PI3K signaling and adherens junctions in invasive lobular breast cancer

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General Introduction

BREAST CANCER

Breast cancer is a disease that results from neoplastic transformation of cells in the breast, that grow autonomously to form a malignant tumor. It is the most frequently diagnosed cancer among women, and the leading cause of cancer-related deaths in women worldwide, with more than 2 million new cases and more than 600,000 deaths in 2018 (1, 2). Breast tumors can be classified into many histopathologic types according to the WHO classification, based on morphologic criteria observed in the tumor tissue and cells (3). Carcinoma, the term for a malignant tumor of epithelial cells, is the most common malignancy of the breast. Carcinomas can be divided into *in situ* carcinomas, that do not invade through the basement membrane of the ducts and lobules of the breast, and invasive carcinomas that do. There are several histologic types of invasive breast carcinomas, the most common being “no special type” (NST), also called invasive ductal carcinoma (IDC), followed by invasive lobular carcinoma (ILC). There are also several less common types of breast cancers, such as metaplastic carcinomas or carcinomas with neuroendocrine features. Breast tumors can also be categorized according to the presence or absence of molecular markers and therapeutic targets. Expression of hormone receptors for estrogen (ER) and for progesterone (PR) can be detected through immunohistochemistry (IHC). Also human epidermal growth factor 2 expression can be detected via IHC, and amplification of the encoding gene (*ERBB2* or *HER2*) is incorporated into testing of breast cancer tissue biopsies. These tests guide clinical decisions for selecting endocrine therapy and HER2-targeted therapy (4). Molecular pathology is becoming increasingly powerful and clinically relevant. Genomics, transcriptional profiling and bioinformatics have created additional options for tumor classification, and molecular signatures can offer information on the prognosis of the disease, or prediction of the response to therapies (5-7).

INVASIVE LOBULAR CARCINOMA

Invasive lobular carcinoma (ILC) is the second most common histological type of breast cancer, after invasive ductal carcinoma (IDC), accounting for 8-14% of all breast cancers (8, 9). ILC forms an ill-defined mass that is difficult to palpate. Calcification is infrequent, reducing the sensitivity of detection via mammography (10). ILCs are generally hormone receptor positive and HER2-negative, and there is often a good response to endocrine therapy. However, compared with IDC, ILC is more likely to be detected at an advanced stage of the disease, and the disease can be highly metastatic (11). Information about

the best treatment specifically for ILC is sparse, and standard treatments are similar to IDC, but the pathologic response to primary chemotherapy appears to be poorer (12). Recent publications indicate that chemotherapy may not improve survival for a large proportion of patients with ILC, especially those with ER-positive, HER2-negative tumors (13-15). In contrast to IDC, the incidence of ILC is rising. Risk factors that are more strongly associated with ILC than with IDC include postmenopausal hormone replacement therapy with estrogens and progestagens, and an increasing age at giving first birth (9, 16-19).

The most common histologic variant of ILC is classic ILC, which is characterized by non-cohesive small and monomorphic cancer cells, with a low mitotic rate, round or ovoid nuclei, and a scant amount of cytoplasm. The cancer cells lack cohesion and can be arranged in a single-file linear pattern in a fibrous stroma (3). Besides the classic type, several other histologic variants of ILC exist. Pleomorphic ILC has a higher degree of cellular atypia and pleomorphism, a higher mitotic rate, and more frequently exhibits HER2 amplification and/or mutant p53 expression. Other types include solid ILCs, with cells arranged in sheets, and alveolar ILCs, where the cells are organized in discrete rounded aggregates of 20 or more cells (11). Histologic subtyping of ILCs can have prognostic value, with classic ILC conferring a better outcome than non-classic ILC (20). The metastatic pattern of ILC is different from IDC, with more frequently observed metastasis to digestive tract, reproductive tract and/or peritoneum (21). Histologic grading of breast tumors is routinely performed according to the Nottingham system, to provide prognostic information (22). This grading system is based on the percentage of tubule formation, the degree of nuclear pleomorphism, and a count of mitotic cells. Most ILCs are scored as grade 2 because tubule formation is rare, nuclear pleomorphism is limited and the mitotic count is low. The relevance of histological grading of ILC is therefore subject of debate (11). Mitotic score may have prognostic value in pleomorphic ILC (23). A histological grade 3 seems to confer an increased recurrence rate, but grade 3 is rare in ILC at 6% (24). Grade 3 is also associated with more axillary lymph node positivity and hormone receptor negative status (25).

A well-known diagnostic trait of ILC is loss of E-cadherin expression (encoded by *CDH1*). Other genes that are altered more frequently in ILC than in other breast cancers include *PIK3CA*, *PTEN*, *AKT1, FOXA1, HER2*, *HER3* and *TBX3* (26, 27). Molecular profiling of tumors is one of the powerful tools in current efforts to improve clinical decision making. Ultimately, the goal is to choose the best treatment for each patient, in what has become known as personalized medicine. Several research groups have identified molecular ILC subtypes (28). Ciriello *et al.* have distinguished reactive-like,

immune-related, and proliferative subtypes (27). Reactive-like tumors appeared to have lower tumor purity and a more dominant stromal response. In immune-related tumors, evidence of higher immune activity was seen, with, for example, high expression of genes related to macrophage activity. Proliferative subtype ILCs had higher levels of cell proliferation, but still lower than in IDC. Michaut *et al.* describe immune- related and hormone-related ILC subtypes (29). The immune-related subtype in this study was characterized by upregulation of lymphoid signaling molecules as well as negative regulators of immune response, namely PD-L1, PD-1 and CTLA-4. The hormone-related subtype was associated with higher expression of estrogen receptor (*ESR1*) and progesterone receptor (*PGR*), and of cell cycle genes. Although these studies may evoke hypotheses regarding a more personalized treatment selection, the subtype classifications have not led to stratification of patients for clinical management of ILC.

THE ROLE OF ADHERENS JUNCTIONS IN INVASIVE LOBULAR CARCINOMA

An important hallmark of ILC is a lack of expression of E-cadherin, occurring in approximately 90-95% of ILCs (11,27, 30). E-cadherin plays a key role in cell-cell contact of epithelial cells through adherens junctions, and suppresses cancer cell dissociation and invasion. This molecular defect in the adherens junction explains the discohesive nature of cancer cells in ILC (31-33). Adherens junctions are important for control of epithelial cell behavior, including survival, proliferation and migration (34), and E-cadherin is an essential protein during embryogenesis and in maintaining tissue architecture (35, 36). The intracellular domain of E-cadherin is connected to several catenins, including α-catenin, β-catenin, γ-catenin and p120 catenin (hereafter p120), and its binding to p120 stabilizes the adherens junction complex (37, 38). In cancer, expression of E-cadherin may be reduced or lost through several mechanisms. Known examples are *CDH1* mutations in hereditary diffuse gastric cancer, *CDH1* mutations, promoter hypermethylation and loss of heterozygosity (LOH) at the *CDH1* locus on chromosome 16q22.1 in lobular carcinoma of the breast, or degradation of E-cadherin as a result of loss of p120 in lung cancer (39, 40). Mutations in *CDH1* occur in 63% of ILCs, and are evenly distributed along the coding sequence (27). The reported frequency of *CDH1* promoter hypermethylation in ILC varies widely, between 21 and 77% (11, 40, 41). LOH and mutations are also found in corresponding in situ lesions (lobular carcinoma in situ, LCIS), indicating that these are early events in the development of ILC (11, 40, 42). A minority of ILCs do express E-cadherin at the cellular membrane, but in those

cases the cadherin-catenin complex appears to be nonfunctional, as catenin complex members, in particular p120, are translocated and can be detected in the cytoplasm through immunohistochemistry (IHC). Because of this, immunohistochemistry for p120 can be a useful diagnostic tool in E- cadherin-positive ILCs (30, 43, 44).

In the mammary gland, E-cadherin loss alone is not tolerated and leads to apoptosis and clearance of luminal epithelial cells (45, 46). Mouse mammary epithelial cells (MMECs) that lose E-cadherin extrude towards the lumen as well as towards the basal lamina. A recent study showed that these cells have increased membrane blebbing and actomyosin contractility, and that progression to ILC after loss of E-cadherin in MMECs depends on partial relaxation of actomyosin (47). The first genetically engineered mouse model (GEMM) of ILC was published in 2006, based on concomitant inactivation of E-cadherin and p53. Mouse ILC (mILC) formation in this model (known as the KEP model) is driven by tissue-specific recombination of floxed *Cdh1* and *Trp53* alleles via Cre recombinase expression under the cytokeratin 14 promoter (K14-Cre) (48, 49). Combined loss of E-cadherin and p53 leads to accelerated tumorigenesis compared to loss of p53 alone. Phenotypically, tumors that are induced by loss of p53 in the mouse mammary gland are relatively noninvasive, but mice with combined knockout of p53 and E-cadherin in the mammary gland developed invasive and metastatic carcinomas with ILC-like tumor growth patterns (48). This model has enabled genetic studies to further elucidate molecular mechanisms that play a role in the survival and behavior of mILC cells.

E-cadherin mediated cell-cell adhesion plays a regulatory role in many oncogenic signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), Wnt and Hippo (50). E-cadherin loss seems to contribute to the activation of PI3K signaling in several ways. For example, loss of E-cadherin has been linked to increased EGFR expression and AKT signaling (51, 52). Homophilic ligation of E-cadherin suppresses cell proliferation and epidermal growth factor signaling (53). E-cadherin may inhibit Wnt signaling by sequestering the proto-oncogene β-catenin at the membrane, preventing its translocation to the nucleus. Nuclear β-catenin inhibits EGR1, which positively regulates expression of the tumor suppressor PTEN, a key negative regulator of PI3K signaling (54). Interestingly, inactivation of E-cadherin in ILC has also been found to hyperactivate PI3K/AKT signaling via autocrine stimulation of growth factor receptors (55).

In addition to its role in the stabilization of adherens junctions, p120 mediates several important intracellular signaling pathways, including Wnt and Rho-ROCK. Upon loss of E-cadherin in the KEP model, the adherens junction is dismantled and

p120 translocates to the cytosol and nucleus, leading to anoikis resistance as well as cell migration via activation of Rho-ROCK signaling (56, 57). In the nucleus, p120 can prevent the transcriptional repressor Kaiso from inhibiting transcription of its target genes (58-60). Similar to E-cadherin, loss of p120 in the mammary epithelium is not tolerated, with elimination and death of p120-deficient cells in the developing mammary gland (61). In the salivary gland, ablation of p120 blocks acinar development and causes intraepithelial neoplasia (62). The tumor suppressive role of p120 has been demonstrated in several tissues. Knockout of p120 in keratinocytes of the oral cavity, esophagus and forestomach results in inflammation, hyperproliferation and abnormal mitosis (63). In the mouse esophagus, p120 deletion has been shown to cause neoplasia and an inflammatory tumor microenvironment (64).

E-cadherin is a general marker of the epithelial nature of normal or neoplastic epithelial cells. Loss of E-cadherin is an important part of a process known as epithelial to mesenchymal transition (EMT). Major transcriptional regulators of EMT are SNAIL, TWIST and ZEB, which repress expression of E-cadherin (65). Although ILC cells typically lack E-cadherin, they do express other epithelial markers, and are often classified as luminal A subtype, associated with well-differentiated epithelial cell morphology (7). Results from mouse models of ILC show that loss of E-cadherin is insufficient to induce complete EMT (46, 48, 66). Similarly, diffuse gastric cancer cells are E- cadherin deficient, but otherwise still epithelial in nature (67). In invasive breast cancer cells, basal epithelial traits have been observed, with expression of cytokeratin 14 (68). In ILC, invading cells as well as metastases are epithelial in nature, and loss of E-cadherin seems to be an early event, whereas in IDC and other cancers, silencing of E-cadherin seems to be a late event during progression (69). E-cadherin is a suppressor of invasion of human carcinoma cells *in vitro* (70). Also *in vivo* activation of E-cadherin with monoclonal antibodies in mouse models of breast cancer can reduce invasion, intravasation, and extravasation in target organs (71). Conversely, experimental inactivation of E-cadherin in mouse models of IDC also increases invasion, but it reduces proliferation and survival of tumor cells, as well as formation of metastases (72). However, such artificial abrogation in cells that robustly express E-cadherin may not reflect the situation in ILC cells in patients, which may have adapted to loss of E-cadherin (73).

EMT is important during development, and allows cells to lose polarity, to dissociate from adjacent epithelial cells, and to become motile (74). Because of this, EMT is viewed as a process that contributes to invasion and metastasis in cancer, allowing cells to dissociate and to become motile (75, 76). Cancer cells can invade and disseminate as single cells, possibly with an EMT phenotype, but invasion and metastasis

as clusters of cells has also been described. Such clusters may contain so-called leader cells, which have EMT-associated motility and proteases to degrade the extracellular matrix (77, 78). Carcinoma cells with a mesenchymal phenotype do seem to occur in human breast cancers as well as in mouse breast cancer models (79). However, if and how EMT actually contributes to metastasis is still incompletely understood and subject of debate (80, 81). Several groups have studied the role of EMT in mouse models of metastatic mammary tumors (82). Fischer *et al.* have used genetically engineered mice with an irreversible activation of green fluorescent protein (GFP) upon expression of *Fsp1* and *Vim*, two genes that are associated with mesenchymal cells, allowing detection and tracing of EMT tumor cells. GFP+ cells were present in primary tumors, but not in the metastases, and experimental inhibition of EMT by overexpression of mIR-200 (resulting in inhibition of ZEB1 and ZEB2 and concomitant re-expression of E-cadherin) did not prevent metastasis (83). However, detecting and tracing EMT is technically and biologically complex, and others have cast doubt over the conclusions drawn by Fischer *et al.*, because not all EMT events are associated with expression of *Fsp1* or *Vim*, and there are EMT-independent effects of mIR-200 on metastatic potential (84). Zheng *et al.* reported that EMT is dispensable for metastasis in a mouse model of pancreatic ductal adenocarcinoma, by inactivating Twist or Snail, both EMT-associated transcription factors, and showing that this prevents EMT, but not metastasis (85). This study triggered critical comments as well, arguing that EMT could still occur despite inactivation of Twist or Snail, and that the methods used to detect EMT cells were insufficient (86). EMT of cancer cells can be viewed as a form of cellular plasticity, with a spectrum of states, rather than an all-or- nothing switch (87). Partial EMT and the ability to revert back to an epithelial state in metastases (mesenchymal to epithelial transition, MET) may contribute to tumor progression and metastatic growth (88). Using intravital imaging, Beerling *et al*. have identified a small population of cells in mouse mammary carcinomas with spontaneous EMT, migratory behavior, and low expression of E-cadherin. Both E-cadherin-high and E-cadherin-low tumor cells were found to circulate and metastasize. The authors reported that the mesenchymal E-cadherin-low cells and the epithelial E-cadherin-high cells have similar potential for metastatic growth, and that E-cadherin-low cells convert to E-cadherin-high cells after arrival at the metastatic sites (89). Since EMT is not a proven requirement for completion of the metastatic cascade for every tumor type, it is not considered an established essential hallmark of metastasis (90).

THE ROLE OF PI3K SIGNALING IN INVASIVE LOBULAR CARCINOMA

PIK3CA and *PTEN* are the second and third most commonly mutated genes in human cancers (91). *PIK3CA* encodes the p110α isoform of the catalytic subunit of class I phosphatidylinositol 3-kinase (hereafter called PI3K). PI3K and PTEN are both key players in the PI3K signaling pathway, important for cell survival, growth, division, and motility. These processes occur as normal aspects of cellular physiology, but they are also hallmarks of cancer (92, 93). In mammals, there are 4 isoforms of the PI3K catalytic subunit: p110α, p110β, p110γ, and p110δ, encoded by *PIK3CA*, *PIK3CB*, *PIK3CG*, and *PIK3CD*, respectively. p110α and p110β are expressed ubiquitously, and p110γ and p110δ expression is found mostly in immune cells. PI3K signaling can be stimulated by several receptor tyrosine kinases. After binding of an extracellular ligand to the receptor, PI3K is recruited to the membrane and activated. PI3K then converts phosphatidyl-inositol-4-5-bisphosphate (PIP2) to phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) (94). This is followed by recruitment of phosphoinositide-dependent kinase-1 (PDK-1) and AKT to the membrane. There, PDK-1 phosphorylates AKT at Thr308 (95), and mTOR complex-2 (mTORC2) phosphorylates AKT at Ser473, both contributing to its full activation (96, 97). Activated AKT phosphorylates mTORC1, as well as many other substrates. Downstream of mTORC1, protein synthesis and lipid, nucleotide and glucose metabolism are promoted. Important effector molecules include p70S6 kinase 1 (S6K1) and eIF4E Binding Protein (4EBP) (98-101). The tumor suppressor phosphatase and tensin homolog (PTEN) negatively regulates PI3K signaling by converting PIP3 to PIP2.

Around 2006, it started to become clear that PI3K signaling activation occurs more frequently in ILC compared with other invasive breast cancers. Findings included more frequent mutations in *PIK3CA* and higher levels of phosphorylated AKT in ILC (102-104). In PIK3CA, two mutation “hotspots” are known, E542K/E545K in exon 9 and H1047R in exon 20 (105, 106). At first, only exon 9 mutations were correlated with worse prognosis in breast carcinomas (107). Later, exon 20 mutations were also reported to confer poor prognosis (108). Mutations in *PIK3CA* and *PTEN* are enriched in ILC compared to other breast cancers, and phosphorylation of AKT is highest in ILC among all breast cancer subtypes (27, 109). In more than half of the cases, ILCs have an alteration in either *PIK3CA*, in *PTEN*, or in *AKT1* (26). PTEN protein expression has been found to be significantly lower in ILC than in IDC. In line with this, ILC has the highest levels of phosphorylation of AKT, both at Serine 473 and Threonine 308, among all breast cancer subtypes, and increased phosphorylation of p27 and p70S6

kinase compared with IDC (27). Local recurrences of ILC after surgical removal have a higher mutation rate in *PIK3CA* than primary tumors (69% vs 36%), indicating a positive selection advantage. Interestingly, such an increased mutation rate was not found in metastases compared with primary tumors (110). *PIK3CA* mutation is also common in LCIS, the *in situ* counterpart and possible pre-invasive stage of ILC, and seems to be an early event, that does not correlate with progression into ILC (111). These findings suggest that PI3K signaling may be one of the driving forces in the pathogenesis of ILC, and that pharmacological inhibition of this pathway is an interesting therapeutic strategy for ILC patients. Several clinical trials exist with PI3K pathway inhibitors for breast cancer, and additional analyses could potentially reveal information about their efficacy specifically for ILC (55, 112). Breast cancer patients, including a group of 110 patients with ILC, are currently being recruited for the POSEIDON trial, in which patients are treated with a combination of tamoxifen, a well-established endocrine therapy for estrogen receptor-positive breast cancer, and the PI3K inhibitor taselisib (113).

PRECLINICAL MODELS OF INVASIVE LOBULAR CARCINOMA

To find better treatments for ILC, a better understanding of the biology of the disease is needed. Based on solely observational clinical data, it would be difficult to identify cause-and-effect relationships in disease mechanisms. Experiments are required to unravel the pathogenesis of tumor initiation and disease progression, and predictive preclinical models are needed to find and validate therapeutic targets, and to test promising therapies. Studies that are performed *in vitro* always lack some of the hallmarks of cancer, such as an immune system or vasculature (92). Even with the most advanced alternatives currently available, complete modeling of all of these hallmarks still can only be achieved with the use of living animals.

Arguably the simplest *in vivo* models are xenografts of human ILC cells in immunodeficient mice. With cell line models, it is important to keep in mind that they represent a subpopulation from a tumor or effusion, selected by artificial growth conditions in a laboratory environment. Only few cell lines are available to model ILC, and their origin is not always authentic or well-established (114). The cell line MDA-MB-134 was originally reported to derive from IDC, and later reclassified as ILC (115). SUM-44PE was obtained from malignant pleural effusion of a presumed but unconfirmed ILC (116). And IPH-926 was derived from ascites fluid of a patient with metastatic ILC (117). These three cell line models are the most intensively used, and they all have a

metastatic origin, mutated *TP53*, and absence of *PIK3CA* hot spot mutations that are common in ILC (114).

Patient-derived tumor xenograft (PDX) models are generated by transplantation of pieces of tissue, taken directly from patient tumors, in immunodeficient mice. In contrast to cell line-based xenograft models, breast cancers propagated in PDX models maintain their original tumor tissue architecture, consisting of both cancer cells and stroma. These grafts also maintain other key features such as histopathologic characteristics and gene expression profiles, but they lack the immune microenvironment of the original tumors. While the cancer cells persist, the human stromal elements are replaced with mouse-derived stroma (118). Unfortunately, ILC tissue xenografts have a low take rate upon transplantation into the mammary fat pad or at subcutaneous sites, as is the case in general for ER-positive breast cancers (119, 120). However, recent studies employing intraductal injection of dissociated tumor cell from patient tumors have reported a much higher success rate for establishing PDX models from ER- positive breast cancers, including ILCs (121, 122).

Three-dimensional culture systems for organoids, recapitulating epithelial architecture, have been developed for both healthy and diseased tissue, including cancer (123). Breast cancer organoids contain histological and genetic features of the original tumors, and allow drug screening and are potentially useful for testing personalized therapy. Hypotheses regarding the contributions of genetic events to biological behavior and drug responses of breast organoids can be tested in gene editing experiments. Sachs *et al.* have generated a biobank with organoid lines from more than 100 primary and metastatic breast cancers, including 18 organoid lines from ILCs. Hormone receptor and HER2 status were maintained in the organoid cultures. Morphologically, ductal carcinoma organoids generated solid organoids, and lobular carcinoma organoids were discohesive (124). It remains however to be established whether these ILC organoids can be stably propagated *in vitro*, and whether they retain typical features of ILC, including estrogen-dependent growth and sensitivity to endocrine therapy.

It has become increasingly clear that many of the hallmarks of cancer involve complex cancer cell-extrinsic mechanisms, such as angiogenesis, the interplay with the immune system, and invasive growth and metastasis (92). Through genetic engineering of human cancer traits into mice, autochthonous models of human cancer can be developed in which *de novo* tumor development occurs in the context of an intact immune system. In genetically engineered mouse models (GEMMs), the dynamics of tumor development, pathogenetic mechanisms, and causal relationships between genotypes and phenotypes can be studied. The KEP mouse model of ILC, based on

tissue-specific inactivation of E-cadherin and p53, has been mentioned earlier in this chapter (46, 48, 49). An important characteristic of the mouse ILCs (mILCs) arising in female KEP mice is the histologic pattern of single files of cancer cells that are negative for E-cadherin, but morphologically epithelial and positive for epithelial markers. The tumors were classified as luminal via mRNA profiling and expression of the luminal marker cytokeratin 8. The KEP mILC model also recapitulates the metastatic behavior of the tumors, including spread to gastrointestinal tract and the peritoneum. This opens up the possibility to model not only the growth of primary ILC, but also the progression of metastatic ILC. Drawbacks of the KEP model include the lack of expression of estrogen receptor in the mammary tumors, and the fact that a proportion of the KEP mice also develop skin tumors due to expression of K14-Cre in the epidermis. In 2011, another mILC model was presented that was based on inactivation of the same two genes, but with Cre recombinase expression driven by the whey acidic protein (*Wap*) promoter. Tumorigenesis in this model (known as the WEP model) specifically occurs in the mammary epithelium (46). For both of these models, it is important to recognize that a spectrum of tumor types can be found in the mammary glands after inactivation of E-cadherin and p53, comprising not only ILC-like tumors, but also poorly differentiated carcinomas and sarcoma-like tumors with plump polygonal or spindle-shaped cells. Also, cancer cells with large high-grade nuclei occur, as may be expected in p53-deficient cells with genomic instability (125).

The most commonly mutated signaling pathway in ILC is the PI3K pathway. To investigate the *in vivo* role of PI3K pathway activation in ILC, several GEMMs have been generated in which tissue-specific inactivation of inactivation of E-cadherin was combined with loss of PTEN or expression of oncogenic PIK3CA mutants. In 2016, we presented a mouse model with combined deletion of *Cdh1* and *Pten* in the mammary gland. PTEN inactivation rescued apoptosis induced by loss of E-cadherin in the mammary gland, and the mice developed tumors resembling classic invasive lobular carcinomas, with expression of estrogen receptor and formation of metastases. The tumors regressed upon pharmacological inhibition of PI3K signaling with BEZ235 (126). In 2018, a genetically engineered mouse model with *Cdh1* deletion and *Pik3ca* activation in the mammary epithelium was published (109). These mice also develop tumors that closely resemble human ILC. One noticeable difference with human ILC was the smaller amount of connective tissue in the mouse tumors, possibly related to the fact that the human breast has much more connective tissue than the murine mammary gland (127). This tumor model was further classified as ILCs of the immune-related subtype, based

on transcriptional profiling and histology (27, 29). These studies helped to establish the causal role of E-cadherin loss in combination with activated PI3K signaling in ILC.

Genetic in vivo screens allow for the discovery and validation of additional genes that may play a role in tumor formation. Insertional mutagenesis screening, using the *Sleeping Beauty* transposon system, has been applied to discover additional drivers of ILC (128). This strategy helped to identify the oncogenic role of several genes that are frequently aberrant in human ILC, notably *MYH9*, *PPP1R12B* and *TP53BP2*. In mice, truncated *Trp53bp2* (also known as *Aspp2*) was recently found to induce actomyosin relaxation and survival of E-cadherin deficient MMECs during initiation of ILC (129). Combining CRISPR/Cas9 technology with intraductal delivery of lentiviral vectors via the nipple enabled the development of somatic GEMMs of ILC, which can be used for rapid *in vivo* validation of putative ILC driver genes (130). These somatic GEMMs can be even further refined by applying modified CRISPR/Cas9 systems for *in situ* base editing, which allows for more precise alterations in endogenous genes in the mammary gland and can be used to introduce somatic point mutations (131).

SCOPE OF THIS THESIS

The PI3K signaling pathway is clearly relevant in the pathogenesis of breast cancer, and receives much attention as a promising therapeutic target. A range of pharmacological compounds have been developed to inhibit PI3K signaling. In **chapter 2**, we review genetically engineered mouse models of PI3K signaling in breast cancer. We discuss the role of PI3K pathway mutations in human breast cancer and relevant genetically engineered mouse models, with special attention to the role of PI3K signaling in oncogenesis, in therapeutic response, and in resistance to therapy. During the last few decades, treatment options for breast cancer have improved, and the 5-year survival rate in metastatic breast cancer has improved to approximately 25% (132). While many preclinical studies focus on the treatment of primary tumors, it is important to keep in mind that 90% of cancer-related deaths are not caused by the primary tumor, but by metastases. The development of antimetastatic agents has been hampered by the paucity of preclinical models of human metastatic disease. In **chapter 3**, we present a mouse model of spontaneous metastasis of invasive lobular carcinoma. For this, we used the KEP model of *de novo* mammary tumor formation, based on tissue-specific inactivation of E-cadherin and p53 (*K14cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$) (48). We harvested KEP tumors, and subsequently transplanted tumor fragments into mammary glands of wild- type syngeneic hosts. After outgrowth of these primary tumors, we removed

them by mastectomy. After surgery, recipient mice succumbed to widespread overt metastatic disease in lymph nodes, lungs, and gastrointestinal tract. This preclinical model of metastatic lobular breast cancer supports the development of more effective treatment strategies for metastatic disease.

We employed the metastatic KEP model to perform a preclinical intervention study targeting mammalian target of rapamycin (mTOR), one of the main kinases in the PI3K signaling pathway. In **chapter 4**, we report that PI3K signaling is activated in the KEP model, and that tumor growth as well as progression of metastatic disease can be blocked by treatment with AZD8055, an inhibitor of mTOR. However, we also found that resistance to this treatment was ultimately acquired, despite continued suppression of mTOR signaling activity. A key finding in this study was that antigen presentation processes seemed to be activated in tumors that were responding to treatment, and that this activation was lost in resistant tumors. In this chapter, we demonstrate that part of the therapeutic effect of mTOR inhibition is mediated by the adaptive immune system.

To investigate the role of PTEN loss in the pathogenesis of ILC, we generated a mouse model with tissue-specific inactivation of E-cadherin and PTEN in mammary epithelial cells, presented in **chapter 5**. Loss of only E-cadherin resulted in cell dissemination and apoptosis, and inactivation of only PTEN induced formation of squamous metaplastic carcinomas. Combined loss of E-cadherin and PTEN resulted in formation of tumor resembling classic invasive lobular carcinomas. These tumors recapitulated the histological growth patterns of human ILC, as well as estrogen receptor positivity and metastatic potential. Pharmacological inhibition of PI3K signaling with BEZ235 resulted in tumor regression. With this study, we provide evidence for the causal role of combined E-cadherin loss and activation of PI3K signaling in ILC, suggesting that pharmacological inhibition of PI3K may be a promising therapeutic strategy.

In **chapters 6 and 7**, we present our findings regarding the role of p120-catenin, an important molecule in the adherens junction complex. In **chapter 6**, we show that somatic loss of p120-catenin (p120) in a conditional mouse model of noninvasive mammary carcinoma, driven by loss of p53, results in formation of stromal-dense tumors that resemble human metaplastic breast cancer and metastasize to lungs and lymph nodes. Loss of p120 in anchorage-dependent breast cancer cell lines strongly promoted anoikis resistance through hypersensitization of growth factor receptor (GFR) signaling. Interestingly, p120 deletion also induced secretion of inflammatory cytokines, a feature that likely underlies the formation of the pro-metastatic microenvironment in p120-negative mammary carcinomas. Using mouse models with mammary gland-

specific inactivation of E-cadherin, p120 and p53, we demonstrate in **chapter 7** that ILC formation induced by E-cadherin and p53 loss is impaired upon concomitant inactivation of p120. Tumors that developed in the triple-knockout mice were mostly basal-like tumors, with an epithelial-to-mesenchymal-transition (EMT) phenotype. We show that loss of p120 in the context of the p53-deficient mouse models is dominant over E-cadherin inactivation and its inactivation promotes the development of basal, epithelial-to-mesenchymal-transition (EMT)-type invasive mammary tumors.

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