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Overexpression of p204 leads to abnormal embryos and osteogenesis in zebrafish

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1. ABSTRACT

p204, an inteferon-inducible protein, is known to play an important role in modulating cell proliferation, cell cycling, and the differentiation of various tissues, including osteoblasts. In order to determine the role of p204 during development *in vivo*, the teleost zebrafish (*Danio rerio*), an established vertebrate model for developmental studies, was employed. *p204* cDNA was introduced into zebrafish by microinjection, and p204 was ectopically expressed throughout the whole embryo during the early stages of zebrafish embryogenesis, then its expression gradually decreased, mainly in ventrally located cells and retina capsules. Importantly, overexpression of p204 in zebrafish resulted in striking malformations such as bent spine and expanded belly. Furthermore, the expressions of some genes (*vent*, *runx2b*, *osn*) involved in dorsoventral patterning and osteogenesis were significantly upregulated after *p204* injection. This study provides not only the *in vivo* evidences demonstrating the role of p204 during embryonic development, but also new insights into the molecular mechanism by which p204 mediate osteogenesis.

2. INTRODUCTION

The p204 gene is a member of the interferon-inducible p200 family (1, 2). Increasing evidences from cultured cells demonstrate that p204 modulates cell proliferation (3,4), cell cycling (5-6), and the differentiation of various tissues and cells, including skeletal muscle myotubes (7, 8), beating cardiac type myocytes (9, 10), osteoblasts (11-13), chondrocytes (14), and macrophages (15). (For more information, see recent reviews (16, 17)). However, the efforts to exploit the actions of p204 *in vivo* and to understand the mechanisms involved are significantly hampered by the fact that mice in which p204 is overexpressed or deleted are still not available.

The zebrafish model is a necessary vertebrate complement to the mouse model. It has several advantages such as transparent embryos, external development, short generation time and efficient genetic manipulation techniques (18). Its osteocyte involved in bone formation and remodeling are similar in many aspects to those found in mammals. While little is known on how these cells

regulate bone matrix formation and remodeling (19). Although zebrafish have not been widely used to study bone development and disease, many studies have shown its potential. Similar to mammals, *runx*, *dlx* and the hedgehog family of genes are expressed in the process of bone formation in zebrafish (20-23). In addition, Manuel *et al.* suggest that fin ray regeneration in zebrafish could be useful for preclinical studies of skeletal tissue disorders although lepidotrichia is not a typical type of skeletal tissue (24, 25). Also, Simoes *et al*. identified a region in the zebrafish collagen X a1 gene (ColXa1) promoter that is responsive to *runx2*, suggesting that zebrafish could be used as a model for studies of bone and cartilage development (26). Finally, a mutation in the collagen IA1 gene, which models the human inherited disorder cleidocranial dysplasia, has been identified through forward genetics (27). In zebrafish there are two orthologs of Runx2, namely Runx2a and Runx2b, which have 86% conservation (78% identity; 8% similarity) (28). The *runx2b* gene induces the expression of *osteocalcin* by binding to its 5'-regulatory region (26). It has been demonstrated that *runx2b* is a materal determinant of ventral zygotic genes in zebrafish and the only known direct regulator of *vent* at the onset of zygotic transcription. Embryos treated with a *runx2b*-specific morpholino (MO) are strongly dorsalized, suggesting that *runx2b* may influence dorsoventral patterning (29).

Genetic comparison and analysis between zebrafish and mice indicates the *runx2b* gene exists in the zebrafish genome (30), nevertheless, we could not find *p204* orthologs in the zebrafish genome. In this study we introduced the *p204* gene into zebrafish embryos by microinjection in order to investigate the effects of p204 on embryonic development, with the special focus on osteogenesis and potential molecular events.

3. MATERIALS AND METHODS

3.1. Larval rearing and maintenance

Zebrafish eggs were obtained from the natural spawning of AB line breeding fish. The eggs were maintained with a photoperiod of 14 h light/10 h dark and supplied with freshwater and aeration. The larva and adult fish were reared using standard methods according to Westerfield (31).

3.2. Construction of expression plasmids

To construct an expression plasmid containing the osteocalcin promoter a Bgl II site was produced at the 5' end and a Hind III site was produced at the 3'end of the osteocalcin promoter with the polymerase chain reaction, using the zebrafish genome as a template. This modified DNA was inserted into the pEGFP-N1 vector that had been digested with Bgl II and Hind III and the construct was named CMV-*post*. A PCR fragment encoding the full-length p204 gene was obtained from CMV-*p204* using primers that added a 5'Hind III site and a 3'BamH I site, and the amplified fragment was subcloned into the Hind III-BamH I sites of CMV-*post*. The resulting construct was named CMV-*post-p204*.

3.3. Microinjection of Embryos with DNA and observation of GFP expression

AB line zebrafish were used for all microinjections. The plasmid constructs were digested with the restriction enzyme Ase I, purified with a gel extraction kit (Omega, USA), and resuspended in 0.1 M KCl at a final concentration of 100 ng/ul. One or two-cell stage embryos were microinjected as described by Culp *et al.* (32), except that the volume of the injections was 2 nl and TE injections were included as a control. Microinjection was performed with a dissection microscope with an Eppendorf injector (Eppendorf, GER). GFP expression was observed and photographed with a TE 2000 microscope system (Nikon, JPN). Images were collected using Scanalytics IP Lab Spectrum software. The pictures showing GFP-positive cells in living embryos were generated by superimposing a bright field image on a fluorescent image using Adobe Photoshop software.

3.4. Quantitative real-time PCR

Total RNA from different stages, ranging from 0 h post-fertilization (hpf) to 3 days post-fertilization (dpf), was extracted with TransZol reagent (TransGen, CHN) following the manufacture's recommendations. There were approximately 50 embryos per test sample. Briefly, one microgram total RNA was primed with an oligo(dT)18 primer and reverse-transcribed using Transcript First-Strand cDNA Synthesis SuperMix (TransGen, CHN) in a 20 µl reaction volume. The reaction was diluted to 1/5 its concentration with ddH_2O and used as a template for qRT-PCR. Samples were amplified using TransStart Green qPCR SuperMix (TransGen, CHN) on a real-time thermal cycler, PTC-200 Peltier Thermal Cycler (Bio-Rad, USA). Efla1 was used as an endogenous control. Primer sequences and information on the probe templates are available in the supplementary information. QRT-PCR was performed in a 25 µl reaction volume containing 1.0 µl template cDNA (equivalent to 10 ng total RNA), 0.2 µM each primer and 12.5 µl 2* TransStart qPCR SuperMix (TransGEN, CHN). Reactions were run on a DNA Engine Opticon 2 continuous fluorescence detection system (MJ Research, USA). The PCR program contained an initial denaturation of 3 min at 95°C followed by 45 cycles of 95°C for 15 s, annealing at 53°C for 15 s and extension at 72°C for 30 s. The fluorescence was measured at the end of each cycle at 80°C for 2 s. After 45 cycles, the samples were run with the dissociation protocol and had a single melting peak. Results were analyzed using Opticon Monitor analysis software (Version 2.03/MJ research, USA), and the $2^{-\Delta\Delta ct}$ method was used to determine various relative levels of expression. The data are reported as the mean of three different experiments and the t-test was used to test the significance of the differences.

3.5. *In situ* Hybridization

The digoxigenin (DIG)-labeled riboprobe was used in whole-mount *in situ* hybridization as previously described (33). The *runx2b* cDNA was generated with the polymerase chain reaction, using zebrafish cDNA as a template. Then the amplified fragment was subcloned into pEASY-T3 (TransGen, CHN) with T7 RNA polymerase and SP6 RNA polymerase. The plasmid was digested with

EcoR I, followed by *in vitro* transcription with T7 RNA polymerase to generate the antisense RNA probe. Then the probe was purified with Quick Spin RNA Columns (Roche, GER).

Staged embryos were fixed with 4% paraformaldehyde in PBS overnight at 4°C and then washed with PBS/0.1% Tween 20. Embryos older than 24 hpf were treated with 10 μg/ml proteinase K for 10 min at room temperature, followed by three washes with PBST for 5 min at RT. Then the embryos were postfixed as above for 20 min and washed with PBST as above. After two washes, the chorions were removed from the embryos using watchmaker forceps. Embryos were then transferred to a pre-hybridization mixture (50% formamide, 5* SSC, 0.1% Tween 20) for 5 min at 65°C and then soaked in hybridization solution (hyb- plus 5 mg/ml yeast RNA, 50 μg/ml heparin) for 4 h at 60°C. The hybridization mixture was then replaced with the fresh hyb+ containing the DIG-labeled RNA probe (treated at 65°C for 10 min and chilled on ice for 5 min) and the embryos were incubated at 60°C overnight. Following probe removal (2* SSCT/50% formamide two times for 20 min at 60°C; 2* SSCT for 15 min at 60°C; 0.2* SSCT twice for 30 min at 60°C; MABT three times for 5 min at RT) was performed. Then the embryos were incubated with fresh blocking solution (2% blocking reagent, 10% sheep serum and 70% MAB) with a 1:2500 dilution of alkaline-phophatase (AP)-conjugated anti-DIG Fab fragments (Roche, GER) at 4°C overnight. After antibody removal with MABT and PBST, twice for 30 min at RT, the embryos were immersed in staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl at pH 9.0, and 0.1% Tween 20) with 0.5 mg/ml levamisol to inhibit endogenous alkaline phosphatase. Dectection was performed with the substrate, nitroblue terazolium (NBT) and 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP), to produce purple and insoluable precipitates. After 1-4 h of incubation in the dark, the embryos were stored in 4% paraformaldehyde/PBS for 2 h at 4°C and then transferred to 70% ethanol overnight at 4°C to remove the background.

4. RESULTS

4.1. GFP expression patterns of zebrafish embryos after CMV-*post-p204* injection

To study the effects of injection of the *p204* cDNA on zebrafish embryos, CMV-*p204* and CMV-*post-p204* vectors were injected into 1-cell stage zebrafish embryos. pEGFP-N1 and TE were injected as controls. After injection, the embryos were cultured to the larva stage and observed under a fluorescent microscope at various stages. p204 expression was first detected at 80% epiboly stage and the fluorescence continued to 10 dpf. The spatial expression was different at various developmental stages. Before 24 hpf, *p204* was expressed throughout the embryo, and then the expression regions gradually decreased to the ventral cells and retinal capsules (Figure 1). The fluorescence rates were different among various injections. The fluorescence rates generated by pEGFP-N1 and CMV-*p204* were not significantly different, while the fluorescence rates of CMV-*post-p204* were increased significantly (Figure 2, D).

4.2. Abnormal embryos induced by *p204* injection and statistical analysis

To investigate the effects of *p204* injection on juvenile zebrafish, the *p204*-injected larva were raised to adult fish. At 10 dpf, malformations ranging from mild to severe were found in the *p204*-injected zebrafish. The mild malformations included slightly bent spines, while the severe malformations had serious spine bends with belly-expansion (Figure 2, B-C). However, the similar malformations were not found in the pEGFP-N1 and TE injection controls. Embryos with severe malformations died at 15 dpf. Statistical results showed the malformation rates of *p204*-injected groups were significantly higher than the control group, while the mortality rates of the four groups were almost the same by 10 dpf (Figure 2, D).

4.3. p204 regulates the expression of *runx2b*, *vent*, and *osn*

To study the effects of *p204* injection on the expression levels of the genes involved in zebrafish dorsoventral patterning and osteoblastogenesis, qRT-PCR was performed, using *efla* 1 as a control. The expression levels of *runx2b* declined from 4 hpf to 1 dpf and then increased from 1dpf to 3dpf compared with groups without *p204* injection. The vent gene, a direct downstream gene of *runx2b*, had a similar expression pattern as *runx2b* (Figure 3). In this study, we also measured the expression levels of *osn*, a mature stage expression marker gene in zebrafish osteoblast differentiation. Expression of *osn* was first detected at 10 dpf and increased from 1 dpf to 3 dpf. The expression pattern of *osn* was consistent with that of *runx2b* (Figure 3).

4.4. p204 injection increases the expression of zebrafish runx2b, assayed by whole-mount *in situ* hybridization

To investigate the changes in expression levels of zebrafish *runx2b* before and after *p204* injection, we performed whole-mount *in situ* hybridization with a digoxigenin-labelled 216 bp RNA probe. Negative control hybridizations were performed with a sense *runx2b* RNA probe. Runx2b transcripts were observed at all the stages of embryogenesis we assayed (Figure 4, A-B). We found the expression of zebrafish *runx2b* was enhanced after p204 injection (Figure 4, C-D), which was consistent with our real-time RT-PCR results. At stages from 1-cell to high-pec, *runx2b* transcripts were widely expressed (Figure 4, A-B). At 2.5 dpf embryos were in the pec-fin stage, and *runx2b* expression was restricted prominently to the hindbrain, midbrain, forebrain and the eye (Figure 4, C-D). *Runx2b* was also strongly expressed in the spine and in the vessels. The results reported here are in line with previous research on *runx2b* expression patterns during the development of *Danio rerio* (28).

5. DISCUSSION

5.1. The expression pattern of *p204-GFP* in zebrafish embryos

In this study, we found that GFP was largely expressed in zebrafish embryos from 8 hpf to the 8 dpf stage. In addition, malformations such as bent spine and

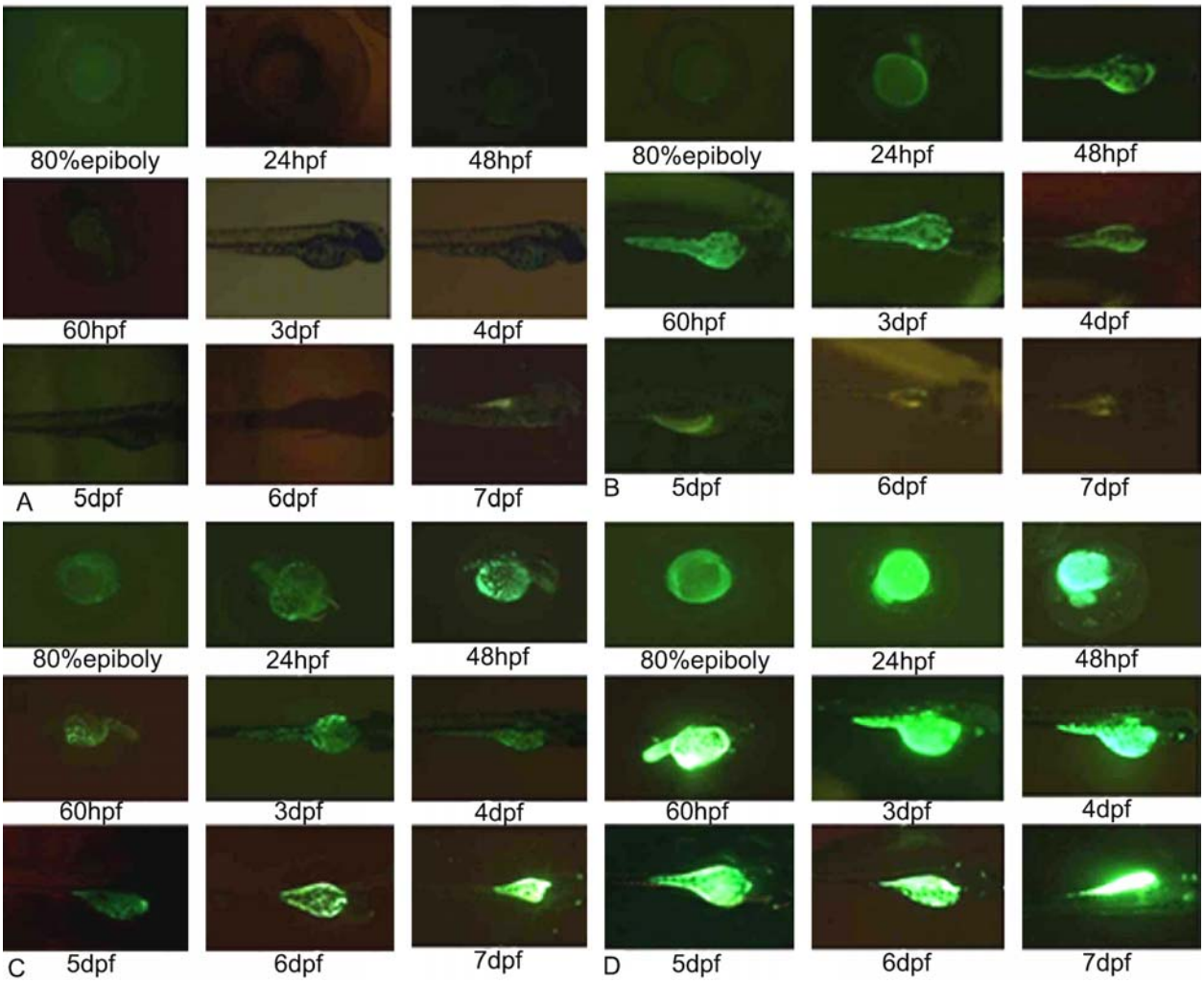

Figure 1. Diagram of GFP-positive embryos derived from injection of *p204*-expression vector. (A) TE injection group (control I). (B) pEGFP-N1 injection group (control II). (C) Expression pattern of *p204* denoted by GFP in CMV-*p204* injection group. (D) Expression pattern of *p204* denoted by GFP in CMV-*post*-*p204* injection group. The development stages were: 80% epiboly, 24 hpf, 48 hpf, 60 hpf, 3 dpf, 4 dpf, 5 dpf, 6 dpf and 7 dpf.

expanded belly were induced at 10 dpf after injection of a *p204*-GFP fusion expression vector.

Temporally, our results showed that *p204*-GFP could be first detected at 80% epiboly stage (about 8 dpf), which is in agreement with Yang's study that shows the foreign gene GATA1-GM2 begins its expression at 8 hpf after injection into zebrafish embryos (Yang *et al.*, 2003). The expression of *p204*-GFP gradually declined after the 8 dpf stage and completely disappeared by 11 dpf. These results seem to suggest that the injected *p204*-GFP fusion vector was not integrated into the zebrafish genome but was gradually distributed to various cells during the process of tissue differentiation and cell division. Simultaneously, the distributed DNA was gradually degraded by endogenous nucleases and finally disappeared. This would support a study by Zhu that showed the integration of a foreign gene into the host genome is a complicted process. Zhu's study revealed that foreign genes injected into fish zygotes undergo profound changes in morphology. Most of the genes are converted into super-tight rings, loose big rings, linear dimers or polymers though some of them maintain a linear confirmation. Meanwhile, the DNA pieces begin slow replication. The speed is accelerated at the mid-blastula stage and peaks at the blastopore closure and neurulation stages. During the process of embryonic development, the expression of foreign genes comes to a temporary halt and only the genes integrated into the genome continue replication. Besides, the stable expression of integrated exogenous genes depends on their location on the chromosome, their promoters and their external environment (34, 35).

Our study demonstrated that a dual-promoter GFP fusion vector generated more intense fluorescence than a single CMV-GFP vector under the same injection

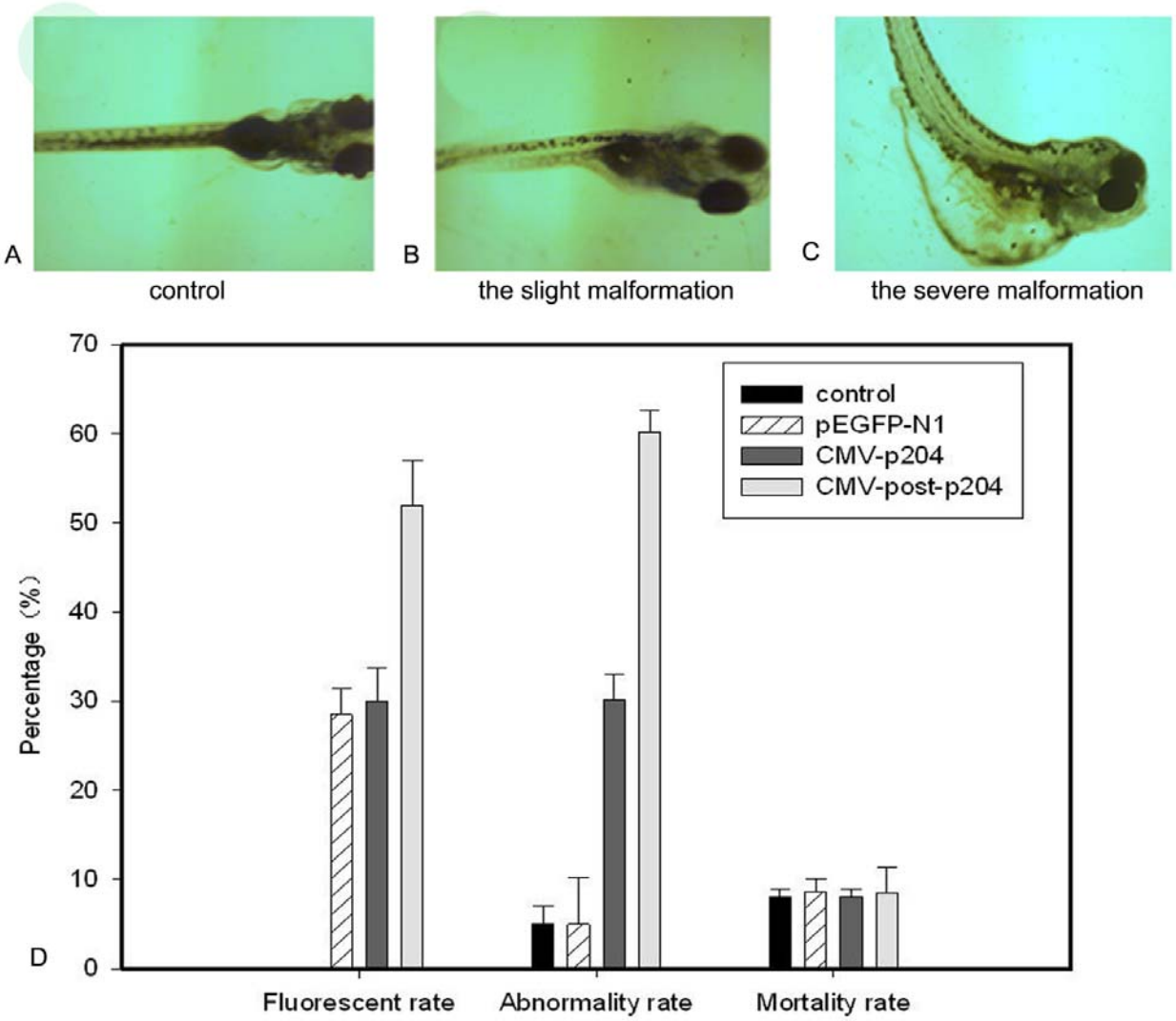

Figure 2. Malformed zebrafish induced by *p204* injection and statistical analysis of fluorescence, abnormalities and mortality rates after injection of the *p204*-expression vector. (A) normal; (B) bent spine; (C) bent spine with expanding belly; (D) *p204*-injection-induced fluorescent rate, abnormality rate and mortality rate differences in p204-injection embryos and controls. CMV-*p204* and CMV-*post-p204* were injected into 1-cell stage embryos. Then they were raised to 11 dpf stage. The TE and pEGFP-N1 groups were controls. Data are expressed as percentages and represent means (±standard deviation, n = 3); *Significant differences from control groups ($P < 0.5$).

conditions. This supports a study by Finn *et al.* that revealed that two tandem promoters initiated more efficient expression of the downstream gene than a mono-promoter (36-38). Our results revealed that the spatial location of the three GFP expression vectors was similar, mainly in the ventral region and in scattered parts of the head. The results do not support the hypothesis that the CMV promoter has no tissue-specific expression (39), and this is similar to a study by Yang (40). It is possible that the expression efficiency of the gene and the spatial location of expression with the CMV promoter differ between different species. In addition, Elwood *et al.* constructed elongation factor 1-a enhancer/promoter-GFP expression vectors and transferred them into zebrafish embryos by microinjection. Their results show that GFP is ubiquitously expressed in zebrafish embryos without tissue specificity (41). The results seem to suggest that different promoters have spatial specificity when they switch on exogenous genes.

5.2. Malformed zebrafish embryos were induced after *p204* injection

It has been demonstrated that the transcriptional hierarchy found in mammals is conserved during zebrafish osteoblastogenesis (42). The study by Blyth *et al.* revealed that Runx genes function as dominant oncogenes in mouse models in which the genes have been identified as common insertion sites for murine leukaemia virus (MLV) in

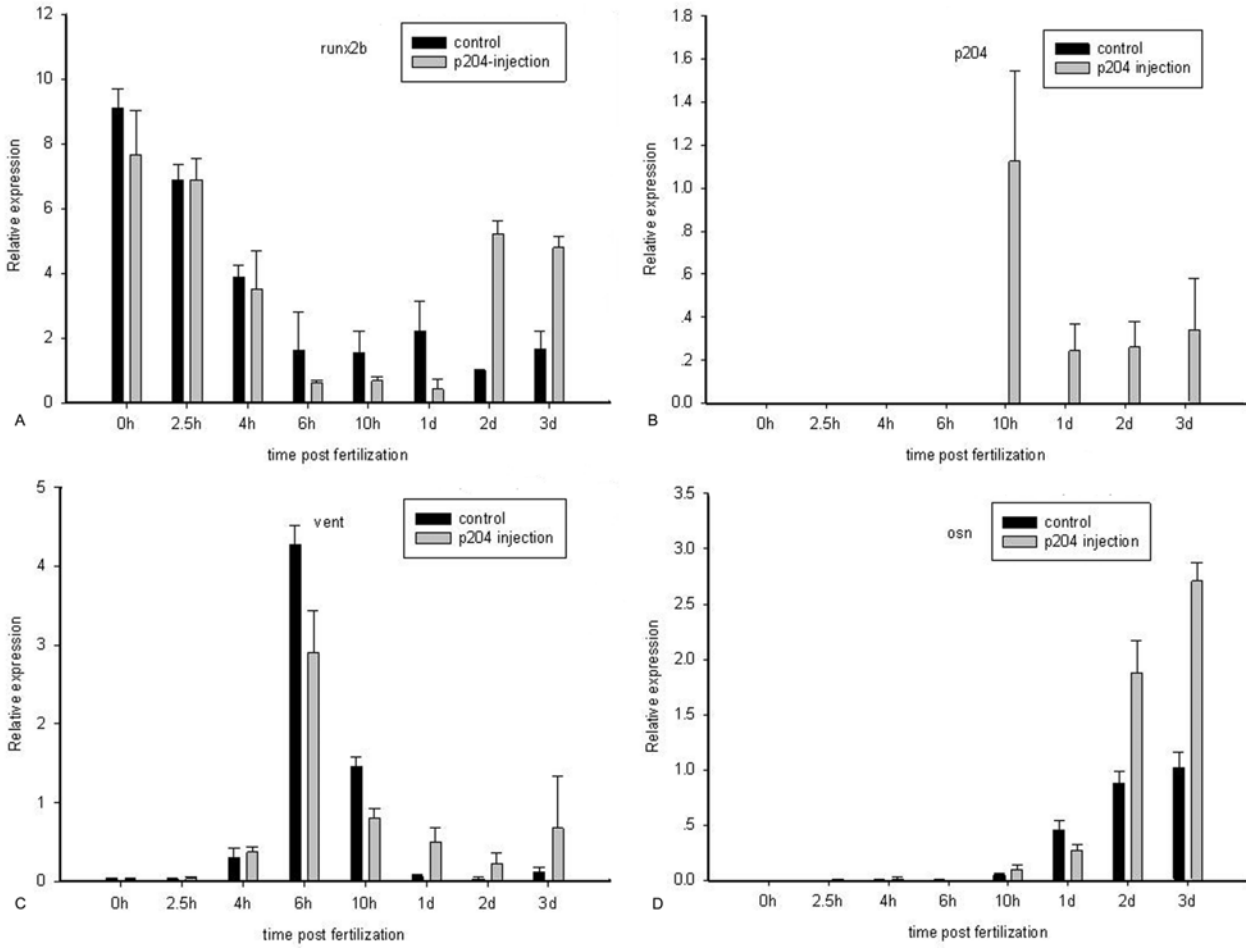

Figure 3. Quantitative RT-PCR assay for the transcription levels of *p204* and the genes involved in dorsoventral patterning and osteoblastogenesis. Total RNA was extracted from various stages of the control and *p204* injection groups. Efla 1 gene was used as an endogenous control and the $2^{-\Delta\Delta ct}$ method was used to determine various relative expression levels. Data were represented as the mean of three different experiments.

haematopoietic tumors (43). In addition, the study by Kanatani seems to suggest that the expression of *runx2b* must be down-regulated for the completion of osteoblast differentiation (44). In mice lacking Runx2b, osteoblasts do not differentiate, but form the initial mesenchymal derivatives normally before death at the embryonic stage (45-47). However, the study by Flores *et al*. showed that depletion of materal Runx2b strongly resulted in zebrafish embryos dorsalization (48). Our results revealed that bent spine and expanded belly zebrafish were induced by *p204* injection (Figure 2); in addition, p204 also upregulated Runx2b expression (Figure 3). These findings, together with the literatures concerning p204 in osteogenesis, we proposed a model for explaining the regulation of osteogenesis by p204 in zebrafish (Figure 5): p204 modulates Runx2b at two levels: (a) directly interacting with Runx2b and enhancing the binding of Runx2b to its target DNA (12), and (b) upregulating the expression of Runx2b (Figure 3 in this study). Then the activated Runx2b upregulates the expression of *vent*, which is a repressor of the dorsal organizer of gene expression and a key zygotic ventralizing factor, and this induces ventralized embryos (29). Moreover, the expression levels of *osn* were upregulated, and *osn* is a bone marker in mature stages (42). The upregulation of *osn* might be attributed to the overexpression of *runx2b* shown in Figure 5. Normally, BMP2 regulates the expression of *runx2b* through the BMP signaling pathway. Then *runx2b* induces its direct downstream gene *vent* to ventralize embryos. The *boz* gene that is induced by the Wnt-β-catenin pathway, is one of the key dorsalizing factors, determining dorsal formation. There is an equilibrium between dorsal and ventral formation, which is accomplished when *vent* inhibits *boz* and *boz* negatively regulates BMP2. Our study reveals that some genes involved in dorsoventral patterning and osteogenesis are also upregulated after *p204* injection; however, the precise mechanisms remain to be delineated. In summary, this study not only

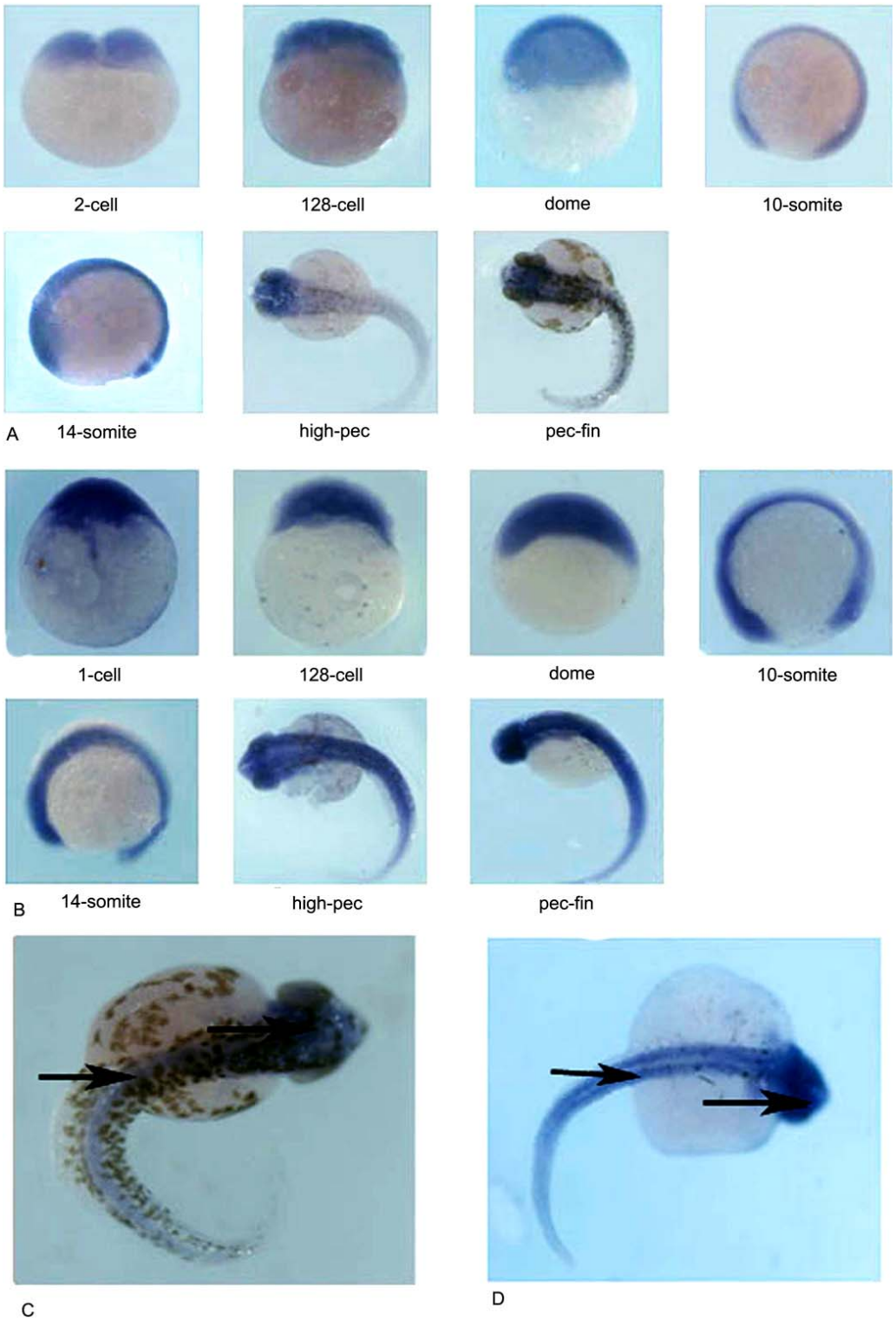

Figure 4. Expression pattern of *runx2b* during early zebrafish embryogenesis before and after *p204* injection by RNA *in situ* hybridization. (A-B) Expression patterns of *runx2b* at seven subsequent stages of embryonic development (1-cell stage, 128-cell stage, dome, 10-somite, 14-somite, high-pec, and pec-fin). (A) control; (B) *p204* injection group. (C) Expression pattern of *runx2b* in the control group at pec-fin stage (2.5 dpf), the arrows indicate the eye and the spine; (D) Expression pattern of *runx2b* in the *p204* injection group at pec-fin stage. The arrowhead shows the deep staining eye and spine parts.

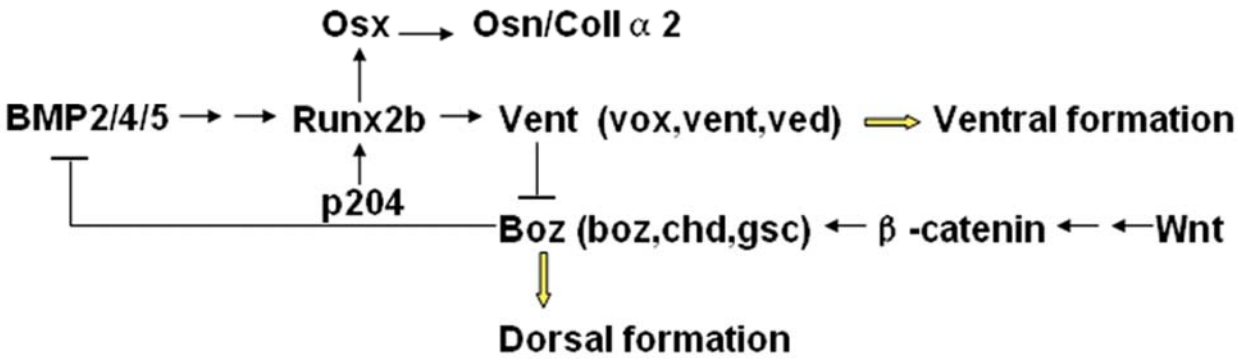

Figure 5. A proposed model for explaining the involvement of *p204* in inducing belly-expansion malformation and altering the expression levels of the genes-related to dorsoventral patterning and osteoblastogenesis. Runx2b is an essential transcription factor that activates the marker genes of osteogenic differentiation, including *osn* and *ColI a2*. Overexpression of Runx2b will activate *vent*, a direct downstream gene of *runx2b* and one of the ventral-specific genes.

provides first *in vivo* evidence demonstrating the importance of p204 in development, but also leads to a better understanding of molecular events by which p204 mediates osteogenesis.

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Abbreviations: AP, alkaline-phophatase; BCIP, 5-bromo, 4-chloro, 3-indolyl phosphate; BMP, bone morphogenetic protein; Cbfa 1, core binding factor a 1; CMV, human cytomegalovirus; DIG, digoxigenin; dpf, day post-fertilization; efla 1, elongation factor a 1; FGF, fibroblast growth factor; GFP, green fluorescence protein; hpf, hour post-fertilization; ISH, *in situ* hybridization; MAB, maleic acid buffer; MLV, murine leukaemia virus; MO, morpholino; osn, osteonectin; PBS, phosphate buffered saline; post, promoter of osteocalcin; pRb, retinoblastoma protein; qPCR, quantitative PCR; RT, reverse transcription; Runx2, Runt-related transcription factor 2; TE, Tris-EDTA buffer; TGF, transforming growth factor.

Key words: p204, Embryonic Development, Runx2b, Osn; Danio Rerio, Osteogenesis

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