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RESEARCH ARTICLE

Sonic hedgehog specifies flight feather positional information in avian wings

Lara Busby¹,*, Cristina Aceituno²,§, Caitlin McQueen¹,§, Constance A. Rich¹,‡, Maria A. Ros²,³,¶ and Matthew Towers¹,¶

ABSTRACT

Classical tissue recombination experiments performed in the chick embryo provide evidence that signals operating during early limb development specify the position and identity of feathers. Here, we show that Sonic hedgehog (Shh) signalling in the embryonic chick wing bud specifies positional information required for the formation of adult flight feathers in a defined spatial and temporal sequence that reflects their different identities. We also reveal that Shh signalling is interpreted into specific patterns of Sim1 and Zic transcription factor expression, providing evidence of a putative gene regulatory network operating in flight feather patterning. Our data suggest that flight feather specification involved the co-option of the pre-existing digit patterning mechanism and therefore uncovers an embryonic process that played a fundamental step in the evolution of avian flight.

KEY WORDS: Avian, Chick, Embryo, Flight feather, Positional information, Shh

INTRODUCTION

Although much is known about the molecular pathways involved in the induction, positioning and morphogenesis of feathers (Chen et al., 2015; Chang et al., 2019), little is known about how different types of feathers are specified. Classical tissue recombination experiments in chickens provide evidence that signals acting at the earliest stages of wing bud development (day 3.5 of incubation or HH20 - see Materials and Methods for staging) specify feather identity (Cairns and Saunders, 1954; Saunders and Gasseling, 1959). Thus, grafts of prospective chick thigh mesoderm made to the wing result in the formation of feathers characteristic of those found in the leg. These findings show that the cells of the morphologically indistinct wing bud mesoderm, which give rise to the dermis, have non-equivalence (have a different intrinsic character), and thus carry positional information that determines feather identity in the overlying ectoderm (Lewis and Wolpert, 1976).

An important signal known to operate at HH20 is Sonic hedgehog (Shh) – a protein produced by a transient signalling centre called the polarising region (also known as the zone of polarising activity or ZPA), which is located in mesoderm at the posterior margin of the limb bud (Riddle et al., 1993). Shh is implicated in the specification of antero-posterior positional values (thumb to little finger, digits 1, 2 and 3) in chick limb bud cells derived from the lateral plate mesoderm in a concentration-dependent manner between HH18 and HH22 (Yang et al., 1997; Towers et al., 2011). Shh is also involved in stimulating proliferative growth along the antero-posterior axis (Towers et al., 2008; reviewed by Tickle and Towers, 2017). Grafts of Shh-expressing polarising region cells made to the anterior margin of host HH20 wing buds (day 3.5) duplicate the antero-posterior axis to produce digit patterns such as 3, 2, 1, 1, 2 and 3 (Fig. 1A; Tickle et al., 1975; Riddle et al., 1993). Other tissues that are not derived from the lateral plate mesoderm, including the nerves and muscles, are duplicated as a secondary consequence (Luxey et al., 2020), and thus show equivalence (Lewis and Wolpert, 1976; i.e. progenitor cells are not intrinsically different in character and do not carry positional information). The pattern of feather buds is also duplicated across the antero-posterior axis (Fig. 1A,B; see also Riddle et al., 1993). [Secondary flight feather buds are marked in Arabic numerals, primaries in Roman numerals and alulars are yet to form.] Therefore, the fact that, like the digit skeleton and other connective tissues, the dermis originates from multipotent lateral plate mesoderm progenitor cells (Pearse et al., 2007) raises the possibility that it is also specified with positional values in response to Shh signalling, and that this could determine feather identity. Alternatively, feathers could be specified independently of antero-posterior polarity or by other signals.

In this study, we show that Shh signalling by the embryonic chick wing polarising region specifies the pattern of flight feathers, which provide most of the flapping, gliding and soaring ability required for airborne locomotion in birds (Matloff et al., 2020). Our data provide evidence that Shh signalling integrates the patterning of digits and flight feathers, and thus provides insights into the co-evolution of these important structures.

RESULTS

Shh signalling is required for flight feather bud formation

To determine whether Shh signalling specifies feather identity in the chick wing, we transiently inhibited it using cyclopamine at day 3 of incubation (HH19) for ∼72 h (Pickering and Towers, 2016). Cyclopamine is a potent inhibitor of the Hedgehog signalling pathway at the level of Smoothened (Taipale et al., 2000). When cyclopamine is systemically applied to chick embryos at HH18-20, it causes the loss of posterior structures in both wings and legs (Scherz et al., 2007; Towers et al., 2008, 2011) and closely mimics the genetic inactivation of Shh signalling in chicken oligozeugogadactylus mutants (Ros et al., 2003). At day 13 of development, we observed abnormal
flight feather bud development. Thus, elongated flight feather buds expressing Ptc1 [a direct target of Shh signalling that is involved in feather morphogenesis (Harris et al., 2002, 2005; McKinnell et al., 2004)] are found along the posterior margin of untreated day 13 wings (arrow in Fig. 1C, schematic shown in E), but not in the wings of embryos that were treated with cyclopamine (Fig. 1D). Here, the feather buds along the posterior margin are identical to those in other places, suggesting that they are not flight feathers (arrow in Fig. 1D). The schematic representation in Fig. 1E,F shows how the feather buds along the posterior border of the cyclopamine-treated wing do not show the typical elongated morphology.

In addition to its function in limb patterning, Shh is also involved in the epithelio-mesodermal interactions that drive feather formation (Harris et al., 2002, 2005; McKinnell et al., 2004). However, the observation that Ptc1 is still expressed in feather buds of wings treated at HH19 with cyclopamine (Fig. 1D) suggests that it is the earlier loss of Shh signalling by the polarising region that prevents flight feather bud formation, rather than the loss of Shh signalling within the buds themselves.

The failure of flight feather bud formation could be interpreted as a secondary consequence of the loss of all posterior tissues – e.g. digit 3 often does not form in wing buds treated with cyclopamine at HH19 (Fig. 1D; Towers et al., 2011). This is an important consideration because, in the case of the muscles, their absence would be due to the loss of migrating myoblasts into posterior regions of the wing. However, wing bud mesoderm, which differentiates into the dermis, is not lost following cyclopamine exposure, but instead contributes to structures that are anteriorised (i.e. cells that would have contributed to digit 3 now contribute to digit 2) (Towers et al., 2011, 2008). In addition, flight feather buds often fail to form in forewing regions that have no overt changes in antero-posterior patterning (which always form a radius and ulna, Fig. 1D,F).

Flight feather buds form along the dorsal-ventral boundary of the wing, which, when disrupted, can result in abnormal flight feather development (Grieshammer et al., 1996). Therefore, to examine whether the loss of Shh signalling affects dorso-ventral patterning of the wing bud, we examined the expression of Lmx1b, which is expressed in the dorsal mesendoderm of untreated (Fig. 1G; n=4/4) and HH19 cyclopamine-treated (H; n=4/4) wing buds at day 9. Scale bars: 2 mm in A,C,D; 1 mm in G,H.

Shh is required for flight feather development during late embryogenesis

Developing flight feather buds become morphologically distinct during late embryogenesis by growing inwards to make ligamentous connections with the skeleton, and by displaying bilateral asymmetry...
to the ulna (showing developing flight feathers in untreated embryos and their connections and the skeleton to which they connect using ligaments. (B-D) Hematoxylin digit skeleton. We used these morphological characteristics to

Fig. 2A shows the flight feather pattern and its association with the

(Fig. 2B-D). In addition, developing flight feather buds can also be identified in transverse section by their asymmetric pattern of Shh expression at day 15 (Kondo et al., 2018; Fig. 2E).

However, in the wings of embryos treated with cyclopamine, flight feather buds that produce ligamentous connections with the ulna frequently fail to form along the posterior border of the wing. The surface of the wing remains covered with natal down buds, none of which invaginates deeply towards the skeleton (Fig. 2F-H). Furthermore, only developing feather buds with symmetrical expression of Shh are observed in forewing regions at day 15, again demonstrating the specific absence of flight feather buds (Fig. 2I). In summary, these observations demonstrate the transient and specific requirement of polarising region-derived Shh signalling for advanced stages of flight feather bud development during late embryogenesis.

RNA sequencing reveals a flight feather bud transcriptome

Recently, molecular markers of the flight-feather forming regions of the chick wing have been identified, including Sim1 (Seki et al., 2017) and Zic1 (Wu et al., 2018). Notably, Sim1 has an avian-specific forelimb expression pattern in the dermis (Coumailleau and Duprez, 2009; Seki et al., 2017). To determine whether ‘memory’ of exposure to early Shh signalling is interpreted into patterns of flight feather bud-associated gene expression, we performed a series of RNA sequencing (RNA-seq) experiments on tissue dissected from day 10 wings. This stage was selected because it is when flight feather buds become morphologically distinct from other feather buds (see expression of a general marker, Bmp7; Michon et al., 2008), in raised flight feather buds at this stage, Fig. S1). We sequenced RNA extracted from soft tissue flanking the posterior margin of the ulna at day 10, because this tissue forms normally in all wings treated with cyclopamine at HH19.

We contrasted sequencing data from the posterior forewing regions of cyclopamine-treated and untreated control Bovans brown wings (Fig. 3A; the top ten genes up- and downregulated by more than fivefold are shown, see Table S3 for more information). We enriched for genes associated with feather bud development by contrasting RNA-seq datasets obtained from Bovans brown wings with one obtained from the corresponding region of Bovans brown legs, which produce scales instead of feathers (Fig. 3B and Table S3). These results were confirmed by qPCR performed on cDNA synthesised from the RNA that was used in the sequencing analysis (Fig. S2). We performed a hierarchical clustering analysis to identify genes that behave similarly across the three comparisons (the expression levels of all genes included exhibit a more than
Fig. 3. RNA-sequencing reveals a flight feather bud transcriptome. (A–C) Schematics showing regions of day 10 limbs that were used to make RNA and the pairwise contrasts made: HH19 cyclopamine-treated Bovans brown wings versus control Bovans brown wings (A), Bovans brown wings versus Bovans brown legs (B) and Pekin bantam legs versus Bovans brown wings (C). The top ten genes up- and downregulated more than fivefold are shown for each comparison (P<0.005). (D) Cluster of genes downregulated in wings by earlier Shh signalling inhibition, upregulated in wings versus legs, and upregulated in Pekin bantam legs versus Bovans brown legs (P<0.005 and a greater than twofold change in at least one contrast; red, upregulated; blue, downregulated. r, radius; u, ulna; t, tibia; f, fibula; mt, metatarsals.

The duration of Shh signalling is interpreted into the later spatial pattern of Sim1 expression

Sim1 appears to be the clearest marker of the flight feather-forming regions of the chick wing from day 8 to day 13 (Fig. 4B and Fig. S3) – compare this with Bmp7 expression in all feather buds (Fig. S1). In order to precisely define the temporal requirement for Shh signalling in specifying the later pattern of Sim1 expression, we applied cyclopamine at different stages. Application at HH119 causes loss of Sim1 expression along the posterior margin of the ulna and digit 2 of day 10 wings, and significantly reduces expression in digit 1 (Fig. 5A). Progressively later treatments at HH19 cause loss of Sim1 expression in the ulna and the proximal part of digit 2 (Fig. 5B), and at HH21, loss of expression in digit 3 only (Fig. 5C). In addition, although Shh is expressed until HH128 (Riddle et al., 1993), treatment with cyclopamine at HH22 does not affect Sim1 expression (Fig. 5D).

To determine whether an ectopic source of Shh signalling is sufficient to induce Sim1 expression, we grafted HH20 polarising regions to the anterior margin of stage-matched host chick wing buds. In day 10 wings, Sim1 is expressed along the anterior margin of the duplicated skeletal elements (asterisks in Fig. 5E), forming a mirror image of its normal pattern (Fig. 4B). Furthermore, we also performed smaller polarising region grafts to reduce the concentration of Shh signalling (see Tickle, 1981). In day 10 wings, Sim1 expression is specifically duplicated along the anterior margin of the additional digit 1 (asterisk, Fig. 5F). Although it is expected that flight feather buds would be duplicated, alular feather buds have still not formed at day 14 (see Fig. 1A) and, owing to the nature of the experiments, we could not obtain ethical approval to look at older specimens. Therefore, lowering the concentration of Shh signalling, either endogenously or ectopically, has the same effect on Sim1 expression (compare Fig. 5F with A).
These findings reveal that Shh signalling from the polarising region between HH18 and HH22 specifies the later pattern of Sim1 expression in a defined spatial and temporal sequence, which can be replicated by polarising region grafts made to the anterior margin of the wing bud in a dose-dependent manner. Thus, in reference to the classical positional information of digit patterning (reviewed by Tickle and Towers, 2017), a short exposure of Shh signalling (equivalent to a low concentration) is sufficient for weak expression
of *Sim1* in digit 1, and progressively longer exposures (higher concentrations) for expression in the distal part of digit 2, the ulna and then digit 3.

**Sim1-expressing cells and flight feather buds are adjacent to the polarising region lineage**

The pattern of *Sim1* expression along the posterior margin of the wing superficially resembles polarising region fate maps (Towers et al., 2011). Therefore, to examine this putative lineage relationship, we replaced normal HH20 polarising regions with stage-matched GFP-expressing polarising regions and analysed the expression of *Sim1* at day 10 (Fig. 6A,B). Transverse sections show polarising region-derived GFP-expressing dermal cells lying immediately ventral to both *Sim1*-expressing cells in forewings at day 10 (Fig. 6C,D). In addition, Hematoxylin and Eosin staining also shows that GFP-expressing dermal cells abut emerging flight feathers at days 11 and 12 (Fig. 6E-H). These findings show that cells positioned dorsally adjacent to the polarising region express *Sim1* and produce flight feather buds. In addition, the GFP-expressing polarising region lineage and *Sim1*-expressing cells also abut one another in anterior regions of mirror image-duplicated wings (Fig. 6I-L′). We often observed a second stripe of *Sim1* expression along the anterior margin of such wings, but the significance of this is unclear (Fig. 6J,K). Taken together, these findings imply that paracrine Shh signalling induces *Sim1* expression and flight feather formation in cells immediately dorsal to the polarising region lineage.

**Shh signalling is required for flight feather formation in hatchlings**

During the stages leading up to hatching (days 18-21 of incubation), the first generation of flight feathers is replaced by the second generation of mature flight feathers (Lucas and Stettenheim, 1972; Kondo et al., 2018). To determine whether polarising region-derived Shh signalling is required for the formation of mature flight feathers, we obtained licensing and ethical permission to allow chicks treated with cyclopamine at HH19 to hatch on day 21 of incubation. In untreated wings, both well-formed flight feathers and dorsal major covert feathers can be observed extending from the posterior margin of the wing (Fig. 7A,A′, most of the natal down is trimmed back). A schematic documenting the association between the flight feathers and the digit skeleton at hatching is shown in Fig. 7B. Hematoxylin and Eosin staining on a transverse section through the forewing region of the specimen in Fig. 7A shows a ligament connecting the flight feather to the ulna (Fig. 7C,C′).

In hatchlings that were treated with cyclopamine at HH19, most flight feathers and dorsal major covert feathers fail to develop at hatching (Fig. 7D,D′, natal down trimmed back, Table S1). A schematic of this pattern of flight feathers with the other feathers completely removed is shown in Fig. 7E [some stunted alulars and secondaries are present but obscured by natal down (see Fig. S4 and Table S1)]. These are the exact locations where restricted expression of *Sim1* is often observed following the inhibition of Shh signalling at HH19 (Fig. 5B). Hematoxylin and Eosin staining of a section from the wing in Fig. 7D shows that natal down feathers still form in areas in

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**Fig. 6. Sim1 and flight feather buds are adjacent to the polarising region lineage.** (A) GFP-expressing polarising regions transplanted in place of normal polarising regions at HH20 contribute to posterior soft tissues of day 10 wings (green labelling, n=2/2; r, radius; u, ulna; 1-3, digits 1, 2 and 3). (B) *Sim1* expression along posterior margin of the ulna and digit 3 (n=2/2; the same limb as in A). (C) *Sim1* expression along posterior margin of the ulna and digit 3 (n=2/2; the same limb as shown in A). (D) Transverse section through forewing shown in A and B reveals adjacent expression of GFP and *Sim1* in dermis (n=2/2; GFP protein and *Sim1* mRNA are detected on same section). Arrowheads indicate the domain of *Sim1* expression. (E-G) Transverse sections through a day 11 wing (E, n=2/2, experiment performed as in A) and a day 12 wing (G, n=2/2) showing GFP expression ventral to emerging flight feather (ff, bv, blood vessel; u, ulna; blue shows DAPI staining). (F,H) Hematoxylin and Eosin staining on serial sections to those in E,G show tissue anatomy. (I) HH20 GFP-expressing polarising region grafts made to the anterior margin of a host wing bud duplicate the distal structures of day 10 wings (n=5/5). (J,K) GFP-expressing polarising region grafts duplicate the pattern of *Sim1* expression (n=5/5; there is a second line of *Sim1* expression, n=3/5; this is the same limb as in I). (L,L′) *Sim1* expression is found adjacent to the polarising region lineage (n=5/5). Unlabelled arrowheads indicate the domain of *Sim1* expression. Asterisks indicate duplicated skeletal elements. Scale bars: 1 mm in A,B,I,J; 150 μm in E-H; 75 μm in C,D,K,L.
which flight feathers are normally present and ligaments connecting feathers to the ulna are not found (Fig. 7F,F′). In addition, proximal primaries along the border of digit 3 are preferentially lost following the application of cyclopamine at HH21 (Table S1); this also matches the loss of Sim1 expression in this domain (Fig. 5C). Furthermore, the flight feather pattern is usually normal following cyclopamine treatment at HH22 (Table S1), just as the Sim1 expression pattern is normal (Fig. 5D). Therefore, Shh signalling by the polarising region specifies the spatial pattern of Sim1 expression and flight feather formation in the same temporal sequence.

**Shh signalling is required for flight feather formation in mature birds**

Hatchlings that were treated with cyclopamine at HH19 did not survive owing to the failure to close the abdominal wall and this prevented the study of their mature wing plumage. As our analyses of hatched chickens shows that dorsal major coverts – which are closely associated with developing flight feathers – are also absent, this raised the possibility that Shh inhibition could affect the later development of other feathers that had not yet replaced the natal down.

To analyse the second generation of feather development in the wings of mature birds, we treated embryos at HH19 with cyclopamine, and then after 10 h at HH20/21, we grafted their right-hand wing buds in place of those of stage-matched untreated embryos (Fig. 8A; see control experiment showing that the grafting procedure does not affect mature postnatal feather development in Fig. S5). This procedure enabled six chicks to survive beyond hatching (Fig. 8B) and they displayed similar patterns of flight feather loss as hatched chicks that were systemically treated with cyclopamine as embryos (Tables S1 and S2). Three birds survived further and their patterns of flight feather loss remained the same as at hatching (Tables S1 and S2). One such example of a postnatal day 22 bird shows that the flight feather pattern is normal in its untreated left-hand wing, but that there is a loss of distal primary flight feathers in its cyclopamine-treated right-hand wing (Fig. 8C). This bird was kept until postnatal day 66, so that its adult feather pattern could be studied in more detail (Fig. 8D-G): 18 secondary flight feathers develop from the ulnar region of its control left wing (green asterisks, Fig. 8D,F) and ten primary feathers from its digital region [eight primaries from digit 3, orange asterisks; two from digit 2, blue asterisks, Fig. 8D,F (one feather was broken)]. Three alular flight feathers extending from digit 1 are also present (red asterisks in Fig. 8D,F). However, in the contralateral cyclopamine-treated wing of this bird, eight primary flight feathers and their overlying dorsal major coverts are absent along the posterior margin of digit 3 (Fig. 8E,G). In addition, two bunched primary flight feathers, which are much smaller than the equivalent ones in its control wing, extend from the margin of digit 2 at the distal tip of the wing (blue asterisks, Fig. 8E,G), and overlying them are dorsal major covert feathers (purple asterisks, Fig. 8E). The development of ventral major covert feathers is unaffected by cyclopamine treatment (Fig. 8G).
pattern of feather loss in the wing of this bird is consistent with the pattern of Sim1 expression in the wings of embryos that were treated with cyclopamine at HH21 (Fig. 5C). It is unclear why we observed stronger effects in cyclopamine-treated embryos at HH19 versus cyclopamine-treated wing buds at HH19 that were transplanted, but it could be a consequence of variability with the effects of cyclopamine treatment. Indeed, the two birds with transplanted wings that survived the longest had the milder flight feather defects (Table S2). These results demonstrate that Shh signalling in the embryo is required for the specification and formation of mature flight feathers, and also their overlying dorsal major coverts in a defined spatial and temporal sequence.

DISCUSSION

Embryonic Shh signalling is required for flight feather formation

We have revealed that Shh signalling by the embryonic chick wing polarising region is required for specifying the adult pattern of flight feathers in dorsally adjacent cells. This process is independent of the later role that Shh signalling fulfils in feather morphogenesis (Harris et al., 2002, 2005; McKinnell et al., 2004). Thus, the transient loss of Shh signalling between HH18 and HH22, but not later, causes the loss of bilaterally asymmetric flight feathers that make ligamentous connections to the skeleton, and also the loss of molecular markers associated with the flight feather forming regions of wing. The Shh signalling pathway (Pichl read-out) is still active during later feather bud morphogenesis, demonstrating that this general process is unaffected.

Detailed fate-mapping experiments have shown that when Shh signalling by the polarising region is transiently blocked, the lateral plate mesoderm-derived cells of the early chick wing bud are not selectively lost, but instead contribute to the development of distal structures (Towers et al., 2011, 2008). Therefore, although digit 3 often fails to form, this is a consequence of its progenitor cells being anteriorised and differentiating into digit 2. The fact that dermal tissue is also derived from the lateral plate mesoderm (Pearse et al., 2007), suggests that, when Shh signalling is inhibited in the early wing bud, this induces the formation of feather types in the overlying ectoderm that are usually found in more-anterior positions (i.e. dermal cells which would normally specify flight feathers instead specify major or median coverts).

We have also shown that the dorsal ventral boundary, which is important for flight feather development (Grieshammer et al., 1996), remains intact following the inhibition of Shh signalling. Together, our data provide molecular insights into classical tissue recombination experiments performed in the chick, which show that feather position and identity are specified by signals acting at around HH20 (Cairns and Saunders, 1954; Saunders and Gasseling, 1959).

Shh specifies flight feather positional information

Our findings can be explained by the classical positional information model of antero-posterior patterning, in which Shh signalling specifies limb bud cells derived from the lateral plate mesoderm, including the presumptive dermis, with a positional value, which when interpreted at a later stage of development allows them to differentiate into the appropriate structure (Tickle et al., 1975; Tickle and Towers, 2017). Thus, the temporal requirement for Shh signalling in specifying the anterior to posterior pattern of Sim1 expression and flight feathers closely follows that for specifying the anterior to posterior pattern of digits (Yang et al., 1997; Towers et al., 2011; Tickle and Towers, 2017) (Fig. 9). Digit 1 and alular flight feathers are specified first by a low concentration/short duration of Shh at HH18, and then increasing concentrations of Shh over time specify the other skeletal elements and flight feathers in the following order: digit 2 and distal primaries at HH19, the ulna and secondaries at HH21, and digit 3 and proximal primaries at HH22 (Fig. 9). It is noteworthy that flight feather specification
closely follows ulna/digit specification, which can be observed by normally patterned skeletal elements forming without associated flight feathers, but with feathers of different identities (Fig. 9). This indicates that a slightly longer exposure to Shh signalling is required for the specification of flight feathers, relative to their associated skeletal elements.

The pattern of Sim1 expression in the wings of embryos treated at HH19, closely matches the pattern of flight feathers present both at hatching and in mature bird wings (Fig. 9). In the treated wing of the oldest bird we studied (P66) eight proximal primaries – which normally form along the border of digit 3 – are absent, yet two distal primaries are present along the border of digit 2 (Fig. 9). This pattern of flight feather loss is also accompanied by the loss of an associated row of overlying dorsal major covert feathers. However, the inhibition of Shh signalling does not affect the development of the remaining feathers in the wing, including the ventral major coverts, implying that their identities are specified by other signals. Our findings therefore indicate that flight feathers and dorsal major covert feathers have similar developmental programmes.

Interestingly, a genetic programme for flight feather development was indicated by naturally occurring mutants that specifically failed to form this feather type (McCready, 1932; Urrutia et al., 1983). Taken together, these observations suggest that the evolution of the flight feather programme involved the co-option of the pre-existing temporal positional information gradient of Shh signalling used in antero-posterior forewing/digit patterning (Fig. 9).

The interpretation of positional information, in which cells ‘memorise’ their positional value to give rise to appropriately patterned and positioned structures at a later stage of development, is generally a ‘black box’ in developmental biology (Wolpert, 2016). Indeed, despite decades of research, the genes acting downstream of the positional information gradient of Shh signalling in the specification of digit identity remain unknown (Tickle and Towers, 2017). However, our RNA sequencing experiments provide molecular insights into a putative gene regulatory network that operates downstream of Shh signalling in determining flight feather identity. These genes include Sim1 and, notably, genes encoding three Zic transcription factors (Zic1, Zic3 and Zic4). Interestingly, Zic transcription factors can bind to sites in promoters that are also recognised by the Gli family of transcription factors – the downstream effectors of Shh signalling (Aruga et al., 1994). This mechanism could involve positional memory, in which polarising region-derived Shh signalling could remove Gli transcriptional repressors from the regulatory elements of genes, thus making them accessible to Zic transcription factors at stages of flight feather bud development. Such directions could be the focus of future studies. In conclusion, as flight feathers were one of the earliest known adaptations associated with the evolution of flight in theropod dinosaurs (Turner et al., 2007; Ortega et al., 2010; Godefroit et al., 2013), our findings have significant implications for this extraordinary transition.

MATERIALS AND METHODS

Chick husbandry

Wild-type and GFP-expressing Bovans brown chicken eggs were incubated and staged according to the Hamburger Hamilton staging table (Hamburger and Hamilton, 1951). Day 3 of incubation is HH18, day 4 is HH21, day 5 is HH24, day 6 is HH27, day 7 is HH29, day 8 is HH30, day 10 is HH36, day 11 is HH37, day 12 is HH38, day 13 is HH39, day 14 is HH40, day 15 is HH41 and hatching (at day 21) is HH46. All experiments involving the use of hatching chicken embryos in this work were conducted in accordance with the EU animal experiment guidelines and reviewed and approved by the Bioethics Committee of the University of Cantabria (PI-20-17).

Wing bud and polarising region grafts

Embryos were dissected in DMEM and wing buds removed using fine tungsten needles, grafted in place of stage-matched host limb buds and held in place with 25 μm platinum pins. Polarising regions were grafted to the anterior and posterior margins of the wing buds of host embryos (tissue sizes 100 μm3 for normal grafts, 25 μm3 for small grafts) and held in place with 25 μm platinum pins (Stanton and Towers, 2018).

Hematoxylin and Eosin staining

Transverse sections (12 μm) of paraffin embedded forewings were mounted on glass slides. Slides were washed twice in xylene for 5 min followed by rehydration through an ethanol series (2×100%, 95% and 70%), and washed...
in H₂O. Slides were stained for 2 min in Harris Haematoxylin followed by differentiation in 0.3% acid alcohol. Blueing was achieved in Scott’s tap water and slides were rinsed in H₂O before staining in Eosin for 5 min. Slides were rinsed in H₂O and dehydrated through an ethanol series (70%, 95% and 100%). Dehydrated slides were cleared of remaining wax with xylene before mounting.

Shh signalling inhibition

Cyclopamine (Sigma) was suspended in a carrier (45% 2-hydropropyl-β-cyclodextrin in PBS, Sigma, to a concentration of 1 mg/ml). 4 µl was pipetted directly onto embryos over the limb bud, after removal of vitelline membranes. In all cases, untreated wings were treated with 2-hydropropyl-β-cyclodextrin only. Digit identities were determined by visualising phalanges under illumination.

Whole-mount RNA in situ hybridisation

Embryos were fixed in 4% PFA overnight at 4°C, dehydrated in methanol overnight at −20°C, rehydrated through a methanol/PBS series, washed in PBS, then treated with protease K for 20 min (10 µg/ml), washed in PBS, fixed for 30 min in 4% PFA at room temperature and then pre-hybridised at 65°C for 2 h (50% formamide/50% 2× SSC). Antisense DIG-labelled (Roche) mRNA probes (1 µg) were added in 1 ml of hybridisation buffer (50% formamide/50% 2× SSC) at 65°C overnight. Embryos were washed twice in hybridisation buffer, twice in 50:50 hybridisation buffer: MAB buffer and then twice in MAB buffer, before being transferred to blocking buffer (2% blocking reagent 20% lamb serum in MAB buffer) for 2 h at room temperature. Embryos were transferred to blocking buffer containing anti-digoxigenin antibody (Roche 1:2000) at 4°C overnight, then washed in MAB buffer overnight before being transferred to NTM buffer containing NBT/BCIP and mRNA distribution visualised using a LeicaMZ16F microscope.

Double RNA in situ hybridisation/immunohistochemistry

Whole-mount RNA in situ hybridisation was performed as above. Embryos were fixed for 20 min at room temperature in 4% PFA, washed twice in PBT for 10 min and then dehydrated through an ethanol series (10 min each wash in PBT) to 100% ethanol and stored at −20°C overnight. Embryos were cleared in xylene until light was visible through the tissue (~2-10 min). Embryos were processed through a series of 30 min wash changes at 60°C (25%, 25%, 50%, 75%, 100% and 100%) and then left in the oven overnight. Limbs were embedded in wax and allowed to set for 4-6 h before being sectioned using a microtome. Sections were floated on a slide rack overnight at 52°C. Slides were washed in xylene for 5 min (2×) in a Coplin jar, rehydrated through an ethanol series (2×5 min washes each) to H₂O and then washed twice in PBT. Slides were blocked horizontally for 1 h in 3% HINGS in PBT and incubated in primary antibody (anti-chick GFP at 1:100) in blocking solution overnight at 4°C or for 4 h at room temperature. Slides were washed in a Coplin jar (three times for 15-30 min) and then incubated with secondary antibody goat anti-chicken conjugated to Alexa 488 at 1:500 in blocking solution in the dark. Slides were rinsed four or five times in the dark in PBS and mounted with Fluoroshield (with DAPI).

RNA sequencing analyses and clustering

Tissue used for making RNA was manually dissected using fine forceps. Three replicate experiments were performed from each condition and the tissue from several embryos was pooled before the RNA was extracted using Trizol reagent (Gibco). Sequencing libraries were prepared using Illumina TruSeq library preparation kit. Samples were sequenced on a HiSeq 2000 (Paired end reads of 50 bp - Instrument: ST300). Reads were aligned to the chicken genome, assembly Gallus_gallus-5.0, using STAR aligner. The raw RNA sequencing data have been deposited in array express under accession number E-MTAB-7520. A total of 12 samples (three replicates for each condition) were QC analysed using automatic outlier detection. This was carried out by manually inspecting the density plot, boxplots, PCA plots, correlation heatmap and distance plot, as well as using several automatic outlier tests, i.e. distance, Kolmogorov–Smirnov, correlation and Hoeffding’s D (all samples passed QC). The read-count data for the samples were normalised using trimmed mean of M-values normalisation and transformed with Voom, resulting in counts per million with associated precision weights. Negative values result when expression is between zero and one before log₂ transformation. Genes were clustered using the cValid R package based on their log₂ fold changes. The Dunn Index was selected as the preferred cluster validation measure. Three clustering methods (hierarchical, k-means and PAM) were tested for up to 20 clusters and the clustering analysis was performed on 906 unique genes that were differentially expressed in these contrasts at the significance threshold of FDR-adjusted P-value <0.005 and fold change ≥2. Using k-means, the clustering of the 906 genes resulted in four groups.

qPCR analysis

cDNA was prepared from RNA that was used in the sequencing analysis using SuperScript II Reverse Transcriptase (Invitrogen). qPCR was performed on an Applied Biosystems StepOne RT-PCR machine using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) and a TaqMan probe and primer set designed against chicken Sim1 (Thermo Fisher Scientific). 5 ng cDNA was used per reaction (20 µl volume) with cycle conditions of 95°C for 20 s, followed by 32 cycles of 95°C for 1 s and 60°C for 20 s. All reactions were carried out in triplicate and normalized against eukaryotic 18S rRNA expression (Thermo Fisher Scientific). Standard errors of the means were generated from the triplicate Ct values. Unpaired t-tests measured significance of expression change between appropriate samples. Applied Biosystems StepOne Software V2.3 was used to analyse the data and generate gene expression comparisons.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.T.; Methodology: M.T., M.A.R.; Validation: M.T., L.B., C.A., C.R., M.A.R.; Formal analysis: M.T., L.B., C.M., C.A., C.R., M.A.R.; Investigation: M.T., L.B., C.M., C.A., C.R., M.A.R.; Resources: M.T., M.A.R.; Data curation: M.T.; Writing - original draft: M.T.; Writing - review & editing: M.T., L.B., M.A.R.; Visualization: M.T., L.B., C.M., C.A., C.R., M.A.R.; Supervision: M.T., C.R., M.A.R.; Project administration: M.T., M.A.R.; Funding acquisition: M.T., M.A.R.

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Data availability

The raw RNA sequencing data have been deposited in array express under accession number E-MTAB-7520.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.188821.supplemental

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