

GENES INVOLVED IN BREAST CANCER PROGRESSION AND METASTASIS

Cancer is the result of sequential accumulation of multiple genetic changes affecting oncogenes and tumor suppressor genes in somatic cells. Despite recent advances in understanding of the molecular basis of oncogenic transformation only a limited number of key players involved have been identified and extensively studied, while this is essential for the development of more effective novel therapeutic strategies. The aim of our laboratory is to identify genes involved in breast cancer by using mouse mammary tumor virus (MMTV) induced insertional mutagenesis in mouse models and characterize these genes and the oncogenic pathways they act in.

Identification of mammary cancer genes in mouse models for breast cancer by MMTV insertional mutagenesis screens MMTV proviral insertion in the genome of murine mammary epithelial cells can activate flanking proto-oncogenes leading to mammary tumor induction. In recent years, we identified approximately 50 common insertion sites (CISs) in mammary tumors in a series of approximately 400 tumors from wild-type Balb/c mice, Balb/c mice conditionally deficient for Tp53 in the mammary gland and MMTV-NeuNT transgenic mice, using the splinkerette PCR and conventional capillary sequencing. Many of the CISs affect genes not previously implicated in cancer or breast cancer in particular. Together with the Jonkers lab, most of the collected MMTV induced mammary tumors were reanalyzed using the new parallel sequencing platforms. Preliminary results indeed show additional proviral insertion sites and some novel CISs.

The proviral insertion mutagenesis screen performed to date, revealed that Wnt and Fgf genes were activated in almost all wild type BALB/c tumors, less frequently in Tp53 deficient tumors and rarely in ErbB2 overexpressing tumors. These results suggest that the initial oncogenic event determines the subsequent oncogenic changes. Two genes belonging to the R-spondin gene family, Rspo2 and Rspo3, and members of the Odz gene family were also frequently activated in tumors from wild type and conditionally deficient Tp53 mice but rarely or not activated in tumors from ErbB2 transgenic mice. In contrast, other genes like Eras were found to be more frequently tagged in tumors from MMTV infected ErbB2 transgenic mice than in wild type mice.

Analysis of genes collaborating with ErbB2. We have identified Eras, Irs4 and Igf2 as frequent MMTV target genes in mammary tumors from ErbB2 TG mice. Overexpression of Eras was found to be most significantly associated with tumors from MMTV infected ErbB2 transgenic mice compared with tumors from MMTV infected wild-type FVB mice. Overexpression of Eras and Irs4 in immortalized normal mouse mammary epithelial cells (NMuMG cells) strongly increased the growth rate in vitro, anchorage independent growth and rendered these cells tumorigenic in nude mice, validating the mammary tumor inducing capacity of these genes. Moreover, both genes strongly accelerated ErbB2 induced tumorigenesis when co-expressed in NMuMG cells and strongly activated the PI₃-kinase pathway. It has been shown that ERBB2 preferentially forms a heterodimer with ERBB3. In this complex, ERBB2 mainly activates the MAPK pathway, while ERBB3 activates the PI₃K pathway. Since ERBB2 driven mammary tumorigenesis is highly dependent on PI₃-kinase activity, we hypothesize that genes like Eras and Irs4 are targeted by MMTV in ErbB2 tumors that lack ERBB3 mediated PI₃K activation (see model in figure 5). Indeed, ErbB3 is highly expressed in all tumors that arise in non-MMTV-infected ErbB2 transgenic mice but only in 57% of the MMTV infected ErbB2 transgenic mice. Thus activation of the PI₃-pathway via ERAS can act as an alternative for the activation of this pathway through ERBB3, PTEN inactivation or PIK3CA mutations. Although Eras is highly homologous to H-Ras and K-Ras, the latter Ras oncogenes do not synergize with ErbB2. Eras mRNA was also expressed to a variable degree in a small percentage of human breast cancers suggesting a possible involvement of this normally silent gene in human breast carcinogenesis.



Group leader John Hilkens

John Hilkens PhD Group leader
Gerjon Ikink MSc PhD student
Mandy Boer Technical staff

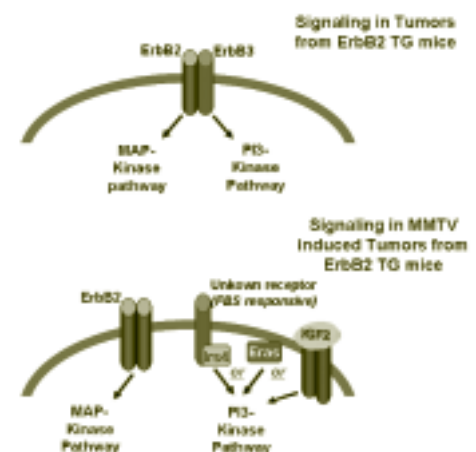


Figure 5: Model of PI₃K signaling pathways in MMTV induced mammary tumors from ErbB2 transgenic mice.



Group leader Jacqueline Jacobs

Jacqueline Jacobs PhD Group leader

Paul-André Genest PhD Post-doc

Marieke Peuscher MSc PhD student

Jaco Van der Torre MSc PhD student

Janet Van Noord Technical staff

CHROMOSOME END PROTECTION BY TELOMERES

Telomeres are protein-DNA complexes that protect natural chromosome ends from being treated as damaged DNA. Telomeres progressively shorten with every cell division until they become too short to function properly. The subsequent recognition of chromosome ends as broken DNA has important consequences for cellular and organismal life span but also for tumor development, and telomere maintenance is therefore target of several recently developed anti-cancer strategies. Our main aim is to increase our understanding of how mammalian cells precisely perceive and respond to loss of telomere function, how telomere maintenance is controlled and how factors involved in the response to telomere dysfunction affect cellular transformation and tumor development.

Telomere-induced cellular senescence Loss of telomere function triggers a DNA damage-like response that causes cells to die or stop proliferating indefinitely (senescence). This response limits the replicative life span of cells and thereby contributes to organismal aging. In addition, it represents an important tumor suppressor mechanism as it prevents unlimited outgrowth of potentially cancerous cells. To investigate the consequences of loss of telomere protection we have performed micro-array analysis of the gene expression changes induced by loss of telomere function upon TRF2 inhibition. Next to genes involved in cancer, cell death and cell cycle, genes involved in inflammatory/immune responses represented gene groups with the most significant changes in expression upon telomere dysfunction. We have subsequently focused on common upstream transcriptional regulators of these genes and have investigated whether these regulators respond directly to telomere dysfunction and contribute to telomere-induced senescence.

As an alternative unbiased approach to identify novel factors involved in DNA damage and/or telomere damage response activation, we performed an siRNA screen to find factors that are involved in ATM activation following DNA damage. Out of 254 different chromatin remodelers, modifiers or DNA helicases, we found 26 genes that upon knockdown led to reduced activation of ATM in a primary screen. We are in the process of validating and characterizing several of these genes.

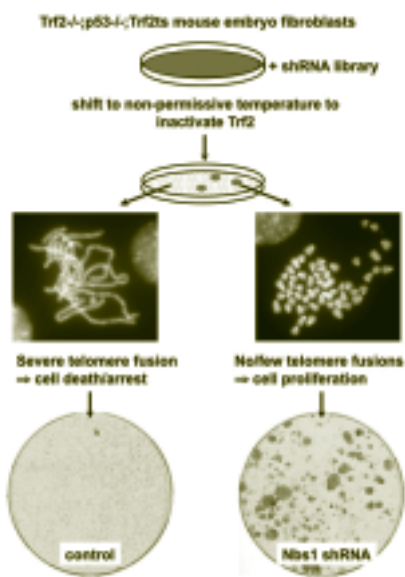


Figure 6: Loss-of-function genetic screening strategy aimed at identifying genes that contribute to telomere-induced genome instability. Mouse embryo fibroblasts with genetic disruption of the telomere component Trf2, but expressing a temperature sensitive Trf2 allele, lose telomere protection on all their chromosome ends when cultured at a non-permissive temperature that inactivates Trf2. This results in severe fusion of chromosome ends and cells are unable to divide or die. However, inhibition of Nbs1 by a shRNA isolated in this screen, allows cells to survive and divide in the presence of uncapped telomeres.

Telomere maintenance and telomere-induced chromosome instability

If cells with uncapped telomeres fail to senesce or die and continue proliferating in the absence of a mechanism that replenishes telomeric repeats, DNA repair activities acting on chromosome ends cause chromosome fusions, anaphase bridges and nonreciprocal translocations. Such cells are at high risk of developing into cancer cells. Although telomere dysfunction is thought to be a major mechanism underlying chromosomal instability in human cancers, little is known about the precise structure of an uncapped telomere, how it is recognized, what precise processing events occur and how these events are controlled. To identify novel factors that contribute to telomere-induced genome instability we have developed an RNAi loss-of-function genetic screen in mouse cells in which we can instantly and reversibly uncap telomeres (see figure 6). The degree of telomere fusion induced in this system is so severe that cells stop proliferating or die. In this screen we have obtained multiple cell clones that survived severe telomere uncapping. The isolation and characterization of shRNA vectors is ongoing, but has already led to the discovery of several genes whose inhibition by RNAi is able to confer resistance to lethal telomere-induced genome instability. One of these is Nbs1, a component of the MRN complex which has recently also been shown by others to indeed contribute to the processing of uncapped telomeres by non-homologous end-joining (see figure), and which validates our screen. The other genes identified so far have not previously been implicated in the response to uncapped telomeres. They include genes that encode for proteins involved in protein ubiquitination and methylation. Their mechanism of action in the telomere damage response is now being investigated, as well as their potential roles in the response to DNA double-strand breaks and in cellular transformation and tumor formation.

THE DEVELOPMENTAL ROLES OF ONCOGENES AND TUMOR SUPPRESSORS IN ZEBRAFISH

Our research focuses on understanding how vertebrates sense and respond to energetic stress, using zebrafish as our central experimental model. We aim to provide insights into how tumors adapt their metabolism to survive, proliferate and grow under nutrient and oxygen-poor conditions. We are following two main lines of research:

Analysis of energy metabolism and hypoxia during development and cancer

During development of an organism, mechanisms that sense nutrient availability are intimately linked with growth control pathways in order to coordinate energy conditions with organ growth and tissue homeostasis. Tumor cells employ several of the same mechanisms that coordinate nutrient availability with growth during development to ensure tumor cell proliferation. To improve cancer therapy, we need a better understanding of the molecular pathways that link energy metabolism with growth control during development and how are those affected in cancer.

Zebrafish (*Danio rerio*) is an ideal system to dissect molecular mechanisms and pathways, largely owing to the rapid development and optical clarity of the embryos. Furthermore, the large progeny size (females typically lay 200 eggs at a time), and availability of readily accessible transparent embryos combine to render the zebrafish an excellent model for high-throughput genetic and chemical screens in the intact organism. The major metabolic pathways are very well conserved between humans and zebrafish.

Mutations in the serine-threonine kinase LKB1 in humans lead to a gastrointestinal polyposis disorder with highly increased predisposition to cancer. Recently, somatic mutations in LKB1 have been found in about 30% of lung carcinomas as well as in endometrial cancer. LKB1 activates AMP-activated kinase (AMPK) the “energy switch” of the cell and that leads to growth suppression through several pathways including inhibition of the mTOR pathway. Since mouse models of *lkb1* deficiency are embryonic lethal, the role of energy metabolism during development has not been explored.

To study control of metabolism during development, we generated and characterized *lkb1*-deficient zebrafish. Importantly, the zebrafish *lkb1* mutants are embryonic viable -unlike their mouse counterparts- and exhibit deregulated metabolism.

We demonstrated that zebrafish LKB1 is required for AMPK activation and found that *lkb1* mutants are unable to sense and respond to energetic stress. *Lkb1* mutant zebrafish display a fast metabolic rate and exhaust their energy reserves prematurely. They exhibit hallmarks of premature starvation such as abnormal lipid accumulation in the liver (figure 7). We showed that attenuation of metabolic rate in *lkb1* mutants, by application of the TOR inhibitor rapamycin, suppresses key aspects of the *lkb1* phenotype.

We are currently exploring the connection between LKB1, energy metabolism and hypoxia. This investigation will lead to a better understanding of the adaptation of metabolic processes in order to meet energy demand that tumor cells employ in order to cope with decreasing nutrient and oxygen supply within the tumor. We aim to gain insight into these processes at the organism level during normal development and during cancer formation.

Analysis of the developmental roles of the Polycomb group proteins (collaboration with Maarten van Lohuizen)

Polycomb group (PcG) protein complexes, which function in the epigenetic regulation of gene expression, control numerous developmental processes. Epigenetic silencing mediated by PcG is implicated in stem-cell fate maintenance and cancer. The PcG member *Ring1b* is an E3 ubiquitin ligase that ubiquitinates histone H2A. This mark correlates with the repression of gene expression of PcG target genes. Targeted inactivation of *Ring1b* in mice leads to very early lethality precluding analysis of the role of *Ring1b* in embryonic development. We have recently succeeded in generating a stable mutant in *Ring1b* by using Zinc Finger Endonuclease technology. We have established that *Ring1b* protein levels and ubiquitination of H2A are dramatically reduced in the mutants. *Ring1b* mutant embryos lack fins and all jaw cartilage elements. We are currently characterizing the mechanism underlying these specific defects.



Group leader Anna-Pavlina Haramis

Anna-Pavlina Haramis PhD Group Leader

Yme van der Velden MSc PhD student

Liqin (Bruce) Wang Research assistant

Publication

Brennan C, Dosch R, Haramis AP, Luckenbach T, Martinez-Morales JR, Moro E, Polok B, Ramesh TM, Russell C, Argenton F, Strähle U. Report of the European zebrafish principal investigator meeting in Padua, Italy, March 18-22 2010. *Zebrafish*. 2010;7:305-10

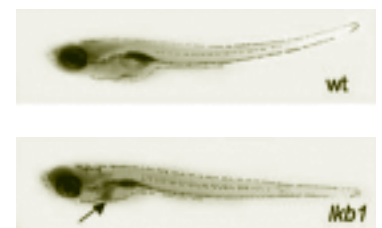


Figure 7: Zebrafish larvae at day 7 post-fertilization stained with Oil Red O, a red dye that binds lipids. No lipids are detected in wt larvae; abnormal lipid accumulation in the liver of *lkb1* mutants (arrow).