Introduction

The vertebrate limb bud arises from the lateral plate mesoderm and then develops into three segments (including the stylopod, zeugopod and autopod) along the proximal-distal (PD) axis through an endochondral ossification mechanism. Limb patterning and morphogenesis are established by diffusible signals originating from different domains of the limb bud, including the Fgf, Wnt and RA signaling pathways [1]. In concert with these instructive morphogens, Hox genes (paralogous groups 9–13) are expressed in restricted domains along the axes of the limb buds in spatial and temporal colinearity, and provide positional information for limb patterning, skeletal condensation and differentiation. Expression of the Hoxd 9-13 genes in the limb bud is activated sequentially from Hoxd9 to Hoxd13 at the posterior border of the limb bud. The Hoxa9-13 genes are activated similarly to the Hoxa0-13 genes. The sequential activation of these genes correlate with the malformations in the limb skeletons of specific Hox mutants. For instance, compound mutants of Hoxa9/d9 have a shorter humerus and a loss of deltoid crest formation in the forelimb stylopod [2]. Quadruple Hox9 [Hox9aabbccdd] mutant mice exhibit severe forelimb defects with a loss of posterior skeletal elements including complete autopod loss and partial zeugopod loss, suggesting a role of Hox9 in forelimb anterior-posterior patterning [3]. The triple inactivation of Hox10 (Hox10aaccdd) results in severe agenesis in the hindlimb stylopod, and defects in the forelimb zeugopod and deltoid crest to a lesser degree [4,5]. Double mutants of Hoxa11/d11 selectively display deformities in the forelimb zeugopod [4,6], and triple mutants (Hoxa11aaccdd) demonstrate dramatic malformations of the fore- and hindlimb [5]. Additionally, double mutants of Hoxa13/d13 exhibit malformations in autopod development [7]. Elimination of all Hox and Hoxd genes results in early arrest of limb outgrowth, with severe truncations in distal elements [8]. These functional analyses highlight the synergistic and redundant role of the Hox genes in limb development: Hox9 participates in stylopod specification, Hox10/11 contributes to zeugopod formation and Hox12/13 regulates autopod development.

While the Hox genes have a universal role in a variety of developmental processes, the specificities of Hox function are achieved by interactions with co-factors such as the members of the TALE (Three Amino acid Loop Extension) superfamily. The TALE superfamily consists of transcription factors with atypical homeodomains including the Pbx, Meis and Pknox proteins. Similar to the Hox genes, these TALE genes are dynamically expressed in the limb bud. Genetic studies reveal that TALE genes regulate limb patterning and development of the skeletal elements. For instance, Pbx1 is exclusively expressed in the proximal limb bud, whereas Pbx2 is expressed throughout the limb mesenchyme [9]. Pbx1-deficient mice have malformations in the proximal limb.
elements, while double Pbx1/2 mutants exhibit distal limb deformities in addition to the presumptive stylopod skeleton in early limb bud [11]. Overexpression of the Meis1 gene in the limb bud shifts limb PD patterning and promotes the formation of proximal limb segments [11,12]. The TALE transcriptional factors could exert their effect by forming a heterotrimeric complex, such as Pbx-Meis-Hox or Pknox-Pbx-Hox, adding DNA binding specificity and affinity to the Hox proteins [10,13]. In addition, Pbx-Hox complex could directly activate the Shh expression as well as Hox expression in the regulation of limb development [9]. The Pknox subfamily members Pknox1/2 are also dynamically expressed in the avian limb bud [14,15]. Pknox1-null mutation is lethal in mice at E7.5 and hypomorphic Pknox1 mutant mice exhibit no obvious alterations in limb development [16,17]. However, the role of Pknox2 protein in limb development remains unclear.

Here, we overexpressed the Pknox2 genes in limb mesenchyme or osteochondroprogenitor cells in the early limb bud in transgenic mice. Misexpression of Pknox2 in the limb bud mesenchyme resulted in deformities in zeugopod elements and a loss of deltoid crest formation. The malformations in these transgenic mice were correlated with the perturbations of Hox10-11 gene expression profiles in the zeugopod elements. Therefore, Hox-dependent patterning alterations underlie, at least in part, the limb zeugopod defect in mice of Pknox2 misexpression.

Materials and Methods

Generation of Transgenic Mice

The 1.4 kb coding sequence CDS of Pknox2 gene (BC050865, ATCC) was cloned into a vector harboring the Prxl promoter [18], Col2a1 promoter [19] or rat Cola1-3.6 kb promoter [20], as previously described. Transgenic embryos were generated by microinjecting the linearized construct into fertilized oocytes of the ICR strain. Transgenic mouse strains were descendants from the founder animals. For genotyping, genomic DNA was extracted from mouse tails. Forward oligo used for Pknox2 transgenic mice: 5’-TCTGTTGGCAGCGAAAGTC-3’; forward oligo used for Col2a1-Pknox2 transgenic mice: 5’-AGGGTGTTGTGTTFTA-GAATGGGA-3’; forward oligo used for Cola1-Pknox2 transgenic mice: 5’-CCTCTGAGTGAAGACACGCCTC-3’; reverse oligo for transgenic mice: 5’-ATGGAGGATAGTTCAGGGCTT-3’.

Ethics Statement

All the mouse embryonic manipulations comply with the guidelines of the Bio-X Institutes of Shanghai Jiao Tong University (SYSK-SH-2011-0112).

Skeletal Preparations

Mouse embryos at P0 were eviscerated, and their skins were removed. Mice were fixed overnight in 95% ethanol followed by staining overnight in Alcian Blue and Alizarin Red solution (SIGMA) as previously reported [21].

Histology, in situ Hybridization and Immunohistochemistry

For histology and in situ hybridization, embryos were sacrificed at various ages, dissected, and fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight. After fixation, the tissues were dehydrated in 100% ethanol and embedded in paraffin. The embedded tissues were cut to generate 8 μm-thick sections and mounted onto slides. HE staining and Safranin O staining were performed following standard protocols. Whole mount in situ hybridization were performed as described [22]. Immunohistochemistry was conducted with antibody MpoD (BD, Cat#554130) and Col1a2 (Millipore, Cat#AB765P).

Results

Pknox2 is Mainly Expressed at the Zeugopod and Partial Stylopod Domains During Limb Bud Outgrowth

Pknox2 expression has been detected throughout the limb mesenchyme and is particularly strong in the mesenchyme underlying the ectoderm in the chick limb bud [15]. We examined Pknox2 expression during mouse limb bud development. Meis1, Hoxa11 and Hoxd13 are markers for the stylopod, zeugopod and autopod, respectively [12,23]. Compared with the expression of those markers, Pknox2 expression was first detected as a stripe in the central region of the hindlimb at E10.5, which was between the Meis1-expression domain and the progress zone (right panel of Fig. 1A). At E11.5, Pknox2 expression mostly overlapped with the Hoxa11-expression domain in the zeugopod, distinct from the Meis1 and Hoxd13-expression domains. Of note, the expression of Pknox2 was strong in the intermediate region, whereas Hoxa11 was preferentially expressed in the anterior and posterior borders of the presumptive zeugopod (Fig. 1B). In addition, Pknox2 was not only detected in similar regions in the forelimb as that in the hindlimb at E10.5 and E11.5, but was also partially present in the stylopod domain of the forelimb (left panel of Fig. 1A, B). At stages E12.5 and E13.5, Pknox2 expression was also detected in the digit joint region in the autopod in addition to the zeugopod domain (Fig. 1C), which was indicated by Gdf5 expression. Taken together, Pknox2 expression was mainly restricted to the zeugopod domains of both the forelimb and hindlimb buds. It was also partially expressed in the stylopod domain of the forelimb as well as the digit joint regions.

Ectopic Expression of Pknox2 in the Limb Mesenchyme Progenitors Impairs Zeugopod Development

To investigate the role of Pknox2 in limb development, we overexpressed Pknox2 cDNA in the mouse limb mesenchyme under the control of the 2.4 kb promoter of Prxl gene [18], represented by the diagram in Figure 2A. The Prxl promoter ectopically drove Pknox2 expression in the whole limb bud mesenchyme from E9.5 onward. Three stable transgenic lines were obtained with different phenotypic severities. According to the skeletal preparations at P0, they displayed obvious defects in the zeugopod elements of the forelimb and hindlimb, including a bent anterior radius/tibia and shortened ulna/fibia with disruption of ossification (black arrows in Fig. 2B-D). Yet the deformities were less severe in the hind limb compared to the forelimb (Fig. 2D). Additionally, the deltoid crest of forelimb stylopod was missing in the transgenic mice (red arrows in Fig. 2B-D). However, the autopod and other stylopod skeletons were largely intact in the transgenic mice. Moreover, the sternum, axial, facial and cranial skeletons were relatively normal in the Pknox2 transgenic mice, even though Prxl promoter has various activity in these tissues. Our findings suggest that Pknox2 misexpression selectively affects limb zeugopod development.

To molecularly characterize the zeugopod defects in the Prxl-Pknox2 transgenic mice, we examined the expression of zeugopod-related Hox genes in the forelimb at E11.5 by whole mount in situ hybridization. By E11.5, limb skeletal progenitor cells begin to condense and undergo chondrogenic differentiation. Compared to wild type (WT) controls, the expression domains of Hoxa10, Hoxd10 and Hoxd11 in Prxl-Pknox2 transgenic mice were anteriorly shifted (arrows in Fig. 3B, B’, C, C’, E and E’) and the posterior
region of the Hoxa11-expression domain was shortened (arrows in Fig. 3D, D') (n = 3/3). In contrast, the expression of Hoxd9 and Hoxd13 were largely unchanged (Fig. 3A, A', F and F') (n = 3/3). In addition, the expression levels of these Hox genes were not significantly altered based on qRT-PCR (Fig. S1). Therefore, the abnormality of zeugopod elements in the Prx1-Pknox2 transgenic mice might be correlated with alteration of the expression domains of zeugopod-related Hox genes.

Ectopic Expression of Pknox2 in Osteochondroprogenitor Cells Recapitulates the Forelimb Defects

To examine whether Pknox2 itself had a preferential activity in the skeletal progenitors, we generated Col2-Pknox2 transgenic mice that ectopically expressed Pknox2 in chondroprogenitor cells and most of the chondrocytes in all the skeletons (Fig. 4A). Three stable Col2-Pknox2 transgenic lines were generated and their skeletal preparations were analyzed at P0 (Fig. 4B). Compared with the Prx1-Pknox2 transgenic mice, similar but less severe phenotypes were observed in the Col2-Pknox2 mice, including partially fused carpals, anteriorly bent and shorter radius, and delayed ossification in forelimb zeugopod elements (Fig. 4C). The olecranon was missing and ectopic cartilage was formed in the upper side of the elbow joint region in Col2-Pknox2 mice (arrows in Fig. 4C). In addition, a small ectopic rib-like cartilage structure was detected in the L1 vertebrae of mutant axial skeletons; this structure was not observed in the control embryos (Fig. 4C). This result suggests that a partial homeotic transformation occurred in the axial skeletons of Col2-Pknox2 mice. In contrast, deltoid crest formation was lost in Prx1-Pknox2 mice but was relatively normal in Col2-Pknox2 mice. Interestingly, we found that the hindlimb skeletons in Col2-Pknox2 mice were also largely intact, suggesting that misexpression of Pknox2 selectively disrupts the forelimb zeugopod development.

To gain insight into the timing and appearance of the defects in Col2-Pknox2 mice, we analyzed forelimb zeugopod development by in situ hybridization and immunohistochemistry (IHC). Safranin O staining on the sections at E15.5 showed that chondrocyte hypertrophy and bone ossification were delayed in the radius and ulna (Fig. 5A). The chondrogenic differentiation was severely suppressed in the ulnas from Col2-Pknox2 mice at E15.5, as indicated by the decreased expression of Col2, Ihh and ColX (Fig. 5A), which are markers for non-hypertrophic, prehypertrophic and hypertrophic chondrocytes, respectively. At E12.5, HE staining showed that no clear condensation of the olecranon was observed and that the cells in this region were loosely organized in the transgenic embryos compared to WT controls (stars in Fig. 5B). In fact, early condensation and chondrogenic differentiation in the zeugopod elements were impaired, as revealed by the decreased Sox9 and Col2 expression in the proximal elbow-joint region of the transgenic mice at E12.5 (arrows in Fig. 5B). We also examined zeugopod-related Hox gene expression patterns in the E11.5 Col2-Pknox2 transgenic embryos, but no visible changes were detected (data not shown). Collectively, these results suggest that misexpression of Pknox2 disrupts zeugopod formation at the condensation stage and during early chondrogenic differentiation.
Bmp signaling is an important regulator of chondrogenesis during endochondral ossification.

Bmp7 and Bmpr1b double knockout mice have a nearly absent ulna and a shortened and bent radius [25]. To test whether overexpression of Pknox2 affects Bmp signaling activity, the protein expression of p-Smad1/5/8 was examined in the ulna and radius of Col2-Pknox2 transgenic embryos at E12.5 via IHC. Interestingly, p-Smad1/5/8 expression levels were markedly decreased in the developing ulna and radius of transgenic mice, whereas p-Smad1/5/8 expression levels were relatively normal in the humerus of transgenic mice and WT controls (Fig. 5B). These findings indicate that the effect of Pknox2 misexpression on zeugopod condensation and differentiation is partially mediated by the Bmp signaling pathway.

Ectopic Expression of Pknox2 in Osteoblasts Blocks Deltoid Crest Formation in the Forelimb

To investigate whether Pknox2 regulates zeugopod development at later stages of osteoblast differentiation, we also ectopically overexpressed Pknox2 in osteoblast lineages by generating Col1-Pknox2 transgenic mice (Fig. 6A). In these transgenic mice, Pknox2 expression was driven by the 3.6 kb rat Collagen I promoter (Colla1 3.6), which was active as early as the osteoblast progenitor stage. Interestingly, there were no apparent defects in other skeletal elements except for the missing deltoid crest in the forelimbs of the Col1-Pknox2 transgenic mice (arrows in Fig. 6B). The disruption of deltoid crest formation can be detected by HE staining at P0, when the deltoid crest primordia are initiated (Fig. 6C). The mesenchymal cells within the presumptive DC domain of the Col1-Pknox2 transgenic mice were accumulated at early stage as indicated by HE staining (Figure 6C). However, the later differentiation of DC cells was altered. IHC examinations using Col1 and MyoD antibodies revealed that the cell population within the deltoid crest of transgenic mice consisted of elongated muscle cells (arrows in Fig. 6D), rather than tendon or osteoblast cells. These results indicate that overexpression of Pknox2 disrupts deltoid crest formation at the stage of osteoblast differentiation.

Discussion

The members of the TALE family of transcriptional factors have pivotal roles in regulating limb patterning and morphogenesis as cofactors for Hox proteins. Previous studies have documented that the Meis1 gene is a good marker for proximal limb mesenchyme and regulates limb stylopod development. The
Pbx1/2 loss-of-function mutation affects the stylopod and autopod in the hindlimb [9]. In this study, we analyzed Pknox2 expression profiles and the effect of Pknox2 overexpression in limb development. Misexpression of Pknox2 in the limb mesenchyme or osteochondroprogenitor cells displayed deformities in zeugopod elements, partially resulting from perturbations of Hox10/11 gene expression domains and decreased Bmp signaling activity in the zeugopod region. Our results suggest that Pknox2 not only has a zeugopod-associated expression pattern, but also might be involved in the regulation of limb zeugopod and deltoid crest development. The further analysis of Pknox2 knockout mice would add our understanding of the function of Pknox2 in limb development.

In our study, misexpression of Pknox2 in the limb bud mesenchyme selectively interrupted zeugopod development. We generated transgenic mice by using three different promoters. Prx1 gene promoter drives gene expression in early mesenchymal progenitor cells including the offspring osteoblasts and chondrocytes [24]. Col2 and Col1 promoters activate gene expression in chondrocytes and osteoblasts, respectively. The transgenic mice driven by Col2 and Col1 promoter resembled partial defects of that in the Prx1-Pknox2 mice. The timing and types of alterations in Pknox2 transgenic mice are similar to those observed in several Hox gene loss-of-function mutants. The perturbation of deltoid crest development, ectopic cartilage formation in the upper elbow joint and presence of rib-like cartilage in L1 vertebrae in Col2-Pknox2 or Col1-Pknox2 mice are also observed in Hoxa9/d9 double deficient mice [2]. However, no obvious perturbations of Hox9 expression were detected in the forelimbs of the Prx1-Pknox2 transgenic mice. On the other hand, Hox10 functions in regulating hindlimb more than forelimb development, based on the defects in the hindlimb of the Hoxa10/d10-deficient mutants [26] and the triple Hox10-aaccd mutants [5]. Moreover, expression of Pknox2 together with Pbx1 or Hoxa10 in 293T cells did not affect Hoxa10-mediated activation of the Osteocalcin promoter based on a luciferase assay (data not shown). Therefore, it is likely that perturbations in Hox10/d10 expression profiles could not fully account for the forelimb zeugopod deformities caused by misexpression of Pknox2.

Figure 4. Skeletal preparations of Col2-Pknox2 transgenic mice. A: Schematic diagram for Col2-Pknox2 construct. B: Whole skeletons of WT and Col2-Pknox2 transgenic mice at P0. C: High-power view of the forelimb, carpal bones, elbow and hindlimb at P0. In comparison to WT, Col2-Pknox2 transgenic embryos exhibit defects including a bent radius and ulna with impaired ossification (black arrows), partially fused carpal bones (star) in the wrist, deformed elbow (black arrow: missed olecranon; red arrow: ectopic cartilage) and ectopic rib formation at lumbar vertebrae (L1, black arrow). N = 3.
doi:10.1371/journal.pone.0064237.g004

Figure 5. Disruption of chondrocyte differentiation and ossification in the zeugopod elements of Col2-Pknox2 mice. A: Chondrocyte differentiation is blocked at an early stage in Col2-Pknox2 transgenic embryos compared to WT, as revealed by Safranin O staining and in situ hybridization of Col2, Ihh and ColX in the ulna at E15.5 (50X, n = 2). B: HE staining showed that the outline of ulna and radius skeleton in the transgenic embryo was not as clear as that of WT (left panel). The expression levels of Col2 and p-Smad1/5/8, but not Sox9, were down-regulated in the ulna and radius skeletons of transgenic mice compared to WT controls at E12.5 (100X, n = 3), indicating that the chondrocyte condensation and differentiation is impaired upon Pknox2 overexpression.
doi:10.1371/journal.pone.0064237.g005
Double mutants of Hoxa11/Hoxd11 exhibit deformities in the forelimb [4,6], similarly to the Prx1-Pknox2 transgenic mice. Several lines of evidence have demonstrated that Pknox2 could form a heterotrimeric complex with the Hox and PBX proteins in vitro [14,27]. Therefore, we speculate that Pknox2 forms complex with Hox11 and Pbx1/2 in controlling Hox gene expression. Ectopic expression of Meis1 in the distal limb bud also results in abnormalities in forelimb zeugopod formation [11], but disrupts radius development in a Pbx1-independent manner [12]. Taken together, at least some of the defects observed in Prx1-Pknox2 mice may result from the combination of Hox10/11 gene activity in zeugopod elements.

The defects in the Prx1-Pknox2 mice were recapitulated to a lesser extent in mice ectopically expressing Pknox2 in osteochondrogenprogenitor cells. On the other hand, early limb patterning was largely intact in both the Prx1-Pknox2 and Col2-Pknox2 mice, which had relatively normal skeletal segmentation in the proximal-distal axis as well as normal anterior-posterior polarity in the autopod skeletal elements. Only chondrogenic differentiation and ossification were strikingly perturbed in the zeugopod elements of transgenic mice at E12.5. Misexpression of Pknox2 in the limb bud mesenchyme altered the expression of Hoxa10/d10 and Hoxa11/d11 in the zeugopod at E11.5. In fact, 5' Hoxa13 (Hoxa 9-15) genes have been shown to function at the mesenchymal condensation stage of skeletal formation [28]. For instance, Hoxa11 functions from the condensation stage onwards and programs chondrogenic and endochondral ossification [29]. Therefore, the anterior shift of the Hoxa10/d10 and Hoxa11 domains and a diminished Hoxa11-expression domain in the posterior side might lead to fewer mesenchymal progenitor cells available for ulna condensation and differentiation. These findings indicate that overexpression of Pknox2 perturbs zeugopod development at the condensation and chondrogenic differentiation stages as a co-factor of Hox proteins.

Another molecular event in mice with Pknox2 misexpression was the decrease of Bmp/Smad signaling in the zeugopod elements. Loss of Bmp7 and Bmpr1b in the mouse limb leads to malformations in the ulna, radius and the deltoid crest, similar to the phenotype observed in Prx1-Pknox2 transgenic mice [25]. Depletion of TGFβ2 also results in a slightly shortened zeugopod lacking a deltoid crest and olecranon [30]. Defects in deltoid crest formation in the Prx1-Pknox2 mice could be recapitulated in the Col1-Pknox2 mice but not in the Col2-Pknox2 mice, indicating that Pknox2 misexpression affects deltoid crest formation at the osteoblast differentiation stage. In fact, the deltoid crest is induced by the Bmp4 signal from neighboring tendon cells at E13.5 and then develops through endochondral ossification [31]. The decrease of Bmp/Smad signaling might be the consequence of altered Hox protein activity upon Pknox2 overexpression. For instance, it has been reported that Hoxa13 directly binds to the enhancer elements of Bmp2 and Bmp7 to regulate their expression [32]. In addition, a variety of Hox proteins differentially suppress the transcriptional activation of Smad proteins directly via protein interaction. Protein interaction of Hoxa13-Smad5 [33], Hoxc8-Smad1 [34], Hoxd13-Smad1 [33] and Hoxa9-Smad4 [35] have been reported in various limb tissues. In addition, a trimeric complex of Smad4-Pbx1-Pknox1 has been reported to regulate the promoter of the FSHβ gene [36]. Therefore, Pknox2 misexpression may suppress Bmp/Smad signaling activity during zeugopod development and deltoid crest formation as a co-factor for Hox genes.

**Supporting Information**

**Figure S1** qRT-PCR analysis of Hox10-11 gene expression in the limbs from the Prx1-Pknox2 embryos. qRT-PCR is performed for Hoxa10, Hoxa11, Hoxd10 and Hoxd11 genes in the limbs from Prx1-Pknox2 embryos at E12.5. No obvious alteration is detected in the expression levels of these genes. (TIF)
Acknowledgments

We thank Prof. Xiao Yang for providing us the plasmid construct with the Col1 (3.6 kb) promoter.

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Author Contributions

Conceived and designed the experiments: XZG. Performed the experiments: WRZ. Analyzed the data: WRZ. Contributed reagents/materials/analysis tools: H. Zhu JZZ HJL YW JJC H. Zhao JY RJZ YYY LLZ LW LH GM ZJY. Wrote the paper: XZG.