breast cancer cell lines. Depletion of AnxA1 in a panel of basal-like breast cancer cells results in reversal of their invasive, migratory phenotype, which is linked to actin reorganization and decreased TGFβ /Smad signaling. Moreover, AnxA1 knockdown results in a reduction of lung metastasis *in vivo*, also *in vivo* this is linked to reduced TGFβ /Smad signaling. We propose that AnxA1 may influence TGFβ signaling by interfering with TGFβ-receptor endocytosis. Other members of the annexin family are involved in the endocytic pathway(17, 18), but it remains unclear if and/or how AnxA1 is involved in endocytosis, and in particular endocytosis of TGFβ-receptors. Alternatively, AnxA1 may influence internalization of TGFβ-receptors indirectly via regulation of the actin cytoskeleton like has been described for AnxA2 (19). Decreased actin cytoskeletal dynamics as observed in our AnxA1 knockdown cells, might stiffen the cell membrane thereby impeding TGFβ-receptor internalization. Compared to other actin-regulatory proteins, AnxA1 has the unique property to bind both phospholipids at the plasma-membrane as well as F-actin, thereby stabilizing and/or regulating membrane-actin interactions (20). Currently, we are investigating whether TGFβ-receptor I and II are able to interact by performing studies using iodinated TGF to induce TGFβ-receptor cross linking. Given the iodinated status of TGF we are able to determine the amount of labeled TGF and subsequent interaction of the TGFβ-receptors. In addition, we use renilla-based luciferase reporter assays to determine if interaction between the TGFβ-receptor I and II is reduced is AnxA1 knockdown basal-like breast cancer cells. Moreover,

we recently performed gene expression analysis on AnxA1 knock down human basal-like breast cancer cells to unravel AnxA1-regulated pathways. In the near future we will complete the analysis and dissect the pathways mediated by AnxA1.

AnxA1-mediated regulation of actin cytoskeletal dynamics may facilitate migration and invasion of tumor cells and thus may contribute to breast cancer progression. Indeed, elevated AnxA1 expression levels correlate with the aggressive basal-like breast cancer subtype. Cytokeratin 5 expression, in combination with triple-negative status (ER, HER2 and PR) is often used to define basal-like breast cancer. Remarkably, in contrast to cytokeratin 5 that is also expressed in some luminal-like ER positive tumors, AnxA1 expression is restricted to triple-negative breast cancers. We propose that AnxA1 could be used as an additional marker to improve diagnostics to better discriminate basal-like breast cancers from other subtypes in the clinic.

Future perspectives

We have implicated a role for FAK in the process of mammary gland development (**chapter 2**) and mammary tumorigenesis (**chapter 3 and 4**). Future research should resolve whether FAK is indeed a potential target for breast cancer therapy. One of the critical points that we revealed is the down-regulation of E-cadherin observed in FAK deficient tumors. Extensive research of all FAK deficient tumors is needed to determine if this is a general mechanism for FAK deficient tumors to develop. In addition, we must investigate what the result is of E-cadherin down-regulation on the metastatic potential of FAK deficient tumors. With regard to the results on AnxA1 in breast cancer progression described in **chapter 5**, further research is necessary to test the potential of AnxA1 as a new marker for basal-like breast cancer. For this we will expand our tissue micro-array study on human breast cancers to better define the link between AnxA1 and basal-like breast cancer.

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