Introduction

Mammalian Twist1 and Twist2 are two members of the Twist subfamily of the basic-helix-loop-helix (bHLH) transcription factors that have been highly conserved during evolution [1]. In Drosophila, a single Twist gene, DTwist, is essential for embryonic gastrulation and mesodermal formation [2,3]. Mouse Twist1 was identified by its high homology with DTwist [4,5], while Twist2, originally called “Dermo1”, was discovered by a yeast-two-hybrid screening using the ubiquitous bHLH protein E12 as bait [6]. The expression patterns of Twist1 and Twist2 show an extensive overlap during mouse embryonic development [6], and their encoded proteins exhibit a high degree (up to 98%) of sequence similarity [7]. Both proteins perform various functions by forming either homodimers or heterodimers with bHLH-E proteins [E12/E47] that bind to DNA canonical regulatory sequences called “E-boxes” (CANNTG) in the promoter region of target genes [8].

In humans, mutations in the TWIST1 gene are associated with Saethre-Chotzen Syndrome (SCS), which is an autosomal dominant disorder characterized by craniosynostosis, brachydactyly, soft tissue syndactyly and facial dysmorphism [9]. The skeletal phenotype of Twist1-heterozygous mouse consistently resembles that of human SCS with premature fusion of the cranial suture [9,10]. As mouse embryonic development progresses, the Twist1 expression declines in the developing bones of the skull [11]. In addition, Twist1 overexpression was found to inhibit osteoblast differentiation in vitro and in vivo [12,13,14]. Together, these observations suggest that Twist1 negatively regulates osteoblast differentiation and bone formation.

Various molecular mechanisms may be responsible for the inhibitory role of Twist1 in osteoblast differentiation. Twist1 may modulate FGF signaling, especially Fgf2 expression in cranial suture development [15,16,17] or it may directly bind to and inhibit the transactivation function of Runx2, a master regulator of osteogenesis [11]. In addition, Twist1 might indirectly regulate the Runx2 expression through modulating FGF2 expression as shown in the ex vivo cultured primary osteoblasts isolated from human
To our surprise, we found that the Twist2 replaced by the Twist1 [22]; therefore, the floxed carry a Cre recombinase that replaces one allele of the associated with reduced FGF signaling as a consequence of the differentiation of osteoprogenitors. The skeletal abnormalities were exhibited reduced bone formation and impaired proliferation and compound Twist1flox/flox [21] and Twist2cre/+ mice. Generation of Twist1flox/+ mice were bred with Twist2 Cre knock-in mice. Thus, the compound Twist1flox/+; Twist2cre/+ mice had one allele of Twist2 replaced by the cre recombinase and one allele of Twist1 deleted specifically in tissues where the Twist2 gene was expressed. To our surprise, we found that the Twist1flox/+; Twist2cre/+ mice exhibited reduced bone formation and impaired proliferation and differentiation of osteoprogenitors. The skeletal abnormalities were associated with reduced FGF signaling as a consequence of the decreased expressions of Fg2 and Fgfrs.

### Materials and Methods

#### Ethical Approval

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University Baylor College of Dentistry. IACUC has specifically given ethical approval for all the procedures in this study.

#### Generation of Twist1flox/+; Twist2Cre/+ mice

Twist1 floxed mice (Twist1flox/+flox) were maintained on a C57/129 mixed genetic background [21] and Twist2 Cre knock-in mice (Twist2cre/+flox) were on a 129 mixed genetic background [22]. The Twist1flox/+flox mice were bred with Twist2cre/+flox mice to generate compound Twist1flox/+flox; Twist2cre/+flox mice. The Twist2cre/+flox mice carry a Cre recombinase that replaces one allele of the Twist2 gene [22]; therefore, the floxed Twist1 allele is deleted in the tissues where the Twist2 gene is active. The Twist1 floxed mice and Twist2cre/+flox mice were genotyped as described previously [21,22]. In this study, we analyzed the skeletal phenotype of 6–8 day-old Twist1flox/+flox; Twist2cre/+flox mice and used age-matched Twist1flox/+flox; Twist2cre/+flox as control mice (counting the day of birth as day 0).

## SCS patients [18]. Finally, it is possible that Twist1 inhibits osteoblast apoptosis via the suppression of TNF-α expression [19].

Twist2 has been shown to have an inhibitory function similar to that of Twist1 in bone formation [11]. While recessive TWIST2 mutations in humans and its inactivation in mice result in a focal facial dermal dysplasia (FFDD) syndrome, there is no Twist2-deficient skeletal phenotype [20]. The phenotypic difference between the Twist1- and Twist2-deficient subjects is indeed intriguing when viewed in the context of their significantly overlapping expression patterns in vivo [6] and their similar functions in bone formation [11]. Thus, it is largely unknown how Twist1 and Twist2 synergistically regulate bone formation and what molecular mechanism is involved.

In this study, we generated a compound Twist1- and Twist2-haploinsufficient animal model, Twist1flox/+; Twist2cre/+ mice, by crossing Twist1 floxed mice with Twist2 Cre knock-in mice. Thus, the compound Twist1flox/+; Twist2cre/+ mice had one allele of Twist2 replaced by the cre recombinase and one allele of Twist1 deleted specifically in tissues where the Twist2 gene was expressed. To our surprise, we found that the Twist1flox/+; Twist2cre/+ mice exhibited reduced bone formation and impaired proliferation and differentiation of osteoprogenitors. The skeletal abnormalities were associated with reduced FGF signaling as a consequence of the decreased expressions of Fg2 and Fgfrs.

### Alcian blue/alizarin red staining of the skeleton

Alcian blue/alizarin red staining was performed to analyze the overall skeletal and mineralization defects, as described previously [23]. Briefly, 6-day-old Twist1flox/+; Twist2cre/+ mice and control mice were sacrificed, skinned, eviscerated and fixed for three days in 95% ethanol. They were then stained with alcian blue for cartilage and alizarin red for bone visualization.

### Plain X-ray radiography and high-resolution microcomputed tomography (μ-CT)

The femurs and tibiae from 6-day-old Twist1flox/+; Twist2cre/+ mice and control mice were dissected free of the skeletal muscles and fixed in 70% ethanol. For plain X-ray radiography, the femurs and tibiae were scanned at 3.5-μm resolution using a μ-CT35 imaging system (Scanco Medical, Baslerdorf, Switzerland), as previously described [24]. The trabecular bone was analyzed at a threshold of 160 in 20 sections underneath the growth plate.

### Histology, immunohistochemistry and in situ hybridization

For histologic analysis, the bone specimens from 6-day-old mice were fixed in freshly prepared 4% paraformaldehyde, decalcified in 10% EDTA with 0.5% paraformaldehyde, and embedded in paraffin using standard procedures [25]. Serial 7-μm sections were cut and mounted on silane-coated slides. The sections were then used for Hematoxylin and Eosin (H&E) staining, Tartrate-resistant alkaline phosphatase (TRAP) staining, immunohistochemistry or in situ hybridization, as described previously [24,25].

The following antibodies were used for the immunohistochemical analyses: anti-Osterix (Santa Cruz Biotechnology, Dallas, TX, USA; 1:400), rabbit anti-biglycan antibody “LF-159” (gift from Dr. Larry Fisher, National Institutes of Health, Bethesda, MD, USA; 1:1000), anti-Fgf2 polyclonal antibody (Abcam, Cambridge, MA, USA; 1:5000), and anti-phospho-p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology, Danvers, MA, USA; 1:100). All the immunohistochemical experiments were performed with a 3, 3-diaminobenzidine kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions.

For the in situ hybridization, the RNA probes for dentin matrix protein (Dmp1), osteocalcin (Ocn) and alkaline phosphatase (Alp) were labeled with digoxigenin (DIG) using a RNA Labeling Kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. DIG-labeled RNA probes were detected by an enzyme-linked immunoassay with a specific anti-DIG-alkaline phosphatase antibody conjugate (Roche, Indianapolis, IN) and

| Table 1. Primers used for real-time PCR. |
|----------------------------------------|
| **Gene** | **Forward primer (5’-3’)** | **Reverse primer (5’-3’)** |
|----------|-----------------------------|-----------------------------|
| Fg2      | cgccgtctacctggaagcatc      | gcgaaggtgatgagatgcc         |
| Fgfr1    | ttgattgcctgcaacacatc      | gcagacgtctacccacacatc      |
| Fgfr2    | gtcggtgcgggaattatca       | gatgactgtacccacacatc       |
| Fgfr3    | cccactctgcaagctttgtag      | ggggtgaacccaggtctattt      |
| Fgfr4    | aagctcatcgctggaaccatc     | cgagagggctgtagtattca       |
| Erm      | gtagcatgcctggaacagct      | ggaggcccatggtcatacg        |
| Pea3     | cctagctctccccccccacaa     | cctccctgcggagactggaa       |

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Figure 1. Reduced bone formation in Twist1flox/+; Twist2Cre/+ mice. (A) Skeletons of 6-day-old control (left) and Twist1flox/+; Twist2Cre/+ (right) mice stained with alcian blue (cartilage) and alizarin red (bone). The skeleton of the Twist1flox/+; Twist2Cre/+ mouse is remarkably smaller. (B) Alcian blue- and alizarin red-stained skull from 6-day-old Twist1flox/+; Twist2Cre/+ mice (right) showed delayed fusion of interfrontal suture and open posterior fontanel (arrows), compared with the control mice (left). (C) Alcian blue- and alizarin red-stained hind foot of 6-day-old control (left) and Twist1flox/+; Twist2Cre/+ (right) mice. Note the delayed ossification in metatarsals (mt) and phalanges (pl), and an additional toe (arrow) originating from the same (or duplicated) metatarsal as the hallux in Twist1flox/+; Twist2Cre/+ mice. (D) Plain X-radiography of the tibiae from 6-day-old control (left) and Twist1flox/+; Twist2Cre/+ mice (right). The Twist1flox/+; Twist2Cre/+ mice had shorter tibiae and reduced radiopacity, compared to the control mice. (E) Representative three-dimensional μ-CT images of tibiae from 6-day-old control (left) and Twist1flox/+; Twist2Cre/+ (right) mice. The Twist1flox/+; Twist2Cre/+ mice showed reduced trabecular (arrowheads) and cortical bones (arrows). (F–H) Quantitative μ-CT data showing that the 6-day-old Twist1flox/+; Twist2Cre/+ mice had a significant decrease in the ratio of bone volume (BV)/total volume (TV) (F) and in apparent bone density (G), compared to the control mice (n = 6, P < 0.001). The Twist1flox/+; Twist2Cre/+ mice also presented reduced material density although no statistically significant difference was observed (H).

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VECTOR NBT/BCIP alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA), which produced a blue color indicating positive signals. Methyl green was used for counterstaining.

Cell proliferation assay
Six-day-old Twist1^lox/+; Twist2^Cre/+ mice and control mice were intraperitoneally injected with 5-bromo-2'-deoxyuridine (BrdU) (10 mg/100 g body weight) at 24 hours and then two hours before sacrifice. The long bones were collected and processed for paraffin sectioning as described above. The incorporated BrdU was detected with a BrdU staining kit (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. The BrdU-positive nuclei and total nuclei were counted in the metaphysis region (trabecular bone) as well as in the midshaft (cortical bone). The data represented the percentage of BrdU-positive nuclei from four individual animals each group.

Quantitative Real-time PCR
Total RNA was extracted using Trizol (Invitrogen, Camarillo, CA, USA) from the long bones of 8-day-old Twist1/2 dHet mice and control mice and reverse-transcribed into cDNA with a Reverse Transcription Kit (QIAGEN, Germantown, MD, USA). Quantitative real-time PCR (qPCR) was performed using a GoTag qPCR Master Mix System (Promega, Madison, WI, USA). Mouse 18S rRNA primers (PPM57735E-200, QIAGEN, Germantown, MD, USA) were used for normalization. The primers for Alp, Ocn, bone sialoprotein (Bsp), osterix (Osx), Dmp1 and Runx2 were reported elsewhere [26,27]. In situ hybridization analyses (signal in blue) of the transcripts of Alp (D), Ocn (E) and Dmp1 (F) in the femurs of one-week-old control and Twist1^lox/+; Twist2^Cre/+ mice, Twist2^Cre/+ mice. (G, H) Immunohistochemical analyses (signal in brown) of the osterix (G) and biglycan (H) protein levels in the femurs of the 6-day-old control and Twist1^lox/+; Twist2^Cre/+ mice. Scale bar = 100 μm. doi:10.1371/journal.pone.0099331.g002

Cell culture, constructs and promoter luciferase assay
The C3H10T1/2 mesenchymal cells and MC3T3-E1 preosteoblast cells were cultured as described previously [29]. A 4.9 kb-Fgf2 promoter luciferase construct and expression constructs for Twist1 and E12 have been reported elsewhere ([30]. An expression construct for Twist2 was generated by cloning Twist2 cDNA into the BamHI and EcoRI sites of pcDNA3 vector (Invitrogen). The

Figure 2. Histological examination of Twist1^lox/+; Twist2^Cre/+ mice. (A–B) Femur sections of 6-day-old control and Twist1^lox/+; Twist2^Cre/+ mice were stained with H&E. The Twist1^lox/+; Twist2^Cre/+ mice displayed reduced metaphyseal trabecular bone (A, red arrows) and a decreased thickness of the periosteum (B, blue arrows) and cortical bone (B, red arrows). (C) TRAP staining of femur sections of 6-day-old control and Twist1^lox/+; Twist2^Cre/+ mice. Note that the osteoclasts (red arrows) appeared to be similar in size and distribution in the control and Twist1^lox/+; Twist2^Cre/+ mice. The osteoclast densities were 0.55 ± 0.06/0.01 mm² in the controls (n = 5) and 0.60 ± 0.02/0.01 mm² in the Twist1^lox/+; Twist2^Cre/+ mice (n = 5, P < 0.05). (D–F) In situ hybridization analyses (signal in blue) of the transcripts of Alp (D), Ocn (E) and Dmp1 (F) in the femurs of the 6-day-old control and Twist1^lox/+; Twist2^Cre/+ mice. Scale bar = 100 μm.
Twist1 and Twist2 in Bone Formation

A promoter luciferase assay was performed as described previously [24,30]. Briefly, C57BL/6J/2 cells and MC3T3-E1 cells were plated in 24-well plates at a density of 3 x 10⁴ cells per well. Then the cells were transiently transfected with 0.1 μg of 4.9 kb-Fgf2 promoter luciferase construct, together with 0.4 μg of various constructs expressing Twist1, Twist2 or E12 using FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA). The total amounts of transfected DNA were balanced by the addition of an empty vector (pCDNA3). A Renilla luciferase expression construct was co-transfected as an internal control to monitor the transfection efficiency. Forty-eight hours later, the transfected cells were analyzed using a dual luciferase reporter assay system (Promega, Madison, WI, USA). The luciferase activities were normalized by the control. All experiments were carried out in triplicate and repeated three times.

Statistical analysis

The statistical analyses were performed with a one-way ANOVA for a multiple group comparison and the Student’s t-test for a two-group comparison. If significant differences were found with the one-way ANOVA, the Student’s t-test was used to determine which groups were significantly different from the others. The quantified results were expressed as mean ± standard deviation (SD). P<0.05 was considered to be statistically significant.

Results

Generation of Twist1- and Twist2-haploinsufficient mice

Twist1flox/+; Twist2Cre/+ mice were generated by breeding Twist1 floxed mice (Twist1floxed) with Twist2 Cre Knock-in mice (Twist2Cre/+), in which one allele of Twist2 was replaced by the Cre recombinase. Twist2-Cre is active in the condensed mesenchyme that will later produce the chondrocytes and osteoblasts [22]; therefore, in the compound Twist1floxed/+; Twist2Cre/+ mice, the floxed Twist1 allele is deleted in the osteoblasts and their precursors. Consequently, both Twist1 and Twist2 were haploinsufficient in the osteoblast lineage of the Twist1floxed/+; Twist2Cre/+ mice. Real-time PCR confirmed that the mRNA levels of both Twist1 and Twist2 were about three folds less in the Twist1floxed/+; Twist2Cre/+ mice than in the control mice (Figure S1). Although the floxed Twist1 allele was conditionally deleted, the compound Twist1floxed/+; Twist2Cre/+ mice had a phenotype similar to that of the mice completely heterozygous for both the Twist1 and Twist2 genes [31]. Most of them died within two weeks after birth but a few of them survived to adulthood and were fertile.

Reduced bone formation in Twist1floxed/+; Twist2Cre/+ mice

We first examined the overall skeletal structures of Twist1floxed/+; Twist2Cre/+ mice in Figure 1. The alcian blue/alizarin red staining showed that Twist1floxed/+; Twist2Cre/+ mice had a much smaller skeleton with delayed fusion of the interfrenal suture, open posterior fontanelles and delayed ossification in the metatarsals and phalanges (Figs. 1A–C). In addition, the Twist1floxed/+; Twist2Cre/+ mice developed an extra toe close to the hallux (Fig. 1C), the hallmark of Twist1 heterozygous mice [32]. Plain X-radiography showed that the Twist1floxed/+; Twist2Cre/+ mice had reduced radiopacity in the tibiae compared to the control mice (Fig. 1D). Micro-CT images further confirmed that the Twist1floxed/+; Twist2Cre/+ mice had reduced trabecular bone and decreased cortical bone thickness (Fig. 1E). The quantitative analyses revealed a significant decrease in trabecular bone volume versus total volume (BV/TV) and in apparent bone density in the Twist1floxed/+; Twist2Cre/+ mice (Figs. 1F–G). The material density was also slightly reduced in the Twist1floxed/+; Twist2Cre/+ mice although the difference in this parameter was not statistically significant compared to the control mice (Fig. 1H). These data demonstrated that bone formation was inhibited in the Twist1floxed/+; Twist2Cre/+ mice.

Defects in osteoblast differentiation in Twist1floxed/+; Twist2Cre/+ mice

We carried out a series of histological and molecular analyses to determine whether there were any abnormalities in osteoclast numbers and/or osteoblast differentiation. First, H&E staining confirmed that the Twist1floxed/+; Twist2Cre/+ mice formed less trabecular bone and thinner periosteum and cortical bone (Figs. 2A–B). Second, TRAP staining showed that the distribution and size of the osteoclasts in the Twist1floxed/+; Twist2Cre/+ mice were similar to those of the control mice (Fig. 2C). Although the osteoclast density was slightly increased in the Twist1floxed/+; Twist2Cre/+ mice, no significant difference was observed between two groups. Third, we examined the expression levels of the osteoblast differentiation markers by in situ hybridization, immunohistochemistry and real-time PCR. These methods revealed that the levels of the osteoblast differentiation markers Alp (Figs. 2D and 3), Ocn (Figs. 2E and 3), biglycan (Fig. 2H) and Bsp (Fig. 3)
were sharply reduced in the Twist1flox/+; Twist2Cre/+ mice, compared to the control mice. In addition, the osteocyte marker Dmp1 was also dramatically decreased (Fig. 2F and 3). Furthermore, the expression levels of Runx2 and Osx, two key transcription factors essential for osteoblast differentiation and bone formation, were remarkably reduced in the Twist1flox/+; Twist2Cre/+ mice, compared to the control mice (Figs. 2G and 3). Taken together, these findings supported the hypothesis that the reduced bone formation resulted from defects in osteoblast differentiation rather than abnormal osteoclast activities.

Reduced cell proliferation in Twist1flox/+; Twist2Cre/+ mice

Since the Twist1flox/+; Twist2Cre/+ mice had a reduced periosteal layer compared to the control mice (Fig. 2B), we performed BrdU labeling to determine whether the proliferation of osteoblasts and their progenitors was affected. We noticed that the BrdU-positive cells in the area of the metaphysis (Figs. 4A), as well as in the mid diaphysial periosteum and cortical bone (Fig. 4D), were significantly reduced in the Twist1flox/+; Twist2Cre/+ mice. However, no difference in the osteoblast apoptosis was observed (Figure S2). These findings suggested that the reduced cell proliferation might...
also contribute to the reduced bone formation in the Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice.

### Reduced FGF signaling in Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice

A variety of studies have indicated interactions between Twist1 and FGF signaling although the outcome of such interactions appears to be context dependent [15,17,18,33]. We analyzed the components of the FGF signaling pathway in the Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice. Quantitative real-time PCR revealed a substantial decrease in the transcript levels of Fgf2 and Fgfrs1, 2, 3 and 4 in the Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice (Fig. 5A). Immunohistochemistry further confirmed that the Fgfr2 protein was markedly reduced in the long bones of the Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice (Fig. 5B). FGF signaling triggered the MAP kinase cascade, resulting in the phosphorylation and activation of p44/42 (Erk1/2) and the expression of two effector molecules, Erm and Pea3 [34]. Consistent with the reduced Fgfr2 and Fgf expression, the immunohistochemistry revealed that the levels of phospho-Erk1/2 were considerably lower in Twist1\textsuperscript{flox/}; Twist2\textsuperscript{Cre/+} mice than in the control mice (Fig. 5C). Accordingly, quantitative real-time
ubiquitously in the epiblast cells at embryonic day 6.5 and together with E12, could regulate the expressed as luciferase activities relative to that of the control. The values represented mean ± SD. n = 3 for each group. "a" indicates significant difference from the control (p<0.05); "b" denotes a significant difference from all other groups (p<0.05).

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Figure 6. Effects of Twist1, Twist2 and E12 on the activity of a 4.9 kb Fgfr2 promoter fragment. C3H10T1/2 (A) and MC3T3-E1 cells (B) were transiently co-transfected with a 4.9 kb Fgfr2 promoter luciferase construct and the indicated expression constructs, along with a pRL-TK construct as an internal control. The luciferase activities were determined by a dual luciferase assay system, and the promoter activities were expressed as luciferase activities relative to that of the control. The values represented mean ± SD. n = 3 for each group. "a" indicates significant difference from the control (p<0.05); "b" denotes a significant difference from all other groups (p<0.05).

PCR demonstrated that the levels of Etm and Psa3 transcripts were significantly downregulated (Fig. 5A). These data demonstrated that FGF signaling was reduced in the Twist1flox/+; Twist2Cre/+ mice, suggesting that Twist1 and Twist2 might upregulate the expressions of Fgfr2 and Fgfr3.

Twist1 and Twist2 stimulated Fgfr2 promoter activity

As described above, Fgfr2 was significantly downregulated at both the mRNAs and protein levels in the Twist1flox/+; Twist2Cre/+ mice. The skeletal phenotype of Twist1flox/+; Twist2Cre/+ mice partially resembles that of the Fgfr2 conditional knock-out mice [22]. Therefore, we examined whether Twist1 and Twist2 could upregulate the Fgfr2 promoter activity in vitro. Our promoter luciferase reporter assay revealed that Twist1 or Twist2 alone was unable to significantly stimulate a 4.9 kb Fgfr2 promoter fragment. However, they strongly enhanced the stimulatory activity of E12, a ubiquitously expressed Twist binding partner, in both the C3H10T1/2 mesenchymal cells and MC3T3-E1 pre-osteoblast cells (Fig. 6). This in vitro evidence implied that Twist1 and Twist2, together with E12, could regulate the Fgfr2 expression.

Discussion

Twist1 and Twist2 are two highly conserved members of the Twist subfamily of bHLH transcription factors. In this study, we generated Twist1- and Twist2-haploinsufficient mice (Twist1flox/+; Twist2Cre/+), and found that these mice displayed delayed closure of the cranial sutures. Furthermore, we found that the Twist1flox/+; Twist2Cre/+ mice presented with reduced bone formation and impaired osteoprogenitor proliferation and osteoblast differentiation as well as compromised FGF signaling.

Previous studies have demonstrated that the cranial sutures of the Twist1 heterozygous mice prematurely fuse [10,11,32]. To confirm this finding, we crossed the Twist1-floxed mice (Twist1flox/+; Sox2-Cre transgenic mice expressing a Cre recombinase ubiquitously in the epiblast cells at embryonic day 6.5 and generated conditional Twist1-haploinsufficient mice (Twist1flox/+; Sox2-Cre) (Figure S5). We found that the Twist1flox/+; Sox2-Cre mice developed craniosynostosis - a phenotype that is similar to the Twist1 heterozygous mice [11,32], characterized by much narrower sagittal and interfrontal sutures and had additional toe originating from a duplicated metatarsal as demonstrated by alizarin blue and alizarin red staining (Figure S2A-D). The plain X-radiography and histological examination did not reveal apparent difference in the radiopacity of the long bones, in the metaphyseal trabecular bones or in the diaphyseal cortical bones between Twist1flox/+; Sox2-Cre and control mice (Figure S3E–G). In contrast, although the Twist2 heterozygous mice are viable and fertile with no apparent phenotype [20,31], the Twist1flox/+; Twist2Cre/+ mice showed reduced growth of both cranial and long bones as well as delayed closure of the cranial sutures. Therefore, the Twist1flox/+; Twist2Cre/+ mice displayed a skeletal phenotype that is opposite to that of Twist1 heterozygous mice. The skeletal phenotype of Twist1flox/+mice is similar to that of Twist2flox/+mice. Thus, the outcomes of the loss of Twist1 and/or Twist2 really depend on the relative expression of both genes in a specific tissue.

Accumulating evidence supports the notion that Twist1 might control cranial suture development through modulating FGF signaling. It was found that the mutations in FGF receptors FGFR1, FGFR2, and FGFR3 in humans are associated with craniosynostosis, a characteristic phenotype of the Saethre-Chotzen Syndrome caused by dominant loss-of-function TWIST1 mutations [35]. In addition, the primary cranial osteoblasts isolated from SCS patients with Twist1 mutations show reduced FGFR2 transcript levels, which can be restored by overexpression of Twist1 [18]. Twist1 haploinsufficiency in mice also results in an altered Fgfr2 expression pattern in the cranial sutures [17]. It has been proposed that Twist1 haploinsufficiency favors the formation of Twist1 homodimers in the osteogenic front of cranial.
sutures, which results in the upregulation of Fgf2 expression and leads to craniosynostosis [16]. Taken together, the data from these human and mouse studies suggest that Twist1 might regulate FGF signaling, particularly the Fgf2 expression, in a context-dependent manner.

Our current studies provide further support that Twist1 and Twist2 regulate FGF signaling in bone formation. We found that Twist1flox/+ Twist2flox/+ mice had reduced FGF signaling in bone as a result of decreased expression of not only Fgf2 but also Fgfr1, Fgfr3, Fgfr4, and even Fgfr2. FGF signaling plays essential roles throughout osteogenesis, including the commitment of mesenchymal cells to osteoprogenitors, the proliferation and differentiation of osteoprogenitors to osteoblasts, and osteoblast apoptosis [22,36]. Indeed, the Twist1flox/+ Twist2flox/+ mice displayed reduced proliferation of osteoprogenitor cells and defective osteoblast differentiation. Some of the skeletal abnormalities even resemble those of Fgf2 conditional knockout mice [22]. Consistent with the in vivo results, our in vitro promoter luciferase assays further supported the role of Twist1 and Twist2 in the upregulation of Fgf2 promoter activity when E12 was present. However, it remains to be determined why only Twist/E12 heterodimers, instead of Twist homodimers, stimulate Fgf2 promoter activity.

In summary, our current study suggested that Twist1 and Twist2 may synergistically enhance the proliferation and differentiation of osteoprogenitors via the upregulation of FGF signaling during skeletal development. However, further studies are necessary to determine whether these two genes perform identical functions and could completely replace each other in vivo. Research is also needed to advance our understanding of how the expression levels of Twist1 and Twist2 are temporally and spatially regulated during development and how fine-tuning achieves the optimal Twist protein level needed for normal skeletal development. Such knowledge is essential for the development of future therapies aimed at correcting the effects of Twist deficiency in humans.

Supporting Information
Figure S1 The mRNA levels of Twist1 and Twist2 in the long bones of Twist1flox/+; Twist2flox/+ mice. Real-time PCR was performed with total RNA isolated from the long bones of the 8-day-old control and Twist1flox/+; Twist2flox/+ mice. The primers used for Twist1 were sense 5'-CAGCGGGGTATGGC-TAAC-3' and antisense 5'-GCAGGACCCTGGTACAGGAAG-3', and for Twist2 sense 5'-AGCAAGAAATCGAGCGAAGA-3' and antisense 5'-CAGCTTGGGGTCTGATCT-3'. The mRNA levels of Twist1 and Twist2 were about three folds less in the Twist1flox/+; Twist2flox/+ mice than in the control mice. The data represented three analyses (n = 3) for each group. (TIF)

Figure S2 Osteoblast apoptosis in the Twist1flox/+; Twist2flox/+ mice. TUNEL assay was used to analyze the osteoblast apoptosis in the long bones of 6-day-old Twist1flox/+; Twist2flox/+ and control mice. Three serial sections from each of four individual Twist1flox/+; Twist2flox/+ mice and control littermates were counted. No significant difference in osteoblast apoptosis was found between the two groups of mice. (TIF)

Figure S3 Skeletal abnormalities of Twist1flox/+; Sox2-Cre mice. (A) The skeletons of 7-day-old Twist1flox/+ (control) and Twist1flox/+; Sox2-Cre mice were stained with alizarin blue and alizarin red. (B-C) Alcian blue and alizarin red stained skulls, femurs and tibiae, and hind feet from 7-day-old control mice and Twist1flox/+; Sox2-Cre mice. The Twist1flox/+; Sox2-Cre mice showed much narrower sagittal and interosseous sutures (arrows; B) and had additional toe (arrow originating from a duplicated metatarsal (MT), D), but the femurs and tibiae showed no apparent difference between two groups (C). (E) Plain X-radiography of the hind limbs from 7-day-old control and Twist1flox/+; Sox2-Cre mice. No apparent difference was noted between the two groups of mice. (F and G) Histological examination of Twist1flox/+; Sox2-Cre mice. Tibia sections of 7-day-old control and Twist1flox/+; Sox2-Cre mice were stained with H&E. No apparent difference was observed in the metaphyseal trabecular bone (F) or in the diaphyseal cortical bone (G) between these two groups. (TIF)

Materials and Methods S1 Generation of Twist1flox/+; Sox2-Cre mice. (DOCX)

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Author Contributions
Conceived and designed the experiments: YL. H. Performed the experiments: YH TM SW. Analyzed the data: YH GM YL. Contributed reagents/materials/analysis tools: HZ CQ JQF RND. Wrote the paper: YH YL.

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