The medaka *rs-3* locus required for scale development encodes ectodysplasin-A receptor

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The bodies of most teleost fish species are covered with specialized subepithelial structures known as scales. The scale is an epithelial appendage that differentiates from the dermal mesenchyme. Mammals, on the other hand, have no scales, but instead their bodies are covered with hair. Although their appearances are quite different, scales and hair can be considered structurally similar in that both of them are epithelial appendages distributed over the body surface in an orderly pattern. This analogy suggests that they may have the same evolutionary origin. But, to date, no molecular evidence has been presented that links scales and hair. A mutation at the *rs-3* locus of medaka (*Oryzias latipes*) leads to almost complete loss of scales. We demonstrated that the *rs-3* locus encodes ectodysplasin-A receptor (EDAR), which is required for the initiation of hair development in mammals. We identified a novel transposon inserted in the first intron of *EDAR*, which causes aberrant splicing. This work shows that EDAR is required for scale development in fish and suggests that it is an evolutionarily conserved molecule that is required for the development of epithelial appendages in vertebrates.

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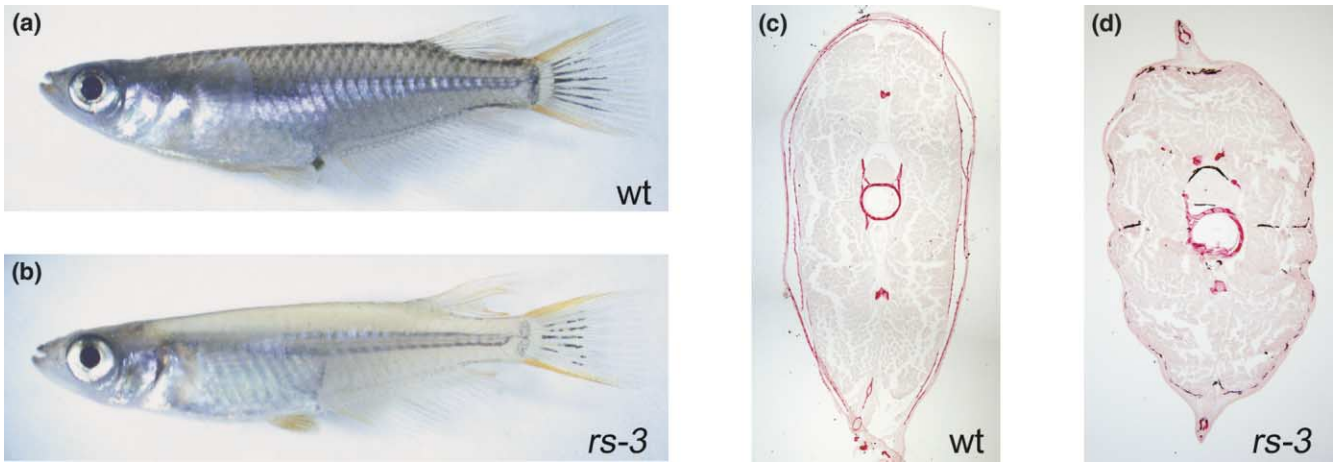
Results and discussion

rs-3 (reduced scale-3) was originally isolated from wild populations by Hideo Tomita in a screen for spontaneous mutants [1, 2]. *rs-3* is a recessive mutation, and homozygous fish are viable and fertile but completely lack scales (Figure 1), except for a few, which are larger in size and irregular in shape. The few scales in *rs-3* mutants usually form around the dorsal fin and sometimes along the lateral lines (data not shown). *rs-3* mutants have no detectable defects other than the loss of scales. The fact that mutant fish do form a few scales, if irregular in shape, suggests that the *rs-3* locus encodes a protein involved in either the proliferation or differentiation of scales rather than a protein that is a structural component of scales per se.

Linkage analysis between known DNA markers and the *rs-3* locus revealed that the *rs-3* locus maps to medaka linkage group (LG) 21. LG21 contains the *HOXDA* cluster, whose orthologs in zebrafish and humans are located on zebrafish LG9 and the long arm of chromosome 2 (Chr 2q), respectively. Studies of the zebrafish genome had identified extensive conservation of synteny between zebrafish LG9 and human Chr 2q [3, 4]. Given evolutionary relationships between humans, zebrafish, and medaka, we also expected synteny to be conserved between medaka and human chromosomes. Therefore, we took advantage of conserved synteny to isolate EST markers closely linked to the *rs-3* locus. We cloned medaka orthologs of 14 human genes on Chr 2q by degenerate PCR and mapped them using a reference panel of 39 male meioses [5]. Ten of them were mapped to LG21, whereas the remaining four were mapped to LG2 (Figure 2b). Although synteny conservation between human Chr 2q and medaka LG21 spans the entire chromosome arm, the gene orders are not conserved at all. A discrepancy in gene order reflects intrachromosomal inversions after the divergence of humans and medaka. Our data support the hypothesis that inversions have been more frequently fixed than translocations during vertebrate genome evolution [4].

Ectodysplasin-A receptor (EDAR) is one of the EST markers cloned during this process and was found not to recombine with the *rs-3* locus by the analysis of 535 meioses. EDAR is a type I transmembrane protein that shows weak similarity to members of the TNF receptor superfamily, acting as a receptor for a TNF-like transmembrane ligand, ectodysplasin-A (EDA) [6]. Mutations

Figure 1



The phenotype of *rs-3* mutant fish. **(a,c)** Wild-type. **(b,d)** *rs-3* mutant. (a,b) Side views of 3-month-old fish. Mutant fish look more transparent, owing to the loss of melanophores associated with scales. (c,d) Transverse sections of 2-month-old fish stained with alizarin. The calcium contained in the scales is stained red in (c) wild-type fish.

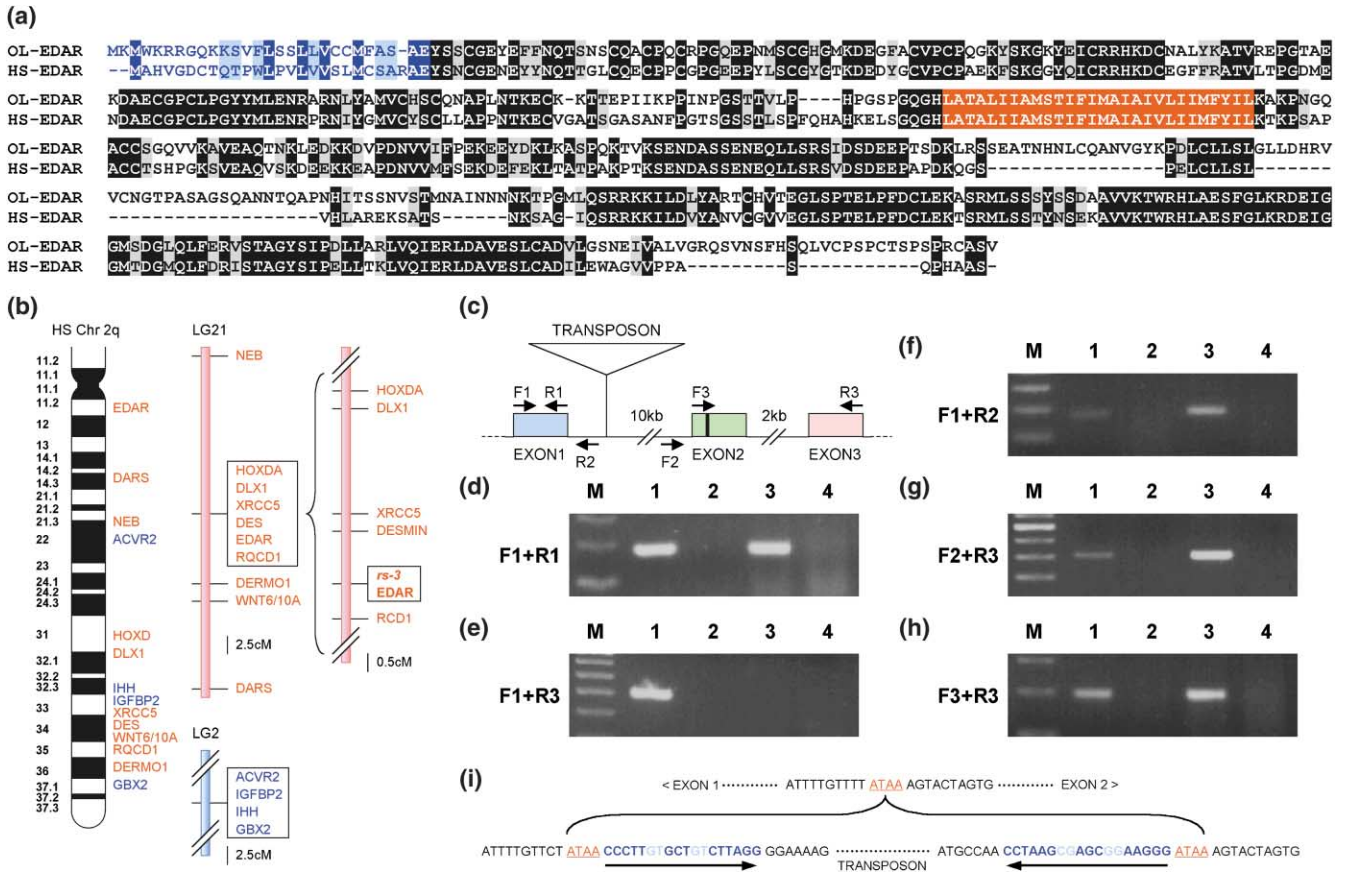
in both the ligand and the receptor are known to result in the loss of certain types of hair in humans and mice. In humans, mutations in *EDA* and *EDAR* cause a clinical syndrome known as hypohidrotic ectodermal dysplasia [7, 8]. In mice, mutants with mutations in *EDA* and *EDAR* are known as Tabby (*Ta*) and downless (*dl*), respectively [6, 9]. We determined the full-length cDNA sequence of medaka *EDAR* and compared it with the human ortholog. The deduced amino acid sequence of medaka EDAR is 56% identical to that of the human ortholog in the entire sequence (Figure 2a). Sequence conservation is high in all of the extracellular, transmembrane, and intracellular domains. It is to be noted that the highly conserved intracellular domain shows no similarity to known proteins, and it will be interesting to unravel how the signal is transduced via EDAR.

The analogy between hair and scales prompted us to test whether EDAR, which is required for hair development in mammals, is disrupted in the scaleless medaka mutant *rs-3*. We compared the full-length coding region sequences of wild-type and mutant *EDAR* cDNAs, but neither missense nor nonsense mutations were identified in the mutant cDNA. We noticed, however, that the 5′ UTR of the mutant cDNA could not be amplified by RT-PCR (Figure 2e). Since exons 1 and 2 are normally amplified from the mutant cDNA (Figure 2d), we assumed that there is an insertion in the 5′ UTR of mutant cDNA that is too long to be amplified by PCR. LA-PCR of a BAC clone containing wild-type medaka *EDAR* revealed an intron of more than 10 kb in the 5′ UTR (data not shown). We designed primers around the splice donor and acceptor and performed RT-PCR to test whether this intron is spliced correctly. Even in the wild-type, we could detect small amounts of unspliced transcripts (Figure 2f,g). However, a significantly larger amount was amplified from the mutant cDNA with primers encompassing the splice acceptor site (Figure 2f). The region containing the splice donor site was also amplified more than the wild-type. But the defect of splicing was more severe at the acceptor site. These results suggest that the mutant *EDAR* transcript harbors several kb of extra sequence in the 5′ UTR that is too long to be amplified by PCR. Since the extra sequence contains out-of-frame start codons and stop codons, the mutant transcript is likely to result in premature initiation and termination of translation and, as a result, the *EDAR* protein is not synthesized.

Next, we asked what causes the aberrant splicing of *EDAR* in *rs-3*. A comparison of the sequences of the mutant and wild-type genomic DNAs revealed that the splice donor and acceptor sites of the mutant were intact. But, we identified an insertion of indeterminate size about 100 bp downstream of the donor site of the first intron of the mutant. We amplified fragments containing the junctions by inverse PCR and determined their sequences. We found that the insertion contains imperfect inverted repeats of 18 bp at the ends and is accompanied by a 4-bp target site duplication, both of which are characteristic of transposon insertion (Figure 2i). In mammals, at least two cases have been reported in which a transposon insertion in an intron causes mutation [10, 11]. In these cases, strong acceptors in the transposable elements impede appropriate splicing. A similar situation may result in the aberrant splicing of *rs-3*. It is also possible that the insertion makes the intron too large to be spliced efficiently.

To determine the expression pattern of EDAR, we per-

Figure 2



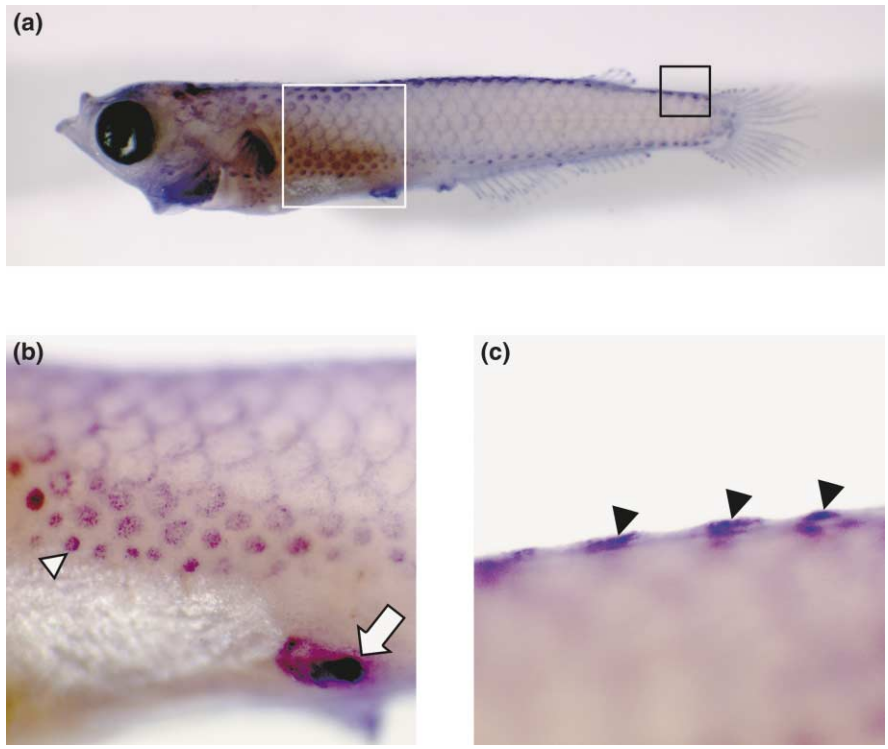
An analysis of medaka EDAR. **(a)** The sequence alignment of the medaka and human EDAR proteins. The residues in blue and red letters are the signal peptide and the transmembrane domain, respectively. **(b)** A comparison of human Chr 2q and medaka linkage groups (LG) 2 and 21. The genes on LG2 and LG21 are written in blue and red letters, respectively. Medaka orthologs of 14 human genes on Chr 2q were cloned and placed on the reference map (middle column; [5]). *HOXDA* and 5 other genes that did not recombine with HOXD in the reference panel were put to linkage analysis with the *rs-3* locus (right column) using 192 meioses of F2 progeny. **(c)** A schematic diagram depicting the genomic structure of medaka EDAR. The vertical line in exon 2 indicates the initiation codon. **(d–i)** The arrows indicate the positions of primers used for RT-PCR analysis. In the *rs-3* mutant genome, a transposon is inserted about 100 bp downstream of the splice donor of the first intron. (d–h) RT-PCR analysis of the wild-type and mutant EDAR transcripts. M, size marker; 1, wild-type RT+; 2, wild-type RT−; 3, *rs-3* RT+; and 4, *rs-3* RT−. (i) The sequence analysis of the transposon insertion in the *rs-3* locus. The 4-bp target site duplication is shown in red (underlined), and the 18-bp inverted repeats are shown in blue and are indicated by arrows.

formed whole-mount in situ hybridization of 8-, 15-, and 30-day-old fish. Expression was barely detectable on the body surface of 8- and 15-day-old fish. The scale development begins around 30 days after fertilization, when the body size reaches 10 mm. In 30-day-old fish, strong expression was detected in patches of epithelial cell clusters and the posterior margins of growing scales (Figure 3a). The clusters of cells expressing EDAR correspond to scale placodes, which are the first morphological sign of scale development (Figure 3b,c). At this stage, we could observe nascent scales at different stages of development, depending on their positions in the body. Mature scales were observed on the lateral flanks, while only placodes were seen in the dorsal and pectoral regions.

On the basis of tight linkage, expression pattern, and transposon insertion that causes mutation, we conclude that the *rs-3* locus encodes EDAR. The phenotypes of *Ta* and *dl* mice also manifest in teeth. We analyzed the teeth of *rs-3* fish using scanning electron microscopy, but no defects were observed in terms of shape and size (data not shown).

In mice, EDAR is the earliest expression marker of hair development and is strongly expressed in clusters of epidermal cells that eventually become hair placodes. Since the expression of early markers including BMP-4 and Sonic Hedgehog is abolished in *dl* mutants, EDAR is required for the initiation of hair development. The ex-

Figure 3



The expression of EDAR. The expression of EDAR in a wild-type 30-day-old fish was visualized by whole-mount in situ hybridization. **(a)** The expression is seen in scale placodes and the posterior margins of growing scales. The expression is also observed in fin rays. **(b,c)** Close-up views corresponding to the white and black boxes in (a), respectively. (b) An example of a scale placode expressing EDAR is indicated by an arrowhead. Developing pectoral fins also express high levels of EDAR (arrow). (c) Epithelial cells of scale placodes (arrowheads) are clusters of cells that express EDAR.

pression of murine EDAR is first uniform throughout the embryonic epidermis and later localized to hair placodes. In mutant mice with a point mutation in the *EDAR* gene, the distribution of its transcript is not localized to hair placodes and remains uniform [6]. Headon and Overbeek suggest that focal upregulation of EDAR expression is achieved by a mechanism of lateral inhibition, and uniform EDAR expression in *dl* mice indicates that it is also required for its own localization by inducing lateral inhibitors. BMP2 and BMP4 are good candidates for the mediator of lateral inhibition in hair development, considering the fact that they are potent inhibitors of chick feather development [12] and that they are expressed in hair and feather placodes [12–14]. Thus, EDAR first localizes the expression of itself to the hair placode and then induces differentiation of the epidermal cells that express EDAR and the dermal cells underneath.

The phenotype of the *rs-3* mutants indicates that EDAR is required for scale development in fish, and its expression pattern suggests that, like the mammalian counterpart, it determines the future positions of scales. To our knowledge, EDAR is the first molecule that has been identified to be required for both hair and scale development. Our findings are the first indication of the possibility that scales and hair use the same molecular machinery during their development at early stages. Although both scales and hair are epithelial appendages that cover the animal body, their structures and modes of development are quite different [15]. But, at least, the two structures share the feature that they are distributed over the body surface in an orderly pattern. We propose that EDAR is an evolutionarily conserved molecule specifying this feature by defining the future positions of vertebrate epithelial appendages in an organized array.

If we take advantage of small teleosts like medaka and zebrafish, the fish scale will serve as an excellent model for mammalian hair development. Forward genetics is probably the most powerful approach that will complement the study using mammalian and avian systems. A screen for mutants with scale defects is expected to identify key molecules of scale and hair development as well as fish models for human hair diseases.

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