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Regulatory elements of *Xenopus col2a1* drive cartilaginous gene expression in transgenic frogs

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ABSTRACT **This study characterizes regulatory elements of *collagen 2α1 (col2a1)* in *Xenopus* that enable transgene expression in cartilage-forming chondrocytes. The reporters described in this study drive strong cartilage-specific gene expression, which will be a valuable tool for further investigations of *Xenopus* skeletal development. While endogenous *col2a1* mRNA is expressed in many embryonic tissues, its expression becomes restricted to tadpole and adult chondrocytes. This chondrocyte-specific expression is recapitulated by *col2a1* reporter constructs, which were tested through I-SceI meganuclease-mediated transgenesis. These constructs contain a portion of the *Xenopus tropicalis col2a1* intron, which aligns to a cartilage-specific intronic enhancer that has been well characterized in mammals. Two overlapping regions of the first intron that are 1.5-Kb and 665-bp long, both of which contain this enhancer sequence, drove EGFP expression in both larval and adult chondrocytes when connected to an upstream promoter. However, neither a truncated 155-bp region that also contains the enhancer, nor a separate 347-bp intronic region that lacks it, was able to drive cartilaginous transgene expression. The two cartilage-specific transgenes are heritable in F1 progeny, which exhibit none of the background expression observed in the injected founders. This study is the first to use the I-SceI technique to characterize an enhancer element in *Xenopus,* and the first to generate chondrocyte-specific gene expression in a non-mammalian vertebrate. The creation of novel cartilage-specific gene expression provides a new tool for further studies of anuran skeletal development.**

KEY WORDS: *col2a1, chondrocyte, meganuclease, Xenopus, sox9*

Introduction

The collagen 2α1 gene (*col2a1*) encodes type-II collagen, the primary collagen protein of cartilage. The gene product, a pro α1(II) polypeptide, forms helical homotrimers through its collagenous domain in the mature type-II collagen protein (reviewed in Boot-Handford and Tuckwell, 2003). The high copy number of individual polypeptides required for the collagen-rich extracellular matrix of cartilage coincides with high copy number of precursor mRNA within individual chondrocytes (Kosher *et al.*, 1986a; Kosher *et al.*, 1986b). *Col2a1* mRNA is also expressed in the notochord, somites, lateral plate mesoderm, and ventral neural tube in *Xenopus laevis* (Su *et al.*, 1991; Kerney *et al.*, 2007) and mouse embryos (Thorogood *et al.*, 1986; Wood *et al.*, 1991). Expression of *col2a1* in *X. laevis* becomes restricted to the cartilaginous skeleton of the tadpole and notochordal sheath during skeletogenesis (Su *et al.*, 1991). An alternatively spliced isoform, which lacks the second exon, also occurs during carti-

lage-forming stages in *Xenopus* (Su *et al.*, 1991) and mouse (Sandell *et al.*, 1991).

Investigations into the regulation of *col2a1* transcription in mammals have concentrated on its mRNA expression in chondrocytes. These studies reveal an enhancer region in the first intron that is necessary for chondrocyte-specific expression of *col2a1* mRNA. Successive truncations have reduced this intronic en-

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hancer sequence from over 3-Kb to 800-bp (Horton *et al.*, 1987), 182-bp (Zhou *et al.*, 1995), 48-bp (Zhou *et al.*, 1998) and 18-bp (Lefebvre *et al.*, 1996) regions, each of which is capable of driving chondrocyte expression when connected to a *col2a1* promoter. The mouse minimal enhancer contains the HMG recognition sequence, CATTCAT, which binds to the HMG-box containing transcription factor Sox9 (Lefebvre *et al.*, 1997). However, multiple copies of the truncated 48 bp and 18 bp enhancer regions are required for detectable expression of reporter genes, indicating the presence of additional regulatory information outside the Sox9-binding region.

The tissue-specificity and high level of expression generated by the *col2a1* promoter/enhancer have provided a valuable tool for studies of gene function within the chondrocyte lineages of mice. Variations of this reporter construct have been used for over-expression of dominant-negative and full-length genes within mouse chondrocyte lineages (Nakata *et al.*, 1993; Tsumaki *et al.*, 1999; Takeda *et al.*, 2001; Ueta *et al.*, 2001; Stricker *et al.*, 2002). The mouse reporter construct also has been fused to both doxycycline and tamoxifen-inducible enhancers, allowing temporal control of chondrocyte-specific gene expression (Grover and Roughley, 2006; Nakamura *et al.*, 2006; Chen *et al.*, 2007).

To date, the use of a cartilage-specific reporter has been limited to studies in mammals. No other vertebrate taxon has a similar tool for driving transgene expression in chondrocytes. The development of a *col2a1* reporter in alternative species (and classes) would allow investigations of both comparative gene

regulation and comparative skeletal development. Metamorphosing anurans are ideal taxa for this type of investigation given their unique skeletal development, which is associated with a biphasic life history.

Metamorphic development of the anuran skeleton differs from that of amniotes in having two discrete periods of skeletogenesis. The first, embryonic phase of skeleton formation yields the strictly cartilaginous tadpole skeleton. The second, metamorphic phase of skeleton formation results in both new cartilage and bone formation in the post-metamorphic froglet (reviewed in Hanken, 1992). Previous investigations have concentrated on the cellular origins of the tadpole skull (Stone, 1929; Sadaghiani and Thièbaud, 1987; Olsson and Hanken, 1996) and the differentiation of tadpole chondrocytes (Spokony *et al.*, 2002; Baltzinger *et al.*, 2005; Kerney *et al.*, 2007). Few studies, however, have investigated the cellular origins and differentiation of chondrocytes during the second—metamorphic—phase of anuran skull development (e.g., Gross and Hanken, 2008). This lack of research is largely attributable to the long period required for tadpoles to complete metamorphosis, and a lack of long-term genetic tools needed to manipulate and analyze metamorphic processes.

This study investigates the regulation of *col2a1* in *Xenopus* using I-SceI meganuclease-mediated transgenics and provides a tool for future investigations of anuran skeletal development. This transgenic technique was originally used in a teleost, the Japanese medaka (*Oryzias latipes*; Thermes *et al.*, 2002), and later adapted for the creation of transgenic *Xenopus* (Ogino *et al.*, 2006 a,b; Pan *et al.*, 2006). The technique allows for the efficient transfection of embryos through simple enzyme digestion and microinjection. This study is the first to employ this transgenic technique for the characterization of an enhancer element in *Xenopus*. The cartilage-specific reporter construct described in this study will facilitate further investigations into the genetic basis of anuran skeletal development and metamorphosis.
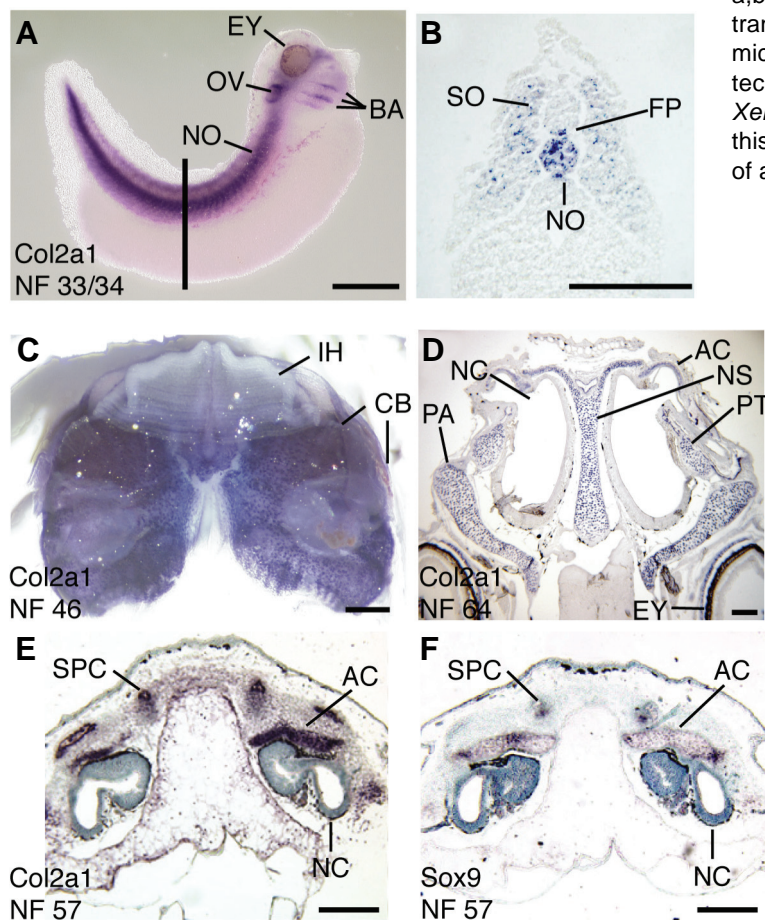
Fig. 1. *In situ* hybridizations during embryonic (A,B), larval (C), metamorphic (E,F) and post-metamorphic stages (D). **(A,B)** *Whole-mount* col2a1 in situ *hybridization of a Nieuwkoop-Faber (NF) stage-33/34 embryo.* **(A)** *Lateral view, anterior is to the right. There is strong expression of* col2a1 *in the notochord (NO), otic vesicle (OV) and branchial arches (BA). Black line points to level of section depicted in (B).* **(B)** *Histological cross section through the trunk. Note diffuse staining in the somites (SO) and floor plate of the neural tube (FP).* **(C)** *Whole-mount* col2a1 in situ *hybridization; stage-46 tadpole head. Ventral view, anterior is to the top. There is strong expression in early larval cartilages, including the ceratobranchials (CB), but not in the interhyoideus muscle (IH).* **(D)** *Sectioned in situ hybridization of a stage-64 froglet nasal capsule. Coronal section, anterior is to the top.* Col2a1 *is expressed again in the adult cranial cartilages, including the post-metamorphic nasal septum (NS), planum terminale (PT) planum antorbitale (PA), and alary cartilage (AC).* **(E,F)** *Mid-metamorphic, stage-57 tadpole. Coronal histological sections of the nasal capsule, stained by in situ hybridizations with* col2a1 *and* sox9 *probes.* **(E)** Col2a1 *expression increases in the alary (AC) and superior prenasal (SPC) cartilages as they form during metamorphosis.* **(F)** *Up-regulation of* col2a1 *corresponds with heightened expression of* sox9 *in these cartilages during metamorphic chondrogenesis. Additional abbreviations: NC, nasal capsule; EY, eye. Scale bar, 0.5 mm.*

Results

In situ *hybridizations*

After an initial distribution in several embryonic tissues, expression of *col2a1* mRNA becomes restricted to the cartilaginous skeleton of the tadpole and later of the adult frog. In stage 33/34 embryos, *col2a1* is expressed in the floor plate of the neural tube, the notochord, branchial arches, somites, and otic vesicles (Fig. 1A, B). By stage 46, *col2a1* is strongly and exclusively expressed in larval chondrocytes (e.g., ceratobranchial cartilages; Fig. 1C). During metamorphosis, new cartilages contribute to the adult nasal capsule and sound-conducting plectral apparatus (Trueb and Hanken, 1992; Gross and Hanken, 2008). Metamorphic formation of alary and superior prenasal cartilages in the nasal capsule corresponds with heightened *col2a1* mRNA expression (Fig. 1E) along with co-expression of the *col2a1* regulator *sox9* (Fig. 1F). By stage 64, *col2a1* is strongly expressed in post-metamorphic cartilages of the froglet, including the nasal capsule (Fig. 1D).

Promoter characterization

Previous research on the chondrocyte-specific expression of *Col2a1* in mammals indicated a synergism between the 5′ promoter and intronic enhancer (Savagner *et al.*, 1990; 1995; Leung *et al.*, 1998; Seki *et al.*, 2003). Therefore, 491 bp of upstream sequence from *Xenopus tropicalis* was used as a promoter in each transgene construct characterized in this study (Fig. 2A). This sequence includes 366 bp of regulatory DNA and 125 bp of 5′-untranslated region (UTR). A LAGAN/Vista analysis revealed no significant sequence similarity between the mouse and *X. tropicalis* promoter regions (data not shown). However, a sequence 13 bp upstream of the transcription start site (TATAACT) in *X. tropicalis* is identical to the TATA-box sequence of the mouse *Col2a1* promoter (Fig. 2A), but not the human *COL2A1* TATA-box (TATAACGG) (Osaki *et al.*, 2003). While both the human and mouse *Col2a1* promoters share a TFIIB recognition sequence (GGGMGCA) upstream of the TATA-box (Osaki *et al.*, 2003), this sequence is not found in the *X. tropicalis* promoter. The *X. tropicalis col2a1* promoter region also contains a putative binding site for the delta EF1 transcription factor, based on a TFSearch analysis (95% minimal threshold score with vertebrate matrices; http://www.cbrc.jp/research/db/ TFSEARCH.html; Heinemeyer *et al.*, 1998), which is not found in the mouse or human promoter (Fig. 2A).

Intron characterization

The LAGAN/Vista alignment of the first *col2a1* intron from mouse and *Xenopus tropicalis* reveals a 144-bp sequence that aligns with 74.3% sequence identity (Fig. 2B, C). This region overlaps only the first 69 base pairs of the 182-bp mouse enhancer (Zhou *et al.*, 1995). However, the truncated 48-bp and 18-bp (Lefebvre *et al.*, 1996) enhancer sequences are con-

tained within the aligned *X. tropicalis* sequence. The 48-bp mouse enhancer contains two inverted repeats that are conserved with the rat and human *Col2a1* intronic sequences (Lefebvre *et al.*, 1996). Only the outer repeat (TGAAT) is conserved with *X. tropicalis* (Fig. 2C). Its inverted repeat (ATTCA) appears 30 base pairs downstream and is within the seven-base Sox9 binding region (CATTCAT; Lefebvre *et al.*, 1997), which is strictly conserved between mouse and *X. tropicalis*. Two additional putative
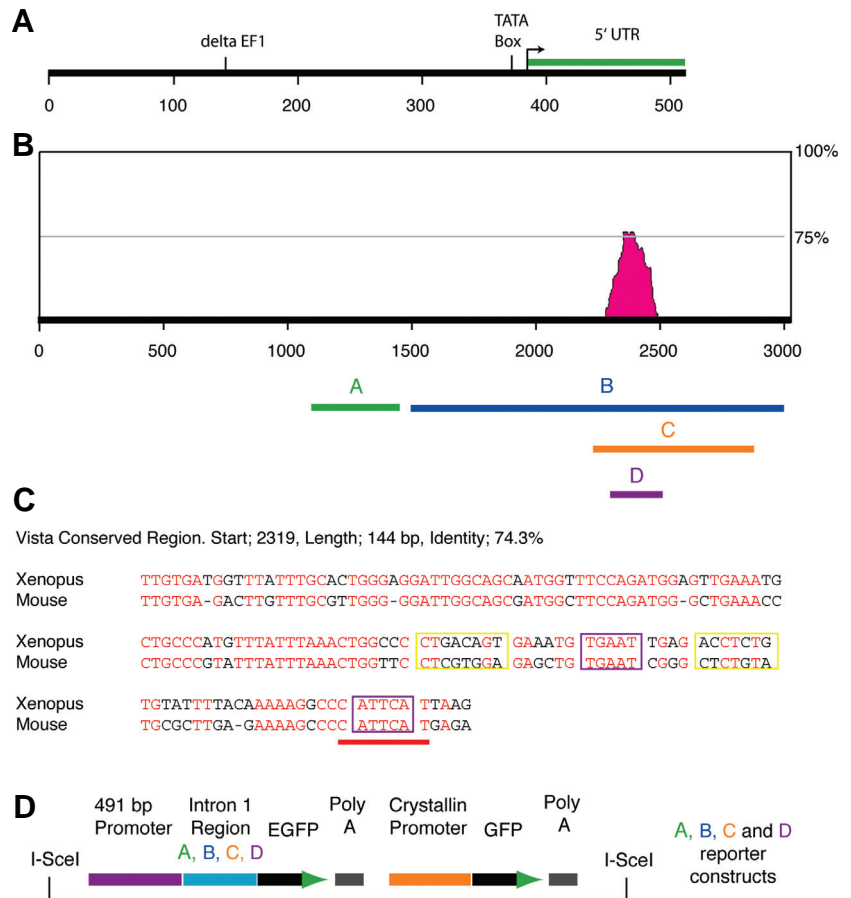
Fig. 2. Diagrams of the regulatory elements used in this study. (A) *The 491-bp, 5′-promoter region used in the reporter includes 366 bp of untranscribed DNA and 125 bp of the 5′ UTR. A TFSearch analysis (Heinemeyer* et al., *1998) reveals a putative transcription-factor binding site for delta EF1 (-243 bp from the transcription start site). A TATA-box sequence occurs -13 bp from the transcription start site.* **(B)** *Lagan/Vista plot of alignments between* X. tropicalis *and mouse* col2a1 *first introns. Base pair positions of aligned sequence along the X-axis; the Y-axis depicts percentage sequence identity. The line graph shows a 144-bp alignment region that begins at* X. tropicalis *position 2319 bp. Intron fragments, represented by colored bars beneath the alignment, were tested in reporter constructs.* **(C)** *Nucleotide sequences of aligned region in (B) with conserved nucleotides shown in red; gaps and substitutions are black. The two yellow boxes correspond to two putative HMG binding sites of weak affinity described from the mouse sequence (Zhou* et al., *1998). The red underline corresponds to the nucleotide region that is essential for binding Sox9 protein to the mouse enhancer (Lefebvre* et al., *1997). The purple boxes correspond to a conserved inverted repeat (Lefebvre* et al., *1996), which is separated by 30 bp and has a downstream portion inside the Sox9 binding region.* **(D)** *Schematic depiction of the reporter constructs used in this study. The* col2a1 *5′ promoter region was connected to the (A, B, C, and D) intronic sequences to drive the expression of EGFP. A separate* gamma-crystallin *promoter was used to drive lens-specific expression of GFP as a positive control for transfection of injected embryos. Two 18-bp I-SceI recognition sites flank each reporter construct.*
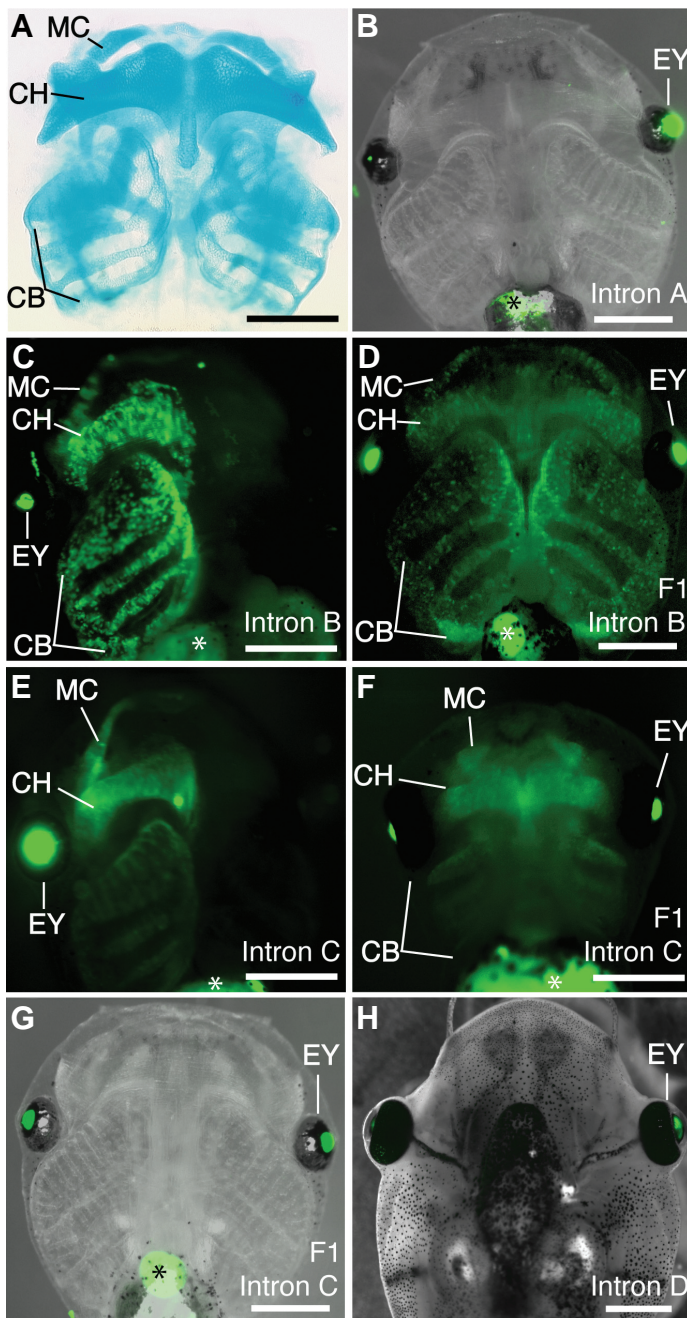
Fig. 3. Distribution of EGFP in transgenic *Xenopus laevis* tadpoles.
(A–G) *Ventral views, anterior to the top.* **(H)** *Dorsal view, anterior to the top.* **(A)** *Cleared-and-stained tadpole showing Meckel's (MC), ceratohyal (CH) and ceratobranchial (CB) cartilages stained with Alcian blue (stage 42). All other images show EGFP/GFP expression in living tadpoles.* **(B)** *Transient transgenic tadpole with a reporter carrying the intronic region A (stage 48). EGFP is not seen in the cartilaginous skeleton of any tadpole carrying reporter construct A. GFP expression is apparent in the lens of the left eye (EY), under the control of the* gamma-crystallin *promoter.* **(C)** *Transient "half-transgenic" tadpole with a reporter carrying the intronic region B (stage 42). Unilateral transgene expression is apparent in the same cartilages labeled in (A), along with GFP expression in the eye.* **(D)** *An F1 transgenic tadpole (stage 48), bred from a half-transgenic mother carrying construct B.* **(E)** *Transient half-transgenic tadpole carrying a reporter with the C construct (stage 42) also exhibits unilateral cartilaginous expression, although expression is weaker than that seen in tadpoles carrying the B construct.* **(F)** *An F1 transgenic tadpole (stage 40), bred from a half-transgenic mother carrying construct C. Early expression of EGFP is strongest as the cartilages are forming.* **(G)** *Stage 48 of the same tadpole in (F). None of the tadpole cartilages continues to express EGFP under construct C during the later larval stages. However, cartilaginous expression of EGFP increases again during the metamorphic formation of adult cartilages (Fig. 4).* **(H)** *Merged bright-field and fluorescent image of a transgenic tadpole carrying a reporter with the smaller intronic region D (stage 48). This region does not drive cartilaginous EGFP expression during any stage in any injected tadpole. Auto-fluorescence is visible in the yolk of stage 40–42 individuals (asterisks in C,E,F) and in the gallbladder of stage-48 tadpoles (asterisks in B,D,G). Scale bar, 1 mm.*

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shared expression may be due to expression driven by the 5´-promoter region itself, or it may be an artifact of the *egfp* injection (Pan *et al.*, 2006). No construct drives detectable EGFP expression similar to the *col2a1* mRNA expression in the notochord, otic vesicle, somites, floor plate of the neural tube, or branchial arches during early development (Fig. 1A, B).

The 347-bp intronic region 'A' does not overlap with the aligned region from our Vista analysis (Fig 2B). This sequence was used as a negative control and compared to the expression generated by intronic sequences that contain the aligned enhancer. A reporter construct containing intronic sequence A does not drive expression outside the lens and early epidermal cells (Fig. 3B, Table 1). Epidermal expression dissipates in later-stage tadpoles. No other pattern of EGFP is found in any additional larval tissue.

The reporter construct that contains the 1,517-bp region 'B' of the first intron (Fig. 2B) drives EGFP expression in the cartilaginous skeleton (Fig. 3C–D, 4A–F) in 24 of 70 transgenic animals (34.3%; Table 1). Twelve of these have bilateral mosaic expres-

TABLE 1

EXPRESSION OF EGFP IN TADPOLES INJECTED WITH FOUR REPORTER CONSTRUCTS

Intronic Region	Length	Total Transgenic	% Chondrocyte Expression
A	347 bp	22	0
B	1,517 bp	70	34
C	665 bp	78	26
D	153 bp	22	0

"Total transgenic" refers to the number of injected tadpoles with GFP expressed in the lens under the *gamma-crystallin* promoter (positive control). Chondrocyte expression in transgenic tadpoles (with lens-specific GFP expression) was scored between stages 42 and 47 for each intronic region assayed.

HMG binding sites (Zhou *et al.*, 1998) are not conserved between mouse and *X. tropicalis* intronic sequences (Fig. 2C).

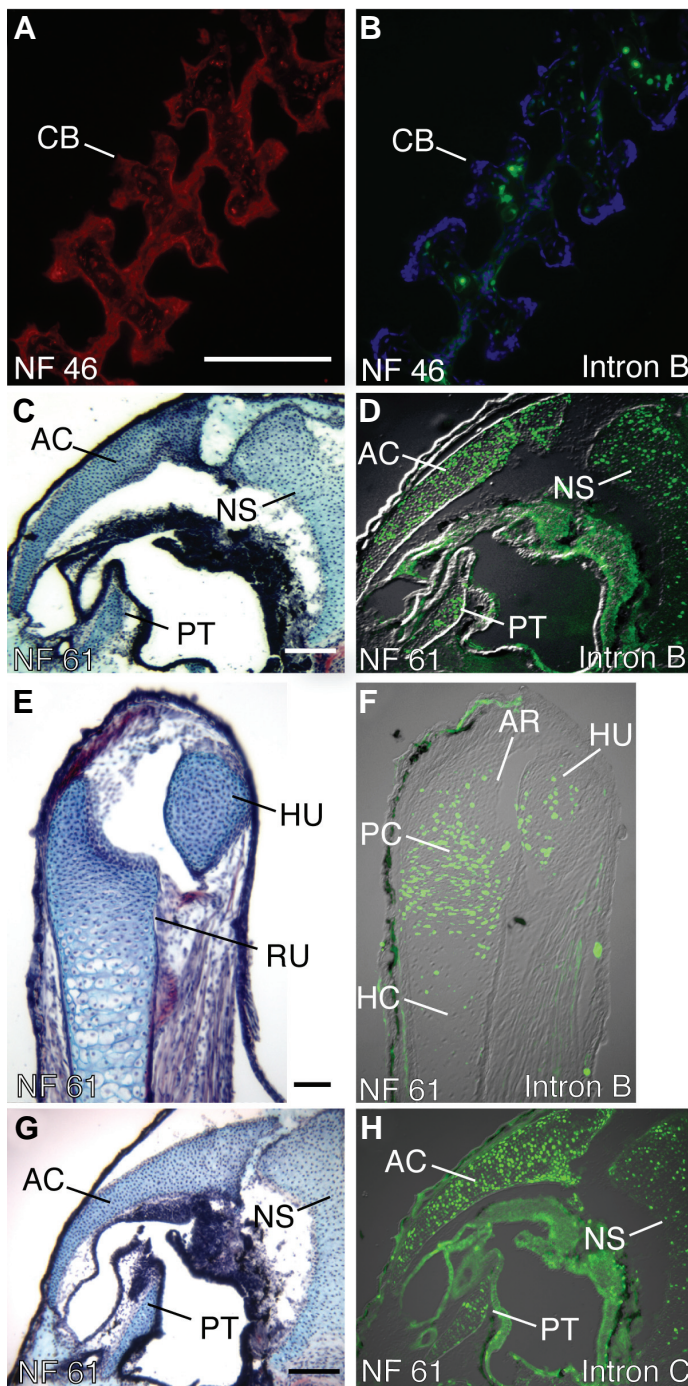### I-SceI meganuclease-mediated transgenesis

A region of the *col2a1* promoter and 5´ UTR was connected to three separate regions of the first *col2a1* intron and linked to *egfp*. Each construct was injected into zygotes of *Xenopus laevis* along with I-SceI meganuclease. The resulting embryos were screened for separate lens-specific GFP expression under the control of the *gamma-crystallin* promoter, which was used as a positive control for transgene uptake (Rankin *et al.,* 2009). Injection of each construct generates EGFP expression in a subset of epidermal cells early in development in most injected animals (Table 1). This

Fig. 4. Chondrocyte expression of reporter constructs in histological sections. (A,B) *Tadpole injected with a reporter construct that contains intronic region B. Coronal sections through a single larval ceratobranchial cartilage stained with anti-collagen II antibody (red in A) or anti-GFP antibody (green in B) with DAPI nuclear counter stain (blue).* **(C,E,G)** *Trichrome and* **(D,F,H)** *merged bright-field and fluorescent images of anti-GFP antibody stained sections.* **(C,D)** *Coronal sections of the nasal capsule of a post-metamorphic froglet that carries the reporter construct with intronic region B.* **(D)** *EGFP is expressed in the alary cartilage (AC), nasal septum (NS) and planum terminale (PT).* **(E,F)** *Longitudinal sections through the leg of a froglet that carries a reporter construct with intronic region B. Cartilaginous expression is seen in the humerus (HU) and proliferating chondrocytes (PC) of the radio-ulna (RU). EGFP is not expressed in the hypertrophic (HC) or articular (AR) chondrocytes of the radio-ulna.* **(G,H)** *Serial coronal sections through the ethmoidal region of a froglet that carries the C.1 reporter. Cartilaginous expression is seen in the same post-metamorphic cartilages shown in D. Scale bar, 0.25 mm.*

sequences in later stages. Cartilaginous EGFP expression increases in post-metamorphic cartilages of the nasal capsule (Fig. 4 C–D), which also express *col2a1* mRNA (Fig. 1D–E). Proliferative chondrocytes of the radio-ulna and humerus also express EGFP under this reporter (Fig. 4 E–F). EGFP is not expressed, however, in either articular or hypertrophic chondrocytes of the radio-ulna (Fig. 4F). *Col2a1* mRNA expression diminishes in hypertrophic chondrocytes of mice and chickens, where type II collagen is largely replaced by type X collagen before endochondral ossification (reviewed in Goldring *et al.*, 2006). Seven tadpoles express EGFP in a small number of axial myocytes; this gradually diminishes in later tadpole stages.

A half-transgenic female carrying the B construct was bred to a wild-type male. Bilateral cartilaginous EGFP expression is observed in 24 of 110 progeny. These 24 individuals consistently express EGFP in the cartilaginous skeleton with no variation among individuals, which indicates a single integration of the transgene (Marsh-Armstrong *et al.*, 1999). Early chondrocyte expression is strong (stages 40–42). Later EGFP expression (stage 48) is reticulate within individual cartilages (Fig. 3D).

The reporter construct with the 665-bp intronic region 'C' also drives EGFP expression in the cartilaginous skeleton, although its expression is less intense than that generated by intronic region B (Figs. 3E–G, 4G–H). EGFP-expressing chondrocytes occur in 20 of 78 tadpoles transfected with this reporter (25.6%; Table 1). Nine of these have stronger unilateral expression (Fig. 3E), while eleven exhibit weaker sporadic expression that is distributed bilaterally in the early cartilaginous skeleton. Early cartilaginous expression of EGFP dissipates by stage 48 and is undetectable in later-stage chondrocytes (Fig. 3G). As with construct B, however, EGFP is strongly expressed in metamorphic cartilages of the nasal capsule in froglets that carry construct C (Fig. 4H). Myocyte expression also occurs in ten of the injected tadpoles, although it dissipates in later stages.

A half-transgenic female carrying the C construct, again with strong unilateral EGFP expression, was bred to a wild-type male. The resulting progeny have bilateral cartilaginous expression of EGFP by stage 40 (Fig. 3F), which dissipates entirely by stage 48 (Fig. 3G). The transgene was inherited in approximately half (18 of 30) of the F1 progeny. The F1 generation of construct C exhibits more variation in the distribution of EGFP and has twice the proportion of transgenic individuals compared to the F1 genera-

sion within cartilage (data not shown), while the other twelve show nearly complete expression within cartilage that is distributed on only one side of the tadpole (Fig. 3C, 4B). The stronger unilateral expression indicates continued integration of the transgene after the first cell division. Cartilaginous EGFP expression co-localizes with collagen-II antibody reactivity in the ceratobranchial cartilages (Fig. 4A–B). Expression of EGFP driven by this construct is strong during early cartilage-forming stages (40–42) but decreases later (stage 48–metamorphosis). This may be due to an increase in production of the extracellular matrix and spreading of the chondrocyte lacunae, or to weaker activation of the *col2a1* regulatory

tion of construct B, which indicates multiple transgene insertions in the maternal founder. Despite this increased number of insertions, cartilaginous expression of EGFP generated by construct C is weaker than that generated by construct B in stage-matched, F1 progeny (Fig. 3D, G).

The reporter construct that carries the 155-bp intronic region 'D' does not drive expression in any tissue outside the early epidermis (Fig. 3H). Although this construct contains the entire aligned intronic enhancer region (Fig. 2B), it fails to drive EGFP expression in myocytes or chondrocytes of 22 transgenic tadpoles, all of which express GFP in the lens under the control of the *gamma-crystallin* promoter (Table 1).

Discussion

This study describes the expression of *col2a1* mRNA in larval, metamorphic and post-metamorphic *Xenopus laevis* and characterizes reporter constructs that use a transcriptional enhancer element from *X. tropicalis' col2a1* in *X. laevis* tadpoles and adults. This enhancer element is located in the 3' region of the first intron and aligns closely with a well-characterized chondrocyte-specific mouse enhancer. The *Xenopus* enhancer, combined with a fragment of the 5' promoter, drives cartilaginous EGFP expression in I-SceI meganuclease-mediated transgenic tadpoles and adults. These transgenes are heritable through the germ line and are expressed both in larval cartilages that form during embryogenesis and in adult cartilages, which form de novo during metamorphosis and are not present in the larval stage.

Whereas several studies examine the early distribution of *col2a1* mRNA in *Xenopus laevis* (Su *et al.*, 1991; Bieker and Yazdani-Buicky, 1992; Larrain *et al.*, 2000; Kerney *et al.*, 2007), this study is the first to investigate its expression through metamorphosis (Fig. 1). An initially widespread embryonic expression of *col2a1* becomes restricted to the cartilaginous skeleton of the larva and continues to be strongly expressed in chondrocytes during subsequent stages. Formation of adult cartilages in the nasal capsule during metamorphosis coincides with an increase in mRNA expression of both *col2a1* and the transcription factor *sox9*. This transcriptional regulator is essential for chondrocyte differentiation (Akiyama *et al.*, 2002) and early *col2a1* expression (Lefebvre *et al.*, 1997) in mice. Expression of *sox9* during metamorphic chondrogenesis may either coincide with the late differentiation of mesenchymal precursors into functioning chondrocytes or reflect an increase of type II collagen production by existing larval chondrocytes to create new cartilage. Post-metamorphic cartilages of the nasal capsule continue to express *col2a1* mRNA after they are fully formed.

Several studies in mammalian cell culture characterize other positive and negative regulatory elements in the *Col2a1* promoter region (Savagner *et al.*, 1990, 1995; Seki *et al.*, 2003; Osaki *et al.*, 2003). An Sp1 binding region (Savagner *et al.*, 1995) and two E-box silencer regions in the rat promoter (CIIS1 and CIIS2; Savagner *et al.*, 1990) are not found in the 3.6 Kb upstream sequence of the *X. tropicalis* promoter (data not shown). However, the presence of a putative delta EF1 binding site in the *Xenopus tropicalis* promoter (Fig. 2A) indicates a possible site of transcriptional regulation. Delta EF1 is a zinc finger homeobox-1 transcription factor (zfhx1). Null mutant mice that lack functional Delta EF1 have fused limb cartilages and fusions of the carpal and tarsal cartilages in neonates (Takagi *et al.*, 1998). *Delta ef1* mRNA is expressed in paraxial mesoderm, migratory cranial neural crest, retina and neural tube of *Xenopus laevis* embryos (van Grusven *et al.*, 2006). Delta EF1 acts as both a repressor of E2-box mediated transcription in mice (Sekido *et al.*, 1994) and Smad-mediated BMP signaling in *Xenopus* (van Grusven *et al.*, 2006). However, studies of the mouse *Col2a1* promoter have not identified Delta EF1 as a potential transcriptional silencer. Further research in the binding of Delta EF1 to the *Xenopus* promoter is needed to validate its role in *col2a1* regulation and anuran skeletogenesis.

Lagan/Vista alignment of the first intron from *col2a1* in mouse and *Xenopus tropicalis* reveals a 144-bp region with 74.3% sequence identity. This sequence contains the seven-base Sox9 binding region from the mouse intron (CATTCAT; Lefebvre *et al.*, 1997), which is strictly conserved with the *X. tropicalis* sequence. Part of this recognition sequence exists as an upstream inverted repeat, which also is found in the mouse enhancer (Lefebvre *et al.*, 1996). Pairs of inverted Sox-binding sites have been characterized in the enhancer of a precursor gene for type XI collagen, *col11a2.* These are essential for chondrocyte-specific transcriptional activity in *col11a2* as well (Bridgewater *et al.*, 2003). However, additional sequences outside the repeated binding domains are required for the *col11a2* enhancer to drive transgene expression in chondrocytes. Other regulatory information may also exist outside the conserved *col2a1* enhancer region. Together, Sox6 and L-Sox5 cooperate with Sox9 to activate *col2a1* expression in chondrocytes (Bell *et al.*, 1997). Their binding sites in the *col2a1* regulatory regions have not been identified in either mammals (Lefebvre and Smits, 2005) or *X. tropicalis*.

While the one established Sox9 binding site in the *col2a1* enhancer is conserved between mouse and *Xenopus*, two additional putative HMG binding sites (Zhou *et al.*, 1998) are not (Fig. 2C). The Sox9 protein does not bind to either of these putative sites in the mouse intron (Lefebvre *et al.*, 1997). Absence of these sites from the *Xenopus* sequence further suggests that these sites are not part of the Sox9 regulation of *col2a1*.

Regulatory ability of the intronic enhancer from *Xenopus tropicalis* was tested in *X. laevis* through I-SceI meganuclease-mediated transgenesis. A large 1.5-Kb region of the intron drove cartilaginous expression in both tadpole and adult cartilages when connected to an upstream promoter (Fig. 2B, region B). While a smaller 665-bp region also drove cartilage-specific expression, its expression was weaker than the larger intronic fragment B (Fig. 2B, region C). A truncated 155-bp region containing the entire aligned sequence from the LAGA/Vista analysis failed to drive any expression (Fig. 2B, region D). These results are similar to previous studies of the mouse *Col2a1* intronic enhancer, in which multiple copies of smaller enhancer regions are required to drive cartilaginous gene expression (Lefebvre *et al.*, 1996; Zhou *et al.*, 1998). Multiple copies of region D were not tested in this study.

The negative-control construct A, which contains the 5'-promoter (491 bp) but not the conserved intronic enhancer, failed to show any EGFP expression in chondrocytes. Intronic region A (347 bp) is shorter than B (1.5 Kb) and C (665 bp) regions that do drive cartilage-specific expression. The small size of A may account for its inability to drive cartilage-specific expression. The decreasing sizes of regions B, C and D correspond with a

decrease in their effectiveness in driving cartilage-specific expression (Table 1). This trend has been observed in studies of mouse *Col2a1* regulation, where smaller enhancer-containing fragments require multiple copies to activate transcription (Zhou *et al.*, 1998; Lefebvre *et al.*, 1996). However no study of a mammalian *col2a1* first intron has revealed regulatory information outside the Sox9-binding enhancer region. The rat *col2a1* 5′-promoter (310 bp) is capable of driving faint expression in chondrocytes without the intronic enhancer (Horton *et al.*, 1987). However, neither 6.1 Kb of the human promoter (Leung *et al.*, 1998) nor 309 bp of the mouse promoter (Zhou *et al.*, 1995) drives chondrocyte expression without the intronic enhancer in transgenic mice. *Xenopus,* like human and mouse, appears to require intronic enhancer sequence for chondrocyte-specific expression.

The mouse enhancer also can drive chondrocyte-specific expression when connected to a minimal human *β-globin* promoter (Zhou *et al.*, 1995). Surprisingly, the *Col2a1* enhancer (4 copies of 264 bp) from the extinct "marsupial wolf" *Thylacinus cynocephalus* can drive chondrocyte-specific transgene expression in transgenic mice when connected to the *β-globin* promoter (Pask *et al.*, 2008). However, a similar *β-globin* promoter construct, with 308 bp of the human *COL2A1* intronic enhancer, does not drive chondrocyte-specific expression in mice (Leung *et al.*, 1998).

Expression of EGFP in *X. laevis* tissues under the control of *X. tropicalis* regulatory information further supports the previously suggested compatibility of the two species (Khokha *et al.*, 2002). Zygotes of *X. laevis* are larger and easier to microinject than those of *X. tropicalis.* However, the longer generation time in *X. laevis* makes germ line transmission of transgene constructs difficult to test within a reasonable time frame. Founder frogs bred in this study required 18 months to reach sexual maturity in the laboratory. Compatibility in regulatory sequence function between these species also suggests the possibility that the *col2a1* reporters described here will drive transgene expression in cartilages of other amphibians. The I-SceI meganuclease method of transgene integration works in the Mexican axolotl, *Ambystoma mexicanum* (Sobkow *et al.*, 2006), and similar microinjection techniques have been developed for the Oriental fire-bellied toad, *Bombina orientalis* (Olsson and Hanken, 1996; Olsson *et al.*, 2001), which suggests the feasibility of testing the *col2a1* reporter in a wide number of alternative amphibian models.

The meganuclease technique does yield some mosaic expression of EGFP among individual chondrocytes for each reporter tested. This mosaic expression is dramatically reduced in "half-transgenic" tadpoles, which strongly express EGFP unilaterally on either the left or right side. EGFP expression is consistently more intense in the cartilages of half-transgenic tadpoles than in "full-transgenic" individuals, which express cartilaginous EGFP bilaterally (Fig. 3). Half-transgenic individuals result from incorporation of the transgene after the first cellular division. The transgene is heritable provided the germ line cells carry the inserted construct (Pan *et al.*, 2006).

Background expression from the reporter construct was observed in epidermal cells and larval myocytes. It is reduced in older tadpoles and post-metamorphic froglets, and absent in F1 progeny. This pattern indicates that the background is a result of injected *egfp* DNA, and not due to integrated transgene expression.

Conclusions

Cartilage-specific expression generated by these reporter constructs provides a useful tool for future investigations of anuran skeletal development. These constructs can be used to drive dominant-negative protein expression or to over-express various genes in the cartilaginous skeleton. Whereas several studies investigate the skeletal morphology of *Xenopus laevis* during development (Bernasconi, 1951; Sedra and Michael, 1957; Sokol, 1977; Trueb and Hanken, 1992), few examine the underlying transcriptional regulators involved in skeletogenesis (Pasqualetti *et al.*, 2000; Spokony *et al.*, 2002; Moriishi *et al.*, 2005; Kerney *et al.*, 2007). Despite ongoing use of *Xenopus* as a model system of organogenesis (Thomsen, 2006) and the quickly growing field of skeletal developmental biology (Karsenty, 2003; Zelzer and Olsen, 2003; Hall, 2005; Goldring *et al.*, 2006) few studies research skeletal development in *Xenopus*. This lack of attention is due largely to the limited array of techniques available for investigations of later development in *Xenopus* (Blitz *et al.*, 2005). Functional experiments through the injection of morpholino oligonucleotides, antisense RNA, or RNA interference are limited to early stages (Nutt *et al.*, 2001; Khokha *et al.*, 2002). Due to these limitations, there are no current investigations into the function of skeletal regulatory genes during anuran metamorphosis. The ability to drive chondrocyte-specific gene expression in *Xenopus* will provide a valuable tool for future investigations of gene function and cell lineage in anuran skeletal development.

Materials and Methods

All animal protocols were approved by the Harvard University Faculty of Arts and Sciences Standing Committee on the use of Animals in Research and Teaching (protocol 99-09) and Dalhousie University's Committee on Laboratory Animals (protocol 07-126).

In situ *Hybridizations*
Antisense mRNA probes were prepared from pCS2-AS-collagen II (Larrain *et al.*, 2000) and pGEMT:Sox9 (Spokony *et al.*, 2002). Whole-mount *in situ* hybridizations were performed on albino embryos and hatchlings following the protocol of Sive *et al.* (2000). Embryos were fixed in MEMFA and stored in 70% methanol at -20 ºC prior to initial use. *In situ* hybridizations on sectioned tissues followed the protocol of Zelzer *et al.* (2001). Sectioned tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), dehydrated and embedded in Paraplast X-tra (Sherwood Medical, Deland, FL), and cut to a thickness of 7 μm on a rotary microtome (Leica 1512, Wetzlar, Germany).

Regulatory Region Analysis
The *Xenopus tropicalis col2a1* promoter and first intron sequence are from scaffold 161 of the Joint Genome Institute *X. tropicalis* draft genome assembly version 4.1 (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Xentr4). The transcription start site was determined by aligning the complete coding sequence of *X. tropicalis col2a1* (Genbank Accession: BC063191) with the genomic sequence. A Lagan/ Vista analysis (Brundo *et al.*, 2003) aligned the enhancer sequence between the first introns of mouse *Col2a1* (NCBI accession NW_001030577.1; base pair positions 19052638–19048835) and *X. tropicalis col2a1* (JGI scaffold 161; base pair positions 125221–121349). The mouse-specific repeat masker was used for the mouse sequence; the "softmask" option was used for the *X. tropicalis* se-

quence (http://lagan.stanford.edu).

Preparation of Plasmid Constructs

Regulatory sequences and *egfp* were inserted into the pDHG_I-SceI plasmid (Daniel Buchholz, unpublished). This plasmid also contains a *gamma-crystallin* promoter that drives *gfp* within the I-SceI recognition sites (Fig. 2D–E). This strong promoter is used to screen injected animals that are successfully transformed with an injected construct (Pan *et al.*, 2006). *Egfp* was taken from the pCS2+*egfp*-Bgl2 plasmid (Turner and Weintraub, 1994). The 5´-promoter region and first intron of the *Xenopus tropicalis col2a1* gene were amplified by PCR. The forward primer 5´-GCA CTG CAC ATG TTC TTG CT-3´ and reverse primer 5´-GCA GAA TCT TTG TGG GGA AA-3´ were used to amplify ~3 Kb of the 5´ promoter region. The forward primer 5´-GTT CGC AGC CAC ACA AGT C-3´ and reverse primer 5´-CCC AGT GTC ACA GAC ACA GAT T-3´ were used to amplify ~3.2 Kb of the first intron. Internal primers were used to amplify 491 bp of the promoter with additional 5´-Hind III and 3´-Sac1 sites. Intron-specific internal primers were used to amplify four regions of the intron with additional 5´-SacI and 3´-HindIII sites. The resulting intronic fragments are 347 bp (A), 1,517 bp (B), 665 bp (C), and 153 bp (D) (Fig. 2B). Whereas regions B–D each overlapped the sequence aligned by Vista analysis, region A did not; it was used as a negative control for enhancer activity. Orientation of each promoter-intron was determined through PCR and sequencing. The four reporter constructs were used for I-SceI meganuclease-mediated transgenics in *Xenopus laevis* zygotes.

I-SceI Meganuclease-Mediated Transgenics

The I-SceI transgenic protocol followed those described previously for *Xenopus* (Ogino *et al.*, 2006 a,b; Pan *et al.*, 2006). A total of 200 ng plasmid DNA was digested for 40 min at 37º C in a 23-µL I-SceI meganuclease reaction (New England Biolabs, Ipswich, MA). During this digest fertilized *Xenopus laevis* eggs were obtained and dejellied using established protocols (Sive *et al.*, 2000). A total of 4.5 nL of the reaction mix was injected into each single-celled zygote (40 pg DNA/ embryo) using a Femtojet microinjector (Eppendorf, New York). Embryos were raised for 2 hr at room temperature. Individuals that failed to undergo normal cell division were culled. Remaining embryos were raised to embryonic stages 36–40 (Nieuwkoop and Faber, 1994) and screened for GFP expression in the lens under the control of the *gamma-crystallin* promoter. Incorporation of the transgene after the first cell division yields "half-transgenic" embryos that exhibit unilateral fluorescence on either left or right sides. Incorporation of the transgene before the first cell division yields "full-transgenic" individuals with bilateral transgene expression. Transgenic embryos were raised to early tadpole stages 42–47 and screened for tissue-specific EGFP expression. Tadpoles with intense EGFP expression were raised through metamorphosis to examine reporter expression in adult cartilages.

Antibody Staining and Histology

Antibody staining was carried out on sectioned material following established *Xenopus* protocols (Gross *et al.*, 2006). Briefly, tadpoles were fixed overnight in 4% PFA at 4º C, embedded in OCT media (Sakura, Torrance, CA), frozen, and sectioned at 10 µm on a Leica CM 3050S cryostat. Primary antibodies included a FITC-conjugated goat polyclonal antibody to GFP (1:2000; Abcam, Cambridge, MA) and an anti-collagen II monoclonal antibody (1:100; Linsenmeyer and Hendrix, 1980). Slides were counterstained with a 2 µg per mL DAPI nuclear stain solution (Invitrogen) before being cover-slipped with Fluoromount-G (Fisher Scientific, Waltham, MA). Serial sections of post-metamorphic tissues were stained with the Hall-Brunt-Quadruple stain, which colors cartilage blue (Hall, 1986).

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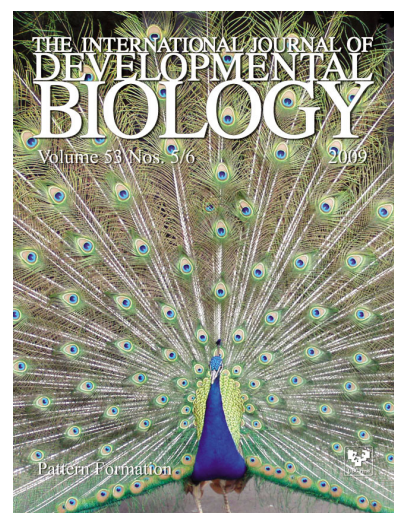**Analysis of the odontogenic and osteogenic potentials of dental pulp in vivo using a Col1a1-2.3-GFP transgene.**
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