Chapter 6

General discussion

Since the early 1900s, *in vitro* cultures of animal cells have been extensively used for biological research. Studies using cell lines have elucidated many molecular mechanisms underlying processes such as cell growth and proliferation, adhesion, motility, apoptosis and metabolism. These studies have had significant impact in the biomedical field as cell cultures can be used to study tissue growth and development, ageing, genetic instability, immunity and cancer biology. In addition, large-scale cell cultures are also important for the manufacture of many products in biotechnology, including viral vaccines, enzymes, synthetic hormones, antibodies and anti-cancer agents. *In vitro* cultured cells can be divided in several categories. First of all they include immortalized cell cultures. These have either been derived from various tissues by transformation with viral genetic information or, alternatively, from cancer samples obtained from patients or test animals. Secondly, use has been made of primary cultures of tissues that have the capacity to differentiate, the so-called stem cells. Of this category currently most attention is focused on the study of omnipotent stem cells derived from embryonic tissues, i.e. cells that can still differentiate into every possible tissue. Cells of each of these two categories have in common that they will change their genetic and epigenetic make-up during culturing. In this respect, a comparison with original tissues isolated directly from test organisms is of great importance. Since in medical studies such comparisons are very difficult or very often impossible due to lack of material or ethical constraints, such comparisons mostly rely on studies in animal models. As the zebrafish, *Danio rerio*, has been increasingly used as an animal model for the study of development and diseases, there are increasing possibilities for the use of *in vitro* zebrafish cell models in such comparisons. The experiments described in this thesis aimed at developing zebrafish cell models for cancer and inflammation studies (Figure 1).

To this goal, the growth properties of the three zebrafish cell lines, ZF4, PAC2 and ZFL were characterized. All of these adherent cell lines can be maintained at 28 degree with atmospheric CO_2 for at least 25 passages using conventional media and serums. Under these culture conditions, embryonic ZF4 cells show typical fibroblast morphology and embryonic PAC2 cells look 'fibroblast-like'. The ZFL cells, derived from adult liver, have typical epithelial morphology. In general, the doubling time of these cell lines (approximately 72 hours) are longer than many mammalian cell cultures such as HEK93. Transfection in these cell lines can be achieved either by lipid-mediated transfection using Fugene and Lipofectamine or by nucleofection, resulting in variable transfection efficiencies (ZFL>ZF4>PAC2). It was found that the membrane structure of PAC2 cells looked different than the other two cell lines in electron microscopy (unpublished data), which might be the reason of the low delivery efficiency of foreign constructs. In order to develop more efficient methods of genetic manipulation, virus transduction, such as adenovirus transduction or lentivirus transduction, could be applied in these cell lines in future research.
Stable transfection can be achieved in all three cell lines by antibiotic selection such as neomycin (G418) and hygromycin (Figure 1B). However, these cell lines showed clear difference in generation of clonal stable cell lines. For this purpose the PAC2 cell line has been most successful, as colonies could be derived efficiently from single cells. The growth of the ZF4 cell line is more dependent on cell density than PAC2 cells and the growth of the ZFL cell line is highly

dependent on cell density. During stable transfection, the ZFL cells have to be rescued by conditional medium (Chapter 4).
In addition to over-expression of foreign gene constructs, expression of genes of interest can also be knocked down by the morpholino treatment. Morpholinos are synthetic molecules of 25-base length which bind to complementary sequences of RNA molecules and thereby block translation into protein. Morpholinos can be efficiently introduced into zebrafish cell lines using the Endo-Porter peptide delivery system or by nucleofection (unpublished data).

A microarray-based transcriptomics approach was used to further characterize these zebrafish cell lines as potential *in vitro* models. We found that the embryonic derived ZF4 and PAC2 cell lines have significant transcriptome overlap with 24 hour zebrafish embryos (85% and 87.4%, respectively, Chapter 2). The ZFL cell line, derived from adult zebrafish liver, largely resembles tissue of healthy zebrafish liver at the transcriptome level (Chapter 4). These findings highlight the possibility to systematically analyze specific signaling events *in vitro* using these cell lines and subsequently validate the findings *in vivo* using zebrafish. In addition, annotation of the serum-responsive transcriptional gene signatures of zebrafish fibroblast cell lines revealed a number of genes involved in wound-healing related programs, similar to human fibroblast serum-responses. The conservation of serum responses between zebrafish and human cell lines serves as an example that the zebrafish cell lines can be used to model essential cellular processes which are evolutionarily conserved.

The Toll-like receptor (TLR) family is one of the most conserved components of the vertebrate immune system [1]. As a family of pathogen recognition receptors, TLRs and their downstream signaling molecules are involved in innate immunity and inflammation responses. In Chapter 3 we studied the TLR signaling in zebrafish cell lines. Many of the TLRs are expressed in the cell lines mentioned above, although the subcellular localization of the endogenous receptors was not characterized yet, due to the lack of suitable antibodies. In addition to the receptors, also other key molecules of the TLR signaling pathway are expressed in the ZF4, PAC2 and ZFL cell lines; these include the TLR adaptors MyD88, Trif and Mal and transcription factors of the NFκB family (Chapter 3). Stimulation of the three cell lines with bacterial flagellin activated the NFκB pathway and induced distinct transcriptional programs. Previous study by Stockhammer *et al.* showed that zebrafish TLR5a and TLR5b are the receptors that recognize bacterial flagellin in zebrafish embryos [2]. Knocking down of essential genes involved in the TLR5 pathway (such as TLR5a, TLR5b and the adapter MyD88) by the morpholino approach, will help to further understand the specificity of flagellin recognition and the downstream inflammation responses in the zebrafish cell lines.
Except flagellin, stimulation with many other typical TLR ligands failed to induce detectable responses in these zebrafish cell lines. It is known that in mammals the recognition of flagellin is mainly carried out by TLR5 expressed on non-professional antigen presenting cells (APCs) in the intestine [3]. Taken together the non-hematopoietic origin of the three tested zebrafish cell lines, our results indicate that these cells exhibit typical non-professional APCs properties. Therefore these cell lines can be valuable models to understand the biological relevance of TLRs in non-professional APCs, which remains unclear in mammalian models. For more immunological studies in the zebrafish model, cell lines with hematopoietic origins will be required.

Inflammation has been strongly associated with cancer as it plays important roles in tumor initiation and progression [4]. It is known that chronic inflammation is often correlated with increased risk of cancer. Release of cytokines and chemokines, usually initiated by non-professional APCs, and subsequent attraction of inflammatory cells can eventually induce cell transformation and support survival and proliferation of the transformed cells. However, the precise mechanisms underlying these processes remain unclear. Owing to its well characterized innate immune system and transparency, the zebrafish is an ideal vertebrate model system to study cancer-related inflammation and tumor progression. To further study cancer related signaling events using zebrafish cells, we constructed zebrafish cell lines with stable over-expression of particular oncogenes as described in Chapter 3. The tamoxifen/ER inducible system was chosen to conditionally turn on the selected oncogenes. Several oncogenes have been tested in zebrafish cell lines. For instance, cMyc-ER was stably expressed in the ZF4 and ZFL cell lines whereas ΔRaf-ER was stably expressed in the ZF4, PAC2 and ZFL cell lines. We have found that the cMyc-ER product specifically localized in the nucleus of both the ZF4 and ZFL cells. These cell lines first acquired mitogenic advantage upon the ectopic expression of cMyc-ER but soon after went into senescence, suggesting oncogene-induced senescence (unpublished data).

In contrast, senescence was not observed when ΔRaf-ER was over-expressed in the zebrafish cell lines. ΔRaf was activated by application of 4-hydroxytamoxifen and induced hyper-activation of the zebrafish MEK/ERK cascade in the ZF4, PAC2 and ZFL cell lines (Chapter 4 for the ZFL cells and unpublished data for the ZF4 and PAC2 cells). This hyper-activation of MAPK signaling led to mitogenic transformation in the ZFL cell line resulting in a self-sufficient growth. ΔRaf activation promoted survival of the ZFL cells and had anti-apoptotic effect. However, these ΔRaf-induced growth advantages were not observed in the ZF4 and PAC2 cell lines. It shows that the activation of the Raf kinases and MAPK signaling does not always induce proliferation or inhibit apoptosis in zebrafish cells and indicates that these effects are dependent on the phenotypic and genetic background of each cell line.
The described ZFL-ΔRaf1-ER cell line is the first reported zebrafish cell line expressing a human oncogene which can be conditionally activated leading to mitogenic transformation. Besides the growth advantages acquired by ΔRaf activation (Chapter 4), we further analyzed the cellular effects of ΔRaf activation and the hyper-activation of the MAPK signaling in the ZFL cells by molecular profiling using the array-based transcriptomics and kinomics approaches (Figure 1C). Using these methods we have identified molecular changes involved in cell survival, adhesion, motility, vesicular transport, metabolism, apoptosis suppression and inflammation responses. The results not only proved the versatility of the described transcriptomics and kinomics approaches in zebrafish cell cultures, but also indicated which type of cellular alterations play important roles in cell transformation.

The MEK inhibitor U0126 was applied to the ZFL-ΔRaf1-ER cell line to specify molecular signatures downstream of the MEK/ERK cascades in the transcriptome and kinome analysis of cellular alterations induced by ΔRaf activation in the ZFL cells. In Chapter 4 and 5 several downstream targets of the MAPK signaling were identified, including both phosphorylation and transcription targets such as *spred2*,

etv5 and *dusp4* confirming previous studies in mammalian cell cultures. Besides these conserved signatures, there were also some novel molecular targets identified in the zebrafish liver cells. These target genes can be further studied in the ZFL-ΔRaf1-ER cells and can help to understand the evolutionary conservation of MAPK signaling in different species.

Our results show that the hyper-activation of the MAPK pathway induces mitogenic transformation in the ZFL cells, but is not sufficient for full transformation of the cells into an aggressive phenotype (Chapter 4). It indicates that the oncogenic transformation of zebrafish liver cells must be the net outcome of a series of combined alterations in complex signaling networks. For example, although ΔRaf activation changed expression of genes involved in cell adhesion and motility, it did not change the epithelial morphology. After implantation of these cells into zebrafish embryos significantly increased motility was not detected. This suggests that more genetic mutations in the liver cells are required to gain an invasive phenotype. The required mutations could be acquired spontaneously during hyper-proliferation of implanted liver cells. It could also be achieved by further genetic manipulation. For example, it is known that disruption of E-cadherin is required for tumor progression in a Raf-1-driven murine lung cancer model [5]. We found that the expression of E-cadherin was reduced by ΔRaf activation in the ZFL-ΔRaf1-ER cells, but this might be not sufficient to transform the cells into invasiveness. In the future, E-cadherin morpholino could be applied in the ZFL-ΔRaf1-ER cell line to further knock-down E-cadherin expression.

One of the interesting findings described in Chapter 4 is that ΔRaf activation resulted in regulation of the expression of many inflammation genes in the ZFL cells, such as *mpx*, *il12α*, *ccr9*, *ccr6a* and a few TLRs. These results strongly suggest crosstalk between growth advantages acquired by the hyper-activation of MAPK signaling and inflammatory responses. One possible mechanism for this observation is that the neutrophil cytosolic factor 1 (NCF1) was phosphorylated by the hyper-activated MAPK cascade, which in turn activated the NADPH oxidase, over-producing reactive oxygen species and leading to inflammatory responses (Chapter 5). The phosphorylation and activation of NCF1 by ΔRaf activation in the ZFL cells which was detected by PamChip peptide array should be further validated by immuno-histochemistry using a specific antibody. It is known that inflammation plays an essential role in cancer and tumor development [4, 6]. Especially in the cases of liver tumors, it is known that in many patients liver tumors result from chronic liver diseases, indicating the involvement of inflammation responses in carcinogenesis [7]. However, the mechanism remains highly hypothetical and the relations between the over-growth of mitogenic transformed cells, inflammation responses and the development of a primary tumor still are to be determined. Our results suggest that the ZFL-ΔRaf1-ER cell line might serve as a platform to study these questions.
It was revealed by both the transcriptomic and kinomic approaches that the hyper-activation of MAPK signaling leads to alterations of cellular metabolism (Chapter 4 and 5). Nuclear magnetic resonance (NMR) can be applied to further characterize the metabolic alterations *in vivo* [8]. After implantation of the cells into zebrafish embryos, the metabolome can also be measured by High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy method. Preliminary results in collaboration with Axel Meissner (Leiden University Medical Center, the

Netherlands) and Eberhard Humpfer (Bruker BioSpin GmbH, Germany) showed that this method can be applied to living zebrafish embryos (Figure 1J).

Previous studies by Gong *et al.* revealed the deregulation of the MAPK signaling in chemically induced zebrafish liver tumors [9]. Based on the transcriptome overlap of the ZFL cells and zebrafish liver tissue, we compared the *in vitro* ΔRaf/MAPK transcriptional signatures in the ZFL cells and *in vivo* zebrafish liver tumor signatures using microarrays (Chapter 4). The resulting common signatures are markers for liver tumor progression that are regulated by hyper-activation of the MAPK cascade. Cross-species comparison between the transcriptome of the zebrafish models and a large set of human liver tumors identified a subset of common signatures conserved in zebrafish and human liver tumors, probably under the control of the MAPK signaling. The kinomic signatures discovered in Chapter 5 should also be validated in zebrafish and human liver tumors, to identify liver carcinogenesis associated proteins that are commonly activated or suppressed by phosphorylation events downstream of the MAPK signaling. The potential of these common gene and protein signatures as markers for liver carcinogenesis and possible therapeutic targets for liver cancer treatment can be further investigated using the zebrafish model. For example, they can be individually studied *in vitro* using the ZFL-ΔRaf1-ER cell line, which will provide basic information towards understanding related *in vivo* events. The molecular signatures can also be studied *in vivo* in zebrafish, either by creating transgenic fish lines, or more simply, by transplantation of zebrafish cell cultures (Figure 1). Altered cell behaviors caused by these molecular changes can be monitored *in vivo* in zebrafish in real time, which in turn can help us to understand early tumor progression events in patients.

In this thesis, several zebrafish cell lines were characterized and applied as models for cancer and inflammation research. Our case studies illustrate that zebrafish cell lines are as reliable models as the widely used mammalian cell cultures. Taking advantage of the transparency of zebrafish and cell implantation protocols, zebrafish cell lines can serve as a bridge platform between *in vitro*, *in silico*, *ex vivo* and *in vivo* studies in order to enhance our understanding of molecular mechanisms underlying disease progression, especially cancer. Zebrafish cells can be genetically manipulated and transformed into aggressive phenotypes. Implantation of these cells into zebrafish will allow us to monitor tumor initiation and progression, cancer-related inflammation responses and cross-talk between tumor cells and their microenvironment *in vivo* in high resolution (Figure 1).

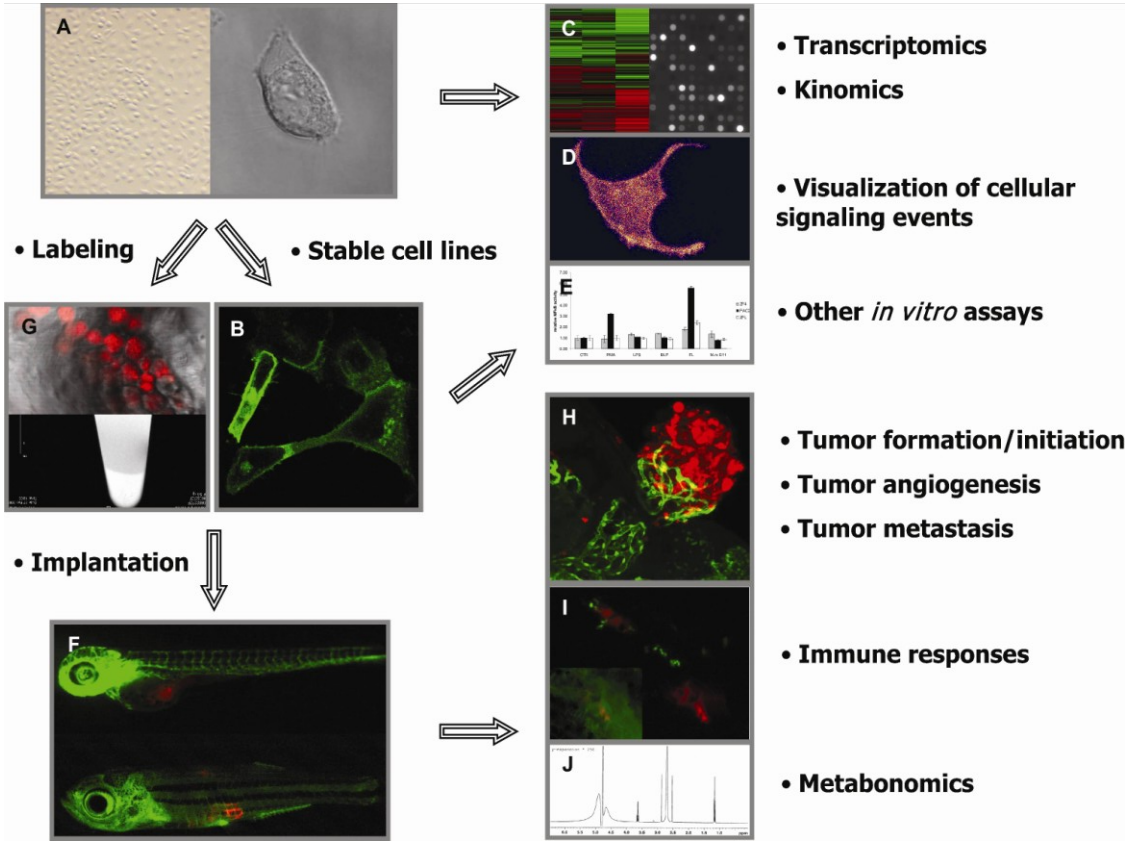
A
C
D
E
G
B
F
H
I
J
• Labeling
• Stable cell lines
• Implantation
• Transcriptomics
• Kinomics
• Visualization of cellular signaling events
• Other *in vitro* assays
• Tumor formation/initiation
• Tumor angiogenesis
• Tumor metastasis
• Immune responses
• Metabonomics

Figure 1. Zebrafish cell lines as models for cancer and immune research.
Zebrafish cell lines (A) can be genetically manipulated and stable cell lines can be generated from well-established cultures (B: ZF4 cells with stable expression of ΔRaf1-ER and YFP-CAAX, unpublished data). The newly-developed array-based transcriptomic and kinomic approaches can be applied to these cells to profile their molecular signatures (C, Chapter 2, 3, 4 and 5). The cellular signaling events caused by the molecular alterations can be visualized. For instance, activation of PI3K in a living ZF4 cell was monitored in real-time by measuring the translocation of the fluorescent PH domain of PKB (D, unpublished data). Application of many other *in vitro* assays also contribute to understand the signaling networks in these cells, including the luciferase reporter assay described in Chapter 3 (E). To validate *in vitro* findings and model diseases *in vivo*, the cells can be implanted into zebrafish embryos or adult fish (G, top: a 2dpf Fli:GFP transgenic embryo showing GFP-labeled vasculature and red cells implanted into the yolk; bottom: a 2-month old Fli:GFP transgenic zebrafish with red cells implanted into the peritoneal cavity and muscle). Various labeling reagents can be applied to zebrafish cells to facilitate *in vivo* visualization by either optical microscopy (F, top: ZFL cells labeled with the red cell tracker CM-DiI which remained fluorescent for 14 days after implantation, unpublished data) or other detection methods such as magnetic resonance imaging (F, bottom: PAC2 cells labeled with gadolinium to enhance contrast for MRI, unpublished data). The transparency of zebrafish embryos makes it possible to study tumor initiation, angiogenesis and metastasis *in vivo* after implantation of malignant transformed cells (H: in a Fli:GFP embryo, implanted malignant cells formed tumor mass and induced formation of neo-vasculature in the host which supported the tumor growth and was also invaded by the malignant cells, unpublished data). Studies of the interface between cellular grafts and the host will help to understand cancer-related inflammation, innate immune responses (I, top: interactions between GFP-labeled neutrophils and implanted cells which stably expressing dsRed in a mpo:GFP transgenic embryo, unpublished data) and adaptive immune responses (I, bottom: accumulation of fluorescence from implanted cells in the thymus of a 2-month zebrafish). In addition, metabolic alterations associated with these processes can be studied using *in vivo* metabolomic approaches (J, example data of the HR-MAS NMR spectroscopy on living zebrafish embryos implanted with zebrafish cells, unpublished data).

Reference

1. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int. Immunol., 2005. **17**(1): p. 1-14.
2. Stockhammer, O.W., et al., *Transcriptome Profiling and Functional Analyses of the Zebrafish Embryonic Innate Immune Response to Salmonella Infection.* J. Immunol., 2009. **182**(9): p. 5641-5653.
3. Uematsu, S. and S. Akira, *Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection.* J Gastroenterol, 2009. **44**(8): p. 803-11.
4. Lu, H., W. Ouyang, and C. Huang, *Inflammation, a Key Event in Cancer Development.* Molecular Cancer Research, 2006. **4**(4): p. 221-233.
5. Ceteci, F., et al., *Disruption of tumor cell adhesion promotes angiogenic switch and progression to micrometastasis in RAF-driven murine lung cancer.* Cancer Cell, 2007. **12**(2): p. 145-59.
6. O'Neill, L.A.J., C.E. Bryant, and S.L. Doyle, *Therapeutic Targeting of Toll-Like Receptors for Infectious and Inflammatory Diseases and Cancer.* Pharmacological Reviews, 2009. **61**(2): p. 177-197.
7. Budhu, A. and X.W. Wang, *The role of cytokines in hepatocellular carcinoma.* J Leukoc Biol, 2006. **80**(6): p. 1197-1213.
8. Kabli, S., et al., *In vivo metabolite profile of adult zebrafish brain obtained by high-resolution localized magnetic resonance spectroscopy.* J Magn Reson Imaging, 2009. **29**(2): p. 275-81.
9. Lam, S. and Z. Gong, *Modeling liver cancer using zebrafish: a comparative oncogenomics approach.* Cell Cycle, 2006. **5**(6): p. 573-7.