Zebrafish as a Model System to Study Skin Biology and Pathology

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INTRODUCTION

Several animal models have been developed to recapitulate the features of specific skin diseases, and such model systems have provided valuable insight into the pathomechanistic details of these diseases. They have also provided useful systems for testing of treatment modalities for a number of conditions. The preferred platform for such model development has been the mouse, often through the development of "knockout" mice or by development of transgenic mice with expression of the mutant genes. Although the mouse models have often demonstrated remarkable similarity to human diseases, mice as a model system have considerable limitations, including relatively long lifespan and high cost of development. Also, the genetic background of the mouse strains can have a major influence on the development of the disease phenotype. In some cases, development of the mouse model is not feasible because of the absence of the corresponding gene in the mouse genome. These considerations, particularly in conjunction with cost-containment issues, have prompted the search for an alternative model system to study skin diseases. In fact, Vanchieri (2001) suggested, "Move over mouse: make way for the woodchucks, ferrets, and zebrafish," and Lieschke and Currie (2007) said, "Animal models of human disease: zebrafish swim into view."

ADVANTAGES OF THE ZEBRAFISH MODEL SYSTEM

- A freshwater vertebrate with a complement of genes very similar to those of mammals.
- Rapid development of transparent embryos, allowing visual inspection of internal organs.
- Easy breeding, with a large number of embryos and larvae.
- Easy manipulation of gene expression with phenotypic consequences.
- A facile and cost-efficient system for large-scale screening.

LIMITATIONS

- Lack of functional stratum corneum and expression of genes necessary for terminal differentiation of epidermis.
- The zebrafish genome database is not complete and contains misassemblies.
- Paucity of antibodies recognizing zebrafish protein sequences.

THE ZEBRAFISH GENOME

The genome of zebrafish, a small freshwater vertebrate, consists of 25 chromosomes and contains essentially the full repertoire of vertebrate genes. Although the zebrafish genome sequencing has not been fully completed, the most recent gene set comprises 26,247 protein-coding genes and 54,869 transcripts, based on integrated whole-genome assembly (Ensembl, Zebrafish Zv9; http://asia.ensembl.org/Danio_rerio/Info/Index). Comparison with the human genome reveals that approximately 70% of our genes have a zebrafish counterpart; perhaps more importantly, about 84% of the genes that cause human diseases have a zebrafish ortholog (McKie, 2013).

A characteristic feature of the zebrafish genome is that a number of genes appear in duplicate, reflecting two sequential rounds of duplication of the genome before divergence of ray-finned and lobe-finned fish. In addition, there is evidence of another round of whole-genome duplication near the origin of teleost fish ~350 million years ago (Postlethwait, 2007). Although in many cases one copy of the duplicated gene has been lost or silenced during evolution, in some cases both copies have survived in a functional state but resulted in distinct function or spatial distribution. The overall conservation in different orthologous genes between species, such as humans and

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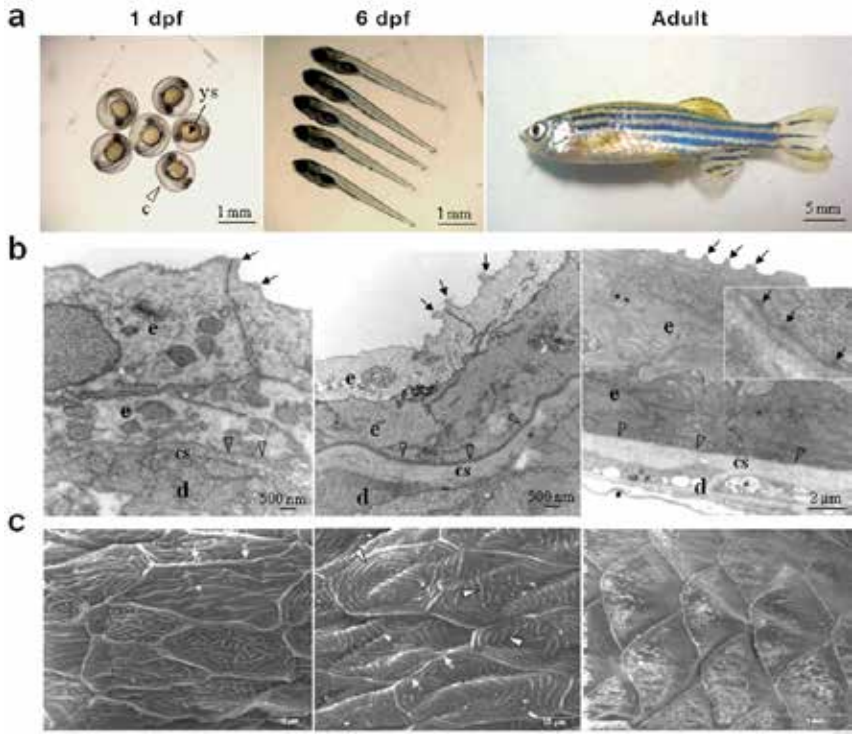

Figure 1. Cutaneous biology of the developing zebrafish. (**a**) The growth of zebrafish from an embryo 1 day postfertilization (dpf), which is surrounded by a transparent chorion (**c**) and displays a prominent yolk sac (ys), to an adult fish. At 6 dpf, pigmentation becomes apparent on the skin. (**b**) Transmission electron microscopy reveals an epidermis (**e**) at 1 and 6 dpf consisting of two cell layers; 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 multilayered 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 that at 6 dpf become well organized (open arrowheads). In an adult fish, the epidermis is covered by scales. Reprinted with permission from Li *et al*. (2011).

zebrafish, can be determined by constructing phylogenetic trees based on precise nucleotide information. The conservation of individual genes between humans and zebrafish is variable, but certain protein domains are well conserved. Nevertheless, the overall conservation of the human and zebrafish genome forms the basis to study the role of distinct genes in cutaneous biology and pathology.

ZEBRAFISH SKIN DEVELOPMENT

The advantages of using zebrafish as a model are that a large number of embryos can be obtained per laying, approximately 50–100 per female, and the development of various organs in zebrafish embryos is easy to visualize *in vivo* because the embryos are optically transparent during the first several days of development (Li *et al*., 2011). Zebrafish has a rapid rate of maturation from embryo to fully developed fish, and at 5–6 days postfertilization (dpf), all important internal organs are largely formed, and skin consists of distinct compartments, as visualized by transmission electron microscopy (Figure 1). Layers representing the epidermis and the dermis can be recognized as early as 1 dpf, but the cutaneous basement membrane zone (BMZ) separating the epidermis and dermis is not evident at that point. However, at 6 dpf, the epidermis is composed of a two-cell layer and is separated from the underlying connective tissue stroma by a basement membrane. On the dermal side, there is collagenous stroma with fibroblastic cells with well-developed rough endoplasmic reticulum. At 6 dpf, the zebrafish skin has a well-demarcated epidermis and dermis, and in a fully developed adult zebrafish, there is a multilayer epidermis that is separated from the underlying collagenous stroma by the basement membrane (Figure 1).

Examination of the zebrafish skin surface as early as 1 dpf by scanning electron microscopy reveals well-demarcated keratinocytes with surface contour containing developing microridges, which become fully developed by 6 dpf. In adult zebrafish the epidermis is covered by scales, which develop at around 30 dpf as a result of a genetic cascade that includes sonic hedgehog expression. In addition to fibroblastic cells adjacent to the collagenous stroma, zebrafish skin has a neural crest–derived pigment cell system that includes melanocytes, potential targets to study the developmental biology and pathology of pigmentation (Ni-Komatsu and Orlow, 2007). Zebrafish also have structures that are specialized for the aquatic environment, including the lateral line that contains 54 neuromasts, topographically highly conserved neural elements consisting of hair cells. This lateral line serves as a sensory organ monitoring the rheological movement of the fish in water. It should be pointed out, however, that in addition to the

scales, zebrafish and human skin are different in that the zebrafish lacks mammalian appendages, including hair follicles and sebaceous glands.

CUTANEOUS GENE EXPRESSION

Considering the similarity of the skin structure, with clearly recognizable epidermis, BMZ, and dermis, we have previously profiled zebrafish skin gene expression by reverse transcription (RT)-PCR for selected genes known to be expressed in human skin (Li *et al.*, 2011). A number of epidermal marker genes, including keratins 1 and 5, the 230-kDa bullous pemphigoid antigen, and plectin, are expressed in zebrafish skin as early as 1 dpf. Several BMZ genes, including subunit polypeptides of type IV collagen, as well as collagens VII and XVII, can be readily detected. Major collagens present in human dermis, including collagens I, V, and VI, are also expressed in zebrafish skin at 6 dpf. Thus, the gene expression profile in zebrafish reveals a repertoire of genes that are also represented in the developing human skin. It should be noted, however, that the zebrafish genome does not appear to contain genes encoding filaggrin, involucrin, or trichohyalin. The absence of these genes may reflect the fact that zebrafish epidermis does not undergo terminal differentiation and does not form stratum corneum with barrier functions similar to that of human skin. This difference clearly limits the study of some human epidermal disorders using zebrafish as a model system.

In addition to analysis of temporal expression of the genes in zebrafish by RT-PCR, spatial expression of these genes can be monitored by *in situ* hybridization, which allows identification and distinct localization of mRNAs at different developmental stages. Collectively, technologies such as RT-PCR and *in situ* hybridization allow determination of the temporal and spatial expression of genes in developing zebrafish. The corresponding techniques at the protein level (i.e., western blot and immunohistochemistry) can also be applied to study gene expression in zebrafish, but these approaches are limited by the paucity of antibodies that recognize zebrafish protein sequences.

GENETIC MANIPULATION OF THE ZEBRAFISH GENOME AND INHIBITION OF GENE EXPRESSION

One approach to study heritable skin diseases using zebrafish as a model system employs forward genetics, in which discrete point mutations are introduced into the genome with ethylnitrosourea (ENU), or random mutagenesis is carried out using retroviral techniques. After mutagenesis, a large number of embryos or larvae are screened for cutaneous phenotypes, facilitated by the transparency of the developing fish (Figure 2). Large-scale forward-genetics screens can also be used to identify mutated genes orthologous to those causing human heritable diseases, with phenotypic similarities. This approach will also allow identification of previously unreported genes that can be tested in patients with similar phenotypes but with no known gene defects.

Another way of utilizing zebrafish as a model system to study heritable skin diseases is to perform "knockdown" of the expression of specific genes by stable morpholino-based antisense oligonucleotides (Figure 3). These morpholinos can target either the sequences around or slightly upstream from the translation initiation codon (AUG) to prevent translation or splice junction sequences to prevent synthesis of a mature mRNA. In the case of morpholinos targeting the upstream regulatory sequences, the efficiency

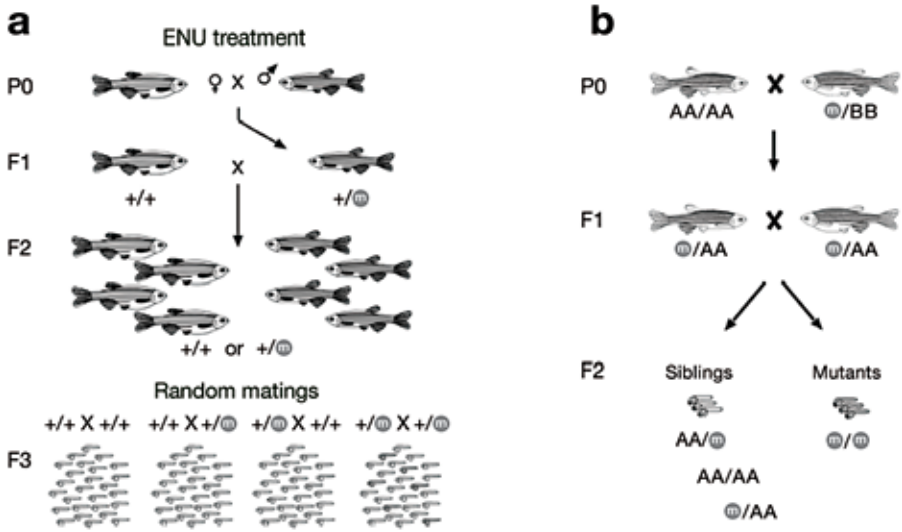

Figure 2. Principles of forward genetics for identification of mutated genes in zebrafish by ethylnitrosourea (ENU) mutagenesis. (**a**) ENU treatment of male fish results in random mutagenesis (m). Mating of mutant males with normal females results in a progeny of mutant fish with a readily observable phenotype in the case of a dominant mutation in F1 and F2 generations. In the case of a recessive mutation, random matings result in a population with the phenotype (gray fish). (**b**) Identification of the recessive mutations resulting in a phenotype (gray fish). The specific mutant genes will be identified by linkage analysis and the mutations can be detected by genome sequencing. SNP, single-nucleotide polymorphism; SSLP, simple sequence-length polymorphism. Modified with permission from Dahm *et al.*, 2005. Copyright Wiley–VCH Verlag GmbH & Co. KGaA.

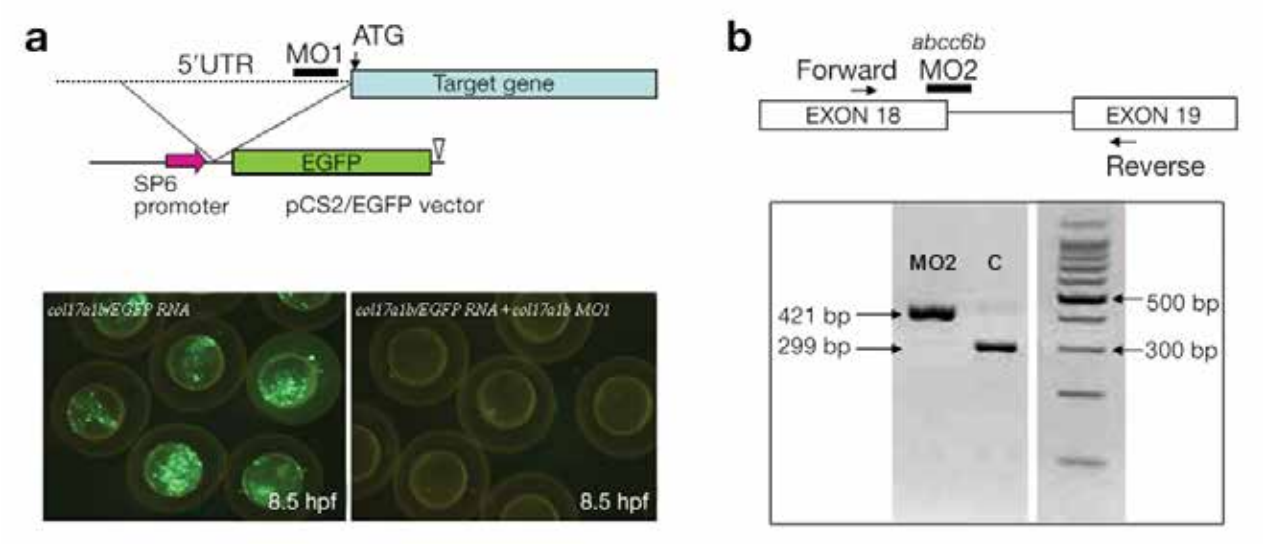

Figure 3. Morpholino-mediated knockdown of zebrafish genes. (**a**) A morpholino (MO1) corresponding to the *col17a1b* gene was used to target the 5′ untranslated region of the corresponding mRNA to prevent translation. To determine the efficacy of morpholino in downregulating the translation, an expression construct consisting of the SP6 promoter, 5′ untranslated region 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). Coinjection of this mRNA together with the MO1 morpholino completely abolished the fluorescence, indicating inhibition of the translation. (**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 reverse transcription (RT)-PCR using primers placed on exon 18 (forward) and exon 19 (reverse). PCR of the genomic sequence resulted in a 421-bp fragment, whereas 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. Because the intron 18 sequence is out of frame, this results in a complete absence of the abcc6b protein product. Reprinted with permission from Li *et al.* (2011).

can be monitored by coinjection of the morpholino with mRNA transcribed from an expression construct containing the 5′ regulatory elements of the corresponding gene linked to green fluorescent protein reporter. In the case of splice junction morpholinos, the efficiency of the gene expression knockdown can be monitored by reverse RT-PCR (Figure 3).

APPLICATION OF THE ZEBRAFISH MODEL FOR SKIN RESEARCH

The advantages of the zebrafish as a model system to study cutaneous biology and pathology are becoming widely recognized, and an increasing number of publications utilize this system (Rakers *et al.*, 2010). For example, several recent articles published in the *Journal of Investigative Dermatology* utilized zebrafish as a model system. One examined wound healing and reepithelialization in adult zebrafish skin following full-thickness wounds inflicted onto the flank of adult zebrafish (Richardson *et al.*, 2013). The results showed that, apart from external fibrin clot formation, all steps of adult mammalian wound repair also exist in zebrafish. Extremely rapid reepithelialization was initiated with no apparent lag phase, followed by migration of inflammatory cells and formation of granulation tissue consisting of macrophages, fibroblasts, blood vessels, and collagen. The granulation tissue later regresses, resulting in minimal scar formation. Further studies suggested that wound reepithelialization occurs independently of inflammation and fibroblast growth factor signaling, essential for fibroblast recruitment and granulation tissue formation. Together, these results demonstrated that major steps and principles of cutaneous wound healing are conserved among adult mammals and adult zebrafish, making zebrafish a valuable model for studying vertebrate skin repair.

Another study demonstrated the utility of forward genetic screening to identify a loss-of-function mutation in the *kindlin-1* gene in mutant zebrafish demonstrating epidermal fragility at 2 dpf (Postel *et al.*, 2013). The phenotype consisted of progressive rupturing and eventual complete loss of medial fins as a result of ENU mutagenesis. Mitotic mapping positioned the causal mutation on chromosome 20 to an interval that contained *kindlin-1* (FERMT1), which encodes Kindlin-1, the zebrafish ortholog of the human protein at fault in Kindler syndrome (Has *et al.*, 2011). Characterization of the zebrafish mutation in this gene revealed a G → T transversion mutation in exon 13, resulting in a premature termination codon at amino-acid residue E565. It was noted that the fin phenotype of the *kindlin-1* mutants resembled that of knockdown embryos injected with kindlin-1-specific morpholino. Thus, the *kindlin-1* mutant zebrafish provides a unique model system to study epidermal adhesion mechanisms *in vivo*.

Another study examined the microphthalmia-associated transcription factor (MITF), the master melanocyte transcription factor with a complex role in melanoma, using a conditional zebrafish model (Lister *et al.*, 2013). Specifically, a temperature-sensitive *MITF* zebrafish mutant was employed to conditionally control endogenous MITF activity. The results showed that low levels of endogenous activity are oncogenic with $BRAF^{V600E}$ to promote melanoma, reflecting the pathology of the human disease. Abrogating MITF activity in $BRAF^{V600E}$ melanoma led to dramatic tumor regression marked by melanophage infiltration and increased apoptosis. These studies suggested that targeting MITF activity is a potent antitumor mechanism, but also showed that caution is warranted because low levels of wild-type MITF activity could be oncogenic.

CONCLUSIONS

Zebrafish is an expedient and cost-effective model system that can be applied to explore the pathomechanisms of a number of human skin diseases. It is conceivable that the zebrafish model will become increasingly popular with increasing recognition of its advantages over conventional model systems.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

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SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching purposes is available at http://dx.doi.org/10.1038/jid.2014.182.

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QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

1. Which of the following statements is applicable to zebrafish?

A. The zebrafish genome has been completely sequenced.

B. There is a multitude of antibodies suitable for western analysis and immunohistochemistry of zebrafish proteins.

C. The zebrafish model system is expensive because of the long developmental delay of embryos.

D. None of the above.

2. The zebrafish model system can be used to study which of the following?

A. Pigment cell biology.

B. Wound healing and skin repair.

C. The genetic basis of skin diseases.

D. Epidermal adhesions.

E. All of the above.

3. Techniques applicable to study genetic diseases in the zebrafish model include which of the following?

A. Morpholino "knockdown" of gene expression.

B. Forward genetics by ENU mutagenesis.

C. *In situ* hybridization to examine spatial expression of genes.

D. All of the above.

4. Zebrafish skin development is different from that of humans because of

A. a lack of terminal differentiation toward formation of stratum corneum.

B. the absence of sebaceous glands and hair follicles.

C. formation of the lateral line containing neuromasts.

D. All of the above.

5. Which layer is absent in zebrafish skin?

A. Basement membrane zone.

B. Collagenous stroma.

C. Stratum corneum.

D. Basal cell layer.

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