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# Visualizing Normal and Defective Bone Development in Zebrafish Embryos Using the Fluorescent Chromophore Calcein

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**Zebrafish have recently become a model of choice among developmental biologists. This unique model enables both modern molecular and genetic studies to be carried out to identify genes involved in a wide variety of developmental processes. The success of the genetic approach depends largely on the application of an easy and effective screening method to identify interesting mutants. In order to develop a method for visualizing skeletal structures in zebrafish embryos that would be suitable for screening skeletal mutants, we investigated the use of the fluorescent chromophore calcein, which binds specifically to calcified skeletal structures. By using this method, we followed the development of the skeletal structures in zebrafish embryos from day 1 to day 21 postfertilization, and analyzed the effect of bone morphogenetic protein-2 (BMP2) on axial skeleton development. We found the development of the calcified skeletal structure to appear in a progressive fashion from head to tail. Calcified structures in the head (i.e., the jaw) developed first, which were then followed by the axial skeleton in the trunk. Interesting to note was that there appeared to be two domains in the calcification of vertebrae within the axial skeleton. The first three vertebrae were in the first domain; the rest being in the second domain. Compared with Alcian blue staining, we found that calcein staining indeed labels calcified skeletal structures, and, moreover, it is a more sensitive and inclusive method for visualizing skeletal structures. To determine whether calcein staining could also be used to detect abnormal bone development, we ectopically expressed BMP2 in zebrafish notochord cells. We demonstrated that ectopic expression of BMP2 in notochord cells inhibited the development of the axial skeleton. Together, these results clearly demonstrated the sensitivity of calcein staining for visualizing bone structures in developing zebrafish embryos and its effectiveness for screening for mutants that have bone structure defects.** 

***Key Words:*** **skeletal structure; bone; calcein; zebrafish; BMP.**

## INTRODUCTION

Bone is a specialized tissue that, together with cartilage, makes up the skeletal system that confers multiple mechanical and metabolic functions, such as providing support site for muscle attachment, protecting vital organs (e.g., brain) or cells (e.g., bone marrow), and serving as a reserve of ions (see review by Sims and Baron, 2000). The important function of bone can be easily recognized in day-to-day life, where millions of people suffer from bone disease such as osteoporosis, which is, in part, caused by an imbalance between bone formation and bone resorption (Rodan and Martin, 2000). The better understanding of the regulation of bone formation will provide new insights into the molecular mechanisms of bone disease and give rise to novel strategies for new drug design. In the past, most of the research on bone formation derived from clinical studies. However, recently, many important insights into bone formation have come from studies of genetically modified mice. These studies have provided fundamental information about growth factors (e.g., BMPs) and the transcription factors (e.g., Cbfa1) that regulate growth and differentiation of chondroblasts and osteoblasts (Hogan, 1996; Karsenty, 2000; Ducy *et al.*, 2000). It is apparent that most of the regulatory factors or pathways that control bone formation are highly conserved in vertebrates during evolution, and signaling molecules required for embryonic skeletal development are also important for adult homeostasis. Therefore, in recent years, there has been an increasing interest in

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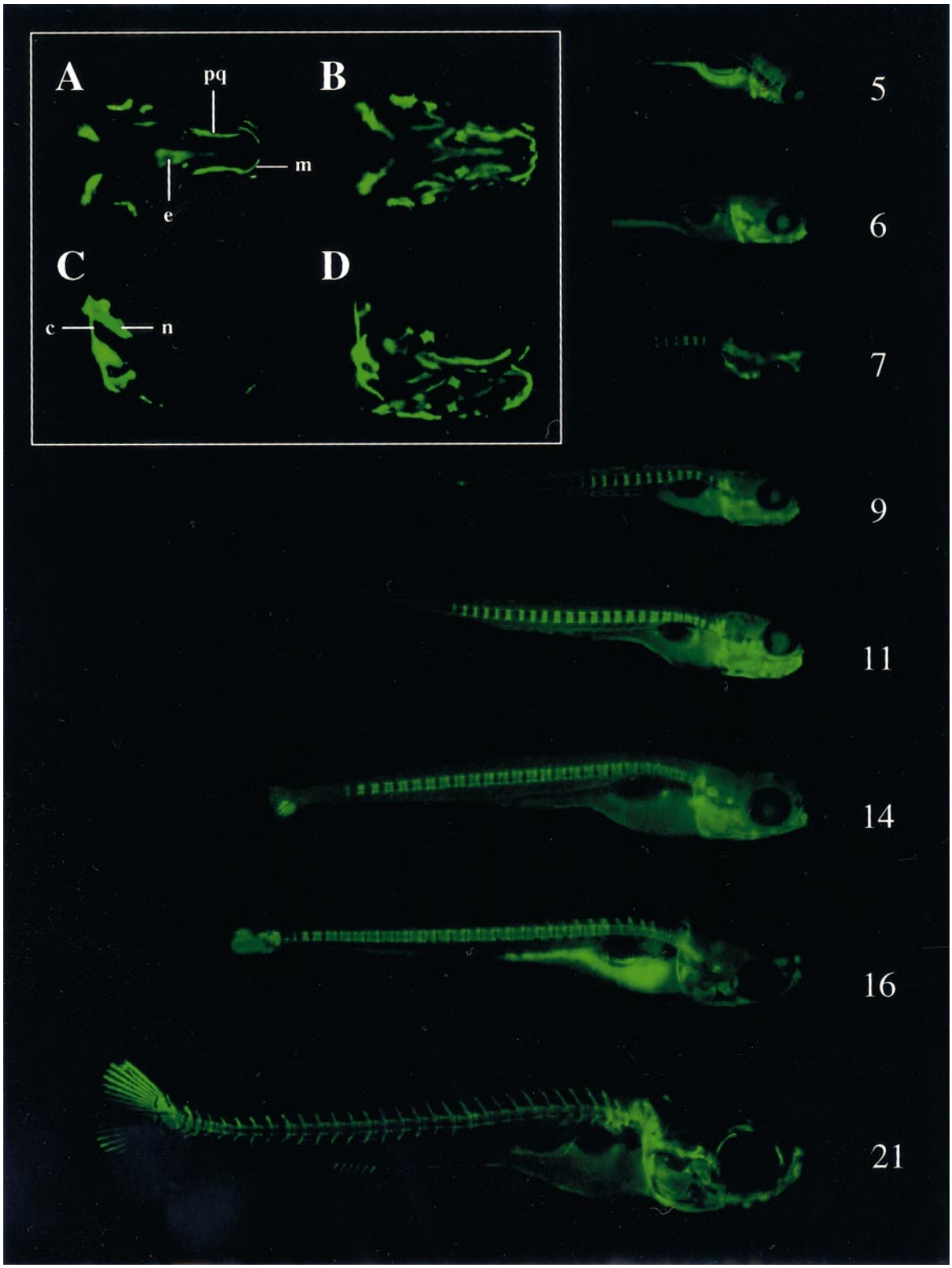


**FIG. 1.** Visualization of calcified skeletal structures in developing zebrafish embryos at 5, 6, 7, 9, 11, 14, 16, and 21 dpf by calcein staining. (A, B) Ventral view of the head skeleton of day-7 (A) and -11 (B) embryos labeled with calcein. m, Meckel's cartilage; pq, palatoquadrate; e, ethmoid plate. (C, D) Side view of the head skeleton of day-7 (A) and -11 (B) embryos labeled with calcein. n, notochord; c, cleitrum.

the search for new genes involved in bone development in other model systems.

Zebrafish have become an important model for developmental studies, having several advantages compared with other model systems. In particular are the easy accessibility of zebrafish embryos for direct observation of their development and their suitability for systematic mutagenesis studies to identify genes regulating the development of various tissues and organs, including the skeletal system (Nusslein-Volhard, 1994; Vogel, 2000; Schilling, 1997). This genetic approach has been shown to be very powerful and productive. In the past few years, several hundred zebrafish mutants have been generated, which possess developmental defects in various tissues or cell types in early embryonic stages (Haffter *et al.*, 1996; Driever *et al.*, 1996). Most of the mutants were, however, identified based on phenotypes that could be easily distinguished under a dissecting microscope. This screening method has obvious limitations for tissues that develop internally and cannot be observed easily, such as the skeletal system. Alternative screening approaches include RNA or antibody staining to detect changes in patterns of gene expression (Currie *et al.*, 1999), staining with specific dyes (Schilling *et al.*, 1996; Piotrowski *et al.*, 1996; Kelsh *et al.*, 1996; Kimmel *et al.*, 1998; Miller *et al.*, 2000), or even the use of X-rays to search for mutants with abnormal bone structures (Fisher and Halpern, 1999). Although several interesting mutants have been identified by using these screening approaches, these methods possess a number of drawbacks. For example, staining methods require the use of fixed embryos, thus valuable mutants have to be sacrificed, and the use of X-rays has limitations for screening of younger embryos. Therefore, the development of a simple and sensitive method to visualize the skeletal structure in live fish embryos is desirable for investigating the bone development in normal embryos and for screening mutantions that affect bone development.

Calcein ($C_{30}H_{26}N_2O_{13}$) is a fluorescent chromophore that specifically binds to calcium. Because the skeletal system is comprised of calcified structures, calcein has been used to label bone structures and to study bone growth (Ducy, *et al.* 2000). Calcein was first developed for the fluorometric detection of calcium (Hoelzl *et al.*, 1959) and has been used in fish as a nonspecific marking compound, where the otoliths were the target-marking site. It has been delivered by injection into Summer flounder *Paralichthys denatus* (Monaghan, 1993), and also by immersion into wild spot *Leiostomus xanthurus* and spotted seatrout *Cynoscion nebulosus* (Beckman *et al.*, 1990) and red drum *Sciaenops ocellatus* (Thomas *et al.*, 1995). More recent investigations on using calcein to mark fish designated for stock enhancement programs have targeted the calcified fin rays, allowing for nonlethal detection of the marks. Calcein marks in the rays have successfully been produced by immersion in Atlantic salmon *Salmo salar* (Mohler, 1997), and more recently in Rainbow trout *Oncorhynchus mykiss*, where calcein uptake was enhanced by first giving the fish a short ultrasound exposure (Frenkel *et al.*, 2000).

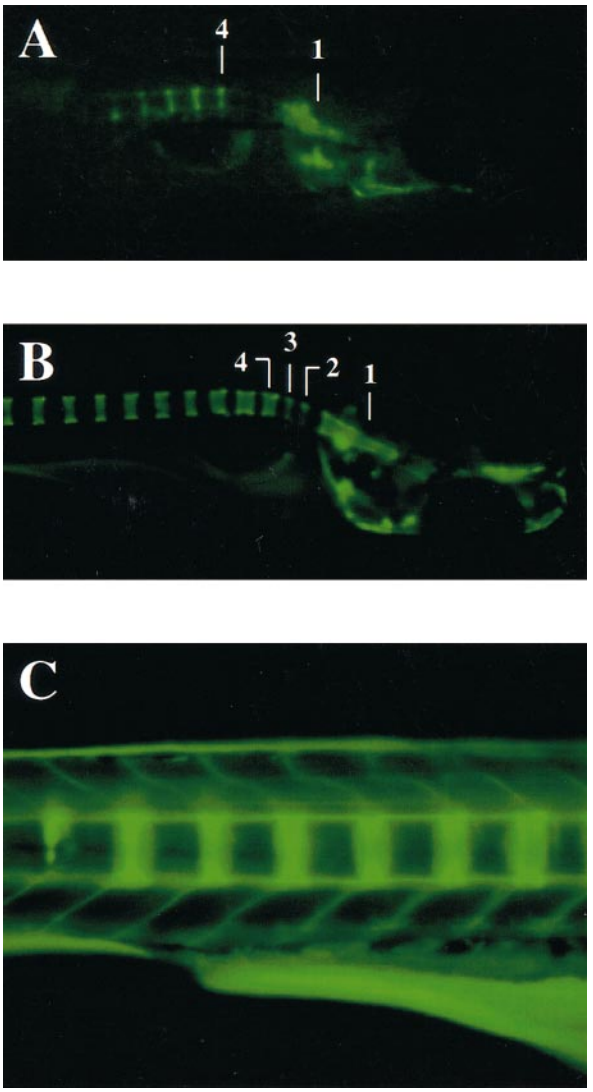


**FIG. 2.** Visualization of calcified axial skeletal structures in developing zebrafish embryos. (A, B) Embryos at 8 and 9 dpf, respectively, showing that calcification of vertebrae number 4 appears earlier than vertebrae numbers 2 and 3. Vertebrae 1, 2, 3, and 4 are indicated. (C) Embryos at 9 dpf, showing the calcification initiation site of the vertebrae, which commences at the boundaries of each segment.

We report here the development of a simple method to fluorescently stain bone structures in live zebrafish embryos and the use of this method to analyze the development of skeletal structures in zebrafish embryos. This method is based on the use of the fluorescent chromophore calcein, which is capable of rapidly penetrating into zebrafish embryos and specifically binds to calcified skeletal structures. By using this method, we followed the develop-

ment of the skeletal system in zebrafish embryos from day 1 to day 21, and analyzed the effect of BMP on axial skeletal development. Our results showed that the skeletal structures developed in a progressive fashion from head to tail in zebrafish embryos, where the axial skeleton appeared to be calcified in two domains. The first domain consists of the first three vertebrae, whereas the second domain consists of the rest. By comparison with Alcian blue staining, we showed that calcein staining indeed labels calcified skeletal structures, and moreover, it is a more sensitive and inclusive method for visualizing skeletal structures. With respect to the effect of BMP2 on skeletal formation, we found that ectopic expression of BMP2 in the notochord inhibited the development of the axial skeleton. These results clearly demonstrated the sensitivity of calcein staining for visualizing bone structures in developing zebrafish embryos and its effectiveness for detecting defective bone structures.

## MATERIALS AND METHODS

### *Collection and Fertilization of Zebrafish Eggs and Rearing of Zebrafish Embryos*

Mature zebrafish were raised at the zebrafish facility of the Aquaculture Research Center, Center of Marine Biotechnology. The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark, in 8-gallon aquaria supplied with freshwater and aeration. Spawning of zebrafish was carried out by pairing one male with two females. Injected and control embryos were raised as described in "The Zebrafish Book" (Westerfield, 1995) and staged according to Kimmel *et al.* (1995).

### *Calcein Immersions*

Immersion solutions (0.2%) were prepared by dissolving 2 g of calcein powder (Sigma Chemical, St. Louis, MO) in 1 liter of deionized water. Due to calcein's strong acidifying affects, an appropriate amount of NaOH (0.5 N) was added to the solution to restore the pH to its original value. Zebrafish embryos to be treated were netted and immersed in the solutions in petri dishes; immersion times varied from 3 to 10 min, depending on the size of the embryos. After the immersions, the embryos were rinsed a number of times in fresh water, and then allowed to stand for 10 min to allow the excess, unbound calcein to diffuse out of the tissues. The embryos were then euthanized in tricaine–methanesulfonate (MS 222) and mounted on glass slides with methyl-cellulose (3%). Observations were carried out at a magnification of ×5 and ×20 by using an Olympus BX60 microscope with a green fluorescence filter set. Images (84,882 pixels) were captured with a Quatrix (Photometrics, Inc.) CCD camera and compatible IPLab 3.0 (Scanalysis, Inc.) software and saved in TIFF format. Composite images of the embryos were produced with Adobe PhotoShop 5.0 and saved in MS-ppt format.

### *Alizarin Red and Alcian Blue Staining*

Alizarin Red and Alcian blue stainings were carried out as described by Fisher and Halpern (1999), Schilling *et al.* (1996), and Piotrowski *et al.* (1996)), respectively.

### *DNA Constructs*

The *twhh-BMP*$_2$ expression construct was constructed by ligating the zebrafish *BMP*$_2$ cDNA (Nikaido *et al.*, 1997) with the 5.2-kb *twhh* promoter (Du *et al.*, 1997). Briefly, the DNA coding region of zebrafish *BMP*$_2$ was amplified by PCR using pfu DNA polymerase (Stratagene), and the PCR product was cloned into *Sma*I site of pBluescript SK vector (Stratagene). The resultant plasmid was then digested with *Bam*HI and *Xho*I to release DNA insert coding the zebrafish BMP2. The DNA insert was then cloned into the *Bam*HI and *Xho*I sites of the pCS-*twhh–β*gal-vec plasmid (Du *et al.*, 1997) to generate the expression construct *twhh-BMP*$_2$. The expression construct, *twhh-dorsalin*, was reported previously (Du *et al.*, 1997).

### *Microinjection in Zebrafish Embryos*

The DNA construct *twhh-BMP*$_2$ or *twhh-dorsalin* was dissolved in distilled $H_2O$ to a final concentration of 50 μg/ml, and approximately 2 nl of DNA solution was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage. Microinjection was carried out under a dissection microscope (MZ8, Leica) using a PLI-100 pico-injector (Medical System Corp., Greenvale, NY).

## RESULTS AND DISCUSSIONS

### *Ontogeny of Skeletal Calcification in Zebrafish Embryos*

To test whether or not calcein could be used to visualize calcified skeletal structures in zebrafish embryos and to follow the ontogeny of bone formation during development, zebrafish embryos of various developmental stages were immersed in a calcein solution and observed under a fluorescence microscope. No fluorescent signals could be detected in embryos up to 4 dpf. Fluorescent signals first became apparent in 5-dpf embryos and were restricted to the head (Fig. 1). By 7 dpf, head skeletal elements, such as Meckel's cartilage, the palatoquadrate, and the ethmoid plate, were clearly distinguishable by calcein staining (Fig. 1A). By day 11, more skeletal elements were visible in the head region (Figs. 1B and 1D). The fluorescent labeling of the axial skeleton in the trunk region first appeared on day 7. The calcification process was found to progress from the anterior to posterior regions in a segmented fashion as the embryos developed. It was interesting to note that the anterior-to-posterior calcification process of vertebrae was not continuous, but instead appeared to be divided into two distinct domains: an anterior domain and a posterior domain. The rationale for dividing them into two domains is based on observations that vertebrae numbers 2 and 3 were always calcified later in time than vertebrae 4 (Figs. 2A and 2B). Hence, the anterior domain covers the first three vertebrae, consisting of the first long vertebra and the two adjacent vertebrae. And the second domain is comprised of the rest of the vertebrae from numbers 4 to 31. Within each vertebrae, the calcification process initiated at the boundary of each segment (Fig. 2C) and then expanded in both directions. As the embryonic development progressed, cal-

cein marks in the rays of the caudal fin began to appear on day 12 (data not shown).

These results demonstrated that calcein could be used to label calcified bone structures in developing zebrafish embryos. To confirm that calcein specifically labeled calcified bone structure in fish embryos, we compared calcein staining with other bone markers, such as Alcian blue or Alizarin Red. Our results showed that, although calcein staining and Alcian blue staining could detect complex skeletal structures in the head region of day 11 and day 23 old embryos, the staining patterns of calcein and Alcian blue were different in the region covering the vertebrae and fin rays. As shown in Fig. 3, calcein staining revealed most if not all calcified skeletal structures in these regions of a 23-dpf embryo, whereas Alcian blue staining could only identify a subset of these structures. In addition, there was little or no staining from Alizarin Red (data not shown). These data indicate that calcein staining indeed recognizes calcified bone structure in zebrafish embryos, being a more inclusive and sensitive method compared with other bone makers such as Alcian blue or Alizarin Red. Moreover, calcein staining is a simple and quick method taking approximately 10 min, whereas the Alcian blue staining requires 2–3 days.

To test whether calcein incubation could pose any toxicity to the zebrafish embryos, embryos of various developmental stages were immersed in calcein solution for 10 min and then allowed to develop. As shown in Table 1, immersion in calcein had no apparent deleterious effects on the zebrafish embryos in terms of their survival and development. This is consistent with the results from studies with fish embryos of other species. Calcein staining had no effects on survival and growth of Rainbow trout *Oncorhynchus mykiss* fry (8 days post-hatch) immersed in calcein when compared to control (nontreated) fish, over a period of 6 months post treatment (Frenkel, V., *et al.*, unpublished observations).

### *Ectopic Expression of BMP Signal in Notochord Inhibits Axial Skeleton Development*

The above results clearly demonstrated the effectiveness of using calcein to visualize the calcified skeletal structures in normal zebrafish embryos. We next investigated whether calcein staining could be used to detect abnormal bone formation in zebrafish embryos, which were caused by misexpression of genes that regulate bone development. BMP molecules have been shown to function in skeletal development (for review, see Karsenty, 2000). To analyze the role of BMPs on the development of the axial skeleton in zebrafish embryos, BMP2 was ectopically expressed in notochord cells of zebrafish embryos, driven by a notochord-specific promoter. The effect of BMP on the development of the axial skeleton was analyzed by using calcein staining at various development stages. We found that, in 57% (38/67) of the injected embryos, ectopic expression of BMP2 in notochord cells inhibited the development of the axial skeleton (Fig. 4). In young embryos (15 dpf), the defects were observed as incomplete formation of vertebrae (Fig. 4B). And in older embryos (24 dpf), the affected vertebrae were clearly narrower than those in controls (Fig. 4D). Moreover, the vertebral processes projecting from the affected vertebrae were shorter than in the controls, and projected abnormally; unlike the normal processes that projected posteriorly, the affected processes projected anteriorly (Figs. 4D and 4F). It appeared that vertebrae in only some regions of the injected embryos were affected. This is likely due to mosaic expression of BMP2 protein in the notochord. We have demonstrated in our previous studies that the *twhh* promoter always drove gene expression in the notochord in a mosaic pattern (Du *et al.*, 1997). To confirm that the bone defects in the injected embryos were not due to toxic effects of injected DNA and/or physical damage from the microinjection, we analyzed the bone development in control embryos injected with an expression construct *twhh–β*gal that specifically expresses *β*-gal in the notochord cells (Du *et al.*, 1997). Our data showed that embryos (n = 147) injected with the *twhh–β*gal DNA construct had no defects in the development of the axial skeleton and were indistinguishable from the wild-type control (Figs. 4A, 4C, and 4E). Consistent with the hypothesis that the skeletal defects in *twhh-BMP2*-injected embryos were caused by ectopic expression of the BMP signal in the notochord, we further demonstrated that expression of a BMP-like protein, Dorsalin, in the notochord produced similar bone defects to those from BMP2 (data not shown). Dorsalin is closely related to the BMP family. It shares 52 and 55% identity with BMP2 and BMP4, respectively, at the protein level, and functionally is a BMP-like protein. Ectopic expression of Dorsalin in frog or zebrafish embryos by mRNA injection ventralized the embryos, which was identical to observations made when BMP2 mRNA was injected (Du, S. J., unpublished observations). Moreover, we observed that the localized bone defects always overlapped with regional pigment-patterning defects, which were caused by the local misexpression of Dorsalin in the notochord (Du, S. J., unpublished observations). These data indicate that there is a correlation between the BMP expression site and the localized vertebrae defects. At present, the mechanism for the defective pigment patterning is not clear. Dorsalin is normally expressed in the dorsal neural tube (Basler *et al.*, 1993), adjacent to the region where the neural crest forms. Dorsalin has been shown to regulate migration and differentiation of neural crest cells (Basler *et al.*, 1993). Thus, extopic expression of Dorsalin in the notochord could affect the migration or differentiation of neural crest-derived pigment cells as they migrate through the lateral regions of the notochord.

These results demonstrated that the BMP signal has an inhibitory effect on axial skeleton formation when ectopically expressed in the notochord. This phenotype is very similar to the mutant phenotype caused by a mutation in the *chordin* gene (Fisher and Halpern, 1999). Chordin func-

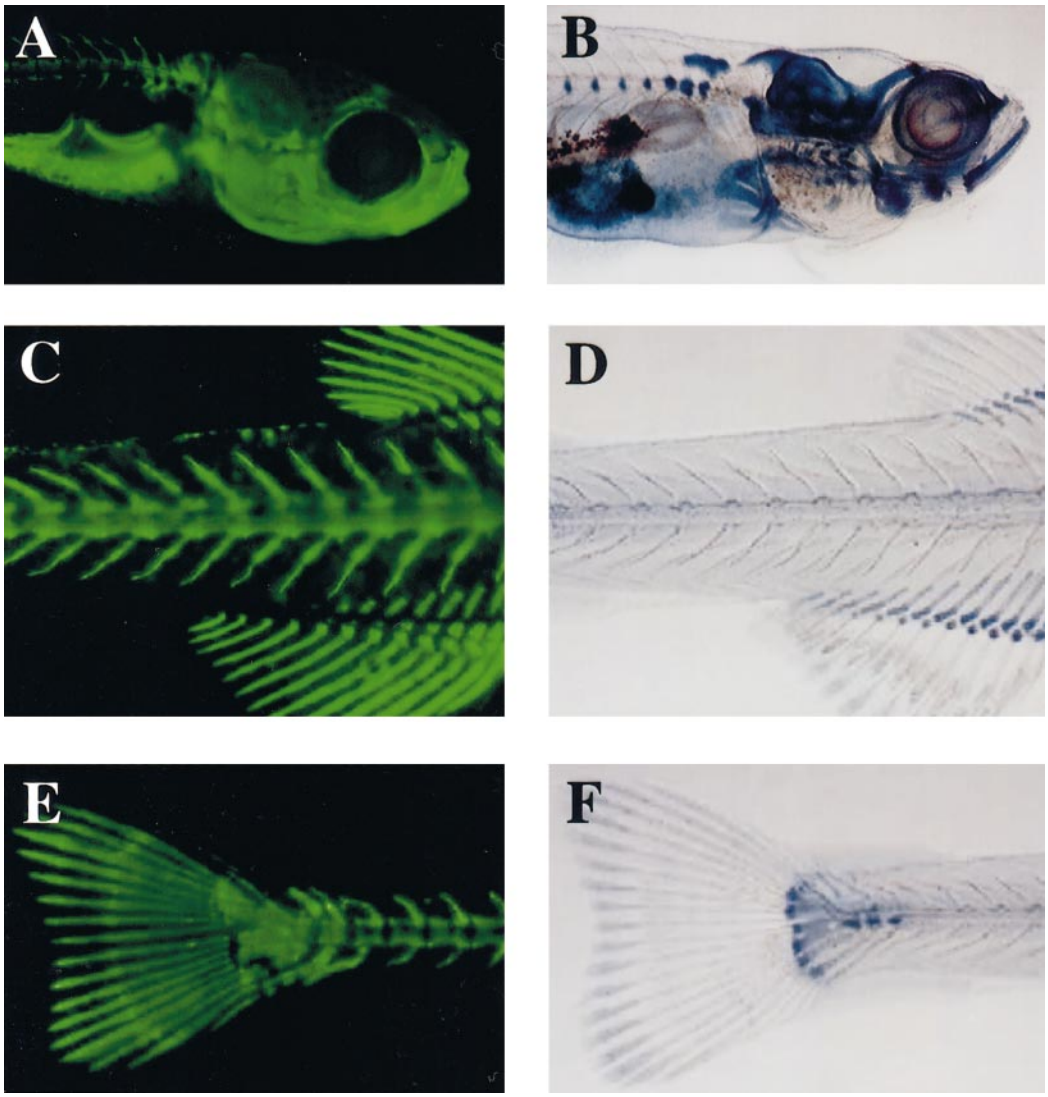


**FIG. 3.** Comparison of calcein staining with Alcian blue staining. (A, C, E) Calcein labeling of calcified structures in the head, trunk, and tail regions of a 23-dpf embryo. (B, D, F) Alcian blue staining in the head, trunk, and tail regions of a 23-dpf embryo.

**TABLE 1**

The Effect of Calcein Incubation on the Survival and Development of Zebrafish Embryos

| Developmental stage (dpf) | Treated | Survived | Abnormal |
|---|---|---|---|
| 7 | 50 | 50 | 0 |
| 14 | 45 | 44 | 0 |
| 21 | 41 | 41 | 0 |

*Note.* Zebrafish embryos at 7, 14, and 21 dpf were incubated in calcein (0.2%) for 10 min and then allowed to develop for 2 days. The survival rate and development of the treated embryos were examined. No developmental defect was observed in treated embryos. Data listed represent numbers of fish.

tions as a BMP antagonist by directly binding to the BMP protein (Piccolo *et al.*, 1996), preventing BMP from interacting with its receptor. Loss of Chordin could lead to up-regulation of BMP activity surrounding the notochord. Thus, it is possible that the inhibitory effect of BMP on skeletal development was due to the inhibition of Chordin activity. This is consistent with the timing of ectopic BMP2 expression in the notochord (14 hpf) that overlaps with the expression of Chordin. The results of Fisher and Halpern (1999), together with those presented here, strengthen the idea that BMP signaling may have an inhibitory effect on skeletal formation in zebrafish. This inhibitory effect of BMP2 on bone formation seems to contradict some of the early studies demonstrating that BMPs are bone-promoting

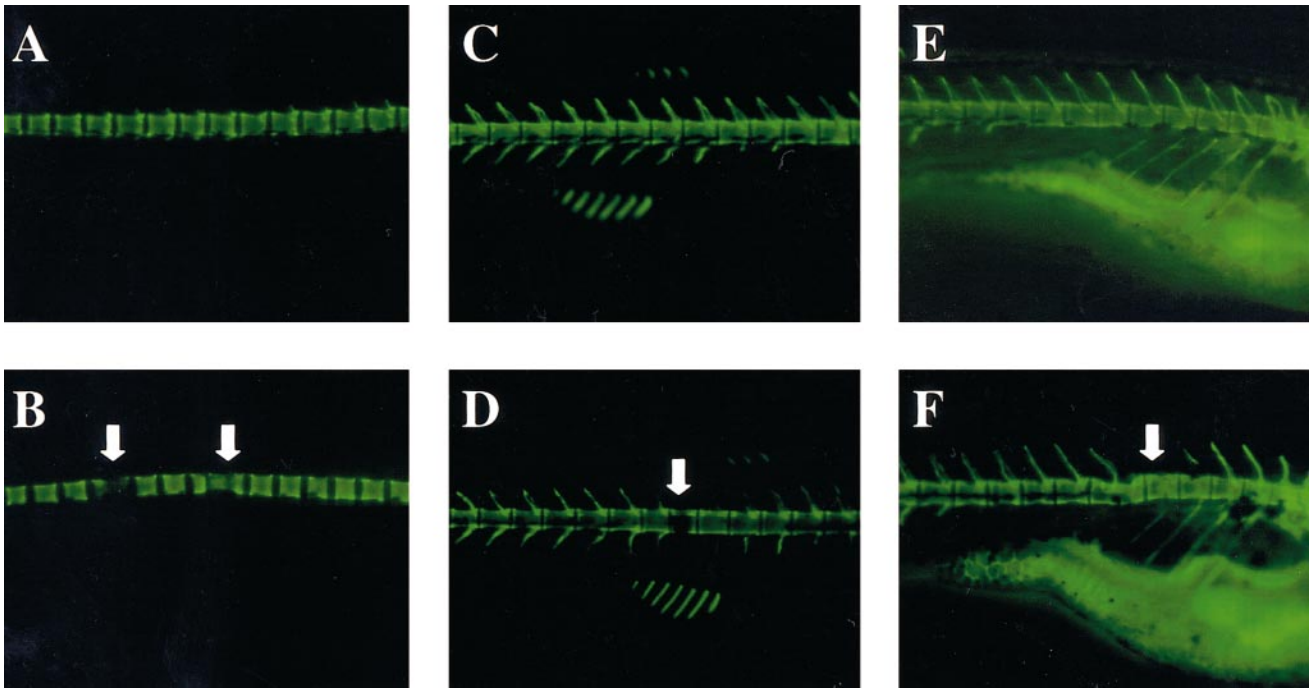


**FIG. 4.** Effects of ectopic expression of BMP2 in notochord cells on the skeletal development of zebrafish embryos. (A, C, E) Control embryos at 15 (A) and 24 (C, E) dpf. (B, D, F) *twhh-BMP2*-injected embryos of 15 (B) and 24 (D, F) dpf. The affected regions are indicated by arrows.

factors capable of inducing the formation of ectopic cartilage and bone when implanted at intramuscular sites in rats (Wozney *et al.*, 1988; Kingsley, 1994) and in chick limbs (Duprez *et al.*, 1996). The different effects could be due to the timing of BMP action. It has been shown that BMP signals have different effects on regulating chondrogensis at different developmental stages. Mutaugh *et al.* (1999) demonstrated that there were at least two distinct phases in the formation of vertebral cartilage from presomatic mesoderm. In the early phase, Hedgehog signal induces presomatic mesoderm to form sclerotome, and subsequently, in the second phase, sclerotome is induced by the BMP signal to differentiate into chondrocytes. It appeared that, in the early phase, the BMP signal could function as an inhibitor in sclerotome formation by inducing naive presomatic mesoderm to assume lateral plate fates (Pourquie *et al.*, 1996; Mutaugh *et al.*, 1999). In this study, because *twhh* promoter drove ectopic BMP2 expression in notochord cells starting at approximately 14 hpf, a time when sclerotome is forming, it could suggest that the bone developmental defects in *twhh-BMP2*-injected embryos might have been caused by the early inhibitory effect of BMP2 on sclerotome formation. In fact, we have shown that expression of a BMP-like protein, Dorsalin, in notochord cells inhibited the formation of slow muscles induced by the Hedgehog signal (Du *et al.*, 1997).

In summary, our results demonstrated both the simplicity and sensitivity of using calcein for visualizing bone structures in developing zebrafish embryos, and the effectiveness of the method to detect embryos with abnormal skeletal structures induced by ectopic expression of BMP signals. This method clearly has a direct application in mutant screening of zebrafish embryos with developmental defects in skeleton structures.

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