ABSTRACT
Chondrichthyan (sharks, skates, rays and holoccephalans) possess paired appendages that project laterally from their gill arches, known as branchial rays. This led Carl Gegenbaur to propose that paired fins (and hence tetrapod limbs) originally evolved via transformation of gill arches. Tetrapod limbs are patterned by a sonic hedgehog (Shh)-expressing signalling centre known as the zone of polarising activity, which establishes the anteroposterior axis of the limb bud and maintains proliferative expansion of limb endoskeletal progenitors. Here, we use loss-of-function, label-retention and fate-mapping approaches in the little skate to demonstrate that Shh secretion from a signalling centre in the developing gill arch establishes gill arch anteroposterior polarity and maintains the proliferative expansion of branchial ray endoskeletal progenitor cells. These findings highlight striking parallels in the axial patterning mechanisms employed by chondrichthyan branchial rays and paired fins/limbs, and provide mechanistic insight into the anatomical foundation of Gegenbaur’s gill arch hypothesis.

KEY WORDS: Sonic hedgehog, Gill arch, Evolution, Skate, Appendage patterning, Leucoraja erinacea

INTRODUCTION
Chondrichthyans are unique among extant jawed vertebrates in possessing appendages, known as branchial rays, which project laterally from their gill arches (Gillis et al., 2009a). This anatomy mirrors the configuration of paired fins (including limbs) and their proximal girdle, and led the comparative anatomist Carl Gegenbaur to propose that paired fins evolved by transformation of a gill arch, with the epi- and ceratobranchial cartilages of the gill arch giving rise to the girdle, and branchial rays giving rise to the fin proper (Gegenbaur, 1878). This hypothesis of serial homology (Fig. 1) would predict that the gill arches of chondrichthyans and the paired fins/limbs of jawed vertebrates share axial patterning mechanisms. However, whereas a great deal is known about the molecular basis of paired fin and limb patterning (Zeller et al., 2009), comparable approaches in the little skate to demonstrate that Shh signalling functions similarly to the limb bud ZPA, both in the establishment of the skate gill arch anteroposterior axis and in the proliferative expansion of branchial ray endoskeletal progenitors.

RESULTS AND DISCUSSION
Shh signalling in skate gill arch development
In order to identify the source and targets of chondrichthyan gill arch Shh signalling, we characterised the expression of Shh and Patched2 [Ptc2; a readout of Shh signalling (Pearse et al., 2001)] by mRNA in situ hybridisation in skate embryos. In vertebrate embryos, pharyngeal arches are delineated by an iterative series of endodermal pouches that outpocket from the foregut and contact overlying pharyngeal ectoderm. In fishes, endodermal pouches fuse with overlying ectoderm, giving rise to gill slits and leaving, between presumptive gill slits, pharyngeal arches filled with neural crest-derived mesenchyme and a central mesodermally derived core (Graham, 2001). In skate embryos at stage 22 (Ballard et al., 1993) (Fig. 2A), Shh expression is observed in the region of the pharyngeal gill slits and pouches (Fig. 2B). Expression analysis on paraffin sections reveals that Shh transcripts localise to the posterior pharyngeal arch epithelium (Fig. 2C,D), consistent with previous reports of Shh expression in the posterior hyoid arches of chick (Wall and Hogan, 1995) and zebrafish (Richardson et al., 2012) embryos. Analysis of Ptc2 expression at stage 22 indicates that the pharyngeal arch Shh signal is transduced posteriorly within the developing gill arches, in pharyngeal arch epithelium, mesenchyme and in the mesodermally derived core (Fig. 2E).

By stage 27, all pharyngeal arches have formed and the hyoid and gill arches are expanding laterally (Fig. 2F). At this stage, Shh expression is restricted to the epithelium along the leading edge of the expanding hyoid and gill arches (Fig. 2G-I), with Shh-expressing cells having the appearance of a ridge [the gill arch epithelial ridge (GAER)]. The GAER is reminiscent of the apical ectodermal ridge (AER) of the developing fin and limb buds, and we have previously shown that, like the fin/limb bud AER, the GAER also expresses the gene encoding the signalling molecule Fgfl8 (Gillis et al., 2009b). Ptc2 expression reveals that the GAER Shh signal is asymmetrically transduced in posterior-distal arch mesenchyme, as well as in cells within the mesodermally derived core and distal arch epithelium (Fig. 2J). By stage 29, the hyoid and...
gill arches continue to expand laterally and have taken on a pronounced posterior curvature (Fig. 2K). Shh expression persists in the GAER of the hyoid arch and gill arches (Fig. 2L-N), and Ptc2 expression indicates transduction of GAER Shh in posterior-distal arch mesenchyme, a few cells at the distal tip of the mesodermally derived core, and in the GAER and adjacent epithelium (Fig. 2O). At stages 27 and 29, cells of the GAER are distinguishable as a pseudostratified epithelial ridge, ~5-6 cells in diameter (Fig. 2P). By stage 30, anlagen of pharyngeal endoskeletal elements appear (Gillis et al., 2009a). In summary, the GAER is a Shh-expressing signalling centre that spans the leading edge of the expanding skate hyoid and gill arches. GAER Shh expression originates within posterior pharyngeal arch epithelium and persists through lateral expansion of the hyoid and gill arches, resolving into a morphologically distinct pseudostratified epithelial ridge, while signalling to posterior arch mesenchyme and epithelium. Thus, although the GAER is distinct from the limb bud ZPA at the tissue level (the former is epithelial, whereas the latter is mesenchymal), both provide a posteriorly localised source of Shh signal that is transduced in adjacent mesenchymal and epithelial cell populations.

**Shh-responsive mesenchyme gives rise to branchial rays**

The gill arch endoskeleton of chondrichthyans consists of proximal epi- and ceratobranchial cartilages, and a series of branchial rays projecting laterally from these (Fig. 3A,B). In the tetrapod limb, it has been demonstrated that ZPA Shh-responsive mesenchyme contributes extensively to the distal limb skeleton (Ahn and Joyner, 2004), and these elements exhibit morphological defects following loss of Shh signalling (Riddle et al., 1993; Chiang et al., 2001; Ros et al., 2003; Stopper and Wagner, 2007; Towers et al., 2008; Zhu et al., 2008). To test the endoskeletal fate of GAER Shh-responsive mesenchyme, we labelled this cell population by microinjecting CM-Dil subjacent to the GAER of gill arches in skate embryos at stages 27 and 29 (Fig. 3C,D; compare injection with Ptc2 expression in Fig. 2J). Injected embryos were reared until stages 31-32 (~8-10 weeks of development, when gill arches and branchial rays have differentiated), and then analysed for the presence and distribution of CM-Dil-positive chondrocytes in histological sections of the gill arch endoskeleton.

Histological analysis of gill arches of skates with labelled GAER Shh-responsive mesenchyme revealed the presence of CM-Dil-labelled chondrocytes in the branchial rays (100% of examined individuals; n=5 each for CM-Dil labelling at stage 27 and stage 29; Fig. 3E). In individuals labelled at stage 27, CM-Dil-positive chondrocytes were distributed broadly throughout the branchial rays, whereas individuals labelled at stage 29 possessed CM-Dil-positive chondrocytes predominantly in the distal tips of the rays. These data indicate that GAER Shh-responsive mesenchymal cells contribute to branchial rays, which are the elements that Gegenbaur serially homologised with the paired fin/limb endoskeleton (Fig. 1).
Shh polarises and maintains proliferative expansion of gill arch endoskeletal progenitors

In the tetrapod limb, Shh signalling from the ZPA functions both in the establishment of the limb bud anteroposterior axis and in the maintenance of proliferative expansion of limb endoskeletal progenitors (Towers et al., 2008; Zhu et al., 2008). To test the patterning function of Shh signalling during skate gill arch development, we conducted a series of loss-of-function experiments by in ovo injection of the hedgehog signalling antagonist cyclopamine (Chen et al., 2002). Skate eggs were injected with cyclopamine to a final concentration of ∼20 μM at either stage 22, 27 or 29, and were then reared until endoskeletal differentiation (∼8-12 weeks). As with digit number in mice (Zhu et al., 2008) and salamanders (Stopper and Wagner, 2007), successively earlier loss of Shh signalling resulted in a progressively greater reduction in the number of branchial rays on each arch. Specifically, cyclopamine treatment at stages 22 and 27 resulted in significant reductions in branchial ray number relative to controls, but there was no significant reduction in branchial ray number with cyclopamine treatment at stage 29 (Fig. 4A,B).

We postulated that reductions in branchial ray number were due to reduced proliferation of gill arch mesenchyme in the absence of gill arch Shh signalling, and to test this we conducted a series of EdU (Salic and Mitchison, 2008) incorporation experiments. Embryos at stage 27 were reared ex ovo in either 20 μM cyclopamine or DMSO.
vehicle in seawater for 24 h, prior to intraperitoneal microinjection with EdU. Injected embryos were left to develop for a further 24 h, then fixed and analysed for EdU retention in gill arch mesenchyme. Embryos treated with cyclopamine showed a significant reduction in the proportion of EdU-positive nuclei in gill arch mesenchyme relative to controls, indicative of reduced DNA replication (and hence cell proliferation) in the absence of Shh signalling (Fig. 4C). Together with our fate-mapping data, these findings indicate that gill arch Shh signalling functions, in part, to maintain the proliferative expansion of branchial ray progenitors during gill arch development.

Finally, we noted a striking anteroposterior patterning defect in the gill arches of the earliest cyclopamine-treated skate embryos. Chondrichthyan gill arches exhibit a clear anteroposterior polarity, with branchial rays invariably articulating with the epi- and ceratobranchial cartilages along their posterior margins (Gillis et al., 2009a). In skate embryos treated with cyclopamine at stage 22, the epi- and ceratobranchial cartilages were severely misshapen, consistently lacking evidence of an anteroposterior axis, with branchial rays articulating along their midlines (n=4/7; Fig. 4D). Notably, this patterning defect was not observed in any embryos treated with cyclopamine at stages 27 (n=0/7) or 29 (n=0/9). These findings suggest that, in addition to its prolonged role in maintaining gill arch proliferative expansion, Shh signalling also functions early in gill arch development to establish gill arch anteroposterior polarity.

Conclusions
Gegenbaur’s gill arch hypothesis of paired fin origins is often regarded as the flawed alternative to the lateral fin fold hypothesis of Thacher (1877), Mivart (1879) and Balfour (1881), which purports that paired fins evolved from a bilateral median fin-like structure. Although neither the gill arch nor lateral fin fold hypothesis is supported by paleontological data (Coates, 2003), consensus has largely shifted toward the latter owing to the discovery of shared expression of developmental patterning genes between paired and dorsal median fins (Freitas et al., 2006; Dahn et al., 2007). Our demonstration of a dual role for Shh signalling in patterning the endoskeleton of chondrichthyan gill arches points to a common molecular mechanism underlying the axial patterning of branchial rays and paired fins/limbs, and highlights chondrichthyan branchial rays as an important feature in the evolutionary story of gnathostome paired appendages. Conserved developmental mechanisms are generally regarded as the basis of serial homology (Roth, 1984; Wagner, 1989, 2007), although it remains to be determined whether developmental mechanisms shared by branchial rays and paired fins/limbs reflect conservation, parallel evolution (i.e. the independent co-option of deep conserved developmental mechanisms, or ‘deep homology’) or convergent evolution (Hall, 2003; Shubin et al., 2009).

In the absence of paleontological data illustrating the stepwise acquisition of the paired fin endoskeleton, comparative studies of axial patterning mechanisms in diverse vertebrate appendages – e.g. fins/limbs, branchial rays, median fins and external genitalia (Cohn, 2011) – will allow us to formulate testable hypotheses of nested relationships among body plan features in order to explain morphological similarity by the extent of shared developmental information.

**MATERIALS AND METHODS**
**Embyro collection and fate mapping**
Skate (Leucoraja erinacea) eggs were obtained at the Marine Biological Laboratory (Woods Hole, MA, USA) and maintained in a flow-through seawater system at ~17°C. CM-Dil fate-mapping experiments were carried out as described (Gillis et al., 2012). All animal work complied with protocols approved by the Institutional Animal Care and Use Committee at the MBL. Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, rinsed three times in PBS, dehydrated into methanol and stored at −20°C. For an overview of embryonic development of the little skate, see http://www.youtube.com/watch?v=eve93mSgM0c.

**Histology and mRNA in situ hybridisation**
Embryos were embedded in paraffin wax and sectioned as described (O’Neill et al., 2007). Sections of CM-Dil-labelled embryos were counterstained with DAPI. In situ hybridisation experiments for L. erinacea Shh (GenBank accession number EF100667) and Ptc2 (GenBank accession number EF100663) were performed as described (Gillis et al., 2012).

**In ovo cyclopamine treatment and skeletal preparations**
To achieve a final in ovo cyclopamine concentration of ~20 μM, 25 μl 9 mM stock solution of cyclopamine in DMSO was injected into 25 skate egg cases at each stage 22, 27 and 29, using a syringe and 30-gauge needle. This volume was determined based on a mean egg volume of ~10 ml. For controls, an equivalent volume of DMSO alone was injected. Embryos were reared for 8-12 weeks. Surviving embryos (n=7, 7 and 9 for cyclopamine treatment at stage 22, 27 and 29, respectively) were analysed for skeletal defects by wholemount skeletal preparation (Gillis et al., 2009a).

**EdU incorporation assay**
For 5-ethyl-2-deoxyuridine (EdU) incorporation experiments, stage 27 embryos were removed from their egg cases and reared in 10-cm diameter Petri dishes in either 20 μM cyclopamine in seawater (n=5) or in seawater with an equivalent volume of DMSO (n=5). After 24 h, embryos received an intraperitoneal microinjection of ~0.5 μl 5 mM EdU (ThermoFisher Scientific) in 1× PBS using a pulled glass capillary needle and a Picospritzer pressure injector. Embryos were then returned to their cyclopamine/DMSO baths for a further 24 h. EdU-injected embryos were fixed and processed for histology as described above. EdU was detected in sections using the Click-iT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher Scientific), and sections were counterstained with DAPI. Counts of EdU-positive nuclei were carried out manually using the Cell Counter plugin for ImageJ (NIH). The mean proportion of EdU-positive nuclei in gill arch mesenchyme was calculated for three individuals per treatment or control (with cell counts from three consecutive sections per individual), and statistical significance was determined by unpaired t-test.

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**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
J.A.G. conceived the study, designed and conducted the experiments, analysed the data and wrote the manuscript with input from B.K.H.

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**References**


