An Odyssey towards personalised medicine in breast cancer

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CHAPTER 5

General Discussion

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5

INSERTIONAL MUTAGENESIS SCREENS

RETROVIRUS (MMTV)-MEDIATED SCREENS

This thesis expands the work of MMTV insertional mutagenesis screens we previously performed (Klijn et al., 2013; Theodorou et al., 2007). Both these screens yielded predominantly CISs within gene loci belonging to the *Wnt* (Wingless-type MMTV integration site family), *Fgf* (fibroblast growth factor) and *Rspo* (R-spondin) gene families. This is not surprising, as these gene families have consistently been associated with MMTV in the past, independent of genetic background of the mouse model or strain of the virus. These gene loci have therefore been referred to as 'core MMTV CISs' (Callahan and Smith, 2008; Callahan et al., 2012). Even in the tumour-predisposed backgrounds carrying the mammary gland-specific deletion of the *Trp53* (transformation related protein 53) gene (*K14cre;Trp53*$^{F/F}$) or deletion of one allele of the *Pten* (phosphatase and tensin homolog) gene (*Pten*$^{+/-}$), primarily these 'core MMTV CISs' were tagged and no significant CISs enrichment compared to wild-type strains was found (Klijn et al., 2013). *Trp53* encodes the P53 protein, an important tumour suppressor, and is the murine equivalent to the human *TP53* (tumour protein p53), a gene mutated or lost in approximately 50% of human cancers and in 30% of breast cancer cases (Bertheau et al., 2013). *Pten* encodes the PTEN protein, a tumour suppressor that acts as the major antagonist of PI3K/AKT/mTOR signalling pathway activity, and *PTEN* is deleted or carries loss-of-function mutations in many cancer types, including breast cancer (Hu et al., 2009; Saal et al., 2008; Stemke-Hale et al., 2008). Due to the lack of specific CISs in the tumour-prone mice with *Pten* haploinsufficiency or tissue-specific deletion of *Trp53*, it was suggested that MMTV is a rather inflexible insertional mutagenesis system, inducing mammary tumorigenesis through a limited number of targets (Klijn et al., 2013). However, in the screen in HER2+ transgenic mouse models (*MMTV-cNeu*) presented in Chapter 2, the 'core MMTV CISs' were significantly less enriched compared to wild-type mice and we identified *MMTV-cNeu* associated (genotype specific) CISs. This shows that MMTV insertional mutagenesis is still a useful tool to identify clinically relevant oncogenes.

Of course, it must be noticed that the value of MMTV as insertional mutagenesis tool is still dependent on its tissue-specificity, which is equally true for Murine Leukaemia Virus (MuLV). This restricted tropism of the retroviruses limits their utility for insertional mutagenesis in other tissue types, which has sparked research into other systems for cancer gene discovery screens, like transposons, lentiviral vectors and library-based screening methods.

TRANSPOSON-MEDIATED SCREENS

Transposons are genetic elements that can 'jump' from one genetic position to another in the genome of their hosts and their sequences are ubiquitously found in both prokaryotes and eukaryotes. The human genome is occupied for 44% with transposon-like elements of which almost all are no longer active (Mills et al., 2007). The 'resurrection' of inactive transposons in vertebrates allowed transposon-mediated insertional mutagenesis, of which Sleeping Beauty (Collier et al., 2005; Dupuy et al., 2005; Horie et al., 2001; Ivics et al., 1997) and piggyBac (Cary et al., 1989; Ding et al., 2005; Fraser et al., 1983; Wu et al., 2007) are the most prominent examples. Compared to retroviruses, transposons have the advantage that they are not restricted to a particular organism or tissue, making them much more versatile in insertional mutagenesis (Collier and Largaespada, 2005; Uren et al., 2005).

Sleeping Beauty has the disadvantages of leaving a 2-5 nucleotide footprint when excising, which can lead to frameshift mutations, and is prone to local transposon mobilisation and reintegration ('hopping') (Liang et al., 2009; Luo et al., 1998). Moreover, Sleeping Beauty is biased to TA-nucleotide containing host sequences and intragenic regions, resulting in more repressing CISs, thus more likely to identify tumour suppressors (de Jong et al., 2014; Liu et al., 2005). Conversely, piggyBac leaves no footprint upon mobilisation and shows less local hopping, but requires a genomic TTAA site to integrate and prefers insertion in transcription start sites, resulting primarily in direct gene activation (Fraser et al., 1996; de Jong et al., 2014; Liang et al., 2009; Meir et al., 2011; Wang et al., 2008).

LENTIVIRAL VECTOR-MEDIATED SCREENS

Lentiviral vectors were originally developed for use in gene therapy. They are engineered from the HIV virus genome, from which all sequences for the structural proteins required for a full viral life-cycle have been removed, while retaining integration-related sequences such as reverse transcription signals (Ranzani et al., 2013a). In an attempt to compare genotoxicity profiles of lentiviral and retroviral vectors to increase biosafety for gene therapy, lentiviral vectors engineered to carry strong promoter/enhancer sequences in their LTRs were found to have a high frequency of integrations near certain classes of growth-control and cancer-related genes (Montini et al., 2009). This allows cancer gene discovery screens by lentiviral vector-mediated insertional mutagenesis.

Lentiviral vectors have the advantage over retroviruses that they can also integrate in non-replicating cells, while retroviruses require cells to proliferate (Lewis and Emerman, 1994; Roe et al., 1993). Moreover, lentiviral vectors are also highly versatile in tissue tropism and because they are engineered to integrate in their hosts' genome once without the ability to replicate, they can uniquely exhibit a single CIS integration in tumours (Ranzani et al., 2013b). On one hand, that results in a strong reduction of passenger integrations, easing retrieval of driver insertions and allowing detailed studying of a single mutagenic event (Ranzani et al., 2013b), but on the other hand it may greatly reduce yield and prevent the discovery of collaborating or mutually exclusive cancer genes.

ALTERNATIVE PRECLINICAL SCREENS

Alternative induced forward-genetic screening methods include knockdown screens using short hairpin RNA (shRNA) (Bernards et al., 2006), induced expression screens by employing ORF (open reading frame) cDNA libraries (Yang et al., 2011) and both loss-of-function and gain-of-function screens using the CRISPR-Cas9 genome editing technology (Chen et al., 2015; Konermann et al., 2015; Sanchez-Rivera and Jacks, 2015; Sanjana et al., 2014; Shalem et al., 2014; Wang et al., 2014). The genomic coverage and resulting bias of these methods is dictated by the used library, which is never completely genome-wide, and the delivery of the library

to the target cells is very challenging, especially *in vivo* (DeNicola et al., 2015). Moreover, significant off-target effects and undesired vector-induced insertional mutagenesis can occur in each of these screening methods (DeNicola et al., 2015). Nevertheless, shRNA and CRISPR-Cas9 mediated screens have a strong advantage in identifying tumour suppressors, as gene knockdown and knockout, respectively, is readily attained. This in contrast to retroviral and transposon-induced screens which requires very rare coincidental biallelic gene disruption. Moreover, the versatility of the CRISPR-Cas9 tool can additionally open doors for new forms of forward-genetic screening.

SCREENING CLINICAL SAMPLES

One could also consider screening human cancers directly, which has a great advantage with regard to immediate clinical relevance. Indeed, whole-genome, whole-exome, whole-transcriptome or gene-panel sequencing of clinical samples can offer unbiased information on tumour genetics and may discover cancer-related genes. However, mutation analysis approaches are still expensive (although increasingly cheaper), time-consuming and challenging to analyse. To acquire sufficient power, sample size and depth of sequence coverage has to be high, which is also difficult and costly to achieve (Garraway and Lander, 2017; Mwenifumbo and Marra, 2013).

RNA sequencing-based approaches can also be used to for cancer gene discovery and have the benefit of simultaneously collecting expression data, thus including the effects of epigenetic alterations, and transcriptome mutation data. This can be used to identify putative oncogenes (upregulated or gain-of-function mutation) and tumour suppressor genes (downregulated or loss-of-function mutation) as well as fusion events and splice variants when performing paired-end sequencing (Kumar et al., 2016; Wang et al., 2009). On the other hand, this combination of sequence data and expression levels also blurs cause and effect of (epi)genetic aberrations in the output, making it more difficult to identify the specific cancer genes (Mattison et al., 2009). Furthermore, RNA-seq is obviously unable to identify chromosomal rearrangement outside the transcriptome.

Cancers typically carry numerous mutations, have high intra-tumour heterogeneity and genomic instability, which is aggravated by cytotoxic therapies (Shyr and Liu, 2013). Most of these genetic alterations will not be driving the cancer, but will rather be passenger mutations. For all mentioned sequencing approaches, distinguishing the driver mutations from the passenger mutations is not trivial and is computationally demanding, especially considering that different types of genetic alterations (i.e. point mutations, indels, rearrangements, etc.) may each require different algorithms and bioinformatic pipelines (Damodaran et al., 2015). Moreover, the identification of copy number variations (CNVs), although recently possible to some extent with next generation sequencing methods, is still predominantly relying on array-based comparative genomic hybridization (CGH) and single-nucleotide polymorphism (SNP) approaches (Liu et al., 2013; Russo et al., 2014). These approaches typically have a low resolution, genome coverage and signal-to-noise ratio, making it difficult to pinpoint the specific cancer genes and to detect infrequent genetic changes. Also with these techniques, it is generally difficult to distinguish driver mutations from passenger mutations, as well as from noise (Mattison et al., 2009).

Overall, when using clinical tumour samples, the quantity and quality of the available specimens are often problematic, as these are commonly very small, formalin-fixed paraffin-embedded (FFPE) and/or contaminated with non-tumour (i.e. 'normal') tissue (Damodaran et al., 2015; Yu et al., 2015). Moreover, clinical samples are also associated with important regulatory, ethical and privacy related considerations, especially when these are sequenced. An insertional mutagenesis screen is a preclinical functional approach and therefore does not have these issues with regard to clinical material, is relatively easier and cheaper to perform. Furthermore, these screens can also shed light on mechanisms that have not yet occurred or been detected in the clinic (e.g. predict putative resistance pathways against new therapeutic agents or genetic variations missed in a biopsy due to intra-tumour heterogeneity). Nonetheless, analyses of human tumour samples are more directly relevant to the clinic, and provide complementary insight in cancer biology as well as essential data for clinical validations. Conversely, insertional mutagenesis screens can greatly inform the translation of the correlative output of clinical screens

to causal relationships between cancer genes and disease (i.e. distinguishing driver and passenger mutations). Hence, both preclinical functional approaches and direct analyses of clinical material are necessary to progress.

VALUE OF MMTV AS INSERTIONAL MUTAGENESIS TOOL

MMTV has found to be by far the least biased system with regard to integration-site preferences compared to other retroviruses and transposons (Faschinger et al., 2008; de Jong et al., 2014). MMTV is probably also less biased than the HIV-1 based lentiviral vectors, considering their strong integration bias towards (regions with) actively expressed genes (Mitchell et al., 2004; Schröder et al., 2002). Besides its highly random integration pattern, MMTV is additionally not limited by the coverage of any library design nor hindered by a technically challenging delivery. Moreover, retroviruses are naturally integrating mutagens, giving them the advantage of a long time of natural evolution for its mechanism of mutagenesis compared to engineered transposons, lentiviral vectors and library-based screening methods. Hence, in the field of breast cancer gene discovery, where its limited tropism is not an issue, MMTV-mediated insertional mutagenesis may still be the best tool to use. Considering that the most clinically relevant heterogeneity in breast cancer is found within each molecular subtype (The Cancer Genome Atlas Network, 2012), employing MMTV-induced cancer gene discovery screens would in particular be recommended in murine models for these molecular subtypes. Taking into account that therapeutic options are primarily based on the breast cancer subtype, assessing heterogeneity within the subtypes clearly also makes sense in relation to therapy resistance, as this heterogeneity is likely a defining factor in therapeutic response.

PI3K/AKT/mTOR PATHWAY-ACTIVATING SCREEN HITS

The screens presented and/or followed-up in this thesis (Chapters 2 and 3; Klijn et al., 2013; Theodorou et al., 2007) have yielded several novel candidate cancer genes. Three of these, *Eras*, *Irs4* and *Igf2*, have a role in activating the PI3K/AKT/mTOR signalling cascade, of which ERAS and IRS4 are shown here to induce hyperactivation of this pathway (Chapters 2 and 3). ERAS has been known to be constitutively active, presumably due to its Ser50, Ala100/Asp100 and Ile101 residues that render it insensitive to GTPase-activating proteins (GAPs) that normally switch off RAS proteins (see Chapter 1). In Chapter 3, IRS4 is reported to have a growth factor-independent activity, in contrast to IRS1 and IRS2 (Figure 1). Specifically, IRS4 has a high basal signal transduction activity and a sustained activity upon upstream stimulation due to a lacking Src homology phosphatase 2 (SHP2)-binding site. In IRS1 and IRS2, this phosphatase is recruited by specific phosphotyrosines in their carboxyl-termini, leading to tyrosine dephosphorylation of the IRSs, consequently preventing docking and further activation of downstream effectors. Hence, IRS4 is, in contrast, unresponsive to this strong feedback regulation and hyperactivates the PI3K/AKT/mTOR pathway even upon no to very weak upstream receptor tyrosine kinase activation (Figure 1).

IGF2 is a known oncogene in breast cancer and *Eras* and *Irs4* are also tagged in a Murine Leukaemia Virus (MuLV)-induced screen (Uren et al., 2008). Moreover, in an analysis aimed at mapping integration biases, a comparison between integrations in unselected cells and those in cells that grew out into tumours *in vivo*, identified *Eras*, *Irs4* and *Igf2* as significant 'true' as opposed to spurious MMTV CISs ($p = 6.58 \cdot 10^{-12}$, $1.13 \cdot 10^{-8}$ and $1.04 \cdot 10^{-4}$, respectively) (de Jong et al., 2014).

ROLES IN EMBRYONIC DEVELOPMENT AND STEM CELLS

Interestingly, *ERAS, IRS4* and *IGF2* are all mainly expressed during embryonic development and/or in embryonic stem cells (see Chapters 1-3). Moreover, their common main downstream signalling cascade, the PI3K/AKT/mTOR pathway, is linked to embryogenesis, stem cell maintenance and pluripotency (Armstrong et al.,

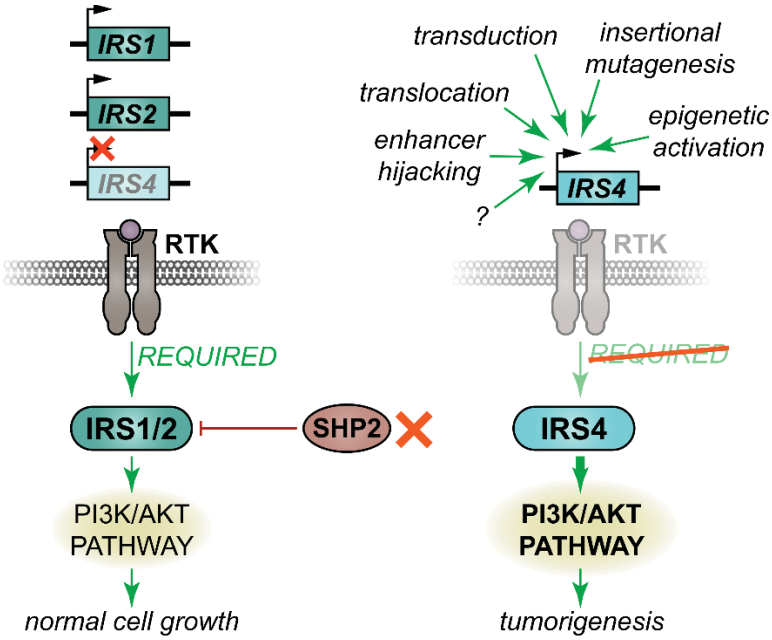

Figure 1 | Overview of insulin receptor substrate (IRS) signalling and regulation

Simplified IRS-induced PI3K/AKT/mTOR signalling cascade in normal cells expressing *IRS1* and *IRS2* (left) or cells expressing *IRS4* (right). In most normal cells, either *IRS1* or *IRS2*, or both *IRSs* are expressed, whereas *IRS4* is rarely expressed. IRS1 and IRS2 activity is kept in check by negative feedback via SHP2-mediated tyrosine dephosphorylation. In cancer cells, various mutagenic events may activate IRS4, which is irresponsive (X) to SHP2-mediated feedback and hyperactivates the PI3K/AKT/mTOR pathway leading to tumour growth. Green arrows indicate positive interactions (e.g. induction or stimulation). Red arrows indicate negative interactions (e.g. deactivation or inhibition).

2006; Dreesen and Brivanlou, 2007; Paling et al., 2004; Riley et al., 2005; Shoni et al., 2014; Storm et al., 2009; Takahashi et al., 2005; Watanabe et al., 2006; Yang et al., 2003). Stem cells and cancer cells in general share many characteristics, including rapid clonal proliferation, anchorage-independent growth, inhibition of differentiation, indefinite cell division by bypassing replicative senescence, but also comparable gene expression signatures and biomarkers, common transcriptional and epigenetic regulators and several shared signalling pathways (Dreesen and Brivanlou, 2007; Hadjimichael et al., 2015; Hanahan and Weinberg, 2011; Monk and Holding, 2001; Oren and Smith, 2017; Reya et al., 2001; Takahashi et al., 2005). These common features have also, in part, contributed to the concept of cancer stem

cells: rare cancer cells that drive tumorigenesis in a similar fashion as normal stem cells give rise to organs (Clevers, 2011; Jordan et al., 2006; Reya et al., 2001). Indeed, cancer progression generally results in a heterogeneous collection of cells comparable, albeit less structured, to that seen in normal tissues during development (Oren and Smith, 2017; Reya et al., 2001). *ERAS* expression has been correlated to side-population cells (Yashiro et al., 2009), which are generally thought to be enriched for cancer stem cells (Christgen et al., 2012; Richard et al., 2013). Furthermore, a murine model of induced pluripotent stem cells (iPS)-derived cancer stem cells was also found to express *Eras* (Chen et al., 2012). Indications of involvement of *IGF2* in cancer stem cells have recently also been reported (Tominaga et al., 2017; Zhao et al., 2016).

The theory of cancer stem cells has important clinical implications. These cells are thought to be refractory to most therapies, resulting in the reoccurrence of tumours driven by the small surviving subpopulation of such cancer stem cells after an initial successful treatment of the tumour bulk (Bütof et al., 2013; Cojoc et al., 2015; Colak and Medema, 2014; Eyler and Rich, 2008; Peitzsch et al., 2017). Effective treatment of the whole tumour would thus additionally require attacking the cancer stem cells, for which targeting the PI3K/AKT/mTOR pathway (among others) seems an appropriate strategy (Chang et al., 2013; Dubrovska et al., 2009; Francipane and Lagasse, 2016; Kolev et al., 2015; Potiron et al., 2013; Schöning et al., 2017; Sharma et al., 2015; Zhou et al., 2007). Considering all this, the link established in this thesis between *ERAS* and *IRS4* with therapy resistance via PI3K/AKT/mTOR pathway activation and their involvement in embryonic development, justifies further studies for a potential role of these genes in (putative) cancer stem cells.

TRANSCRIPTIONAL REGULATION OF ERAS, IRS4 AND IGF2

In adult tissues, *ERAS*, *IRS4* and *IGF2* are all strictly regulated on transcriptional level (see Chapter 1-3). Although transcriptional control of *IGF2* is thoroughly investigated, the mechanisms underlying the repression of *ERAS* and *IRS4* are unclear. Both genes are located on the X-chromosome, theoretically resulting most likely in monoallelic expression in males, but also in females due to X-chromosome

inactivation by lyonization. *IGF2* expression is silenced by genomic imprinting, similarly resulting in monoallelic silencing (Chapter 1). Both loss-of-imprinting as well as escape from X-inactivation are mechanisms known to predispose to and cause cancer (Balaton and Brown, 2016; Chaligné and Heard, 2014; Jelinic and Shaw, 2007; Lim and Maher, 2010). However, in most to all adult human and mouse tissues, including all normal stages of the breast, *ERAS* and *IRS4* are not expressed at all (Chapters 2 and 3; Giovannone et al., 2000; Kameda and Thomson, 2005; Takahashi et al., 2003), indicating that additional epigenetic repression is present. Therefore, it is remarkable that both *ERAS* and *IRS4* were found to be expressed (independently) in a relatively small but significant subset of human breast cancers (Chapters 2 and 3).

How *ERAS* expression is activated in these tumours is not known. Previous studies first identified human *ERAS* as a pseudogene and later to produce only a truncated non-coding transcript (Kameda and Thomson, 2005; Miyoshi et al., 1984; Zhan et al., 2005). Perhaps consequently, human *ERAS* and its transcriptional regulation has not received much further attention. Very recently, high *ERAS* expression was found in one case of primary colon cancer, where the *ERAS* gene had come under control of the highly expressed housekeeping gene *USP9X*. This was caused by a USP9X-ERAS gene fusion resulting from a highly local chromothripsis event on chromosome X (Kloosterman et al., 2017). It is possible that *ERAS* expression could be activated via a similar mechanism in breast cancer, including a translocation event, especially considering that genomic instability is a hallmark of most cancer cells.

Alternatively, earlier studies that reported *ERAS* mRNA expression in several human cancer cells, revealed some insights in its epigenetic activation that pointed to histone acetylation and promoter-localised DNA demethylation using inhibitors (Figure 2) (Yashiro et al., 2009; Yasuda et al., 2007). The involvement of epigenetic dysregulation in cancer is well-known, but predominantly entails the inverse: histone deacetylation and DNA methylation leading to the deactivation of tumour suppressors (Zahnow et al., 2016).

DNA methylation entails the addition of a methyl group to a cytosine (C) base in the DNA, generally preventing replication. In mammals, the methylated cytosine is always followed by a guanine (G), which is therefore known as a CpG dinucleotide

or simply CpG (Esteller, 2002; Herman and Baylin, 2003). Although CpG dinucleotides are overall quite rare in the genome, clusters of CpGs, known as CpG islands, are common in promoter regions. These CpG islands are predominantly unmethylated, but are known to be fully methylated by X-chromosome inactivation and genomic imprinting, leading to the stable transcriptional silencing of genes (Bird, 2002). Hypermethylation of such CpG islands, associated to the silencing of tumour suppressor genes, is also observed in many types of cancer (Baylin and Herman, 2000; Esteller, 2002).

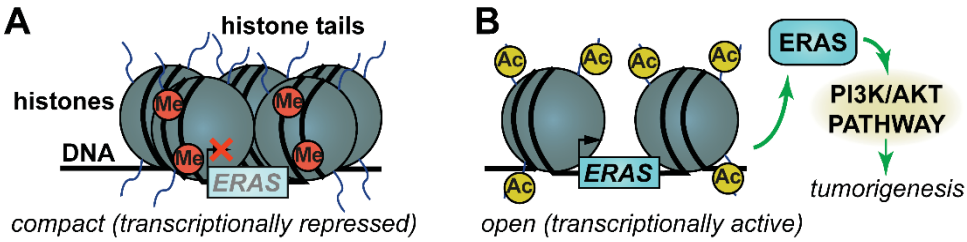

Figure 2 | Putative epigenetic regulation of *ERAS*

(**A**) Promoter-localised DNA methylation of CpG islands is likely to cause the transcriptional silencing of *ERAS*, which is reinforced by the absence of acetylation of histone tails, which keeps the chromatin in a condensed, transcription impermissible conformation.

(**B**) Histone acetylation and promoter-localised DNA demethylation may allow *ERAS* expression, thus leading to the production of the constitutively active ERAS protein.

Acetylation of the DNA packaging proteins, the histones, increases gene transcription by opening up condensed chromatin to a more accessible and transcription permissible conformation (Zentner and Henikoff, 2013). The levels of histone acetylation are controlled by the counteracting activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In many cancers, the balance between the activities of HATs and HDACs is disrupted due to various molecular aberrations, which can be germline as well as somatic and include mutations, amplifications and chromosomal translocations (Miremadi et al., 2007).

Histone deacetylation and DNA methylation are interconnected and are even thought to collaborate synergistically to establish gene silencing (Cameron et al., 1999; Grewal and Moazed, 2003; Meng et al., 2011; Zahnow et al., 2016). Both mechanisms of epigenetic regulation are extensively studied in relation to tumour

suppressor silencing, which is most prevalent in cancers (Zahnow et al., 2016). The epigenetic silencing of microRNAs (miRNAs) that inhibit translation or induce mRNA degradation of oncogenes, effectively activating these oncogenes, has also been reported (Lujambio et al., 2007; Saito and Jones, 2006). Additionally, (proto-)oncogene activation through these same epigenetic alterations has been described in the case of the maternally imprinted *IGF2* (see Chapter 1). *IGF2* is shown to become activated after hypermethylation of the insulator region between its promoter and a distant enhancer of the paternally imprinted *H19* gene (Bell and Felsenfeld, 2000; Moulton et al., 1994; Schoenherr et al., 2003; Steenman et al., 1994). Direct *IGF2* upregulation after promoter-specific demethylation has been found in cases of human hepatoblastoma (Li et al., 1998). Other cases of direct epigenetic activation of oncogenes have only rarely been reported (Akiyama et al., 2003; Cho et al., 2001; Nishigaki et al., 2005; Oshimo et al., 2003; Sato et al., 2003; Toyota et al., 2000). As activation of *ERAS* expression has been linked to histone acetylation and DNA demethylation, the gene may prove to be a new case of direct epigenetic upregulation of an oncogene, but this requires further investigation. This is important, as the clinical effectiveness of HDAC inhibitors (e.g. Panobinostat, Belinostat and suberoylanilide hydroxamic acid, SAHA, also known as Vorinostat) and inhibitors of DNA methylation (e.g. Azacytidine and Decitabine) could be compromised by the unintended epigenetic (re)activation of oncogenes. Histone modifications and DNA methylation status also have other profound therapeutic implications as the plasticity and reversibility of these epigenetic changes could explain acquired resistance in absence of obvious connections to genetic mutations (Brown et al., 2014; Wilting and Dannenberg, 2012). Our observations of ERAS-induced therapeutic resistance (Chapter 4) correspond with this concept.

The mechanism behind *IRS4* upregulation in the IRS4+ breast tumours has also not been elucidated, but in paediatric T-cell acute lymphoblastic leukaemia (T-ALL), strong *IRS4* upregulation has been reported due to chromosomal translocation, bringing the gene under the transcriptional control of T-cell receptor β regulatory elements (Karrman et al., 2009). A somewhat related mechanism was reported recently, revealing *IRS4* as a candidate pan-cancer gene that is activated due to "enhancer hijacking" in ten different tumour types, most prominently lung squamous

carcinomas and cervical squamous carcinomas (Weischenfeldt et al., 2016). Here, *cis*-regulatory elements such as enhancers were found to be rearranged and juxtaposed to *IRS4*. This resembles the activation of *Irs4* by MMTV proviral integrations in our insertional mutagenesis screens (Chapter 3; Klijn et al., 2013; Theodorou et al., 2007), where the proviral transcriptional enhancers interact with the *Irs4* promoter, upregulating the gene. Finally, epigenetic regulation of *IRS4* expression by DNA methylation and histone modifications should also be considered, especially in the light of acquired therapy resistance (Figure 1).

CLINICAL IMPLICATIONS: THERAPY RESISTANCE

HER2-targeting therapy using monoclonal antibodies Trastuzumab or Pertuzumab, or the tyrosine kinase inhibitor Lapatinib, greatly improves the prognosis of HER2+ breast cancer patients (Figure 3A). However, it is well recognised that a hyperactivated PI3K/AKT/mTOR pathway can induce resistance to various therapies in cancer (Brown and Toker, 2015). Both primary and secondary resistance are common in HER2+ breast cancer and are often associated with PI3K/AKT/mTOR

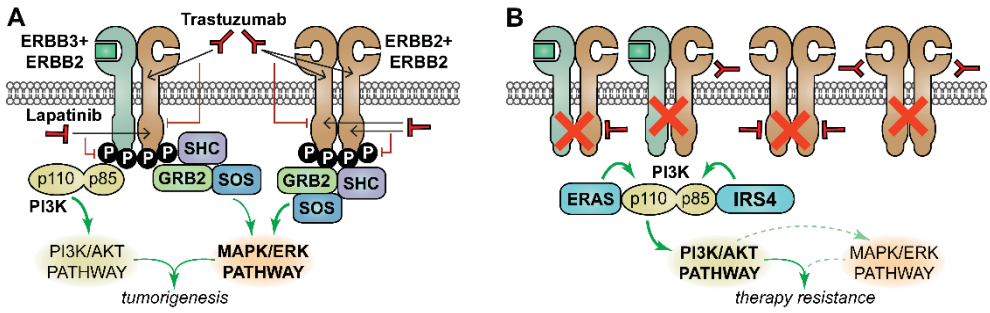

Figure 3 | ERBB2-ERBB3, ERAS and IRS4 signalling in malignant cells and therapy resistance

(**A**) Tumorigenesis requires continued stimulation of the PI3K/AKT/mTOR and RAF/MEK/ERK/MAPK pathways. The ERBB2-ERBB3 heterodimer may provide both these signals in a subset of tumours, where ERBB2 provides the RAF/MEK/ERK/MAPK signal and ERBB3 the PI3K/AKT/mTOR signal.

(**B**) Trastuzumab (humanised monoclonal antibodies against ERBB2) and lapatinib (a tyrosine kinase inhibitor inhibiting HER2 kinase activity) prevent the oncogenic signals of ERBB2 and ERBB3, but this is circumvented by ERAS/IRS4-induced hyperactivation of the PI3K/AKT/mTOR pathway, leading to therapy resistance. Red "X" indicates no activity or signalling. Thickness of the arrows indicates strength of signalling. Semi-transparency and dashed arrows indicate weak interactions.

pathway hyperactivation (Appert-Collin et al., 2015; Berns et al., 2007; Chandarlapaty et al., 2012; Cizkova et al., 2013; Eichhorn et al., 2008; Esteva et al., 2010; Hanker et al., 2013; Junttila et al., 2009; Loibl et al., 2016a, 2016b; Nagata et al., 2004; Park et al., 2014; Razis et al., 2011; Serra et al., 2008; Wang et al., 2011, 2013). Indeed, expression of *ERAS* or *IRS4* in various cell lines with *ERBB2* overexpression greatly reduced the sensitivity to HER2-directed therapeutic agents (Chapter 4). Moreover, ERAS as well as IRS4 synergistically accelerated tumorigenesis *in vitro* and *in vivo* when co-expressed with *ERBB2*, most likely due to the combined potent activation of the PI3K/AKT/mTOR pathway by ERAS and IRS4 with the RAF/MEK/ERK/MAPK pathway activation facilitated by ERBB2 (Figure 3B).

As mentioned in Chapter 1, ERBB2 has a strong binding preference for ERBB3 and in this heterodimer, it is ERBB3 that is mainly responsible for PI3K/AKT/mTOR pathway activation. As such, the ERBB2-ERBB3 heterodimer is oncogenic and even suggested to be essential for HER2+ breast cancer transformation (Holbro et al., 2003; Lee-Hoeflich et al., 2008; Pinkas-Kramarski et al., 1996; Siegel et al., 1999; Tzahar et al., 1996). However, in Chapter 2, 42% of the MMTV-induced tumours from MMTV-infected *ErbB2*-transgenic mice lacked *ErbB3* expression (whereas all tumours from non-infected mice expressed *ErbB3*), indicating that ERBB3 is not essential for oncogenesis in these tumours. *ErbB3* was furthermore found significantly less expressed in *Eras*-expressing tumours compared to ERAS-negative tumours in MMTV-infected *ErbB2*-transgenic mice and co-expression of *ERAS* and *ERBB3* was also not found in human breast cancer samples (Chapter 2). As ERAS and ERBB3 have the activation of PI3K/AKT/mTOR pathway in common, co-expression is indeed not expected. IRS4 is also a potent activator of the PI3K/AKT/mTOR pathway and can therefore also be expected to functionally replace ERBB3 as PI3K/AKT/mTOR pathway activator in HER2+ tumours, which would be worth investigating in the future. Replacement of ERBB3-driven activation of the PI3K/AKT/mTOR pathway by ERAS or IRS4 also fits well with the finding of ERAS- and IRS4-induced therapy resistance (Chapter 4), considering that absence of ERBB3 in HER2+ breast cancer cells and tumours has been reported to reduce sensitivity to HER2-targeted therapy (Holbro et al., 2003; Lane et al., 2000; Münster

et al., 2002; Yakes et al., 2002). As PI3K/AKT/mTOR pathway activity is essential in HER2 oncogenic signalling (Santa-Maria et al., 2016), the identification of other constitutive activators that circumvent the need for ERBB3 in activating this pathway, may reveal additional putative biomarkers for treatment options and resistance.

Evidence that both *ERAS* and *IRS4* expression may be involved in acquired resistance to HER2-targeted therapy is presented in Chapter 4. The expression of both genes could rapidly be attained in naive HER2+ breast cancer cell lines by culturing the cells for several passages in medium with increasing concentrations of Trastuzumab or Lapatinib, indicating selection for cells expressing these genes under the pressure of the drugs. Hence, Chapter 4 suggests that ERAS and IRS4 can cause both primary resistance to Trastuzumab or Lapatinib, as well as acquired resistance during treatment, and are therefore likely to play a role in relapse in breast cancer patients.

POTENTIAL TREATMENT OPTIONS

ALTERNATIVE HER2-TARGETING AGENTS

Although only Trastuzumab and Lapatinib were investigated in this thesis, treatment efficacy of other HER2-targeting drugs, and specifically Pertuzumab (monoclonal antibody inhibiting ERBB2-ERBB3 heterodimerization), is likely to be impeded by ERAS and IRS4 as well. Both ERAS and IRS4 activate the PI3K/AKT/mTOR pathway downstream of the ERBB2-ERBB3 heterodimer that Pertuzumab targets. Hence, the signalling and oncogenic effect of ERAS and IRS4 are not expected to be affected by this drug. Indeed, similar to Trastuzumab and Lapatinib, there is evidence that the effectiveness of Pertuzumab is hampered by high PI3K/AKT/mTOR pathway activity (Baselga et al., 2014; Bianchini et al., 2012; Majewski et al., 2015; Schneeweiss et al., 2014; Wuerkenbieke et al., 2015).

In contrast, another HER2-targeting therapeutic agent, the Trastuzumab-emtansine conjugate (T-DM1) may be a viable treatment option in ERAS and IRS4-positive cancers. Several studies in cancer cells, xenografts and human tumours suggest that anti-tumour and cytotoxic activity of T-DM1 is independent of PIK3CA mutation, PTEN status and ERBB3 expression (Baselga et al., 2016; Junttila et al., 2011; Kim et al., 2016; Krop et al., 2012; Nonagase et al., 2016). Although resistance to the effects of the Trastuzumab-component of the conjugate likely still exists (Junttila et al., 2011; Nonagase et al., 2016), the emtansine-component (DM1) is hypothesised to induce cell death due to mitotic arrest and apoptosis or due to mitotic catastrophe upon cytoplasmic accumulation (Barok et al., 2011; Lewis Phillips et al., 2008). The cytoplasmic concentration of the potently cytotoxic DM1 is presumed to be increased by its Trastuzumab-mediated delivery in T-DM1 though drug-bound ERBB2 internalisation and subsequent release upon lysosomal degradation (Erickson et al., 2006). This is unlikely to be affected by PI3K/AKT/mTOR pathway activity and may thus provide a therapeutic avenue in ERAS and IRS4 expressing HER2+ breast cancers.

HER2 AND PI3K/AKT/MTOR DUAL TREATMENT

The main clinically relevant conclusion of this thesis is that both *ERAS* and *IRS4* expression can be considered as biomarker for Trastuzumab and Lapatinib resistance, and by extrapolation also to other HER2-directed therapies. This would implicate that breast cancers expressing either of these genes should not be treated with HER2-targeted drugs, as this would only expose the patient to the toxicity of the drugs, without any expected treatment benefit.

However, considering that both ERAS and IRS4 induce this resistance through PI3K/AKT/mTOR pathway hyperactivation by acting directly on PI3K, combined treatment with a drug targeting ERBB2 and a drug targeting PI3K and/or its downstream effectors may be feasible. Currently, the only European Medicines Agency (EMA) and US Food and Drug Administration (FDA) approved PI3K/AKT/mTOR pathway inhibitor for breast cancer is the Rapamycin analogue (rapalogue) Everolimus (previously known as SDZ RAD and RAD001). Treatments with this drug in combination with HER2-targeted therapy has already been under investigation in the clinic.

In the BOLERO-1 trial, a phase-III randomised double-blind study in HER2+ advanced breast cancer, Everolimus versus placebo was tested in combination with Trastuzumab and Paclitaxel. Overall, no difference in progression-free survival was observed between the Everolimus and placebo arms (Hurvitz et al., 2015a). However, within the 43.3% of patients that were hormone receptor negative (HR-), a 7.2 month improvement in progression-free survival (PFS) was observed, although this was just outside the prespecified criteria for statistical significance (Hurvitz et al., 2015a).

The phase-III, randomised and double-blind trial BOLERO-3, tested Everolimus versus placebo, both combined with Trastuzumab plus Vinorelbine, in HER2+ Trastuzumab-resistant advanced breast cancer previously subjected to taxane treatment. Progression-free survival was slightly improved by Everolimus and, similar to the BOLERO-1 trial, mostly in HR- patients (André et al., 2014). Interestingly, patients with indicators for high PI3K/AKT/mTOR pathway activity showed more benefit with Everolimus than placebo. More specifically, both patients

with low PTEN levels and with high phosphorylated S6K levels both showed significant improvement with Everolimus and patients with *PIK3CA* mutations showed a trend towards a benefit from Everolimus.

Combined biomarker data from the BOLERO-1 and BOLERO-3 trials suggested that patients with tumours with a hyperactive PI3K/AKT/mTOR pathway (defined as: with known *PIK3CA*-activating mutations and/or low/no/mutated *PTEN* and/or *AKT1* E17K mutation) consistently derived significant benefit from Everolimus with regard to progression-free survival (André et al., 2016). This progression-free survival benefit with Everolimus was also observed for *PIK3CA* mutations or PTEN loss when these were analysed separately. In contrast, patients with wild-type *PIK3CA*, normal levels of PTEN or normal PI3K/AKT/mTOR pathway activity, did not benefit from Everolimus (André et al., 2016). Interestingly, hormone receptor status was not associated with benefit from Everolimus treatment in this combined dataset of BOLERO-1 and BOLERO-3 (André et al., 2016). Although pooling of two independent trials with dissimilar Everolimus doses and different chemotherapy regimens has clear limitations, the conclusions deserve further clinical investigation, specifically by preselecting patients that have tumours with high PI3K/AKT/mTOR pathway activity. This especially considering the encouraging results of preclinical studies with Everolimus (Hurvitz et al., 2015b; Lu et al., 2007; Mabuchi et al., 2007; Weigelt et al., 2011). Moreover, when taking into account the strong, sometimes even lethal, toxicity associated with Everolimus (André et al., 2014), further stratification of patients is a compelling aim to improve the, so far limited, clinical efficacy of the drug.

Everolimus specifically inhibits downstream signalling of the mTORC1 complex, similarly to rapamycin by binding FKBP1A (FK506 binding protein 1A; previously FKBP12) and subsequently disrupting mTOR from interacting with RPTOR (regulatory associated protein of MTOR complex 1) (Sedrani et al., 1998). Although RPTOR is not present in the mTORC2 complex, rapamycin and rapalogues may context-dependently and after prolonged treatment also partly inhibit mTORC2 signalling by sequestering free mTOR, which subsequently prevents mTORC2 complex assembly (Barlow et al., 2012; Macaskill et al., 2011; Sarbassov et al., 2006; Zeng et al., 2007). Still, inhibition of mTORC1 but not (or only indirectly and

limited) mTORC2 signalling by rapamycin and its analogues often results in the induction of AKT phosphorylation. This among others due to the release of negative feedback loops on IRSs, primarily the phosphorylation of serine residues of IRS1 (Chapter 1; Yoon, 2017), and mTORC2, which is observed with rapamycin and its analogues (Cloughesy et al., 2008; Dibble et al., 2009; Harrington et al., 2004; Julien et al., 2010; O'Reilly et al., 2006; Shi et al., 2005; Sun et al., 2005; Tabernero et al., 2008). That could explain why the therapeutic success of rapamycin and analogues, including Everolimus, has so far been modest (Xie et al., 2016).

Inhibitors of the PI3K/AKT/mTOR pathway void of this feedback loop issue have been developed and include PI3K inhibitors (p110 catalytic subunit isoform-specific or pan-PI3K), catalytic and allosteric AKT inhibitors, catalytic mTORC1/2 dual inhibitors, and dual PI3K/mTOR inhibitors. Some of these drugs have already shown promising results in preclinical studies in HER2+ breast cancer models (Berns et al., 2007; Eichhorn et al., 2008; Hanker et al., 2013; Junttila et al., 2009; Miller et al., 2009; Nagata et al., 2004; Serra et al., 2008), including the results presented in Chapter 4. Clinical trials with numerous of these inhibitors, also in combination with Trastuzumab, Lapatinib, Pertuzumab and/or T-DM1, are currently pending approval, are ongoing or have even been completed and are in data analysis (Dey et al., 2017; Guerrero-Zotano et al., 2016; Luque-Cabal et al., 2016).

There is some evidence that treatment with PI3K/AKT/mTOR inhibitors, results in a compensatory upregulation of ERBB signalling (Chakrabarty et al., 2012; Serra et al., 2011). Targeting ERBB2/ERBB3 and PI3K/AKT/mTOR signalling simultaneously may therefore have a combined, enhanced effect. Indeed, the results in Chapters 2 and 4 suggest a synergy between ERBB2 and PI3K inhibition, in agreement with previous reports (Chakrabarty et al., 2012; Crafter et al., 2015). As early clinical studies suggest that PI3K, AKT and mTOR inhibitors can have severe adverse events (Dey et al., 2017; Guerrero-Zotano et al., 2016; Harbeck et al., 2016), such a therapeutic synergism between these and HER2-targeted drugs could offer a welcome reduction of effective dose. Still, a substantial treatment benefit will be required for these inhibitors to be incorporated into clinical practice.

IMPROVING DIAGNOSIS FOR PERSONALISED TREATMENT

BIOPSIES

In the human disease, therapy resistance may be a consequence of intratumour heterogeneity, with effectively a selection for resistant clones. However, treatment decisions are commonly based on a single sample of the primary tumour, e.g. a core needle biopsy in breast cancer. Such single biopsies do not take into account the heterogeneity within various locations of a tumour mass and between the primary bulk and in metastases (spatial heterogeneity), nor changes over time (temporal heterogeneity), e.g. due to selective pressure during treatment (Gerlinger et al., 2012; Ryška, 2016; Yates et al., 2015). Repeated multiregional biopsies before, during and after treatment, could provide highly valuable information for both clinicians and researchers. Unfortunately, such biopsies are rarely available, if done at all, which was certainly a major challenge for the research presented in this thesis. Indeed, intratumour heterogeneity can have major clinical implications. For example, it has been reported that there is a discordance in HER2-status between core needle biopsies and resection samples of the primary tumour, which in the majority of cases would have led to a change in treatment choice (Striebel et al., 2008; Wu et al., 2010). The same has been described for discordances in HER2-status between primary tumour and metastases, which also would have led to an alternative treatment decision in some cases (Amir et al., 2012; Niikura et al., 2012; Wilking et al., 2011). Hence, repeated multiregional biopsies could provide more comprehensive clinical information essential for optimal management of the disease. For the researcher, more comprehensive biopsies may provide essential data to model tumour evolution, possibly enabling the prediction of common paths that tumours take to escape therapy (Greaves, 2015; Lipinski et al., 2016), but it will also provide material to validate newly identified biomarkers.

Repeated multiregional biopsies may have clear benefits, but it also carries practical issues and a burden to the patients. Much less invasive and more practical are 'liquid biopsies': the analysis on circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA; more broadly: cell-free DNA, cfDNA) in blood samples (Alix-Panabières and

Pantel, 2013; Crowley et al., 2013; Diaz and Bardelli, 2014). However, both techniques are still under development and have technical challenges, and importantly with ctDNA/cfDNA, any non-genetic information is not available. Still, CTCs and ctDNA could provide a practical avenue to follow tumour evolution and heterogeneity in real-time before, during and after treatment, knowledge which is currently sorely lacking.

BIOMARKERS

To guide personalised treatment decisions for breast cancer the need for associated biomarkers is high. Unfortunately, despite much effort, ER and HER2 status are still the only validated predictive markers in breast cancer. Although preclinical data strongly links hyperactivation of the PI3K/AKT/mTOR pathway to HER2-targetted therapy resistance, data from the clinic have not consistently supported this link, in particular for *PIK3CA*-activating mutations (Barbareschi et al., 2012; Dave et al., 2011; Esteva et al., 2010; Gianni et al., 2012; Loi et al., 2013; Pogue-Geile et al., 2015; Razis et al., 2011). This may be due to the use of different assays and analyses (Wang et al., 2013), but could also be explained by the limitations of looking only at genetic aberrations. Indeed, *PIK3CA*-activating mutations do not always lead to a concomitant activation of the pathway, based on the more accurate readout of phosphorylation status of key members of the pathway (Stemke-Hale et al., 2008; The Cancer Genome Atlas Network, 2012). The molecular and cellular effects of genetic alterations can thus be context-dependent. Moreover, the majority of mutations found in the exome of tumours may not even be expressed, considering that (in triple-negative breast cancers) only a mere 36% of genomic mutations found in the exome were observed in the transcriptome as well (Shah et al., 2012). Also, only a handful of cancer genes are commonly mutated and these explain only a part of all breast cancer cases, whereas a myriad of infrequently mutated genes causes or contributes to the substantial remainder of cases (Stephens et al., 2012).

Hence, it makes sense to consider other, more comprehensive, tools that allow for looking beyond merely DNA sequences, but also take into account the effects of gene expression levels and post-translational modifications, including activation of

signalling pathway. Analysing PI3K/AKT/mTOR pathway activation directly, e.g. though the phosphorylation status of AKT, S6 and 4EBP1, may have the additional benefit of evaluating the common signalling effect of multiple genetic and epigenetic aberrations, like mutations and genomic rearrangements, altered RNA or protein degradation, changes in histone acetylation and DNA demethylation, etc., without the need of analysing all these separately.

Assessing the pathway activation status of tumours is also useful since some contributing genetic events may be very infrequent and could therefore be missed during analysis of genomic data. For example, mutations in the gene encoding the PI3K regulatory subunit p85, *PIK3R1*, are detected in only 4% of HER2+ breast cancers and even less in other subtypes (The Cancer Genome Atlas Network, 2012). Conceivably therefore, *PIK3R1* has only recently been identified as a significantly mutated gene in breast cancer, which was in the comprehensive analysis of the Cancer Genome Atlas Network. Both *ERAS* and *IRS4* expression were also not very frequently observed in breast cancers (Chapters 2 and 3) and were never implicated in breast cancer before.

Advancing research methods, technological developments and bioinformatics are likely to improve the detection of increasingly rare oncogenic drivers, but this may pose problems for the clinical validation of such rare biomarkers. If only a very low percentage of tumours e.g. carry a specific oncogenic mutation or, in the case of this thesis, express a certain oncogene, it is virtually impossible to find a clinical dataset large enough to perform any survival analysis with sufficient samples in the test group. Hence, the requirement of Kaplan-Meier plots for oncogenic validation on gene-level may not be feasible.

Similarly, clinical trials investigating treatment of infrequent oncogenic drivers would require the screening of an enormous number of patients before a meaningful population can be enrolled. It is because of this that innovative clinical trial designs, such as basket and umbrella trials, are currently being developed to cope with rare genetic alterations. Basket trials test the effectiveness of certain therapies in a pool of patients with the same genetic alteration, but which can comprise completely different tumour types. In contrast, umbrella trials are designed to guide patients of one tumour type, but with diverse genetic alterations, towards a matching (putative)

treatment. Here, assessing oncogenic pathway activation to determine treatment options may provide an additional means to address the clinical implications of infrequent oncogenic drivers and to prevent too rigid stratification based only one or a few genetic aberrations. In the case of HER2+ breast cancer, this would mean pooling all patients with tumours having PI3K/AKT/mTOR pathway hyperactivation, due to any sort of driver, and administering HER2-targeting agents combined with PI3K, AKT and/or mTOR inhibitors, when these become available and are confirmed to be clinically effective.

FURTHER CONSIDERATIONS

In Chapters 2 and 3 of this thesis, *Eras* and *Irs4*, respectively, are validated as oncogenic drivers, both in the murine *in vivo* setting and *in vitro* in human breast (cancer) cell lines. Moreover, both genes are shown to be expressed in the human disease, whereas they are normally silent in the normal adult breast. The evidence in this human material (both human cell lines and primary carcinomas) is important, as there is not always a strong overlap between mice and humans on cellular and molecular level, even if the genetics are very similar. For example, the human orthologue of the first gene identified by MMTV insertional mutagenesis, *WNT1*, is not found overexpressed human breast carcinomas, probably due to redundancy in the *WNT* family and other molecules that equally result in WNT pathway activation (Brown, 2001). As hyperactivity of the WNT pathway is associated with human breast cancer (Howe and Brown, 2004), this is exemplary for the notion that the tumorigenic effect of oncogenes and tumour suppressors may differ between mice and humans, but oncogenic pathways are often shared. Moreover, looking at pathway activity can provide a broader 'biomarker' with a more direct insight in possible therapeutic avenues. Subsequently, following basket trials, resulting therapies are more likely to be effective on many patients' tumours that share the same oncogenic pathway, but have distinct underlying oncogenic drivers. This is especially important as some of these drivers may be very rare and thus unfeasible to target individually. Chapters 2 and 3 show that the frequency of *ERAS* and *IRS4* expression in human breast cancers is quite limited, so this is likely to apply to these oncogenes as well. All this is important to take aboard for any screen that seeks to translate results from the murine setting to the clinic. ERAS, IRS4 and IGF2 seem to be oncogenic drivers in both mice and humans, but more important is the identical molecular mechanism of action in mice and humans: the activation of the PI3K/AKT/mTOR signalling cascade, which is highly relevant in (both murine and human) cancer.

Still, the most direct confirmatory model for a causal relationship between ERAS or IRS4 and human tumorigenesis, their synergism with ERBB2 and induction of therapy resistance, would be the use of patient-derived xenografts (PDX) models. But although establishment of a PDX model is already a technical feat, considering

the limited frequency of tumours presenting both *ERBB2* amplification/overexpression and *ERAS* or *IRS4* expression, it would be practically challenging to establish ERBB2+ERAS or ERBB2+IRS4 PDX models as well. Indeed, none of the existing models express one of these combinations, to our knowledge. Alternatively, assessing if there is an enrichment for *ERAS* or *IRS4* expression in therapy resistant tumours (i.e. tumour samples obtained after failed treatment instead of the original tumour biopsy of refractory patients) would be useful and provide a strong confirmation to use the expression of these genes as a clinical biomarker. The *ex vivo* culturing of primary tumour cells may be very useful in the context of a direct prediction of therapeutic response for an individual patient and can take into account the influence of the tumour microenvironment (Corben et al., 2014; Majumder et al., 2015), but is still in early development and has similar logistical challenges to PDX models.

Additional directions for further research that arise from this thesis are the role of these genes in putative cancer stem cells and the exact mechanism behind their expression activation in human tumours. Importantly, the assessment of viable therapeutic options for patients with HER2+ breast cancer that expresses *ERAS* or *IRS4* warrants investigation. In particular the clinical development of PI3K, AKT and/or mTOR inhibitors requires continued effort to hopefully fulfil their prospect to overcome HER2-targetting treatment resistance. This especially when the tumours exhibit PI3K/AKT/mTOR pathway hyperactivation due to *PIK3CA* mutations, *PTEN* loss, *AKT1* amplification, *ERAS* or *IRS4* expression or any other means.

Finally, this thesis has shown that MMTV-induced insertional mutagenesis is still a highly valuable tool for oncogene discovery and to study heterogeneity in breast cancer. Also because mammary tumours in mice resemble human breast tumours well (Dimri et al., 2005). This may inspire future screens employing MMTV again, which may especially be useful in murine models for specific molecular subtypes, potentially combined with the selective pressure of a drug used in the clinic to screen for drivers of resistance. The knowledge derived from such screens, in combination with continuing advances in the screening of clinical material, will undoubtedly contribute further to the odyssey towards personalised medicine.

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