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Special Review

Zebrafish for modeling skin disorders

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Abstract

The experimental advantages of zebrafish make this model system highly amenable to the field of dermatology. Zebrafish skin development is similar to humans and its genome is ~70% orthologous to the human genome. Its external developmental process allows for genetic manipulation and analysis of embryogenesis within a short time frame with all important internal organs and skin compartments formed within 6 days. Zebrafish models of cutaneous human diseases offer insight into pathogenesis and a unique platform for testing of potential therapies. This review details the specific advantages of zebrafish and highlights its use in dermatological research.

Keywords: Zebrafish, Dermatology, Animal Models

Introduction

Animal models help probe cutaneous pathophysiology and facilitate testing of treatments. Human diseases have been modeled in mice, through “knock-out” (KO) animals or transgenic mice mutants, but mice as a model system have limitations including high cost of model development, limited litter size, and a relatively long generation time [1]. Alternative animal model systems may help address these limitations.

The zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the minnow family (Figure 1). Zebrafish are useful models due to ease of embryological manipulation and *in vivo* transparency of cell-biological events and have been used in modeling several human diseases [1]. Forward genetic screens have generated zebrafish models of monogenic human disease, and

transgenic approaches enable zebrafish to model acquired diseases [2]. Zebrafish can also be used for drug screening, target identification, and toxicology [3].

Advantages of the Zebrafish Model System

Zebrafish have a remarkable similarity to humans in genetics and skin development. Approximately 70% of human genes have a zebrafish counterpart, and 84% of the genes that cause human diseases have a zebrafish orthologue [4]. The zebrafish model system also has the advantage of approximately 50–100 embryos obtained per laying.

Zebrafish embryos are optically transparent during the first several days (Figure 1) [5]. The external development process facilitates testing embryos with potential therapies. Zebrafish are easily maintained in the laboratory setting with a rapid rate of maturation from embryos to fully developed fish. Organ structures are identifiable just 24 hours post fertilization (hpf), and the epidermis and dermis can be recognized. At 5–6 days post fertilization (dpf), organogenesis is largely completed, and skin consists of distinct compartments.

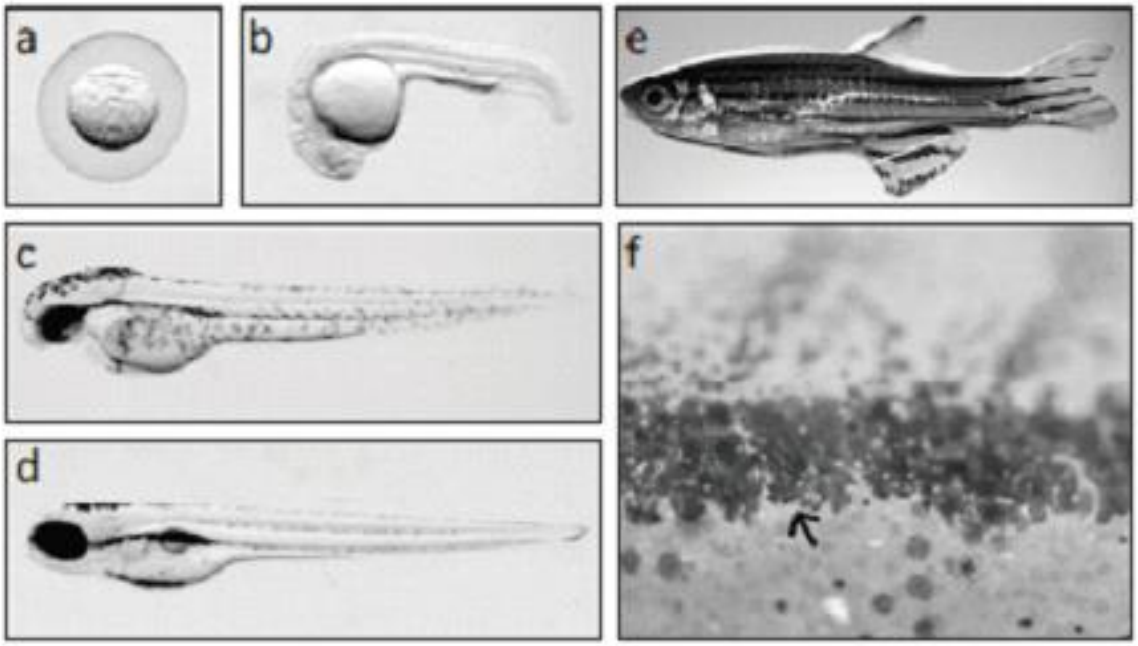

Figure 1. Timeline of Zebrafish Development a) Zebrafish egg, which is surrounded by a transparent chorion 10 minutes post fertilization. b) Dechorionated zebrafish 24 hours post fertilization. c) Zebrafish 48 hours post fertilization. d) Zebrafish 4 days post fertilization. e) Adult zebrafish. f) Close up of adult zebrafish showing melanophores (arrowhead)

Zebrafish Skin Development, Structure and Organization

Similar to humans, zebrafish skin has three compartments: epidermis, dermis and hypodermis (Table 1). The entire skin development can be comprehensively studied within a very short time window. 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 [6].

Table 1. Epidermal Organization in Zebrafish Skin

Structure	Location	Key Function (s)	Human Equivalent
Cuticle	Epidermis	Protection, antimicrobial factors	Keratinized epithelium
Microridges	Epidermis	Support cuticle, improve secretion	No equivalent
Superficial stratum	Epidermis	Stability, gas exchange, shock protection	Stratum granulosum
Intermediate stratum	Epidermis	Mucous goblet cells, club cells, sensory cells, non-differentiated cells	Stratum spinosum
Basal stratum	Epidermis	Attachment to dermis, hemidesmosomes	Stratum basale
Lamina lucida	BMZ	Contains laminin and integrin	Lamina lucida
Lamina densa	BMZ	Contains collagen	Lamina densa

Hemidesmosomes	Epidermis, BMZ Dermis	Attach basal layer to extracellular basal lamina	Hemidesmosomes
Desmosomes	Epidermis	Adhesive junctions of epithelia	Desmosomes
Lateral line	Epidermis	Sensory organ of movement and vibrations	Inner ear hair cells
Melanocytes	Dermis	Pigmentation, protection against UV-radiation	Melanocytes
Scales	Dermis	Protection, calcium source	Hair, teeth
Blood vessel	Dermis	Blood transport, supply nutrition	Blood vessel
Adipocyte	Hypodermis	Insulation, calorie reservoir, absorbs trauma	Fat cells

BMZ: Basement Membrane Zone

At 1 dpf, well-demarcated keratinocytes are present with distinct cell-cell borders. In the middle of the epidermal surface are developing microridges, which become well organized by 6 dpf [5]. Pigmented melanocytes develop from neural crest cells, appear at 24 hpf [7, 8]. The number of melanocytes increases through 60 hpf, reaching approximately 400 melanocytes. Unlike mammalian melanocytes that transfer melanosomes to keratinocytes, zebrafish melanocytes retain melanin (Figure 1F)[9].

At 6 dpf, the zebrafish skin has a fully distinguished epidermis and dermis. The epidermis is composed of two cell layers: a basal layer and a superficial layer of keratinocytes [6]. The epidermis is readily noticeable and clearly separated from the underlying connective tissue stroma by a basement membrane. On the dermal side there is a collagenous stroma with adjacent fibroblastic cells with well-developed rough endoplasmic reticulum [6].

By 20 dpf, the epidermis increases to three layers: the superficial stratum, the intermediate stratum, and the basal stratum (Table 1). The superficial stratum acts as a seal between the animal and its surroundings. Its surface has actin-rich microridges that maintain the cuticle layer on the surface of the fish [10, 11]. The cuticle is a mucous layer heavily enriched with antimicrobial factors, including antibodies, complement, lysozyme, C-reactive protein, lectins, proteases, transferrin, and polypeptide antibiotics [12]. Beneath this protective mucus, cell-cell junctions of the superficial epithelial cells provide epithelial coherence. The functions of these tight junctions in fish skin may be relevant for dermatologists investigating the role of tight junctions expressed by keratinocytes and Langerhans cells [13, 14].

Unlike mammalian skin, zebrafish epithelial cells are only replaced upon death or injury, and all epidermal cells appear capable of proliferation [6]. In the intermediate stratum, most epithelial cells remain non-differentiated, providing a potential stem cell reservoir for keratinocytes . Also in this intermediate layer are club cells, sensory cells, and mucous cells that provide the mucous layer on the zebrafish surface. [15]. The third, deep region of the epidermis is the basal stratum, with hemidesmosomes attaching epidermis to the underlying dermis.

Cellular Junctions in the Zebrafish Epidermis

The zebrafish genome contains homologues of all mammalian hemidesmosome components (Table 1) [16]. Hemidesmosomes are found in the dorsal and lateral epidermis of zebrafish, connecting the basal layer to the extracellular basal lamina. Integrin α6 (Itga6) is the membrane receptor of zebrafish hemidesmosomes. Itga6 localizes to the lateral and basal domains of basal epidermal cells at 2.5 dpf and clusters with intermediate filaments prior to hemidesmosome formation [17]. Hemidesmosomes first become visible in the basal epidermal cells at 4 dpf, and mature in size and shape at 5.5 dpf. Once hemidesmosome clusters appear, the laterally-localized Itga6 molecules move to the basal side of the epidermis via the cell polarity protein Lgl2 and vesicle trafficking protein Clint1[18, 19]. Improper transport of Itga6 results in defective hemidesmosome assembly, which manifests as epidermal detachment [17].

The adhesion molecules of desmosomes, desmocollin (Dsc) and desmoglein (Dsg), are expressed early in zebrafish development. Zebrafish contain an orthologue of mammalian desmocollin 1, and two closely related orthologues of mammalian desmoglein 2 [20]. Fully formed desmosomes are present between epidermal cells beginning at 12 hpf. *Dsc* and *Dsg* knockdowns have retarded head development, altered somite morphogenesis, blebbing of the epidermis and some embryonic lethality [20].

Zebrafish Basement Membrane

Zebrafish cutaneous basement membrane is present by 32hpf and fully differentiated by 48hpf. The lamina lucida accumulates granular material while collagen fibrils insert into the lamina densa, facilitating the dermal-epidermal support and binding (Table 1) [6]. The basement membrane is composed of collagen IV, VII, XII and XIV, which link the dermis to the basement membrane [5, 21, 22]. Also present are laminin, α3-integrin, α6-integrin, and Fras1 [5, 23].

Differences between Zebrafish and Mammalian Epidermal Development

Around 30 dpf, the epidermis of adult zebrafish is covered by scales resulting from a genetic cascade that includes sonic hedgehog expression [24]. In contrast to human epidermis, the epidermis of zebrafish is non-keratinized. Keratinization is uncommon in fish, occurring only in specific sites that are subjected to abrasion, such as adhesive organs, lips and pads, and the epidermal surface of some species capable of emerging from the water [25].

Zebrafish lack hair follicles, and sebaceous glands but have specialized aquatic structures including mucous secreting cells and the lateral line containing mechanosensory neuromast hair cells that sense movement and vibrations in the water (Table 1) [26]. The hair cells are functionally analogous to mammalian inner ear cells, providing a model system for studying hair cell migration and regeneration [27].

Zebrafish and Human Cutaneous Gene Expression

The architecture of fish skin is highly homologous to the epidermis of mammals [28, 29]. Markers for studying zebrafish epidermal development have been defined that compare to mammalian epidermal development, revealing common developmental pathways, such as sonic hedgehog (SHH), bone morphogenetic protein (BMP), and Wnt signaling pathways [30, 31].

Reverse transcription (RT)-PCR of zebrafish for selected genes expressed in human skin showed several epidermal marker genes, including keratins 1 and 5, the 230kDa bullous pemphigoid antigen1, and 500kDa plectin, are expressed in zebrafish skin as early as 1 dpf. Basement membrane genes, including collagens VII and XVII (bullous pemphigoid antigen 2), and subunit polypeptides of type IV collagen are detected. Collagens present in human dermis, including collagens I, V, and VI, are also expressed in zebrafish skin at 6 dpf, while collagens XII, XIV, XV, XVI, XVIII, and XIX are detected at different stages of zebrafish development [5]. Molecular genetic substances including the retinol-binding protein 4 and apolipoprotein Eb are in both zebrafish and humans in epidermal and dermal development [32]. Thus, the zebrafish gene expression profile reveals a wide repertoire of genes also present in the developing human skin.

The zebrafish genome does not appear to contain genes encoding filaggrin, involucrin, or trichohyalin granules [5]. The absence of these genes may reflect the lack of keratinization. This difference clearly limits the use of zebrafish as a model for some human epidermal disorders.

Genetic Manipulation in the Development of a Zebrafish Model

The availability of spontaneous and engineered zebrafish mutants with defined skin phenotypes has made this model system an attractive alternative to mutant mice for cutaneous *in vivo* experimentation. One approach involves use of ethylnitrosourea (ENU) or random mutagenesis is carried out using retroviral techniques to introduce discrete point mutations into the genome. Following mutagenesis, embryos are screened for cutaneous phenotypes, easily facilitated by the transparency of the developing fish. Large-scale forward-genetics screens also identify mutated genes orthologous to those causing human heritable diseases with phenotypic similarities.

One such example is the zebrafish orthologue to human kindlin-1, mutations of which result in Kindler Syndrome, a congenital disorder characterized by skin fragility, photosensitivity, and blistering [33]. A forward genetic screen identified a zebrafish Kindlin-1 loss-of-function mutant that develops the same mechanical trauma-induced epidermal fragility as seen in Kindler syndrome [34]. This mutant zebrafish provides a unique model system to study epidermal adhesion mechanisms *in vivo*. Such large scale mutagenesis and screening strategies generate zebrafish models of a wide variety of skin disorders, and are now being increasingly employed in drug discovery and drug development programs [1].

In addition to traditional chemical mutagenesis and insertional mutagenesis methods, there is the more recently developed technology of gene-breaking transposon (GBT). GBT mutagenesis system integrates a gene-breaking transposon containing a protein trap that efficiently disrupts gene expression with >97% knockdown of normal transcript amounts (Figure 2a) [35]. This

method allows for systematic conditional mutant alleles using Cre recombinase or morpholinos targeted toward the splice site. This technology has identified and characterized new genes, expression patterns, and phenotypes in the vasculature, muscle, and even skin development [36-38].

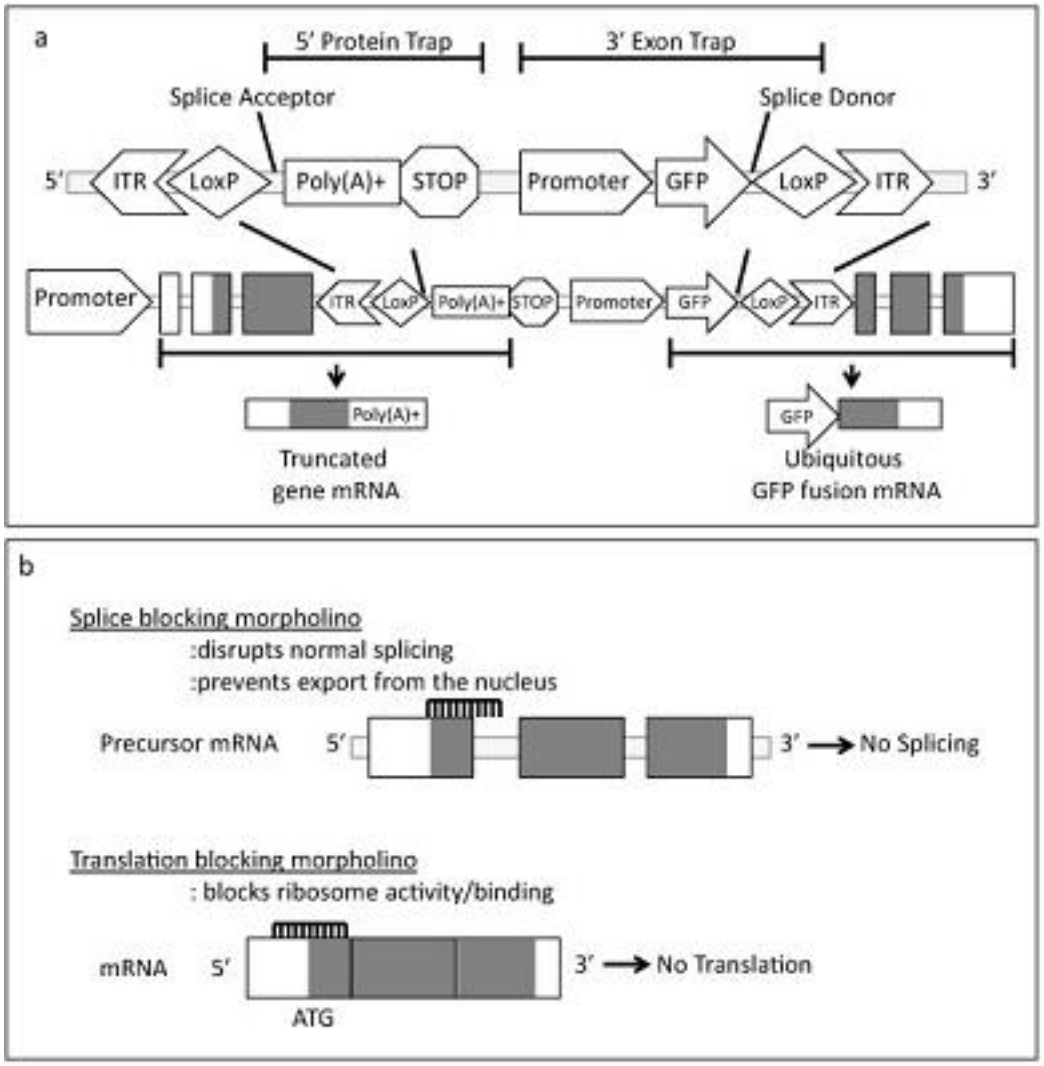

Figure 2. Gene manipulating technologies in zebrafish. (**a**) Diagram of Gene-Breaking Transposon (GBT). Gene-breaking occurs during transcription of an endogenous locus with a GBT insertion. The splice acceptor in the 5' protein trap cassette intercepts splicing machinery, resulting in a truncated transcript. The 3' exon trap cassette uses the splice donor to create a GFP fusion transcript with the remaining downstream endogenous transcript. GBT alleles are revertible because LoxP sites flank the cassettes, allowing for Cre recombinase excision of mutagenic elements. ITR, inverted terminal repeat; Poly(A)+, polyadenylation signal; STOP, extra transcriptional terminator. (**b**) Morpholinos: Splice blocking morpholinos bind at the exon-intron junction, preventing normal splicing activity and preventing mRNA export from the nucleus. Translation blocking morpholinos bind to the 5'-untranslated region of mRNA, thus preventing ribosomal activity and binding.

The Zebrafish Integument Project aims to identify new skin genes and phenotypes in zebrafish development [38]. *In vivo* selection for skin-specific gene expression identified GBT alleles of genes involved in skin development and human skin diseases, such as *fras1*. In humans, loss-of-function mutations in *FRAS1* cause the rare congenital disorder Fraser Syndrome, which is characterized by epidermal or epithelial blistering during fetal development [39]. An ENU-induced zebrafish mutant of Fras1 has been defined, but lethal craniofacial defects limit investigations into epidermal blistering [23]. GBT alleles of *fras1* fully phenocopy the mutant zebrafish model of Fraser Syndrome and allows for allele reversion spatially and temporally. The ability to revert can rescue the lethal craniofacial defects of *fras1* mutants, thus allowing for later investigations of epidermal blistering [40].

Another approach to zebrafish model development is specific gene expression "knockdown" by morpholino-based antisense oligonucleotides [41, 42]. Morpholinos are injected into a 1-to-4 cell embryo and allow for manipulation of expression of specific genes. Morpholinos inhibit translation or splicing of a mature mRNA by targeting and blocking the translation initiation codon (AUG) or splice junction, respectively (Figure 2b). Morpholino knockdown specificity can be confirmed by co-injection of the

corresponding mRNA or protein from another species to counteract the phenotype development [41]. Additionally, microinjection of purified mRNA (without morpholinos) results in gene overexpression.

Morpholinos have a short half-life (up to 5 days), and therefore this approach is most suitable for evaluation of early zebrafish development. Therefore, the morpholino knockdown phenotype is most likely to reproduce the clinical manifestations in human diseases that develop during prenatal development or shortly after birth. One example includes the morpholino knockdown of the *abca12* gene that is associated with harlequin ichthyosis and a variant of lamellar ichthyosis [43]. In harlequin ichythyosis, newborns are born encased in a thick collodion membrane that distorts their facial features, limits mobility of the extremities as well as the chest wall, and results in a reduced lifespan with death in childhood or adolescence if they survive the immediate post-natal period. Knockdown of the *abca12* resulted in morphant fish with absent microridge and development of scale-like spicules on the skin surface. These phenotypic manifestations resemble the scales seen in human ichthyosis [43].

Dermatological Research in the Zebrafish Model System

Zebrafish are advantageous in studying skin development and pathology because they can mimic human skin disease. One instance is the morpholino knockdown of the col17a1a gene expressed in skin hemidesmosomal complexes. Knockdown fish manifest blistering of the dermis similar to junctional epidermolysis bullosa due to mutations in the COL17A1 gene [44]. The zebrafish system is becoming widely recognized as more and more dermatological studies use zebrafish. (Table 2)

Table 2. Zebrafish Models of Human Skin Conditions

Human Condition	Zebrafish Model	Reference
Kindler Syndrome	*kindlin-1*	Postel et al. 2013
Fraser Syndrome	*fras1*	Carney et al. 2010, Talbot et al. 2012
Human Ichythyosis	*abca12*	Li, Frank, Akiyama, et al. 2011
Epidermolysis bullosa	*col17a1a*	Kim et al. 2010
Melanoma	*mitfa-BRAF*^{V600E}	Patton et al. 2005
Psoriasis	*clint1*	Dodd et al. 2009
	penner	Sonawane et al. 2005
	psoriasis	Webb, Driever, and Kimelman 2008
	hai1	Carney et al. 2007

Certain aspects of zebrafish skin development also make this model system appealing to research. Zebrafish have a neural crest-derived pigment cell system that includes melanocytes, allowing for investigations into the development and pathology of pigmentation [45]. The increase in melanocyte number from 60 hpf to 2 weeks provides a window to explore melanocyte regeneration independently of normal ontogenetic mechanisms for melanocyte development. Furthermore, the fact that zebrafish melanocytes retain melanin serves as a reliable and useful cell-type marker. A number of zebrafish pigmentation mutants that affect melanocyte specification, differentiation, and function are available. A transgenic zebrafish model for melanoma overexpresses activated human *BRAF* oncogene in neural crest cells, and inactivation of *p53* in these fish results in melanoma formation [46]. Moreover, xenografting of human melanoma cells into early zebrafish embryos allow for observation of tumorigenesis and tumor-host interactions [47]. These models yield valuable insight into melanoma research, serving as a tool for testing therapies in preventing and treating melanoma.

Other zebrafish mutants have provided clues to epidermal development by exhibiting psoriasis-like phenotypes [17, 19, 30, 48]. The *clint1* mutant has chronic inflammation characterized by increased interleukin 1β expression, leukocyte infiltration, and phagocytosis of cellular debris [19]. These mutants display keratinocyte hyperproliferation, the development of epidermal aggregates, and inflammation similar to psoriasis seen in humans.

Another area of research is vertebrate skin repair. Zebrafish show regeneration in fins, spinal cord, optic nerve, heart and skin [49]. Research of mammalian wound healing may benefit from zebrafish tissue regeneration, such as the regrowth of amputated caudal fins, or zebrafish blood vessel formation [50, 51]. Based on studies of full-thickness wounds inflicted onto the flank of adult zebrafish, the major steps of cutaneous wound healing are conserved among adult mammals and adult zebrafish [52]. However, the mechanisms of fish cutaneous regeneration still have a long way to go before they can be harnessed in healing human skin.

Future research may investigate the role of the fish epidermis in innate protective functions against infections. Antimicrobrial peptides (AMPS), such as hepcidin, defensin-like peptides, certain apolipoproteins, and piscidin provide an antimicrobial defense system against pathogenic bacteria, fungi, algae, viruses or parasites [53-56]. Research may provide insight into the innate immune system of mouse and human skin [57, 58]. AMPs in fish skin may reveal potential AMPs in the search for mammalian homologues. Fish-derived AMPs could be used in human skin therapy, such as patients with atopic dermatitis whose skin displays pathologically heavy colonization by bacteria [59].

Systematic exploration of zebrafish skin models is biologically, clinically, and technologically relevant. The zebrafish model system has contributed to investigative dermatology and offers opportunities for future research. Zebrafish allow for use of molecular tools, genetic manipulation, and a platform for therapeutic testing. Through the use of these technologies, zebrafish have become a valuable animal model for the study of cutaneous human disease.

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