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The Edar subfamily in feather placode formation

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Abstract

A subgroup of the TNF receptor family, composed of Edar, Troy and Xedar, are implicated in the development of ectodermal appendages, such as hair follicles, teeth and sweat glands. We have isolated chicken orthologues of these three receptors and analysed their roles in early feather development. Conservation of protein sequences between mammalian and avian proteins is variable, with avian Edar showing the greatest degree of sequence identity. cXedar differs from its mammalian orthologue in that it contains an intracellular death domain. All three receptors are expressed during early feather morphogenesis and dominant negative forms of each receptor impair the epithelial contribution to feather bud morphogenesis, while the dermal contribution appears unaffected. Hyperactivation of each receptor leads to more widespread assumption of placode fate, though in different regions of the skin. Receptor signaling converges on NF-κB, and inhibiting this transcription factor alters feather bud number and size in a stage-specific manner. Our findings illustrate the roles of these three receptors during avian skin morphogenesis and also suggest that activators of feather placode fate undergo mutual regulation to reach a decision on skin appendage location and size.

Keywords: TNFR; Placode; Feather; Skin; Evolution; Death domain; Edar; Troy; Xedar

Introduction

Skin appendages, such as hairs, feathers, scales and glands, are class-defining features in vertebrates. While the skin itself forms a barrier, its appendages play a wide variety of roles, from defence and display to insulation and aerodynamics. During development these cutaneous appendages are laid out in a periodic pattern in the embryonic ectoderm, the cells of which must all choose between appendage and surface keratinocyte fates. The cells that will become appendages first condense to form dense patches, called placodes. After placode specification, cell proliferation generates downgrowths in mammals to produce hair follicles (Hardy, 1992), or outgrowths in birds to produce a feather bud (Lin et al., 2006). The process of appendage formation in all vertebrate classes relies on a series of reciprocal interactions between the epidermis and its underlying dermis (Sengel, 1990; Hardy, 1992; Fuchs et al., 2001; Millar, 2002).

In mouse, hair follicles are generated across the entire surface of the embryo in a series of temporally defined pulses late in gestation, the later forming follicles filling in the gaps that open up between older follicles as the skin grows. In contrast, in avian skin, several tracts are formed first; following which a defined morphogenetic wave moves across specific tracts, leaving a very regular array of placodes in its wake (Jiang et al., 2004). Despite the similarities between early hair and feather follicle morphogenesis, they appear to be convergently evolved structures (Wu et al., 2004).

The formation of feather buds takes place in hierarchical levels (Chang et al., 2004). In the first level, feather fields (which later become feather tracts) form from presumptive dermis and ectoderm. In the second level, periodic patterning takes place and the originally homogeneous feather field breaks into individual feather buds and interbud regions. In the subsequent levels, feather buds undergo morphogenetic events

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to form an anterior–posterior axis and branches (Yu et al., 2004; Lin et al., 2006).

The expression pattern of genes involved in periodic pattern formation can be categorized into two distinct modes, 'restrictive' and 'de novo', reflecting the successive stages of their functions (Jiang et al., 2004). Molecules with 'restrictive' expression are involved in negotiating placode position. They are initially expressed homogeneously at a moderate level. As appendage locations are specified, these genes become restricted to or upregulated in the placodes and downregulated in the surrounding regions, or vice versa. *β-Catenin* is an example and is considered to be required for establishing the competence of feather field. Molecules with 'de novo' expression, such as *Shh*, appear directly in the placode once its position has been defined. They serve to regulate bud growth, shaping, axis determination and outgrowth morphogenesis (Jiang et al., 1999; Widelitz et al., 2000). The molecules involved in pattern formation fall into two functional categories, i.e. activators and inhibitors of placode fate. Whether direct or indirect, interactions between these inhibitors and activators are responsible for breaking the symmetry of the early skin into the hexagonal feather pattern that emerges (Jiang et al., 2004).

The tumour necrosis factor receptor (TNFR) family is expanded in the vertebrate lineage, with mammalian genomes containing about 30 members (Locksley et al., 2001), compared to a single gene in *Drosophila* (Kanda et al., 2002). This expansion appears to be correlated with acquisition of roles in vertebrate evolutionary novelties, such as the adaptive immune system, bone, mammary gland, and skin appendages (Locksley et al., 2001). The TNFR family can be subdivided in two ways. Several subfamilies are defined by sequence similarities in the receptors' extracellular ligand binding domains, with each subfamily being a product of gene duplication and divergence (Locksley et al., 2001). Alternatively, TNFR family members can be allocated to one of two functional classes according to their mode of signaling, which depends on whether they contain an intracellular death domain or not. Eight receptors (Edar, p75 NGFR, TNFR1, Fas, DR3, DR4, DR5, and DR6), which are scattered among the subfamilies, contain a C-terminal death domain which is used to recruit cytoplasmic death domain adaptor proteins (Wajant, 2003). These adaptors in turn recruit members of the Traf family to transduce signals, commonly resulting in activation of the transcription factor NF-κB, and sometimes initiating apoptosis. A majority of TNFRs do not contain a death domain and initiate signaling by recruiting Trafs directly to their cytoplasmic tails (Inoue et al., 2000).

Ectodysplasin (Eda) is a member of the TNF family of ligands and it was initially implicated in appendage development by the cloning of a gene underlying hypohidrotic ectodermal dysplasia (HED) in mouse and human (Kere et al., 1996; Thesleff and Mikkola, 2002). HED is characterized by agenesis or malformation of ectoderm-derived appendages, such as teeth, sweat glands and hair follicles, while the skin itself develops normally. Positional cloning identified a receptor for Eda, a member of the TNFR superfamily called Edar (Headon and Overbeek, 1999), and a cytoplasmic transducer of Edar signals called Edaradd (Headon et al., 2001; Yan et al., 2002). Like the ligand, Eda, both receptor and adaptor are mutated in mouse and human HED. Mice with genetic lesions that affect this signaling pathway display a phenotype in which primary hair follicles, normally developing between embryonic day 14 (E14) and E16, are entirely absent, while the later developing secondary hair follicles are almost normal (Headon and Overbeek, 1999; Laurikkala et al., 2002). Therefore, the Eda pathway is required specifically for initiation of primary hair follicles, while whiskers and secondary follicles are minimally affected by its absence. A reciprocal phenotype is caused by mutation of the transcription factor Lef-1 or the BMP inhibitor Noggin, which are required to initiate development of secondary, but not primary, hair follicles (van Genderen et al., 1994; Botchkarev et al., 2002; Plikus et al., 2004). Thus two genetically distinct pathways are utilized to activate hair follicle development at different stages of mouse development. Interestingly, Lef-1, Noggin and the Eda pathway components are all expressed in both primary and secondary follicle placodes, and so their expression characteristics do not indicate their functional roles in a given follicle subtype. Though the evolutionary relationships between ectodermal appendages in different vertebrate classes are unclear, a conserved role for Edar signaling in their development is indicated by the finding that its mutation underlies a spontaneous fish mutant that lacks scales (Kondo et al., 2001).

Following identification of Edar, two novel TNFRs, Troy and Xedar, were cloned and found to be expressed in the embryonic epidermis and appendages (Kojima et al., 2000; Yan et al., 2000). The extracellular domains of Edar, Troy and Xedar mark them out as a distinct subfamily within the TNFRs, though their intracellular domains are unrelated to one another (Locksley et al., 2001). Xedar binds to a specific splice variant of Eda, EdaA2, which differs by two amino acids from the Edar binding variant, EdaA1 (Yan et al., 2000). No TNF ligand has been identified for Troy (Bossen et al., 2006). All three receptors employ Trafs, which ultimately leads to activation of NF-κB (Kojima et al., 2000; Yan et al., 2000). However, while Edar contains a death domain used to recruit Edaradd for signal transduction, this domain is entirely absent from the mammalian Troy and Xedar proteins. Recent studies of null mutations in Xedar and Troy have reported an absence of gross skin or appendage phenotypes (Newton et al., 2004; Shao et al., 2005).

Previous work has described the expression of *cEda*, *cEdar* and *cEdaradd* in the forming feather field and showed that ectopic activation of cβ-catenin was sufficient to induce *cEdar* expression (Houghton et al., 2005). Here we describe the effects of suppression and activation of Edar and its related receptors in developing chicken skin *in vitro* and *in vivo*, and the effects of suppression of NF-κB activity. This work defines functional roles for signaling from these receptors, and for NF-κB, in feather development. Our findings also suggest a mutual feedback regulation among activators in the periodic patterning process. It is perhaps the summation of these activities that specify the placode and inter-placode fates, rather than the linear cβ-catenin–cEdar axis proposed in previous studies (Houghton et al., 2005).