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

Shuning He, Enrique Salas-Vidal, Saskia Rueb, S.F. Gabby Krens, Annemarie H. Meijer, B. Ewa Snaar-Jagalska, Herman P. Spaink

Institute of Biology, Leiden University, Einsteinweg 55, 2333 CC, Leiden, the Netherlands

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 *Pimephales promelas*
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) GTAGGCAAGAAGAAGTGCTCTGTGATCAGAGACAGCCTTCAGGTGGAGGGAGACTGGACAATGGACATCAGGACA
ZF4 profilin 2A     (1) GTAGGCAAGAAGAAGTGCTCTGTGATCAGAGACAGCCTTCAGGTGGAGGGAGACTGGACAATGGACATCAGGACA
PAC2 profilin 2A    (1) GTAGGCAAGAAGAAGTGCTCTGTGATCAGAGACAGCCTTCAGGTGGAGGGAGACTGGACAATGGACATCAGGACA
FHM profilin 2A     (1) TTAGGCAAAAAGAAGTGCTCTGTGATCAGAGACAGTCTTGCACTGGAAGGCGACTGGACAATGGACATCAGGACA
```

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.

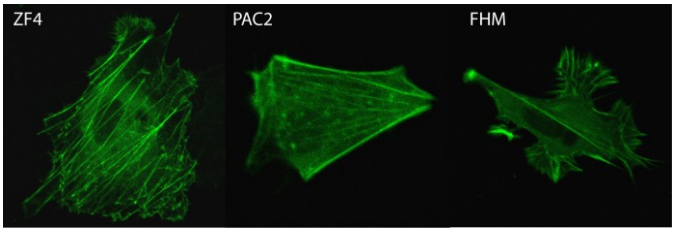
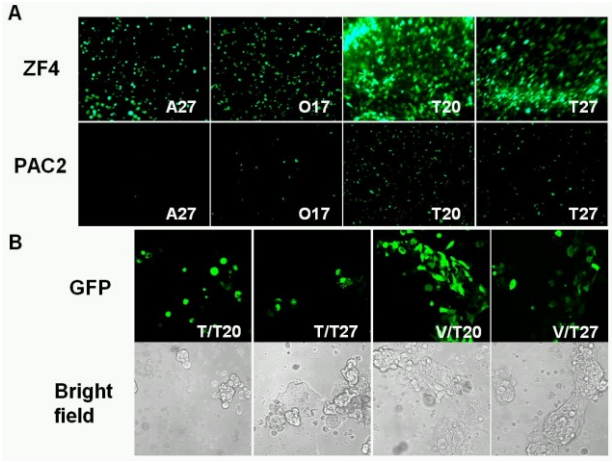


Figure 3. Nucleofection in zebrafish cell lines.
(A) Optimization of nucleofection in zebrafish cell lines using different preprogrammed electroporation programs. ZF4 and PAC2 cell lines were nucleofected with pmaxGFP in Nucleofector solution V and 24 hours after nucleofection, fluorescence was analyzed 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 oligo nucleotide 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.

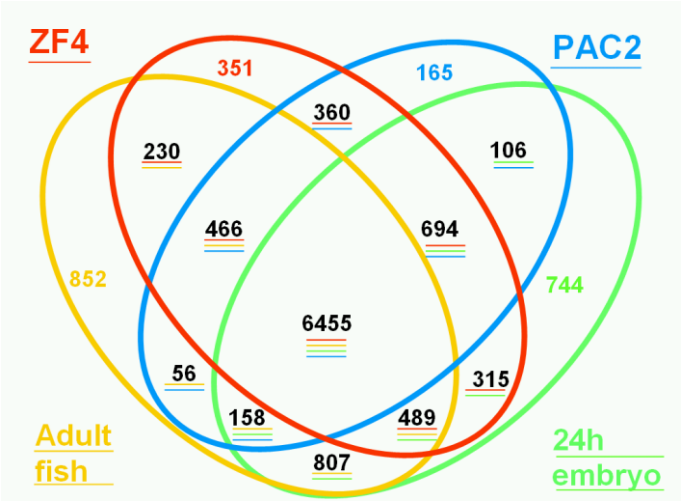


Table 3. Distribution of cell line-specific genes over different categories.

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;GO0006950	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.

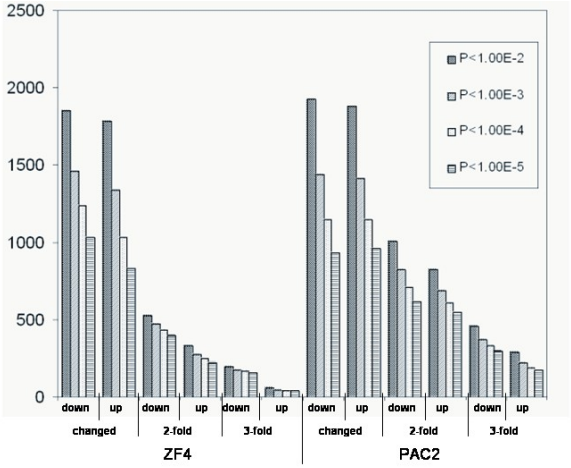
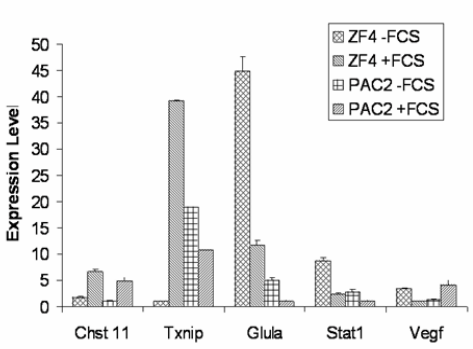


Figure 6. Confirmation of microarray results by quantitative real-time PCR.
(A) Quantitative real-time PCR was performed on five genes that showed differential expression in response to serum in ZF4 and PAC2 cell lines: Chst11 (carbohydrate sulfotransferase 11, NM_212824), Txnip (thioredoxin interacting 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.

A

	Fold-change in ZF4 by FCS		Fold-change in PAC2 by FCS	
	microarray	qPCR	microarray	qPCR
Chst11	+ 6.9	+ 4.8	+ 8.7	+ 3.7
Txnip	- 1.4	- 1.7	+ 38.4	+39.3
Glula	- 3.3	- 4.9	- 2.3	- 3.8
Stat1	- 2.4	- 2.8	- 2.4	- 3.6
Vegf	+ 3.0	+ 3.0	- 1.5	- 3.3

B



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 CAII 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. $2x10^6$ 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 $MgCl_2$, 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'- GGCCTTTTTCACCAGTGGACTGACT and 5'- GAAACATTGTATGTCGGCTCTCCATT (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 µl volume comprised of 1 µl cDNA, 12.5 µl 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 coefficiencies 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 glula (glutamate-ammonia ligase a, accession no. NM_181559), 5'-CGGAGGTGACCAGAGTGATG and 5'-CTGTTGGATGTTCGTCTAGTTGG 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).

References

1. Pichler, F., et al., *Chemical discovery and global gene expression analysis in zebrafish.* Nat Biotechnol, 2003. **21**(8): p. 879-83.
2. Meijer, A., et al., *Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish.* Mol Immunol, 2004. **40**(11): p. 773-83.
3. van der Sar, A., et al., *A star with stripes: zebrafish as an infection model.* Trends Microbiol, 2004. **12**(10): p. 451-7.
4. van der Sar, A.M., et al., *MyD88 Innate Immune Function in a Zebrafish Embryo Infection Model.* Infect. Immun., 2006. **74**(4): p. 2436-2441.
5. Lee, L., et al., *The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation.* Dev Dyn, 2005. **233**(4): p. 1560-70.
6. Driever, W. and Z. Rangini, *Characterization of a cell line derived from zebrafish (Brachydanio rerio) embryos.* In Vitro Cell Dev Biol Anim, 1993. **29A**(9): p. 749-54.
7. Collodi, P., et al., *Culture of cells from zebrafish (Brachydanio rerio) embryo and adult tissues.* Cell Biol Toxicol, 1992. **8**(1): p. 43-61.
8. Miranda, C., et al., *Regulation of cytochrome P450 expression in a novel liver cell line from zebrafish (Brachydanio rerio).* Arch Biochem Biophys, 1993. **305**(2): p. 320-7.
9. Ghosh, C., Y. Zhou, and P. Collodi, *Derivation and characterization of a zebrafish liver cell line.* Cell Biol Toxicol, 1994. **10**(3): p. 167-76.
10. Ghosh, C. and P. Collodi, *Culture of cells from zebrafish (Brachydanio rerio) blastula-stage embryos.* Cytotechnology, 1994. **14**(1): p. 21-6.
11. Fan, L., et al., *Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture.* Zebrafish, 2004. **1**(1): p. 21-6.
12. Fan, L., et al., *Production of zebrafish germline chimeras from cultured cells.* Methods Mol Biol, 2004. **254**: p. 289-300.
13. Fan, L., J. Crodian, and P. Collodi, *Production of zebrafish germline chimeras by using cultured embryonic stem (ES) cells.* Methods Cell Biol, 2004. **77**: p. 113-9.
14. Peppelenbosch, M., et al., *Ionic responses to epidermal growth factor in zebrafish cells.* Exp Cell Res, 1995. **218**(1): p. 183-8.
15. Amsterdam, A., et al., *A large-scale insertional mutagenesis screen in zebrafish.* Genes & Dev., 1999. **13**(20): p. 2713-2724.
16. Chen, W., et al., *High-Throughput Selection of Retrovirus Producer Cell Lines Leads to Markedly Improved Efficiency of Germ Line-Transmissible Insertions in Zebra Fish.* J. Virol., 2002. **76**(5): p. 2192-2198.
17. Paw, B. and L. Zon, *Primary fibroblast cell culture.* Methods Cell Biol, 1999. **59**: p. 39-43.
18. Miklos, G. and G. Rubin, *The role of the genome project in determining gene function: insights from model organisms.* Cell, 1996. **86**(4): p. 521-9.
19. Cornelis K.D.Breek, F.v.I., Susan Wijting, Nico Stuurman, Herman P.Spaink, *Expression of multicolor fluorescent fusion proteins in zebrafish cell cultures: a versatile tool in cell biology. .* 2002.

20. Patricio Villalobos, M.V.R., Pablo Conejeros, Sergio H.Marshall, *Lipopolyamine-mediated transfection of reporter plasmids into a fish cell line.* Electronic Journal of Biotechnology, 1999. **2**.
21. Chang, H., et al., *Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds.* PLoS Biol, 2004. **2**(2): p. E7.
22. Iyer, V.R., et al., *The Transcriptional Program in the Response of Human Fibroblasts to Serum.* Science, 1999. **283**(5398): p. 83-87.
23. Mizgireuv, I.V. and S.Y. Revskoy, *Transplantable Tumor Lines Generated in Clonal Zebrafish.* Cancer Res., 2006. **66**(6): p. 3120-3125.
24. Grabher, C. and A. Look, *Fishing for cancer models.* Nat Biotechnol, 2006. **24**(1): p. 45-6.
25. Haldi, M., et al., *Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish.* Angiogenesis, 2006. **9**(3): p. 139-51.
26. Gloghini, A., et al., *RT-PCR Analysis of RNA Extracted from Bouin-Fixed and Paraffin-Embedded Lymphoid Tissues.* J Mol Diagn, 2004. **6**(4): p. 290-296.

Acknowledgements

This work was supported by a European Communities 6th framework grant (contract LSHGCT-2003-503496) and a grant from the Dutch government (CYTTRON within the Bsik program, Besluit subsidies investeringen kennisinfrastructuur).
We thank Dr. D. Zivcovic (Hubrecht laboratory, Utrecht, The Netherlands) for providing us with the FHM cell line, Dr. G. Otto (Max Planck Institute, Tuebingen, Germany) for sending us zebrafish embryos from the Tuebingen line and Dr. N. Foulkes (Max Planck Institute, Tuebingen, Germany) for the PAC2 cell line.