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Developing an ancient epithelial appendage: FGF signalling regulates early tail denticle formation in sharks

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Abstract

Background: Vertebrate epithelial appendages constitute a diverse group of organs that includes integumentary structures such as reptilian scales, avian feathers and mammalian hair. Recent studies have provided new evidence for the homology of integumentary organ development throughout amniotes, despite their disparate final morphologies. These structures develop from conserved molecular signalling centres, known as epithelial placodes. It is not yet certain whether this homology extends beyond the integumentary organs of amniotes, as there is a lack of knowledge regarding their development in basal vertebrates. As the ancient sister lineage of bony vertebrates, extant chondrichthyans are well suited to testing the phylogenetic depth of this homology. Elasmobranchs (sharks, skates and rays) possess hard, mineralised epithelial appendages called odontodes, which include teeth and dermal denticles (placoid scales). Odontodes constitute some of the oldest known vertebrate integumentary appendages, predating the origin of gnathostomes. Here, we used an emerging model shark (Scyliorhinus canicula) to test the hypothesis that denticles are homologous to other placode-derived amniote integumentary organs. To examine the conservation of putative gene regulatory network (GRN) member function, we undertook small molecule inhibition of fibroblast growth factor (FGF) signalling during caudal denticle formation.

Results: We show that during early caudal denticle morphogenesis, the shark expresses homologues of conserved developmental gene families, known to comprise a core GRN for early placode morphogenesis in amniotes. This includes conserved expression of FGFs, sonic hedgehog (shh) and bone morphogenetic protein 4 (bmp4). Additionally, we reveal that denticle placodes possess columnar epithelial cells with a reduced rate of proliferation, a conserved characteristic of amniote skin appendage development. Small molecule inhibition of FGF signalling revealed placode development is FGF dependent, and inhibiting FGF activity resulted in downregulation of shh and bmp4 expression, consistent with the expectation from comparison to the amniote integumentary appendage GRN.

Conclusion: Overall, these findings suggest the core GRN for building vertebrate integumentary epithelial appendages has been highly conserved over 450 million years. This provides evidence for the continuous, historical homology of epithelial appendage placodes throughout jawed vertebrates, from sharks to mammals. Epithelial placodes constitute the shared foundation upon which diverse vertebrate integumentary organs have evolved.

Keywords: Homology, Shark, Epithelial appendage, Anatomical placode, Dermal denticle

Background

The diversity of phenotypes among vertebrate epithelial appendages is vast and includes disparate structures of the integument such as feathers, hair, scales and teeth [1, 2]. These organs have evolved to facilitate wide-ranging aspects of survival and reproduction. Despite such diversity, these structures generally develop from patterns of reciprocal interactions between two adjacent tissue layers: the epithelium and underlying mesenchyme [1]. Where scale-like structures also arise from more derived

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mechanisms, for example the physical cracking of highly keratinised crocodile skin to form randomly spaced, polygonal head scales, placode-derived scales are also present on the body [3].

Recent research has revealed shared ancestry among amniote epithelial appendages, based on the observation that reptilian scales, avian feathers and mammalian hair share a common foundation during early development [4]: the anatomical placode. This structure is characterised by conserved molecular markers and columnar epithelial cells with a reduced rate of proliferation. Placodes constitute a localised thickening of the epithelium together with an underlying dermal condensate (mesenchyme) [5, 6]. Morphogenesis of the placode results in the adult form [7] and is controlled by molecular signals that participate in a complex gene regulatory network (GRN). This placode GRN is thought to be largely conserved throughout amniotes [4, 8]. However, there is a gap in our knowledge regarding the developmental processes guiding placode morphogenesis in non-amniote vertebrates. It is not known whether this GRN is conserved across all jawed vertebrates [9].

Chondrichthyans (sharks, rays and chimaeras) are the sister lineage of osteichthyans and occupy a basal position in jawed vertebrate phylogeny. They possess hard, mineralised epithelial appendages known as odontodes. Odontodes include both teeth and dermal denticles and have been observed in early vertebrates that lived as long as 450 million years ago [10, 11]. Odontodes consist of a central pulp cavity surrounded by a dentine layer, encased within an enameloid (enamel-like) covering [12, 13]. Recent work has provided new genetic evidence for the old hypothesis that teeth and denticles share deep homology and that their development is controlled by a common odontode GRN [14]. Since their likely origin as a form of body armour [15], denticles have evolved to fulfil a plethora of functions: they reduce abrasive damage [16], aid feeding [17], deter parasites [18], enable communication [19] and improve hydrodynamic efficiency [20–22]. Chondrichthyan denticles exhibit broad morphological variation to facilitate these roles [13].

In the S. canicula embryo, this variation can broadly be categorised into 3 classes: (1) the precocious embryonic denticles of the caudal tail, (2) the dorsal trunk and (3) adult type general body denticles (Fig. 1) [14]. Dorsal denticles (Fig. 1d, e) appear in two polarised rows at approximately 60–80 days post-fertilisation (dpf; Stage 31) and may trigger the subsequent emergence of general body denticles [23], as observed during feather tract patterning [24]. They are subsumed into general scalation soon after hatching [14]. General body denticles (Fig. 1f, g) are the most prevalent denticle type, appearing just before hatching at 145–175 dpf (Stage 34) [23], covering the skin in an intricate pattern when space is available and not in discrete rows [25, 26]. Before dorsal and body denticles appear, four rows of caudal denticles emerge at 52–60 dpf (Stage 30) [23]; two rows are present (dorsal and ventral) laterally on either side of the tail fin tip (Fig. 1b, c, j–m) [27]. Caudal denticle number can vary between 9 and 13 units which form on either dorsal row, and between 5 and 10 units which form on either ventral row [23]. The placodes of these denticles consist of a squamous epithelium overlying a basal epithelial layer of columnar cells, with condensing underlying mesenchyme (Fig. 3). They develop sequentially from posterior to anterior, approximately equidistant from one another [23, 27–29]. During morphogenesis, these denticles also mineralise in a posterior to anterior progression [23]. Despite being patterned in rows similarly to dorsal denticles, they display an irregular petaliform shape with variation in cusp number and have a less restricted polarity than other denticle types (Fig. 1l, m). These units have a dentine collar fusing the main cusp to the simple base [27], anchored within the mesenchymal dermis via connective tissues. Caudal denticles are transient epithelial structures that are lost before or during the hatching phase when general body denticles develop to take over their positions. This morphological disparity between caudal denticles and other denticle types from the dorsal trunk and general body extends beyond their macrostructure.

Caudal denticles contain a dentine type that shares histological similarity to dentine from odontodes of the Ordovician and Silurian Periods [10, 11, 27]. Unlike the orthodentine observed in the general body denticles of S. canicula, the tubules of this dentine exhibit a branching pattern [27, 30]. The combination of this primitive-type

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**Fig. 1** Odontode diversity of the pre-hatchling Catshark (S. canicula). Samples a–i are cleared and stained for calcium-rich tissue using alizarin red dye. Samples j–k are computerised tomography (CT) scans of a Stage 32 whole embryo, and samples l–m are light sheet fluorescence microscopy (LSFM) images of caudal denticles of a Stage 31 embryo, stained with alizarin red. The pre-hatching (a) possesses three major external denticle types. The caudal denticles are the first to emerge, appearing on either side of the tip of the tail in dorsal and ventral rows (b, c, j–m) [23]. These denticles are not strongly polarised, although cusps generally point towards the posterior [27]. Next, the dorsal denticles emerge along the trunk of the embryo in two polarised rows (d, e). Finally, general body denticles emerge just before hatching, covering the whole body (f, g). These denticles are also highly polarised. Teeth emerge in the jaws at a similar stage to general body denticles (h, i). The scale bar for a = 1000 µm, b, c, g and f = 200 µm, d and h = 2500 µm, e and i = 500 µm.
dentine composition and the regulated, iterative patterning mechanism of these denticles, which are found in sharks at least across families Scyliorhinae and Heterodontidae, indicates that caudal denticles may have a deep phylogenetic history and have been retained in extant sharks over 450 million years of evolution [27, 28]. However, little is known about the developmental processes or GRN underlying the formation of caudal denticles or indeed other denticle types in chondrichthians.

Fibroblast growth factor (FGF) signalling is essential for various aspects of both embryogenesis and adult homeostasis, such as tissue repair and regeneration [31]. FGFs have essential roles throughout vertebrate organogenesis, for example in limb, lung and brain development [32–34]. They mediate their responses by activating cell surface tyrosine kinase FGF receptors (FGFRs) [31]. FGF signalling is also widely involved in the development of taxonomically diverse epithelial appendages of the integumentary system, such as hair, feathers, scutes, scales and teeth [35–39]. Relative to the epithelial appendages of amniotes, little is known about the GRN controlling shark denticle placode formation, although some recent work has documented signalling during shark tooth development [40] and compared it to development of other odontodote types [14, 41, 42].

During feather placode development, ligands of the FGF signalling family (such as Fgf4) work together with sonic hedgehog (Shh) in a positive feedback loop, that promotes expression of both Fgf4 and Shh whilst also inducing expression of bone morphogenetic protein 4 (Bmp4) [36, 43]. Bmp4 then has an inhibitory effect upon both Shh and Fgf4, downregulating their expression to control patterning by limiting placode formation exclusively to the site of future organs [36, 44, 45]. This inhibitory action of mesenchymal Bmp4 has also been observed during mouse hair development [46]. The mesenchymal expression of Bmp4 is conserved during morphogenesis throughout amniote epithelial appendage development [4]. It is unknown whether this FGF, Shh and Bmp4 signalling feedback system is conserved throughout all vertebrate epithelial appendage placode GRNs, although conservation of these markers is widely observed during amniote placode formation [4].

This study examines whether the molecular signalling observed during early morphogenesis of amniote integumentary organs is conserved within the development of caudal denticles of the shark (S. canicula). By comparing gene expression to the development of other epithelial appendages and using functional experiments to examine gene interactions, it is possible to infer putative GRN relationships [14]. A combination of anatomical, histological and molecular techniques including whole mount in situ hybridisation and immunohistochemistry was used to examine the development of shark caudal denticles, focusing on the role of the FGF signalling pathway and associated members of the putative core conserved placode GRN, inferred from studies in amniotes. To study the conservation of placode GRN members between different odontodote types, gene expression was also examined during development of general body denticles. The fibroblast growth factor receptor (FGFR) inhibitor SU5402 was used to examine the effect of suppressing signalling of this major developmental pathway. By examining the role of FGF signalling during epithelial appendage development in a chondrichthyan model and its effects upon the expression of other putative GRN members, it will be possible to elucidate the degree to which epithelial integumentary organ GRNs are conserved across jawed vertebrates, and evaluate their potential homology.

Results
Caudal denticle placode development reveals conserved morphogenetic mechanisms for integumentary organ formation
To determine the earliest time of caudal denticle morphogenesis in the shark (S. canicula), we chart the sequential development of these units. It has been documented that caudal denticles in S. canicula develop from a posterior to anterior direction in dorsal and ventral rows, on both sides of the caudal-most tip of the tail (Fig. 2) [23, 27]. Previous reports suggest their emergence occurs

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Fig. 2 Sequential development of caudal denticles in the Catshark. As the embryo develops from Stage 27 (a) to Stage 33 (i), the gills proliferate, the eyes are encircled with pigment of increasing darkness and the rostrum protrudes anterior to the mouth [23]. During this period, caudal denticles develop from posterior to anterior in dorsal and ventral rows, on either side of the tail tip. At early Stage 27, no placodes can be detected (a2–a2). Epithelial thickenings then form posterior to anterior (b2–b3, c2–c4). c4 shows a magnified view of c3, highlighting an individual placode (marked with an arrowhead). These placodes then accumulate their first layers of mineralised tissue during morphogenesis (d2–d4). d4 shows a magnified view of d3, highlighting a mineralising placode (marked with an arrowhead). Mineralisation of denticles also occurs sequentially from posterior to anterior (e2–e3, f2–f3, g2–g3, h2–h3 and i2–i3) and can be highlighted with alizarin red staining for calcium-rich tissue (e4–e5, f4–f5, g4–g5, h4–h5 and i4–i5). For the axis, D dorsal, V ventral, P posterior and A anterior. Scale bars are 1000 µm for a1, b1, c1, d1, e1, f1, g1, h1 and i1 and 200 µm for all other images.
at 52–60 dpf (Stage 30) [23] (Fig. 2f, g). However, our observations suggest placode development begins earlier, between 42 and 46 dpf (Stage 27) [23] (Fig. 2b, c), although some variation in timing of denticle initiation was noted. One explanation for such variation in development is temperature of the surrounding environment [23]. Caudal denticles arise from distinct placodes (Figs. 2c4, 3a), which form from a thickened condensation of epithelial cells with an underlying mesenchymal condensate (Fig. 3). The first denticle placodes to form (most posterior) are also the first in the sequence to mineralise. This progresses in a posterior–anterior fashion (Fig. 2d4) and can be visualised using alizarin red staining (Fig. 2e4–i5).

The sequential development of this morphogenetic placode unit bears remarkable similarity to feather bud development in chicks [4, 36, 47, 48] (Fig. 2). We wished to test whether members of the amniote epithelial appendage placode GRN are conserved in chondrichthysans [4, 8]. Therefore, a selection of well-known GRN components assembled from the literature regarding feather, hair and tooth development were chosen [36, 49, 50], and their expression during early placode morphogenesis of caudal denticles in S. canicula was examined.

**Gene expression from integumentary appendage development is conserved in sharks**

Recent research has revealed ectodysplasin signalling is conserved throughout development of amniote epithelial appendages [4]. Ectodysplasin-A (Eda) and its receptor (Edar) comprise some of the earliest markers of placode morphogenesis in vertebrates (including zebrafish, chick and mouse) [49, 51–54]. During early morphogenesis of shark caudal denticles, eda and edar expression is detected in the localised epithelial thickening (Fig. 4a–f). eda is also expressed during later denticle morphogenesis in epithelial cells in the signalling centre of the putative enameloid knot (EK) (Fig. 4b, bi). This shares similarity to mammalian tooth development, during which interactions between Eda/Edar and other signalling molecules (e.g. Shh, Fgf4 and Bmp4) regulate morphogenesis of the enamel knot [55]. During hair morphogenesis in mammals, Eda and Edar signalling induces expression of other signalling molecules, such as Shh [56, 57].

Shh is a ligand of the Hedgehog (Hh) signalling pathway that marks early stages of epithelial morphogenesis in a diverse range of integumentary organs [50, 58, 59], including shark teeth and chick feathers [14, 40, 59, 60].
**shh** is expressed throughout morphogenesis of shark caudal denticles (Fig. 4g–i). During early morphogenesis, **shh** is first observed in the epithelium during early placode morphogenesis, before becoming restricted to the basal epithelium later in morphogenesis (Fig. 4g–i). **glI2** is also seen in the epithelium early during placode formation (Fig. 4j–l). Expression of **fgf3** is first seen in the epithelium, before moving to both the epithelium and mesenchyme later in placode morphogenesis (Fig. 4m–o). The dashed lines show where in the WMISH the section was taken. WMISH Section 1 represents a younger stage specimen than WMISH Section 2. For the WMISH, D dorsal, V ventral, A anterior and P posterior. For WMISH sections, R right, L left, D dorsal and V ventral. For scale bars, a, b, d, e, g, h, j, k, m, n = 200 µm, ai, bi, di, ei, gi, hi, ji, ki, mi, ni = 100 µm, and c, f, i, l, o = 50 µm.

**Fig. 4** Gene expression analyses of early morphogenesis of caudal denticles. Expression of **eda** and its receptor **edar** are observed in the epithelium during early placode morphogenesis (Fig. 4a–f). **eda** can also be seen in tissue undergoing mineralisation later in morphogenesis (Fig. 4b–bi). **shh** is first observed in the epithelium during early morphogenesis, before becoming restricted to the basal epithelium later in morphogenesis (Fig. 4g–i). **glI2** is also seen in the epithelium early during placode formation (Fig. 4j–l). Expression of **fgf3** is first seen in the epithelium, before moving to both the epithelium and mesenchyme later in placode morphogenesis (Fig. 4m–o). The dashed lines show where in the WMISH the section was taken. WMISH Section 1 represents a younger stage specimen than WMISH Section 2. For the WMISH, D dorsal, V ventral, A anterior and P posterior. For WMISH sections, R right, L left, D dorsal and V ventral. For scale bars, a, b, d, e, g, h, j, k, m, n = 200 µm, ai, bi, di, ei, gi, hi, ji, ki, mi, ni = 100 µm, and c, f, i, l, o = 50 µm.

**Fgf3** expression is mesenchymal during early morphogenesis of both feathers [67] and teeth, although in later tooth morphogenesis it is present in the epithelium of the primary enamel knot [68]. In shark caudal denticles, **fgf3** expression is initially epithelial, although it is later seen in both the epithelium and mesenchyme, in a pattern similar to shark tooth and body denticle development [14, 40] (Figs. 4m–o, 6g–i, 7). **Fgf8** is an epithelial initiatory signal of mammalian tooth morphogenesis [69]. In the shark, **fgf8** expression is observed in the epithelium during early caudal denticle morphogenesis, at a similar stage to **shh** (Figs. 5a–c, 6j–l, 7), and remains in the epithelium during later morphogenesis of the denticle cusps (Fig. 5i).
Studies from teleosts (medaka) and mammals (mouse) have indicated that Fgf8 is a ligand of Fgfr1 [70–72] that regulates enamel formation during mammalian tooth morphogenesis [73]. During early caudal denticle morphogenesis, we observed expression of fgfr1 in the squamous epithelium of placodes (Fig. 5d–f). Fgfr1 is also expressed throughout the epithelium later in morphogenesis during caudal denticle mineralisation (Fig. 5e–i), which may be indicative of a conserved role regulating enameloid formation. Fgfr2 can transduce Fgf3 during mammalian development [74, 75]. During early morphogenesis of caudal denticles, fgfr2 is expressed in the squamous epithelium of the early developing placodes in S. canicula (Fig. 5g–i). This pattern is similar to epithelial expression of fgf3, suggesting the role of fgfr2 as a fgf3 signal transducer could be conserved.

Dlx2 is a member of the Dlx homeodomain transcription factor family, which is widely important throughout various aspects of vertebrate development, including epithelial appendage formation [76, 77]. Fgf8 regulates Dlx2 expression in the underlying mesenchyme during both mouse tooth and branchial arch development [78, 79]. Previously, dlx gene expression has been documented during caudal denticle morphogenesis in S. canicula [41]. Our results confirm dlx2 is expressed in caudal denticles, and additionally we show that expression is restricted to the mesenchyme throughout early morphogenesis (Figs. 5j–l, 6d–f, 7), as observed during mouse tooth development [78].

Mesenchymal Bmp4 has an inhibitory role during amniote epithelial appendage development [1, 36, 46]. Consistent with expression observed during feather, shark tooth and body denticle development [14, 36, 46],
**bmp4** is expressed in the mesenchyme during early morphogenesis of caudal denticle placodes (Figs. 5m–o, 6a–c, 7). bmp4 may also be acting as an internal inhibitor here [1, 36], helping to define the size of the placode and therefore the adult caudal denticle.

In addition to examining caudal denticle development, gene expression of these putative core GRN members was also examined in general body denticles to compare signalling between different odontode types (Figs. 1, 8). Expression of **shh** is restricted to the epithelium throughout early morphogenesis of general body denticles (Fig. 8a–c), whereas **fgf3** is first observed most strongly in the epithelium (Fig. 8d) before being expressed in both the epithelium and underlying mesenchyme (Fig. 8e, f). Epithelial **fgf3** overlaps with **shh** expression in the putative enameloid knot, whereas **bmp4** is restricted to the mesenchyme throughout morphogenesis (Fig. 8g–i). These results show conservation of gene expression patterns between caudal and general body denticles.

Proliferating cell nuclear antigen (PCNA) immunoreactivity marks several phases of cell division from late G1 to mitosis [80]. Developing body denticles are highly proliferative units similar to teeth and dental lamina [40, 42] (Fig. 8). During early morphogenesis, the columnar cells of these placodes are characterised by reduced proliferation (Fig. 8j), as also observed throughout amniote skin appendage development [4]. Interestingly, a region of the apical denticle cusp also shows marked reduction in PCNA immunoreactivity (Fig. 8k, l) that corresponds to a putative signalling centre comparable to the enameloid knot in shark teeth [40]. This set of cells appears to overlap with the region of **shh** and **fgf3** expression in the polarised cells that will become the apical cusp (Fig. 8a–f). Caudal denticles display comparable PCNA immunoreactivity during morphogenesis (Fig. 6p–r), including reduced proliferation of columnar cells during early morphogenesis compared to younger anterior epithelial tissue in which placode formation has not begun (Fig. 6p). However, the region of reduced proliferation that occurs in columnar epithelial cells later during morphogenesis appears to be positioned more centrally than observed in body denticles, which have a distinct polarity (Fig. 6q, r). This region overlaps with expression of **fgf3** and **shh** (Fig. 6k–n), and could also be indicative of a putative primary enameloid knot, as observed in general body denticles (Fig. 8) [40]. The positional variation of this enameloid knot could reflect a shift in the morphology of these units, as caudal denticles display a less definitively polarised cusp than general body denticles (Fig. 1).

In the absence of functional data, it is not possible to test for the conserved action of GRN members, which could yield important clues regarding the putative homology of denticles and amniote epithelial appendages. We have therefore initiated a small-molecule-based targeted signalling pathway-knockdown screening assay in *S. canicula* to test the function of putative epithelial appendage GRN members, based on published results from other vertebrates.

**Small molecule inhibition reveals dependency of caudal denticle development on FGF signalling**

To elucidate the specific roles of FGF signalling during early caudal denticle placode morphogenesis, in vivo pathway perturbation assays were undertaken using SU5402. This chemical inhibits FGF signalling by blocking FGFR activity [81–83]. Stage 28 *S. canicula* embryos were treated in their sealed egg cases by injection with SU5402 to a final concentration of ~10 µM for 25 days and then allowed to develop for a further 35 days following the opening of their egg cases and washing with fresh artificial seawater. Treatment with SU5402 resulted in a single denticle knockout in 40% of the treated samples (*n* = 5) and none of the DMSO-treated control samples (*n* = 5) (Fig. 9). These units normally form equidistant from each other [23, 28, 29]; however, in the drug treated specimen shown (Fig. 9e–h) the 6th denticle was missing in the left-side dorsal row. This corresponds to the time at which treatment took place, when approximately 5 caudal denticle placodes had developed in sequence on each row (Fig. 2c). Therefore, ~10 µM SU5402 appears to prevent placode formation and subsequent morphogenesis, indicating this process is dependent upon FGF signalling.

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**Fig. 6** Gene expression/PCNA analysis of early caudal denticle morphogenesis. Gene expression is shown in 30-µm transverse sections of wild-type *S. canicula* embryo tails post-WMISH, to highlight progressive stages of caudal denticle morphogenesis from the initial epithelial thickening. **bmp4** and **dlx2** expression is restricted to the mesenchyme throughout morphogenesis (Me) (a–f). **shh** and **fgf8** are first observed in the squamous epithelium (SE) before becoming restricted to the basal epithelium (BE) (m–o, j–l). Expression of **fgf3** begins in the squamous and basal epithelium and is subsequently observed throughout the epithelium and mesenchymе (q–r). PCNA immunofluorescence is observed in the epithelium and mesenchymе throughout morphogenesis (p–r). Reduced activity (marked with an *arrowhead*) was noted in columnar cells of the epithelium during early morphogenesis (p) and in a central region of columnar cells of the basal epithelium during later morphogenesis (q–r). This region (q) overlaps with **fgf3** and **shh** expression in the basal epithelium (k, n) (marked with an *arrowhead*) and may be indicative of a basic primary enameloid knot. a is anterior, and p is posterior. Dashed lines separate the squamous epithelium (SE), basal epithelium (BE) and mesenchyme (Me). All scale bars are 50 µm in length.
As only a single denticle was lost from the sequence, it is likely the chemical either diffused out of the egg case or decomposed within it after its initial inhibitory action. We observed a similar result in our preliminary SU5402 treatment trial (see Additional file 1), which revealed a vestige when stained with alcian blue, indicative of denticle abortion. The relatively short window of sensitivity to FGF inhibition by SU5402 treatment coupled with the offset in developmental timing of individual caudal denticle rows is likely to provide an explanation for the unilaterality of this denticle knockout. As subsequent placodes developed, the field of initiatory competence is likely to have already been in place, enabling the sequential, iterative patterning to proceed beyond the disturbance once the effect of SU5402 had subsided.

We sought to confirm a specific effect of SU5402 upon the FGF signalling pathway and the placode-forming GRN by examining expression of participating network members. The prior assay required longer-term development of embryos to observe morphological effects (60 days) and subsequently used a lower drug concentration to avoid mortality, as this was an issue in preliminary trials. Experimental perturbation of FGF signalling was therefore repeated using a higher concentration of SU5402 (1 × 50 µM injection every 24 h, over a 96-h period), in line with previously published assays [37, 84, 85], and embryos were fixed immediately after the 96-h period. Specimens were then processed for in situ hybridisation for a selection of the same putative GRN members examined previously in wild-type embryos (Figs. 4, 5, 6). This allowed us to test whether perturbation of FGF signalling in shark denticles disrupted other members of the placode GRN in a manner consistent with a conserved relationship between network members.

FGF ligands exhibit positive feedback loops with Shh during many aspects of vertebrate embryogenesis, including epithelial appendage, limb bud and gill arch development [36, 65, 66, 86, 87]. We observed a dramatic downregulation of fgf3, fgf8, shh and dlx2 expression in the SU5402-treated individuals compared the DMSO-treated controls, in all but the youngest (most anterior) denticles (Fig. 10c–ji). Expression intensity of bmp4 was also notably reduced compared to the control (Fig. 10a–bi). Two SU5402-treated specimens were used for WMISH for each marker, along with one DMSO control specimen. These results suggest that SU5402 blocked FGF/FGFR signalling [81–83], thereby reducing expression of fgf3 and fgf8 (Fig. 10e–hi). This is likely due to SU5402 blocking earlier FGF signalling required for expression of these ligands [37], and interrupting the FGF–Shh positive feedback loop, which consequently limited expression of shh, bmp4 and dlx2 (Fig. 10a–di, i–ji) [36, 66, 78, 85]. Dlx family members have a role downstream of FGFs during feather bud development [77], indicating that this downregulation of dlx2 is likely a result of FGF inhibition. These results suggest that during caudal denticle formation, the function of FGF signalling in the GRN which guides epithelial appendage morphogenesis is conserved between sharks and other vertebrates.

**Discussion**

An FGF-dependent GRN constructs the placodes of epithelial appendages throughout jawed vertebrates

Our results suggest that a conserved core GRN, which includes eda/edar, shh, gli2, fgf3, fgf8, bmp4 and dlx2, underlies the development of epithelial integumentary appendage placodes across jawed vertebrates (Figs. 4, 5, 6). These placodes possess columnar epithelial cells with a reduced rate of proliferation (Fig. 6p), which is considered a structural characteristic of amniote skin appendage development [4]. Functional experiments revealed that expression of these GRN members is influenced...
by the FGF signalling pathway and that normal denticle development is perturbed upon inhibition with SU5402 (Figs. 9, 10). Caudal denticles are considered an ancient epithelial appendage that may have originated in early vertebrates over 450 million years ago and have been retained in some extant chondrichthians [10, 11, 27]. The historical continuity of the anatomical placode and underlying GRN in both amniotes [4] and
chondrichthyans provides evidence for the historical homology of all vertebrate epithelial appendages [88].

Previously, researchers have speculated that epithelial appendages have evolved independently in mammals, reptiles and birds and that therefore molecular similarity of GRNs could be a result of independent genetic co-option or deep homology [9, 89–92]. However, recent evidence has suggested that integumentary epithelial appendages
are historically homologous, at least throughout all amniotes on the basis of the anatomical placode with conserved expression and function of GRN members [4]. Our results suggest this historical homology extends even further into vertebrate phylogeny and may encompass the integumentary epithelial appendages of all extant jawed vertebrates. During both mouse and zebrafish tooth morphogenesis, Fgf8 signalling can promote Dlx2 expression [37, 78, 79].

**Fig. 10** Genetic effect of FGF inhibition via SU5402 treatment (4 × 50 µM) on caudal denticle development. There was a reduction in staining intensity of bmp4 (a–b), dix2 (c–d), fgf3 (e–f), fgf8 (g–h) and shh (i–j) in SU5402-treated specimens compared to DMSO-treated controls. We propose this resulted from the interruptions to the following GRN interactions. SU5402 inhibits FGF activity by blocking FGFR activity, thereby reducing expression of fgf3 and fgf8 (e–h). This reduced shh and dix2 expression as a FGF—shh positive feedback loops that would normally promote shh and dix2 expression (as observed during feather development) were interrupted (i–j, c–d) [78]. The fgf4–shh positive feedback loop that promotes bmp4 was also interrupted by the SU5402 treatment, reducing shh and bmp4 expression (i–j, a–b) [36]. SU5402-treated and DMSO control specimens both underwent the colour reaction of the WISH protocol for the same length of time. The dashed lines show where the section was taken from. Scale bars for WISH are 200 µm in length, and for the WISH sections they are 100 µm in length.
Recent research has revealed chondrichthyan general body denticles and teeth are deeply homologous developmental units, despite differences in their regenerative capacities [14, 40]. Our findings suggest this odontode GRN additionally encompasses caudal denticles, as conserved expression patterns were observed throughout early placode morphogenesis between caudal denticles and general body denticles. Caudal denticles are morphologically disparate from general body denticles, dorsal denticles and teeth (Fig. 1), and positional alterations to the putative enameloid knot may contribute to this variation (Figs. 6, 8). As predicted by an hourglass model of development [94], divergence in the GRN later in morphogenesis is also likely to result in alterations to the adult form, constructing different structures upon a homologous foundation: the anatomical placode. Such divergence of networks is known to relate to variation in the adult structure of feathers and teeth [95, 96].

Conclusion
The early morphogenesis of vertebrate epithelial appendages is likely to be a universal and highly conserved process retained over evolutionary time and modified to form the plethora of diverse skin appendages observed throughout all vertebrates, from sharks to mammals. The placodes of vertebrate epithelial appendages constitute the conserved foundations upon which integumentary structures have evolved, via alterations to an otherwise conserved GRN that take effect during later morphogenesis. The shark caudal denticle system provides an ideal set of sequentially developing integumentary epithelial appendages that can be studied further to decipher both complex functional GRNs and patterning mechanisms.

Combining techniques such as small molecule signalling pathway perturbation with gene expression analyses can help us begin to interpret the roles of putative GRN members. The set of genes investigated here were chosen due to their importance in the development of other epithelial appendages; however, there are many important molecules and interactions to further investigate, for example those associated with the Wnt/β-catenin and Notch pathways [97, 98]. A focus on investigating downstream GRN components responsible for later morphogenesis will enable us to elucidate how historically homologous placodes develop into the diverse range of epithelial appendages observed throughout vertebrates.

Methods
Shark husbandry and fixation
The University of Sheffield is a licensed establishment under the Animals (Scientific Procedures) Act 1986. All animals were culled by approved methods cited under
Schedule 1 to the Act. Embryos were imported from ‘Station Biologique’ in Roscoff, France, and housed in tanks at The University of Sheffield, Animal and Plant Sciences, at 16 °C. Salinity was adjusted to replicate sea water using ‘Instant Ocean’ salt dissolved in dechlorinated water. Water was oxygenated with a submerged airflow. 50% water changes were undertaken on a weekly basis. Embryos were removed from their egg cases, anaesthetised using MS-222 (Tricaine) and fixed overnight at 4 °C in 4% paraformaldehyde (PFA). Samples were dehydrated through a graded series of PBS and MeOH and stored at −20 °C in MeOH.

Alizarin red clear and staining
Fixed specimens were rehydrated through a graded series of MeOH and PBS. Staining took place in darkness overnight in 0.01% alizarin red dissolved in 0.5% KOH. Specimens were treated with trypsin in saturated sodium borate and distilled water. For Additional file 1, the sample was stained with 0.1% alcian blue in EtOH and acetic acid before the alizarin red stain was applied. Samples were then run through a graded series of KOH and glycerol solutions, before imaging took place in glycerol, using a Nikon SMZ1500 stereomicroscope.

Haematoxylin and eosin (H&E) staining
Paraffin-embedded sections were deparaffinised in xylene and rehydrated through a graded series of MeOH and PBS, before staining with haematoxylin. Sections were then rinsed in ddH2O, washed with HCl in EtOH and washed with 0.001 M Tris–HCL. Finally, sections were stained with eosin, dehydrated to MeOH and mounted used DePex mounting medium (VWR). Samples were imaged using an Olympus BX51 microscope and Olympus DP71 Universal digital camera attachment.

Micro-computerised tomography (MicroCT) and light sheet fluorescence microscopy (LSFM)
High-resolution MicroCT scanning was carried out upon a Stage 32 embryo stained with 0.1% PTA (phosphotungstic acid) in 70% EtOH for 3 days, using an Xradia Micro-XCT scanner at the Imaging and Analysis Centre of the Natural History Museum (London). Scans were rendered using the 3D volume exploration tool Drishti (www.github.com/nci/drishti) (Fig. 1j, k). LSFM was carried out upon alizarin-stained samples. A Zeiss Z1 light sheet microscope with two sCMOS cameras and an acquisition PC running Zen Black 2014 software was used to scan the tail of a Stage 31 embryo. Rendering was undertaken using the image analysis software Imaris (www.bitplane.com/imaris/imaris) by creating a signal intensity-based isosurface (Fig. 1l, m).

Small molecule gene perturbation experiments
For the first SU5402 treatment trial, Stage 28 S. canicula embryos [23] were treated with the FGF-receptor inhibitor SU5402 (Sigma). At this stage, the egg case is sealed from the external environment, allowing administration of drugs via injection into the vitelline fluid. The egg case acts as a natural treatment chamber. 100 µl of a 500 µM stock solution of SU5402 in 1% DMSO in PBS was injected into 5 egg cases, to achieve a ~10 µM concentration of SU5402 assuming an approximate egg case size of 5 ml. 5 control samples were treated with 100 µl of 1% DMSO in PBS. At Stage 31 of development, the corners of the egg cases naturally open, allowing water to enter the case and replace the vitelline fluid and the chemical gene inhibitor. Once the first egg case had opened, others were artificially opened to ensure that the treatment period remained constant between replicates. Egg cases remained sealed for 25 days before opening and were then allowed to develop for a further 35 days before fixation and morphological examination. After observing inhibition of denticle development (Fig. 9), a second round of drug treatments was conducted to examine the genetic effect of FGF inhibition via SU5402 treatment. WMISH was undertaken to examine caudal denticle morphogenesis for SU5402-treated samples and compared to control samples (treated with DMSO). The concentration of SU5402 was increased, with 10 specimens receiving a 50-µl injection of a 5 mM stock solution of SU5402 in 1% DMSO in PBS, once every 24 h for 96 h, with each individual injection resulting in a ~50 µM concentration. 5 control samples were treated with one 50 µl injection of 1% DMSO in PBS, every 24 h for 96 h. Embryos were immediately fixed after the treatment period, before dissection and WMISH took place. Two SU5402-treated tails and one DMSO control tail were used to investigate expression of each gene. The concentrations used for chemical treatments were gleaned from studies undertaking similar gene perturbation experiments in teleosts and chondrichthians, and honed using preliminary drug treatment trials in S. canicula [37, 84, 85] (see Additional file 1).

Whole mount in situ hybridisation (WMISH)
Digoxigenin-labelled (DIG) antisense riboprobes were designed using partial skate (Leucoraja erinacea) and catshark (Scyliorhinus canicula) EST assemblies [99] (SkateBase; skatebase.org) and the Vertebrate Time-Capsule (VTCap; transcriptome.cdb.riken.go.jp/vtcap). Riboprobes were cloned from S. canicula cDNA, and DIG-labelled antisense riboprobes were generated using the Riboprobe System SP6/T7 kit (Promega). WMISH was carried out in accordance with Fraser et al. [84]. Samples were rehydrated through a graded series of
MeOH and PBS, and treated with proteinase K (1 µl/mg ProK for 60 min), to facilitate probe penetration. Next, samples were refixed in 4% PFA in PBS and incubated in pre-hybridisation buffer for 1 h at 61 °C. For the hybridisation stage, samples were placed in a shaker incubator overnight at 61 °C in 2 ml tubes (Eppendorf) containing 1 ml aliquots of hybridisation buffer and DIG-labelled antisense RNA probe. Samples were then washed in saline sodium citrate with 0.1% Tween-20 (SSCT), before incubation in blocking reagent (Roche). Antibody labelling occurred overnight at 4 °C in Maleic Acid Buffer with Tween-20 (MABT), using anti-DIG-ALP (0.2 µl/ml) (Roche). This was followed by a series of washes and 48-h incubation in MABT at 4 °C. For the colour reaction, BM purple (Roche) was applied at room temperature, until the staining was sufficiently strong to represent gene expression. For WMISH undertaken upon SU5402 treated specimens, the colour reaction was run for the same length of time for SU5402-treated animals and DMSO controls. Samples were stored and imaged in 10% EtOH in PBS using Nikon SMZ1500 stereomicroscope. After WMISH and imaging, embryos were post-fixed with 4% PFA and embedded in chick albumin cross-fixed with 2.5% glutaraldehyde. A Leica Microsystems VT1000 vibratome was used to cut sections at 30 µm. Vibratome sections were then mounted with Fluoromount (Sigma-Aldrich) and imaged using a BX51 Olympus Microscope.

Section in situ hybridisation (SISH)
Fixed, dehydrated specimens were processed through a graded series of MeOH, chloroform and hot wax before being embedded in paraffin, and sectioned at 14 µm with a microtome (Leica RM2145). Sections were rehydrated from MeOH, and SISH was carried out with solutions as described for WMISH. Sections were incubated in pre-hybridisation buffer, before overnight incubation with a DIG-labelled antisense RNA probe. Sections were then run through post-hybridisation washes. Antibody labelling occurred overnight incubation with anti-DIG-AP (Roche). After post-antibody washes, BM purple (Roche) was used for the colour reaction. Sections were counterstained with haematoxylin and imaged using an Olympus BX51 Microscope and Olympus DP71 Universal digital camera attachment.

Immunofluorescence
Sections were rehydrated from MeOH or EtOH as previously described for SISH. Antigen retrieval occurred in hot 0.01 M sodium citrate (pH 6.0) for 10 min, before blocking and antibody labelling. Primary antibody labelling was undertaken using mouse anti-PCNA antibody (ab29; Abcam), overnight at 4 °C. Secondary antibody incubation was undertaken with goat anti-mouse AlexaFluor-488 (Thermo Fisher), before counterstaining with DAPI (Sigma-Aldrich). Slides were mounted with Fluoromount (Sigma-Aldrich). Imaging was undertaken with an Olympus BX61 upright epifluorescent microscope and Olympus DP71 Universal digital camera attachment, and visualised with the software Volocity 6.3.

Additional file

Authors' contributions
RLC, KJM and GJF designed the research. LJR established GH protocols and undertook SISH. KJM undertook the additional data collection. RLC undertook all other experiments. RLC, KJM and GJF analysed and interpreted the results. RLC, KJM and GJF wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The dataset supporting the conclusions of this article is included within the article.

Consent for publication
This work does not involve human subjects.

Ethics approval and consent to participate
This work does not involve human subjects, human material, or human data.

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