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Establishment of a Bone-Specific *col10a1:GFP* Transgenic Zebrafish

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During skeletal development, both osteogenic and chondrogenic programs are initiated from multipotent mesenchymal cells, requiring a number of signaling molecules, transcription factors, and downstream effectors to orchestrate the sophisticated process. *Col10a1*, an important downstream effector gene, has been identified as a marker for maturing chondrocytes in higher vertebrates, such as mammals and birds. In zebrafish, this gene has been shown to be expressed in both osteoblasts and chondrocytes, but no study has reported its role in osteoblast development. To initially delineate the osteogenic program from chondrogenic lineage development, we used the zebrafish *col10a1* promoter to establish a transgenic zebrafish expressing a GFP reporter specifically in osteoblast-specific bone structures that do not involve cartilaginous programs. A construct harboring a ~2.2-kb promoter region was found to be sufficient to drive the reporter gene in osteoblast-specific bone structures within the endogenous *col10a1* expression domain, confirming that separable cis-acting elements exist for distinct cell type-specific expression of *col10a1* during zebrafish skeletal development. The ~2.2-kb *col10a1:GFP* transgenic zebrafish marking only bone structures derived from osteoblasts will undoubtedly be an invaluable tool for identifying and characterizing molecular events driving osteoblast development in zebrafish, which may further provide a differential mechanism where *col10a1* is involved in the development of chondrocytes undergoing maturation in other vertebrate systems.

INTRODUCTION

Collagen type 10A1 (COL10A1) is a short alpha-chain collagen and is considered as a reliable marker for chondrocyte maturation in higher vertebrates [reviewed in (Komori, 2010; van der Kraan and van den Berg, 2012)]. Due to its expression in hypertrophic chondrocytes, which are closely related to osteoarthritis, much attention has been drawn to the regulation of *COL10A1*. Analyses of the *Col10a1* promoter sequences in higher vertebrates have shown that the promoter contains multiple binding sites for various transcription factors that may collectively act to induce cell type-specific *col10a1* expression (Beier et al., 1996; 1997; Dourado and LuValle, 1998; Jacenko et al., 1993). One of the best-studied upstream regulators of COL10A1 is RUNX2, a master regulator that directs pluripotent mesenchymal cells toward development into osteoblast and chondrocyte lineages. Numerous studies have shown the dynamic nature of RUNX2 expression during skeletal development in higher vertebrates, including birds and mammals (Drissi et al., 2003; Enomoto et al., 2000; Higashi-kawa et al., 2009; Li et al., 2011; Zheng et al., 2003; 2009). During chondrocyte maturation, RUNX2 upregulates COL10A1 expression by directly binding to core sequences located in the promoter, showing that the RUNX2-COL10A1 pathway is an indispensable step that leads to chondrocyte hypertrophy (van der Kraan and van den Berg, 2012). During osteoblast differentiation, RUNX2 is initially required to specify the osteoblast lineage program but needs to be downregulated during osteoblast maturation (Komori, 2010). Consistent with its role in skeletal development, *Runx2*-knockout mice develop a cartilage-only skeleton with no bone components (Komori et al., 1997; Otto et al., 1997). In addition to RUNX2 as a positive regulator, other transcription factors such as c-FOS and DLX also regulate *Col10a1* expression in a tissue-specific manner (Hassan et al., 2004; Riemer et al., 2002). Therefore, detailed analysis on the regulation of *Col10a1* expression in both osteogenic and chondro-genic lineages may provide valuable information regarding the upstream regulators of *Col10a1* acting specifically to induce either bone structures or cartilages.

In contrast to the pattern seen in tetrapods, *col10a1* expression is detected in both chondrocytes and osteoblasts in zebrafish (Eames et al., 2012). Along with the advantages of using zebrafish as a model organism, the generation of a transgenic zebrafish that expresses a reporter gene in a lineage should facilitate the identification of the molecular pathways leading to osteoblast-

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and chondrocyte-specific differentiation.

In this study, we generated a stable transgenic zebrafish by using the *col10a1* promoter that drives GFP reporter expression in bone structures derived directly from osteoblasts. Since the endogenous zebrafish *col10a1* is uniquely expressed in both osteoblasts and chondrocytes, this bone-specific transgenic zebrafish will provide a useful tool for identifying molecular pathways that regulate osteoblast-specific programs not involving transient chondrocyte development.

MATERIALS AND METHODS

Constructs

To isolate the zebrafish *col10a1* promoter, we searched for the *col10a1* gene sequence in a genome database (http://www.ensembl.org/index.html). Based on the sequence, a forward primer (5′-GGTCGACGCAATGACAAGAACCTGTTCTTGGCC-3′) and a reverse primer (5′-GGATCCACCTACCGGCATACTT GGGTTCCA-3′) were designed, and the ~2.2-kb promoter region was amplified using a standard PCR protocol. The PCR product was inserted into the pGEM T-easy vector (Promega), and the insert was recovered and subcloned into the mini Tol2 mGFP vector (detailed information and protocol regarding construct construction are available upon request) (Jung et al., 2010; Kim et al., 2008; Park et al., 2000).

Zebrafish care and transgenesis

The zebrafish and their embryos were handled (Jung et al., 2012) and staged according to a standard protocol (Kimmel et al., 1995). Mini Tol2 *col10a1:mGFP* constructs were injected together with *transposase* mRNA at the 1-cell stage of zebrafish embryos, as previously described. The injected embryos were selected based on transient GFP expression, and the sorted embryos were raised to sexual maturity. Each zebrafish was outcrossed to wild-type zebrafish, and the resulting embryos were selected again based on the GFP expression in the bone structures (F1). The F3- and F4-generation embryos were analyzed in this study.

Alizarin Red staining

Embryos at various developmental stages were fixed with 4% paraformaldehyde at 4°C overnight and washed 3 times with PBT. The pigments were removed with a bleaching solution (1% KOH, 3% H_2O_2 in PBT), and the embryos were rinsed 3 times with PBT. The samples were processed for Alizarin Red staining (0.4 ml of Alizarin Red S solution in 10 ml of 0.5% KOH) overnight at room temperature. Alizarin Red S solution was obtained by dissolving 0.1 g of Alizarin Red (Sigma, A5533) in 100 ml of 96% ethanol. After developing Alizarin Red bone staining, the background staining was removed by treating samples with 1% KOH overnight at room temperature.

In situ hybridization

In situ hybridization was performed as described previously (Choe et al., 2009). The *col10a1* antisense probe was synthesized using a standard protocol.

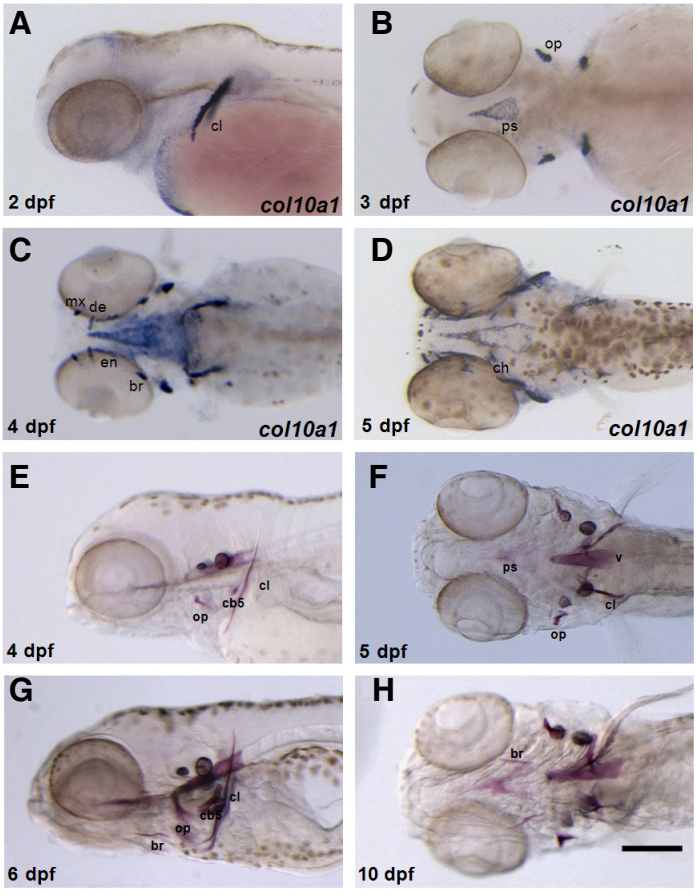

Fig. 1. Spatiotemporal *col10a1* expression and bone formation in developing zebrafish embryos. (A-D) Endogenous *col10a1* expression was visualized by *in situ* hybridization. Embryos are shown in lateral (A) or ventral views (B-D) with the anterior region to the left. (A) *col10a1* is expressed in the cleithrum at 2 dpf. (B) At 3 dpf, *col10a1* expression is also found in the parasphenoid and opercle. (C) *col10a1* expression is present in the maxilla, branchiostegal ray, and entopterygoid at 4 dpf. (D) At 5 dpf, *col10a1* expression is also found in the cartilaginous ceratohyal. (E-H) Alizarin Red staining showed the head skeleton. Embryos are shown in the lateral (E, G) or ventral views (F, H) with the anterior region to the left. (E) At 4 dpf, the cleithrum, opercle, and ceratobranchial 5 bone structures are visible. At 5 dpf, the parasphenoid bone is formed (F), and at 6 dpf, the branchiostegal rays are visualized (G). These bone structures are detected at 10 dpf (H). cl, cleithrum; op, opercle; ps, parasphenoid; v, vertebrate; br, branchiostegal ray; mx, maxilla; en, entopterygoid; ch, ceratohyal; hm, hyomandibula; cb5, ceratobranchial 5. Scale bar, 200 μm.

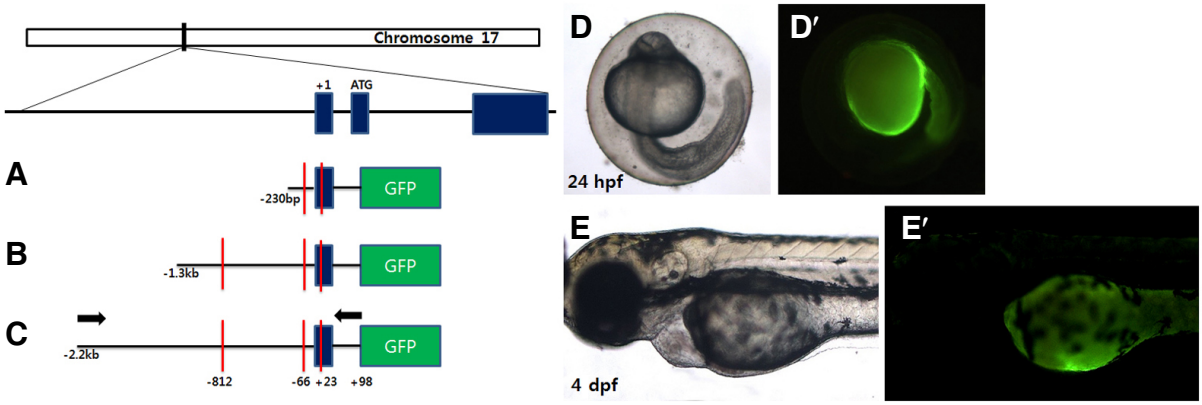

Fig. 2. Schematic view of the transgenic constructs. *Col10a1* is located on chromosome 17 and is composed of 3 exons with the translational start codon present in exon 2 as depicted. The first construct contains 2 predicted Runx2-binding sites and includes the *col10a1* promoter region from the -230 position to the +98 position, relative to the transcriptional start site (TSS) fused to the GFP open reading frame (A). The second construct contains 3 putative Runx2-binding sites and includes a ~1.3-kb promoter fragment (B). The third construct contains 3 Runx2-binding sites and an additional ~900-bp region upstream of the second construct, yielding an ~2.2-kb *col10a1* promoter region (C). The arrows in (C) indicate a primer set to initially isolate the promoter region by PCR. Runx2-binding sites are marked by red bars. (D-E') Microinjection of *transposase* mRNA together with the construct (A) or (B) did not induce transient GFP expression at 1 dpf or 4 dpf as indicated.

RESULTS

Zebrafish *col10a1* is expressed in all bone structures during development

A previous study suggested that *col10a1* is expressed in both chondrocyte and osteoblast lineages during zebrafish development (Eames et al., 2012). In order to confirm *col10a1* expression in zebrafish, we performed in situ hybridization by using various stages of developing zebrafish embryos. At 2 days post fertilization (dpf), *col10a1* expression was first detected in the cleithrum (cl), which is formed by intramembranous ossification (Fig. 1A). This expression was maintained during the later stages of development. At 3 dpf, the intramembranous opercle (op) and parasphenoid (ps) showed *col10a1* expression, which was also maintained during the later stages of development (Fig. 1B). At 4 dpf, *col10a1* expression was also seen in the maxilla (mx), dentary (de), branchiostegal ray (br), and entopterygoid (en) (Fig. 1C). At 5 dpf, *col10a1* expression was additionally detected in the cartilaginous ceratohyal (ch) and hyomandibula (hm) (Fig. 1D). Thus, *col10a1* expression was found in both osteoblasts and chondroblasts in zebrafish. Next, we performed Alizarin Red staining to determine the extent to which *col10a1* expression is related to osteoblast differentiation. Alizarin Red-positive signals seemed to be somewhat delayed by 1-2 days after initial *col10a1* expression, but the signals became visible in bones belonging to a subset of *col10a1*-expressing structures (Figs. 1E-1H). These results suggest a strong correlation between *col10a1* expression and bone induction during zebrafish development.

The zebrafish *col10a1* promoter was used to generate constructs for producing a transgenic zebrafish line to study osteoblast lineage development

To identify the *col10a1* promoter region responsible for osteoblast-specific gene expression in zebrafish, we first analyzed the nucleotide sequence of the *col10a1* promoter. As reported previously (Simoes et al., 2006), we found that the *col10a1* promoter contains 2 Runx2 consensus binding sequences (PuACCPuCA/TGTGGT) upstream of the predicted transcriptional start site (TSS, -812 and -66 positions) and an additional binding site at the +23 position from the TSS (Fig. 2). Moreover, we also identified other consensus sequences for transcription factors implicated in skeletal development (e.g., RAR/RXR and ERE). Based on this information obtained from the amino acid sequence and on the fact that Runx2 is essentially required for normal osteoblast development, we generated several transgenic constructs by using different lengths of the *col10a1* promoter regions, including the putative Runx2-binding sites (Fig. 2): the first construct contained the immediate proximal promoter containing 2 Runx2-binding sites at -66 and +23; the second construct included an ~1.3-kb promoter sequence containing 3 binding sites at -812, -66, and +23 bp; and the third construct harbored an ~2.2-kb region containing the same Runx2-binding sites as those present in the second construct and an additional upstream ~900-bp fragment. It has been suggested that the *col10a1* promoter contains positive and negative elements for its gene expression *in vitro* (Simoes et al., 2006); therefore, these constructs were used to test whether different parts of the *col10a1* promoter are necessary to render promoter activity in the bone primordia.

Transient GFP expression with various promoter lengths helped identify the *col10a1* promoter domain responsible for *col10a1* expression in zebrafish bone

Since Tol2-based transgenesis has been reported to effectively introduce a transgene in the zebrafish genome, resulting in germline transmission, we used the Tol2 backbone in our constructs to determine their ability to induce the expression of the GFP reporter (Figs. 2A-2C). Each construct along with *transposase* mRNA was microinjected into the 1-cell stage of zebrafish embryos, and transient GFP expression was analyzed to test whether the expression of the GFP reporter recovered within the endogenous *col10a1* expression domain during zebrafish development.

Embryos injected with the first construct harboring an immediate proximal promoter region (from approximately -230 bp to +98) did not induce any GFP expression when analyzed at ~24 h post fertilization (hpf) and 4 dpf during development (Figs. 2A and 2D). Similarly, the second ~1.3-kb construct containing the promoter region with 3 predicted Runx2-binding sites also seemed

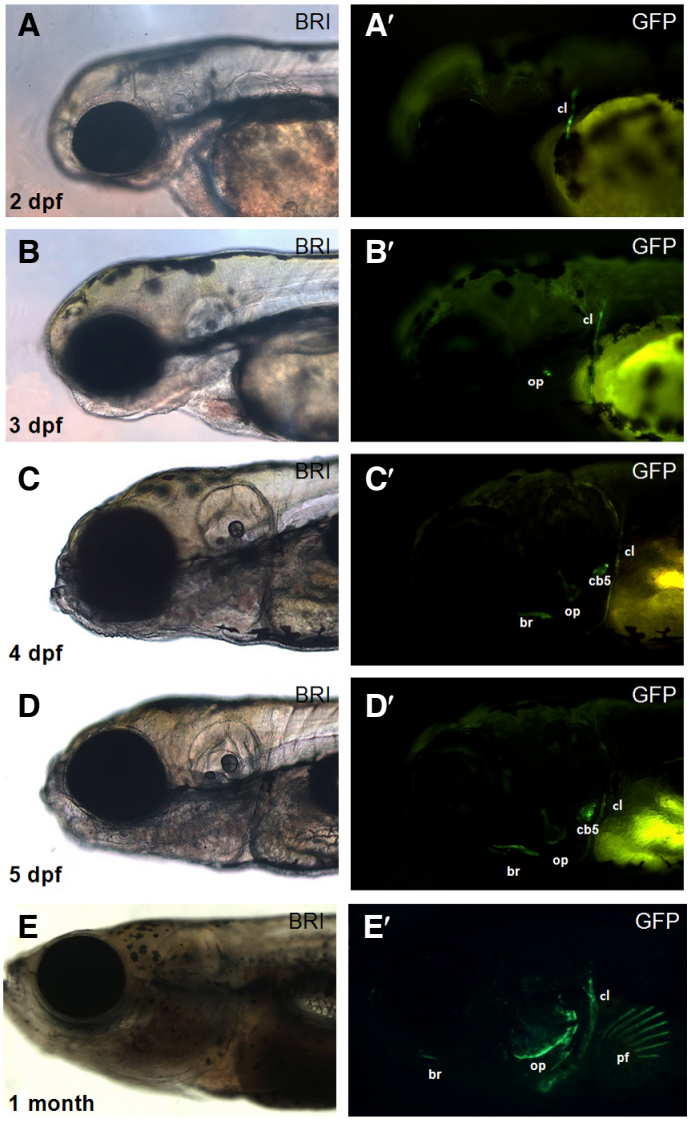

Fig. 3. Transient GFP expression generated using the ~2.2-kb *col10a1:GFP* construct in developing zebrafish embryos. (A-D) A bright field image and an image showing GFP expression are shown as a pair for embryos at each stage. The ~2.2-kb *col10a1* promoter induces GFP expression in the cl at 2 dpf (A, A′); in the cl and op at 3 dpf (B, B′); and in the cl, op, br, and cb5 at 4 dpf (C-D′). This expression pattern was maintained until the zebrafish was at least 1 month old (E). Ectopic GFP expression, for example, in the pectoral fins, was detected in some embryos (E′). cl, cleithrum; op, opercle; br, branchiostegal ray; cb5, ceratobranchial 5; pf, pectoral fin.

to be inactive because no GFP expression was detected in the injected embryos (Figs. 2B and 2E). Since both constructs contain a promoter region with at least 2 Runx2 consensus binding sequences that are thought to be important for *col10a1* expression, it was somewhat surprising to detect no obvious GFP-expressing structures during the entire course of embryonic development.

In contrast, the third construct (~2.2-kb *col10a1:GFP*), which contained 3 Runx2-binding sequences and an additional 900-bp region upstream of the second construct, induced robust GFP expression in the cl at 2 dpf and in the op at 3 dpf (Figs. 3A and 3B). GFP expression in the ceratobranchial (cb) and branchiostegal ray (br) was also evident at 4 dpf (Figs. 3C and 3D). Importantly, this GFP expression pattern was maintained at least until the embryos were 1 month old (Fig. 3E). Although some embryos showed ectopic GFP expression in non-*col10a1* domains such as the pectoral fin and dorsal fin (Fig. 3E), the GFP expression pattern in most embryos injected with the ~2.2-kb *col10a1:GFP* construct was strikingly similar to the endogenous *col10a1* expression in the bone structures of developing zebrafish, suggesting that an early genome integration event of the introduced construct had occurred to activate the bone-specific ~2.2-kb *col10a1* promoter.

We noted that the ~2.2-kb construct contained 3 putative Runx2-binding sequences that were also present in the ~1.3-kb construct and that only the ~2.2-kb construct induced GFP expression in transient transgenic zebrafish. Since Runx2 has been found to be essentially required for both bone and cartilage development, this result suggests that Runx2 may not be the sole regulator but requires other transcription factors (or positive cis-regulatory elements) to induce *col10a1* expression in osteoblast-associated structures in zebrafish, further emphasizing the collective roles of various regulators in directing cell-type specific *col10a1* expression. Nonetheless, all the constructs that we used failed to generate *col10a1*-dependent GFP expression in chondrogenic tissues in developing zebrafish, suggesting that cartilage-specific *col10a1* expression may be suppressed or not sufficiently activated in our constructs.

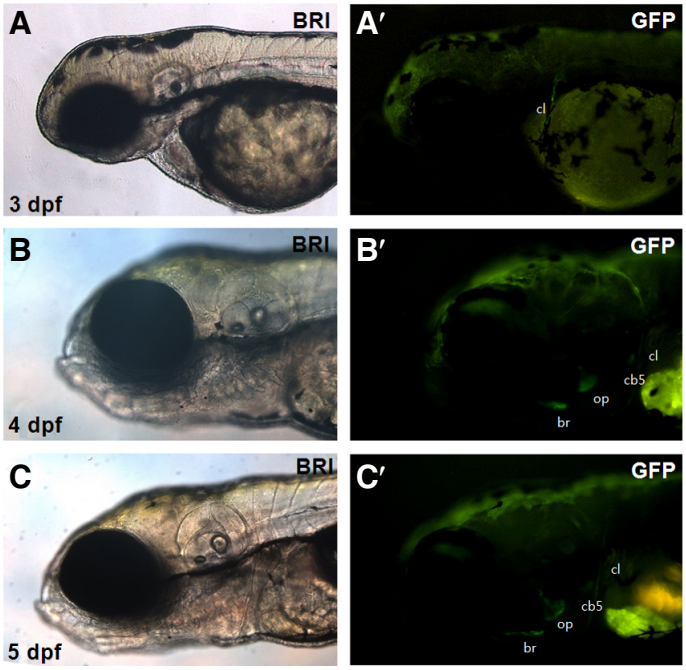

Fig. 4. GFP expression in the stable transgenic zebrafish with the ~2.2-kb *col10a1:GFP*. Expression of the GFP reporter in the stable transgenic zebrafish with *col10a1:GFP* was detected in the cl at 3 dpf (A-A′); in the cl, op, br, and cb5 at 4 dpf (B-B′); and in the cl, op, br, and cb5 at 5 dpf (C-C′). Images taken from both the bright and fluorescent field channels are shown as a pair at each stage. cl, cleithrum; op, opercle; br, branchiostegal ray; cb5, ceratobranchial 5.

Transgenic zebrafish with the ~2.2-kb *col10a1:GFP* construct expressed GFP in the region where endogenous *col10a1* was also expressed

Next, we examined GFP expression in the stable transgenic zebrafish (F3 and F4) in which the third construct (i.e., ~2.2-kb *col10a1:GFP*) was integrated in the genome. In particular, we compared the expression pattern of the GFP transgene with that of the endogenous *col10a1* in bone structures. We observed strong GFP expression starting at 3 dpf in the cl, op, br, and cb where endogenous Col10a1 presumably acts to induce bone formation directly from osteoblasts (Fig. 4) (Cubbage, 1996; Li et al., 2009). This GFP expression pattern was faithfully maintained in the same structures during the entire course of development (Fig. 4 and not shown), and, importantly, the ectopic GFP expression in the pectoral and dorsal fin regions observed in the transient transgenic zebrafish completely disappeared in the stable transgenic line. We noted that GFP expression in the transgenic zebrafish is restricted to a subset of endogenous *col10a1* expression domains and that all the GFP-expressing structures did not originate from the chondrocyte-associated skeleton but were derived directly from osteoblasts.

Taken together, we have generated a bone-specific transgenic zebrafish by using the ~2.2-kb *col10a1* promoter that drives GFP expression in a subset of endogenous *col10a1*-expressing structures. Unlike other vertebrates such as mammals and birds, *col10a1* expression is atypically observed in osteoblasts during zebrafish development. Furthermore, no study describing the exact role of *col10a1* during osteogenic lineage development has been published yet. Therefore, our *col10a1:GFP* transgenic zebrafish will provide a useful tool for identifying upstream regulators and/or downstream effectors of *col10a1* acting specifically on osteogenic pathways during zebrafish development.

DISCUSSION

Previous studies have suggested that the *col10a1* promoter of vertebrates may contain several components that direct its tissue-specific gene expression, such as the proximal basal regulatory region, and negative and positive regulatory elements (Higashikawa et al., 2009; Li et al., 2011; Simoes et al., 2006; Zheng et al., 2003). Our study also supports the existence of different regulatory elements within the zebrafish *col10a1* promoter at least for the osteoblast-specific program. In particular, the use of a transgenic construct containing an ~2.2-kb fragment of the *col10a1* promoter successfully induced the expression of the GFP reporter in osteoblast-specific bone structures, while other constructs (both the ~1.3-kb fragment and the immediate proximal promoter region alone) did not induce the expression of the GFP reporter (Figs. 2 and 3). Therefore, the zebrafish *col10a1* promoter may contain a positive regulatory element between -2.1 and -1.2 kb from the transcriptional start site for activating its unique expression in the osteoblast lineage. Additionally, a negative element present in the -1.2-kb region may act when no positive signal is delivered, which could be a general mechanism for suppressing *col10a1* expression in most cell types. Consistent with this possibility, the ~1.1-kb *col10a1* promoter (similar to our ~1.3-kb construct) showed only a small amount of transactivation of the luciferase reporter in the *Xenopus* A6 kidney epithelial cell line (Higashikawa et al., 2009; Li et al., 2011; Simoes et al., 2006; Zheng et al., 2003). Therefore, tissue-specific *col10a1* expression in vertebrates may depend on the activity of different elements within the promoter. Further investigation on the molecular interplay among the transcription factors implicated in osteoblast differentiation, including Osterix, β-catenin, HDACs, Twist1, Twist2, ATF4, and Dlx, should shed light on the mechanism underlying the regulation of *col10a1* expression during development.

The zebrafish has emerged as a powerful developmental model organism due to the ease of microscopic observations at the embryonic level as well as other advantages in cellular and molecular studies. In recent years, numerous chemical screens have been performed using developing embryos of zebrafish, exemplifying its value as a live model organism [reviewed in

(Lieschke and Currie, 2007)]. The transgenic zebrafish generated in this study will provide an invaluable tool in addition to the previously-reported bone-specific transgenic zebrafish lines that are used to dissect the molecular pathways involved in osteogenic activities (Hammond and Moro, 2012). Since endogenous *col10a1* is expressed in both osteoblasts and chondrocytes in zebrafish but only in chondrocytes in mammals, our bone-specific transgenic zebrafish can be used, for example, to delineate the molecular regulators acting in osteoblast function/differentiation from those in chondrocyte development. Recently, a *col10a1* transgenic line whose GFP expression is almost similar to endogenous *col10a1* was reported (Mitchell et al., 2013). Since reporter expression was also found in chondrocytes, this *col10a1* transgenic line could be used to develop an osteoarthritis model, which completely distinguishes the use of the transgenic zebrafish from the one generated in this study.

In summary, we have established a transgenic zebrafish by using a defined length of the *col10a1* promoter that drives the GFP reporter in zebrafish head bones induced specifically from osteoblasts.

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