Chapter 1

General introduction:

Zebrafish cells as models for biomedical research

The zebrafish, *Danio rerio*, is a small subtropical fish that has emerged as an important model of vertebrate development and human disease. Due to the amenability of zebrafish to large scale forward and reverse genetic screens, this model vertebrate organism is ideal for discovery of novel gene functions in disease processes at a throughput level that can not be matched by rodent models. Furthermore, owing to its small size and optical transparency, disease symptoms and resulted immune responses can be studied at the whole organism level. Particularly advantageous in this context are fluorescence multicolor labeling techniques that allow *in vivo* visualization of important factors in disease such as cancer cells, immune cells and microbes.

Zebrafish as a model for inflammation and infectious diseases

The innate and adaptive immune system of zebrafish has been shown to be very similar to the human system and therefore it has been recognized as an important model for the study of inflammation and infectious diseases in the last decades [1-3]. Zebrafish embryos possess a functional innate immune system after one day of development, comprised primarily of embryonic macrophages and neutrophils in the embryonic blood circulation [4, 5]. Transgenic reporter zebrafish lines have been generated to visualize specific leukocyte lineages and their involvement in inflammatory responses *in vivo*. It was found that the zebrafish embryonic neutrophils steadily circulate within the tissues and are quickly attracted to tissue damage and/or infection sides (Figure 1A) [5-7]. Subsequently macrophages are recruited to the inflamed tissues, where they are able to phagocytose pathogens and tissue debris (Figure 1B) [5, 8].

The primary defense mechanisms against microbial agents of zebrafish are similar to those of mammals and many signaling molecules and pathways are conserved [2]. For example, Toll-like receptors (TLRs), a family of key pathogen recognition receptors of the innate immunity, were identified in zebrafish and the TLR signaling mechanisms are already functional in early embryos (Figure 2) [9-11]. Homologs of all five members of the mammalian NFκB transcription factor family were also identified in zebrafish, which play key roles in regulation of the immune response to infection [12].

It is known that zebrafish are naturally susceptible to infection by Gram-positive and Gram-negative bacteria, mycobacteria, protozoa and viruses [1]. Recently many infection models have been developed for experimental infections of embryos and adults [3]. For example, adult zebrafish are susceptible to tuberculosis caused by *Mycobacterium marinum* and the recent zebrafish mycobacterial infection models have provided a basis for undertaking genetic dissections of the host- and pathogen-related determinants of active tuberculosis [1, 13].

Notably, the adaptive immune system is not functionally active in zebrafish during the first 3 weeks of development [2]. This clear temporal separation in zebrafish embryos provides a convenient system for *in vivo* study of the vertebrate inflammation and innate immunity independently from the adaptive immune responses.

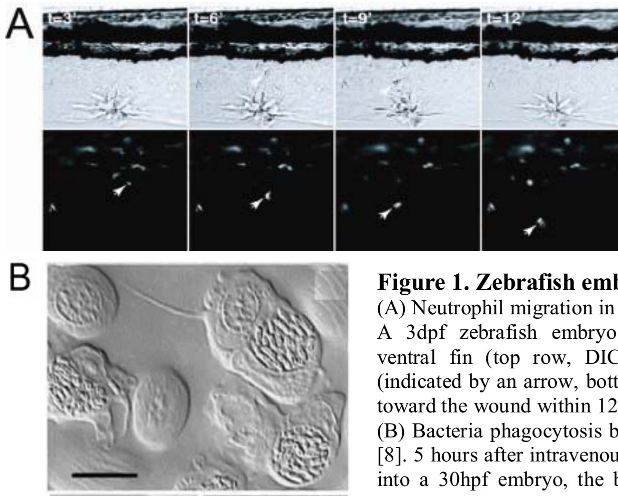


Figure 1. Zebrafish embryonic leukocytes.
(A) Neutrophil migration in response to a wound [7]. A 3dpf zebrafish embryo was wounded in the ventral fin (top row, DIC). A single neutrophil (indicated by an arrow, bottom row, GFP) migrated toward the wound within 12 minutes after wounding. (B) Bacteria phagocytosis by primitive macrophage [8]. 5 hours after intravenous injection of *B. subtilis* into a 30hpf embryo, the blood was cleared from bacteria and macrophages are full of bacteria gathered in a single large vacuole.

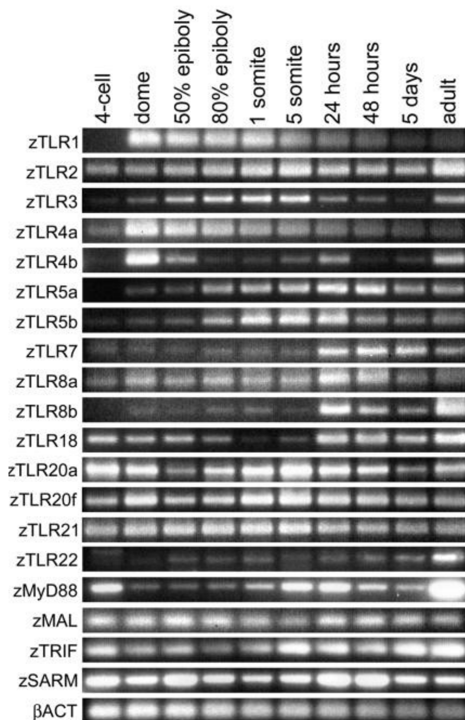


Figure 2. Expression of zebrafish TLR and adaptor genes at different developmental stages [9].

Zebrafish as a model for cancer

In recent years, the zebrafish has vaulted to the top as a laboratory model organism for cancer research. The strikingly similar molecular and histopathological features of fish and human tumors strengthen the rationale for using zebrafish as a cancer model [14, 15]. Like all vertebrate species, spontaneous tumors have been found in wild zebrafish, with incidence increased by age [15, 16]. Genetic screening studies have identified zebrafish mutant strains with genomic instability and enhanced tumor susceptibility [15, 17]. One example is that zebrafish homozygous for an inactivating p53 mutation spontaneously developed malignant peripheral-nerve sheath tumors whereas no tumor were found in wildtype fish [15].

Tumors can also be induced in zebrafish by chemical carcinogenesis, resulting in various benign and malignant tumors developed in virtually all organs after exposure to water-borne carcinogens [16]. Exposure of zebrafish to carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA), dibenzo(a,l)pyrene (DBP) and *N*-nitrosodiethylamine (DEN) induced liver tumor formation, which histologically share characteristic with human hepatocellular carcinoma (HCC) [18, 19]. Comparative transcriptome analysis revealed that the human and zebrafish liver tumors share a molecular framework which is dysregulated during tumorigenesis (Figure 3) [19, 20], suggesting the importance of zebrafish in modeling human liver carcinogenesis.

Using a transgenic approach, zebrafish models of specific cancer types can be generated by expression of known oncogenes in specific organs of interest. The first transgenic tumor model in zebrafish developed T-cell acute lymphoblastic leukemia, which resulted from the expression of mouse Myc oncogene driven by a *rag2* promoter in lymphoid cells [21]. A few zebrafish melanoma models have been generated using the BRaf or Ras oncogenes targeted to melanocytes by melanocyte-specific promoters [22, 23]. Expression of the human KRas oncogene driven by the *rag2* promoter induced embryonic rhabdomyosarcoma in zebrafish which were externally visible at 10 days post fertilization (dpf) [24]. In addition, liver-specific expression of zebrafish KRas induced zebrafish HCC (Gong *et al.*, in preparation). Studies using these transgenic zebrafish models have revealed striking similarity between mechanisms of carcinogenesis in mammals and zebrafish and have expanded our understanding of tumor biology. These models can also be applied as screening tools for genes and drugs that involved in tumor progression and suppression.

Xeno-transplantation in the transparent zebrafish model

Animal models are essential tools for biomedical research, allowing to investigate manifestations of human disease that are inaccessible in patients, to decipher molecular interactions involved in the disease and to perform preclinical testing of therapeutic interventions. The use of biomedical animal models has largely improved our knowledge about cancer and other human chronic diseases. However, due to the limitations of many existing animal models, it is difficult to directly visualize the processes of disease progression in living organisms, such as the initiation of tumor formation and the early stages of metastasis. It also limits the development of effective therapeutic strategies. In this aspect, the zebrafish has

become an important model organism, because the transparency of zebrafish embryos allows direct high quality time-resolved imaging at the subcellular level *in vivo*. For example, the first high resolution observation of *in vivo* tumor formation and tumor-induced vascular remodeling at the subcelluar level was achieved by implantation of human tumor cells into 1 month old zebrafish juveniles (Figure 4 and 5D) [25, 26].

The xeno-transplantation approach has been widely used in the zebrafish where human or mouse tumor cells were transplanted into zebrafish juveniles and embryos. Human malignant melanoma cells transplanted into blastula-stage embryos survived, exhibited motility, divided and retained their dedifferentiated phenotype, suggesting the utility of the zebrafish early-embryonic model to study tumor cell plasticity and tumor-microenvironment interactions (Figure 5A) [27]. A different metastatic melanoma cell line showed proliferation, migration, melanin production and formation of cell masses which stimulated angiogenesis in the embryos within a few days after implanted into the yolk of 2 dpf zebrafish embryos (Figure 5B) [28]. Similar results were reported from colorectal and pancreatic cancer cell lines, demonstrating the embryonic yolk transplantation as a rapid approach for assessing human cancer cells at various stages of tumorigenesis [28]. To investigate tumor angiogenesis, a zebrafish/tumor xenograft angiogenesis assay was developed in which mammalian tumor cells were xenograft into the perivitelline space of 2dpf zebrafish embryos where the xenograft could induce neovascularization (Figure 5C) [29, 30]. Taking advantage of the zebrafish embryos, these xeno-transplantation assays can be applied for high-throughput anti-tumor drug screenings.

Potential of allo-transplantation in the zebrafish model

One limitation of the application of xeno-tranplantation in zebrafish is the different biological backgrounds of mammalian xenografts and the host zebrafish, which generates unpredictable variations when studying disease related signaling events at molecular level. To overcome this limitation and fully explore the power of the zebrafish model, allo-transplantation of zebrafish cells becomes a complementary method where the graft and host share the same genetic background.

Vertebrate cell lines have been used extensively and successfully in a broad range of fields from embryology to immunology and cancer research. They can be applied for obtaining sufficient amounts of tissues that are hard to isolate or for which little tissue is available. Validation of the information acquired from these *in vitro* studies into *in vivo* animal models has largely enhanced our knowledge about human disease. As in the mammalian model organisms, *in vitro* cell models can bridge the knowledge gained from different organisms to the research in zebrafish, and can be used to dissect the findings in zebrafish embryos or adult fish at detailed molecular and cellular level. In addition, because of the transparency of zebrafish and versatile cell implantation protocols, zebrafish cell cultures can be used not only for *in vitro* cellular analysis, but also for *in vivo* studies after cell implantation.

Oncogenic transformation of zebrafish cell lines can be achieved by genetic manipulation with oncogenes or tumor suppressors. Various methods have been established to control targeted gene expression in mammalian models, including the

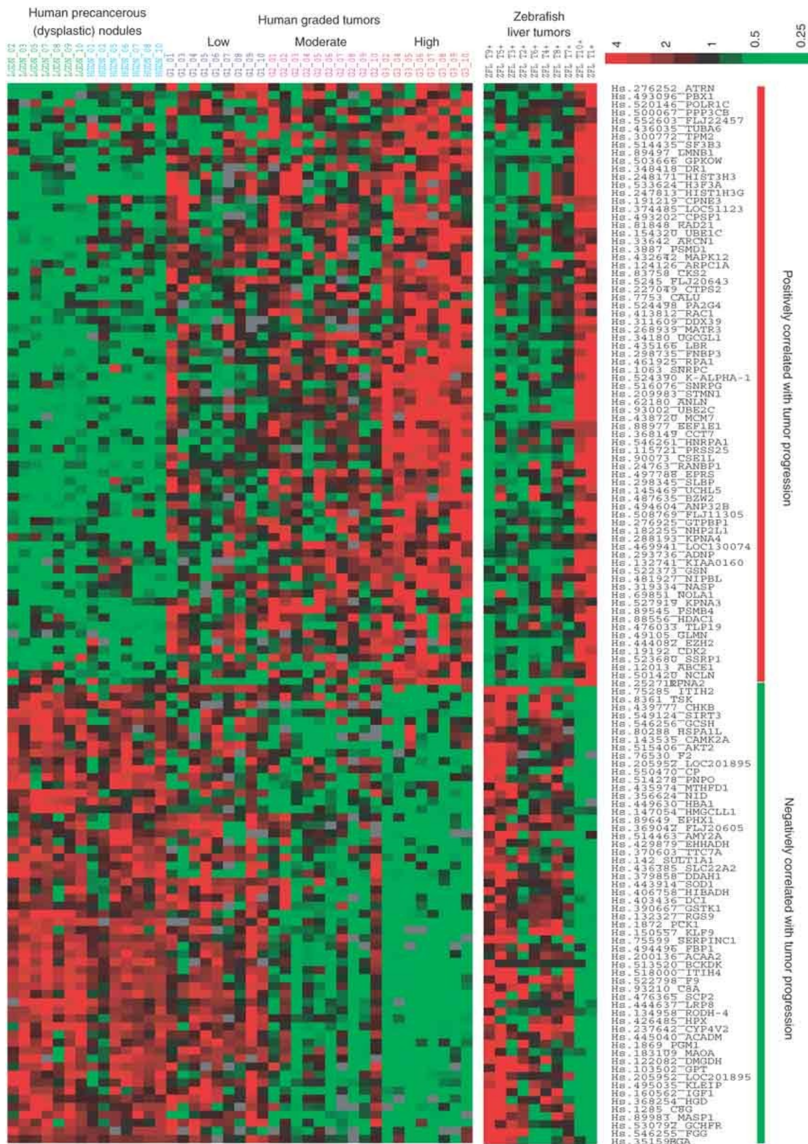


Figure 3. Genetical conservation between human and zebrafish liver tumors [19].

Expression profiles of 132 genes showing similar correlation with tumor progression in both zebrafish and human liver tumors. The color in each cell reflects the expression level of the corresponding gene in the corresponding tissue sample.

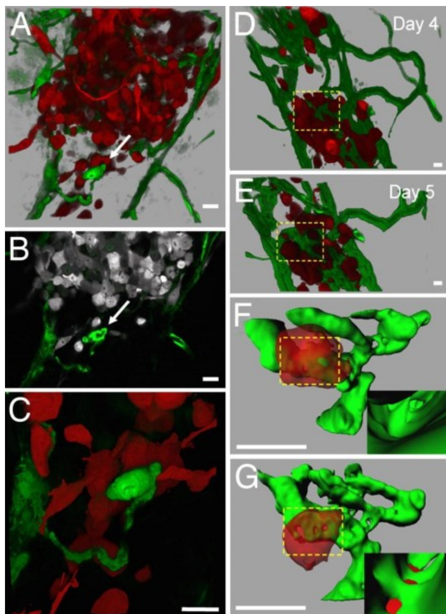
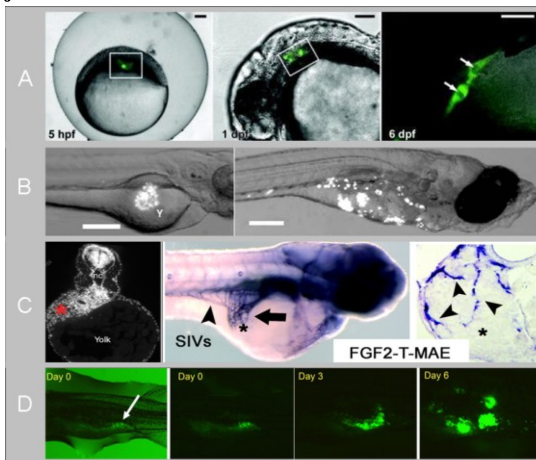


Figure 4. High-resolution visualization of tumor-induced angiogenesis and tumor cell–vascular interactions [25].
(A) 3D-reconstruction of MDA-435 cell microtumor (shown in red) 5 days post implantation in a 1-month Tg(fli1:egfp) zebrafish (The vasculatures are shown in green). The white arrow indicates the remodeling vessel. (B) Single optical section of the microtumor in A. (C) Zoom-in of the dotted square in A. (D and E) 3D-reconstruction of MDA-435 tumor cells secreting human VEGF, 4 (D) and 5 (E) days post implantation in the same 1-month Tg(fli1:egfp) zebrafish. (F and G) 3D-reconstructions of digitally isolated tumor cells in contact with host vessels and the vessel interior at sites of vessel openings and tumor cell membrane integration, from D and E, respectively.

Figure 5. Different xeno-transplantation assays in zebrafish embryos and juveniles.



(A) Approximately 10 C8161 human metastatic melanoma cells were transplanted into the blastodisc halfway between the margin and the animal pole of a 4 hpf embryo (in white box). Some melanoma cells remained in the embryo till 6dpf, which were non-tumorigenic in this environment [27]. (B) Implantation of human melanoma cells into the yolk of 2dpf embryos [28]. Approximately 50 cells were injected (a and b), which proliferated and spread at 7dpi. (C) Tumorigenic murine FGF2-T-MAE cells were injected in the perivitelline space of 2 dpf zebrafish embryos (*), which attracted neovessels originating from the SIV basket that migrated and infiltrated the graft [29, 30]. (D) MDA-435 tumor cells expressing GFP were injected into the peritoneal cavity of 1-month-old zebrafish and imaged daily with a fluorescence stereomicroscope. Arrow shows the injection site [25].

Cre/lox system, the heat shock system and systems inducible by application of compounds such as tamoxifen, tetracycline or miferpristone. These inducible systems can also be applied in zebrafish and zebrafish cells to control the cellular transformation according to spatial or temporal request, which will help to dissect the minimum number of cellular and molecular events required for malignant transformation *in vivo* after implantation. The studies on the interface between grafts and host microenvironment in transparent zebrafish embryos will also bring new insights into understanding of tumor growth, invasion and cancer-related inflammation.

Development and use of zebrafish cell lines

Zebrafish cell cultures can be maintained at room temperature in atmospheric CO_2. It results in cost efficiency and lower chance of contaminations compared to mammalian cell cultures, which makes zebrafish cell cultures very attractive to many laboratory applications. However, since zebrafish is a relatively new model organism for biomedical research, only a few zebrafish cell lines have been generated in the last decades. Most of the known zebrafish cell lines were derived from zebrafish embryos.

In the early 1990's, Collodi *et al.* developed methods for culturing cells from early stage zebrafish embryos and organs of adult fish such as caudal and pelvic fin, gill, viscera and liver [31]. The ZEM2 cell line was isolated from blastula-stage embryos by the described method [31]. Subsequently, the ZEM2S cell line was derived from ZEM2 by selection of growth in a basal nutrient medium. It was reported that zebrafish embryonic cell lines derived from blastula and gastrula stages remained pluripotent and germ-line competent for multiple passages in culture [32]. Zebrafish germ-line chimeras can be generated using short-term primary embryo cell cultures [33, 34]. To support the growth of the blastula cell lines (for example, ZEB2J), a zebrafish spleen cell line, ZSSJ, was developed and can be used as a feeder cell line for zebrafish embryonic stem cell cultures [35, 36].

The ZF4 cell line was derived from 1 dpf embryos. It is the first reported zebrafish cell line that can be maintained in conventional medium containing mammalian serum (Figure 6A) [37]. The fibroblast-like cell lines ZF13 and ZF29 were generated from 20h zebrafish embryos. They were first used in the study of the early cellular ionic response to EGF [38]. The PAC2 cell line was isolated from 24h zebrafish embryos. Retroviral infection was performed in the PAC2 cell line as the first success of retroviral vector technology in zebrafish [39, 40]. A few fibroblast cell lines were derived from amputated caudal fins of adult zebrafish of the AB and SJD strains [41].

The ZFL cell line was derived from a pool of approximately 10 normal adult zebrafish livers [42, 43]. It is the only zebrafish cell line showing typical epithelial morphology (Figure 6B).

Several groups attempted to isolate continuous stable cell cultures from zebrafish tumors. 19 transplantable zebrafish tumor lines were generated from N-nitrosodiethylamine (DEN) induced primary tumors in two homozygous diploid clonal zebrafish lines [18]. These cell lines can be kept in culture for as long as 3 to

25 passages, but it’s hard to maintain them after cryopreservation (personal communications with S.Y. Revskoy). The only reported successful zebrafish tumor cell line was a cell line expressing krt8::EGFP-KRasB^{V12}, derived from a large eye tumor by S. Parinov *et al.*. This cell line has successfully passed through more than 24 passages after cryopreservation (personal communications with S. Parinov).

A few laboratories have attempted to isolate zebrafish hematopoietic cells, but the establishment of continuous stable cell cultures has not been reported yet. Recently, a method was developed to create zebrafish kidney stromal (ZKS) cell lines, which supported the *in vitro* maintenance of hematopoietic precursor cells isolated from adult whole kidney marrow [44]. The ZKS cells were required for the *in vitro* growth of hematopoietic precursor cells and their differentiation into different lineages [44].

Zebrafish cell lines: opportunities and challenges

Compared with the increasing usage of zebrafish as model organism in many laboratories to replace or to supplement studies in higher vertebrate models such as rodents, zebrafish cell lines are still unexploited and limited in applications. One of the bottlenecks for further applications of zebrafish cell cultures is that a detailed characterization and comparison of the existing zebrafish cell lines is lacking. Although some of the zebrafish cell lines were established over a decade ago, their genetic and physiological properties are still not well characterized. Moreover, the fact that general gene expression profiles of zebrafish cell lines have not been analyzed also makes it difficult to perform advanced gene expression assays in zebrafish cell lines. Therefore a good characterization of zebrafish cell lines is required to build up cellular model systems and to broaden the applications, as in the case in mouse and human cell lines. It will not only add value to the zebrafish as model organism, but also provides a novel platform to expand basic cancer research at the molecular and cellular level towards the tissue, organ and the entire organism level.

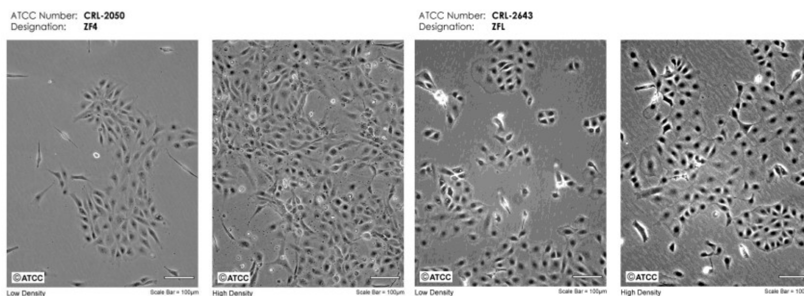


Figure 6. Zebrafish cell lines.
Left: ZF4 is an embryonic fiboblast cell line. Right: the epithelial ZFL cell line. Images were adapted from ATCC.

Outline of this thesis

In this thesis, we aimed to establish zebrafish cell line models for inflammation and cancer studies. To select the good candidate lines for this aim, a few established zebrafish cell lines were characterized and their genetic and physiological properties were compared. We also developed a set of tool methods to investigate cellular signaling events in zebrafish cells, including laboratory applications such as stable transfection, the luciferase reporter assay, the tamoxifen-inducible protein activation system, etc. In order to systematically investigate specific signaling pathways, the transcriptomics and kinomics approaches were applied to the zebrafish cell lines. The technique of allo-transplantation into zebrafish embryos was established, to validate *in vitro* findings and to enable studies of cellular transformation processes *in vivo*.

In **Chapter 2**, we characterized two zebrafish embryonic fibroblast cell lines, ZF4 and PAC2. Their properties as transfection hosts were tested and methods of lipid-mediated transfection and nucleofection method were optimized, enabling future biological studies on gene expression and cell signaling. These two cell lines were classified by their transcriptome profile, compared with adult zebrafish or 24-hour embryos, at which stage the cell lines were derived. By comparison with human fibroblast cell lines, we also found that the transcriptional responses to serum growth factor exposure of the zebrafish fibroblast cell lines showed interesting similarities with the transcriptional responses involved in wound-healing and cancer.

Because many signaling molecules in the TLR signaling pathway are expressed in these cell lines, TLR signaling was studied in the ZF4 and PAC2 cell lines, together with the epithelial ZFL cell line (**Chapter 3**). In all three cell lines, the transcription factor NFκB, which controls expression of many inflammatory genes, can be activated by TLR signaling, but in a cell-line specific manner. The result show that there are large differences in the intracellular signaling networks in these cell lines, which might reflect their different origins. Stimulation with flagelin, which is recognized by TLR5, activated NFkB in all cell lines. Microarray analysis revealed that the same flagelin stimulation induced distinct transcriptome programs in different cell lines, indicating that zebrafish cell lines can be used to study specific signaling events involved in pathogen recognition and inflammation at cellular level. The transcriptome analysis also showed that some of infection-driven inflammation responses were associated with cancer, suggesting the possibility to study cancer-related inflammation in zebrafish cell lines.

Because of the known genetic conservation between human and zebrafish liver tumors, we used the epithelial ZFL cell line to start the establishment of *in vitro* cancer cell line models, which largely represents normal zebrafish liver tissues at the transcriptome level (**Chapter 4**). We studied the Raf/MEK/ERK signaling pathway in this cell line, as this MAPK pathway is essential in cell survival and growth and it was deregulated in zebrafish liver tumors. Oncogenic human Raf-1 (ΔRaf1) was stably expressed in the ZFL cell line, which can be post-transcriptionally activated by 4-hydroxytamoxifen (4HT). The ΔRaf1 activation in turn activated the zebrafish MEK/ERK cascade, resulting in a series of growth advantages, suppression of apoptosis and mitogenic transformation of the ZFL cells, which were confirmed by *in vivo* allo-transplantation and *in silico* microarray

analyses. By transcriptome comparison with zebrafish liver tumors, we identified a set of genes transcriptionally regulated by hyperactive MAPK signaling, which can also be linked to zebrafish liver tumor progression. A subset of these common genes have been reported in human HCC, suggesting that the *in vitro* zebrafish liver cell model can be used for further studying of the molecular basis of human HCC.

In order to better understand the signaling involved in the mitogenic transformation caused by the hyperactive MAPK signaling, we also profiled the kinase activity and phosphorylation events controlled by the ΔRaf1 activation in zebrafish liver cells using newly developed serine/threonine peptide microarrays (**Chapter 5**). The *ex vivo* results suggested that a few peptides were specifically phosphorylated by hyper-activation of the ΔRaf1/MEK/ERK cascade. The function of the proteins whose activation/suppression were regulated by these phosphorylation events are indeed involved in cellular alterations which were suggested by the transcriptome study in Chapter 4. It showed that zebrafish cell lines can be used to bridge studies at different levels, and future studies using these cell lines should improve our understanding of cancer and inflammation.

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