The presence of an embryonic opercular flap in amniotes

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The operculum is a large flap consisting of several flat bones found on the side of the head of bony fish. During development, the opercular bones form within the second pharyngeal arch, which expands posteriorly and comes to cover the gill-bearing arches. With the evolution of the tetrapods and the assumption of a terrestrial lifestyle, it was believed that the operculum was lost. Here, we demonstrate that an embryonic operculum persists in amniotes and that its early development is homologous with that of teleosts. As in zebrafish, the second pharyngeal arch of the chick embryo grows disproportionately and comes to cover the posterior arches. We show that the developing second pharyngeal arch in both chick and zebrafish embryos express orthologous genes and require *shh* signalling for caudal expansion. In amniotes, however, the caudal edge of the expanded second arch fuses to the surface of the neck. We have detailed how this process occurs and also demonstrated a requirement for thyroid signalling here. Our results thus demonstrate the persistence of an embryonic opercular flap in amniotes, that its fusion mirrors aspects of amphibian metamorphosis and gives insights into the origin of branchial cleft anomalies in humans.

Keywords: opercular flap; amniotes; tetrapod evolution; thyroid hormone; branchial cleft

1. INTRODUCTION

The operculum is a large flap consisting of several flat bones that covers and protects the gills, and which is found throughout the actinopterygians. Within extant sarcopterygians, however, it is found in coelacanth and lungfish but not in tetrapods. Palaeontological evidence, however, shows that in the tetrapod stem group, an operculum was found in *Panderichthys* but was not present in *Tiktaalik*, although this animal did possess a gill chamber, and with the further evolution of the tetrapods the internal gills were also believed to have been lost [1–3]. More recently, we have shown that the internal gills were not lost by tetrapods, but rather were transformed into the parathyroid gland [4]. There are also a number of reasons to believe that the opercular flap was not completely lost during tetrapod evolution, but that it persists and is important in internalizing the posterior pharyngeal, ‘gill-bearing’ arches during development. Significantly, although dermal ossifications homologous to those of actinopterygians—such as the opercle—do not form within the second pharyngeal arch in amniotes, in both groups the embryonic second arch expands posteriorly. This raises the possibility that, while adult amniotes clearly do not possess an opercular flap, aspects of opercular development may have been retained in amniotes. If amniotes do have an embryonic opercular flap, the caudally expanding second arch, alterations to its development will also have been evolutionarily

significant. In particular, although the caudal edge of the opercular flap in actinopterygians is open, the posterior edge of the second arch in amniotes fuses to the surface of the neck. This results in the internalization of the posterior arches.

The development of the second pharyngeal arch in amniotes is noticeably distinct from that of the other arches. This arch grows disproportionately and expands caudally, covering the posterior arches (electronic supplementary material, figure S1). In the chick, the second arch is of a similar size to the first arch until stage 19, when it begins to expand posteriorly. After a period of sustained outgrowth, the caudal edge of the second arch subsequently fuses with the cardiac eminence [5]; consequently, the posterior pharyngeal arches become enclosed in a cavity that eventually becomes obliterated by the apposition and fusion of its walls, resulting in the smooth contour of the external surface of the neck. We have, however, almost no detailed knowledge of the cellular and molecular mechanisms that direct the caudal expansion of the second arch and its fusion to the cardiac eminence.

2. MATERIAL AND METHODS

(a) General zebrafish husbandry and egg harvesting

Fertile hen's eggs were incubated at 38°C to the required stages (HH st) [6]. Embryos were fixed in 4 per cent paraformaldehyde (PFA). Zebrafish embryos were obtained by natural spawning and grown at 28.5°C in egg water (0.3 g l⁻¹ Instant Ocean Salt, 1 mg l⁻¹ Methylene Blue). Embryos were staged as described [7] and fixed in 4 per cent PFA.

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(b) Immunohistochemistry

Whole-mount antibody staining was carried out as described previously [8]. Primary antibody used was rabbit anti-laminin at 1:100 (Sigma); Secondary antibody was Alexa 488 conjugated anti-rabbit IgG used at 1:1000 (Molecular Probes). For sectioning, embryos were washed into PBS, then embedded in gelatin–albumin, fixed and vibratomed at 50 µm slices.

(c) In situ hybridization

Whole-mount *in situ* hybridization was carried out on chick [9] and zebrafish embryos [10] as described previously.

(d) LysoTracker staining

The vital lysosomal dye, LysoTracker red (molecular probes L-7528 RED), was diluted to 1:100 in PBS, and pre-warmed at 37°C. This was then added to an equal volume of fresh PBS on each embryo and incubated at 37°C in the dark for 30 min. Embryos were then rinsed 4× in PBS and fixed in 4 per cent PFA overnight at 4°C. Embryos were rinsed in PBS then dehydrated into 100 per cent methanol and viewed under fluorescence.

(e) Cyclopamine treatment

Chick embryos were treated with cyclopamine as previously described [11]. Cyclopamine (Sigma) was complexed with 45 per cent 2-hydroxypropyl-β-cyclodextrin (HBC; Sigma), to a concentration of 1 mg ml⁻¹. Embryos were treated at HH 16–17 and harvested at HH 21–22. Control embryos were treated with HBC alone. Zebrafish embryos were treated with cyclopamine as previously described [12]. Cyclopamine was dissolved in 95 per cent ethanol and embryos were treated with a final concentration of 80 µM cyclopamine or 95 per cent ethanol control. Embryos were left to develop until 5 days post-fertilization (dpf), changing the solution for fresh drug at 4 dpf.

(f) Carboxydichlorofluorescein diacetate succinimidyl esters staining

Carboxydichlorofluorescein diacetate succinimidyl ester (CCFSE; 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, succinimidyl ester; Invitrogen) was dissolved in anhydrous dimethyl sulfoxide to 50 mM (stored at -20°C). A working concentration of 250 µM in PBS was used for all chicken embryo treatments; approximately 10 µl was applied *in ovo* as described for the cyclopamine treatment, either in isolation or in conjugation with thyroid hormone antagonists.

(g) Thyroid hormone inhibition

Chicken eggs were treated as outlined for cyclopamine. Eggs were windowed at HH 25–26, treated, re-sealed and harvested at HH 29–30. For all treatments, CCFSE was concurrently applied to aid visualization by fluorescently labelling the fusing epithelia, and as confirmation of drug delivery. Thyroid antagonists used were methimazole [13] and amiodarone [13,14].

3. RESULTS**(a) The second arches of chick and zebrafish embryos express orthologous genes**

The disproportionate expansion of the second arch in amniotes suggests that it may share developmental similarities with that of the developing opercular flap of actinopterygians. To address this question, we have

examined the caudal expansion of the second arch in both zebrafish and chick embryos. We show that in both these species the expanding second arches express *Hox2* paralogues within the mesenchyme but are negative for *Hox3* paralogues. The more posterior arches express both of these genes (figure 1 *a–d*). In both chick and zebrafish, the expanding second arch comes to overlie the *Gcm2*-expressing pharyngeal pouches (figure 1*e,f*), which will form the internal gill buds in fish and the parathyroid in amniotes [4]. We have previously shown that a unique feature of the second pharyngeal arch in chick is its elevated expression of *Shh* [15]. We therefore assessed whether elevated *Shh* expression is also associated with the second arch in zebrafish, and we found that this is the case [16]. We further show that strong *Shh* expression coincides with the onset of extensive outgrowth in both zebrafish and chick (figure 1*g,h*) and persists throughout the phase of caudal expansion (figure 1*i,j*), remaining detectable until at least E5 in the zebrafish and E6 in the chick (data not shown).

(b) *Shh* signalling is required for the caudal expansion of the second arch in both zebrafish and chick embryos

Shh is a proliferative driver in many epithelia, such as the lung [17] and, given the correlation between expression and outgrowth, is a good candidate as the signal driving the posterior expansion of the second arch in both chick and zebrafish. We therefore tested the requirement for *Shh* signalling for the posterior growth of the second arch. In both zebrafish and chick embryos treated with cyclopamine, which blocks *shh* signalling, there is a loss of *Ptc* expression in the pharyngeal region (data not shown) and in both species the outgrowth of the second arch is reduced (figure 2). In zebrafish, the distance between the edge of the opercular flap and the edge of the eye is much reduced when compared with the distance between the edge of the eye and anterior edge of the pectoral fin (figure 2*a,b,e*). In chick embryos treated with cyclopamine, the size of the second arch is also much reduced (figure 2*c,d,f*) to a proportionately greater degree than the development of other structures of the head, such as the otic vesicle.

(c) The fusion of the caudal edge of the second arch in chick embryos and the requirement for thyroid signalling

In amniotes, unlike in teleosts, the posterior edge of the second arch eventually fuses, internalizing the posterior pouches and ultimately leading to a smooth external surface to the neck. We have detailed this fusion process and found that there is an intercalation between the inner endodermal surface of the expanding second arch with the outer ectodermal surface posterior of the pharynx. To analyse the ectodermal–endodermal interface of the expanding second arch, we used the fluoresceinated lipid soluble marker, carboxydichlorofluorescein diacetate succinimidyl esters (CCFSE) [18]. Once internalized by the cells, this dye is chemically altered and cannot further diffuse. Thus, local application at stage 16 to the exterior surface of the arches results in ectoderm-specific labelling and reveals the position of the ectodermal–endodermal interface at the leading edge of the expanding second

Figure 1. The second arches of fish and amniotes are homologous. (a) *hoxa2b* expression in the outgrowing opercular flap in a 3 dpf zebrafish embryo. (b) *HOXA2* expression in PA2 of an E5 chick embryo. (c) *hoxa3a* is expressed in the developing gill arches (PA3+) but not in the opercular flap covering them in a 3 dpf zebrafish embryo. (d) *HOXB3* expression in an E5 chick embryo, longitudinal section. The non-expressing second arch covers *HOXB3*+ve posterior pharyngeal region. (e) Longitudinal section through a 4 dpf zebrafish embryo showing *gcm2* expression in the developing gill buds and inner edge of the expanding opercular flap. (f) Longitudinal section of an E5 chick embryo showing *GCM2* expression in pouches 3 and 4, with the non-expressing expanding second arch. (g) Expression of *shh* at the caudal edge of the second arch in a 53 hpf zebrafish embryo. (h) Expression of *shh* at the leading edge of the expanding second arch of a 4 dpf zebrafish embryo, (i) *SHH* expression in an E3 (HH18) chick embryo at the posterior edge of the growing second arch. (j) Pronounced *SHH* expression at the caudal edge of the growing PA2 in an E4 (HH23) chick embryo. The edge of the second arch is outlined in (a–f).

arch at E3 (figure 3a). At E4 of chick development, an intact and continuous basal lamina is associated with the epithelia of the expanding second arch and the underlying tissue (figure 3b). By E5.5, however, the inner surface at the caudal edge of the second arch has begun

to fuse with the subjacent ectoderm (figure 3c). The fusion continues internally until there is now a contiguous basal lamina running between these tissues. A sinus is formed that is obliterated as the inner surfaces completely fuse (figure 3d,e). We have also found that slightly

Figure 2. Shh inhibition reduces the outgrowth of the second arch in chick and zebrafish. (a,b) Five dpf zebrafish embryos treated with ethanol (carrier, a), or cyclopamine (b), and probed with *shh* to visualize the flap. Numbers indicate the measurements taken to calculate the size of the flap. (c,d) Bisected chick embryos (4 dpf) treated with (c) HBC—carrier or (d) cyclopamine. The second arch (PA2) and otic vesicle (OV) are outlined in red. (e) Graph showing the size of the opercular flap (OF) in treated fish as a percentage of total eye–pectoral fin length. (f) Graph showing the area of the second arch (PA2) and otic vesicle (OV; internal control to account for any generalized growth reduction) in squared micrometre. There is a significant reduction in PA2 compared with the OV for the cyclopamine-treated embryos versus HBC (carrier) control-treated embryos (two-tailed *t*-test, $p < 0.001$ for both d and e). Blue, 15 μl HBC control; pink, 15 μl cyclopamine.

elevated levels of cell death are associated with this interface during the fusion process (figure 3f). Apoptosis is also seen more prominently post-fusion, from E6 onwards, and most significantly at E7, on the external surface of the arch (figure 3i). It is likely that this apoptosis serves to remove excess tissue post-fusion. By E9, this tissue has been obliterated, leaving a smooth external surface to the neck (data not shown).

The internalization of the gills and the eradication of the external pharyngeal opening is seen in amphibian metamorphosis and is driven by thyroid hormone. Therefore, to determine whether the fusion of the second arch to the cervical surface in chick is in fact a cryptic form of metamorphosis that also requires thyroid signalling, we employed two different thyroid blockers: amiodarone, a thyroid receptor antagonist, and methimazole, which inhibits thyroid hormone synthesis by blocking the uptake of iodine [13,14]. Chick embryos were treated at HH25–26 with one of the two thyroid hormone inhibitors and the fluorescent dye CCFSE, pipetting directly under the second arch, and harvested at HH29–30, after which fusion has ordinarily completed. The CCFSE labelling enabled identification of embryos that had received the drug in the appropriate region, and also aided with visualization of the phenotypes. Owing to the primarily internal nature of the fusion process, it was necessary to section and analyse treated embryos by confocal microscopy.

We found that, in embryos treated with either amiodarone or methimazole, complete fusion of the caudal edge of the second arch to the underlying ectoderm was never observed (figure 3g). Of those treated with

amiodarone at 5 μM, 4/4 showed a partial fusion of the flap, resulting in an expanded sinus, and often contorted epithelial structures at the site of caudal fusion. However, with treatment at a slightly higher dose, 10 μM, 2/4 embryos were now unfused, with 2/4 still showing a partial fusion (figure 3h). Amiodarone and methimazole interfere with thyroid signalling at different points, and we therefore also treated embryos with both drugs to substantiate that the effects we are seeing with amiodarone alone are due to blocking of thyroid signalling. We found that when embryos were treated with amiodarone (10 μM) and methimazole (20 mM) 3/5 embryos were unfused, and 2/5 showed partial fusion (figure 3g). Contrastingly, out of nine PBS-treated control embryos at HH29/30, seven (7/9) showed complete fusion and eradication of the cavity while two showed fusion with a remnant of cyst still visible (2/9).

4. DISCUSSION

Collectively, our results establish that an embryonic opercular flap persists in amniotes. The expanding second arches of both chick and zebrafish embryos express the same genes and both require Shh signalling for the posterior expansion of this arch. In both teleosts and amniotes, the second arch comes to overlie the *Gcm2*-expressing pharyngeal endoderm of the posterior arches, which will form the internal gills/parathyroid gland. Thus, the mechanisms that act to drive the development of the embryonic operculum are likely to be conserved across osteichthyans. However, amniotes differ in that the caudal aspect of the expanded second arch fuses

Figure 3. Morphogenesis of the amniote second arch and inhibition of fusion. (a) Longitudinal section of ectoderm labelled with CCFSE. The inner surface of the second arch is endoderally derived. The leading edge of the second arch is indicated by the white arrow. (b) Laminin immunofluorescence at E4; the second arch has not fused. Leading edge indicated by white arrow. (c) Laminin staining shows the beginning of fusion of the inner surface of the second arch to the subjacent epithelium of the posterior arches at E5 (white arrow). Posterior edge of the second arch indicated by the white arrow. (d) Longitudinal section at E6 shows the second arch has broadly fused; internally, a small gap remains where the sinus is in the process of closing up (white asterisk). (e) These internal surfaces eventually fuse; the line of fusion marked by a white asterisk. (f) Lysotracker staining labelling apoptotic cells. Some dying cells are present along the line of fusion. (g) Treatment with thyroid-blocking drugs (amiodarone and methimazole) leads to failure of fusion (white arrowhead) and persistence of cyst (white asterisk). Longitudinal section at E6 (HH29). (h) A partial failure of fusion after treatment with thyroid hormone-blocking drugs (methimazole only). There is a persistent cyst (white asterisk) and an involuted structure where the ectodermal interfaces have failed to properly meet and fuse (white arrowhead). Longitudinal section at E6 (HH29). (i) Whole-mount chick embryo (E7) stained with lysotracker, showing a line of cell death at the remaining edge of the fused second arch, expanding medially from the lateral edges. ecto, ectoderm; endo, endoderm; PA2, pharyngeal arch 2.

with the subjacent epithelium and thus internalizes the posterior pharyngeal arches. We further show that, in the chick, this fusion event requires thyroid hormone. This fusion of the posterior edge of the second arch and the corresponding internalization of the posterior pharyngeal arches is similar to what is observed during amphibian metamorphosis. The gills are internalized and the external opening is covered and thyroid hormone signalling drives these events. Thus, our analysis uncovers a cryptic metamorphic event in amniotes: the fusion of the posterior of the second arch to the subjacent epithelium that mirrors what is seen during amphibian metamorphosis.

Intriguingly, a recent study has shown that opercular development in a holocephalan, *Callorhincus milli*, is also associated with sustained *Shh* expression in the second pharyngeal arch [19]. In holocephalans, the

operculum is supported by endoskeletal appendages and it is suggested that the pronounced outgrowth of these elements is facilitated by elevated *Shh* expression. This contrasts with what is seen in the other chondrichthyans, the elasmobranchs, in which *Shh* is expressed at similar levels in hyoid and gill arches and is required for the formation of gill rays in these arches [20]. Our work, together with this recent analysis of holocephalan development, suggests parallels between this situation and the scenario we describe here for osteichthyans. However, it should be noted that there are significant differences in that the osteichthyan operculum is not supported by endoskeletal elements but rather by dermal ossifications, suggesting convergence in skeletal support. Yet, both studies emphasize the point that elevated *Shh* expression can result in the promotion of outgrowth of a particular pharyngeal arch. It is possible that the ancestral

gnathostome condition was for *Shh* expression to be involved in driving the outgrowth of all pharyngeal arches and that this became separately modified in holocephalans and osteichthyans, such that *Shh* driven expansion became associated with the second arch.

Finally, our results also help to explain the aetiological basis of the majority of branchial cleft anomalies. Ninety-five per cent of these are associated with the second pharyngeal arch, and involve the presence of cysts and/or fistula [21]. These would result from failure in the fusion of the opercular flap and the eradication of the internal walls. Thus, internalization of the gills was an important event. It would have facilitated the colonization of land by tetrapods and is still replayed during amphibian metamorphosis. Significantly, however, it still occurs during amniote development and if aspects of this programme fail they will contribute to the prevalence of second arch-associated branchial clefts in humans.

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