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Cancer and inflammation studies using zebrafish cell lines

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Cancer and inflammation studies

using zebrafish cell lines

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“It never will rain roses. When we want to have more roses we must plant trees.”

George Eliot

“One has to be an optimist in science because most of the time it doesn't work.”

Oliver Smithies

For my grandparents and parents

献给我的爷爷奶奶爸爸妈妈

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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 sites (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 NF κ B 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.

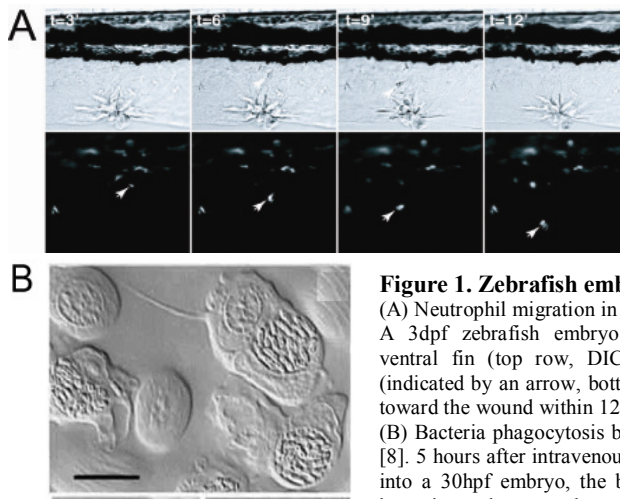


Figure 1. Zebrafish embryonic leukocytes.
 (A) Neutrophil migration in response to a wound [7]. A 3dpf zebrafish embryo was wounded in the ventral fin (top row, DIC). A single neutrophil (indicated by an arrow, bottom row, GFP) migrated toward the wound within 12 minutes after wounding.
 (B) Bacteria 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.

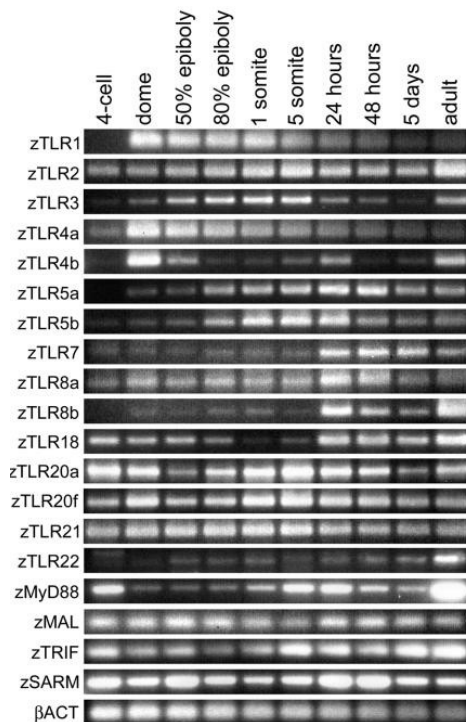


Figure 2. Expression of zebrafish TLR and adaptor genes at different developmental stages [9].

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 subcellular 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-transplantation 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

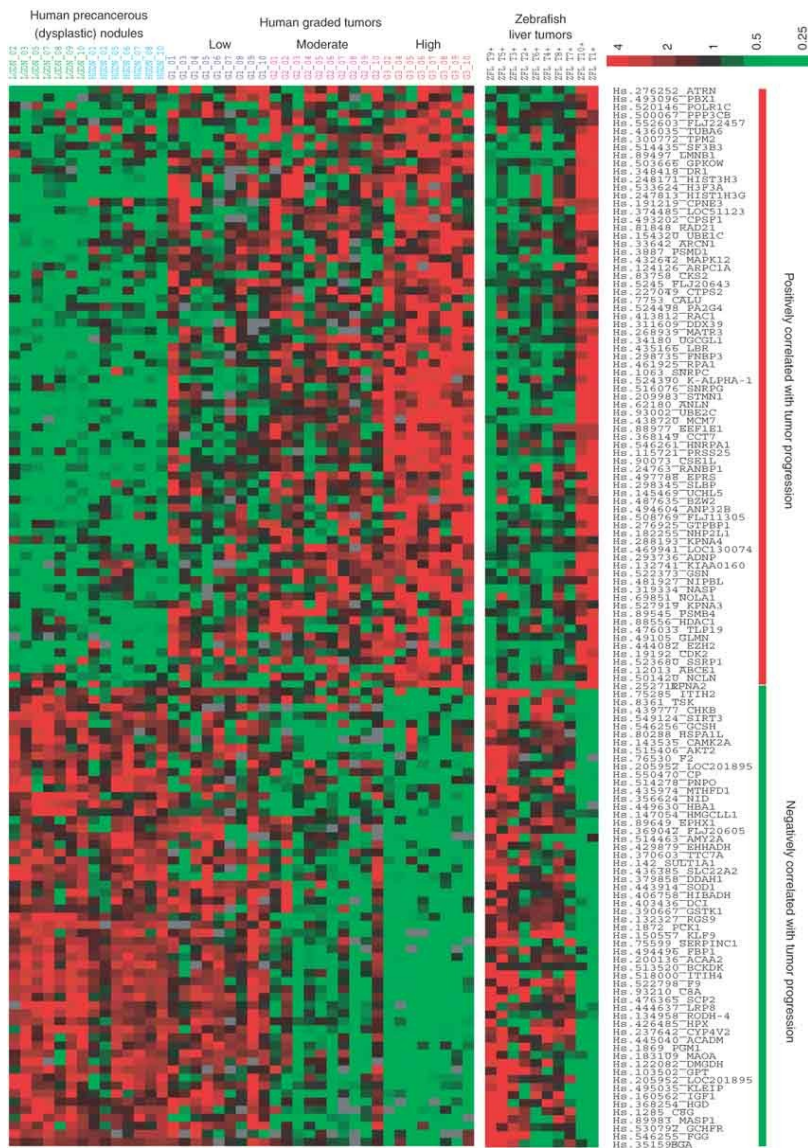


Figure 3. Genetical conservation between human and zebrafish liver tumors [19].

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.

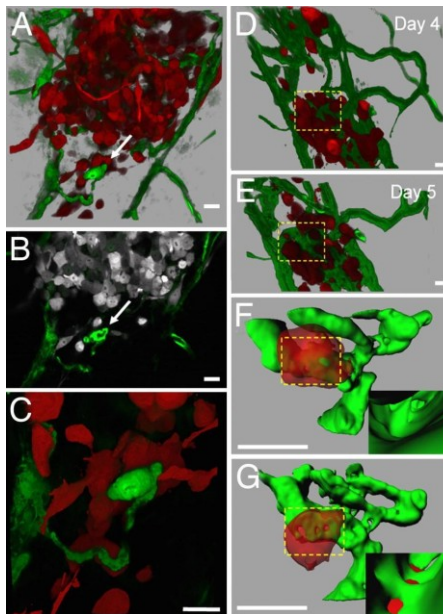
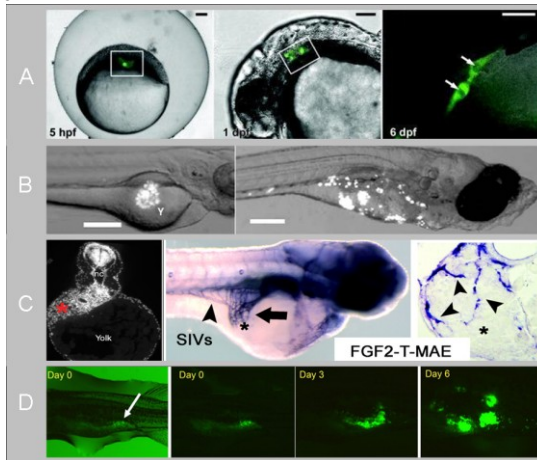


Figure 4. High-resolution visualization of tumor-induced angiogenesis and tumor cell-vascular interactions [25].

(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 mifepristone. 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 CO₂. 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.

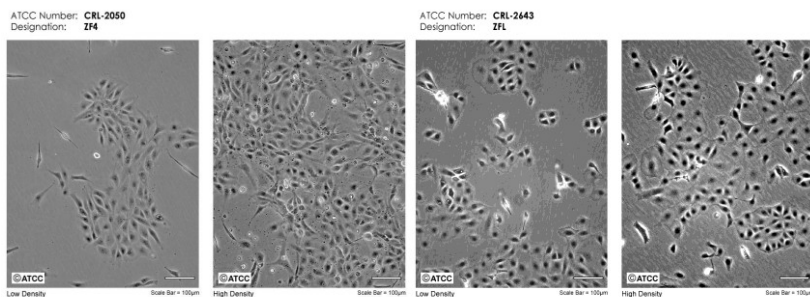


Figure 6. Zebrafish cell lines.

Left: ZF4 is an embryonic fibroblast 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 NF κ B, 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 NF κ B 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 (Δ Raf1) was stably expressed in the ZFL cell line, which can be post-transcriptionally activated by 4-hydroxytamoxifen (4HT). The Δ Raf1 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 Δ Raf1 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 Δ Raf1/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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Chapter 2

Genetic and transcriptome characterization of model zebrafish cell lines

Compared with the increasing usage of zebrafish as model organism in many laboratories, zebrafish cell lines are still unexploited and limited in applications, partly due to their unknown genetic and physiological properties. In this paper we characterized two zebrafish embryonic fibroblast cell lines, ZF4 and PAC2. We demonstrated the genetic stability of these two zebrafish cell lines and achieved genetic manipulation by either lipid-mediated transfection or an electroporation-based nucleofection method. Data from Affymetrix zebrafish chip analysis demonstrate unique characteristics of these two cell lines in gene expression levels, showing that different zebrafish cell lines can be classified by their transcriptome profile. Their transcriptional responses to serum growth factor exposure suggested that zebrafish fibroblast cell lines may be used for studying processes related to wound-healing or cancer.

Keywords: *Danio rerio*, transfection, nucleofection, transcriptome, microarray, serum-response

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Introduction

The zebrafish, *Danio rerio*, has been used as a successful model for the study of developmental genetics of vertebrates because of its small size, rapid generation time, powerful genomic resources and optically transparent embryos [1]. Furthermore, since zebrafish has innate and adaptive defense mechanisms against microbial infections that are very similar to those of mammals, it also has been recognized as an attractive experimental model for infectious disease and immunity [2-4]. Although zebrafish has been used in many laboratories to replace or to supplement studies in higher vertebrate models such as rodents, *in vitro* analyses using zebrafish cell cultures are still not as advanced as in other model systems. Owing to the transparency of zebrafish embryos and versatile cell implantation protocols [5], zebrafish cell cultures can be used not only for *in vitro* cellular analysis systems, but also as a powerful tool for *in vivo* studies after cell implantation into embryos.

Vertebrate cell lines have been used extensively and successfully in a broad range of fields from embryology to immunology and cancer research. They can have applications for obtaining sufficient amounts of tissues that are hard to isolate or for which little tissue is available. However, many of the most commonly used human or murine cell lines are transformed, exhibiting different gene expression and cell cycle profiles than those of cells in the living organism. In contrast, most of the known zebrafish cell lines are untransformed embryonic cell lines. As zebrafish is a relatively new model organism, only a few zebrafish cell lines have been generated. ZF4, the first reported zebrafish cell line that can be maintained in conventional medium containing mammalian serum, was established from 1-day-old embryos [6]. 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 [7]. They derived the ZFL cell line from a pool of approximately 10 normal adult zebrafish livers [8, 9], and the ZEM2 cell line from blastula-stage embryos [10]. ZEM2S cells were 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 [11]. Recently it was reported that some zebrafish germ-line chimeras could be generated using short-term primary embryo cell cultures [12, 13]. Fibroblast-like cell lines ZF13 and ZF29 were generated by Zivkovic *et al.* [14] and they were first used in the study of the early cellular ionic response to EGF. Around the same time, retroviral infection was performed in the embryonic PAC2 cell line as the first success of retroviral vector technology in zebrafish [15, 16]. Later several fibroblast cell lines were derived from amputated caudal fins of adult zebrafish of the AB and SJD strains in 1999 [17].

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 more than 10 years ago, their genetic and physiological properties are still not well known, which limits their application. 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. The recent advances in microarray gene expression profiling offer an excellent opportunity to further characterize zebrafish cell lines.

In this study, we describe the morphology and physiology of two zebrafish embryonic cell lines, ZF4 and PAC2. Their properties as transfection hosts were tested and optimized, providing information for future biological studies for instance on gene expression and cell signaling. To obtain a stable reference data set that can be used as a public resource for comparison to data from other research projects, we chose the Affymetrix zebrafish GeneChip platform for transcriptome characterization of the cell lines. Our microarray data demonstrated unique characteristics of ZF4 and PAC2 cell lines in gene expression levels compared with adult zebrafish or 24-hour embryos, and as well as their transcriptional programs in response to serum growth factor exposure. Comparable to the results obtained in human cell lines, serum treatment of fibroblast cell lines was shown to have interesting similarities with the transcriptional responses in wound-healing and cancer.

Results

Biologic characteristics of zebrafish cell lines

In this study two zebrafish embryonic cell lines ZF4 and PAC2 were investigated as potential models. The ZF4 fibroblast cell line was established from 1-day-old zebrafish embryos by Driever *et al.* [6] and showed typical fibroblast morphology (Figure 2). The PAC2 cell line was isolated from 24-hour-old zebrafish embryos by Chen and Amsterdam *et al.* [15, 16]. Although it was described as a fibroblast cell line [16], it didn't show a clear fibroblast morphology in our experiments and therefore it is not certain they are fibroblasts (Figure 2). Both cell lines adhered tightly to culture flasks in a monolayer sheet under growth protocols that are listed in Table 1.

In order to confirm identity and exclude possible contaminations of the used cell lines we cloned and sequenced one of their profilin genes. We compared these sequences with the profilin 2A gene cloned from a cell line from another fish species, *Pimephales promelas* (fat head minnow; FHM), which has been grown for many passages in our laboratory. The comparison of a 83 nucleotides fragment shows that the profilin 2A genes cloned from the ZF4 and PAC2 cell lines are identical to profilin of the Tuebingen genomic sequence, whereas the profilin 2A sequence from the FHM cell line showed a difference of 10 nucleotides (Figure 1).

Flow cytometry analysis was performed to test genetic stability of the zebrafish cell lines. As a control we used the FHM cell line. Somatic cells from adult zebrafish muscle were taken as reference and it showed a nuclear DNA content (2C) of 3.86 pg, close to the value calculated from genomic sequence data [18]. The nuclear DNA contents of PAC2 and ZF4 cells were 3.84 pg/2C and 3.76 pg/2C, respectively, similar to the muscle cells. However, the FHM cell line showed a significantly lower amount of DNA, yielding 1.86 pg/2C.

Table 1. Biological properties & culture conditions of cell lines analyzed in this study

	ZF4	PAC2	FHM
Source	1-day-old zebrafish embryos	24-hour-old zebrafish embryos	Adult <i>Pimephales promelas</i>
Growth properties	adherent	adherent	adherent
Morphology	fibroblast	fibroblast	epithelial
Growth medium	1:1 mixture of DMEM and F12 medium with 10% FCS	Leibowitz-15 medium supplemented with 15% FCS	67% L-15 medium with 10% FCS
Subculture ratio	1:2-1:4	1:4	1:2-1:3
Freeze condition	Growth medium + 10%FCS + 5%DMSO; 3.5x10 ⁶ cells/ml	Growth medium + 30%FCS + 10%DMSO; 2x10 ⁶ cells/ml	Growth medium + 10%FCS + 10%DMSO; 3.5x10 ⁶ cells/ml
nuclear DNA content	3.76 pg/2C	3.84 pg/2C	1.86 pg/2C

Figure 1. Comparison of fragments of profiling sequences from zebrafish and fat head minnow cell lines.

For accession number of the sequences see the material and method section. The profilin 2A sequence of the FHM cell lines was shown to be identical to the EST with accession number DT354999.

	(1)	1	10	20	30	40	50	60	75
Zv6 genomic profilin 2A	(1)	TACG	AA	AAGAAGTGCCTCTGTGATCAGAGACAG	CTTC	GGTGGAGGG	GACTGGACAATGGACATCAGGACA		
ZF4 profilin 2A	(1)	TAGG	AA	AAGAAGTGCCTCTGTGATCAGAGACAG	CTTC	GGTGGAGGG	GACTGGACAATGGACATCAGGACA		
PAC2 profilin 2A	(1)	TAGG	AA	AAGAAGTGCCTCTGTGATCAGAGACAG	CTTC	GGTGGAGGG	GACTGGACAATGGACATCAGGACA		
FHM profilin 2A	(1)	TACG	AA	AAGAAGTGCCTCTGTGATCAGAGACAG	CTTC	CAC	TGGAGGG	GACTGGACAATGGACATCAGGACA	

Figure 2. Microscopic analysis of EGFP-actin fusion in zebrafish cell lines.

Shown are confocal laser scanning microscopic analysis of ZF4 and PAC2 cell lines transfected with a pEGFP-actin construct as described in the text. As a control fat head minnow cell line FHM was used.

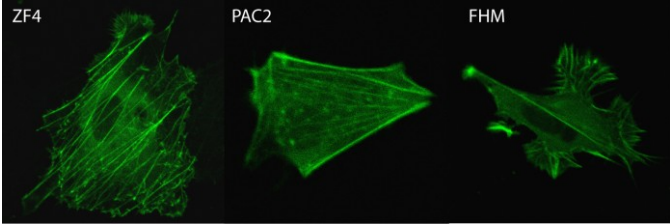
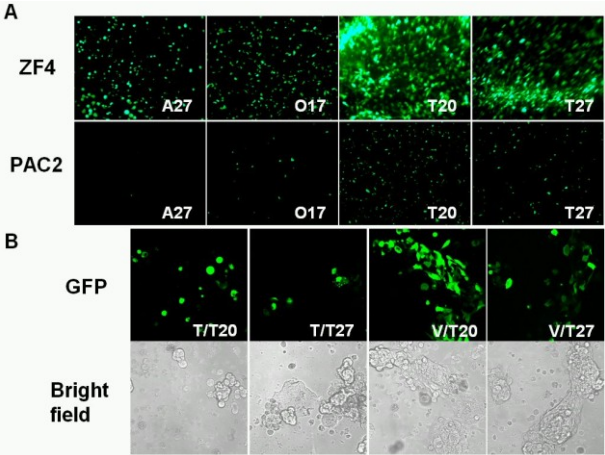


Figure 3. Nucleofection in zebrafish cell lines.

(A) Optimization of nucleofection in zebrafish cell lines using different preprogrammed electroporation programs. ZF4 and PAC2 cell lines were nucleofected with pmaxGFP in Nucleofector solution V and 24 hours after nucleofection, fluorescence was analyzed by fluorescent stereo microscopy. All shown photos were recorded using a Leica DC500 camera using the same settings. (B) Optimization of nucleofection in ZF4 cell line using different Nucleofector solutions. ZF4 cells were nucleofected with pmaxGFP in Nucleofector solution T and V, by electroporation programs T20 or T27. Fluorescence was analyzed by confocal laser scanning microscopic 24 hours after nucleofection. All fluorescent images were taken with the same settings.



Transfection of zebrafish cell lines using a lipid transfection reagent

Transfection of foreign DNA into vertebrate cell lines has been used as an essential tool for numerous biological studies. Many methods have been developed to achieve gene delivery, such as calcium phosphate, liposome-mediated gene transfer, electroporation or viral methods. It was reported that certain zebrafish cell lines can be used as transfection hosts [6, 16, 19]. In this study, we tested the expression of a GFP-fused actin marker construct in ZF4, PAC2 and FHM cell lines using the lipid transfection reagent Fugene 6 (Figure 2, Table 2). The GFP fluorescence localized in actin filaments in all cell lines. Transfection efficiency was determined by semi-quantitative analysis using confocal laser scanning fluorescence microscopy (CLSM). More than 10% of transfected ZF4 cells showed a fluorescent signal, which is slightly lower than the efficiency achieved in FHM cells. In the PAC2 cell line only 5% of the cells showed detectable fluorescence.

Stable integration of foreign DNA in cell lines can be obtained by using antibiotics selection, neomycin (G418) being the most widely used selection reagent. We tested the cellular response of ZF4 and PAC2 cell lines to different doses of G418 (0.2, 0.4, 0.6, 0.8 and 1 mg/ml in complete growth medium). ZF4 cells were killed within 9 days by 0.8 and 1 mg/ml G418, and PAC2 cells were killed within 10 days by 1 mg/ml G418. Using 1 mg/ml G418 we were successful in obtaining stable transfected cell lines of ZF4 expressing a GFP marker gene after 20 days of selection (data not shown).

Efficient gene transfer in zebrafish cell lines by nucleofection

Nucleofection is a relatively new electroporation-based transfection method. Cells are suspended in specific Nucleofector Solutions providing cell-friendly environments and foreign DNA is delivered directly into the nucleus by electric pulses, which largely increases the transfection efficiency in hard-to-transfect cell lines. We performed this technique in ZF4 and PAC2 cell lines using the fluorescent protein-expressing vector pmaxGFP, which is provided by Amaxa as a positive control for nucleofection optimizations. We tested nucleofection solution T and V as supplied by the company Amaxa, and eight electroporation programs with different strength of electric field and length of electric pulses (Table 2, Figure 3). Nucleofection efficiency was determined by semi-quantitative analysis using CLSM. Results showed that nucleofection can be used for transfection in ZF4 and PAC2 cells, providing a higher efficiency than liposome-mediated transfection (Figure 3). Optimal nucleofection solutions and electroporation programs for both cell lines are listed in Table 2.

Table 2. Transfection efficiencies of Fugene transfection and nucleofection in zebrafish cell lines.

Zebrafish cell lines were nucleofected with pmaxGFP, using two nucleofection solutions (T and V) and eight electroporation programs (A23, A27, G16, O17, T01, T16, T20 and T27).

	ZF4	PAC2	FHM
Transfection efficiency by Fugene	15%-20%	5%	≥20%
Transfection efficiency by nucleofection	≥70%	40%-50%	Not tested
Optimized nucleofection solution	V	V	-
Optimized nucleofection program	T27	T27	-

Affymetrix microarray analysis of gene expression in zebrafish cell lines

Transcriptome analyses of ZF4 and PAC2 cell lines were performed using the Affymetrix GeneChip Zebrafish Genome Array (GeneChip 430). There are 15502 oligonucleotide sets on each Affymetrix chip, 14895 of which can be linked to a UniGene assignment (Unigene data set 06-12-2005). Since both cell lines were derived from 24-hour zebrafish embryos, microarray data of the cell lines were compared with data obtained from adult zebrafish as well as 24-hour zebrafish embryos and analyzed using the same Affymetrix GeneChip. Our results showed that the number of genes for which significant expression was detectable in ZF4, PAC2, adult zebrafish and 24-hour embryos are different. The number of oligonucleotide sets with detectable signal in ZF4, PAC2, adult zebrafish and 24-hour embryos are 9360, 8460, 9513 and 9768, respectively. The overlap of the expression data for all RNA sources is shown by a Venn diagram in figure 4. There are 360 oligonucleotide sets, which represent 349 unique genes, that gave a significant signal for both cell lines while the signal for these genes in adult fish or 24-hour embryos was not detectable. 351 oligonucleotide sets (337 genes) were detected only in the ZF4 cell line, not in any other RNA sources, whereas 165 oligonucleotide sets (161 genes) were only detected in the PAC2 cell line. These genes were manually mapped to their putative human homologs and annotated based on the public Gene Ontology (GO) annotation. Table 3 shows the distribution of these cell line-specific genes over different functional categories. The detailed annotations and data for these genes are presented in Supplementary Table 1.

Gene expression profiles of ZF4 and PAC2 cell lines in response to serum treatment

We also examined the gene expression profile of ZF4 and PAC2 cell lines under different culture conditions. Usually serum present in the medium is required for maintaining a cell culture, but it is possible to maintain zebrafish cell cultures in viable condition in the absence of serum for over three days. In this study, ZF4 and PAC2 cells were seeded in 0.5% or 1% FCS, respectively, and grown to 85% confluence and subsequently cultured for 24 hours without serum. Then they were treated with either medium without serum or medium with serum (ZF4 in 10% FCS and PAC2 in 15% FCS). After 6 hours, RNA was extracted from the cells and analyzed using the Affymetrix chip as described above. The resulting datasets were analyzed using the Rosetta Resolver software package. The numbers of differentially expressed genes detected at different P-values are shown in Figure 5. The results show that ZF4 and PAC2 cell lines had different expression profile responses in the absence or presence of serum (Figure 6). For example, Txnip (thioredoxin interacting protein, NM_200087) was 1.4-fold lower expressed in ZF4 cells treated with FCS compared to the serum-starved ZF4 cells, but 38.4-fold higher expressed in PAC2 cells treated with FCS compared to the serum-starved PAC2 cells. Vegf (vascular endothelial growth factor, NM_131408) was 3-fold up-regulated in ZF4 cells in the presence of FCS, and 1.5-fold down-regulated in the presence FCS in PAC2 cells.

Quantitative real-time PCR (qPCR) was performed to verify the data obtained by microarray analysis. We selected five genes that showed differential expression and β -actin was taken as reference (Figure 6). The results of quantitative real-time PCR

analysis confirmed the expression change of the selected genes demonstrated by microarray analysis and they confirmed the unique gene expression profiles of ZF4 and PAC2 cell lines.

Figure 4. Venn diagrams showing comparison of the number of sequences expressed in zebrafish cell lines, 24-hour embryos and adult fish.

Microarray data sets from different RNA sources are represented by ellipses outlined in different colors with numbers of the genes in each cluster. Numbers of genes present in unions of different data sets are underlined with colors representing each RNA source.

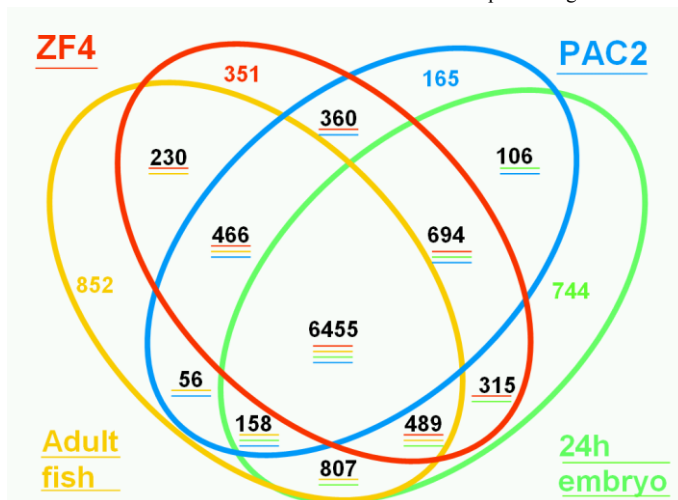


Table 3. Distribution of cell line-specific genes over different categories.

Functional category	GO ID	ZF4 only	PAC2 only	Joint
Apoptosis or cell proliferation	GO:0006915; GO:0008283	9	2	13
Cell cycle	GO:0007049	5	1	5
Cell differentiation	GO:0030154	6	1	4
Cytoskeleton	GO:0005856	9	2	5
Extracellular matrix metazoa	GO:0005578	3	3	1
Membrane function	GO:0016020	8	7	10
Metabolism protein	GO:0019538	11	4	7
Metabolism nucleotide	GO:0009117	13	2	11
Metabolism other	GO:0044237	18	18	32
Signal transduction	GO:0007165	31	19	40
Stress or immune response	GO:0006955; GO:0006950	2	6	5
Transcription	GO:0006350	18	6	17
Transporter activity	GO:0005215	6	1	7
Other		12	10	20
Unknown	GO:0000004; GO:0005554; GO:0008372	186	79	172

Figure 5. Statistical analysis of microarray data.

The graph displays the number of genes in ZF4 and PAC2 cell lines that showed ≥ 2 - or ≥ 3 -fold upregulated or downregulated expression in response to serum at different P-values determined by Rosetta Resolver software analysis.

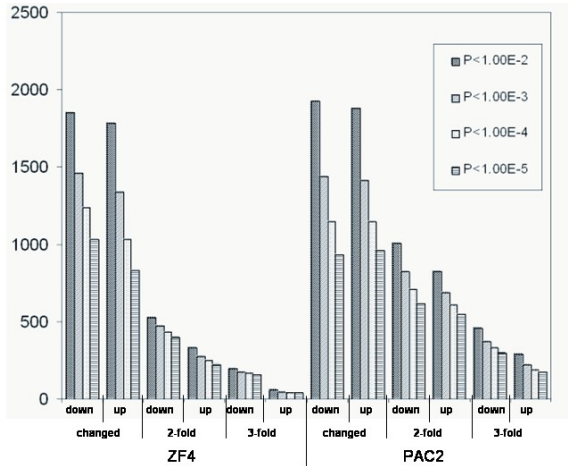
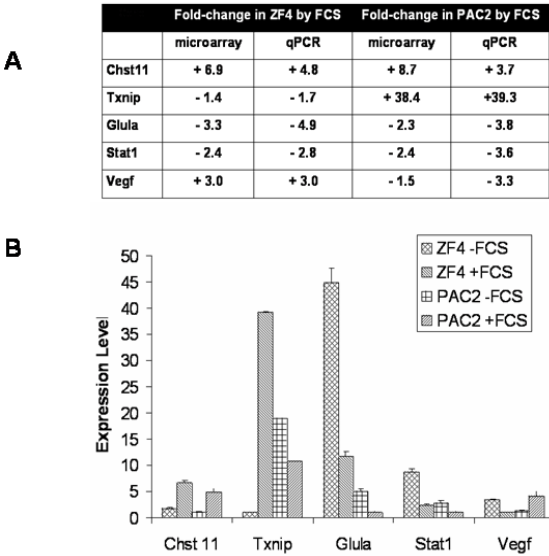


Figure 6. Confirmation of microarray results by quantitative real-time PCR.

(A) Quantitative real-time PCR was performed on five genes that showed differential expression in response to serum in ZF4 and PAC2 cell lines: Chst11 (carbohydrate sulfotransferase 11, NM_212824), Txnip (thioredoxin interacting protein, NM_200087), Glula (glutamate-ammonia ligase a, NM_181559), Stat1 (signal transduction and activation of transcription 1, NM_131480) and Vegf (vascular endothelial growth factor, NM_131408). Their fold-changes detected by microarray and qPCR assay are listed in the table. Induction by serum is indicated by '+' and repression by serum is indicated by '-'. (B) Bars represent the expression level compared to the β -actin housekeeping gene.



Discussion

In the present study we characterized the genomic stability and the transcriptome profile of two zebrafish cell lines, ZF4 and PAC2, under different cell culture conditions. Our detailed analyses make these cell lines very valuable to be used as model cell lines for zebrafish research. Since zebrafish cell lines are easy to maintain at room temperature they are also very attractive for general microscopy applications outside the zebrafish field. The availability of the ZF4 cell line in the ATCC collection makes this cell line very attractive for standard analyses.

Transfection is an essential tool for numerous *in vitro* applications including studies of gene expression and intracellular cell signaling. In comparison with many mammalian cell lines, zebrafish cell lines have not been intensively exploited for genetic manipulations. One reason is that most cultured fish cells appear to be sensitive to transfection reagents commonly used in mammalian cell lines [20]. We showed that transient and stable transfections can be performed in ZF4 and PAC2 cell lines by lipid mediated transfection reagents. However, we noticed that under the same condition, transfection efficiencies achieved in ZF4 and PAC2 lines are lower than the efficiencies achieved in other fish cell lines, such as FHM and ZFL cell lines, and some widely used mammalian cell lines such as HEK293 and Jurkat cell lines (data not shown). Since efficient gene transfer is required for many applications in cell lines, we analyzed the use of nucleofection, a recently developed electroporation-based transfection method in ZF4 and PAC2 cell lines. Here we report the successful and efficient introduction of a GFP marker construct in ZF4 and PAC2 cell lines by nucleofection. Therefore nucleofection appears to be a highly efficient gene transfer method for the introduction of genes into zebrafish cell lines, which offers new opportunities for using zebrafish cell lines in various research applications.

By comparing the gene expression profiles of the ZF4 and PAC2 cell lines to the expression profiles of 24-hour zebrafish embryos and adult fish, we revealed 847 genes that were only detected to be expressed in cell lines, including genes involved in cell cycle and proliferation, cell differentiation, metabolism, cytoskeleton dynamics, signal transduction and transcription. These results are very valuable for researchers interested in further studies of these particular sets of genes. In addition, our data showed both similarity and cell-type specific differences at the transcriptome level between the ZF4 and PAC2 cell lines. About eight thousand genes present on the Affymetrix zebrafish genechip are commonly transcribed in these two cell lines, whereas 1385 genes transcribed in ZF4 cell line were not transcribed in PAC2 cell line. On the other hand, 485 genes transcribed in PAC2 were not detected to be transcribed in ZF4. It could be considered surprising that these cell lines are quite different in their expression profiles. However, their appearance in cell cultures and their transfection efficiencies are also quite different (Figure 2) and therefore it is likely that the PAC2 cell line does not represent the same cell type as ZF4. Further studies of these cell lines should indicate the functional relevance of these different expression patterns. Our data has been submitted to ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) to present a resource data set that can be used as an open reference for comparison to data of other groups. It will be useful to compare our data to future expression data

obtained from many different isolated tissues, and in a complementary approach, with data obtained from many other cell cultures.

Serum, the soluble fraction of coagulated blood *in vivo*, is normally encountered by cells involved in wound healing response, which has a proposed link with cancer progression [21]. Study of genomic response of human fibroblast cells to serum and the wound-like gene expression pattern in human cancers showed that the many fibroblast serum-response genes are coordinately regulated in diverse types of cancers. For example, Chang *et al.* reported that a set of core serum response (CSR) genes repressed by serum in human fibroblasts were mostly expressed in a reciprocal pattern in tumors [21, 22].

In this study, we examined the gene expression profiles of the fibroblast ZF4 and PAC2 cell lines in cultures with and without the presence of serum, and discovered sets of genes activated or repressed by serum in zebrafish fibroblast cell lines. Comparison of their profiles showed that more than 1500 genes were regulated in a common manner in both cell lines, whereas around 100 genes were regulated in a cell-line-specific pattern.

Ontology annotation of the zebrafish fibroblast serum-response genes showed that a number of these genes are involved in wound-healing related programs such as cell cycle and proliferation, epithelial cell migration and angiogenesis, similar to the human fibroblast serum response. For example, we found that zebrafish VEGF was 3 folds induced by 6 hours of serum-treatment in ZF4 cell line, which is very similar to the 2 folds induction of VEGF after 6 hours of serum-treatment in human foreskin fibroblasts reported by Lyer *et al.* [22]. In contrast, VEGF was repressed in the PAC2 cell line by the serum treatment indicating that links with cancer gene expression should be regarded in a tissue specificity context where even fibroblast cell types might differ.

In recent publications [21, 23, 24], it was suggested that, due to the conservation of expression profiles at different levels between fish and human tumors, applying comparative transcriptome profile analysis among evolutionary distant species can reveal specific gene expression signatures contributing to the molecular pathogenesis of human cancer. We believe that the further study on responses of zebrafish fibroblast cell lines to growth factors such as those present in serum will benefit from the advantages of the zebrafish cell implant system [5, 25]. This will, for instance, contribute to cross-species validation of models for molecular control mechanisms of cancer.

Materials and Methods

Cell culture

ZF4 cells (ATCC CRL-2050) were grown at 28 °C in a mixture of 90% 1:1 mixed Dulbecco's modified Eagle's medium and Ham's F12 medium (containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate, Invitrogen-Gibco) and 10% fetal calf serum (FCS, Invitrogen-Gibco). PAC2 cells (supplied by Nick Foulkes) were grown at 28 °C in Leibovitz L-15 medium supplemented with 15% FCS. FHM cells (ATCC CCL-42) were maintained at 28 °C in medium consisting of 67% Leibovitz L-15 (Invitrogen-Gibco) and 10% FCS.

Flow cytometric DNA measurement

Cells were plated in 6-well culture plates (Greiner Bio-one GmbH) and cultured to confluency. Cells were washed with PBS-EDTA (PBS + 1mM EDTA, Invitrogen-Gibco), resuspended in 100 µl PBS and 900 µl 100% ethanol and maintained at -20°C for at least 30 min. Prior to analysis, the cells were washed again with 1 ml PBS-EDTA, resuspended to a single cell suspension in 500 µl PBS-EDTA and treated with 7.5 µM Propidium Iodide (Sigma-Aldrich) and 10 µg/ml RNase A (Sigma-Aldrich). After incubation at room temperature for 20 min in darkness, cells were analyzed on a CAIL flow cytometer (Partec GmbH, Munster, Germany). *Agave stricta* leaf was used as internal standard (2C = 7.84 pg).

Transfection in zebrafish cell cultures

Transfections were carried out using the Fugene 6 Reagent (Roche) according to the manufacturer's instructions. Cells were seeded in 4-well cover slide chambers (Lab-Tek II, German Coverglass system) and allowed to grow to 70% confluency. Before transfection, medium was removed from the cells and replaced by serum-free medium. For all transfections, 1 µg of DNA and 3 µl of Fugene 6 Reagent were combined in serum-free cell-specific medium. After a 15 min incubation at room temperature, the DNA:Fugene mixtures were applied to the cells. After 4-6 hours, the cells were washed with fresh medium to remove Fugene.

Nucleofection in zebrafish cell cultures

DNA for nucleofection was prepared using the GenElute Endotoxin-free plasmid kit (Sigma-Aldrich) according to the manufacturer's instructions. 2×10^6 cells were harvested and resuspended in 100 µl Nucleofector Solution (Amaxa, Cologne, Germany) containing 5 µg DNA for each nucleofection. The cell suspension was transferred into a kit-provided cuvette and positioned into a Nucleofector device. The nucleofections were performed with a single pulse using the preprogrammed nucleofection programs according to the manufacturer's instructions (see Table 2). After the nucleofection cells were transferred into 4-well cover slide chambers (Lab-Tek II) containing prewarmed medium using kit-provided plastic pipettes.

RNA isolation from adult zebrafish, embryos and cell cultures

ZF4 and PAC2 cells were seeded in T75 flasks (Greiner Bio-one) and sampled at 95% confluence. Adult zebrafish or 24-hour zebrafish embryos (provided by Georg Otto) were homogenized in liquid nitrogen. Total RNA samples were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. To remove genomic DNA, RNA samples were incubated at 37 °C for 15 min with 10 units of DNaseI (Roche). Next the RNA samples were purified over RNeasy columns (Qiagen) according to the RNA Cleanup protocol in the RNeasy Mini Handbook (Qiagen). Total RNA concentrations were determined spectrophotometrically using a Biophotometer (Eppendorf). Optical density A260/A280 ratios of all samples ranged from 1.8-1.9, indicating high purity.

cDNA synthesis

cDNA synthesis was performed using a TGradient Thermocycler 96 (Whatman Biometra) according to the manufacturer's instructions. RNA samples were identical to those used for microarray hybridization. Reactions were performed in a 20 µl mixture of 150 ng RNA, 4 µl of 5x iScript Reaction mix (Bio-Rad) and 1 µl of iScript Reverse Transcriptase (Bio-Rad). The reaction mixtures were incubated at 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min.

Polymerase Chain Reaction (PCR)

PCR reactions were performed using PerkinElmer PCR Machine (PerkinElmer) according to the manufacturer's instructions. Reactions were performed in a 50 µl volume comprised of 1 µl cDNA, 4 µl of 2.5mM dNTP (Bio-Rad), 3 µl MgCl₂, 25 pmol of gene-specific primers, 1 µl Taq polymerase (Roche) and 5 µl 10x Tag polymerase buffer. Cycling parameters were 94 °C for 3 min, 40 cycles of 94 °C for 30 sec followed by 55 °C for 1 min and 72 °C for 1 min and final elongation for 10 minutes at 72°C. Sequences of forward and reverse primers for profilin 3/8 were 5'-GGCCTTTTTCACCACTGGACTGACT and 5'-GAAACATTGTATGTCTGGCTCTCCATT (accession no. AW422722).

Hybridization of Affymetrix microarrays

Total RNA (2 µg) was used for first and second strand cDNA synthesis as described in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix). For each condition biological duplications were taken. The resulting double-stranded cDNA was used as a template for biotin labeling with the MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. Hybridization and scanning were performed according to standard Affymetrix protocols. Equal amounts of labeled cRNA were used per zebrafish GeneChip array. The 3'/5' ratio values for GADPH were below the acceptable level of 2 in all experiments. The number of present calls varied between 60 and 75%.

Microarray data analysis

Affymetrix GeneChip data were extracted and normalized using Affymetrix GCOS software. The data has been submitted to ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>). For analysis, data outputs were imported into Rosetta Resolver 5.0 (Rosetta Inpharmatics LLC). Individual arrays were normalized using default settings. After that, replicate intensity profiles were combined using the default intensity experiment builder implemented in the Rosetta Resolver system (for details see <http://info.rosettabio.com/>). For the dataset shown in fig 5, cells treated with FCS were compared against pre-starved cells and ratio experiments were built using experiment definition in the Rosetta Resolver system.

Quantitative PCR

Quantitative real-time PCR was performed using the Chromo4 Four-color Real-time PCR detection system (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions. Gene-specific primers for quantitative real-time PCR were designed to generate single gene-specific amplicons of 75-150 nucleotides. Reactions were performed in a 25 μ l volume comprised of 1 μ l cDNA, 12.5 μ l of 2x iQ SYBR Green Supermix (Bio-Rad) and 10 pmol of each primer. Cycling parameters were 94 °C for 3 min to activate the polymerase, followed by 40 cycles of 94 °C for 15 sec and 59 °C for 45 sec. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were done in duplicate or triplicate and the threshold cycle C_T values [26] were plotted against the base 10 log of the amount of cDNA by using Opticon Monitor 3.1 (Bio-Rad) according to the manufacturer's instructions. For evaluation of PCR efficiencies of all primers sets standard curves were generated using serial diluted cDNA samples (dilution factors of 1, 5, 25, 125 and 625) and strong linear correlations between the C_T values and the log of input cDNA amount were obtained, indicating correlation efficiencies ranging from 98% to 101%. Data were normalized using the Genex macro provided by Bio-Rad. β -actin was taken as reference and it showed unchanged expression level between starved and FCS-treated cells based on both microarray and quantitative real-time PCR data. Sequences of forward and reverse primers were 5'- CGAGCAGGAGATGGGAACC and 5'- CAACGGAAACGCTCATTGC for β -actin (accession no. AF057040), 5'- AGGAAATGCGGGAAGATGGTG and 5'- CATAGGTGCGGATGTGATAGTTG for glul (glutamate-ammonia ligase a, accession no. NM_181559), 5'-CGGAGGTGACCAGAGTGATG and 5'- CTGTTGGATGTTTCGTCTAGTTGG for txnip (thioredoxin interacting protein, accession no. NM_200087), 5'- AGTATGAGACTCTGGAGGATGATG and 5'- TTGGCAAACGATGGGAAGC for chst11 (carbohydrate sulfotransferase 11, accession no. NM_212824), 5'- GCGGCTCTCCTCCATCTG and 5'- ACATCCATGAAGGGAATCACATC for vegf (vascular endothelial growth factor, accession no. NM_131408), 5'- CAAGACAATCCTGTTTCAATGGC and 5'- TCGGTGTTGGACTCTCTGAC for STAT1 (signal transduction and activation of transcription 1, accession no. NM_131480).

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Chapter 3

Toll-like Receptor signaling in zebrafish cell lines

Toll-like receptors (TLRs) are a family of key pathogen recognition receptors which recognize microbial molecules and transduce intracellular signaling via recruitment and activation of adapter molecules e.g. TRAF6, which in turn deliver the signal to activate the transcription factor NF κ B and the mitogen-activated protein kinases (MAPKs), which subsequently regulate transcription of many genes involved in inflammation and the immune response. Reverse transcription PCR revealed unique expression profiles of a set of immune-related genes in three zebrafish cell lines (ZF4, PAC2 and ZFL), including TLRs, their downstream signaling molecules, transcription factors and cytokines. The transcription factor NF κ B can be activated by either TRAF6 over-expression or by stimulation with TLR ligands, in a cell-line dependent manner. Stimulation with flagellin, which is recognized by TLR5, activated NF κ B in all cell lines. Microarray analysis revealed distinct transcriptome response in different cell lines upon flagellin stimulation, suggesting that zebrafish cell lines can be used to study specific signaling events involved in pathogen recognition at cellular level.

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Introduction

The innate immune system of vertebrates acts as the first line of host defense against microbial pathogens by detecting and responding to a broad range of cues of invading pathogens directly after infection [1]. Toll-like receptors (TLRs) form a family of key pathogen recognition receptors of the innate immunity and each member recognizes a restricted subset of molecules produced by microbes [2]. The TLR family can be divided into two subfamilies: one subfamily of cell surface TLRs recognizes microbial membrane components such as lipids, lipoproteins and proteins, and the other subfamily, localized on endosomal compartments, mainly recognizes microbial nucleic acid species [3]. After recognition, transduction of intracellular signaling depends on the recruitment of adapter molecules such as MyD88, TIRAP and TRIF and the activation of IRAK family kinases and TRAF6. In turn, activated TRAF6 can deliver the signal to activate the transcription factor NF κ B and the mitogen-activated protein kinases (MAPKs), which regulate transcription of many genes involved in inflammation and the immune response [2, 4]. It is known that different TLRs can exert distinct but overlapping sets of biological effects, which may attributed to both common and unique aspects of the signaling mechanism [4]. However, there is limited understanding of how specificity in the activation of downstream targets is achieved.

The zebrafish, *Danio rerio*, has been recognized as an attractive experimental model for infectious disease and immunity studies because of the small size, rapid generation time, vertebrate biology and the optically transparent embryos [5]. The primary defense mechanisms against microbial agents of zebrafish are similar to those of mammals and TLR signaling mechanisms are already functional in early embryos [5-7]. Especially the transparency of zebrafish embryos allows imaging of cellular behavior related to pathogen recognition and defense, which is impossible or very hard to achieve in mammalian systems.

The 19 putative TLRs have been found in the zebrafish genome, including orthologs of mammalian TLRs 2-5, 7-9 and a group of fish specific TLRs [8]. Previous work in our laboratory characterized the human TLR homologue genes in zebrafish and found up-regulated expression in response to the tuberculosis-inducing pathogen *Mycobacterium marinum* infection [8]. Analysis of the zebrafish embryonic innate immune response to *Salmonella* infection showed that *Salmonella* flagellin was recognized by the zebrafish homologs (tlr5a/b) of the mammalian flagellin receptor TLR5 [7]. Together with fact that exposure of zebrafish to snakehead rhabdovirus or *Edwardsiella tarda* led to up-regulation of mRNA expression of TLR3 and TRAF6 [6], it demonstrated that zebrafish possess TLR-signaling pathways conserved to the mammals. It was also found that multiple TIR domain-containing adaptors, including MYD88, MAL, TRIF and SARM, were expressed in myeloid leukocytes which contributed to wound healing and bacteria phagocytosis in zebrafish embryos [9]. These findings underscore the suitability of analysis of the vertebrate innate immune system in fish. In addition, zebrafish TRIF exhibits sequence divergence from its mammalian orthologs and did not interact with TRAF6 [10]. Together with the fact that zebrafish also lacks the homologue of mammalian TIR domain-containing adaptor TRAM [10], it suggested that evolution of vertebrate TLR pathway and zebrafish can be used as an important model for understanding the evolution of the vertebrate immune system.

One of the current bottlenecks of using zebrafish for biomedical research to supplement studies in other vertebrate models is the lack of *in vitro* zebrafish cell models. As in 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. Owing to the transparency of zebrafish embryos and versatile cell implantation protocols, zebrafish cell cultures can also be used for *in vivo* studies after cell implantation into embryos, to fully explore the power of zebrafish system.

As zebrafish is a relatively new model organism, only a few zebrafish cell lines have been generated. Methods were developed to culture cells isolated from early stage zebrafish embryos and organs of adult fish such as caudal fin and liver [11]. A few laboratories have tried to isolate zebrafish hematopoietic cells, but the establishment of continuous cell cultures has not been reported yet. Most of the established zebrafish cell lines were non-professional antigen presenting cells, described as fibroblast cell lines or fibroblast-like cell lines [12]. Previous reports have shown that signaling pathways involved in innate immunity can be activated in these cell lines [6, 13]. However, their genetic and physiological properties are still not well known, which limits their application.

In order to better characterize the existing zebrafish cell lines and to evaluate their application for immunological research, we analyzed three previously described cell lines, ZF4, PAC2 and ZFL for their response to external bacterial stimuli. The ZF4 and PAC2 cell lines were established from 24 hour post fertilization (hpf) embryos [14, 15]. The ZFL cell line was derived from adult fish liver as the first zebrafish cell line showing a typical epithelial morphology [16]. These three cell lines can be easily maintained for at least 50 passages in conventional medium containing mammalian serum. Transfection and stable cell lines can be achieved in all three cell lines following lipid-mediated methods or by nucleofection. In this work, these three zebrafish cell lines were further characterized for their expression of TLR-signaling compounds and their function in the pathogen recognition. The stimulation with flagellin, recognized by TLR5 in mammals, activated NFκB in all three zebrafish cells lines.

Results

Expression of genes involved in the TLR signaling

Expression of a set of immune-related genes was examined by semi-quantitative reverse transcriptional PCR in three zebrafish cell lines: ZF4, PAC2 and ZFL (Figure 1). In mammals, TLR1, TLR2, TLR4, TLR5 and TLR6 are mainly expressed on the plasma membrane and recognize microbial membrane components [3]. Homologs of TLR1, TLR2, TLR4, TLR5 and TLR6 have been identified in the zebrafish genome [8] and their expression was checked in the zebrafish cell lines. The results showed that TLR1, TLR4a, TLR4b, TLR5a and TLR5b are commonly expressed in all three cell lines. TLR2 had highest expression level in the ZFL cell line and the lowest level in the PAC2 cell line. The expression of TLR18 (a second homologue of human TLR1) was not detected in the PAC2 cell line. Among the TIR-domain containing adaptors downstream of TLRs, MYD88 and TRIF were expressed in all cell lines, whereas the expression of TIRAP was only detected in the ZFL cells. Some other important molecules involved in the TLR signaling, such as TRAF6, ERK1 and ERK2, were also expressed in the three cell lines.

Five transcription factors of the NF κ B family have been found in the zebrafish genome. Four of them, NF κ B2, RelA, RelB and cRel are detected in all three cell lines. NF κ B1 was only detected in the ZFL cell line. Several cytokines known to be downstream of the TLR signaling in human and mouse were also expressed in all cell lines, including IL1 β , IL8, IL12 and TNF α . In general, most of the genes are commonly expressed in all three zebrafish cell lines, with only variations in their expression level.

Over-expression of TRAF6 activates NF κ B

TRAF6 is an important signaling molecule in the TLR signaling, downstream of the TLRs and adaptors such as MYD88, TIRAP and TRIF. It can be activated by TLR-mediated signal transduction and in turn activate the TAK/TAB-I κ B kinase cascade, which leads to activation of NF κ Bs. Activation of TRAF6 can be mimicked by its over-expression in cells [17]. It was reported that over-expression of TRAF6 activated NF κ B in the ZFL cell line [6]. In this study we over-expressed TRAF6 by nucleofection in the three zebrafish cell lines to mimic its activation by TLR signaling and measured the downstream NF κ B activation using a luciferase reporter assay (Figure 2). Independent experiments were performed using two different NF κ B-Luc reporters that were widely used in mammalian cell cultures. No response after TRAF6 over-expression was detected using the reporter NF κ B-Luc-A (containing 5x GGGGACTTTC repeats of κ B enhancer) in any cell line (data not shown). Using the reporter NF κ B-Luc-B (containing 4x GGGAATTTC repeats of κ B enhancer), hyperactivation of NF κ B induced by the over-expression of TRAF6 was detected in the ZFL cell line, but not in the ZF4 and PAC2 cell lines (Figure 2B), strongly indicating the variation in the signal transduction downstream TLR and TRAF6 activation in the three zebrafish cell lines.

Figure 1. Expression of a set of immune-related genes in three zebrafish cell lines.

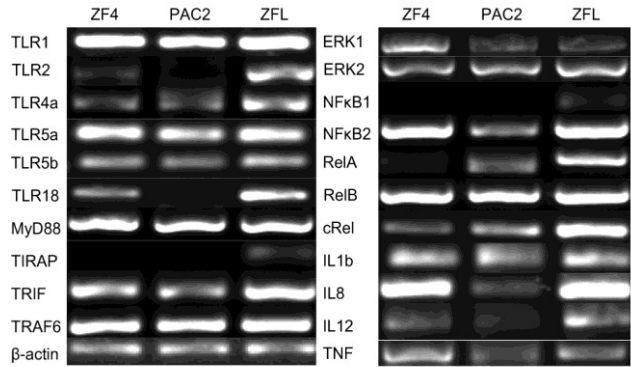


Figure 2. Over-expression of TRAF6 activated NFκB in the ZFL cell line.

A: TRAF6 was over-expressed in zebrafish cell lines by nucleofection. Semi-quantitative RT-PCR was performed to check the TRAF6 expression after nucleofection in the ZFL cells. Data of ZF4 and PAC2 cell lines are not shown.

B: Over-expression of TRAF6 activated NFκB in the ZFL cell line, not detected in the ZF4 and PAC2 cell lines. These results are representative of at least 3 independent experiments with at least 3 biological controls in each cell line.

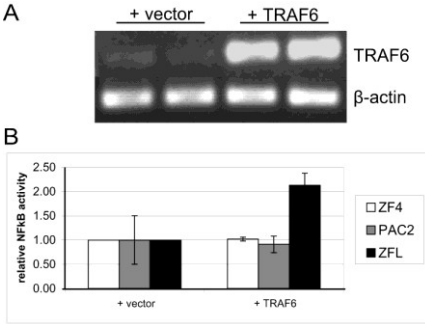
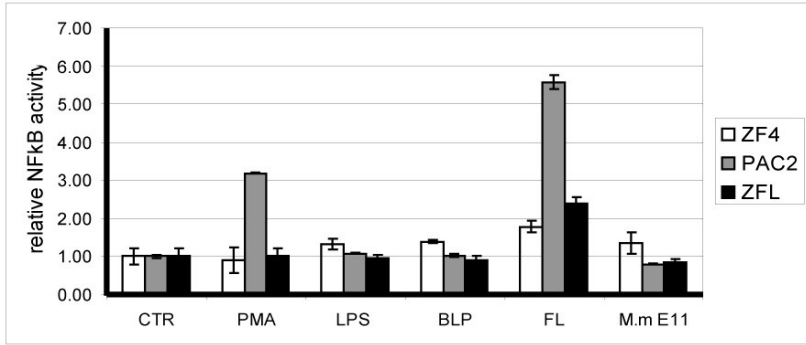


Figure 3. Stimulation with flagellin activated NFκB in zebrafish cell lines.

The zebrafish cell lines were transfected with NFκB-Luc reporters and stimulated for 24 hours with PMA (25 ng/ml), LPS (1 μg/ml), BLP (1 μg/ml), flagellin (100 ng/ml) or extracts of *M. marinum* (5 μg/ml). These results are representative of at least 3 independent experiments with at least 3 biological controls in each condition.



Flagellin stimulation leads to NFκB activation

It is known that the NFκB transcription factors can be activated by TLR signaling upon pathogen recognition [2]. To characterize the functionality of the zebrafish TLRs in zebrafish cell lines, PAC2, ZF4 and ZFL cell lines were stimulated with compounds that commonly function as TLR ligands in mammalian cell cultures. The subsequent NFκB activation was measured after 24 hours of stimulation, using the luciferase reporter assay (Figure 3). Independent experiments were performed using the two NFκB-Luc reporters and experiments using the reporter NFκB-Luc-B did not show a response upon any stimulation in each of the listed cell lines (data not shown).

Using the reporter NFκB-Luc-A, stimulation with PMA, a known activator of human NFκB, resulted in elevated NFκB activity in the PAC2 cell line. The other two cell lines did not respond to PMA, suggesting cell-line specificity of intracellular signal transduction. Despite the expression of TLR2 in all three cell lines, the stimulation with bacterial lipoprotein (BLP), the ligand of human and mouse TLR2, did not induce NFκB activity in any of these lines. In addition, no NFκB activation was detected upon stimulation with lipopolysaccharide (LPS), the ligand of mammalian TLR4. The cells were also stimulated with extracts of *Mycobacterium marinum* (strain E11), a known pathogen of zebrafish [8], but no significant response was detected in the zebrafish cell lines (Figure 3), whereas the same extracts induced NFκB activation in a HEK293 cell line stably expressing human TLR2 (data not shown). In contrast, stimulation with flagellin, recognized by TLR5 in mammals, activated NFκB in all three zebrafish cell lines which was detected by the reporter NFκB-Luc-A.

Distinct transcriptional programs activated by flagellin stimulation

The cell-line dependent activation pattern of NFκB strongly suggested that although many genes involved in the TLR signaling are commonly expressed, the cellular responses towards extracellular stimulation and intracellular signaling downstream of TLR activation are highly cell-line specific.

To further characterize the cellular response upon activated TLR signaling of each cell line, the cells were stimulated with flagellin, which activated NFκB in all cell lines (Figure 3), and a microarray approach was taken to investigate the responses at the transcriptome level after one hour of stimulation. Comparing with non-stimulated cells, expression of 2668 Unigene clusters were significantly up- or down-regulated in the ZF4 cell line, 1224 Unigene clusters regulated in the ZFL cell line and 855 Unigene clusters regulated in the PAC2 cell line ($P \leq 1.0 \times 10^{-5}$, Figure 4A). The overlap of up- or down-regulated Unigene clusters between the three cell lines was limited (Figure 4B). This result indicates that although the NFκB transcription factors were commonly activated in all cell lines after flagellin stimulation, the transcriptome responses of these cell lines were very distinct. 2D-hierarchical cluster analysis showed that the ZFL and PAC2 cell lines had more similarity in their transcriptional response to flagellin than the ZF4 cell line (Figure 5). Many genes involved in immune and wounding responses were regulated in the zebrafish cell lines upon flagellin stimulation, but the common signatures in the three cell lines were very limited (Figure 6). The ZF4 cell line had more immune

and wounding related signatures than the ZFL cell line, whereas the PAC2 cell line had the least signatures (Figure 6). This difference between cell lines was also reflected by the significance of expression level changes in the flagellin-signature sets that more percentage of signatures was regulated ≥ 1.5 fold in the ZF4 cell line than the other two cell lines (Figure 4).

Flagellin stimulation in the three zebrafish cell lines activated distinct genes involved in TLR signaling

To examine the transcriptional responses upon flagellin stimulation at the level of the TLR signaling pathway, map-based pathway analysis was performed using the GenMAPP software [7]. GenMAPP analysis showed that numerous genes involved in the TLR signaling were differentially regulated in the three zebrafish cell lines (Figure 7). In the ZF4 cell line, several genes involved in signal transduction downstream of TLR activation were up-regulated, such as *trif*, *ikbkkg*, *rel*, *rela*, *relb*, *nfkbb2*, *jun* and *atf3*, whereas *irf5* was down-regulated. Several negative regulators of the TLR signaling were also up-regulated, including *traf1*, *socs3a*, *socs3b*, *nfkbiaa*, *nfkbiab*, *dusp1* and *dusp2*. Up-regulation of the negative regulators of the TLR signaling was also observed in the ZFL cell line, where *pik3cg*, *irak3*, *dusp1* and *dusp2* were up-regulated. Different than in the ZF4 cell line, the ZFL cell line showed more genes involved in the TLR signal transduction to be down-regulated (*trif*, *tab3*, *irf5*, *rel*, *relb* and *atf3*) than up-regulated (*tpl2*). Compared to the other two cell lines, less genes involved in the TLR signaling were transcriptionally regulated in the PAC2 cell line: *trif* and *mapk1* was down-regulated and *nfkbiaa* was up-regulated.

It is known that activation of the TLR signaling leads to transcription of pro-inflammatory genes [4], which was also found in the three zebrafish cell lines upon flagellin stimulation. The expression of *il15l*, *ifn* and *tnf β* was up-regulated in the ZF4 cell line. The expression of *il8*, *ifn*, *cox2a* and the two homologs of human TNF α (*tnfa*, *tnfb*) were up-regulated in the ZFL cell line. In the PAC2 cell line, *ifng1.2* and *tnf β* were up-regulated.

Among all these genes involved in the TLR signaling, *tnfb* was the only commonly regulated gene in all three cell lines.

Genes transcriptionally regulated by flagellin stimulation are predicted to be involved in different biological processes

To further characterize the transcriptional responses upon flagellin stimulation in the three zebrafish cell lines, GO term analysis was performed to analyze the functionality of the signatures in an unbiased manner. This revealed cell-line-specific distributions of the signatures over different functional categories (Figure 8). In the ZF4 cell line, the up-regulated signatures were enriched in biological processes such as apoptosis, protein kinase cascade, response to stress, negative regulation of cellular protein metabolic process and regulation of immune system process. The down-regulated signatures were enriched in biological processes including receptor linked signal transduction, cell proliferation, cell projection organization and negative regulation of transcription. In the ZFL cell line, many

Figure 4. Expression profiles of genes responsive to flagellin stimulation in zebrafish cell lines.

A: Total numbers of Unigene clusters that were significantly up- or down-regulated compared with un-stimulated cells ($P \leq 1.0 \times 10^{-5}$).
 B: Venn diagrams showing the over-lap of Unigene clusters up- or down-regulated in the three cell lines ($P \leq 1.0 \times 10^{-5}$).

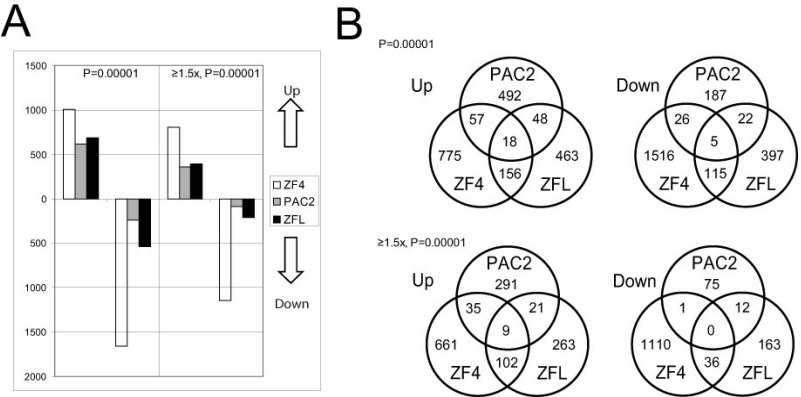
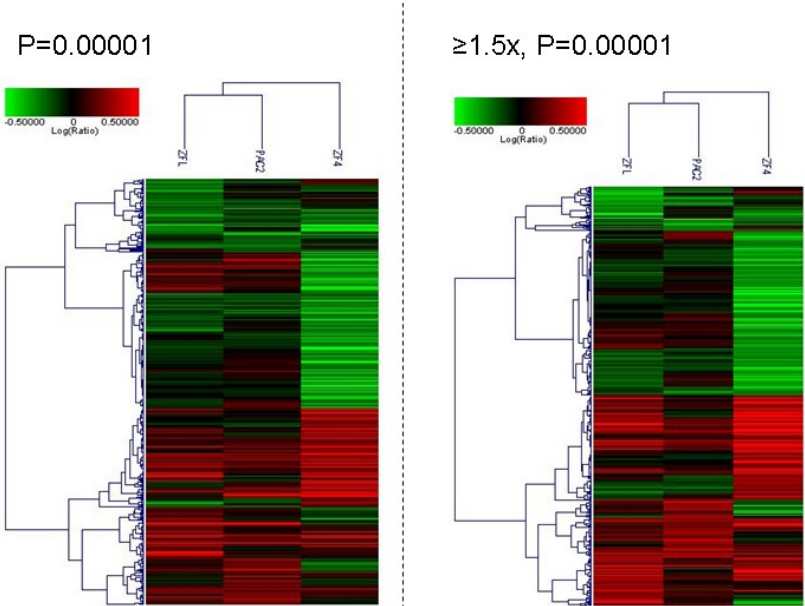


Figure 5. 2D- hierarchical cluster analysis of genes significantly regulated upon flagellin stimulation in the zebrafish cell lines.

Only the Unigene clusters that were significantly regulated by flagellin in at least one cell line ($P \leq 1.0 \times 10^{-5}$) were included for the clustering.



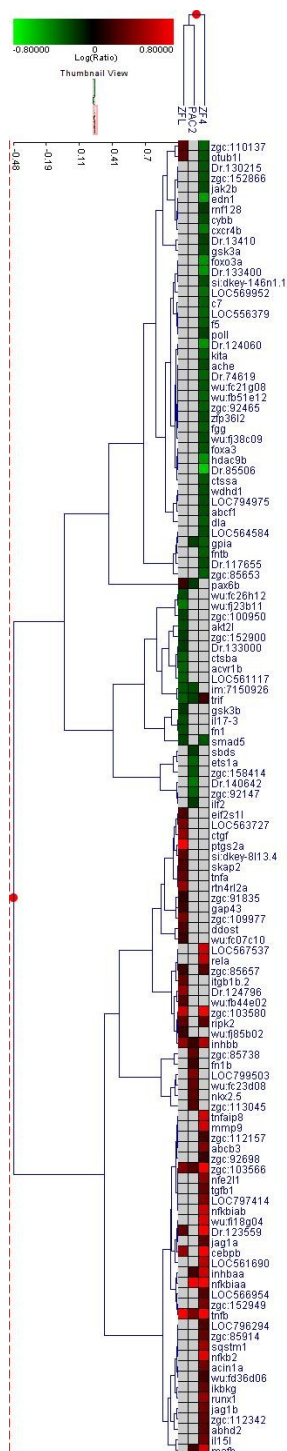
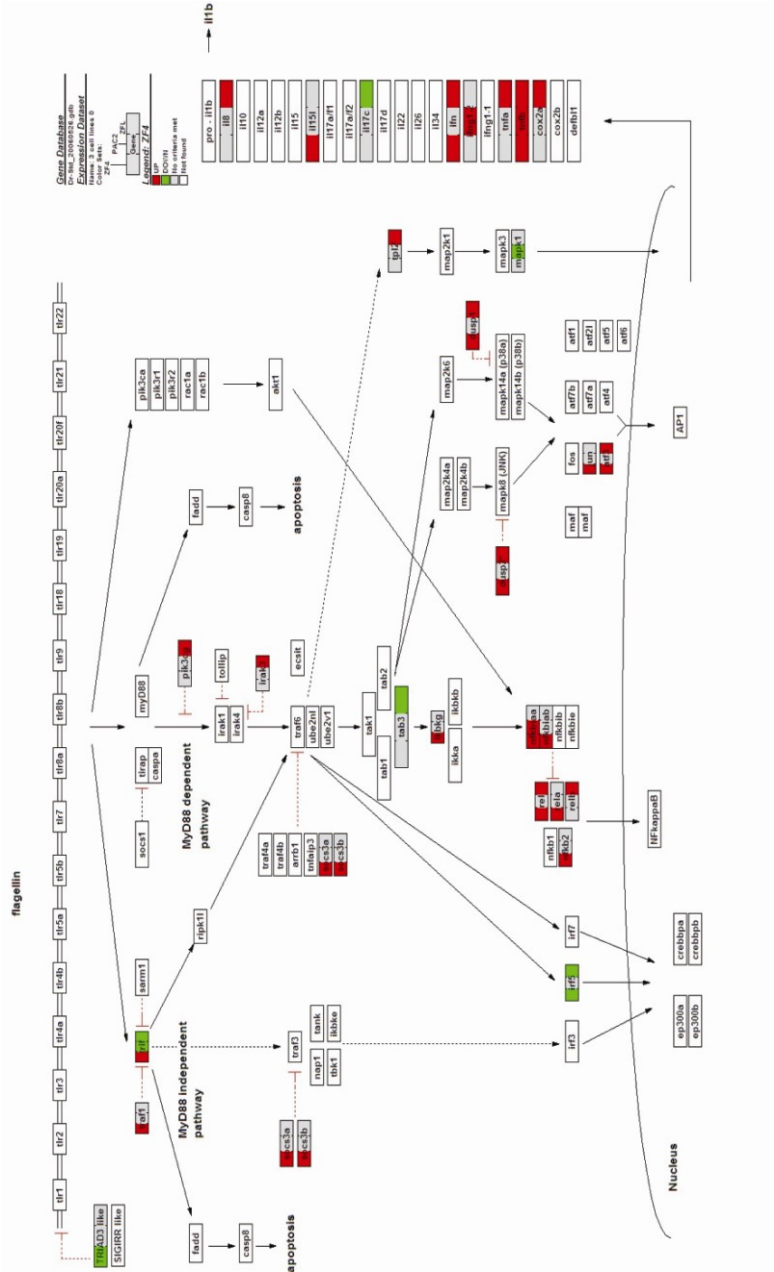


Figure 6. 2D- hierarchical cluster analysis of immune and wounding related genes significantly regulated upon flagellin stimulation in the zebrafish cell lines.

Only the Unigene clusters that were significantly regulated by flagellin in at least one cell line ($P \leq 1.0 \times 10^{-5}$) were included for the clustering. The data points with $P > 1.0 \times 10^{-5}$ are displayed in gray.

Figure 7. GenMAPP analysis of transcriptome responses in the TLR signaling pathway upon flagellin stimulation in the zebrafish cell lines.

Expression profiles of the three zebrafish cell lines upon flagellin stimulation ($P \leq 1.0 \times 10^{-5}$) were simultaneously mapped on the TLR pathway. Gene boxes are colored from left to right with expression data of the ZF4, PAC2 and ZFL cell lines. Up-regulated genes are indicated in red and down-regulated genes are indicated in green. The map is based on knowledge of the TLR signaling in mammalian species. It should be noted that not all interactions have been experimentally confirmed in zebrafish.



deregulated genes were involved in processes related to cellular transport and localization. For example, up-regulated signatures were enriched in transport, endocytosis, protein targeting, regulation of cellular localization, neurotransmitter transport and regulation of vesicle-mediated transport, and down-regulated signatures were enriched in intracellular protein transport, nucleocytoplasmic transport and RNA localization. In the PAC2 cell line, many genes involved in transcription regulation were up-regulated. Other up-regulated signatures were enriched in biological processes such as signal transduction, cell differentiation and cell adhesion. The down-regulated signatures were mainly enriched in metabolic related process, including oxidation reduction, generation of precursor metabolites and energy, protein and cofactor metabolic processes.

In addition to the GO term analysis, enrichment analysis of KEGG pathways was also performed to sort the flagellin-signatures depending on which biological pathways they belong. The three zebrafish cell lines showed cell-line-specific KEGG pathway enrichment patterns (Figure 9). In the ZF4 cell line, the top-three pathways with enriched up-regulated signatures were the MAPK signaling pathway, apoptosis and small cell lung cancer. The top-three pathways with enriched down-regulated signatures were basal cell carcinoma, long-term depression and DNA replication. In the ZFL cell line, many deregulated genes were associated in pathways related to cancer, especially the small cell lung cancer which was enriched by both up- and down-regulated signatures. The focal adhesion pathway was also deregulated in both directions in the ZFL cell line, and many genes involved in the Wnt signaling pathway were down-regulated. In contrast, many genes involved in the Wnt signaling pathway were up-regulated in the PAC2 cell line. TGF β and Hedgehog signaling pathways were also enriched by up-regulated signatures in PAC2 cells, as well as pathways of focal adhesion and adherence junction. It indicates that the cellular inflammation responses in these zebrafish cell lines also involve alterations in signaling pathways associated with cancer.

Figure 8. GO analysis of transcriptome responses upon flagellin stimulation in the three zebrafish cell lines.

Flagellin-signatures ($P \leq 1.0 \times 10^{-5}$) in each cell line were categorized based on GO term analysis at the level 6 of 'biological process'. The functional categories with highest enrichment of regulated transcripts are listed.

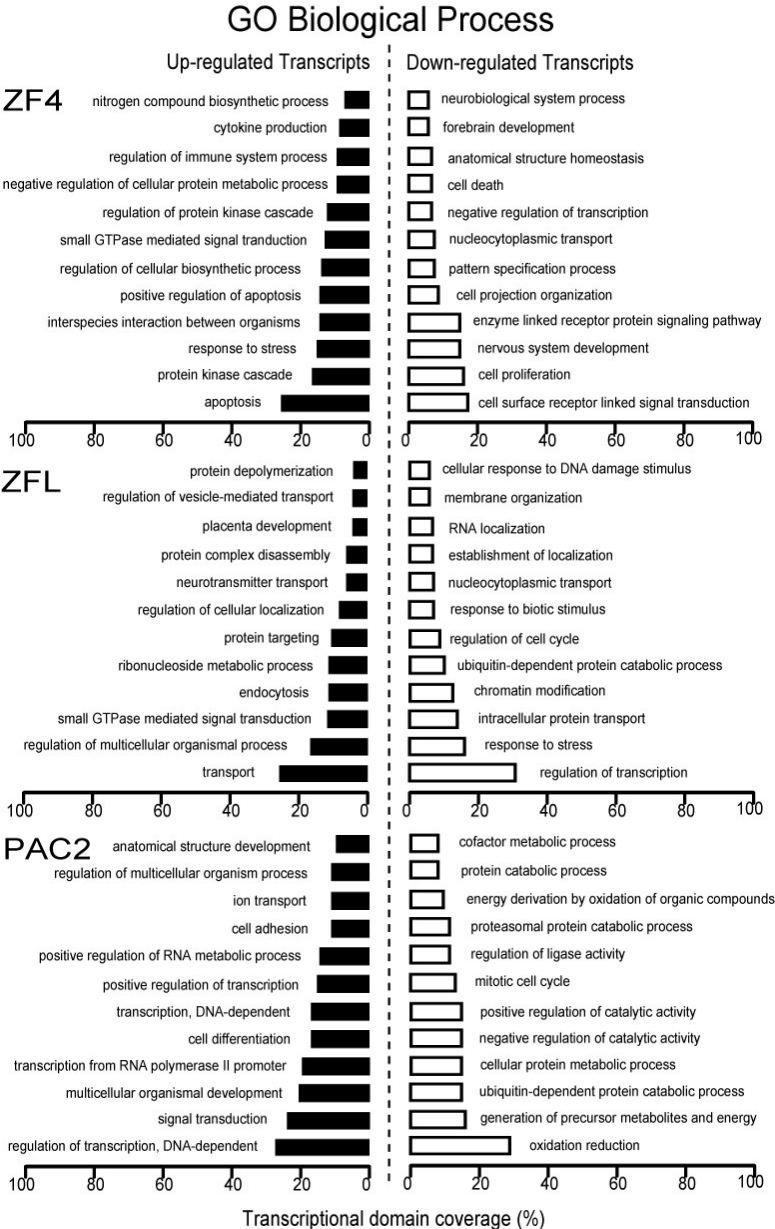
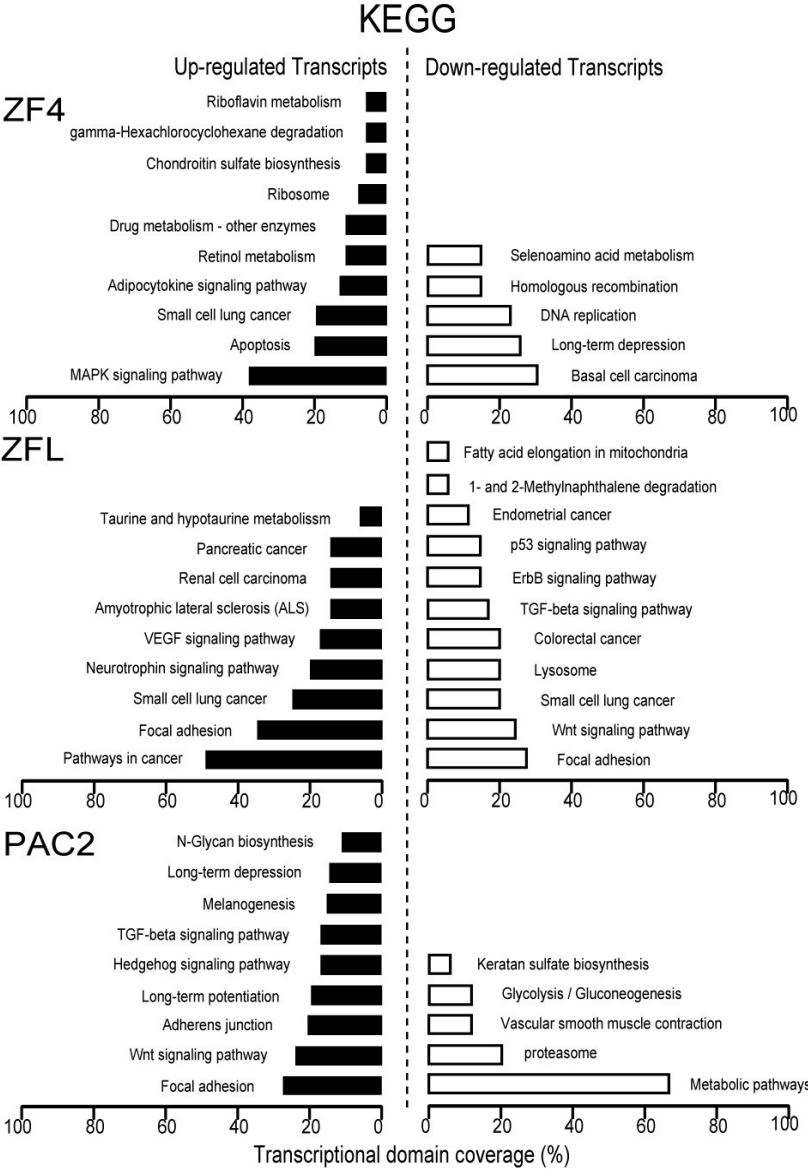


Figure 9. KEGG pathway enrichment analysis of transcriptome responses upon flagellin stimulation in the three zebrafish cell lines.

Flagellin-signatures ($P \leq 1.0 \times 10^{-5}$) in each cell line were analyzed for their enrichment in the KEGG pathways. The KEGG pathways with highest enrichment of regulated transcripts are listed.



Discussion

To explore the possible application of three zebrafish cell lines to study pathogen recognition and innate immunity, the ZF4, PAC2 and ZFL cell lines were first characterized for expression of genes involved in the TLR signaling pathways, such as TLRs, their downstream adaptors and cytokines. Most of the examined genes were expressed in all three cell lines, including homologs of human TLRs that recognize microbial membrane components (zTLR1, TLR2, TLR4a/b, TLR5a/b and TLR18).

TRAF6, the crucial molecule mediating the signal transduction from TLRs to NFκBs, is an ubiquitin ligase that catalyzes its auto-ubiquitination when activated by the TLR signaling [20]. The over-expression of TRAF6 also leads to its auto-ubiquitination [21]. Therefore TRAF6 over-expression mimics its activation and in turn activates NFκBs [6, 21], which was observed in the ZFL cell line. However, it was recently discovered that TRAF6 can also activate NFκB in an autoubiquitination-independent manner [20]. This indicates that the over-expression of TRAF6 does not fully represent its activation via TLR signaling. Since the NFκB activity induced by flagellin stimulation and TRAF6 over-expression were detected by different NFκB-Luc reporters, it suggested that different intracellular signaling pathways were activated after TLR activation by flagellin recognition and over-expression of TRAF6, perhaps by activation of different members of the zebrafish NFκB family. Considering the facts that the expression of NFκB1 was only detected in ZFL cell line but not in ZF4 and PAC2 cells, it is possible that NFκB1 might be activated by TRAF6 over-expression and its activity can be recognized by the NFκB-Luc-B reporter in the ZFL cells.

To characterize the functionality of the TLRs in zebrafish cell lines, several bacterial ligands were used to stimulate the cells. Stimulation with flagellin induced activation of the transcription factor NFκB in all three cell lines. Flagellin was the only compound that induced NFκB activation in all three cell lines. Flagellin is the primary structural component of the bacterial flagellum and in mammals, it is known to be recognized by TLR5 [18]. In zebrafish, it was recently found that TLR5a and TLR5b are required for flagellin recognition in zebrafish embryos [7]. Both TLR5s are expressed in the zebrafish cell lines, suggesting that TLR5a and TLR5b are putative recognition receptors of flagellin in these cells.

Mycobacterium marinum is a fish pathogen genetically closely related to the human pathogen *Mycobacterial tuberculosis*, which causes tuberculosis in fish. In human, TLR1 and TLR2 have been associated with mycobacterial recognition. In zebrafish, it was found that TLR1, TLR2, TLR5a/b, TLR9, TLR18, TLR20a and TLR22 were expressed at higher levels in the adult fish infected by *M. marinum*, compared with un-infected controls [8], suggesting that some of these TLRs might contribute to the mycobacterium recognition by the zebrafish innate immunity system. However, when the cell lines were stimulated with extracts of *M. marinum*, no induced NFκB activation was detected, despite the expression of TLRs in the cell lines. One explanation is that the response towards the pathogen in the three tested *in vitro* cell lines might be different than the *in vivo* situation in adult fish. It also possible that the *M. marinum* was actually recognized but the downstream response was not detected due to lack of sensitivity of the luciferase reporter assay.

In this study two NF κ B-Luc reporters with different κ B enhancers were used. These two reporters were widely used in mammalian cell cultures and induced NF κ B activity was detected by both reporters upon BLP stimulation in HEK cells overexpressing TLR2 (unpublished data). However, only few responses were detected using these reporters in the zebrafish cells. For example, despite the expression of TLR2 and TLR4 in the cell lines, no induced NF κ B activation was detected in any cell line after stimulation with ligands of human TLR2 and TLR4. It is probably due to the specificity of the enhancer recognition by the NF κ B dimmers. The specificity of target gene transcription by NF κ B is determined by the dimerization of different NF κ B subunits and their interaction with other co-activators [19]. TLR activation can result in formation of various NF κ B dimers in the zebrafish cells and not all of the dimers might be able to recognize the enhancer sites in the two reporter constructs used in this work.

Cell-line specificity in responses to activated TLR signaling in the three zebrafish cell lines was suggested by their NF κ B activation pattern upon either ligand stimulation or TRAF6 overexpression. It was further confirmed by transcriptome analysis of the immediate transcriptional responses to flagellin stimulation. Although similar NF κ B activation was induced in all cell lines upon flagellin stimulation, the transcriptome alterations were very different, with limited overlapping gene signature sets. The different transcriptome responses might reflect the different origins of the cell lines. The ZFL cell line is an epithelial cell line derived from adult zebrafish liver [16]. The ZF4 and PAC2 cell lines were derived from 24 hour zebrafish embryos in different laboratories and their exact tissue origins are not known. ZF4 cells have typical fibroblast morphology [14]. Although the PAC2 cell line was originally described as a fibroblast cell line [22], it showed epithelial-like morphology in culture [12]. 2d-cluster analysis showed that the ZFL and PAC2 cell lines shared more common transcriptional responses to flagellin, suggesting that the PAC2 cell line might be closer to an epithelial cell type rather than a fibroblast line.

It has been found that TLRs are widely expressed in most cell types, not only in professional antigen presenting cells (APCs) such as macrophages, dendritic cells and B-cells, but also in non-professional APCs including fibroblasts and epithelium [1]. However, the pattern of their expression and functionality in the non-professional APCs remain poorly characterized. Recently it was found that in mice, TLR5 is not expressed on macrophages and conventional DCs, but on lamina propria cells (LPCs) in the small intestine [23]. These LPC cells induced immune responses and secreted pro-inflammatory cytokines upon flagellin recognition by TLR5 [23]. The fact that flagellin induced inflammatory responses in the three zebrafish epithelial or fibroblast cell lines strongly suggested that these cell lines can be used to understand TLR5 signaling and the biological relevance of TLRs in non-professional APC cells. However, additional cell lines of myeloid origin will also be needed for further immunological studies in the zebrafish model.

Enrichment analysis of KEGG pathways of the flagellin-signatures in the three cell lines has revealed cell-line specific KEGG pathway enrichment patterns. Although the patterns varied in the three cell lines, pathways associated with cancer were among those with highest enrichment of flagellin-signatures in all cell lines. In the ZF4 cell line, genes involved in basal cell carcinoma progression were down-

regulated. Many up-regulated genes were in the MAPK signaling pathway and apoptosis, which are essential events in tumor initiation and progression. In the PAC2 cells, many up-regulated signatures were enriched in focal adhesion, Wnt, Hedgehog and TGF β signaling pathways, the deregulation of which are frequently found in cancer. The association of flagellin-signatures with cancer is the most profound in the ZFL cell line, where the top pathways with enriched signatures were involved in many cancer types, as well as deregulation in the VEGF, Wnt, p53, ErbB and TGF β signaling pathways. These results are consistent with the well known association of inflammatory responses with cancer. It was reported that infection-driven inflammations are linked to 15-20% of human cancers [24]. Recent experimental studies have associated inflammatory pathways to tumor pathogenesis and identified key factors in cancer-related inflammation, such as cytokines and NF κ B transcription factors [24, 25]. A lot remains unclear in this field and more experimental and clinical studies are required. The zebrafish cell lines can be used to further study the connection between inflammation and cancer as *in vitro* models. They can be transformed into tumorigenic cell lines. After implantation into transparent zebrafish embryos or adult fish, the zebrafish cells can also be used to monitor the interactions of the inflammatory microenvironment and tumor development *in vivo* at molecular and cellular level.

Acknowledgement

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Material and Methods

Plasmids and compounds

The NFκB-Luc reporter A (NFκB-Luc-A, containing 5x GGGGACTTCC repeats) was purchased from Straatgene. The NFκB-Luc reporter B (NFκB-Luc-B, containing 4x GGGAATTCC repeats) was purchased from Clontech. Zebrafish TRAF6 coding sequence from ATG to stop codon was amplified from clone NM_199821 using primers TRAF6-ATG and TRAF6-STOP (5'-CGGGATCCTCACCATGGCTTGCAACGACGTGGACAAG-3', 5'-CGAGGAATTCTCAAATTGAAGGTCTGGTCCCTCGAG-3', respectively), and subsequently cloned into BamHI-EcoRI-digested pCS2+ vector. Purified LPS from *Escherichia coli* and flagellin from *Salmonella typhimurium* were purchased from Invivogen. Synthetic bacterial lipoprotein (BLP, Pam3Cy-OH) was purchased from EMC Micro Collections. Phorbol 12-myristate 13 acetate (PMA) was purchased from Sigma. Extracts of *Mycobacterium marinum* (strain E11) was kindly provided by Dr. B.J. Appelmelk.

Cell culture

The ZF4 cell line (ATCC CRL-2050) was grown at 28 °C in a mixture of 90% 1:1 mixed Dulbecco's modified Eagle's medium and Ham's F12 medium (containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate, Invitrogen-Gibco) and 10% fetal calf serum (FCS, Invitrogen-Gibco). The PAC2 cell line (supplied by Nick Foulkes) was grown at 28 °C in Leibovitz L-15 medium supplemented with 15% FCS. The zebrafish liver cell line (ZFL; ATCC, CRL2643) was cultured in complete growth medium (CGM; 50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium, 15% Ham's F12 medium, supplemented with 15 mM HEPES, 0.01 mg/ml insulin, 50 ng/ml EGF and 5% fetal bovine serum; all purchased from Invitrogen) at 28°C without additional CO₂.

Nucleofection

DNA for nucleofection was prepared using the GenElute Endotoxin-free plasmid kit (Sigma-Aldrich) according to the manufacturer's instructions. 1×10^6 cells were harvested and resuspended in 100 µl Nucleofector Solution V (Amaxa, Cologne, Germany) containing 5 µg DNA for each nucleofection. The cell suspension was transferred into a kit-provided cuvette and positioned into a Nucleofector device. The nucleofections were performed with a single pulse using the preprogrammed nucleofection programs T27 according to the manufacturer's instructions.

Luciferase reporter assay

For the luciferase reporter assay, 1×10^6 cells were nucleofected with 2 µg of the NFκB-Luc reporter and 3.6 µg of TRAF6 (or 2.5 µg empty vector as control). After nucleofection the cells were divided into 4 wells of a 24-well plate and incubated for 40 hours. The lysis and luciferase measurement was performed using the Dual-Luciferase reporter assay system (Promega) and a luminescence counter (Wallac Jet 1450, PerkinElmer) according to the manufacturer's instructions.

RNA isolation

Total RNA was isolated from cells using Trizol reagent (Invitrogen). After DNase treatment (DNaseI, Roche) RNA samples were purified using MiniElute columns under the manufacturer's instruction (Qiagen).

RT-PCR

RT-PCR was performed using SuperScript™ III One-Step RT-PCR (Invitrogen).

RNA preparation and microarray hybridization

For RNA isolation, cells were prepared as described before [12]. Agarose gel electrophoresis showed the good quality of isolated and purified RNA. cDNA and amino allyl-modified aRNA were synthesized using Amino Allyl MessageAmpII aRNA Amplification Kit (Ambion). The aRNA was coupled with Cy3/Cy5 (GE Healthcare) and purified using NucleoSpin RNAII (Macherey-Nagel). RNA concentration and coupling efficiency was determined by NanoDrop (Thermo Scientific). 500 ng of labeled aRNA was used for each hybridization on the custom-designed Agilent 4x44k zebrafish microarray containing 43365 oligos (19122 unigene clusters, build 105). Hybridization and scanning were performed according to standard Agilent protocols. The feature extraction software version 9.5, protocol ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction the option 'No background subtraction and spatial detrend' was used. The arrays were scanned twice with 10% PMT and 100% PMT laser power and the XDR function was used to extend the dynamic range with 10-fold.

Microarray data analysis

Microarray data was imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modeling. Data analysis was performed at $P \leq 1.0 \times 10^{-5}$ for unigene clusters. The Unigene and Entrez Gene records of the functionally related human homologs of our zebrafish unigene list of gene signatures were automatically retrieved from the NCBI HomoloGene database. GenMAPP analysis was performed as described before [7]. General Gene Ontology (GO) analysis at the level of 'biological process' and the KEGG pathway analysis were performed using the FunNet software (www.funnet.info) [26] using the Entrez Gene codes of human orthologs.

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Chapter 4

An inducible oncogenic zebrafish liver cell model to study hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The molecular conservation between zebrafish and human liver tumors makes zebrafish a valuable model to study HCC. We established the first reported inducible oncogenic zebrafish cell model, in which oncogenic human Raf-1 can be post-transcriptionally activated in zebrafish liver cells by administration of 4-hydroxytamoxifen (4HT). The Raf-1 activation promoted zebrafish liver cell growth and proliferation, compensated the absence of growth factors, and inhibited apoptosis.

The anti-apoptotic effect of Raf-1 was also observed *in vivo* in zebrafish embryos treated with 4HT after cell implantation. Gene expression profiling of cells treated by 4HT and a MEK-inhibitor identified Raf/MEK-dependent signature sets. This transcriptome response was compared to zebrafish and human liver cancer transcriptome responses and transcriptional markers of hepatocarcinogenesis were identified, showing that the ZFL- Δ Raf1-ER cell line is a valuable model for HCC.

Keywords: zebrafish, hepatocellular carcinoma, Δ RafER, ZFL, transcriptomics, allo-transplantation

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Introduction

Hepatocellular carcinoma (HCC), the major type of liver cancer, is one of the most common malignancies worldwide, accounting for more than 500 000 new cases annually [1]. Recently, a small molecule inhibitor Sorafenib was approved for advanced HCC treatment [2-4]. Sorafenib is a multi-target inhibitor that inhibits both receptor tyrosine kinases and the serine/threonine kinase Raf-1 [2, 3]. Since the Raf pathway is misregulated in majority of cancer cells including liver tumors, it is an important target for the development of therapeutic drugs. Therefore further study of the function of this pathway in cancer is of great importance.

It is known that Raf-1 and its downstream MEK/ERK cascade play essential roles in cell proliferation, differentiation and apoptosis. Deregulation of the Raf/MEK/ERK pathway contributes to pathogenesis and progression of many types of human cancers [5, 6]. In HCC, the Raf/MEK/ERK pathway can be activated by HBV and HCV infection and Raf-1 overexpression has been found in a high percentage of HCC patient tumors, suggesting the involvement of Raf-1 in hepatocarcinogenesis, which is also supported by the success of Sorafenib treatments [7-10]. However, the underlying mechanisms still remain unclear and Sorafenib inhibits not only Raf-1. In order to improve diagnosis and to develop new specific therapies for HCC, it's crucial to overcome limitation of existing animal models and further understand the molecular events involved in HCC, for example, the role of Raf-1 [4, 9, 11].

The zebrafish, *Danio rerio*, has been recognized as an important model for biomedical research in the last decades. The *ex utero* development and manipulative genetics of zebrafish allow large-scale genetics or chemical screenings to discover biomarkers for cancer diagnoses, candidate cancer genes for targeted therapies and lead compounds for new cancer treatments. The transparent zebrafish embryos offers great opportunities to monitor cancer, especially the events during early stages of tumor initiation and progression [12, 13]. Importantly, zebrafish develops liver tumor spontaneously or by chemical carcinogenesis [12-15]. Recent studies showed that zebrafish liver tumors are highly similar to human liver tumors at both histology and transcriptome level, highlighting the potential of using zebrafish to model human HCC [14-16].

Deregulation of the Ras/Raf/MEK/ERK pathway in the carcinogen-induced zebrafish liver tumors was revealed by previous transcriptome analysis [15]. It was also reported that HCC was induced in a transgenic zebrafish line with liver specific expression of oncogenic KRas (Gong *et al.*, in preparation). To develop a new model suitable for studying the roles of Raf/MEK signaling in HCC at molecule and cellular level, we established a stable zebrafish liver cell line (ZFL) expressing oncogenic human Raf-1, which is 83.6% identical to the zebrafish ortholog. In the obtained ZFL-ΔRaf1-ER cell line, the kinase domain of human Raf-1 (ΔRaf1) can be post-transcriptionally activated by 4-hydroxytamoxifen (4HT), which has been validated using different mammalian models [17, 18].

After addition of 4HT, zebrafish MEK-ERK cascade was activated in the ZFL-ΔRaf1-ER cells and the cells acquired a series of survival and growth advantages. The Raf-1 activation promoted ZFL cell growth and proliferation, compensated the

absence of growth factors, and inhibited apoptosis, which was also observed *in vivo* after cell implantation into zebrafish embryos. Microarray analysis confirmed cellular transformation following the Δ Raf1 activation in the ZFL cells. Transcriptome comparison between the Raf/MEK signatures in ZFL cells and zebrafish liver tumor signatures identified a set of genes transcriptionally regulated by the hyperactive Raf/MEK signaling, which also involved in 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.

Results

Establishment of the ZFL-ΔRaf1-ER stable cell line

The ZFL cells were transfected with a ΔRaf1-ER-neo construct and a fluorescent membrane marker YFP-CAAX [19]. Because ZFL cell survival and growth is highly dependent on cell density, probably due to an unknown paracrine mechanism, the transfected cells were maintained in conditioned medium collected from 90% confluent wildtype ZFL cells after 24 hour incubation, which allowed growth of cells in low density during G418 selection procedure. After selection the stable transfected cells were FACS sorted for the YFP-positive cells (Figure 1 and 2A). ΔRaf1-ER expression in the stable cell line remained stable for at least 25 passages (Figure 1).

4HT-dependent ΔRaf1 activation leads to hyper-activation of the zebrafish MEK/ERK cascade

1 μM 4-hydroxytamoxifen (4HT) was applied to the ZFL-ΔRaf1-ER cells to sufficiently activate ΔRaf1 without detectable toxicity effect on the cell growth (Figure 3A). The 4HT administration and ΔRaf1 activation significantly induced phosphorylation of zebrafish MEK1/2 and ERK1/2 in serum-starved ZFL-ΔRaf1-ER cells (Figure 2A). Addition of the MEK inhibitor U0126 to the ZFL-ΔRaf1-ER cells completely abolished induction of the ERK1/2 phosphorylation by 4HT administration, indicating that the ΔRaf1-induced ERK1/2 phosphorylation is dependent on the MEK1/2 activity (Figure 2B). The MEK1/2-dependent ERK1/2 phosphorylation was detected in the ZFL-ΔRaf1-ER cells after short term 4HT administration such as 15 and 30 minutes (Figure 1C). The hyper-activation of ERK1/2 can be sustained for at least 5 days in the presence of 4HT (Figure 2C). 4HT administration didn't induce MEK1/2 or ERK1/2 phosphorylation in wildtype ZFL cells (data not shown).

The 4HT-mediated ΔRaf1 activation promotes cell survival and proliferation *in vitro*

The pro-survival and anti-apoptotic role of Raf-1 has been reported in many cell types [20] and it was examined in the ZFL-ΔRaf1-ER cell line, which has the characteristic growth dependence on cell density. Significant cell loss was observed in wildtype ZFL cells within 2 days when cultured in the absence of growth factors with less than 500 cells/mm², which cannot be rescued by 4HT administration (Figure 3A). In the ZFL-ΔRaf1-ER cells, 4HT administration significantly delayed and reduced the cell loss (Figure 3A). After 5 days, over 95% of the remained ZFL-ΔRaf1-ER cells in the absence of 4HT were positively stained by trypan blue, indicating cell death, and no colony was observed. In the presence of 4HT, colonies formed by healthy ZFL-ΔRaf1-ER cells remained in the culture which later expanded into confluent cell layers over longer periods, despite of the loss of surrounding cells and the lack of growth factors (Figure 3B). It showed that the 4HT activated ΔRaf1 signaling promoted survival of the ZFL-ΔRaf1-ER cells and rescued the cell arrest and death, suggesting the anti-apoptotic function of ΔRaf1.

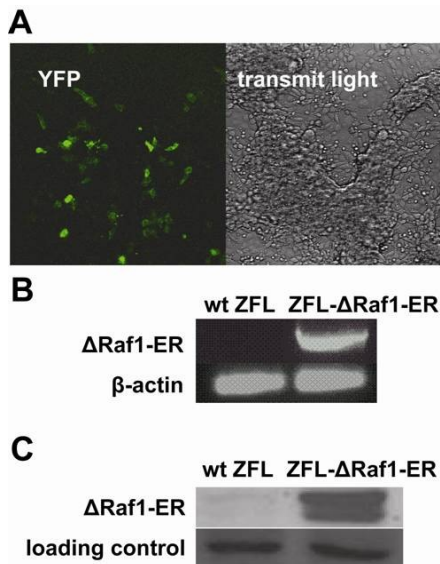


Figure 1. Establishment of the ZFL-ΔRaf1-ER stable cell line.

The ZFL cells were transfected with the ΔRaf1-ER-neo and YFP-CAAX constructs. After G418 selection, the YFP positive cells were sorted by FACS (A). The mRNA expression of ΔRaf1-ER in the ZFL-ΔRaf1-ER stable cell line was detected by One-Step RT-PCR using primers designed against full-length ΔRaf1-ER (B). ΔRaf1-ER protein was detected by western blot using antibody against human ER (Santa Cruz, 1:6000 dilution; C). The wildtype ZFL cells were used as control.

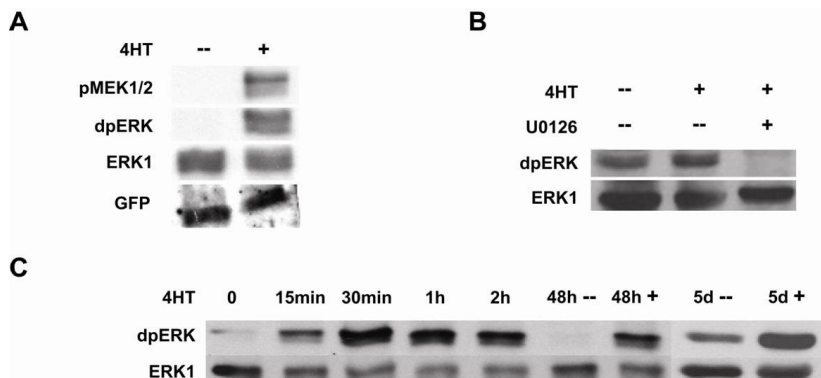


Figure 2. 4HT-dependent ΔRaf1 activation leads to activation of MEK1/2-ERK1/2 cascade.

1 μ M 4-hydroxytamoxifen (4HT) was applied to serum-starved ZFL-ΔRaf1-ER cells to activate ΔRaf1. Phosphorylation of MEK1/2 and ERK1/2 was detected by western blot analysis (A) using antibodies against phosphorylated MEK1/2 (Cell Signaling, 1:1000 dilution) or phosphorylated ERK1/2 (Cell Signaling, 1:1000 dilution). The total amount of ERK was visualized using a ERK1 antibody (Santa Cruz, 1:2000 dilution). Expression of YFP-CAAX in the ZFL-ΔRaf1-ER cells was detected using a GFP antibody (Clontech, 1:1000 dilution). Addition of 30 μ M MEK inhibitor U0126 (Cell Signaling) abolished ERK1/2 phosphorylation in presence of 4HT in ZFL-ΔRaf1-ER cells (B). The ERK1/2 hyperphosphorylation can be detected in the serum-starved ZFL-ΔRaf1-ER cells either after short-term 4HT administration (from 15 minutes to 2 hours, C) or after long-term 4HT administration such as 48 hours or 5 days (D).

These results are representative of at least three independent experiments.

Besides the pro-survival function, the Raf/MEK/ERK signaling plays important roles in cell cycle and proliferation [5]. To quantify effects of the Δ Raf1 activation on ZFL- Δ Raf1-ER cell proliferation, the cell population doubling time (T_d) were measured. 4HT administration largely promoted ZFL- Δ Raf1-ER cell proliferation and significantly shortened the doubling time, from 104.48 to 62.54 hours (Figure 3C). The same 4HT administration didn't give a significant effect on wildtype ZFL cell growth (T_d = 99.33 and 97.07 hours, respectively, in the absence and presence of 4HT; Figure 2A), indicating that the promoted cell proliferation was specifically induced by the 4HT mediated Δ Raf1 signaling.

When cultured in plain medium, in absence of serum and growth factors, the ZFL- Δ Raf1-ER cells were forced into a plateau growth phase (T_d = 232.20 hours; Figure 3D). With 4HT administration, activated Δ Raf1 signaling promoted the cells to proliferate (T_d = 73.53 hours; Figure 3B), which was not observed in wildtype ZFL cells. Thus, the 4HT mediated Δ Raf1 signaling promoted the ZFL- Δ Raf1-ER cell proliferation and transformed the cells to the growth-independence of serum and growth factors.

The 4HT-mediated Δ Raf1 activation promotes cell survival and inhibits apoptosis *in vivo*

To investigate the role of 4HT inducible Δ Raf1 activation *in vivo* in zebrafish embryos, the ZFL- Δ Raf1-ER cells were labeled with the cell tracker CM-DiI and implanted into the yolk of 2dpf TG(fli1:EGFP) zebrafish embryos, which has endothelial-specific GFP expression (Figure 4) [21, 22]. 5 days post implantation (dpi), over 90% of implanted cells went on apoptosis, indicated by formed apoptotic bodies (Figure 4C). When the embryos were incubated with 1 μ M 4HT after implantation, apoptotic bodies were only observed in less than 10% of implanted ZFL- Δ Raf1-ER cells (Figure 4E). The majority of implanted ZFL- Δ Raf1-ER cells remained integrity and showed close association with the host blood vasculatures in 4HT-administrated embryos at 7dpi (Figure 4H). The rescue of cell death by 4HT administration was not observed in embryos implanted with wildtype ZFL cells, where all implanted cells appeared fragmented at 7dpi (Figure 4G). It showed that Δ Raf1 was activated by 4HT in the embryos, which promoted ZFL- Δ Raf1-ER cell survival and inhibited apoptosis *in vivo* after implantation.

Transcriptome analysis of the 4HT-mediated hyperactive Raf/MEK signaling in the zebrafish liver cells

To identify the transcriptional alterations downstream of the hyperactive Raf/MEK signaling in the ZFL- Δ Raf1-ER cells, microarray analysis was performed after 12 hours incubation with 4HT, with or without application of the MEK inhibitor U0126. Expression of 1418 zebrafish unigene clusters was specifically up-regulated by the hyperactive Raf/MEK signaling in the ZFL- Δ Raf1-ER cells and expression of 913 unigene clusters was down-regulated ($P \leq 1.00E-05$; Figure 5A; Supplementary Table 1). 66% of the Raf/MEK-specific signatures (1541/2331, Figure 6A) have been annotated, including homologs of some known transcriptional targets of the MEK/ERK signaling [23], such as *spred2*, *etv5*, *dusp4*, *b4galt6* and *pycr1* (Supplementary Table 1). GO term analysis of these signatures revealed enrichment of the up-regulated transcripts in biological processes such as

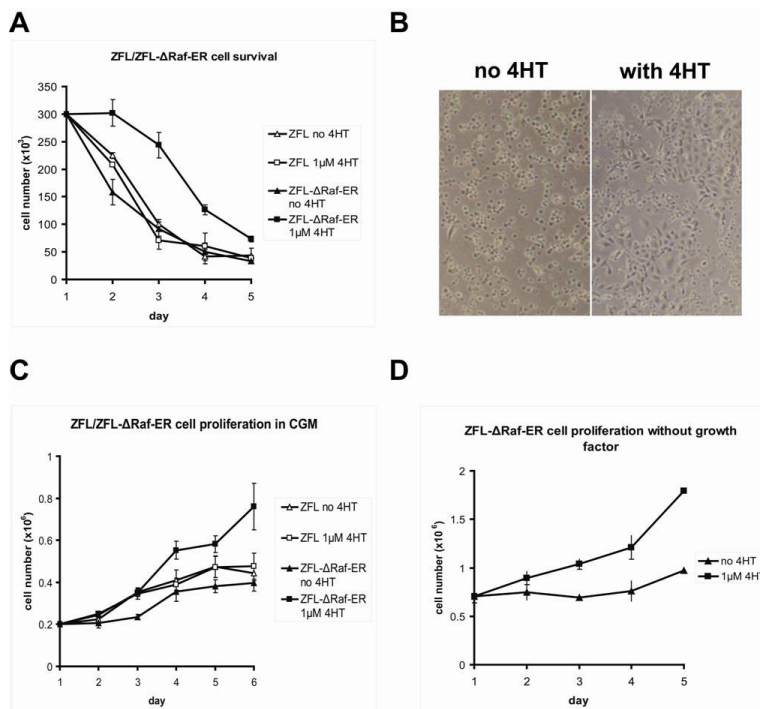


Figure 3. 4HT mediated Δ Raf1 activation promotes cell survival and proliferation *in vitro*.

A: The ZFL- Δ Raf1-ER and wildtype ZFL cells were seeded at a density of 750 cells/mm² in CGM to allow attachment. 12 hours after seeding, CGM was replaced by plain medium with or without 1 μ M 4HT. The cells were counted daily to record the cell loss. 4HT partially rescued cell loss in the ZFL- Δ Raf1-ER cells, whereas not rescuing the wildtype ZFL cells.

B: After 5 day in the lethal condition, healthy cell colonies were only observed in the ZFL- Δ Raf1-ER cells with 4HT administration, which later expanded into confluent cell culture despite the absence of growth factors (data not shown).

C: The ZFL- Δ Raf1-ER and wildtype ZFL cells were cultured in CGM in absence or presence of 1 μ M 4HT and counted daily. ZFL- Δ Raf1-ER cell proliferation was promoted by 4HT administration.

D: When cultured in plain medium without serum and growth factors, the ZFL- Δ Raf1-ER cells stopped proliferation in absence of 4HT, whereas cells proliferated in presence of 1 μ M 4HT. The effect of 4HT administration was not observed in the wildtype ZFL cells in plain medium (data not shown).

These results are representative of at least three independent experiments.

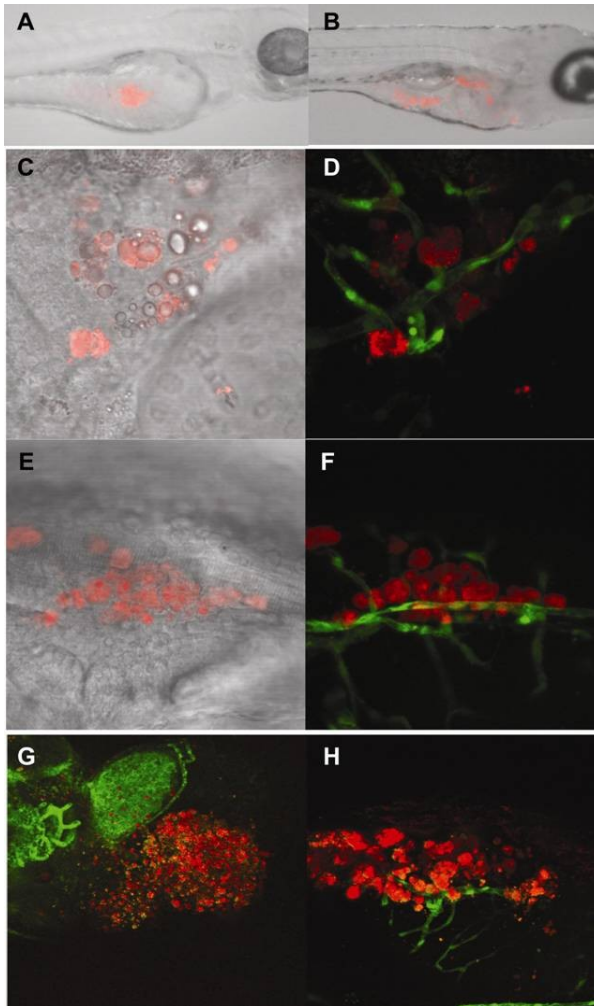


Figure 4. 4HT mediated Δ Raf1 activation promotes cell survival and inhibited apoptosis *in vivo*.

A-B: The ZFL- Δ Raf1-ER cells were labeled with CM-DiI and injected into yolk of 2 dpf zebrafish Fli:GFP embryos. Representative embryos 1 day and 5 days post implantation are shown in A and B, respectively (images taken by Leica fluorescent stereo microscope).

C-F: 5 days post implantation, apoptotic bodies were observed in over 90% of the remained implanted cells in embryos without 4HT administration (C, D), whereas only detected in approximately 5% of remained implanted cell in the embryos administrated with 1 μ M 4HT (E, F).

G-H: 7 days post implantation, in 4HT administrated embryos, all the implanted wildtype ZFL cells were fragmented (G), whereas the implanted ZFL- Δ Raf1-ER cells remained integrity and showed close association with host vasculature in the Fli:GFP embryos (H).

Images in C-H were taken by Leica confocal microscope.

These results are representative of two independent experiments with at least 5 embryos in each condition.

signal transduction, transcription, protein synthesis, cell migration, angiogenesis and cell differentiation. A high percentage of the down-regulated transcripts was involved in the oxidation reduction and cellular metabolic processes, especially carbohydrate, lipid and coenzyme metabolic processes (Figure 5B). Such transcriptional alterations are consistent with the molecular characteristics of HCC. GO analysis also showed the deregulation of the Wnt receptor signaling pathway, which is frequently associated with human HCC [24, 25]. For example, the expression of *wnt4a*, *wnt4b*, *wnt8a*, *wnt10b*, *fzd3*, *fzd6*, *fzd8c* and *fzd10* was significantly altered (Supplementary Table 1). Taken together, the results indicated that the ZFL cells were transcriptionally reprogrammed towards a malignant phenotype after the Δ Raf1 activation. The microarray analysis also identified a set of novel genes which were transcriptionally regulated by the hyperactive Raf/MEK signaling, potentially as transcriptional targets of the Raf/MEK/ERK signaling. It is known that activation of Raf-1 can deliver signal to downstream molecules other than MEK [5, 6]. The use of MEK inhibitor showed that 311 unigene clusters were transcriptionally regulated in a MEK-independent manner (Figure 5A). It was also found that the phosphorylation of histone H3 induced by the Raf-1 activation was not completely abolished by addition of U0126, suggesting that the cell proliferation enhanced by the Δ Raf1 activation is partially due to MEK-independent signaling.

Genes regulated by the hyperactive Raf/MEK signaling in the zebrafish liver cells are associated with zebrafish hepatocarcinogenesis

It is known that the ZFL cell line showed characteristics common to hepatocytes [26], which make up 70-80% of the cytoplasmic mass of the liver. Gene expression profiling indicated that 97% (41987/43365) of the oligonucleotides present on the microarray were commonly expressed in the ZFL cells and livers isolated from healthy adult zebrafish, suggesting the ZFL cells can be used to model signaling in zebrafish liver *in vitro*.

To further dissect the involvement of Raf/MEK signaling in zebrafish hepatocarcinogenesis, the gene signatures regulated by the hyperactive Raf/MEK signaling in the ZFL- Δ Raf1-ER cells were compared with the transcriptome of zebrafish liver tumors (Figure 6A) [14, 15]. This comparison identified 556 unigene clusters commonly regulated in both the zebrafish liver tumors and by the hyperactive Raf/MEK signaling in ZFL cells (513 annotated zebrafish genes and 43 novel transcripts). Significance of the intersections was confirmed by hypergeometric test ($P=0.00E+00$), indicating that these genes regulated by the hyperactive Raf/MEK signaling in ZFL cells were associated with zebrafish hepatocarcinogenesis. The proteins coded by these common gene signatures were involved in biological processes associated with tumor initiation and progression, including cell cycle and proliferation, cell death and apoptosis, cell adhesion and motility, angiogenesis and inflammation responses (Figure 6B), as well as transcription, RNA processing and protein synthesis, transport, metabolism and signal transduction (Supplementary Table 2). The molecular association of the Δ Raf1-reprogrammed ZFL cells and the zebrafish liver tumors validates the ZFL- Δ Raf1-ER cell line as an *in vitro* model to study hepatocarcinogenesis using zebrafish.

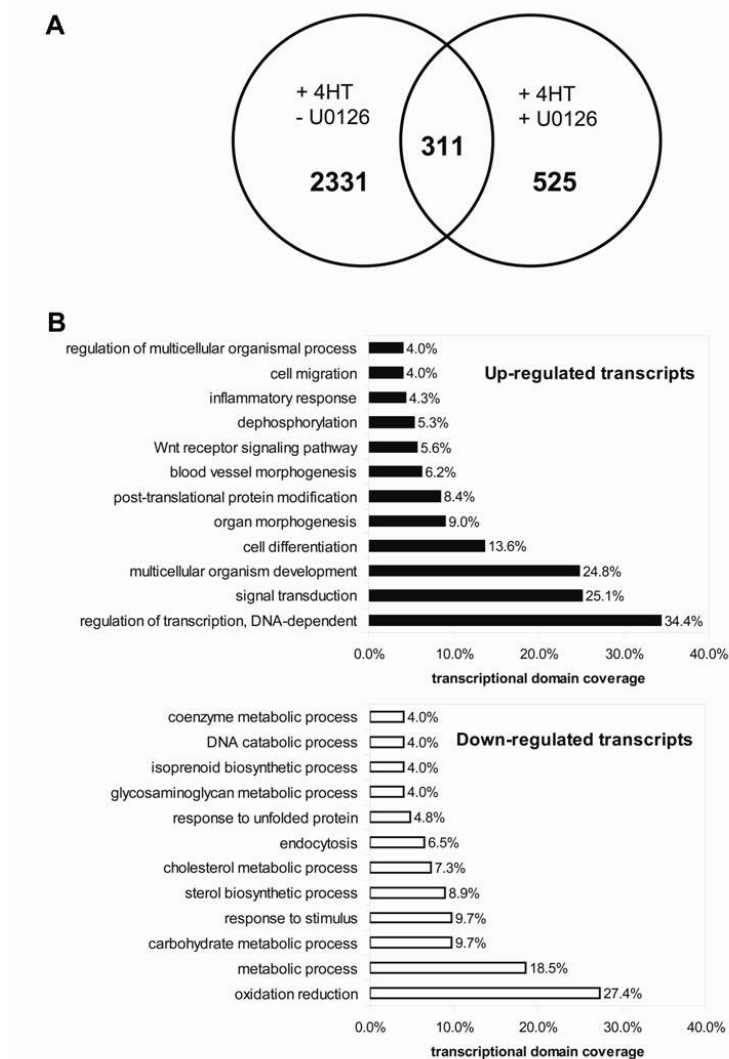


Figure 5. Transcriptome analysis of hyperactive Raf/MEK signaling in the ZFL-ΔRaf1-ER cells.

A: After 12 hours administration of 1 μ M 4HT, expression of 1563 unigene clusters was up-regulated in the ZFL-ΔRaf1-ER cells, whereas expression of 1079 unigene clusters was down-regulated ($P \leq 1.00E-05$). The MEK inhibitor U0126 was used to specify the gene signatures downstream of MEK-independent signaling. After subtraction of MEK-independent transcripts, a set of gene transcripts specifically downstream of the Raf/MEK signaling was identified and highlighted.

B: GO functional analysis of the 2331 genes regulated by the Raf/MEK signaling. Genes were categorized based on GO term analysis at the level of 'biological process'. The functional categories with highest enrichment of regulated transcripts are listed.

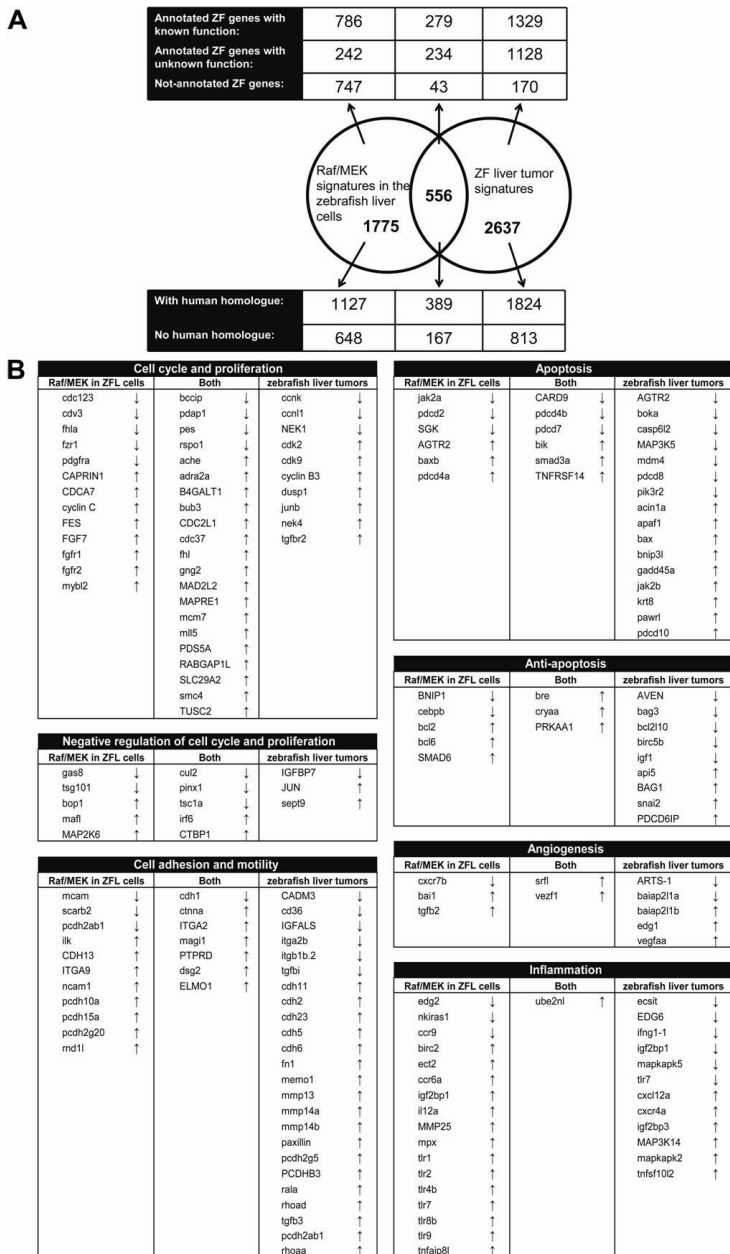


Figure 6. Genes regulated by Raf/MEK signaling in the ZFL cells are associated with zebrafish hepatocarcinogenesis. Comparison of the transcriptome of zebrafish liver tumors to the Raf/MEK signaling induced gene signatures in the ZFL-ΔRaf1-ER cells revealed a pool of 556 common transcripts (A), suggesting their association with zebrafish hepatocarcinogenesis. The signatures were analyzed for gene functionalities involved in tumor progression. Examples of genes are listed (B). The genes in capital letters were analyzed based on functionality of their human homologs.

Common genes regulated by the hyperactive Raf/MEK signaling in the zebrafish liver cells and liver tumors are associated with human HCC

Previous comparative microarray analysis has revealed the molecular conservation between the zebrafish and human hepatocarcinogenesis, suggesting the usefulness of zebrafish to model human HCC [15]. To investigate whether the ZFL- Δ Raf1-ER cells can be used to model human HCC, the 556 common gene signatures regulated by the hyperactive Raf/MEK signaling in ZFL cells and zebrafish liver tumors were analyzed for their human homologs. 389 zebrafish genes were identified with human homologs in the NCBI HomoloGene database. Their human homologs were in turn compared to a number of selected human HCC feature genes, which have been collected in the database of Encyclopedia of Hepatocellular Carcinoma genes Online [27, 28]. The comparison resulted in a subset of 57 genes commonly regulated by the hyperactive Raf/MEK signaling in ZFL cells and in zebrafish liver tumors, which were also correlated to known genetic features of human HCC, comprising 36 up-regulated genes and 21 down-regulated genes (Table 1, Supplementary Table 3). The up-regulated common genes are involved in biological processes such as cell cycle and proliferation (MCM7, POLD1, SMC4, BUB3, NDRG3, MAPRE1), cell adhesion and motility (ITGA2, ACTR3, ELMO1, B4GALT1, CTNNA1, CTTN), transcription (UBE2N, WHSC1, RUVBL2, PAX8), RNA processing and translation (FUS, RPS16, SCNM1) and signal transduction (AGRN, TNFRSF14, CDC37, TBC1D13). Most down-regulated common genes are involved in oxidation reduction (DHTKD1, DHRS1) and cellular metabolic processes (SUCLG2, PANK1, PHYH, ALDH2, ACO1, FAH, OAZ1, UPP1). Some of the common genes are novel and have not been associated with known functionality yet. The common regulation of these genes in zebrafish liver cells, zebrafish liver tumors and human liver tumors suggested the strong association and fundamental importance of these genes in hepatocarcinogenesis across species, which can be further studied in the ZFL- Δ Raf1-ER cell line model.

Table 1. Genes commonly regulated by the Raf/MEK signaling in the ZFL cells, in the zebrafish liver tumors and in human HCC.

A, up-regulation

Unigene	Gene symbol	Gene name	GO
Dr.116619	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	cell adhesion
Dr.114415	FUS	fusion (involved in t(12;16) in malignant liposarcoma)	nuclear mRNA splicing, via spliceosome
Dr.114603	UBE2N	ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	positive regulation of NF-kappaB transcription factor activity
Dr.119248	RNF5	ring finger protein 5	modification-dependent protein catabolic process
Dr.119918	WHSC1	Wolf-Hirschhorn syndrome candidate 1	transcription
Dr.12263	SDPR	serum deprivation response (phosphatidylserine binding protein)	unknown
Dr.132819	CALM2	calmodulin 2 (phosphorylase kinase, delta)	unknown
Dr.24686	RPS16	ribosomal protein S16	translational elongation
Dr.31674	STX5	syntaxin 5	intracellular protein transport
Dr.31830	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	cell motion, regulation of action polymerization
Dr.33356	RRM1	ribonucleotide reductase M1	DNA replication, deoxyribonucleotide biosynthetic process
Dr.35479	RUVBL2	RuvB-like 2 (E. coli)	regulation of transcription, DNA-dependent
Dr.361	C11orf31	chromosome 11 open reading frame 31	cell redox homeostasis
Dr.138569	AGR1	agrin	signal transduction
Dr.47436	MCM7	minichromosome maintenance complex component 7	cell proliferation, transcription
Dr.114210	POLD1	polymerase (DNA directed), delta 1, catalytic subunit 125kDa	DNA synthesis during DNA repair, S phase of mitotic cell cycle
Dr.51273	DAG1	dystroglycan 1 (dystrophin-associated glycoprotein 1)	morphogenesis of an epithelial sheet, protein complex assembly
Dr.76816	SCN1M1	sodium channel modifier 1	mRNA processing
Dr.76826	AKAP8L	A kinase (PRKA) anchor protein 8-like	unknown
Dr.77687	UBAP2L	ubiquitin associated protein 2-like	unknown
Dr.77769	SMC4	structural maintenance of chromosomes 4	cell cycle
Dr.78038	ELMO1	engulfment and cell motility 1	apoptosis, cell motion, Rac protein signal transduction
Dr.78272	C20orf149	chromosome 20 open reading frame 149	cell differentiation
Dr.97335	VPS28	vacuolar protein sorting 28 homolog (S. cerevisiae)	protein transport
Dr.78291	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	cell adhesion and motility
Dr.78635	BUB3	budding uninhibited by benzimidazoles 3 homolog (yeast)	cell proliferation, mitotic cell cycle checkpoint
Dr.79080	TNFRSF14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	apoptosis, cell surface receptor linked signal transduction
Dr.79208	WDR68	WD repeat domain 68	unknown
Dr.80005	NDRG3	NDRG family member 3	negative regulation of cell growth
Dr.80218	CDC37	cell division cycle 37 homolog (S. cerevisiae)	regulation of cyclin-dependent protein kinase activity
Dr.80917	CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	cell adhesion
Dr.8177	PAX8	paired box 8	positive regulation of transcription, DNA-dependent
Dr.82226	CTTN	cortactin	cell adhesion and motility
Dr.83913	MAPRE1	microtubule-associated protein, RP/EB family, member 1	cell cycle, cell proliferation
Dr.89520	TBC1D13	TBC1 domain family, member 13	regulation of Rab GTPase activity
Dr.89609	URM1	ubiquitin related modifier 1 homolog (S. cerevisiae)	modification-dependent protein catabolic process

B, down-regulation

Unigene	Gene symbol	Gene name	GO
Dr.105268	SUCLG2	succinate-CoA ligase, GDP-forming, beta subunit	metabolic process
Dr.11467	PANK1	pantothenate kinase 1	coenzyme A biosynthetic process
Dr.115953	CDH1	cadherin 1, type 1, E-cadherin (epithelial)	cell adhesion
Dr.117252	DHDKD1	dehydrogenase E1 and transketolase domain containing 1	glycolysis, oxidation reduction
Dr.139822	PHYH	phytanoyl-CoA 2-hydroxylase	lipid metabolic process
Dr.14625	COPB2	coatamer protein complex, subunit beta 2 (beta prime)	intracellular protein transport
Dr.28434	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	cellular alcohol metabolic process
Dr.32174	DHRS1	dehydrogenase/reductase (SDR family) member 1	oxidation reduction
Dr.3514	COX4NB	COX4 neighbor	unknown
Dr.3583	SULF6B1	sulfotransferase family, cytosolic, 6B, member 1	unknown
Dr.40063	ACO1	aconitase 1, soluble	metabolic process
Dr.124584	FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	metabolic process
Dr.74875	OAZ1	ornithine decarboxylase antizyme 1	polyamine biosynthetic process
Dr.75635	PINX1	PIN2-interacting protein 1	negative regulation of cell cycle
Dr.76347	C19orf63	chromosome 19 open reading frame 63	unknown
Dr.76704	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	cellular alcohol metabolic process
Dr.78089	COX5A	cytochrome c oxidase subunit Va	unknown
Dr.82487	CCDC94	coiled-coil domain containing 94	unknown
Dr.83978	UPP1	uridine phosphorylase 1	pyrimidine nucleotide metabolic process
Dr.86220	ETFA	electron-transfer-flavoprotein, alpha polypeptide	transport
Dr.116730	C21orf59	chromosome 21 open reading frame 59	unknown

Discussion

Cancer genetics have been studied in human and rodent cell cultures for many years. Validation of the information acquired from these *in vitro* studies into different animal models has largely enhanced our knowledge about cancer. The zebrafish has entered the field of cancer research recently, as a cost-effective model organism sharing the basic molecular cancer biology with human [12, 13]. A few approaches, such as chemical carcinogenesis, forward and reverse genetics, transgenesis and tumor transplantation, have already been used in the zebrafish to model specific cancer types. Comparative oncogenomic studies have validated the carcinogen-induced zebrafish liver cancer as a model to study HCC [14, 15]. In addition, transgenic fish lines have been generated that the KRas oncogene induced liver tumorigenesis and HCC (Gong *et al.*, in preparation). However, an *in vitro* inducible tumor system is not available yet in zebrafish.

In this study, we used the ZFL cell line to establish an *in vitro* HCC model. The ZFL cell line is an epithelial cell line derived from adult zebrafish liver [26]. We showed that it largely represents normal zebrafish liver tissues at the transcriptome level. In the ZFL- Δ Raf1-ER cell line, Δ Raf1 and its downstream signaling was activated by 4HT administration, which validated the usage of the 4HT/ER system to control oncoprotein expression and function for the first time in zebrafish cells. After 4HT administration, hyperactivation of the Raf/MEK signaling in the ZFL- Δ Raf1-ER cells resulted in survival and proliferation advantages, acquired self-sufficiency with respect of growth factors, and rescued cell death. The unconstrained proliferation and impaired apoptosis are both key hallmarks of cancer, suggesting that the zebrafish liver cells were (partially) transformed by the hyperactive Raf/MEK signaling.

The 4HT treatment also promoted cell survival and inhibited apoptosis of the ZFL- Δ Raf1-ER cells after implantation into zebrafish embryos, which validated the usage of the 4HT/ER system *in vivo*. The growth advantage resulted from the hyperactive Raf/MEK signaling allowed studying of longer term events such as cell proliferation and primary tumor formation following the implantation approach. In addition, the close association of implanted cells with the host vasculature (Figure 4), together with the transcriptional regulation of angiogenesis-related genes by hyperactive Raf/MEK signaling (Figure 6B), suggests the usefulness of the implantation approach to study cellular interaction with the host vasculature and angiogenesis in context of oncoprotein activation.

It has been suggested that cancer is the net outcome of series of incorporated alterations in complex signaling networks. Accelerated cell division and proliferation promoted by the Raf/MEK activation increases susceptibility to the step-wise acquisition of additional genetic mutations necessary for malignant transformation [24]. To further understand the involvement of Raf-1 in the zebrafish liver cell transformation, microarray analysis was performed to systematically identify signaling networks and target genes downstream of the hyperactive Raf/MEK signaling. Specificity of the transcriptome analysis was determined by application of the MEK inhibitor U0126.

Because cell transformation is usually due to long-term exposure to cellular alterations caused by the genetic changes, we analyzed the cellular transcriptome after 12 hours of Δ Raf1 activation. The transcriptional alterations resulted from the hyperactive Raf/MEK signaling were involved in many biological processes related to cellular transformation of the ZFL cells. Up-regulation of genes with human homologues known as transcriptional targets by the Raf/MEK signaling indicated the molecular conservation between zebrafish and human cell cultures. A subset of novel zebrafish genes was discovered, as potential transcriptional targets of the Raf/MEK signaling (Figure 6). Longer term of 4HT administration, such as 24 hours, resulted in similar gene signatures.

After identifying a pool of transcripts downstream of Raf/MEK signaling in ZFL- Δ Raf1-ER cells, we performed comparative microarray analysis to systematically validate these gene signatures to zebrafish liver tumors. We discovered that many genes involved in cell cycle, proliferation and apoptosis were commonly regulated by hyperactive Raf/MEK signaling in ZFL cells and in zebrafish liver tumors, indicating the essential role of cell survival and hyper-proliferation in hepatocarcinogenesis. Common genes involved in angiogenesis, cell adhesion and motility were also identified, suggesting their roles in zebrafish liver tumor progression. Strikingly, many genes involved in inflammation, especially the Toll-like receptors, were transcriptionally regulated by hyperactive Raf/MEK signaling in ZFL cells but not in zebrafish liver tumors. It might reflect the dynamic association of inflammation and cancer progression, but the mechanism is currently not understood yet.

Our study has shown that the zebrafish cell lines can be used to bridge cancer approaches at different levels. For example, the anti-apoptotic role of Δ Raf1 activation in the ZFL- Δ Raf1-ER cells was demonstrated *in vitro* by cell growth in culture (Figure 3), *in vivo* by survival of implanted cells in zebrafish embryos (Figure 4), and *in silico* by microarray analysis which showed alteration of apoptosis related gene expression (Figure 6B). It not only adds 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. In addition, we found hundreds of novel transcripts that were specifically regulated by hyperactive Raf/MEK signaling in the ZFL- Δ Raf1-ER cells (Figure 6A). Many of these transcripts were not previously annotated. Most of them have been linked to human homologs, but for many of these the function remains unknown. The ZFL- Δ Raf1-ER cell line can be used for the future functional characterization of these genes, which will bring new insight to understand Raf/MEK signaling and the molecular mechanism of HCC. We believe that modeling HCC using the zebrafish and ZFL cells will provide more knowledge about the molecular and preclinical phases of HCC, and help us to identify a subset of key regulatory genes, which may be subsequently chosen for evaluation as candidate targets for HCC therapies.

Material and Methods

Plasmids

A neomycin resistant vector was generated from the pECFP-N1 (Clontech) by replacing CFP with a short piece from the pEYFP-C1 (Clontech) using BamHI and MfeI. To generate the Δ Raf1-ER-neo construct, primers were designed against the Δ Raf1-ER fragment in pLNC- Δ Raf1-ER (kindly provided by Bob van de Water): forward primer, GGTAGCTGACTGTGTGAAGA; reverse primer, ACCTACAGGTGGGGTCTTTC. Δ Raf1-ER was cloned into pCR[®]4Blunt-TOPO[®] (Invitrogen) following PCR with Phusion (Finnzymes) and subsequently cloned into the neomycin resistant vector using EcoRI.

Cell culture

The zebrafish liver cell line (ZFL; ATCC, CRL2643) was cultured in complete growth medium (CGM; 50% Leibovitz's L-15 medium, 35% Dulbecco's modified Eagle's medium, 15% Ham's F12 medium, supplemented with 15 mM HEPES, 0.01 mg/ml insulin, 50 ng/ml EGF and 5% fetal bovine serum; all purchased from Invitrogen) at 28°C without additional CO₂. Cell population doubling time (T_d) was calculated using Doubling Time Online Calculator (<http://www.doubling-time.com/compute.php?lang=en>). For stable transfection, ZFL cells were nucleofected using Amaxa nucleofector under the manufacturer's instruction (Amaxa, program T27 and buffer V) and followed by G418 selection (1 mg/ml). Stable transfected cells are maintained in CGM with 0.5 mg/ml G418.

Immunoblotting

Cells were lysed in 1x Loading Buffer (Cell Signaling). Proteins were electrophoresed in SDS-polyacrylamid gel and transferred onto nitrocellulose membranes (Schleicher & Schuell). The membranes were subsequently blocked with 5% skim milk in TBS-Tween 20 (TBST) and incubated with appropriate antibodies in TBST with 3% bovine serum albumin (Sigma). Signal was detected using a 1:5000 dilution of horseradish peroxidase-conjugated antibodies and the enhanced chemiluminescence methods (Amersham). Western Re-probe kit (Omnilabo) was used to stripe the blot.

Generation of zebrafish liver tumors

Three-week-old zebrafish fry were treated with 0.75 ppm 7,12-dimethylbenz(α)anthracene (DMBA) or dimethyl sulfoxide (DMSO, vehicle) for 24 h and the treatment was repeated once at 5 weeks old for another 24 hours with 1.25 ppm DMBA or DMSO. Treated fish were rinsed three times in fresh water and transferred into new tanks for maintenance. Fish were sampled during 6-10 months after the onset of DMBA exposure. Tumor samples used for the microarray study were all bigger than 3 mm in diameter. Partial liver tumors were sampled for RNA exaction and the rest of liver tissues were examined by histopathological diagnosis.

RNA preparation and microarray hybridization

For the ZFL-ΔRaf1-ER cells, biological triplications were taken for each condition. 5 zebrafish liver tumors and 6 healthy zebrafish livers were used to detect gene signatures in zebrafish liver tumors. Total RNA was isolated and prepared as described [29]. Hybridization and scanning on the custom-designed Agilent 4x44k zebrafish microarray containing 43365 oligos (19122 unigene clusters, build 105) were performed according to standard Agilent protocols. The feature extraction software version 9.5, protocol ge2_V5_95 from Agilent was used to generate the feature extraction data. For the background subtraction the option 'No background subtraction and spatial detrend' was used. The arrays were scanned twice with 10% PMT and 100% PMT laser power and the XDR function was used to extend the dynamic range with 10-fold. The raw data will be submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo).

Microarray data analysis

Microarray data was imported into Rosetta Resolver 7.0 (Rosetta Biosoftware) and subjected to default ratio error modeling. Data analysis was performed at $P \leq 1.00E-05$ for unigene clusters. The Unigene and Entrez Gene records of the functionally related human homologs of our zebrafish unigene list of gene signatures were automatically retrieved from the NCBI HomoloGene database. General Gene Ontology (GO) analysis at the level of 'biological process' was performed using the GeneTools eGOn v2.0 software (www.genetools.microarray.ntnu.no) [30] and the FunNet software (www.funnet.info) [31] using the Entrez Gene codes of human orthologs.

The featured human HCC gene list was obtained from the database of Encyclopedia of Hepatocellular Carcinoma genes Online (<http://ehco.iis.sinica.edu.tw>), based on 13 microarray studies and PubMed researches related to HCC [27, 28]. The genes regulated by Raf/MEK signaling in the zebrafish liver cells and liver tumors were manually compared with the human HCC gene list using the Entrez Gene codes of human orthologs. The functionalities of the resulted common genes were manually analyzed by searching the NCBI database, PubMed and the Gene Ontology Consortium (www.geneontology.org; Supplementary Table 3).

Cell implantation and zebrafish embryo handling

Cells were labeled with CM-DiI according to the manufacturer's instruction (Invitrogen). After labeling, the cells remained fluorescent up to 14 days, without detected deficiency in survival and proliferation. The labeled cells were injected into the yolk of 2 dpf zebrafish embryos [21]. Zebrafish embryos were raised according to the standard procedures [32]. After implantation, embryos were incubated in daily-refreshed egg water supplemented with 5% DMSO and 0.003% PTU, with or without 1 μ M 4HT.

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Chapter 5

Profile of phosphorylation events controlled by hyper-activation of the MAPK signaling pathway in zebrafish liver cells

We have shown that that hyper-activation of the mitogen-activated protein kinase (MAPK) signaling cascade leads to mitogenic transformation of zebrafish liver cells. To dissect the signaling events involved in this transformation, we profiled the kinase activity and the phosphorylation events caused by hyper-activation of this pathway, using newly developed serine/threonine peptide microarrays. We found that phosphorylation of 7 peptides was strongly affected. Related proteins include radixin (RADI), vasodilator-stimulated phosphoprotein (VASP), cystic fibrosis transmembrane conductance regulator (CFTR), liver glycogen phosphorylase isoform 1 (PYGL), chorionic gonadotropin beta polypeptide (CGHB), forkhead box O3 (FOXO3) and neutrophil cytosolic factor 1 (NCF1). We have discussed the possible functions of these peptides in signal transduction. The findings have validated the application of peptide microarray based kinomics approaches in zebrafish cells, and confirmed the cellular alterations predicted by previous transcriptome study.

Keywords: serine/threonine, phosphorylation, Raf/MEK/ERK, ZFL

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Introduction

Protein phosphorylation is an essential mechanism of eukaryote cellular signaling [1]. It controls the activity, localization, stability and interactions of many proteins. At least 30% of all human proteins can be phosphorylated [2]. The phosphorylation events are catalyzed by protein kinases, which act as regulators of almost all cellular processes by phosphorylation of tyrosine, serine and threonine residues in target proteins, and are regulated by specific phosphatases, which remove the phosphate group added by the kinases. The existence of hard-wired, cell type-specific kinase requirements has been demonstrated in normal healthy cells [3]. Deregulation of the kinases and phosphorylation events often leads to diseases, especially cancer. A large number of kinases are established proto-oncogenes whereas several phosphatases are established tumor suppressors. Currently, protein kinase targets are subjects in 20-30% of the drug discovery programs in pharmaceutical industry [4]. It is estimated that over half of the 518 human protein kinases will be found to be essential in at least one cancer cell line [3]. Hyperactive signaling by kinases can drive cancer growth as well as metastasis. The specific downstream target events of most kinase and their regulation mechanism remain largely unknown, especially those *in vivo* events leading to cell transformation.

In this study we have investigated the specific protein targets of the mitogen-activated protein kinase (MAPK) signaling pathway. This pathway was chosen because its aberrant activation is found in most types of human as well as zebrafish tumors and the MAPK pathway provides a broad target area for cancer drug intervention [5, 6]. However, the downstream signaling events of MAPK pathway remain elusive. Since the recent discovery of all MAPK homologs in the zebrafish, it is considered as a very versatile model to study this protein family. Previous research revealed a high level of genetic and functional conservation of this cascade between zebrafish and human [7, 8]. The serine/threonine kinase Raf and its downstream MEK/ERK cascade play essential roles in cell proliferation, differentiation and apoptosis. To obtain more insights in this pathway, we generated a ZFL-ΔRaf1-ER stable cell line where the inducible activation of human oncogenic Raf-1 activates zebrafish MEK/ERK cascade (He *et al.*, submitted). The hyper-activation of the ΔRaf1/MEK/ERK pathway resulted in a number of survival and growth advantages in the zebrafish liver cells, including the self-sufficiency with respect to growth factors. Unconstrained proliferation and impaired apoptosis are both key hallmarks of cancer, suggesting that the zebrafish liver cells were (partially) transformed by the hyperactive MAPK signaling.

In order to dissect the complex MAPK signaling network and to identify the direct/indirect protein targets regulated via phosphorylation by the MEK/ERK cascade, we took an innovative approach to analyze *ex vivo* kinase activity profiling directly in cell lysates. Pamchip serine/threonine peptide microarrays were applied to profile the phosphorylation events in the zebrafish liver cells transformed by the hyperactive Raf/MEK/ERK signaling. This is a novel non-radioactive technology to profile kinase activity using peptides encoding known phosphorylation sites as substrates spotted onto porous chips (Figure 2) [9, 10]. Application of cell or tissue lysates containing active kinases allows the peptides to be phosphorylated *ex vivo*, which can be detected by fluorescent antibody against the phosphorylated tyrosine or serine/threonine residues. It is a convenient and powerful technology, allowing

detection of kinase activity profiles in the cells and direct identification of the phosphorylated protein targets of the kinase of interest, which facilitate investigation of pathway specific phosphorylation patterns and prediction of the downstream kinase targets for drug discovery. As a novel non-radioactive technology allowing high throughput analysis of kinase reactions, the PamChip® peptide microarrays have been applied to analyze activity of synthetic kinases or lysates from mammalian cell cultures and tissues. It was also used to profile endogenous phosphotyrosine signaling in zebrafish embryos [10]. This study, presents the first time that this technology was applied to zebrafish cell cultures.

Results

4-hydroxytamoxifen-dependent Δ Raf1 activation leads to hyper-activation of the MEK/ERK cascade in the ZFL- Δ Raf1-ER stable cell line

1 μ M 4-hydroxytamoxifen (4HT) was applied to the ZFL- Δ Raf1-ER cells to activate Δ Raf1. The Δ Raf1 activation significantly induced phosphorylation of zebrafish MEK1/2 and ERK1/2 in serum-starved ZFL- Δ Raf1-ER cells (Figure 1). The 4HT administration was performed at the time points 1, 3, 6, 12 and 24 hours, where the most significant hyper-activation of the ERK1/2 was induced by 1 hour 4HT administration. Addition of the MEK inhibitor U0126 to the ZFL- Δ Raf1-ER cells completely abolished the induction of the ERK1/2 phosphorylation, indicating that the ERK1/2 phosphorylation induced by Δ Raf1 activation is dependent on the MEK1/2 activity (Figure 1).

Figure 1. Activation of Δ Raf1 by 4-hydroxytamoxifen induced hyper-activation of the MEK/ERK cascades in the ZFL- Δ Raf1-ER cells.

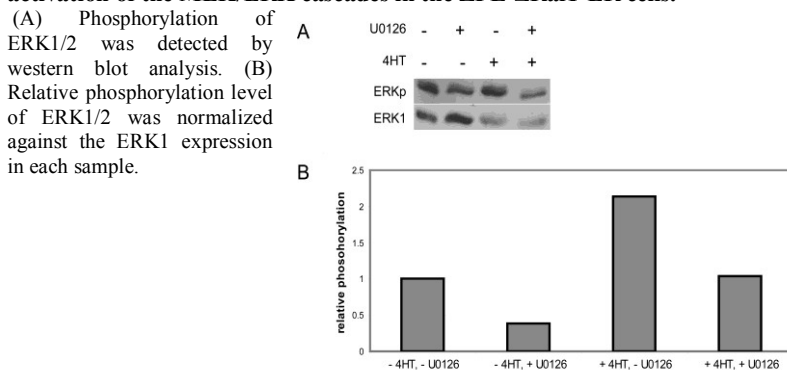
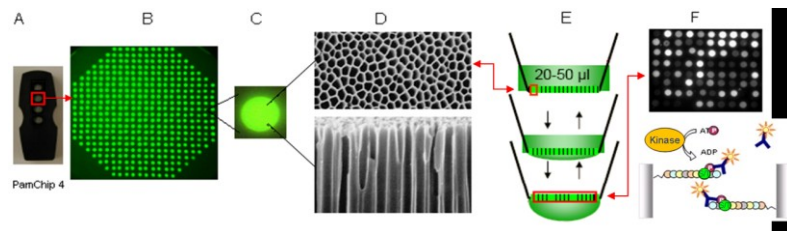


Figure 2. The PamChip® peptide microarray.

The PamChip® serine/threonine peptide microarray (A) contains 144 peptide substrates for serine/threonine kinases (B). Each unique peptide was spotted (C) and coupled to a porous 3D surface made of a microporous material containing millions of 0.2 x 60 micron pores (D). After sample application, cell lysates containing kinases were actively “pulsed” backwards and forwards (E) through these pores where they interact by phosphorylating the immobilized peptides. Phosphorylation of the peptides on the chip is detected by a cocktail of fluorescently labelled anti phosphoserine/threonine antibodies and monitored by CCD camera (F).



PamChip serine/threonine kinase microarrays were applied to profile the kinase activity and phosphorylation events in zebrafish cell lysates

In order to measure the cellular phosphorylation events upon the hyper-activation of the MEK/ERK cascade induced by the Δ Raf1 activation at the kinome level, the PamChip® peptide microarrays were applied using the zebrafish liver cell lysates. The PamChip® peptide microarrays are aligned in multi-well plates (Figure 2A). Each microarray contains 144 peptide substrates serine/threonine kinases (Figure 2B). It incorporates a micro-fluidic system where unique peptides coupled to a porous 3D surface are spotted (Figure 2C). This microporous material contains millions of 0.2 x 60 micron pores in parallel orientation connecting the top and bottom surfaces (Figure 2D). Peptides are pre-coupled to the large internal surfaces of these pores. For kinase activity measurement, samples containing kinases are actively “pulsed” backwards and forwards through these pores where they interact by phosphorylating the immobilized peptides (Figure 2E). Such “pulsing”, combined with minimization of diffusion distances, results in significantly reduced assay times. Due to the high efficiency of this method, usually 1 μ g of total protein is sufficient to monitor 144 phosphorylation reactions. Phosphorylation of the peptides on the chip is detected by fluorescently labeled anti phospho-tyrosine or a cocktail of anti phosphoserine/threonine antibodies and monitored by a CCD camera (Figure 2F). To establish the assay condition, lysates of zebrafish liver cells with various amount of protein contents were loaded on the PamChip® peptide microarrays in the presence or absence of ATP, and the resulted signals were kinetically measured (Figure 3). The results showed that 0.5 μ g protein is optimal, provides significant signal without early saturation and allows kinetic measurement of kinase activities in zebrafish liver cell lysates.

7 peptides were phosphorylated by hyper-activation of the MAPK signaling cascade in the ZFL- Δ Raf1-ER stable cell line

In order to measure the cellular phosphorylation events upon the hyper-activation of the MEK/ERK cascade induced by the Δ Raf1 activation at kinome level, lysates of the ZFL- Δ Raf1-ER cells untreated and treated for 1 hour with 4HT were loaded on PamChip® serine/threonine kinase array. Data processing of the PamChip peptide microarrays using Bionavigator software showed that 7 peptides were specifically hyper-phosphorylated due to 4HT administration and Δ Raf1 activation (Figure 4). Information on these peptides RAD1, NCF1, CGHB, VASP, PYGL, FOXO3 and CFTR is summarized in Table 1.. All of these targets were inhibited by application of the MEK inhibitor U0126, indicating that the hyper-phosphorylation was dependent on the MEK/ERK cascade downstream of the Δ Raf1 activation (Figure 4). The identified targets are involved in cellular processes such as cell adhesion and motility, cellular transport, apoptosis and metabolism, indicating that these processes can be regulated via phosphorylation events controlled by the MAPK signaling. It also suggests that the activation or suppression of these target proteins can be involved in cell transformation.

Figure 3. Optimization of applying the PamChip® peptide microarray to zebrafish cell lysates.

ZFL cell lysates with different amount of protein content were loaded on PamChip® serine/threonine peptide microarrays. Background signal on the arrays was identified using conditions without applying ATP to the lysates.

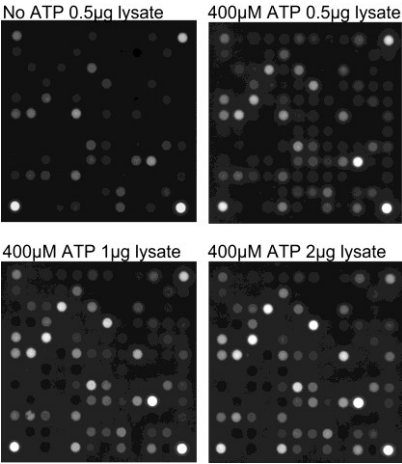


Figure 4. Hyper-phosphorylated of 7 peptides upon ΔRaf1/MEK/ERK activation in the ZFL-ΔRaf1-ER stable cell line.

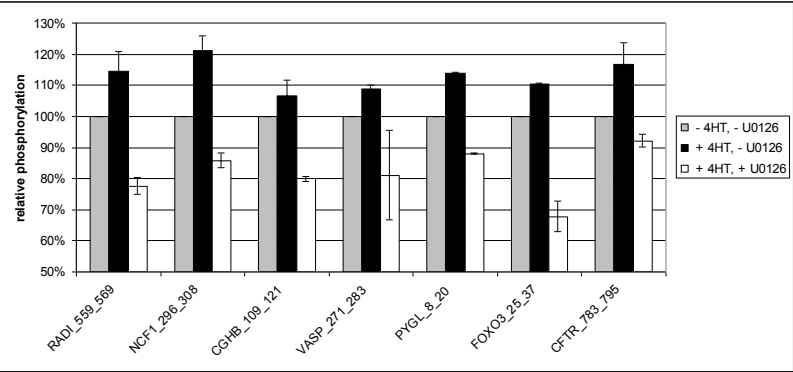


Table 1:

Gene	Full name	Phosphorylated residue	
RADI	radixin	559-569	rdkykTlrqir
NCF1	neutrophil cytosolic factor 1	296-308	rgapprSSirma
CGHB	chorionic gonadotropin beta polypeptide	109-121	qcalcrrSTtdcg
VASP	vasodilator-stimulated phosphoprotein	271-283	larrkaTqvgek
PYGL	liver glycogen phosphorylase isoform 1	8-20	qekrrqiSirgiv
FOXO3	forkhead box O3	25-37	qSrprScTwplqr
CFTR	cystic fibrosis transmembrane conductance regulator	783-795	ihrktttaStrkvS

Description of the proteins represented by the identified target peptides

Radixin (RADI) is a cytoskeletal protein that belongs to the ezrin-radixin-moesin (ERM) protein family. ERM proteins function as cross linkers between the membrane proteins and actin filaments and they are involved in many processes such as cortical cytoskeleton organization, cell survival, adhesion, motility and proliferation [11]. Radixin is the dominant ERM protein in the liver, essential for the formation of the hepatocyte microvilli and maintenance of apical canalicular membrane structure and function in rat hepatocytes [12]. Deregulation of radixin has been associated to liver diseases such as cholestasis [12]. It is known that the activation of radixin is dependent on the phosphorylation of the Thr⁵⁶⁴ in the C-terminal domain, which leads to the conformational alterations of radixin and its binding to the cellular cytoskeleton [11].

In the ZFL-ΔRaf1-ER cell lysate, the hyper-activated MAPK signaling cascade led to the phosphorylation of Thr⁵⁶⁴, which suggests that radixin can be activated by the MAPK cascade in zebrafish liver cells. Thus the MAPK signaling could affect the cytoskeleton organization via regulation of radixin, which in turn affects cell adhesion, motility and vesicular transport.

Vasodilator-stimulated phosphoprotein (VASP) is a member of the Ena-VASP protein family. It was initially characterized as a proline-rich substrate of protein kinases A and G in human platelets and later shown to be a scaffold protein associated with filamentous actin formation and involved in cell adhesion and motility [13]. The hyper-activated MAPK cascade in zebrafish liver cells phosphorylated Thr²⁷⁸, which is located in the EVH2 domain of VASP. This phosphorylation may have an inhibitory role on F-actin binding/bundling by VASP, which in turn affects cellular adhesion to extracellular matrix [14].

Cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) transporter super family, which belongs to the MRP subfamily that is involved in multi-drug resistance. CFTR functions as a chloride channel and controls the regulation of other transport pathways. Its activity is controlled by a series of phosphorylation events, mainly in the central regulatory (R)-domain [15]. In the zebrafish liver cell lysate, phosphorylation of Ser⁷⁹⁰ and Ser⁷⁹⁵ was detected, which are known to be functionally involved in activation of CFTR and modification of its activity. It was reported that the phosphorylation of Ser⁷⁹⁵ (by PKA) was involved in activation of CFTR and the phosphorylation of Ser⁷⁹⁰ (by PKC) might function to modulate the activation [15]. Besides PKA and PKC, other serine/threonine kinases may also phosphorylate and regulate CFTR activity [15]. Our result indicates that the MAPK signaling can induce the chloride influx and affect many signaling events controlled by the chloride. Further experiments are required to investigate whether the MAPK cascade phosphorylate CFTR in a direct manner.

Liver glycogen phosphorylase isoform 1 (PYGL) is a homodimeric protein that catalyses the cleavage of alpha-1,4-glucosidic bonds to release glucose-1-phosphate from liver glycogen stores. It is known that PYGL is switched from inactive phosphorylase B state to active phosphorylase A state by the phosphorylation of Ser¹⁵ [16, 17], which was phosphorylated by the hyper-activated MAPK cascade in zebrafish liver cells. It strongly indicates the essential role of the MAPK signaling

in hepatic glycogen metabolism and support to elevated proliferation of the liver cells.

Deregulation of liver metabolism is often associated with human diseases, especially liver tumorigenesis. In zebrafish liver tumor, many genes involved in metabolism were transcriptionally deregulated, indicating that the cells undergo mitogenic transformation without differentiating into functional hepatocytes [6]. Similar genetic alterations were found in the zebrafish liver cells with hyper-activation of the MAPK signaling (Chapter 4). The phosphorylation profiling in the same cell line has found that a key player of liver glycogen metabolism can be activated by the MAPK signaling.

Chorionic gonadotropin beta polypeptide (CGHB) is a member of the glycoprotein hormone beta chain family and encodes the beta 3 subunit of chorionic gonadotropin (CG). Ectopic expression of CGHB is frequently found in many common epithelial carcinomas and is often associated with high aggressiveness of the cancer and the resistance to therapy [18]. Various studies have shown that the free CGHB may stimulate tumor growth or inhibit its apoptosis [19]. The free CGHB can be phosphorylated on Ser¹¹⁶ and Thr¹¹⁷ [20], which were detected in the zebrafish cell lysate. But the function of these phosphorylation events remains unclear, which might be further characterized in the ZFL-ΔRaf1-ER cell line.

Forkhead box O3 (FOXO3) belongs to the forkhead family of transcription factors. It is involved in the transcriptional activation of proapoptotic molecules [21]. Inhibition of the transcriptional activity of FOXO3 can promote cell transformation, tumor progression, and angiogenesis during tumor development [21]. In response to growth factor and insulin stimulation, FOXO3 can be phosphorylated by kinases including AKT, IKK, and ERK. The three kinases phosphorylate FOXO3 at different phosphorylation sites, which all lead to FOXO3 translocation from the nucleus to the cytoplasm and the subsequent degradation [21]. It is known that Thr³² of human FOXO3 can be phosphorylated in an AKT-dependent manner, which enhances FOXO3/14-3-3 interaction and promotes FOXO3 nuclear export to the cytoplasm, resulting in the repression of FOXO3 transcriptional function [21]. In the zebrafish liver cells, the hyper-activated MAPK cascade also phosphorylated Thr³² of FOXO3, resulting in suppression of its function. It is not clear yet whether the phosphorylation was dependent on AKT. Previous study has shown that the activation of ΔRaf1 inhibited apoptosis in the zebrafish liver cells (Chapter 4). The phosphorylation of FOXO3 identified in this study indicates that the anti-apoptotic role of the ΔRaf1 activation in the zebrafish liver cells might (partially) rely on the suppression of FOXO3.

Neutrophil cytosolic factor 1 (NCF1) also named as p47^{phox}, is a cytosolic subunit of the NADPH oxidase which produces superoxide anion. NADPH is a large membrane bound multi-protein enzyme consisting of six subunits. The NCF1 subunit is the key controlling the assembly and activation of the NADPH complex. In the inactive state, NCF1 stays in an auto-inhibited conformation which prevents its interaction with other subunits [22]. When NCF1 are phosphorylated on Ser³⁰⁴ and Ser³⁰⁴, it can be released from the auto-inhibited conformation and lead to the NADPH activation [22]. The detected phosphorylation of these two serine residues suggests that the NADPH oxidase can be activated by the hyper-activated MAPK cascade in the zebrafish liver cells. It is known that deregulated activation of the

NADPH oxidase can lead to overproduction of reactive oxygen species and inflammatory responses [22]. Thus the phosphorylation of NCF1 and activation of the NADPH oxidase may contribute to the transcriptional alteration of inflammatory genes in neutrophils. Besides the anti-apoptotic function, previous transcriptome study also revealed that the Δ Raf1 activation in zebrafish liver cells led to deregulated expression of many genes involved in inflammation (Chapter 4) perhaps providing a link with this protein.

Discussion

Because deregulated activity of selected kinases is an important feature of cancer cells, kinase activity profiling can provide mechanism-based cancer biomarker identification. To this goal the zebrafish cell model and the activated MAPK signaling pathway was selected for the case study in the search for cancer drug targets using the PamChip peptide microarray application. This kinomics approach provides the unique ability to kinetically profile *ex vivo* kinase activity in cell lysates. It allows rapid assessment of changes of protein activity and signal transduction associated with biological alterations and transformation, providing more insights for drug target candidates.

Our previous study showed that Δ Raf1 was activated by 4HT administration in the ZFL- Δ Raf1-ER cells, which in turns activated the MEK/ERK cascade. The hyper-activation of the MAPK cascade inhibited apoptosis and promoted cell survival and growth. In order to identify the downstream protein targets causing the partial mitogenic phenotype, the kinase activity of these cells were analyzed using the PamChip® serine/threonine kinase array.

The results showed that 7 peptides were specifically phosphorylated by the cell lysates. It indicates that the activity of proteins containing these peptide sequences can be regulated by the hyper-active Δ Raf1/MEK/ERK cascade in zebrafish liver cells. Cellular processes affected by regulation of these protein activities include cell adhesion, motility, vesicular transport, glycogen metabolism, apoptosis suppression and inflammation response. The same biological alterations were revealed by previous transcriptome study using the same cell line, showing that analysis at different level using different approaches can be bridged together and point to the same direction.

The identified phosphorylation events strongly suggest that the related proteins should be involved in mitogenic transformation of zebrafish liver cells. Furthermore, it was discovered using the same kinase array that phosphorylation of Ser^{303/304} of NCF1, Ser⁷⁹⁵ of CFTR and Thr³² of FOXO3 was suppressed by ERK knockdown in zebrafish embryos (data not shown). The fact that these phosphorylations were induced by active MAPK signaling and suppressed by inactivated MAPK signaling shows that these three proteins indeed participate in the signaling network downstream of MAPK in zebrafish liver cells, with high potential as direct protein targets of ERK.

It is also noted that 4 out of the 7 peptides were reported to be phosphorylated by synthetic PKA on the same serine threonine kinase array, including Ser^{303/304} of NCF1, Ser¹⁵ of CGHB, Thr²⁷⁸ of VASP and Ser¹⁵ of PYGL [23]. It had been suggested that Ser⁷⁹⁵ of CFTR can be phosphorylated by PKA [15], but this phosphorylation was not detected *in vitro* on the PamChip. The detection of phosphorylation of PKA substrates in the cell lysate containing activated MAPK cascade can be due to the fact that multiple serine/threonine kinases share the same target. It can also due to the activation of PKA by MAPK signaling, suggesting the crosstalk of the ERK and PKA signaling pathways.

Similarly it was observed that the Thr³² of FOXO3 was phosphorylated upon hyper-activation of the Δ Raf1/MEK/ERK cascade. FOXO3 can be phosphorylated by ERK, AKT and IKK, all leading to inhibition of the transcriptional activity of FOXO3. In human, it is known that ERK, AKT and IKK phosphorylate FOXO3 at different specific sites: ERK on Ser²⁹⁴, Ser³⁴⁴, and Ser⁴²⁵, AKT on Thr³², Ser²⁵³ and Ser³¹⁵, and IKK on Ser⁶⁴⁴ [21]. The detected phosphorylation of FOXO3 Thr³² might be due to the activation of AKT by MAPK signaling. Alternatively, it is possible that the molecular structures/mechanisms of FOXO3 are evolutionally different in zebrafish and in human.

Taken together, we showed that it is feasible to effectively profile kinomic events of zebrafish cells using the PamChip peptide microarray approach. We profiled the kinase activity and phosphorylation events caused by hyper-activation of the MAPK signaling cascade in the zebrafish liver cell line. The results indicated activation of proteins involved in cell adhesion, motility, cellular transport and metabolism, together with suppression of transcription activator of apoptotic factors. The same alterations were observed in the previous transcriptional study on the same cell line, suggesting that the regulation of these proteins can play a role in cell transformation, which can be further dissected using zebrafish liver tumor models. It suggested that the peptide microarray based kinomics approach can be combined with widely used transcriptomics approaches to better analyze signal network involved in cell transformation and malignancies and to select candidate drug targets. In the future, responsible genes and protein targets will be validated using gene knockdown technology or chemical inhibitors of selected protein targets identified by the kinomics and transcriptomics approaches. Such pathway specific mechanistic studies will help to improve our understanding of the basic kinase biology and contribute to drug discovery.

Material and Methods

Cell culture

See chapter 3.

Sample preparation and analysis

The ZFL-ΔRaf1-ER stable cells were seeded in 12-well plate and grew into confluence after 24 hours. The cells were starved for 24 hours in plain medium. After 30 minutes pretreatment in 30 μ M U0126, the cells were incubated in plain medium supplemented with 1 μ M 4-hydroxytamoxifen (Sigma) in absence or presence of 30 μ M U0126. The cells were washed twice with ice-cold PBS and lysed with 100 μ l lysis buffer supplemented with phosphatase inhibitor and protease inhibitor according to manufacture's instruction (PamGene). The lysates were stored at -80°C prior to measurement. Proteins electrophoresis and Western blot protocols are described in chapter 3.

In vitro phosphopeptide profiling

The phosphopeptide profiles were identified using the PamChip® peptide microarrays containing 144 peptide substrates for serine/threonine kinases, according to manufacture's instruction (PamGene)[23]. 0.5 μ g protein of each cell lysate and 400 μ M ATP were loaded on the array. Incubations and kinetic reading of the PamChip peptide microarrays were performed on a PamStation 4 instrument (PamGene), which allows fully automated incubation, washing, and imaging of 4 microarrays simultaneously. Each sample was loaded on two independent arrays for technical duplicates.

Data analysis

Data generated on PamChip peptide microarrays were processed with Bionavigator software (PamGene) [23]. Because most peptides were over-exposed at exposure time of 50 ms while only 1~2 pre-phosphorylated control peptides were over-exposed at 20 ms, the data at exposure time of 20 ms was used for analysis. The average MedBg signal is 361 with a CV of 9% and a STDEV of 32. The peptides with signal above 64 (2x STDEV) were used for comparisons across conditions.

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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 CO₂ 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 NF κ B family (Chapter 3). Stimulation of the three cell lines with bacterial flagellin activated the NF κ B 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 Δ Raf-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 Δ Raf-ER was over-expressed in the zebrafish cell lines. Δ Raf 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. Δ Raf activation promoted survival of the ZFL cells and had anti-apoptotic effect. However, these Δ Raf-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- Δ Raf1-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 Δ Raf activation (Chapter 4), we further analyzed the cellular effects of Δ Raf 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- Δ Raf1-ER cell line to specify molecular signatures downstream of the MEK/ERK cascades in the transcriptome and kinome analysis of cellular alterations induced by Δ Raf 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- Δ Raf1-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 Δ Raf 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 Δ Raf activation in the ZFL- Δ Raf1-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- Δ Raf1-ER cell line to further knock-down E-cadherin expression.

One of the interesting findings described in Chapter 4 is that Δ Raf activation resulted in regulation of the expression of many inflammation genes in the ZFL cells, such as *mpx*, *il12a*, *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 Δ Raf 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- Δ Raf1-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* Δ Raf/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- Δ Raf1-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).

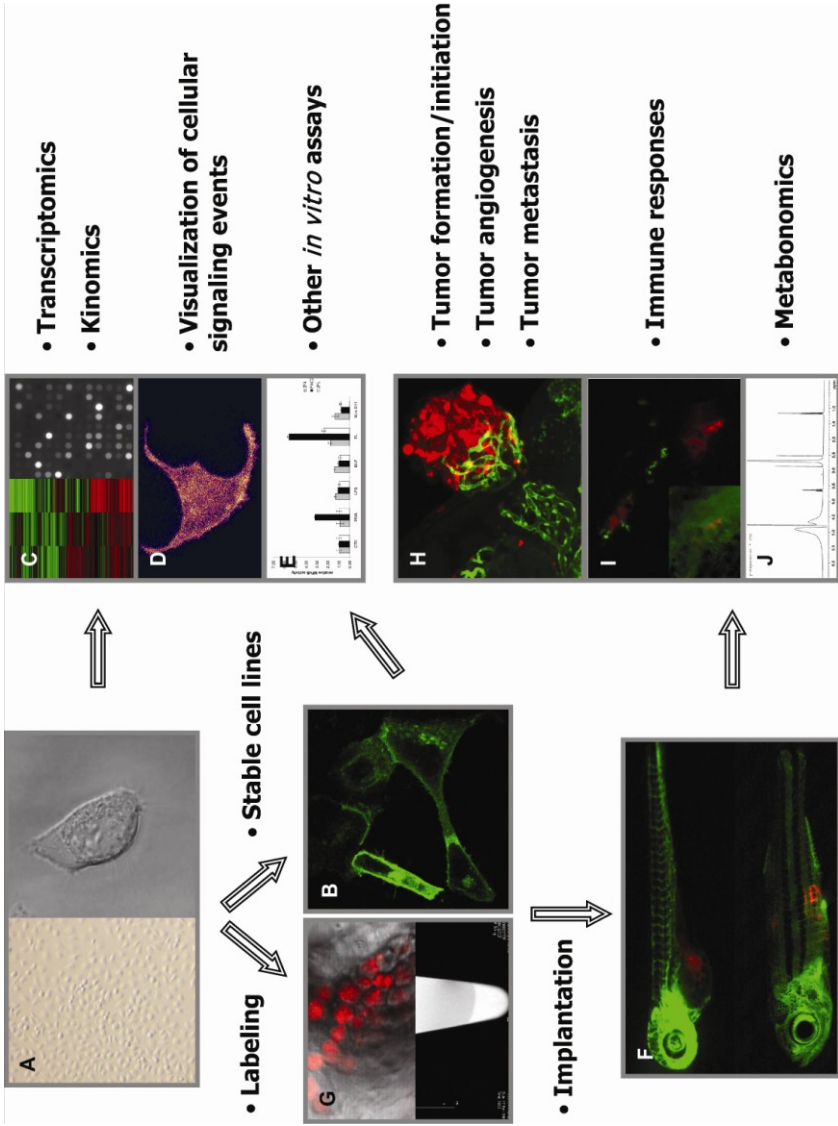


Figure 1. Zebrafish cell lines as models for cancer and immune research.

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 Δ Raf1-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).

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Samenvatting

Dierlijke celcultures worden breed en intensief toegepast als modelsysteem in biologisch onderzoek. Door het onderzoeken van specifieke celcultures zijn veel moleculaire mechanismen opgehelderd die betrokken zijn in cellulaire processen zoals celgroei, celdeling, celadhesie, celmigratie, celdood en metabolisme. De kennis die is vergaard met deze onderzoeken heeft een grote impact op zowel biologisch als op medisch vlak.

De zebravis, *Danio rerio*, is een model organisme dat steeds vaker wordt gebuikt voor het uitvoeren van onderzoek naar medisch-biologische processen, zoals het ontstaan van ontstekingen, infecties en kanker. Het gebruik van de zebravis als model organisme, en met name de eigenschap dat de zebravis embryo's transparant zijn, in combinatie met het gebruik van fluorescente zebravis celcultures, biedt nieuwe mogelijkheden om ziekteprocessen te volgen op een moleculair en cellulair niveau (Hoofdstuk 1). Het onderzoek dat in dit proefschrift wordt beschreven concentreert zich op de optimalisatie en de toepassing van zebravis celcultures als modelsysteem voor studies naar ontsteking- en oncologische processen.

Met dit als doel zijn de groei-eigenschappen gekarakteriseerd van verschillende zebravis cellijnen, waaronder twee embryonale cellijnen (Hoofdstuk 2 en 3) en een lever epitheel cellijn (Hoofdstuk 3 en 4). De mogelijke toepasbaarheid van deze cellijnen als *in vitro* modelsysteem voor verschillende biologische processen is onderzocht door gebruik te maken van gentranscriptie analyse. Uit deze analyse blijkt dat de verschillende cellijnen zeer geschikt zijn voor een systematische analyse van verschillende signaal transductie processen. Dit wordt versterkt door het feit dat de verkregen resultaten zijn gevalideerd door ze te vergelijken met de *in vivo* situatie, door ze te vergelijken met specifieke weefsel monsters van zebravissen (Hoofdstuk 2 en 4).

Als voorbeeld is een belangrijke signaal transductie route in immuun- en ontstekingsreacties, de 'Toll-like receptor' signaal transductie route, bestudeerd in drie verschillende zebravis cellijnen (Hoofdstuk 3). De resultaten laten zien dat deze cellijnen ten eerste geschikt zijn voor de analyse van specifieke signaal processen die betrokken zijn bij herkenningsmechanismen van ontstekingen en pathogenen in cellen die normaliter geen antigenen presenteren aan de celoppervlakten.

Vanwege de sterke associatie van oncologische processen met het ontstaan van ontstekingsreacties is getracht een *in vitro* kanker model te ontwikkelen die gebruik maakt van de zebravis cellijnen. In Hoofdstuk 4 en 5 wordt voor de eerste keer gerapporteerd over het genereren en gebruiken van een induceerbare carcinogene zebravis cellijn. Hiervoor is gebruik van een gemuteerde induceerbare carcinogene versie van menselijk Raf-1 (Δ Raf1) die stabiel tot overexpressie wordt gebracht.

Activatie van de induceerbare Δ Raf1 in de zebravis levercellen leidt tot hyperactivatie van de MAPK signaal transductie route en leidt tot mitogene transformatie van de cellen. De verdere effecten en moleculaire veranderingen in

deze cellijn zijn met behulp van gentranscriptie- en eiwit fosforylatie- analyse in kaart gebracht. In Hoofdstuk 4 worden de verkregen gentranscriptie-profielen van de geïnduceerde Δ Raf1 zebrafish levercellen vergeleken met de gentranscriptie-profielen van oncologische levers monsters van de mens en de zebravis. Uit deze vergelijkende analyse komt een specifieke overeenkomst naar voren in de regulatie van genen die betrokken zijn bij leverkanker.

Het effect van de geïnduceerde Δ Raf1 hyperactivatie op eiwit fosforylatie in de gekweekte zebravis levercellen wordt gerapporteerd in Hoofdstuk 5. Uit deze studie blijkt dat de geïnduceerde oncologische veranderingen in de zebravis cellijnen op verschillende moleculaire niveaus bestudeerd kunnen worden.

In hoofdstuk 6 worden de meest belangrijke resultaten en een aantal pionierstudies met het gebruik van de zebravis cellijnen beschreven, waarnaast een aantal mogelijke toekomstige toepassingen voor zebravis cellijnen worden gesuggereerd om nieuwe inzichten te verkrijgen in de moleculaire mechanismen die betrokken zijn bij het ontstaan van kanker en ontstekingsreacties.

Curriculum Vitae

Shuning He was born on September 11th, 1980 in Nanjing (Jiangsu Province), China. In 1998 she completed her high-school education in Jinling High School, Nanjing, China. At the same year, she started her bachelor study at the Biology Department of Nanjing University, China. During her studies she was involved as research trainee in ongoing projects in the Laboratory of Zoology under the supervision of Cheng Huang and Prof. Jianxiu Chen. After her graduation in 2002, she started her MSc internship with the research topic “*Toll-like receptors: in human and zebrafish*” at Prof. Dr. Herman P. Spaink’s group in the Molecular and Cell Biology department of the Institute of Biology (IBL), Leiden University, Leiden, the Netherlands. She obtained her MSc degree in 2004 (*cum laude*). From September 2004, she started a PhD project under supervision of Dr. B. Ewa Snaar-Jagalska and Prof. Dr. Herman P. Spaink. The topic of the project was “*Cancer and inflammation studies using zebrafish cell lines*”. Since September 2008 she is working as a post-doc fellow in the same group, in the ZF-Cancer project coordinated by Dr. B. Ewa Snaar-Jagalska and funded by the European Commission as part of its Seventh Framework Programme. The aim of the project is to develop clinically relevant high-throughput bioassays for human cancer progression in zebrafish.

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I came to the Molecular Cell Biology section of Institute Biology of Leiden University when Prof. Herman Spaink's group just switched direction and started to set up the research line of using zebrafish as a model organism for biomedical related fundamental studies. This brought me the unusual valuable experience of watching and participating in the development of the section.

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THANKS!

Shuning He

致谢

这篇论文得以完成，首先要感谢的就是我最亲爱的家人，爸爸妈妈爷爷奶奶。不仅仅是这篇论文，你们所给予我的，包括生命、教育、爱、支持和期待，都是我这个人这一生的基础和出发点。

回想起大约八年以前离开南京初到莱顿的时候，对于未来一无所知，刚进实验室的时候也是全然外行。这样一路走下来，到今天能够完成这篇论文，并且继续将生命科学研究作为兴趣和职业，这与周围许多人的帮助和推动是分不开的。

机缘巧合之下，我得以加入莱顿大学生物系分子细胞生物学专业，见证和参与了本专业在 Herman Spaink 教授的领导下转换研究方向，从零开始逐步建立起以斑马鱼为模式生物、在分子细胞层面上研究免疫以及癌症机理的新课题，这本身就是非常难得的宝贵体验和经历。

Herman，多谢你对我的支持和信任。正如你以前讲过的那样，通往博士的道路的确不平坦，充满曲折。在你的鼓励之下我也走过来了，这过程很是值得回味。你总是有一种鼓舞人心的力量，好几次我在迷惑或者情绪低落的时候都是从和你的谈话中获得了继续前进的推动力。非常感谢你给我机会去接触和尝试许多不同的课题和技术。还有，你对我强调过的实验重复性和选择参照物，令我受益匪浅。

Ewa，你对于我来说绝对不只是一位硕士博士期间的指导老师。这些年来，我在各个方面都得到你的指引。你经常都是从我的个人发展为出发点考虑问题，而并非局限于眼下手边的实验工作。一直以来你都在推着我向前，同时向我展示那些原本被我忽略掉的未来的可能和当前的局限。你带着我去领略这个美妙的科学世界，在我还很懵懂的时候就开始给我提供了去参加各种讲座和会议的机会，并且把我介绍给其他的科学工作者们，给我空间去尝试做不同的事情，并且时常提醒我要好好考虑未来的发展前景……不胜枚举。还有，那如母爱一般的关怀总是格外令人感动！

Gabby，有时候我真的不知道怎么说才好，从你这里学习到的东西实在是太多了。在我刚开始读硕士的时候，能够在实验室里和你相遇并且成为你的学生，真是命运的奇妙安排啊。我很幸运的能够在六年的时间里在你的身旁向你学习，而你一向都是慷慨地不吝于分享自己摸索出来的心得和窍门。至今我的很多实验操作还是用着从你那里学来的方法。我们的合作是难忘的，友情是无价的！多谢了！另外还要感谢你担任我的答辩助手并且帮我把论文简介翻译成荷兰语。

许多困难在克服之后都会变成有益的经历，然而在当时却并非易事。我多次在遇到麻烦的时候从 Kick 和 Marlon 那里得到了真诚细致的指导和鼓励，不仅帮助我顺利度过了当时的难关，而且获益至今。Helmi，你总是给我以热情鼓励！谢谢你教了我做味噌汤！崔超，多谢你的帮助和支持，无论是作为同事还是朋友你都是太棒了哟。

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萨拉戈萨以及雅典这些地方，还有数不清的小东西小事情，正是由于你们的关系才有了特别的意义。啊，对了，别忘了你们还欠我一个同游巴黎的旅行！

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亲爱的陈娟以及胡安同学，很抱歉我已经记不太清楚我们是在哪一年认识的，也总是看不出来你其实新换了发型和衣服，更糟糕的是经常因为太忙而没法接你的电话……请务必透过这些浮云般的表面现象看到本质上的深情厚意吧！这些年来有你在真的是太好了，能够和你分享，不管是欢喜还是悲伤都是弥足珍贵。

Hugo，很高兴能够认识你。和你在一起不管是做什么还是吃什么，一直都是轻松愉快。多谢你的关心，以及经常从实验以外的角度给我提供意见和建议。谢谢你担任我的答辩助手！还有啊，这些年来你到中国去的次数比我的多，时间也比我的长，对于这点，我始终都有点心理不平衡……什么时候一起去吧？

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黄老师，一直以来都承蒙你的关心照顾！虽然我目前不再做卤虫和龙虾的工作，但是从你那里学到的许多方式方法以及做人的道理并不受专业的局限。自从本科毕业以后还能够有机会回去看望你并且聆听教诲，真是太好了！

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何舒宁