PI3K signaling and adherens junctions in invasive lobular breast cancer

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General discussion and future perspectives

The main goals of the work presented in this thesis are to develop a better understanding of the etiology and pathogenesis of invasive lobular carcinoma (ILC), and to identify possible new treatment strategies. In this final chapter I discuss our results in a broader context, including recent and possible future developments. ILC is the most common "special type" of breast cancer, with distinct morphological and biological traits. Loss of E-cadherin and disintegration of the adherens junction complex, leading to a characteristic discohesive growth pattern of the tumor cells, is a frequently observed and well-recognized hallmark of ILC. Thanks to experiments with genetically engineered mice, we know that tissue-specific loss of E-cadherin in the mammary gland is not sufficient to cause tumor formation. Clinical observational studies have demonstrated that, compared to invasive ductal carcinoma (IDC), ILC tumors are enriched for activation of PI3K signaling, supporting further investigations into the role of PI3K as a driving mechanism and therapeutic target.

PRECLINICAL MODELS OF ILC

Cultured human cancer cell lines are very common and convenient models for cancer research. In addition to *in vitro* experiments, cell suspensions can be used *in vivo* by grafting them per injection in immunodeficient mice into the subcutis or the mammary fat pad, or via the nipple into the mammary duct system, after which they can grow out in the epithelial tissue layer of the mammary gland of the mouse (1, 2). Using human-specific markers such as Ku-80, cells of human origin can be distinguished from mouse cells via immunohistochemistry. Evidently, cultured cells are grown under very artificial conditions, and these conditions may select for additional acquired mutations that are not as relevant in the clinical situation. Human ILC cell lines are scarce, and the available lines are from metastatic origin and have mutated p53, which is uncommon in ILC. In a recent study, tumor tissue from ILCs and other invasive breast cancers was dissociated to single cells, and injected intraductally in mice. These xenografted cells grew out successfully, and micrometastases were observed in various organs including bones and the brain, but invasive growth in the mammary gland was less pronounced than in the clinical counterparts, and no typical ILC patterns were seen (2). Transplantation of human ILC tumor tissue as patient-derived xenografts into mice has a very low efficiency, limiting the feasibility of xenograft modeling experiments. ILC is generally of the luminal molecular type, with expression of estrogen receptor, and the take rate of breast cancer xenografts tends to be lower for ER+ tumors and for luminal-type

tumors (3). In addition, the low take rate is possibly related to the low cellularity and slow growth speed of ILC tumors (4, 5).

Currently, there is a lack of xenograft models that closely resemble ILC regarding origin, genetics, and hormone receptor expression. The endocrine system differs between humans and mice in several ways that are relevant for breast cancer research. Firstly, luminal progenitor cells in the human mammary gland express higher levels of estrogen receptor (ER) than in mice (6). Secondly, the mammary stroma in mice expresses ER, and may act upon the epithelium in a paracrine fashion, while there is reportedly no estrogen receptor expression in the human mammary stroma (7). And thirdly, the molecular biology of estrogen receptor is not the same in mice and humans. ERα is expressed in the developing mouse mammary gland, but is reduced in the adult mammary epithelium as well as in mouse mammary tumors. In humans, ERα dependency is driven by the pioneer factor FOXA1, which facilitates binding of estrogen receptor alpha to DNA. In mice this is not the case, and experimental introduction of FOXA1 does not restore ERα-responsiveness (8). This suggests that the mouse is not the most suitable organism to model the role of estrogen and estrogen receptor in breast cancer. Researchers have recently been turning to the rat as a model organism for ER+ breast cancer. And although ILC seems to be a human-specific disease, other types of mammary tumors are frequently seen in pet animals such as dogs and cats, which may encourage researchers and clinicians in human and veterinary medicine to join efforts.

Immunodeficient mice are routinely used as recipient animals for preclinical *in vivo* experiments with human xenografts. The importance of the immune system in cancer progression and metastasis is well-recognized, as reviewed recently for example by Garner and de Visser (9). The lack of a fully functional immune system in xenograft studies may therefore lead to failures in the translation of preclinical findings to patient care. In chapter 4, we report that activation of the adaptive immune system is important for the effect of mTOR inhibition on tumor growth in a genetically engineered mouse ILC model. Inhibition of mTOR led to significantly increased protein expression of MHCII and of transcripts related to antigen presentation. After two months, this immunostimulatory effect was lost, and tumor progression resumed, despite continued suppression of mTOR signaling. This illustrates the importance of immunocompetent animal models for preclinical intervention studies. The use of humanized mice, which carry human cytokines and/or immune cells, is technically challenging, but does offer the possibility to study the interaction between the tumor and at least some components of the human immune system (10). Even with a humanized immune system, other elements of the tumor environment, such as blood vessels and fibroblasts, are

of murine origin, and the interaction between the murine host and the human tumor may be affected by species differences.

Through genetic engineering, *de novo* formation of autochthonous tumors can be studied in a fully immunocompetent host organism. In preclinical genetically engineered mousemodels (GEMMs) for ILC, the common theme is inactivation of *Cd1h*, the gene encoding E-cadherin, combined with at least one other oncogenic event to drive tumor formation. The *Krt14Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (KEP) and *WapCre;Cdh1$^{F/F}$;Trp53$^{F/F}$* (WEP) models, based on tissue-specific loss of E-cadherin and p53, are characterized by robust formation of tumors with metastatic capacity, which can be efficiently transplanted. In chapter 3, we present a model of *de novo* breast cancer metastasis based on orthotopic transplantation and outgrowth of a KEP tumor, followed by surgical removal and subsequent development of metastatic diseases. This model facilitates preclinical studies focusing on the treatment of metastatic ILC. It should be noted that metastatic disease is responsible for the large majority of cancer-related deaths, and crude models of metastasis, such as intravenous injection of tumor cells, are not a very realistic or complete representation of the full metastatic process.

As all models, the KEP and WEP models have certain caveats and limitations. A drawback of working with the KEP model is that it develops epidermal abnormalities and skin tumors, because the keratin 14 promoter, used to drive genetic recombination via the cre-lox system, is active in the mammary gland but also in the epidermis (11). The occurrence of skin tumors is absent in the WEP model and in studies with transplanted KEP mammary tumors into syngeneic wildtype mice. While GEMMs enable us to study all stages of tumor development, transplantation-based models generally represent end stage tumors. Furthermore, most classic ILCs are not p53 deficient, and the KEP and WEP mice can be more precisely classified as models of the pleomorphic ILC subtype, where mutated p53 is more common. It should also be recognized that not all tumors in the KEP and WEP models are ILC-like. A considerable percentage of the tumors in these models have a mesenchymal-like morphology, with polygonal or spindle-shaped cells and expression of vimentin, often while still retaining some expression of the epithelial marker cytokeratin 8, consistent with an epithelial-to-mesenchymal transition (EMT). This EMT phenomenon is not seen on this scale in human breast cancer.

The *WapCre;Cdh1$^{F/F}$;Pten$^{F/F}$* (WEPten) mouse model is presented in chapter 5 as a closer approximation of the classic type of ILC. Compared with the WEP and KEP tumors, the WEPten tumor cells are smaller, more discohesive, and consistently form invasive carcinomas with single cell filing and fibrous stroma. WEPten tumors grow rather slowly, and outgrowth after transplantation is inefficient. These traits make it a

less practical model for preclinical treatment experiments. Human ILC growth is often very slow, making it likely that ILCs take many years from their initiation to becoming clinically apparent. Clearly, mouse models cannot fully replicate the duration of ILC development in humans, due to the mouse life span of only a few years. Still, GEMMs of ILC are an important tool to discover mechanisms that drive tumor initiation and progression, and may guide us towards therapies that target these mechanisms. An ILC mouse model with a *WapCre*-based tissue-specific deletion of *Cdh1* and an activating *Pik3ca* H1047R mutation in the mammary gland epithelium has been published by An *et al.*, characterized by formation of ILCs with immune cell infiltrations (12). Compared with the WEPten model, the model with *Cdh1* deletion and *Pik3ca* mutation was reported to have higher expression levels of gene signatures for invasion (with higher expression of matrix metalloproteinases) and for the signaling pathways Rac1, Yap and Oct-4. *In vivo* genetic screens have further contributed to the discovery of genes and pathways that play a role in ILC. Kas *et al.* have used the Sleeping Beauty transposon system to identify *Muh9* and *Ppp1r12b* as drivers of ILC formation in the context of E-cadherin loss in the mouse mammary gland (13).

To speed up the development of novel GEMMs of breast cancer, various methods have been developed to induce somatic mutations in the mouse mammary gland. Cre recombinase can be delivered via intraductal injection of a Cre-expressing viral vector, leading to recombination of floxed alleles. Via engineered nuclease systems such as TALEN and CRISPR, addition, deletion and replacement of DNA sequences is possible. In a study in our research group by Annunziato *et al.*, intraductal injection of a lentiviral vector to deliver sgRNA targeting *Pten* in the mammary gland of mice with tissue-specific inactivation of E-cadherin and a Cre-conditional Cas9 knock-in allele (*WapCre*;*Cdh1*$^{F/F}$;*Cas9*) resulted in ILC formation. We also tested lentiviral delivery of CRISPR-Cas9 and Cre via intraductal injection into *Cdh1*$^{F/F}$ mice to target PTEN and E-cadherin. This approach led to formation of tumors that did not resemble ILC, with incomplete loss of E-cadherin, and with a marked immune response that was likely directed against Cas9 (14). This illustrates the importance to carefully validate mouse models, and to be aware of possible unwanted artifacts, such as an immune response against an exogenous protein.

In the mouse ILC models that we have studied, the first small lesions are often characterized by discohesive cells that leave their normal anatomical location (11). In chapter 5, we describe early formation of fibrous stroma in small mouse ILC lesions. Historically, tumors have been likened to wounds that do not heal (15). In invasive carcinomas, the epithelial tissue architecture is disturbed, and tumor cells break out

of their normal tissue layer. In mouse ILC models, inactivation of E-cadherin leads to disintegration of the adherens junction complex, disruption of the integrity of the epithelial tissue layer, activation of fibroblasts, and an immune cell response. This disruption of an epithelial tissue layer, and the biological response that follows, is indeed reminiscent of a wound and the ensuing healing reaction. In humans, lobular carcinoma in situ (LCIS), a non-invasive proliferative lesion, has been suggested to be a precursor of ILC. The existing ILC mouse models do not represent LCIS, but display discohesion and invasion of the mutated epithelial cells even in very small and early lesions. In parallel to currently ongoing efforts to create animal models of ductal carcinoma in situ (DCIS), the development of preclinical models for LCIS should also be considered, to study if and how LCIS progresses to ILC.

Several questions still remain. We still don't quite understand the consequences of E-cadherin loss for the cellular biology in the mammary epithelium. Does loss of E-cadherin reprogram the transcriptome to activate oncogenic pathways? Does it somehow contribute to secondary mutations that activate survival and cell proliferation mechanisms? What drives progression of LCIS to ILC? The development of ever more sensitive and precise methods, visualizing not only the morphology of cells and tissues, but also transcripts and proteins, even *in vivo*, may hopefully enable us to dissect the mechanisms that lead to early ILC development in further detail.

THE ROLE OF PI3K SIGNALING IN ILC

ILC is typically characterized by loss of E-cadherin, but also by frequent activation of the PI3K signaling network. Alterations in *PIK3CA*, *PTEN*, or *AKT1* occur in more than 50% of ILC cases, and levels of phosphorylated AKT and p70S6 kinase are higher in ILC compared with other breast cancer types (16-20). In addition, mutated *AKT1* is associated with early relapse of ILC in patients (20). Loss of E-cadherin can contribute to PI3K signaling activation via various mechanisms, including increased EGFR expression, Wnt-mediated inhibition of PTEN expression, and autocrine stimulation of growth factor receptors (21-25). ILC cells have the ability to acquire independence from anchorage to the extracellular matrix, allowing them to escape from the primary tumor and survive in the circulation to form distant metastases. *In vitro*, ILC cell lines grow more efficiently in ultra-low attachment (ULA) suspension compared to IDC cells. Compared with classic 2D cell culture, culturing ILC cells under ULA conditions activates PI3K signaling. In contrast, PI3K signaling activity is downregulated in IDC cells under ULA conditions (26). All these findings suggest that PI3K signaling is relevant for ILC progression.

By genetically inactivating PTEN and E-cadherin in the mammary gland epithelium, we confirmed that PI3K signaling is indeed a driving causal mechanism for ILC in mice (chapter 5). We observed consistent formation of mammary tumors that resemble classic invasive lobular carcinoma, with round discohesive tumor cells of moderate size, forming single files, and fibrous stroma. Compared with the KEP and WEP models, the tumor cells in the E-cadherin-PTEN model are smaller and much more monotonous in their morphology. We also showed that pharmacological inhibition of PI3K signaling inhibits tumor growth in this mouse ILC model. Others have reported that mutations in *Cdh1* (encoding E-cadherin) and *Pik3ca* (encoding the catalytic subunit of PI3K) cooperate in the pathogenesis of ILC (12). Annunziato *et al.* have combined inactivation of E-cadherin and activation of AKT (*Akt-E17K* mutation), which also leads to ILC formation, further illustrating the relevance of PI3K/AKT/mTOR signaling in ILC (14). Even though tumors of the KEP model do not have a mutation in the PI3K pathway, they do respond to mTOR inhibition (chapter 4). These findings indicate that PI3K signaling is a driver of ILC progression and is a candidate therapeutic target even in the absence of activating genetic mutation in this pathway. The currently available clinical and preclinical data are in support of clinical trials with inhibitors of PI3K signaling for patients with ILC. The ongoing POSEIDON phase I/II clinical trial (NCT02285179), with an estimated study completion date of July 2022, is designed to evaluate the PI3K inhibitor taselisib (GDC-0032) combined with tamoxifen in hormone receptor positive, HER2 negative metastatic breast cancer, and the study includes a group of patients with ILC (27).

While we may be hopeful that PI3K pathway inhibition in ILC will lead to improved outcomes, we have not been able to cure even a single mouse of ILC. In our preclinical intervention studies with the mTOR inhibitor AZD8055 (chapter 4) and with the PI3K/mTOR inhibitor BEZ235 (chapter 5), we only achieved slowdown or temporary halt of disease progression. Importantly, we observed that the anti-tumor effect of mTOR inhibition is in part mediated by the adaptive immune system. It is indeed known that PI3K signaling is relevant for tumor immunology, and this could guide us to more successful treatment strategies. Activated mTOR signaling in tumor cells is known to modulate the tumor microenvironment, as reviewed for example by Guri *et al.* (28). Loss of PTEN in tumor cells in melanoma reduces T cell-mediated tumor killing, and correlates with reduced efficacy of anti-PD1 therapy in patients. Interestingly, the efficacy of immune checkpoint blockade was shown to improve by treatment with a PI3Kβ inhibitor in a preclinical mouse model (29). In a mouse model of lung cancer, activation of mTORC1 increases PD-L1 expression, allowing cancer cells to escape from

the immune system (30). Inhibition of mTORC2 in dendritic cells augments their capacity to stimulate T cell activity against tumor cells (31). While the effects of PI3K signaling inhibitors in the tumor microenvironment are complex and may be immunostimulatory as well as immunomodulating, there are several benefits, including increased CD8+ T cell activity, decreased regulatory T cells, and inhibition of angiogenesis (32). Our discovery that the adaptive immune system is partly responsible for the anti-tumor effect of mTOR inhibition in the KEP model should encourage us to consider preclinical combined PI3K signaling inhibition and immunotherapy in this model, aimed at stimulating and prolonging an anti-tumor response of the immune system.

THE ROLE OF P120 IN ILC

E-cadherin and p120 are both main components of the adherens junction complex, and both proteins have tumor suppressive functions. Venhuizen *et al.* have recently reviewed the complex roles of these two proteins in tumor biology (33). E-cadherin and p120 contribute to maintenance of cell-cell adhesion, epithelial polarity, and apical extrusion of oncogenic cells. P120 promotes the stability of E-cadherin by preventing endocytosis and docking of the ubiquitin ligase Hakai, and via inhibition of Rho signaling by binding Rho and by recruiting the Rho inhibitor p190RhoGAP (33-36). P120 has many isoforms, and invasion and metastasis of breast cancer is reportedly associated with upregulation of isoform p120-3 and downregulation of p120-1 (37). It has recently become clear that the phosphorylation status of p120 is important for controlling homophilic E-cadherin binding strength, and E-cadherin adhesion activation has a dephosphorylating effect on p120. In experiments with 4T1 mouse mammary tumor cells, a phosphorylation dead p120 mutant increased E-cadherin adhesion strength and reduced the ability of the cells to migrate and invade *in vitro* as well as the ability to metastasize *in vivo* to the lungs after orthotopic mammary fat pad injection (38). P120 regulates the orientation of cell extrusion (*i.e.* the removal of surplus or unhealthy cells from their tissue layer) via S1P signaling. In a mouse model of pancreatic cancer, loss of p120 resulted in basal extrusion and survival of Ras-mutated cells (39). In addition, p120 controls the site of the cytokinetic cleavage furrow in mitotic cells by localizing Rho-dependent actomyosin contraction, thus preventing multinucleation (40). From this, it can be concluded that in the adherens junction complex p120 functions as a tumor suppressor as well as a suppressor of cancer cell invasion and metastasis.

Loss of E-cadherin leads to disintegration of the adherens junction complex and translocation of p120 catenin to the cytosol and the nucleus (41). Diagnostically,

cytoplasmic p120 is a useful finding that helps to discern between ductal and lobular carcinomas, particularly in small or early lesions (42). Translocated p120 can have a tumor-promoting effect, and contributes to ILC progression in several essential ways. Cytoplasmic p120 can contribute to cancer cell migration and invasion via activation of Rac1 and Cdc42 (43). In addition, cytosolic p120 activates Rho-Rock signaling, leading to anchorage independence and driving tumor growth and metastasis. Rho-Rock might therefore be an interesting potential therapeutic target in ILC (44). In the nucleus, p120 relieves transcriptional repression by Kaiso. One of the relevant Kaiso targets is *Wnt11*, which promotes anoikis resistance via RhoA activation (45). In chapter 6, we report that loss of p120 promotes anoikis resistance through hypersensitization of growth factor receptor (GFR) signaling, and secretion of cytokines. Based on these results, it may be hypothesized that GFR signaling inhibition is a therapeutic option for p120-negative breast cancer, which calls for further investigation. Through the work described in chapter 7, we demonstrated that ILC does not simply result from the disintegration of the adherens junction after the loss of any member of the adherens junction. Loss of p120 does lead to invasive tumor growth in p53 knockout tumors in the mouse mammary gland; however these tumors are high-grade, basal-like, and have mesenchymal characteristics, and they do not resemble ILC. Therefore it seems that expression of p120 is essential in the pathogenesis of ILC. In addition to suggestions above to investigate Rho-Rock targeting in ILC and GFR signaling in p120-negative breast cancer, other questions about the role of p120 in cancer include the role of its many isoforms and its possible phosphorylation events (46).

PATHOLOGY IN RESEARCH

Pathology is a medical specialty as well as a bioscience: it is the study of disease. The traditional task of a pathologist is to detect and interpret macroscopic and microscopic changes in the morphology of organs and tissues, and to establish a diagnosis based on visual information. Histopathology, which is the study of abnormalities in tissue sections, is generally based on brightfield microscopy using the H&E stain. H&E is a combination of two dyes: hematoxylin (blue, predominantly staining nuclei), and eosin (pink, predominantly staining proteins). H&E is a principal stain, used to visualize the microscopic anatomy of tissues and to reveal morphologic abnormalities. Special stains, such as histochemistry or immunohistochemistry, are used to label specific molecules, cell types or other tissue components. Multiplex labeling can be used to simultaneously detect different molecules and markers, allowing the visualization of interactions and

spatial relationships of many types of cells and molecules. Digital image analysis helps us with quantifications and measurements, and artificial intelligence can be a tool to detect patterns and correlations of clinical relevance within large amounts of complex data. Studying disease also means that we try to understand the mechanisms that cause and drive disease processes. We use experiments to achieve this, using the toolboxes available for biomedical research. The breadth and depth of knowledge and skills needed to find out how disease works necessitate interdisciplinary collaboration. Within such a collaboration, a pathologist can be a specialist as well as a generalist. By limiting the focus of research to one organ, a single molecular pathway, or the lab's newest and favorite technology, we can easily miss what is actually going on in a study object as complex as a mammalian organism. Local changes in tissues are affected not only by genes and molecules, but by all the organs and systemic processes in the body. A striking example of this is the discovery of abnormalities in placentae of retinoblastoma (Rb) deficient mice. The detection of trophoblast proliferation and disruption of placental tissue architecture pointed to an extra-embryonic role of retinoblastoma (Rb) in embryonic development and viability, which was indeed confirmed by follow-up experiments (47). If we experiment with live animals, we have a scientific as well as an ethical obligation to examine the entire organism instead of just one organ or a local disease process. Modern analytic technologies may be powerful and sensitive, but they are also often very specifically aimed at a certain type of readout. With cheap and simple H&E stains representing all organ systems we can detect unexpected findings and phenotypes, "background pathology" unrelated to the experiment, adverse treatment effects, and complications such as infections. The latest technologies cannot and do not have to replace methods that have been around for more than a century but remain very powerful.

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