Developmental expression of chicken FOXN1 and putative target genes during feather development

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ABSTRACT FOXN1 is a member of the forkhead box family of transcription factors. FOXN1 is crucial for hair outgrowth and thymus differentiation in mammals. Unlike the thymus, which is found in all amniotes, hair is an epidermal appendage that arose after the last shared common ancestor between mammals and birds, and hair and feathers differ markedly in their differentiation and gene expression. Here, we show that FOXN1 is expressed in embryonic chicken feathers, nails and thymus, demonstrating an evolutionary conservation that goes beyond obvious homology. At embryonic day (ED) 12, FOXN1 is expressed in some feather buds and at ED13 expression extends along the length of the feather filament. At ED14 FOXN1 mRNA is restricted to the proximal feather filament and is not detectable in distal feather shafts. At the base of the feather, FOXN1 is expressed in the epithelium of the feather sheath and distal barb and marginal plate, whereas in the midsection FOXN1 transcripts are mainly detected in the barb plates of the feather filament. FOXN1 is also expressed in claws; however, no expression was detected in skin or scales. Despite expression of FOXN1 in developing feathers, examination of chick homologs of five putative mammalian FOXN1 target genes shows that, while these genes are expressed in feathers, there is little similarity to the FOXN1 expression pattern, suggesting that some gene regulatory networks may have diverged during evolution of epidermal appendages.

KEY WORDS: chick, forkhead, WHN, HFH11, thymus, nudee
arose after the last shared common ancestor between mammals and birds in the late Carboniferous period, it is not surprising that proteins in these two lineages have functionally diverged (Furlong, 2005). For example, hair is characterized by a preponderance of soft alpha keratin (Eckhart et al., 2008), whereas feathers are characterized predominantly by the harder beta keratins (Glenn et al., 2008). Alpha keratins are found in the feather sheath and barb ridges but are rapidly overwhelmed by deposition of beta keratins (Alibardi, 2013). Since one function of transcription factor FOXN1 in mice is the activation of alpha keratins 2-6 (Schlake et al., 2004), and birds appear primarily to use beta keratin proteins in feathers, it has not been tested whether feathers express FOXN1. We previously showed that another Forkhead transcription factor, FOXE1, is transcribed in the feather filament (Yaklitchkin et al., 2011), and its mammalian ortholog is implicated in hair morphogenesis (Brancaico et al., 2004). This suggests the hypothesis that FOXN1 and homologs of its mammalian regulatory targets might also be expressed in developing feathers.

In this report we show that, whereas FOXN1 and its putative regulatory targets are indeed expressed in developing feathers, some of the gene regulatory pathways downstream of FOXN1 in epidermal appendage growth appear to have diverged since the division between mammal and bird ancestors about 310 million years ago (Kumar and Hedges, 1998).

Results

Phylogenetic and sequence analysis of avian FOXN1 sequences

The chick FOXN1 gene encodes a 653 amino acid protein with a molecular weight of 71,957 Da. The chick FOXN1 gene shares synteny with mammalian FOXN1, thus providing unambiguous evidence for orthology. Of non-avian orthologs, chick FOXN1 protein sequence overall shares the highest sequence identity, 75%, with turtle and lizard FOXN1, although the lizard protein sequence is incomplete (Fig. 1A). For comparison, Chick FOXN1 protein shares 59% and 53-55% sequence identity with opossum and placental FOXN1 (Fig. 1A). To determine the evolutionary relatedness of avian FOXN1 gene to orthologs, a phylogenetic analysis was performed using the Neighbor-joining method (Kumar et al., 2004). The phylogenetic tree shows that avian FOXN1 genes are clustered within the FOXN1 group and the closest phylogenetic relatedness is observed to the reptilian and amphibian FOXN1 genes, which is supported by the high bootstrap values (Fig. 1B).

Expression of chick FOXN1 gene in developing thymus

We performed whole mount in situ hybridization analysis to examine FOXN1 expression during chick thymus development from stages ED1-16. No expression was detected from ED1-4.5 (not shown). In chicken, thymus is derived from the third and/or fourth pharyngeal pouch endoderm at stage ED5. At about ED9 thymus primordia detach from the pharynx and migrate to their mature locations in the neck, where they form bilateral multi-lobed glands near the jugular vein. We found that FOXN1 mRNA was strongly expressed bilaterally in ED9 thymus (Fig. 2A). A section through the ED9 thymus primordia shows that FOXN1 labeling is stronger in the prospective cortex with uniform lower intensity label in the medulla (Fig. 2B). This is consistent with the proposed function of mouse FOXN1 in regulation of keratin gene expression, which is present in medullary and cortical thymus epithelial cells during early thymus development (Rodenwald, 2008; Nowell et al., 2011). As chick embryonic development progresses (stage ED13) the thymus lobes migrate laterally and expand; expression of FOXN1 persisted (Fig. 2C-D). A section through the thymus at this stage shows increased intensity of FOXN1 mRNA labeling in the cortex (Fig. 2E). This is consistent with the observation in mouse that a FOXN1 mutant phenotype is more severe in the cortical compartment, although both cortex and medulla are affected (Nowell et al., 2011); and expands on the published FOXN1 expression at older stages in chick; (ED18; Neves et al., 2012).

Expression of chick FOXN1 gene in developing feathers

Feathers are unquestionably the most complex vertebrate epidermal appendages and the specifics of their embryology are well described (e.g., Yu et al., 2002, 2004; Alibardi, 2009, 2013). In short, a feather placode in the epidermis with an underlying dermal condensation sprouts into a feather filament with a dermal pulp separated from the epidermal sheath by the pulp epithelium. Barb

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**Fig. 1** (left and above). Comparison of amino acid sequences of avian FOXN1 with orthologous proteins and phylogenetic tree of FOXN1/FOXN4. (A) Predicted amino acid sequences of avian FOXN1 proteins aligned with orthologous protein sequences using T-coffee algorithm. The conserved forkhead DNA-binding (Kaestner et al., 2000) and transcriptional activation domains (Schüddekopf et al., 1996) are underlined in blue and red, respectively. Similar amino acid residues are shaded, threshold 70%. (B) A phylogenetic tree of the FOXN1 proteins, with FoxN4 protein sequences used as an outgroup. A neighbor-joining method was used to construct the tree topology based on the forkhead DNA-binding domain.
ridges begin to form in one side of the epidermis and are displaced laterally to create two vertical half-spirals or arcs between the base and their distal merger into the feather stem or rachis on the opposite side (downy feathers have no rachis). Each barb ridge appears as an arch in cross section with paired stacks of barb plates at its base and a growth zone at its apex, all surrounded by marginal cells. At maturity, a medullary core forms in each barb, the feather sheath is lost and the barbs separate.

Because FOXN1 is important for normal hair development in mammals, we used in situ hybridization to examine expression of FOXN1 in developing chicken feathers. FOXN1 expression was at, or barely above, background levels in initial feather buds at ED9 (Fig. 2A), but strong transient expression was observed in feather filaments at stages ED13-16 (Fig. 2 F-G, I-M). Strong expression of FOXN1 mRNA in the feather filament was detected nearly from base to apex of the feather at stage ED13 (Fig. 2F). Weaker FOXN1 label was observed in the most proximal part of the feather filament and the most distal tip. This may indicate the beginning of the maturation of the distal region of the filament. By stage ED14 FOXN1 transcripts become restricted to the proximal feather filament (Fig. 2G), which is a less differentiated and morphologically more active portion of the feather filament (Haake et al., 1984). To verify that the failure to hybridize in the distal filament was not due to exclusion of the color reaction or another artifact, in situ hybridizations were performed on comparable feather filaments using a probe for another transcription factor, FOXE1 (Fig. 2H; Yakluchkin et al., 2011). FOXE1 showed a complementary expression pattern to that of FOXN1 in proximal/distal distribution at this stage, indicating that both proximal and distal filaments were able to label. By stage ED16, FOXN1 mRNA was no longer detected in the feather filament (Fig. 2I, J), which can be explained by either down-regulation of FOXN1 expression in a differentiated feather filament or increased keratinization in the feather sheath, which may block FOXN1 probe penetration at this stage. Small flecks of trapped color precipitate (artifact, arrowheads Fig. 2J) indicate that probe and labeling solution were able to penetrate. A transverse section near the base (K), midpoint (L) or tip of the shaft (M) of feathers similar to those shown in panel (F) indicates a transition in both morphology of the feather and label.

Expression of FOXN1 and its putative target genes in cutaneous appendages

Putative downstream target genes of FOXN1 have been identified in mouse thymocytes (Newell et al., 2011) and human embryonic stem cells (hESNet). In addition to regulating keratins (e.g., Mecklenburg et al., 2004), the regulation of MHC Class II genes, BRD2 (Bromodomain-containing gene 2), CTSL (Cathepsin L, a lysosomal protease), CD40 (TNF-related receptor), and DLL4 (delta-like gene 4) was positively correlated with FOXN1 dosage (Nowell et al., 2011; hESNet). To determine if these components represent a gene regulatory network that is conserved in other FOXN1-expressing
tissues, we used in situ hybridization to evaluate the expression of chick homologs of these genes in feathers and other epidermal appendages. Chick BLA2 represented the MHC Class II genes, while other chick gene symbols are similar to mouse. Comparisons of expression in whole mount and feather section are shown in Figures 3 and 4. Our section photos were taken near the base of the feather filament where barb ridges are first forming on one side (Fig. 4. ED12), or at the proximodistal level corresponding to the densest label for the majority of genes (ED14, ED17). While each of the potential targets exhibited expression in feather filaments and overlapped in expressing regions with FOXN1, none of the patterns were highly similar to FOXN1 in either whole mounts (Fig. 3) or cross sections of feathers (Fig. 4). FOXN1 expression was not detected as extensively in wing feathers or skin at ED12 (Fig. 3A), whereas the other five genes were strongly expressed (Fig. 3 B-F). At ED14, four of the putative target genes were expressed in

![Image](https://via.placeholder.com/150)

**Fig. 3. Expression of FOXN1 and putative FOXN1 regulatory targets in embryonic day 12, 14 and 17 epidermal appendages.** Photo columns are labeled with the gene name for the probe, rows indicate the days of incubation and tissue or region shown. All in situ probes labeled in feathers on embryonic day 12 (A-E, M-R), 14 (G-L, S-X) and 17 (Y-D’). (A-F) Embryonic day 12 wing tips. (A) FOXN1 was expressed in some feather buds with stronger expression at the midpoint of the shaft (arrowhead) and lower expression at the base and tip. Some smaller buds (arrows) and the skin did not express. (B-F) The other five genes were also expressed more intensely in the mid-shaft (arrowhead) than at the base or tip, but also in skin and all feather buds. The distal wing tip had less expression for all genes tested (arrow in B). In wing tips at day 14, some feather buds still had no detectable FOXN1 (G, arrows) but most feather buds showed stronger gene expression (G-L). At day 14 of incubation, wing skin had no detectable expression of FOXN1 (G) and BLA2 (H), but expressed other genes (I-L). Day 12 (M-R) and 14 (S-X) feathers from the back of the neck expressed all six genes similarly at this gross level. By day 17, feathers all showed probe binding proximally (all probes, arrowheads, Y-D’). Where the sheath had been bent or cut (e.g., arrows in I’, D’), damaged tissue stained. No skin label was detected. ED14 foot: An evaluation of foot scales and nails, two other epidermal appendages. (E’) FOXN1 was expressed solely in the nails. Two genes were expressed in the scales (F’, G’). CTS (H’) was expressed in both scales and nails. CD40 (I’) and DLL4 (J’) were expressed in neither scales nor nails, although it appears that CD40 may have been expressed in some deeper tissue (I’). Final row shows close ups of (E’-J’).
To investigate specific localization of FOXN1 mRNA and these five candidate genes within feather filaments, hybridized feathers were paraffin sectioned transversely. At ED12 at the base of the feather where barb plates were just beginning to form, FOXN1 transcripts were predominantly localized in the feather sheath (Fig. 4A), and more distally in the ends of the marginal plates (not shown) whereas transcripts of the five FOXN1 candidate targets did not share this pattern (Fig. 4B-F). For example, at ED12 BLA2 (and somewhat less so BRD2) mRNA was expressed in the pulp epithelium and within proximal barb plates. CTSL mRNA was ubiquitously expressed with stronger intensity in the sheath and distributed cells (Fig. 4D, arrow), whereas slightly more distally strong expression was uniquely seen in the barbule plates outside the growth zone (Fig. 4D'), a pattern not shared with any of the other probes. DLL4 and CD40 mRNA were weakly expressed in the outside layer of the feather sheath and in the pulp epithelium (Fig. 4 E,F). At ED13-14, in a more distal part of the filament, the overlap in expression patterns was more similar but with identifiable differences (Fig. 2L, 4G-L). FOXN1 was still expressed in the sheath, and also in the lateral barb plates and marginal cells.

Labeling of BLA2 appeared stronger in the barb ridge (Fig. 4H), BRD2 labeled lateral barb plates, medial barb ridges and marginal cells equally (Fig. 4I) whereas CTSL was strongly expressed in the barb plates (Fig. 4J). Expression of CD40 and DLL4 persisted fairly ubiquitously at this level (Fig. 4 K,L) with the exception of the dermal pulp for DLL4. By ED17 the strongest FOXN1 label was in a thin line of unknown fate between the barbs, with barb and marginal cells labeling but no label in sheath, medullary cells of the axial plate or pulp (Fig. 4M). A similar labeling pattern was observed for the other genes with the exception of the inter-marginal line, and that BRD2 and CTSL also labeled in the axial plate of the barbs (Fig. 4 N,R). Based on these data, the putative FOXN1 target genes show dynamic and interesting expression patterns in feather compartments. However, the expression of each of the genes is distinctly different from that of FOXN1 at similar stages and proximodistal levels.

**Discussion**

In the present study, we confirm that chick FOXN1 is expressed in the developing thymus and demonstrate its expression in some epidermal appendages, suggesting a homologous, evolutionarily conserved role for FOXN1 across amniotes. We have identified a novel expression pattern of FOXN1 in embryonic feathers. Chick FOXN1 initially is expressed along the length of the feather filament, with the exception of the base and tip. As the filament elongates between ED 12 and 17, FOXN1 expression becomes restricted to the proximal end of the feather. Although others have published differences in proximal-distal gene expression (e.g., Yu et al., 2002, Chodankar et al., 2003), to our knowledge FOXN1 and FOE1 (Yaklichkin et al., 2011), the two FOX genes we have characterized are the only known differentially and dynamically expressed genes along the proximal-distal axis of the elongating feather filament.

The murine ortholog of FOXN1 regulates expression of specific
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alpha keratins (Mecklenburg et al., 2004). Although hair forms from soft alpha-keratins and feathers form predominantly from hard beta-keratins, it is possible FOXN1 could be modulating expression of feather-specific keratins. Feather-specific beta-keratin expression coincides with differentiation of the barb ridges (Haake et al., 1984, König and Sawyer 1985). Images of feather filaments in the Haake paper indicate the proximal to distal pattern of beta-keratin expression correlates well with the expression pattern of FOXN1 shown here (Fig. 2). Specifically, beta-keratin expression is initiated at stage 38 (ED12) and expressed in the ring sheath cells similar to our image in Fig. 2K, whereas more distal expression is similar to Fig. 2L. Patterns of alpha-keratins also correlate with FOXN1 expression (Alibardi, 2013). Based on these observations and the expression of both mouse and chick FOXN1 in the nails and thymus, other keratinized epidermal structures, chick FOXN1 may regulate expression of both alpha and beta-keratins.

Finally, expression of FOXN1 and the other genes investigated in the feather raises more questions than it answers about the evolution of FOXN1 function, considering that the feather is thought to have evolved from scutate scales of reptiles. Loss and gain of function studies will be required to establish a precise role for FOXN1 transcriptional regulation in epidermal evolution and development.

Materials and Methods

Sequence, data and phylogenetic analysis

The protein sequences used in the analysis were obtained from the NCBI database (ncbi.nlm.nih.gov) and Ensemble database 69 (ensemble.org). The accession numbers of FOXN1/N4 protein sequences analyzed are provided in Supplementary Table S1. Multiple sequence alignments were constructed using T-COFFEE, version 7.7.1. (tcoffee.vital-it.ch/cgi-bin/tcoffee/tcoffee_cgi/index.cgi). Aligned FOXN1/N4 sequences were edited using the alignment editor BioEdit 7.0.4.1. (mbio.ncsu.edu/bioedit/page2.html). A phylogenetic tree for FOXN1/N4 proteins was generated based on the forkhead DNA-binding domain sequences (~100 residues). A neighbor-joining method was used to construct the tree topology. The phylogenetic tree was converted into a cladogram using MEGA 4 (Kumar et al., 2004) (metamereme.sdsc.edu).

Incubation and isolation of chicken tissues

Fertilized white leghorn eggs (Gallus gallus) were incubated in a forced-draft incubator at approximately 38°C with high humidity. Slight variations in temperature between incubators or over the course of the experiment make staging approximate due to long incubation times, therefore days of expression are given. For older embryos, neck/thorax and skin/feathers were isolated prior to fixation with paraformaldehyde.

Molecular cloning and in situ hybridization with gene probes

To clone chicken FOXN1 cDNA sequences for the in situ probe template, mRNA was isolated from day 5 chicken embryos using the Qiagen RNasey Mini Kit (cat. number: 74104) and cDNA synthesized using standard procedures. Template for probe generation was generated by PCR with the GC-rich PCR System Kit (cat. number: 12 140 306 001), Roche Applied Science; 34 cycles of annealing at 50°C and the following primers: forward primer, 5’TATAAAAGCTTGGCGCGCAAGATATTCACTACCCCTACCAAGGATGGC3; reverse primer, 5’GCTCTAGAAATTACCTCCAC-TAAGGGCTCTCGGCTCCAGAACAGATTTG-3’. Chick FOXN1 sequences are in bold, the T3 RNA polymerase binding site is italicized. The PCR generated template was sequenced to confirm identity (NCBI Accession number: XM_415816.3, 904-1622 bp). A 719 nt RNA probe of FOXN1 was produced using T3 polymerase and in situ hybridizations were performed using the GEISHA mRNA Detection Protocol (geisha.arizona.edu/geisha/protocols.jsp). FOXE1 in situ hybridization was performed as previously described (Yakichkin et al., 2011). Probe for BLA2 (NCBI Acc. #: NM_001245061) was generated by GEISHA plasmid W34 with Not I using T7 polymerase. Probe for BRD2 was generated from plasmid CHEST826a15 (NM_001030674.1) with NotI and T3 polymerase. Probe for Cathepsin L, CTSL2 (NM_001168009) was generated from GEISHA plasmid S12 with NotI and T7 polymerase. Probe for CD40 (TNFRSF5, NM_204665) was generated by PCR: Forward primer: 5’-CTGACAGAGCATGAGCACAAGG-3’, Reverse primer: 5’-ATTAACCTGCTAAGGGAAGGATGCCACCTCCTCGG-3’, where the bold sequence allows for directional amplification of the 666 nt probe using the T3 polymerase. Probe DLL4 (XM_421132) was generated from plasmid pgfl2.pk001.e7 (www.chickest.uered.edu) using NotI and T3 polymerase. Probe sequences and additional images are available at http://geisha.arizona.edu/geisha.

Acknowledgments

We are thankful to Tatiana Yatskievych and Terry Seseapasara for technical assistance. This work was supported by NIH grant GM085226 and NIH NICHD grant P41HD064559.

References


FURLONG RF (2005), Insights into vertebrate evolution from the chicken genome sequence. Gen Biol 6: 207.


LEE J, TUMBAR T (2009). Haired tale of signaling in hair follicle development and


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