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Characterization of zebrafish mutants with defects in bone calcification during development

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ABSTRACT

Using the fluorescent dyes calcein and alcian blue, we stained the F3 generation of chemically (ENU) mutagenized zebrafish embryos and larvae, and screened for mutants with defects in bone development. We identified a mutant line, *bone calcification slow* (*bcs*), which showed delayed axial vertebra calcification during development. Before 4–5 days post-fertilization (dpf), the *bcs* embryos did not display obvious abnormalities in bone development (i.e., normal number, size and shape of cartilage and vertebrae). At 5–6 dpf, when vertebrae calcification starts, *bcs* embryos began to show defects. At 7 dpf, for example, in most of the *bcs* embryos examined, calcein staining revealed no signals of vertebrae mineralization, whereas during the same developmental stages, 2–14 mineralized vertebrae were observed in wild-type animals. Decreases in the number of calcified vertebrae were also observed in *bcs* mutants when examined at 9 and 11 dpf, respectively. Interestingly, by 13 dpf the defects in *bcs* mutants were no longer evident. There were no significant differences in the number of calcified vertebrae between wild-type and mutant animals. We examined the expression of bone development marker genes (e.g., *Sox9b*, *Bmp2b*, and *Cyp26b1*, which play important roles in bone formation and calcification). In mutant fish, we observed slight increases in *Sox9b* expression, no alterations in *Bmp2b* expression, but significant increases in *Cyp26b1* expression. Together, the data suggest that *bcs* delays axial skeletal calcification, but does not affect bone formation and maturation.

1. Introduction

Zebrafish have been used for studying the genetic mechanisms involved in animal development and human diseases [1–3]. The zebrafish models are particularly suitable for screening gene mutations, i.e., those that affect the development of heart, kidney, the central nervous system and the motor system [4–9]. Zebrafish can also serve as an excellent model for studying the development of the skeletal system. Zebrafish are characterized by rapid bone development, simple bone structure, external embryonic development, and body transparence. The latter permits easy observations of bone morphology in live embryos [10–12].

The mechanisms involved in skeletal development are highly conserved between zebrafish and the mammalian species, such as the expression and regulation of the major signaling transduction pathways involved in bone development [13–15]. In the past, a number of mutations that affect zebrafish bone development have been identified [16,17]. Some of them are highly conserved in vertebrate species (sequence, expression profiles and function). However, there are some differences in skeletal development between zebrafish and mammals. For example, zebrafish lack bone marrow hematopoietic tissues and osteoclasts [10].

In this study, we characterized a zebrafish mutant line (*bone calcification slow*, *bcs*) that was recently isolated from our mutant screens. We performed chemical (ENU) mutagenesis and screened the resulting F3 generation for gene mutations that cause bone development defects. We stained the embryos and larvae with alcian blue and calcein, respectively, and examined the morphology of developing cartilage and vertebrae. In addition, we examined the expression of bone development marker genes (e.g., *Sox9b*, *Bmp2b* and *Cyp26b1*) in isolated candidates to determine the possible involvement of the mutant genes in cellular signaling pathways in bone development. The *bcs* fish showed defects in bone mineralization in larval stages, between 7 and 11 days post-fertilization (dpf). This mutant line may provide a tool for genetic studies of diseases related to pathological mineralization, such as osteoporosis or osteomalacia [18].

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2. Materials and methods

2.1. Animals

AB strain zebrafish were used in this study. Breeding colonies were maintained in 28.5 °C with a 10/14-h dark/light cycle [19]. Embryos were kept at 28.5 °C and staged by hours post-fertilization (hpf) or days post-fertilization (dpf). Protocols for experimental procedures were approved by Nankai University IACUC and according to NIH guidelines.

2.2. ENU mutagenesis

Methods for ENU mutagenesis were similar as previously described [20]. Twenty males (4–6 months old) were used for mutagenesis. Four weeks after the completion of ENU treatment, the fish were mated to wild-type females to generate the F1 fish. After sexual maturity, F1 fish were mated to wild-type females to generate F2 families. F3 embryos were obtained from individual F2 sibling crosses.

2.3. Calcein and alcian blue staining

Calcein (Sigma, St. Louis, MO) was dissolved in deionized water at a concentration of 0.2% (pH 6.5–7.5). Zebrafish larvae (5–13 dpf) were stained with calcein for 15 min at room temperature. After removing calcein solutions, the fish were washed 2–3 times with system water (10 min per wash), anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate salt, viewed and imaged using a dissecting fluorescence microscope.

Alcian blue (Sigma, St. Louis, MO) was dissolved in 70% ethanol containing 1% hydrochloric acid at a concentration of 0.1% (pH 2.0–2.5). The zebrafish embryos (48–96 hpf) were fixed in 4% paraformaldehyde overnight at 4 °C, and washed with PBST. The embryos were stained with alcian blue overnight at 4 °C. After removing alcian blue solutions, the embryos were washed with acidified alcohol (HCl–EtOH, 5% concentrated hydrochloric acid and 70% ethanol), bleached with HCl–EtOH/H_2O_2 to remove nonspecific staining, and washed again in 30%/70% and 50%/50% of glycerol/PBS. The embryos were viewed and imaged using a dissecting microscope.

2.4. Quantitative RT-PCR

Total RNA was extracted from 7 dpf embryos using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), and was reverse-transcribed by M-MLV reverse transcriptase (Promega, Madison, WI) using the oligo (dT) primers. qRT-PCR was performed using the SYBR Green Labeling System (BioRad, Hercules, CA). Reaction procedures included a denaturing step at 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Primer sequences included: *cyp26b1*, forward 5′-TTTGGTGGTGGCGTTC GTT-3′, reverse 5′-CACAGTGGCG TCAAGCATT-3′; *sox9b*, forward 5′-ACGACTGGTCTCTGGTGCC-3′, reverse 5′-TCCACAAA CGGACGC TTCT-3′; *bmp-2b*, forward 5′-TCTCACGGTGCTGTTGCT CG-3′, reverse 5′-GATTTGCTTGGGGTGGG TTT-3′; *actin*, forward 5′-TTCAC CACCACAGC CGAAAGA-3′, reverse 5′-TACCGCAAGATTCCATACC CA-3′.

3. Results

3.1. Delayed axial vertebra calcification in bcs mutants

Calcein is a fluorescent dye that binds to calcium [21]. Because the skeletal system consists of calcified structures, calcein can be used for staining the developing bone tissues. Using calcein staining, we screened the F3 embryos from 51 F2 families that were derived from ENU mutagenized founders. We obtained a mutant line that showed skeletal dysplasia. This mutant line was designated *bone calcification slow* (*bcs*) (Fig. 1).

In wild-type zebrafish, mineralization of vertebrae occurs after 4 dpf, when the perichordal calcification of the basioccipital starts [22]. At 5 dpf, calcification increases in the cleithrum, pharyngeal teeth, and extends to the opercular bones. At 5 dpf, we did not detect obvious calcein stains in axial vertebrae in either wild-type or mutant fish (Fig. 1A and B). At 7 dpf, calcein-stained vertebrae were observed in all wild-type larvae examined ($n = 367$), but only in 47% of *bcs* mutants examined ($n = 288$). During this stage of development, calcified vertebrae were mainly found in the anterior part of the axial backbone. In wild-type fish, 6.2 ± 2.7 calcified vertebrae were detected (Figs. 1C, 2A, 3A), whereas in *bcs* mutants, only 1.4 ± 1.9 calcified vertebrae were detected (Figs. 1D and 2B, 3A).

During development, the number of calcified vertebrae increased. In wild-type fish, by 9 dpf, 7–26 calcified vertebrae were observed, and they were found in both anterior and posterior axial backbone (Figs. 1E and 2C; $n = 151$). In *bcs* fish, however, at 9 dpf, only 85% of the fish ($n = 153$) examined showed calcified vertebrae (1–20), primarily in the anterior axial backbone (Figs. 1F and 2D). By 11 dpf, 21–28 calcein-stained axial vertebrae were observed in wild-type larvae ($n = 90$), and the skeleton became thicker and wider with the formation of spur and coccyx (Figs. 1G and 2E). In *bcs* fish, all the examined larvae ($n = 73$) showed less calcified vertebrae (6–26). Also, the vertebrae appeared thinner in comparison to wild-type fish at the same development stages (Figs. 1H and 2F). The formation of vertebral spur and coccyx was also observed in the mutants. At 7, 9 and 11 dpf, the number of calcified vertebrae in *bcs* fish were significantly less than wild-type fish (Fig. 3; $^{*}p < 0.001$). Interesting, by 13 dpf, the defects in bone calcification in *bcs* mutants were no longer evident. The number of calcified vertebrae was similar between wild-type and *bcs* larvae. The morphology of the vertebrae was also similar in wild-type and *bcs* larvae (Fig. 1I and J).

3.2. Normal cartilage development in bcs mutants

We examined the development of cartilage by staining the embryos with alcian blue. Alcian blue is a cationic dye that binds to mucopolysaccharide under acidic conditions [23]. Cartilage is a strongly sulfated connective tissue, which contains mucopolysaccharide, and thus can be stained with alcian blue. We stained wild-type and mutant embryos at 72 and 96 hpf, respectively. No differences in alcian blue staining were detected between wild-type and *bcs* embryos (data not shown), suggesting that the development of cartilage in the mutants was not affected.

3.3. Expression of bone development marker genes in wild-type and bcs mutant fish

To investigate the possible involvement of *bcs* in cellular signaling pathways in bone development, we examined the expression of *Sox9b*, *Bmp2b* and *Cyp26b1* in developing wild-type and mutant larvae. *Sox9b*, *Bmp2b* and *Cyp26b1* are involved in different stages of bone development. In the early stages of bone development, *Sox9b* plays an important role in chondrogenic differentiation, whereas *Bmp2b* is involved in postnatal bone formation [24–27]. In later stages, *Cyp26b1* regulates skeletal formation and calcification by suppressing retinoic acid activity [28,29].

We performed qRT-PCR to examine the expression of *Sox9b*, *Bmp2b* and *Cyp26b1* using total RNA isolated from 7 dpf wild-type and *bcs* larvae (Fig. 4A–C; $n = 4$ for each qRT-PCR). The expression of *Sox9b* in *bcs* embryos slightly increased in comparison to the

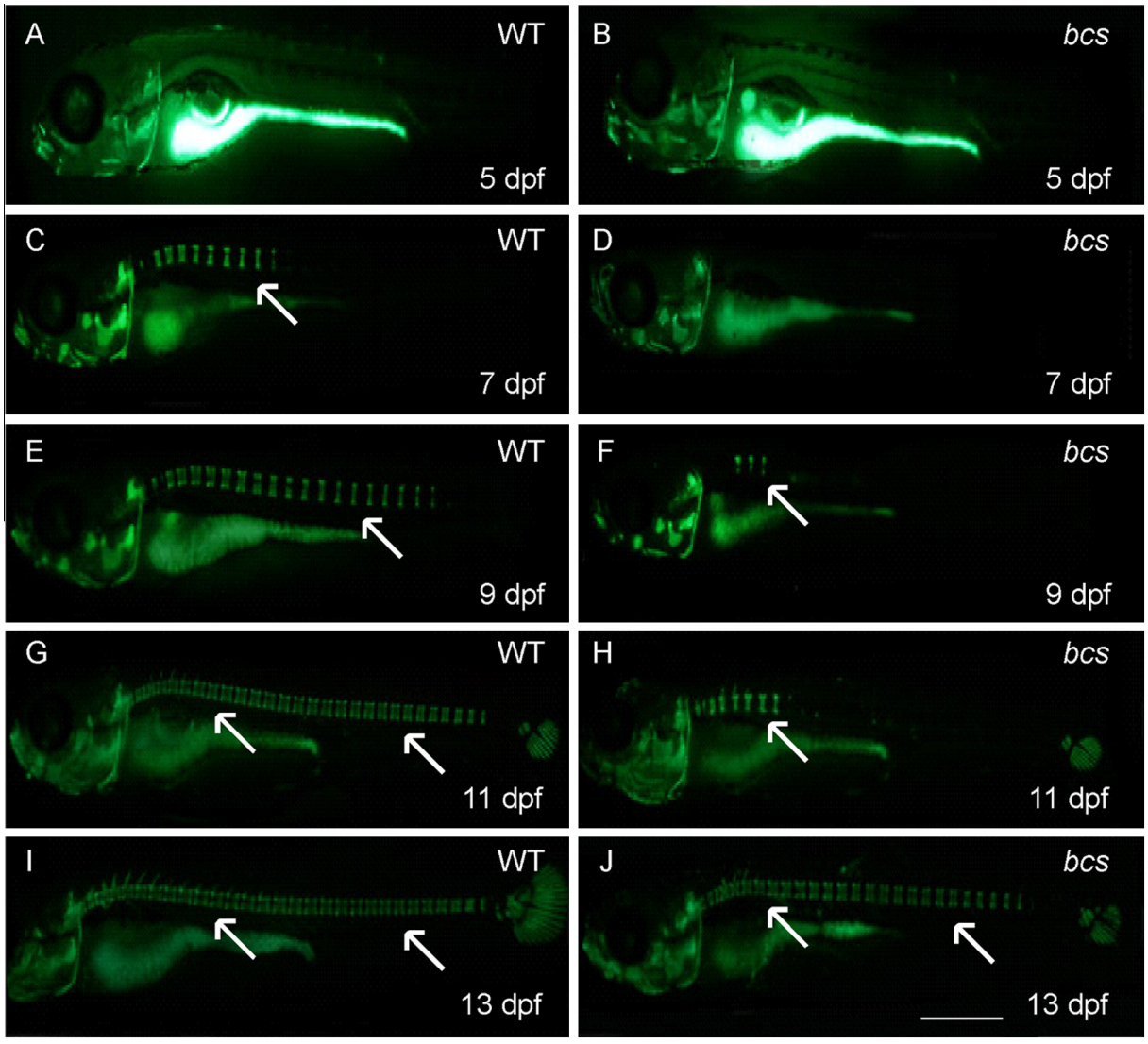

Fig. 1. Fluorescent images of wild-type and *bcs* larvae stained with calcein (lateral view, anterior is to the left). At 5 dpf, no specific calcein staining was observed in wild-type (A) or *bcs* fish (B), except non-specific staining in yolk and gut. At 7 dpf, calcein stained vertebrae were detected in wild-type fish (C, arrow). However, in many *bcs* mutants, no staining was detected (D). At 9 dpf, the number of calcein stained vertebrae increased in wild-type fish (E, arrow), whereas in the mutant, only a few calcein-positive vertebrae were detected (F, arrow). At 11 dpf, in wild-type fish, mineralized vertebrae were seen in both anterior and posterior axial backbone (G, arrows), whereas in the mutant, calcein-positive vertebrae were mainly observed in the anterior axial backbone (H, arrow). By 13 dpf, the number of calcein stained vertebrae were similar in wild-type fish (I) and mutants (J). Scale bar: 0.25 mm.

expression in wild-type embryos, but the difference was not statistically significant (paired *t*-test, $p = 0.06$). The expression of *Bmp2b* was similar in mutant and wild-type embryos ($p = 0.42$). However, the expression of *Cyp26b1* significantly increased in *bcs* mutants than in wild-type fish ($p < 0.05$).

4. Discussion

We developed an assay (calcein and alcian blue staining) for screening zebrafish mutants with defects in bone development. Calcein and alcian blue staining methods are quick and simple, highly sensitive, low toxic, and easy to detect [21,23,30–32]. In this research, we screened the F3 generation embryos and larvae derived from ENU mutagenized founders. We isolated a mutant line (*bcs*) that shows defects in bone calcification during development. In *bcs* mutants, the early development of bone tissues was not affected. Staining with alcian blue revealed no obvious differences in cartilage morphology between wild-type and mutant animals. The defects in *bcs* mutants became evident at 7 dpf, when axial vertebrae calcification occurs. In *bcs* mutants, between 7 and 11 dpf, the number of calcified vertebrae (stained by calcein) significantly decreased in comparison to wild-type siblings. Interestingly, after 13 dpf, the defects in *bcs* mutants were no longer evident, and the mutant and wild-type fish showed similar numbers of mineralized vertebrae. The data suggest that the mutant defects in bone mineralization is not related to chondralloplasia, and that the *bcs* mutation only delays (not inhibits) vertebrae calcification for a specific period of time during development.

In zebrafish, there are two skeletal development centers: the Weber formation center, which generates crania and vertebrae, and the Coccyx formation center, which produces coccyges [13]. We did not observe abnormalities in crania and coccyx in the mutants, suggesting that the *bcs* mutation may specifically affect vertebrae mineralization. It is important to note that the defective phenotypes varied among individuals. At 7 dpf, for example, most of the mutants did not show mineralized vertebrae, but for the mutant fish that showed mineralized vertebrae, 10% of them similar numbers of calcified vertebrae as seen in wild-type animals. This may be due to incomplete penetrance of the *bcs* gene. Worth noting also is the recovery of bone calcification when the mutants

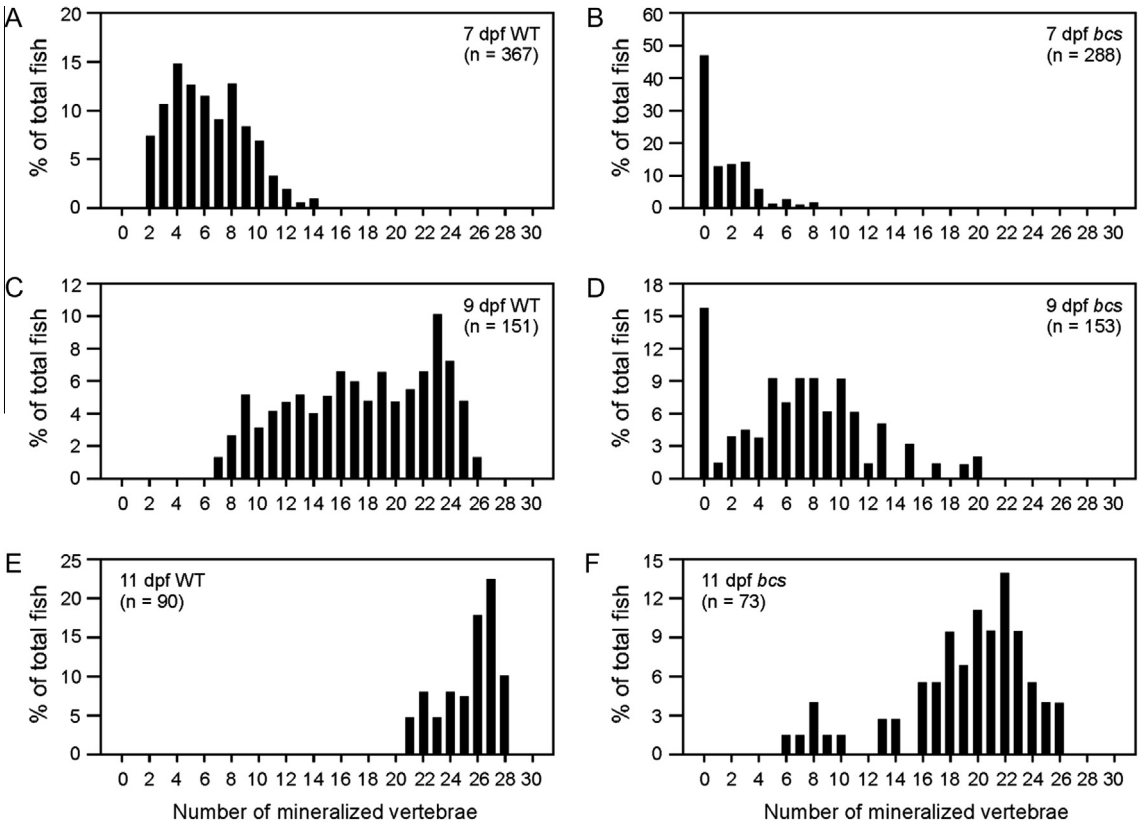

Fig. 2. Histograms showing the distribution of calcified vertebrae in wild-type and *bcs* larvae at different ages. (A, B) 7 dpf; (C, D) 9 dpf; (E, F) 11 dpf. Note the shift of the histograms to the right of the graphs when the fish became older (A, C and E), which demonstrates the graduate increase of calcified vertebrae during development. The same shift pattern was seen in mutants (B, D and F).

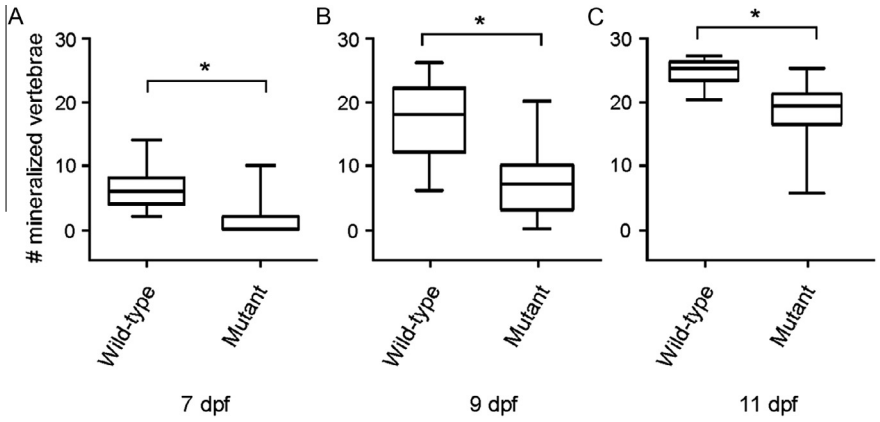

Fig. 3. Quantitative analysis of calcified vertebrae in wild-type and mutant fish at different ages. (A) 7 dpf; (B) 9 dpf; (C) 11 dpf. During development, the number of calcified vertebrae increased in wild-type and mutant fish. However, at each developmental stage, there were significantly more calcified vertebrae in wild-type fish than in mutants (paired *t*-test, $^{*}p < 0.001$).

became older (e.g., >13 dpf). It is possible that the *bcs* locus gradually loses function during ontogenesis or late developmental stages. It is also possible that the function of *bcs* is compensated or replaced by other genes which function in the same or different signaling transduction pathways in bone development.

The results from gene expression experiments support our notion that *bcs* specifically affects bone mineralization, but not other aspects of bone development (e.g., bone cell proliferation and differentiation). The expression of *Sox9b* was similar in wild-type and mutant larvae, as well as the expression of *Bmp2b*. This was consistent with findings from the alcian blue staining experiments (i.e., normal development of cartilage and bone formation). Conclusive results regarding the effects of *bcs* on early bone development cannot be drawn before the completion of detailed anatomical studies (e.g., morphological examinations by electron microscopy).

Previous studies have shown that deletion of the *Cyp26b1* locus led to excessive mineralization of axial and craniofacial bones, and caused fusions of vertebrae [33,34]. In this study, we revealed a linkage between high *Cyp26b1* expression and decreased bone mineralization. In *bcs* mutants, the expression of *Cyp26b1* significantly increased in comparison to wild-type animals. In has been demonstrated that in immature and/or less active osteoblasts, *Cyp26b1* expression was elevated, whereas in fully differentiated and/or

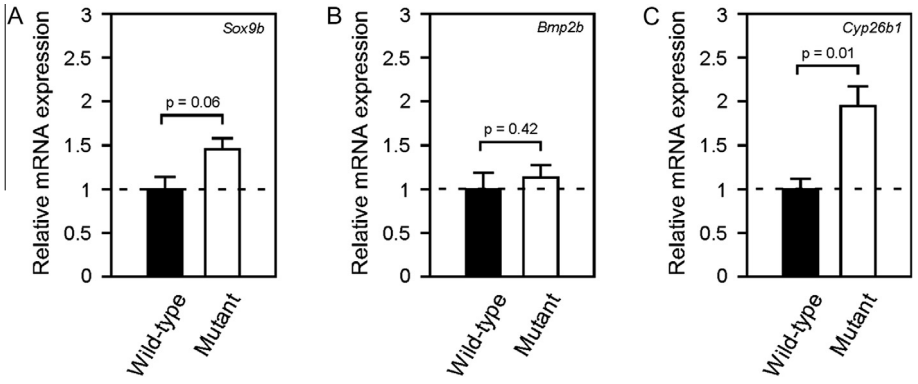

Fig. 4. Relative *Sox9b*, *Bmp2b* and *Cyp26b1* mRNA expression in wild-type and *bcs* fish at 7 dpf. The expression of *Sox9b* (A) slightly increased in *bcs* mutants (white bar) than in wild-type fish (black bar), but the difference was not statistically significant (*t*-test, $p = 0.06$, means ± SE, $n = 4$). The expression of *Bmp2b* (B) was similar in *bcs* and wild-type fish ($p = 0.42$; means ± SE, $n = 4$). By contrast, the expression of *Cyp26b1* (C) significantly increased in *bcs* mutants than in wild-type fish ($p = 0.01$; means ± SE, $n = 4$).

highly active osteoblasts, *Cyp26b1* expression decreased [35]. In *bcs* mutants, between 7 and 11 dpf, the vertebrae were under mineralized, or "immature" in comparison to wild-type vertebrae, thereby explaining the elevated *Cyp26b1* expression. Our data suggest that *bcs* may function up-stream of *Cyp26b1*, but it only plays a role during a specific time in development, i.e., between 7 and 11 dpf.

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