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## Zebrafish: A Model System to Study Heritable Skin Diseases

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### Abstract

Heritable skin diseases represent a broad spectrum of clinical manifestations due to mutations in ~500 different genes. A number of model systems have been developed to advance our understanding of the pathomechanisms of genodermatoses. Zebrafish (*Danio rerio*), a freshwater vertebrate, has a well-characterized genome, the expression of which can be easily manipulated. The larvae develop rapidly, with all major organs having developed by 5–6 days post fertilization, including the skin, consisting of the epidermis comprising two cell layers and separated from the dermal collagenous matrix by a basement membrane. This perspective highlights the morphologic and ultrastructural features of zebrafish skin, in the context of cutaneous gene expression. These observations suggest that zebrafish provides a useful model system to study molecular aspects of skin development, as well as the pathogenesis and treatment of select heritable skin diseases.

### The Spectrum of Heritable Skin Diseases

Genodermatoses manifest with a broad spectrum of clinical conditions where at one end of the spectrum the cutaneous findings can be relatively minor, limited to the skin, hair and nails, while at the other extreme of the spectrum the cutaneous manifestations can be part of multi-system pathology with significant morbidity and mortality (for reviews, see Pulkkinen *et al*, 2002; Feramisco *et al*, 2009; Uitto, 2009). With the advent of technologies in molecular genetics in general and completion of the human genome database, an increasingly large number of gene defects has been linked to specific heritable disorders with cutaneous manifestations. In fact, mutations are now known to occur in as many as 500 different genes in a manner that these genetic variations explain the phenotypic manifestations characteristic of a heritable disease with skin involvement (Feramisco *et al*, 2009).

### Mutation Databases of the Candidate Genes

The genodermatosis mutation databases have revealed both obvious candidate genes and a number of surprises. For example, approximately 20 years ago, based on advances in the knowledge of the cutaneous basement membrane zone (BMZ), we made a prediction that mutations in the structural components of the basement membrane at the epidermal-dermal interface could explain the fragility of skin in different forms of epidermolysis bullosa (EB) (Uitto and Christiano, 1992). This prediction has since been proven correct by the

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#### Conflict of Interest

The authors state no conflict of interest.

demonstration of mutations in as many as 14 distinct genes encoding the structural components of the skin in different variants of EB (Uitto *et al*, 2010a).

In contrast to the apparent candidate genes, many of the mutated genes have turned out to be surprisingly unpredictable, with their role in skin biology being largely unrecognized prior to the identification of these specific mutations. For example, pseudoxanthoma elasticum (PXE), which was initially classified as a prototypic connective tissue disorder affecting the elastic fiber network in the skin, the eyes and the cardiovascular system, was subsequently shown to result from mutations in the *ABCC6* gene, expressed primarily in the liver and the kidneys and at very low level, if at all, in tissues affected with mineralization in PXE (Li *et al*, 2009; Uitto *et al*, 2010b). This condition is now thought to be a metabolic disorder in which the absence of circulating anti-mineralization factors allows ectopic calcification of the connective tissues to ensue (Jiang *et al*, 2009).

## Model Systems to Study Heritable Skin Diseases

In order to gain insight into the pathomechanistic details of heritable skin diseases and to provide model systems for testing of treatment modalities, a number of animal models that recapitulate features of a specific disease have been developed. Traditionally, mice have provided the preferred platform to develop models of human diseases, often through the development of “knock-out” (KO) animals by targeted ablation of the corresponding genes. While often the KO mice show remarkable similarity to the human phenotype both at the genetic, gross morphologic, histopathologic, and ultrastructural levels, mice as a model system can have considerable limitations (Lieschke and Currie, 2007). The drawback of the mouse model is its relatively long lifespan, and it may take several years to develop a KO mouse. In some cases, development of the KO mouse as a model system of the corresponding human disease is not feasible due to the absence of the corresponding gene in the mouse genome, as in case of the *SAMD9* gene underlying normophosphatemic familial tumoral calcinosis in humans (Li *et al*, 2007; Sprecher, 2010). These considerations, together with cost containment issues, have prompted the search for alternative model systems to study heritable skin diseases (Vanchieri, 2001).

## Biology of Zebrafish Skin Development

There are several characteristics that favor choosing zebrafish (*Danio rerio*) for genetic testing in developmental studies (Table 1) (Lieschke and Currie, 2007; Brittijn *et al*, 2009; Rakers *et al*, 2010). This small freshwater fish is easily maintained in the laboratory setting with a rapid rate of maturation from embryos to fully developed fish (Fig. 1a). A single female can lay 50–100 eggs per laying. The development of various organs is easy to visualize *in vivo* because the embryos are optically transparent during the first several days. By day 5–6, all important internal organs as well as skin compartments are largely formed, as can be visualized by transmission electron microscopy (Fig. 1b). At 1 day post-fertilization (dpf) different skin layers representing the epidermis and the dermis can be recognized, although the cutaneous BMZ at the dermal-epidermal junction is not yet developed. However, at 6 dpf the epidermis, composed of two cell layers, is readily noticeable and clearly separated from the underlying connective tissue stroma by a basement membrane. At the surface of the epidermal contour, there are spicule-like protrusions which correspond to microridges of the epidermal cells. On the dermal side there is a well developed collagenous stroma with adjacent fibroblastic cells with well developed rough endoplasmic reticulum (Le Guellec *et al*, 2004). In fully developed adult zebrafish skin, there is a multi-layer epidermis separated from the underlying collagenous stroma by the BMZ and at high magnification, hemidesmosomal structures can be visualized (Fig. 1b), (Sonawane *et al*, 2005). Thus, zebrafish skin has a clearly demarcated dermal-epidermal

BMZ, separating epidermis from the underlying dermis. Previous studies have shown that the basement membrane may be formed as early as 32 hpf, and lamina lucida and lamina densa can be identified by transmission electron microscopy at 48 hpf (Le Guellec et al, 2004).

Examination of the developing zebrafish skin surface at 1 dpf by scanning electron microscopy reveals well demarcated keratinocytes with a surface contour containing microridges, which are well organized by 6 dpf (Fig. 1c). In adult zebrafish the epidermis is covered by scales, which form under the control of a genetic cascade, including *sonic hedgehog* expression, at around 30 dpf (Sire and Akimendo, 2004). However, early on, at least up to 6 dpf as shown here, the developing zebrafish epidermis has characteristic landmark features that can be altered by perturbed keratinocyte gene expression.

In addition to the structural elements of the epidermis, BMZ, and the collagenous dermis, zebrafish skin has a neural-crest-derived pigment cell system, including the presence of melanocytes, that can serve as a target to study developmental biology and pathology of pigmentation (O'Reilly-Pol and Johnson, 2009; Lee *et al*, 2010). Zebrafish skin also has structures that are specialized for the aquatic environment, such as scales, presence of mucous secreting cells, and the lateral line. The latter organ contains 54 neuromasts that are topographically highly conserved neural elements consisting of hair cells, serving as a sensory organ regarding the rheological movements of the fish (Froehlicher *et al*, 2009). Of interest is our recent finding that the gene product of *coll17a1b* is localized to neuromasts, and “knockdown” of the corresponding gene by a morpholino abolishes the formation of functional neuromasts (Kim *et al*, 2010). A key difference between the zebrafish and human skin is, however, the lack of mammalian appendages, including hair follicles and sebaceous glands.

## The Zebrafish Genome

Significant progress has recently been made in sequencing the zebrafish genome encompassing 25 chromosomes. This diploid genome essentially contains the full repertoire of vertebrate genes, and the most recent gene-set comprises 24,147 protein-encoding genes based on the integrated whole genome shotgun assembly at 6.5–7-fold coverage (Ensembl, Zebrafish Zv8; [http://uswest.ensembl.org/Danio\\_reio/Info/Index](http://uswest.ensembl.org/Danio_reio/Info/Index)). In spite of the extensive coverage, this assembly is still considered preliminary because there are contigs that are of lower sequence quality, and the assembly contains misjoins, misassemblies and artificial duplications.

One of the characteristic features of the zebrafish genome is that there are duplicate copies of a number of genes (see Postlethwait, 2007; Roch *et al*, 2009). The current hypotheses put forward suggest that the whole genome underwent two sequential rounds of duplication in the vertebrate stem well before the divergence of ray-finned and lobe-finned fish. Evidence suggests another further round of whole genome duplication occurring near the origin of teleost fish, *i.e.*, about 350 million years ago. In many cases, one gene copy of the duplicate genes may have been lost or silenced, but if both copies survive in a functional state, these duplicates in some cases have evolved a novel function in distinct spatial distribution. For example, as indicated below, we have identified two copies of the zebrafish type XVII collagen genes, *coll17a1a* and *coll17a1b*, which by *in situ* hybridization and morpholino knockdown technologies were shown to have either epidermal or neural distribution, respectively (Kim *et al*, 2010). In higher vertebrates, including human and mouse, type XVII collagen can display either an epithelial or a neural isoform, which, however, are apparently products of the same gene (Seppänen *et al*, 2006; Has and Kern, 2010).

The overall conservation of different orthologous genes in different species, such as in human and zebrafish, can be determined by constructing phylogenetic trees based upon the nucleotide information on these genes. For example, the two zebrafish type XVII collagen genes are phylogenetically close, yet they show a high degree of divergence compared to other *col17* orthologs in different species (Kim *et al*, 2010).

The conservation of individual genes between human and zebrafish can be variable, and certain protein domains may be well conserved. For example, the overall sequence homology at the amino acid level between the human and zebrafish *ABCC6* genes is 47 percent, yet certain critical domains, such as the nucleotide binding folds, indispensable for the function of this transmembrane efflux transporter, are conserved by ~60 percent (Li *et al*, 2010).

## Cutaneous Gene Expression Profiles in Zebrafish

Since ultrastructural survey of the developing zebrafish skin showed clearly recognizable epidermal structures separated from the underlying dermis by BMZ, we have profiled the zebrafish skin gene expression by RT-PCR for selected genes that are known to be expressed in human epidermis, BMZ or dermis, respectively (Table 2). RT-PCR using total RNA isolated from zebrafish as a template revealed expression of selective epidermal markers, including keratins (*krt1* and *krt5*), and 230-kD bullous pemphigoid antigen (*bpag1*) at 6 dpf, and the expression of these genes continued into adulthood. The plectin gene (*plec1*) encoding an intracellular epidermal adhesion molecule, was expressed as early as 1 dpf, and its expression increased at later stages. Among the BMZ genes, mRNA expression corresponding to subunit polypeptides of type IV collagen, as well as collagens VII and XVII, was readily detected. Furthermore, expression of the genes encoding subunit polypeptides of the integrin  $\alpha6\beta4$  (*itga6*, *itgb4*) was noted at 1 or 6 dpf. A number of human collagen genes, as exemplified by collagens I, V and VI, the major collagens in human dermis, were also expressed at 6 dpf. These collagen mRNAs, as well as those corresponding to collagens XII, XIV, XV, XVI, XVIII, and XIX, could be temporally detected at different stages of zebrafish development (Table 2). It is important to note, however, that a search of the latest zebrafish genome database does not reveal the presence of the filaggrin, involucrin, envoplakin and trichohyalin genes. These observations may well reflect the fact that the zebrafish epidermis does not undergo terminal differentiation towards formation of stratum corneum with barrier function similar to humans. This difference limits the study of human epidermal disorders using zebrafish as a model system.

The spatial gene expression during zebrafish development can be monitored by *in situ* hybridizations that allow identification and location of mRNAs both during different developmental stages and also at their cellular locations (Thisse and Thisse, 2008). For example, temporal expression of the *col1a1* gene can be detected as early as at ~10 hpf (Fig. 2a), and *in situ* hybridization localizes the corresponding mRNAs to the cellular elements in the developing skin (Fig. 2b, c). The precise identity of these cells remains to be explored, but it has been reported that the collagenous stroma of the zebrafish skin is acellular until 26 dpf and that up to this time point collagen is synthesized by keratinocytes (Le Guellec *et al*, 2004). Thus, the available technologies, such as RT-PCR and *in situ* hybridization, readily allow determination of the temporal and spatial expression of genes in developing zebrafish. Analogous techniques at the protein level, including Western blot analysis and immunohistochemistry, can also be used to study the gene expression, but these approaches are often hampered by the paucity of antibodies recognizing the zebrafish protein sequences.

## Morpholino Knockdown of Zebrafish Gene Expression

One of the convenient features of zebrafish as a model system to study heritable skin diseases is that the expression of specific genes can be manipulated by injection of 1–4 cell embryos with morpholino-based antisense oligonucleotides (Eisen and Smith, 2008; Shan, 2010). These oligonucleotides can be targeted either to correspond to the sequences around or slightly upstream from the translation initiation codon (AUG) to prevent translation of the mRNA, or to the splice junction sequences to prevent processing of the pre-mRNA to the corresponding mature mRNA (Fig. 3). In case of morpholinos targeting the upstream regulatory sequences, the efficacy of the down-regulation of the corresponding gene expression can be monitored by co-injection of the morpholino with an expression construct containing the 5' regulatory elements linked to a green fluorescent protein reporter (Fig. 3a). In the case of splice junction morpholinos, the efficiency of the morpholino-mediated knockdown of the gene expression can be monitored by RT-PCR of the RNA sequences to determine whether the morpholino has been effective in preventing the splicing of the targeted intron (Fig. 3b). For example, we have been successful in efforts to knockdown the *coll17a1b* gene expression in zebrafish by a specific morpholino targeting the 5' regulatory sequences, with essentially 100 percent efficiency, as shown in Fig. 3 (Kim *et al*, 2010). Similarly, the efficacy of 100 percent of a morpholino placed on the exon 18/intron 19 junction of the *abcc6b* gene is illustrated in Fig. 3 (Li *et al*, 2010).

The specificity of the morpholino knockdown can be confirmed by co-injection of the corresponding mRNA or protein from another species, such as mouse or human, which counteracts the development of the phenotype (Eisen and Smith, 2008). Furthermore, use of a standard control morpholino, which is biologically inactive since there is no target sequence for it in the zebrafish genome, can confirm the specificity of a morpholino (Robu *et al*, 2007). It should be noted that microinjection of purified mRNA (without morpholinos) can also be used to monitor the effects of overexpression of the corresponding gene during zebrafish development.

It should be noted that the morpholinos have a relatively short half life (up to 5 days), and therefore this approach is most suitable for evaluation of the effects of a morpholino on the early zebrafish development. Thus, the morpholino knockdown phenotype is most likely to reproduce the clinical manifestations in human diseases which develop during prenatal development or shortly after birth. An example of such situation is the development of epidermal perturbations in zebrafish as a result of knockdown of *abca12*, the gene harboring mutations in the harlequin ichthyosis (Frank *et al*, 2010). Specifically, the morphant fish demonstrated absence of microridges and development of scale-like spicules on the surface of the skin, somewhat resembling the scales in human ichthyosis (Frank *et al*, 2010). Another example of zebrafish phenotype mimicking a human skin disease is the morpholino knockdown of the *coll17a1a* gene expressed in the skin in hemidesmosomal complexes. These knockdown fish manifest with blistering of the epidermis similar to a form of junctional EB due to mutations in the *COL17A1* gene (Kim *et al*, 2010). In contrast, diseases that are of late onset or slowly progressive may not be evident in the zebrafish model system. An example of such conditions is PXE, a slowly progressive, ectopic mineralization disorder with late onset. Specifically, the clinical diagnosis of PXE in humans is not frequently made until an individual is in the teenage years or early twenties. Similarly, KO mice, in which the *Abcc6* gene has been inactivated by targeted ablation, characteristic mineralization is not evident until at 5–6 weeks of life (Jiang *et al*, 2007, 2009). Injection of the *abcc6a* morpholino in the zebrafish resulted in an early phenotype of pericardial edema and curled tail, associated with death by 8 dpf, but there was no evidence of ectopic mineralization at this stage (Li *et al*, 2010). The mineralization phenotype, if developmentally corresponding to human or mouse pathogenesis of PXE, might occur later



in life. Therefore, the discordance between the zebrafish phenotypes with that in human and mouse, raises the possibility that the *ABCC6* gene products might have different biological functions depending on the species, in which case the *abcc6* knockdown in zebrafish is not an appropriate model for PXE (Li *et al*, 2010).

An unexplored facet of the zebrafish as a model system for heritable skin disorders is the potential influence of the genetic background. The genetic background of the mouse strains may have a major influence on the development of the disease phenotype, as has been shown, for example, in the case of PXE-like mineralization of connective tissues in the *Abcc6* KO mice (Li and Uitto, 2010; Hovnanian, 2010). In case of zebrafish, the genetic background of strains (and substrains) has been reported to be highly variable, thus possibly leading to modulation of the phenotype (Guryev *et al*, 2006).

## Forward Genetics for Identification of Disease-causing Genes

An approach complementary to morpholino knockdown in using zebrafish as a model system to study heritable skin diseases involves forward genetics, in which the fish can be screened for cutaneous phenotypes after ethylnitrosourea (ENU) induced mutagenesis to induce point mutations. Similar random mutagenesis can also be carried out in zebrafish using retroviral methods, which have the advantage of tagging each insertion site, thus facilitating the identification of mutated genes (Jao *et al*, 2008). Following mutagenesis, a large number of embryos and larvae can be screened for phenotypic manifestations in the skin, allowing a large scale screen without elaborate infrastructure or equipment. Such a screen, which is facilitated by transparency of the developing larvae, has an advantage over other vertebrate systems including the mouse (Table 2). These large-scale forward-genetic screens have allowed identification of a number of mutated genes orthologous to those causing human heritable diseases, with phenotypic similarities (Amsterdam and Hopkins, 2006; Lieschke and Currie, 2007). In addition, this approach can be used to identify novel candidate genes that can then be tested in patients with similar phenotypes but with no known mutations. Once the zebrafish disease model has been established, the embryos are readily amenable to further investigation for the molecular and cellular bases of the disease. It should be noted that these approaches have also been used to examine more complex pathological pathways, such as those involved in carcinogenesis and melanoma formation, and the zebrafish models of human diseases can also be used for small-molecular-weight compound screening and for drug discovery programs.

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## Abbreviations

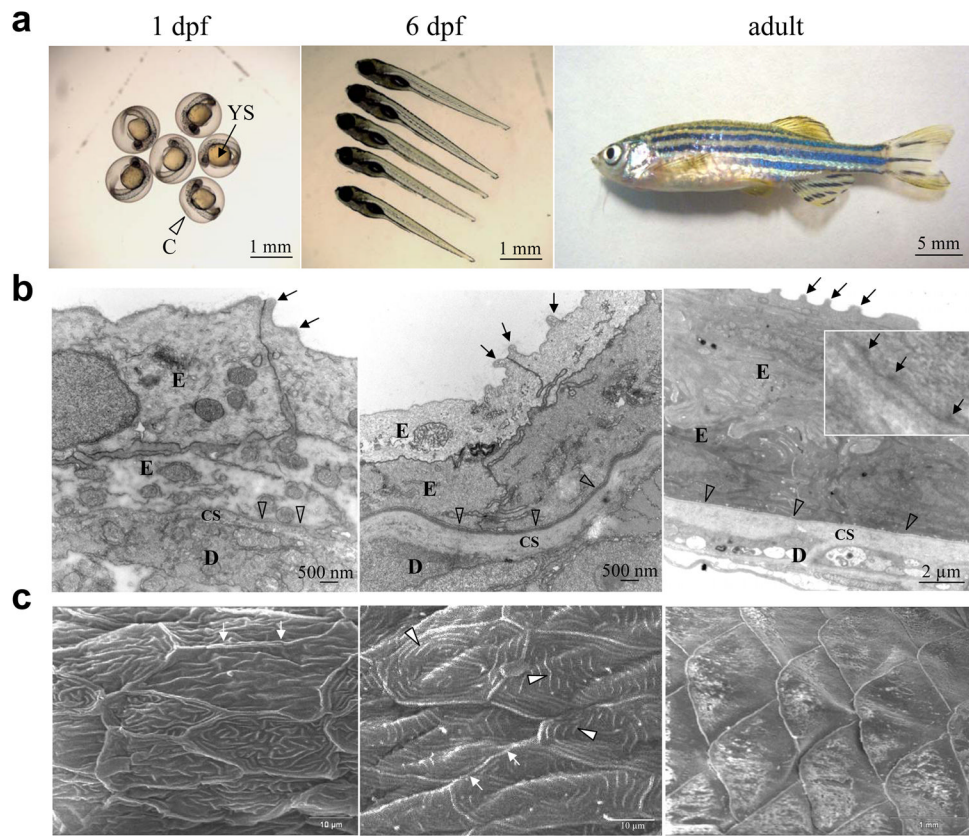
<b>BMZ</b>	basement membrane zone
<b>EB</b>	epidermolysis bullosa
<b>PXE</b>	pseudoxanthoma elasticum
<b>KO</b>	knock-out
<b>hpf and dpf</b>	hours and days post-fertilization, respectively

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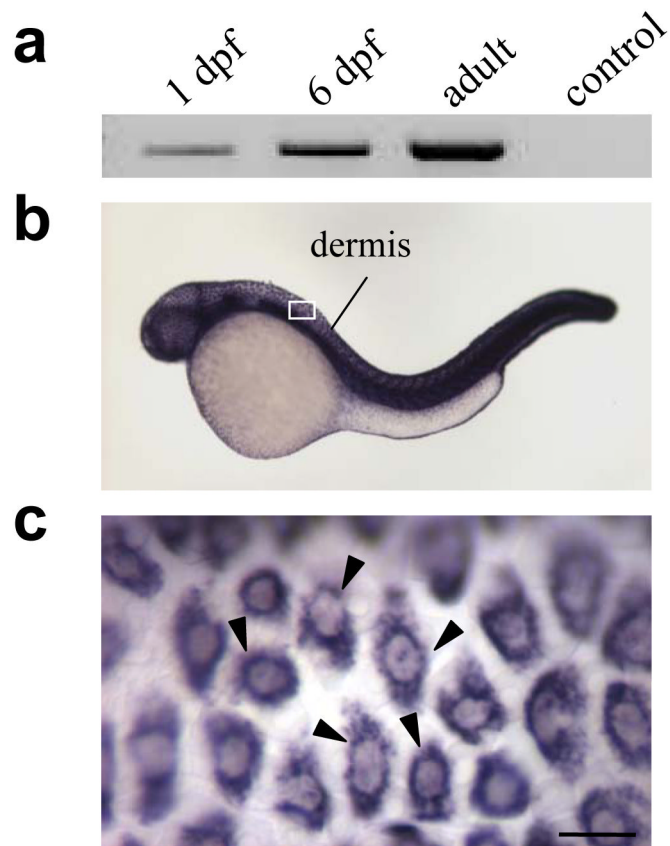
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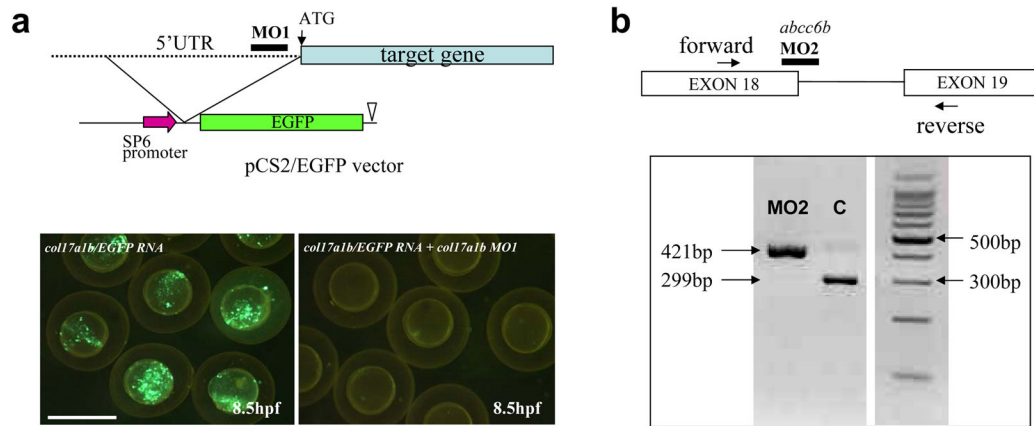




**Figure 1.** Cutaneous biology of the developing zebrafish. (a) The figure illustrates the growth of zebrafish from 1 dpf embryos, which are surrounded by a transparent chorion (C) and display a prominent yolk sac (YS), to an adult fish. At 6 dpf pigmentation becomes apparent on the skin. (b) Transmission electron microscopy reveals at 1 and 6 dpf an epidermis (E) consisting of two cell layers, and at 6 dpf the epidermis is separated from the underlying collagenous stroma (CS) and dermis (D) by a clearly demarcated basement membrane (open arrowheads). In adult fish, there is a multi-layered epidermis, and higher magnification of the basement membrane zone reveals the presence of hemidesmosomes (arrows in the inset). The spicule-like extensions of the surface of the skin (arrows) correspond to microridges. (c) Scanning electron microscopy reveals well demarcated keratinocytes with distinct cell-cell borders (small arrows). In the middle of the keratinocyte surface, there are developing microridges which at 6 dpf become well organized (open arrowheads). In an adult fish, the epidermis is covered by scales.



**Figure 2.** Demonstration of temporal and spatial expression of the *colla1* gene in the developing zebrafish. (a) Total RNA isolated from zebrafish at 1 or 6 dpf or from a ~2-year old adult fish was examined for the presence of *colla1* mRNA by RT-PCR. The control lane was amplified without RNA. (b) *In situ* hybridization with a *colla1* antisense RNA probe on a 42–48 hpf zebrafish embryo reveals the presence of the corresponding mRNA (purple color). (c) Higher magnification of the area within the rectangle in (b) shows the presence of mRNAs within distinct cells in the skin (arrowheads). (Scale bar = 10  $\mu$ m).



**Figure 3.**

Morpholino-mediated knockdown of zebrafish genes. (a) A morpholino (MO1) corresponding to the *col17a1b* gene was used to target the 5' untranslated region of the corresponding mRNA to prevent translation. To determine the efficacy of morpholino in down-regulating the translation, an expression construct consisting of SP6 promoter, 5' UTR of the *col17a1b* gene, and downstream enhanced green fluorescent protein (EGFP) reporter gene was generated. Microinjection of mRNA transcribed *in vitro* from the pCS2/EGFP vector to 1–4 cell stage embryos, shows green fluorescence at 8.5 hpf (lower left panel). Co-injection of this mRNA together with the MO1 morpholino completely abolished the fluorescence, indicating inhibition of the translation (Scale bar = 1 mm). (b) A morpholino (MO2) corresponding to the zebrafish *abcc6b* gene was placed on the exon 18/intron 18 splice junction. Efficiency of the morpholino in preventing splicing of the *abcc6b* pre-mRNA into mature mRNA was monitored by RT-PCR using primers placed on exon 18 (forward) and exon 19 (reverse). PCR of the genomic sequence resulted in a 421 bp fragment while fully spliced cDNA yields a 299 bp fragment devoid of intron 18 (122 bp). RT-PCR of morpholino (MO2)-treated zebrafish embryo reveals the presence of the 421 bp mRNA sequence only, indicating complete inhibition of the removal of intron 18 by splicing. Since intron 18 sequence is out-of-frame, this results in complete absence of the *abcc6b* protein product. (Figures 3a and b are reproduced from Kim *et al*, 2010, and Li *et al*, 2010, respectively, with permission).

**Table 1**

Comparison of selected attributes for the use of zebrafish or mouse as a model system to study human diseases<sup>1)</sup>

Attribute	Zebrafish	Mouse
<i>Practical Issues</i>		
• Husbandry infrastructure	\$	\$\$\$
• Cost per animal per year	\$	\$\$
• Characterized inbred strains	+	++++
• Outbred laboratory strains	+++	++
• Anatomical similarity <sup>2)</sup>	+	++
• Genetic similarity <sup>2)</sup>	++	+++
• Pathological similarity <sup>2)</sup>	++	+++
<i>Molecular Biology Tools</i>		
• Transgenesis <sup>3)</sup>	++	++
• Targeted gene modification <sup>3)</sup>	-	++++
• Transient <i>in vivo</i> assays <sup>3)</sup>	++++	+
• Feasibility of large-scale screens	+++	++
• Affordability of large-scale screens	+++	+
• Genome sequencing progress	++	+++
• Genome annotation progress	++	++++
<i>Cell Biology Tools</i>		
• Cell lines and tissue cultures	+	+++
• Antibody reagents	+	++++

<sup>1)</sup> Designations: \$ and \$\$\$; and -, +, ++ and +++ refer to the relative cost and strength of the model system in each category; ++++ signifies outstanding strength of the model. (Adapted with modifications from Lieschke and Currie, 2007).

<sup>2)</sup> In comparison to human.

<sup>3)</sup> Reverse-genetics approach.

**Table 2**Expression of selected epidermal, BMZ and dermal genes in zebrafish at different stages of development <sup>1)</sup>

Gene	Developmental stage		
	1 dpf	6 dpf	adult
<b>Epidermal</b>			
<i>krt1</i>	-	+	+
<i>krt5</i>	-	+	++
<i>bpag1</i>	-	++	+
<i>plec1</i>	+	+	++
<b>BMZ</b>			
<i>col4a1</i>	+	+	++
<i>col4a2</i>	-	++	+
<i>col4a3</i>	-	+	+
<i>col4a4</i>	-	+	+
<i>col4a5</i>	-	-	-
<i>col4a6</i>	++	++	+
<i>col7a1a</i>	+	+	+
<i>col7a1b</i>	+	+	+
<i>col17a1a</i>	-	+	+
<i>col17a1b</i>	+	++	+
<i>itga6</i>	-	+	++
<i>itgb4</i>	+	++	+++
<b>Dermal</b>			
<i>col1a1</i>	+	++	+++
<i>col1a2</i>	-	++	+
<i>col1a3</i>	+	++	+++
<i>col5a1</i>	+++	++	+
<i>col5a2</i>	-	-	-
<i>col6a1</i>	+	+	-
<i>col6a2</i>	-	+	+
<i>col12a1</i>	-	+	++
<i>col14a1</i>	-	+	+
<i>col15a1</i>	-	++	+
<i>col16a1</i>	-	+	+
<i>col18a1</i>	+	++	+++
<i>col19a1</i>	++	+	+
<b>Reference gene</b>			
<i>β-actin</i>	+	+	+

<sup>1)</sup>Total RNA was isolated from developing zebrafish at 1 and 6 dpf, as well as from an ~ 2-year old adult. The presence of gene expression was determined by RT-PCR with specific primers using 35 cycles. The presence (+) or absence (-) of the PCR product was assessed by 2% agarose gel electrophoresis. The relative amount of each PCR product at different developmental stages was semi-quantitatively indicated by +, ++ or +++.