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**Using insertional mutagenesis to identify breast cancer drivers and therapy resistance genes in mice = Insertie mutagenese voor het identificeren van genen betrokken bij de ontwikkeling van borsttumoren en therapie resistentie in muizen**

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# Chapter 1

## **General introduction**

## Breast cancer heterogeneity

Breast cancer is the most common cancer in women worldwide, with an estimated 1.67 million newly diagnosed patients in 2012<sup>1</sup>. This disease includes a diverse spectrum of tumors that can be divided in distinct biological entities based on histopathological and molecular characteristics, responses to therapy and clinical outcome. Multiple taxonomies have been used to classify the tumors in distinct subgroups, which are used to guide clinical decision-making.

Histopathological classification divides the tumors according to morphology, biomarker expression, size and grade<sup>2,3</sup>. Based on morphology, the most frequent histological subtype is invasive ductal carcinoma (IDC), which is a heterogeneous group of tumors lacking special differentiation patterns. The remaining breast cancer subtypes, of which invasive lobular carcinoma (ILC) is the most common special subtype, are classified based on the dominating growth pattern. In addition, estrogen receptor (ER) expression status (assessed by immunohistochemistry) is routinely used in clinical diagnostics, as this is the major discriminating factor with prognostic and predictive value for clinical outcome of women with breast cancer<sup>4</sup>.

In the early 2000s, molecular classification based on gene expression divided breast tumors in two main clusters (distinguished by ER expression), which were further subdivided into five intrinsic subtypes : luminal A and B, basal-like, ERBB2 (HER2) and normal-like<sup>5,6</sup>. Over the years, several studies confirmed these intrinsic subtypes in independent breast cancer cohorts<sup>7-11</sup>, and provided evidence that these insights are useful to predict therapy response and clinical outcome in women with early-stage breast cancer. Hence, a 50-gene RT-qPCR test (PAM50 gene signature assay) based on the original intrinsic subtypes has recently been introduced in the clinic for subtype classification and prediction of risk of relapse for ER positive breast cancer patients<sup>12</sup>.

In parallel with the identification of intrinsic subtypes based on gene expression, genome-wide copy number analyses defined several subgroups of breast cancer according to similarities in DNA copy number alterations (CNAs)<sup>13,14</sup>. Interestingly, these subgroups encompass tumors with different intrinsic molecular subtypes, indicating that CNAs can further refine the classification of breast cancers. Therefore, several studies have performed an integrative approach and combined genome-wide copy number alteration data with gene expression data<sup>15,16</sup>. Of these, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) study used 2000 breast tumors to define 10 integrative clusters with distinct clinical outcomes. This implies that specific genomic driver events are crucial for the prognosis of breast cancer patients.

Large scale DNA sequencing studies have given additional insight in the mutational landscape underlying the development of human breast cancer<sup>17-19</sup>. Based on the frequency of specific mutations, these studies can distinguish between putative tumor driver mutations and non-pathogenic passenger mutations to gain knowledge in the evolutionary process that drives malignant transformation of

mammary epithelial cells. However, these correlative associations in end-stage tumors need to be investigated in clinically-relevant models to prove the cause-effect relation of candidate driver genes in the development and progression of breast cancer. For this purpose, genetically engineered mouse models (GEMMs) have shown to be valuable tools. As mice are genetically very similar to humans, GEMMs can be used to study tumor initiation, progression and responses to therapies in biologically relevant contexts<sup>20</sup>.

Altogether, recent genetic insights indicate that breast cancer is a complex and heterogeneous disease that depends on specific genomic driver events. Consequently, the identification of true driver events and associated signaling networks may be exploited therapeutically to improve clinical outcome of breast cancer patients. In this work, we focused on the identification of novel cancer driver genes and pathways in ILC formation using genetically engineered mice.

## Invasive lobular breast carcinoma

ILC is the second most common subtype of breast cancer, representing 8-14% of all breast cancer cases<sup>21-24</sup>. ILCs display a low grade histology, low proliferation rate and belong to the luminal breast cancer subtype, which are all features associated with good prognosis<sup>25</sup>. Although ILCs are less responsive to chemotherapy than IDCs<sup>26-29</sup>, the majority of ILCs are ER and progesterone receptor (PgR) positive and show an overall good response to endocrine-based therapies<sup>30</sup>. However, ILCs are also characterized by an infiltrative growth pattern of noncohesive small epithelial tumor cells that invade the surrounding tissue in single files. As a consequence, ILCs are difficult to diagnose by palpation or mammography and are prone to metastasize to distant sites, including the ovaries, peritoneum and gastrointestinal tract<sup>25,31</sup>.

The majority of ILCs are characterized by the functional loss of the cell adhesion protein E-cadherin (encoded by the *CDH1* gene), which is a key component of the adherens junctions that maintain epithelial integrity<sup>24</sup>. E-cadherin is a transmembrane protein that binds to itself on neighboring cells and interacts with the actin cytoskeleton via its intracellular domain to form adherens junctions between epithelial cells. In ILCs, functional loss of E-cadherin is mainly resulting from mutational inactivation, loss of heterozygosity in the chromosomal region of 16q22.1 (which harbors the *CDH1* gene) or impaired interactions between components of the E-cadherin-catenin complex<sup>32-35</sup>.

The genomic profiles of ILCs are considered to be more diploid and generally less complex compared to IDCs<sup>25</sup>. Several studies used microarray-based comparative genomic hybridization (aCGH) data to identify recurrent gains of chromosomes 1q, 16p and chromosomal loss of 16q<sup>36,37</sup>. Other focal chromosomal gains and losses were identified in various regions, including high-level amplifications of 8p12-p11.2 and 11q13<sup>38</sup>. These amplifications harbored *FGFR1* and *CCND1*, which were therefore suggested to be candidate ILC driver genes. Furthermore,

several studies identified recurrent inactivating mutations in *TP53* and activating mutations in *PIK3CA*<sup>39-41</sup>, which may have clinical implications for PI3K inhibitor-based therapy in ILCs.

Next-generation sequencing of large ILC collections have further characterized the mutational and molecular landscape of this breast cancer subtype<sup>35,42,43</sup>. Beyond frequent mutations in *CDH1* and *PIK3CA*, these studies showed mutations in *FOXA1*, *TBX3* and additional genes involved in PI3K-AKT signaling, including *PTEN* and *AKT1*. Molecular profiling confirmed increased AKT phosphorylation in ILCs, supporting the fact that activation of the PI3K-AKT signaling pathway is important in this breast cancer subtype<sup>35</sup>. Furthermore, these studies identified distinct ILC subtypes with different clinical outcomes, which might help to identify novel therapeutic vulnerabilities.

## The role of GEMMs in cancer research

The first transgenic mice were generated by the introduction of cloned oncogenes in the genome of mice, which could be transmitted to progeny and in some cases resulted in hereditary cancer predisposition<sup>44</sup>. These landmark studies provided strong evidence for the hypothesis that the expression of oncogenes in normal cells could lead to tumor development in mice. To investigate the contribution of oncogenes in tumor formation in a particular tissue, mice were generated in which oncogenes were under the transcriptional control of tissue-specific promoters, such as the mouse mammary tumor virus long terminal repeat (*Mmtv-ltr*) and the whey acidic protein (*Wap*) promoters. Using this approach, several studies showed that oncogenes such as *Myc*, rat *ErbB2* (also known as *Neu*) and *Hras* could induce mammary tumor formation<sup>45-48</sup>. In 1992, gene knockout technology allowed researchers to study the role of tumor suppressor genes (TSGs) in the process of tumorigenesis in mice. This was pioneered by a report which showed that mice carrying two *p53* null alleles developed normally, but were prone to develop a diverse spectrum of tumors<sup>49</sup>.

Although these conventional GEMMs provided valuable biological insights in the function of oncogenes and TSGs in tumor development, there were also limitations. For example, conventional transgenic or knockout mice either expressed oncogenes or inactivated TSGs in all cells of a specific tissue, whereas tumorigenesis in human is characterized by the progressive acquisition of genetic aberrations in a single cell resulting in a stepwise malignant transformation within a healthy organ. Furthermore, tumor formation driven by the loss of TSGs requires the inactivation of both chromosomal copies, as described by the two-hit hypothesis of Knudson<sup>50</sup>. However, germline biallelic inactivation of several TSGs in mice led to embryonic lethality, as was shown for *Brca1* and *Rb*<sup>51-55</sup>. This phenotype precluded the analysis of the consequence of losing both alleles in the process of tumorigenesis in adult mice.

To overcome these limitations, various strategies were developed to induce somatic mutations in a tissue-specific and time-controlled fashion in adult mice. Strategies to somatically inactivate TSGs in a limited number of cells include Cre/*loxP* or FLP/*FRT* site-specific DNA recombination systems<sup>56,57</sup>. In mice harboring these systems, the target gene, or part of it, is flanked by recombinase recognition sites (*loxP* or *FRT*) without altering its function in the absence of the recombinase. When the respective recombinase (Cre or FLP) is expressed, recombination of the recognition sites is catalyzed resulting in the deletion of the intervening DNA segment and thereby the inactivation of the target gene. The advantage of these systems is that the recombinase can be expressed at any time and any place, by intercrossing transgenic mice expressing recombinase under the control of tissue-specific promoters, via somatic delivery of viruses encoding the recombinase or via inducible recombinases. Several inducible systems were developed to achieve temporal regulation of Cre-recombinase expression, which can be controlled by the administration of tetracycline<sup>58</sup>, interferon or tamoxifen<sup>59,60</sup>. The Cre/*loxP*-system is commonly used in mice to inactivate one or multiple TSGs resulting in tumor formation in a wide range of tissues, including the mammary gland<sup>61–66</sup>, brain<sup>67,68</sup>, prostate<sup>69</sup>, colon and lung<sup>70,71</sup>. Furthermore, this system is also employed to ‘turn on’ oncogenes by inserting a synthetic ‘stop’ element (*lox-stop-lox* cassette) in front of a mutant allele. Upon expression of Cre recombinase, this stop element is removed resulting in the expression of the mutant allele under the transcriptional control of its endogenous promoter. This approach has been used to express the oncogenic KRAS<sup>G12D</sup>-mutant – a mutation frequently observed in human cancer<sup>72–74</sup> – in lung and pancreas<sup>75–79</sup>.

Conditional mouse models carrying site-specific DNA recombination systems to study tumor initiation are limited by the fact that the genetic perturbations are irreversible. This precludes the analysis of the role of oncogenes in maintaining the malignant phenotype of the tumor. Several strategies that allow spatiotemporal control of target-gene expression have been used to study the dependence of the tumor on the activated oncogene, also known as oncogene addiction<sup>80</sup>. The most widely used binary transcription transactivation system in mice is based on the tetracycline-controlled transactivator (tTA) that regulates the transcription of its ‘target’ transgene<sup>81,82</sup>. The original system is based on the transcriptional inactivation of the target transgene (‘tet-off’) in the presence of the inducer tetracycline (or its non-toxic analogue doxycycline), whereas a modified version of this system (reverse tTA) induces transgene activation in the presence of the inducer (‘tet-on’)<sup>83</sup>. A drawback of using binary transcription transactivation systems is that it is rather difficult to induce oncogene expression comparable to physiological levels. For example, low levels of MYC expression induced increased proliferation of cells and tumorigenesis, whereas too high levels of MYC led to the induction of apoptosis in these cells<sup>84,85</sup>. Nevertheless, mouse studies using these systems provided unambiguous proof that different tumor types depend on the constitutive expression of oncogenes for their initiation and maintenance, such as HRAS in melanoma<sup>86</sup>, ERBB2 in breast cancer<sup>87</sup>, MYC and the BCR-ABL1 fusion in leukemia<sup>88,89</sup> and KRAS in lung adenocarcinoma<sup>90</sup>.

In GEMMs, TSGs are mainly inactivated by germline or somatic biallelic deletions of one or more exons, which results in the functional loss of the protein. However, these large deletions do not mimic the missense and nonsense mutations that are frequently observed in human cancers. Missense mutations can result in gain-of-function properties of proteins that might have novel oncogenic activities compared to loss of tumor suppression function induced by homozygous deletion. In this regard, mice carrying *Trp53* missense mutations developed distinct tumor phenotypes compared to *Trp53* knockout mice<sup>91–93</sup>, which suggests that gain-of-function properties of mutant p53 promoted tumorigenesis via different mechanisms than simple loss of p53 function. In contrast, in a conditional mouse model for *BRCA1*-associated breast cancer the introduction of patient-relevant *Brca1* mutations revealed no differences in tumor suppressive function compared to conditional *Brca1* knockout alleles<sup>94,95</sup>. However, tumors induced by inactivation or loss of the *BRCA1* RING domain developed resistance to DNA-damaging therapies more rapidly compared to *Brca1*-null tumors due to residual homologous recombination (HR) activity of mutant *BRCA1* protein. Thus, generating mouse models with patient-relevant mutations is essential to understand their contribution to tumor formation and therapy response.

## Forward genetic screens to identify candidate cancer genes

Characterization of the mutational landscape of human tumors has revealed many genes that are involved in tumor formation. The biological consequences of genetic aberrations are not always apparent, and therefore functional validation studies are required to investigate their role in the process of tumorigenesis. Reverse genetic approaches in mice are used to study the contribution of genes in tumor initiation, progression and other aspects of tumor biology. In addition, forward genetic screens with insertional mutagenesis systems can be employed to identify novel tumor promoting genes and pathways in mice that are not readily apparent in human cancer. These insertional mutagenesis approaches are based on random insertion of genetic elements in the mouse genome, that can activate or inactivate genes and thereby induce cancer formation. These genetic elements are then retrieved from independent end-stage tumors and mapped to the genome to identify common insertion sites (CISs) that contain candidate cancer genes<sup>96</sup>.

The first insertional mutagenesis screens in mice were performed using slow transforming retroviruses such as the moloney murine leukemia virus (MMLV) and the mouse mammary tumor virus (MMTV)<sup>97,98</sup>. Although these screens identified many genes and pathways involved in the formation of leukemias and mammary tumors<sup>99</sup>, this approach predominantly activated oncogenes and was limited to tissue types that could be easily infected. To overcome these limitations, DNA transposons were developed as insertional mutagens. The *Sleeping Beauty* (*SB*) transposon system is the most commonly used system in mice and is based on the mobilization of transposons via a cut-and-paste mechanism<sup>100</sup>. In this two-component system, concatemers of mutagenic transposons are integrated at specific positions in the mouse genome. When transposon carrying mice are crossed with *SB* transposase



expressing mice, the SB transposase enzyme recognizes specific DNA sequences that flank the transposon and 'cuts' the transposon from its original donor locus. The excised transposon can then be reintegrated elsewhere in the genome. *SB* transposons have been engineered to contain a unidirectional promoter upstream of a splice donor (SD) and splice acceptors (SAs) followed by polyadenylation signals (pA) in both orientations<sup>100</sup>. Therefore, integration of *SB* transposons within a gene can lead to premature termination of transcription via splicing of the gene into the SA-pA elements. Conversely, integration of the *SB* transposon upstream of a gene can result in the activation of gene expression via the promoter and SD sequences. Therefore, the orientation and pattern of transposon integrations often already suggests whether the affected gene is an oncogene or TSG.

In 2005, two landmark studies showed that *SB* transposon mutagenesis was capable of inducing tumor formation in wild-type and cancer-predisposed mice<sup>101,102</sup>. Both studies identified several known and novel candidate cancer genes and pathways relevant for human tumors, demonstrating the utility of transposon-based insertional mutagenesis for cancer gene discovery. To avoid transposition-induced embryonic lethality and direct *SB* transposition to specific tissues of interest, Cre-conditional *SB* transposase carrying mice were generated. Taking advantage of tissue-specific Cre recombinases, multiple studies employed *SB*-mediated insertional mutagenesis screens in mice to identify candidate cancer genes in a wide spectrum of tissues, including the liver<sup>103,104</sup>, intestine<sup>105–107</sup> and pancreas<sup>108,109</sup>. The hepatocyte-specific albumin-Cre (*Alb-Cre*) was used to restrict *SB* transposition to the liver and resulted in the formation of preneoplastic liver nodules in predisposed (carrying a conditional dominant-negative *Trp53* transgene) and non-predisposed males<sup>103</sup>. Analysis of CISs identified 19 genes that were strongly implicated in the formation of hepatocellular carcinoma (HCC), which was supported by distinct copy number gains and losses of several of these genes in human HCC samples. Furthermore, analysis of the insertions in *Egfr* revealed the expression of a truncated EGFR variant, which was confirmed to induce neoplastic growth *in vivo*. A similar screen was performed to identify candidate genes for colorectal cancer (CRC) by using *Villin-Cre* transgenic mice to confine transposon mutagenesis to the gastrointestinal tract epithelium<sup>105</sup>. In this screen mice with active *SB* transposition died faster compared to control animals due to the formation of intestinal lesions. CISs analysis of the tumors identified 77 candidate genes of which 60 were mutated and/or dysregulated in human CRC. The significant overlap of mouse and human candidate genes supports the relevance of these findings for human disease and thereby implies that the remaining 17 candidate genes might also be involved in CRC formation. Another study used *SB*-mediated insertional mutagenesis to identify genes that cooperate with oncogenic *Kras*<sup>G12D</sup> to accelerate and promote progression of pancreatic ductal preneoplasia<sup>109</sup>. In this study, active *SB* transposition accelerated the formation of pancreatic ductal adenocarcinomas (PDACs) in *Kras*<sup>LSL-G12D</sup>; *Pdx1-cre* mice compared to control animals. The list of candidate cancer genes identified from these tumors contained novel candidates as well as known genes and pathways involved in human PDAC. In over half of the tumors, insertions were found in the X-linked deubiquitinase *Usp9x* and conditional deletion of *Usp9x* cooperated

with *Kras*<sup>G12D</sup> to accelerate PDAC formation in mice. Furthermore, USP9X loss was correlated with poor survival after surgery in human patients, highlighting the prognostic and therapeutic relevance of this TSG in PDAC.

Another transposon-based insertional mutagenesis tool in mice is the *piggyBac* (*PB*) transposon system, which has been used to identify candidate cancer genes in both solid and hematopoietic tumors<sup>110,111</sup>. *PB* transposons have been shown to differ from *SB* transposons in several aspects, including a different integration preference and a weaker tendency for local hopping<sup>110,112</sup>. For example, *PB*-mediated transposon mutagenesis in *Kras*<sup>LSL-G12D</sup>; *Pdx1-cre* mice induced distinct types of tumors, which contained CISs in a large number of transcription factors that were not previously implicated in pancreatic cancer<sup>111</sup>. Furthermore, comparison between retroviral, *SB*- and *PB*-mediated insertional mutagenesis screens showed that 42% of the CISs identified in *PB*-induced hematological tumors were not reported in other large-scale insertional mutagenesis screens in mice<sup>110</sup>. These studies illustrate the utility of *PB*-mediated transposon mutagenesis in mice to complement other insertional mutagenesis approaches to identify novel cancer drivers and pathways relevant for human cancer development.

In summary, transposon-mediated insertional mutagenesis screens in mice identified many known and novel candidate cancer genes and pathways that were implicated in a wide spectrum of different human cancers. Furthermore, these screens were important in prioritizing candidate genes that were not readily apparent in human tumors and thereby confirm that insertional mutagenesis screens in mice complement large scale sequencing studies performed in end-stage human tumors. This comparative genomics approach makes it possible to distinguish between tumor driver and passenger genes and to identify new targets for therapeutic intervention.

Despite the identification of many candidate cancer genes using insertional mutagenesis, one drawback is that this approach is not suited to recapitulate all genetic alterations observed in human tumors, such as sequence specific driver mutations. Furthermore, the plethora of somatic mutations detected in human cancer genomes is in marked contrast to the relatively simplified somatic genotypes observed in GEMM tumors, as exemplified by comparing EGFR-, MYC- and KRAS-driven GEMMs of lung cancer to their human counterparts<sup>113</sup>. These data suggest that acquiring additional mutations over time might affect tumor development and progression, which could be tested by carcinogen-induced tumorigenesis in GEMMs followed by analyzing the whole-exome sequencing data of the resulting tumors. Using this approach, the induction of NSCLC in wild-type mice using two different carcinogens resulted in tumors harboring an increased number of non-synonymous mutations compared to tumors that were induced by the genetic activation of mutant *Kras*, which were characterized by an increased level of aneuploidy and CNAs<sup>114</sup>. The difference in genetic alterations observed in these tumors suggest that malignant transformation of lung epithelium in these different models occurred via distinct routes, thereby supporting the use for carcinogen models to mimic the complexity of the mutational landscape observed

in human tumors.

## GEMMs for fast-track validation of candidate cancer genes

*In vivo* insertional mutagenesis screens in mice have been proven to be a powerful approach to discriminate between tumor driver and passenger genes in different human cancer types. However, this approach is limited by the generation of mice carrying multiple conditional alleles and the candidate cancer genes still require *in vivo* validation in independent models. Furthermore, the list of mutated genes identified in large scale sequencing studies of human tumors is exponentially increasing. Therefore, novel mouse modeling strategies are required for rapid testing of multiple candidate cancer genes for their contribution in tumor initiation and progression.

To evaluate the contribution of candidate cancer genes in tumor development in GEMMs, the genetic modification of embryonic stem cells (ESCs) can be used to rapidly generate cohorts of non-germline GEMMs with a specific genetic alteration<sup>115,116</sup>. The GEMM-ESC strategy is an extension of this approach that has been developed to test the contribution of specific genetic alterations in the context of complex genetic backgrounds<sup>117</sup>. In this strategy, ESC clones derived from existing GEMMs harboring multiple conditional alleles enable the rapid introduction of additional gain-of-function or loss-of-function alleles. For example, *Myc1* and *Nfib* were introduced in an existing GEMM for small cell lung cancer (SCLC) and were validated to be driver genes for SCLC development<sup>118,119</sup>. Furthermore, it has been shown that Cre-inducible expression of MET accelerated the development of BRCA1-deficient mammary tumors, thereby validating *Met* as a driver of BRCA1-associated breast cancer<sup>120</sup>. In addition to testing the contribution of proto-oncogenes to tumorigenesis in established GEMMs, the GEMM-ESC strategy was also used to study the role of TSGs in tumor progression and maintenance in a broad range of tissues. To this end, the doxycyclin-inducible RNA interference (RNAi) technology was used to show that the loss of APC and p19(ARF) are important for the development and maintenance of T cell acute lymphoblastic leukemia/lymphoma and lung adenocarcinoma, respectively<sup>121</sup>. Similar approaches were used to demonstrate essential roles for the oncogene *c-Myc* and the TSG *Pten* in the progression and maintenance of pancreatic cancer<sup>122</sup>.

The generation of novel GEMMs was boosted by the development of the CRISPR (clustered regularly interspaced short palindromic repeats for efficient gene-targeting)/CRISPR-associated (Cas) system for precise genome editing. This system is based on the prokaryotic adaptive defense mechanism against foreign genetic elements<sup>123–126</sup>, which has been adapted for efficient gene editing in mammalian cells<sup>127</sup>. In this approach, single guided RNAs (sgRNAs) are used to direct the Cas9 nuclease to a specific site in the genome, where it generates DNA double strand breaks (DSBs). The repair of DSBs by the error-prone non-homologous end joining (NHEJ) pathway frequently results in small insertions and deletions, which leads to gene inactivation. This extremely powerful technology

has been optimized to generate mice carrying the genetic alterations frequently observed in human tumors, including deletions and point mutations in single or multiple alleles<sup>128,129</sup>. Furthermore, the utility of CRISPR/Cas9 for introducing conditional alleles and endogenous reporters expands the researchers toolbox to generate novel GEMMs that faithfully recapitulate human disease<sup>130</sup>.

To speed up the validation of candidate cancer genes in adult mice, a number of studies used (inducible) CRISPR/Cas9 somatic gene editing in several tissue types<sup>131,132</sup>, including the liver<sup>133–135</sup>, lung<sup>136–138</sup>, brain<sup>139</sup> and pancreas<sup>140,141</sup>. This approach to generate non-germline GEMMs does have some limitations, including the risk to induce off-target effects, the low efficiency of targeting multiple genes in the desired tumor-initiating cell population and the risk of developing a Cas9-specific immune response. Moreover, despite optimizations of a CRISPR/Cas9 system to induce target gene activation (CRISPRa)<sup>142–144</sup>, this approach is not yet suited to validate putative oncogenes. Notwithstanding these limitations, these studies demonstrated that CRISPR/Cas9-based somatic gene editing can introduce genetic alterations that are frequently observed in human tumors, such as the inactivation of (multiple) TSGs, chromosomal rearrangements and activating point mutations. Therefore, this highly versatile approach can be used to rapidly validate large numbers of candidate cancer genes in a broad range of tissues and enables the generation of non-germline GEMMs to study the molecular biology in tumors that closely resemble their human counterparts.

## **GEMMs to study the crosstalk of tumor cells and their microenvironment**

Besides for the analysis of specific oncogenes and TSGs in the development of tumors, conditional GEMMs are also used to study the complex interactions between cancer cells and their microenvironment in tumor progression and metastasis formation. Using GEMMs, both cancer-associated fibroblasts (CAFs) and immune cells were shown to have tumor promoting and repressing roles. For instance, CAFs were capable to promote tumor progression in a mouse model for squamous skin carcinogenesis by stimulating inflammation, extracellular matrix remodeling and neovascularization in the microenvironment<sup>145</sup>. In contrast, two independent studies using GEMMs of PDAC showed that tumor progression was restrained rather than supported by certain stromal components, such as myofibroblasts or the soluble sonic hedgehog (Shh) ligand<sup>146,147</sup>. In a mouse model of *Myc*-induced lung cancer it has been shown that reprogramming of the microenvironment by cancer cells can drive tumor progression<sup>148</sup>. Activation of *Myc* in KRAS<sup>G12D</sup>-driven adenomas promotes an immediate highly inflammatory (recruitment of macrophages), angiogenic and immune-suppressed stroma resulting in the development of highly proliferative and invasive adenocarcinomas. Deactivation of *Myc* in these adenocarcinomas reversed all stromal changes and induced tumor regression, providing strong evidence that epithelial-derived signaling can directly reprogram the microenvironment to promote tumor progression. Besides the contribution of stromal components in tumor progression, the role of immune

cells in metastasis formation has also been studied in mouse models for different cancer types, including melanoma and breast cancer<sup>64,149–151</sup>. The complex interactions between mammary tumor cells and their original microenvironment in metastasis formation was investigated using a preclinical model for breast cancer metastasis<sup>152,153</sup>, which is based on the orthotopic transplantation of GEMM-derived mouse ILC fragments followed by surgical resection of the primary tumor once established. Using this approach, it was demonstrated that the tumor-induced expression of interleukin (IL)-1 $\beta$  elicited IL-17 expression from gamma delta ( $\gamma\delta$ ) T cells, resulting in the systemic expansion of neutrophils in tumor-bearing mice. These tumor-induced neutrophils promoted metastasis formation by suppressing the activity of cytotoxic T lymphocytes, which normally play an important role in preventing the establishment of metastases. A follow-up study showed that the systemic expansion of neutrophils is dependent on CCL2-mediated induction of IL1 $\beta$  in tumor-associated macrophages<sup>154</sup>.

In conclusion, these studies demonstrate that GEMMs can provide valuable biological insights in the interactions between cancer cells and their micro-environment in tumor initiation, progression and metastasis formation, which might be exploited therapeutically to improve the clinical outcome of human cancer patients.

## GEMMs to study drug responses

Over the years, many novel anti-cancer therapies have been tested in conventional preclinical mouse models, which are based on the transplantation of human or mouse tumor-derived cell lines. Nevertheless, the majority of the phase 3 clinical trials based on these findings failed to show favorable clinical responses in human patients<sup>155</sup>. The poor predictivity of cell line-based transplantation models is most likely due to the fact that the tumor-derived cell lines used for these models have been extensively cultured *in vitro*, during which they acquired additional mutations and biological properties that were not present in the original tumors. To circumvent this problem, patient-derived xenograft (PDX) models have been developed, which are based on direct transplantation of fresh human tumor biopsies in immunodeficient mice, thereby maintaining the original tumor architecture and mutational profile. PDX models of a broad range of human tumors have been valuable tools for the prediction of responses to clinical drug candidates<sup>156–159</sup>. However, both PDX models and cell line-based xenograft models lack an intact immune system and can therefore not be used for preclinical testing of immunotherapies. The development of anti-cancer therapies should preferably be performed in autochthonous tumor models that contain an intact immune system and capture all cell-autonomous and non-cell autonomous processes underlying *de novo* cancer development and progression. GEMMs that faithfully recapitulate human disease meet these requirements and are therefore expected to increase the translatability of preclinical drug responses and guide personalized medicine for human cancer patients.

In several GEMMs of human cancer, genetic and pharmacological approaches have been used to investigate the dependency of tumors on key signaling pathways. In GEMMs for lung cancer, drug intervention studies have been performed with clinically relevant inhibitors of PI3K, EGFR and KRAS signaling. It was shown that tumors expressing mutant PIK3CA<sup>H1047R</sup> regressed upon treatment with NVP-BEZ235, a dual pan-PI3K and mammalian target of rapamycin (mTOR) inhibitor<sup>160</sup>. In contrast, mutant KRAS-driven lung tumors only showed regression when NVP-BEZ235 was combined with MEK inhibition. Furthermore, genetic and pharmacological approaches were used to show that expression of mutant EGFR is essential for tumor maintenance in different EGFR-driven lung tumors<sup>116,161</sup>, whereas KRAS<sup>G12V</sup>-driven tumors showed no response to the EGFR inhibitor AV412. These findings accurately reflect the observed clinical responses to reversible EGFR inhibitors in non-small cell lung cancer patients with mutations in *EGFR* and *KRAS*<sup>162–165</sup>, illustrating that GEMMs that closely mimic human disease are powerful tools to predict the efficacy of targeted therapies in human cancer patients.

To further improve and accelerate the translatability of drug responses in preclinical mouse models, several groups performed co-clinical trials in which drug intervention studies in mice are performed in parallel with human clinical trials<sup>166</sup>. The information obtained in preclinical mouse studies is used to predict the response to targeted therapies in patients and to identify a subpopulation of patients who would benefit most from a specific treatment regimen. Using this approach, it was shown that loss of *LKB1* markedly impairs the response of *Kras*-mutant lung tumors to combination therapy with docetaxel and the MEK inhibitor selumetinib<sup>167</sup>, which highlights the loss of *LKB1* in human lung cancer patients as a genetic determinant for poor response to this combination therapy. Furthermore, a co-clinical trial performed in mice with *EML4-ALK*-driven tumors showed higher response rates to the ALK inhibitor crizotinib compared to chemotherapy<sup>168</sup>, which was shown to be predictive for therapy responses to the same drugs in patients with lung cancer harboring *EML4-ALK* fusions. The preclinical mouse model was subsequently used to explore novel therapies to improve the clinical outcome of patients with *EML4-ALK*-driven tumors and to overcome intrinsic and acquired resistance to crizotinib treatment. Another co-clinical trial identified genetic determinants for resistance to androgen deprivation therapy, leading to development of a combination therapy for castration-resistant prostate cancer<sup>169</sup>. Together, these studies illustrate the utility of co-clinical trials in mice to guide and to improve personalized treatment strategies in human cancer patients.

### **GEMMs to study therapy resistance**

Despite the fact that numerous anti-cancer drugs show promising results in both mouse models and human, tumor cells have sophisticated ways to escape being killed by the treatment and thereby limit the effectiveness of the drug. Tumors can be insensitive from the start of the treatment (intrinsic resistance) or develop resistance during treatment (acquired resistance). Drug resistance is the most

challenging problem observed in the clinic, as tumors are highly adaptable. This is reflected by the broad range of known resistance mechanisms to anti-cancer drugs, including mutations of the drug target, increased rates of drug efflux, the reactivation of downstream signaling via alternative receptor tyrosine kinases, inactivation of apoptosis-induced signaling, epigenetic changes and the impact of the tumor microenvironment<sup>170</sup>. Furthermore, tumors are characterized as heterogeneous masses of cells<sup>171</sup>, which might also explain the fact that several mechanisms of resistance are observed in a single tumor. The identification of resistance mechanisms is necessary to uncover novel therapeutic vulnerabilities and rational drug combinations for selected groups of patients and improve the clinical outcome of human cancer patients.

The use of GEMMs to study therapy resistance provided valuable insights for the underlying mechanisms of resistance to several targeting therapies. For instance, in two independent mouse models of HER2-driven breast cancer it was shown that tumors were sensitive to the pharmacological inhibition of HER2<sup>172,173</sup>, which resulted in tumor regression or tumor stasis. Therefore, these models were amenable to identify loss of PTEN or retained expression of cyclin-D1 and CDK4 as mechanisms of resistance to HER2-targeted therapies. An inducible model for breast cancer was used to show that the genetic inactivation of *PIK3CA*<sup>H1047R</sup> in *PIK3CA*<sup>H1047R</sup>-driven mammary tumors resulted in recurrent tumors with amplifications of *Met* and *Myc*<sup>174</sup>. Functional validation confirmed that elevated MYC expression conferred resistance to PI3K inhibition in *PIK3CA*<sup>H1047R</sup>-driven tumors, which might have clinical relevance for PI3K-targeted therapies as *MYC* amplification and *PIK3CA* mutations frequently co-occur in human breast tumors. Furthermore, genome wide approaches have also proven to be powerful tools to identify genes in therapy resistance in GEMM. For example, *SB*-mediated insertional mutagenesis was used to show that ERAS expression in *Braf*<sup>V618E</sup>-mutated melanomas induced resistance to the BRAF inhibitor PLX4720<sup>175</sup>. Another study employed a *Piggybac*-transposon screen in *Trp53* wild-type tumors to identify several potential mechanism of resistance to MDM2-targeted therapy<sup>176</sup>, which were mostly involved in abrogating functional p53 signaling. Finally, the use of focused shRNA libraries in a mouse model for liver cancer revealed that elevated MAPK14-ATF2 signaling predicted poor response to the multikinase inhibitor sorafenib<sup>177</sup>, suggesting that sorafenib combined with inhibition of MAPK14 might improve the clinical outcome of liver cancer patients.

Altogether, these studies provided mechanistic insights in the development of resistance to targeted therapies, which could have clinical implications for their use in human cancer patients. However, these studies often require the generation of large cohorts of tumor-bearing mice. Furthermore, genetically engineered mice frequently develop multiple independent tumors which might show different responses to targeting agents and thereby hamper the rapid collection of drug resistant tumors. An approach to overcome these limitations is to perform orthotopic transplantation of tumor fragments into syngeneic mice, which allows the rapid generation of large cohorts of mice bearing a single tumor that maintains its genetic and drug sensitivity profile<sup>178</sup>. Using this approach, large-scale intervention studies

could be performed to study the response and resistance to DNA-damaging drugs or other targeting therapies in both primary and metastasized tumors of different breast cancer subtypes<sup>152,178–182</sup>. For example, *BRCA1*-associated mammary tumors showed long-term responses to PARP inhibitor (PARPi) treatment due to the induction of synthetic lethality in these HR-deficient tumors. Despite these initially good responses, therapy resistant tumors eventually emerged that showed partial restoration of HR due to the somatic loss of 53BP1 or REV7<sup>180,182</sup>. The promising therapeutic efficacy observed in these and other preclinical studies resulted in the evaluation of PARPi treatment in a diverse range of human cancers, which demonstrated significant clinical benefit in *BRCA1/2*-mutation carriers with breast and ovarian cancer<sup>183–186</sup>. Despite this, acquired resistance to PARPi treatment has also been observed in patients, underscoring the need for the identification of resistance mechanisms.

Although the use of GEMMs provided valuable mechanistic insights in PARPi resistance, the relevance and prevalence of the observed mechanisms remain to be determined in human tumors. Furthermore, other studies showed that acquired resistance to platinum drugs can also be mediated by reactivation of *BRCA1/2* via genetic reversion<sup>187–189</sup>, which cannot be investigated in GEMMs for *BRCA1/2*-associated breast cancer carrying large irreversible intragenic deletions of *Brca1* or *Brca2*. These and other mechanisms can be investigated in PDX models of *BRCA1*-deficient breast cancer<sup>190</sup>.

Another strategy to pursue potential resistance mechanisms is to collect tumor biopsies of human patients before treatment and when tumors progress during treatment and compare their genetic and molecular characteristics. Using this approach, several studies showed that reversion mutations in HR-related genes could be detected in post-progression tumor biopsies and circulating free DNA (cfDNA) of patients enrolled in clinical PARPi trials<sup>191–193</sup>.

Altogether, the above studies demonstrate the utility of GEMMs to study response and resistance to anti-cancer drugs in tumors that mimic their human counterparts. Nevertheless, GEMMs and humans also have certain biological discrepancies that might affect therapy response and the route to develop resistance, which means that complementary approaches in mouse models and human cancer patients are necessary to dissect all mechanisms underlying therapy resistance.

## Concluding remarks

Breast cancer is a heterogeneous and complex disease that is characterized by distinct biological entities based on genetic features. Comprehensive analyses of human breast tumors are essential to identify candidate cancer genes and pathways that are involved in tumor initiation, progression and metastasis formation. The validation of correlative associations observed in end-stage tumors requires adequate models that faithfully recapitulate human disease. To this end, GEMMs provided compelling evidence for the contribution of many oncogenes and TSGs in the process of tumorigenesis. The power of CRISPR/Cas9 gene



editing enables rapid somatic testing of single or multiple candidate cancer genes in a wide range of tissues and we expect that this technology will further boost the validation of novel TSGs. Despite the advent of CRISPRa, optimization of CRISPR/Cas9 technology is necessary for a similar impact on the speed and efficacy of the validation of candidate oncogenes in the process of tumor formation.

To reliably gain biological insights in various aspects of tumor biology, GEMMs should have minimal discrepancies with human disease and faithfully recapitulate the cell-intrinsic and cell-extrinsic features underlying tumor formation, progression and responses to therapy. First of all, GEMMs should contain patient-relevant mutations that drive malignant transformation of single cells in a normal organ, thereby reflecting sporadic tumor formation in a healthy microenvironment. Second, the tumor heterogeneity observed within a single tumor and between human cancer patients should be captured within these preclinical mouse models. Finally, for translational tests of drug responses in preclinical models, it is necessary that the model reflects the stage of disease (primary tumor or metastatic disease) for which therapy is intended in human patients.

Despite all biological insights provided by GEMMs, promising results in preclinical studies do frequently not match with the poor success rates observed in clinical trials. This could be explained by several differences between tumors treated in mice and in human, which should be considered in the design of preclinical studies. For example, clinical trials are performed with human patients harboring tumors that progressed during multiple standard treatments, whereas mouse tumors are often still treatment-naïve. Although mouse tumors and human tumors may have similar initial genetic alterations, preceding drug treatments of human tumors might have introduced additional mutations which might affect the responses to the novel therapy.

Current advances in modeling of human cancer in GEMMs are expected to improve the similarities between tumors in mice and human. Nevertheless, any model will have limitations, urging the need for other approaches to gain biological insights in tumor development and responses to therapy and complement GEMMs. These approaches include sequencing of clinical tumor and blood samples of human patients, the use of PDXs and high-throughput genetic or drug screens in tumor organoid or cell line cultures. Altogether, the use of different, complementing strategies is expected to be essential for the analysis of biological mechanisms in tumor development, progression and metastasis formation. These insights can subsequently be used for the identification and testing of novel therapeutic vulnerabilities in human tumors, which should ultimately lead to better clinical outcomes of human cancer patients.

## Thesis outline

In this thesis, we used genetically engineered mouse models to identify genes and pathways that are involved in ILC formation and in the development of resistance to FGFR-targeted therapy. These mice carry conditional alleles of *Cdh1*, which result in the inactivation of the cell-adhesion molecule E-cadherin when Cre-recombinase is expressed. As mice with mammary-specific inactivation of E-cadherin alone were not prone to develop mammary tumors, they were used to investigate the contribution of additional genetic mutations to the development of ILCs using different genetic approaches.

In **chapter 2** we used non-germline modeling to study the role of PI3K-AKT signaling in the development of ILCs. By performing intraductal injections of high-titer lentiviruses, we were able to transduce basal and luminal cells in mammary epithelium. Furthermore, we show that the combined inactivation of E-cadherin and activation of an oncogenic AKT variant or the somatic inactivation of the tumor suppressor gene *Pten* resulted in the development of mouse ILCs that faithfully recapitulated their human counterparts.

In **chapter 3** we employed a *Sleeping Beauty* (*SB*)-based insertional mutagenesis screen in conditional *Cdh1* knockout mice to identify novel genes and pathways involved in the development of ILC. We show that active transposon mutagenesis drives ILC formation and analysis of common insertion sites in *SB*-induced tumors identified a mutually exclusive group of four genes, of which three are frequently aberrated in human ILCs. These genes are involved in the regulation of the actin cytoskeleton, which implicates that this is a novel oncogenic pathway in ILCs and might therefore be a new therapeutic target in patients.

In **chapter 4** we developed IM-Fusion, an approach for the identification of transposon insertion sites from gene-transposon fusions in RNA-sequencing data. Using this approach, we show that we can reliably identify candidate cancer genes in two separate transposon screens. In addition, IM-Fusion was also able to predict the effect of the insertions on their target genes and can therefore be used to prioritize the candidate driver genes to be investigated in functional validation studies.

In **chapter 5** we show that transposon insertional mutagenesis screens in mice can also provide additional insight in mechanisms of gene activation. We observed strong clustering of transposons in the last intron of *Fgfr2*, which resulted in the expression of C-terminal truncated *Fgfr2* transcripts. In contrast to full-length FGFR2, mammary-specific expression of two different FGFR2 truncation variants induced rapid ILC formation in mice. Furthermore, we developed a FACS-based assay to measure S6 phosphorylation levels induced by FGFR2 variants in mammary epithelial cells, which correlated with their tumorigenic potential in mice. Using this approach, a large number of FGFR2 variants can be tested to identify which domains in the C-terminal tail of FGFR2 are essential to suppress tumor formation.

In **chapter 6** we used active mobilization of transposons in transplanted mouse ILCs to identify genes involved in acquiring resistance to the FGFR inhibitor AZD4547. We show that mouse ILCs with active FGFR signaling regress upon FGFR inhibitor treatment, which increased the overall survival of mice treated with AZD4547 compared to controls. However, the majority of the tumors were not completely eradicated and AZD4547-resistant tumors emerged during treatment. Analysis of the transposon insertions in AZD4547-resistant tumors identified several candidate resistance genes, which all converged to the reactivation of the MAPK-ERK signaling pathway.

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## Chapter 1

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