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# Self-regulated left-right asymmetric expression of Pitx2c in the developing mouse limb

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ABSTRACT

The transcription factor Pitx2c is expressed in primordial visceral organs in a left-right (L-R) asymmetric manner and executes situs-specific morphogenesis. Here we show that *Pitx2c* is also L-R asymmetrically expressed in the developing mouse limb. Human *PITX2c* exhibits the same transcriptional activity in the mouse limb. The asymmetric expression of *Pitx2c* in the limb also exhibits dorsal-ventral and anterior-posterior polarities, being confined to the posterior-dorsal region of the left limb. Left-sided *Pitx2c* expression in the limb is regulated by Nodal signaling through a Nodal-responsive enhancer. *Pitx2c* is expressed in lateral plate mesoderm (LPM)–derived cells in the left limb that contribute to various limb connective tissues. The number of Pitx2c$^+$ cells in the left limb was found to be negatively regulated by Pitx2c itself. Although obvious defects were not apparent in the limb of mice lacking asymmetric *Pitx2c* expression, Pitx2c may regulate functional L-R asymmetry of the limb.



## Introduction

Left-right (L-R) asymmetry of the vertebrate body is most obvious for visceral organs such as the heart and stomach. The process by which L-R asymmetry of visceral organs in the mouse is established can be divided into four phases: (1) initial determination of L-R polarity, which is achieved as a result of leftward fluid flow in the node (nodal flow); (2) transfer of an asymmetric signal from the node to lateral plate mesoderm (LPM); (3) asymmetric expression of signaling molecules such as Nodal and Lefty as well as of the transcription factor Pitx2 in LPM; and (4) asymmetric morphogenesis governed by Pitx2 (Shiratori and Hamada, 2006).

Visceral organs begin to develop anatomic asymmetries in distinct manners, such as directional looping for the heart and digestive tract, differential lobation for the lung, and unilateral regression for the vascular system. The main player in regulation of asymmetric organogenesis is Pitx2 (Davis et al., 2008; Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Logan et al., 1998; Lu et al., 1999; Piedra et al., 1998; Shiratori et al., 2006; Yashiro et al., 2007; Yoshioka et al., 1998), the asymmetric expression of which is induced by Nodal (Shiratori et al., 2001). The cellular basis of asymmetric organogenesis is not well understood, however. It thus remains unclear how each organ primordium interprets L-R information, or how Pitx2 regulates situs-specific morphogenesis of various visceral organs by seemingly different cellular mechanisms.

We now show that *Pitx2c* is asymmetrically expressed in the developing mouse forelimb. Although its biological significance is not clear, this asymmetric gene expression may be linked to functional L-R asymmetry of the limb.

## Material and methods

### *Animal care and treatment*

Animals were maintained in the Animal Facility of Graduate School of Frontier Biosciences, Osaka University under 14:10 h light/ dark cycle and were provided with food and water ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee of the Osaka University.

### *Generation of lacZ constructs and transgenic mouse assays*

Permanent transgenic mouse lines harboring *17-P1* (P26 and P44) or *Pitx2-Cre* (PC16) were established previously, and the *ASE0.9-P1* and *Human PITX2 ASE-lacZ* constructs were previously described (Shiratori et al., 2001, 2006). The *Pitx2c-lacZ/BAC*, *Pitx2c-lacZ/BAC2*, and *Pitx2c-lacZ/BAC3* transgenes, in which *lacZ* is positioned downstream of the

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*Pitx2c* promoter, were constructed from BAC clones containing mouse *Pitx2* (RP23-194G9, RP24-358M10, and RP23-317I22, respectively) with the use of the highly efficient phage-based recombination system for *Escherichia coli* (Copeland et al., 2001). The *Pitx2c-lacZ/BAC-ΔASE* transgene was constructed by deleting the 0.6-kb left side-specific enhancer (ASE) region from *Pitx2c-lacZ/BAC*. The *Human PITX2c-lacZ/BAC* transgene, in which *lacZ* is inserted downstream of *PITX2c* promoter, was constructed from BAC clone containing human *PITX2* (RP11-729A1). BAC DNA was prepared for microinjection as described (Gong et al., 2003). Various transgenes were also constructed from a vector (*P1-lacZ*) consisting of the 1.3-kb *P1* promoter region of mouse *Pitx2c* linked to *lacZ*. Test fragments of *Pitx2* ASE were individually subcloned into *P1-lacZ* at the 3′ end of *lacZ*. For generation of site-directed mutants, ASE fragments were first subcloned in pKF18 (Takara). Fragments containing mutated C1 (CTCTGGGGCGA→CTA-GATTTAGA) and C2 (TGGGGGGTGGGGG→CTTAATTGTTAAC) sites were similarly subcloned into the *P1-lacZ* vector. *Cryptic-CreER* and *Pitx2c-CreER* were constructed by linking *CreER* (Danielian et al., 1998) either to an 11-kb genomic fragment containing the LPM-specific enhancer of mouse *Cryptic* and the *Hsp68* promoter (Oki et al., 2007) or to a 7.5-kb genomic fragment containing ASE and the *P1* promoter of mouse *Pitx2c*, respectively. Transgenic mice were generated by injection of these various *lacZ* constructs into the pronucleus of fertilized eggs as described previously (Shiratori et al., 2001). Embryos were recovered at the indicated stages and were examined for *lacZ* expression by X-gal staining as well as for the presence of transgenes with the polymerase chain reaction. Permanent lines harboring *Pitx2c-lacZ/BAC*, *Pitx2c-CreER*, or *Cryptic-CreER* (PZB24, PCE23, and CCE28, respectively) were generated.

### *Generation of a Pitx2c$^{lacZ}$ knock-in mouse*

A targeting vector was designed to introduce *lacZ* downstream of the *Pitx2c* promoter (Fig. S1). Targeted embryonic stem cells were cultured and used for aggregation with ICR fertilized eggs at the 8- to 16-cell stage by standard methods. Male chimeras were bred with *CAG-Flpe* females (Kanki et al., 2006) to yield male offspring without the *PGK-neo* sequence, which were then mated with ICR females. The resulting heterozygous embryos were used for X-gal staining.

### *Tamoxifen treatment*

*Rosa26R* mice and *mTmG* mice (Soriano, 1999, Muzumdar et al., 2007) obtained from The Jackson Laboratory or *CAG-CAT-EGFP* (Kawamoto et al., 2000) mice provided by Jun-ichi Miyazaki (Osaka University) were crossed with *Pitx2c-CreER* or *Cryptic-CreER* mice. Tamoxifen (2.5 mg, Sigma) was mixed vigorously with 250 μl of sesame oil and administered to pregnant females by oral gavage at the indicated developmental stages.

### *In situ hybridization and immunohistofluorescence analysis*

Whole-mount in situ hybridization was performed according to standard protocols with a probe specific for all isoforms of *Pitx2*. For fluorescence in situ hybridization or immunohistofluorescence staining, embryos at E9.5 to E14.5 were fixed in 4% paraformaldehyde, immersed in phosphate-buffered saline containing 30% sucrose, and then embedded in OCT compound for preparation of frozen sections (thickness of 10 μm). In situ hybridization was performed according to standard protocols with 2,4-dinitrophenol– or digoxigenin-labeled riboprobes specific for *Pitx2*, *LacZ*, or *EGFP* mRNAs. Hybridized complexes were detected as Cy3 or Cy5 fluorescence with the use of a tyramide signal amplification system (Perkin-Elmer). Immunohistofluorescence staining was performed with rabbit antibodies to GFP (1:500 dilution, Molecular Probes), chicken antibodies to β-galactosidase (1:500 dilution, Abcam), mouse antibodies to skeletal muscle MHC (1:500 dilution; My32, Sigma), and rabbit antibodies to phosphorylated histone H3 (1:500 dilution, Merck). Immune complexes were detected with Alexa Fluor 488–conjugated antibodies to rabbit IgG, Alexa Fluor 568–conjugated antibodies to chicken IgG, or Alexa Fluor 647–conjugated antibodies to mouse IgG (each at a dilution of 1:500, Molecular Probes). Sections were examined with a laser-scanning confocal microscope (FV1000, Olympus).

### *X-gal staining*

*LacZ* transgenic embryos were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside) as described previously (Saijoh et al., 1999). E18.5 embryos were stained after the skin of the limb had been removed. For histological analysis, some embryos subjected to X-gal staining were embedded in paraffin, sectioned at a thickness of 10 μm, and then counterstained with nuclear fast red.

## Results

### *Pitx2c-lacZ transgenes are L-R asymmetrically expressed in the developing limb*

A *Pitx2c-lacZ* transgene (*17-P1*) that contains a 17-kb region of the mouse *Pitx2* gene with *lacZ* inserted immediately downstream of the *Pitx2c* promoter (Fig. 1A) was previously found to manifest L-R asymmetric expression in primordial visceral organs (Shiratori et al., 2001). We also now show that *17-P1* is expressed in a L-R asymmetric manner in the developing limb. This asymmetric expression of *17-P1* was first apparent in the forelimb bud at embryonic day (E) 9.5 (Fig. 1B), was subsequently evident in the forelimb and hindlimb at E11.5 (Fig. 1C), and persisted in the forelimb and hindlimb until E14.5 (Fig. 1D). Expression of the transgene exhibited not only L-R asymmetry but also dorsal-ventral (D-V) and anterior-posterior (A-P) polarities. Staining with the LacZ substrate X-gal was thus confined to the dorsal region on the posterior side in the left forelimb and hindlimb (Fig. 1B–E). Similar asymmetric expression was also observed with a bacterial artificial chromosome (BAC)–based *Pitx2c* transgene (*Pitx2c-lacZ/BAC*) in which *lacZ* is again positioned downstream of the *Pitx2c* promoter (Fig. 1F–M). The *Pitx2c-lacZ/BAC* embryos showed L-R asymmetric expression in the posterior-dorsal region of the developing limb as well as in primordial visceral organs at E9.5, E11.5, E14.5, and E18.5 (Fig. 1F–M). At E14.5 and E18.5, the left-sided expression of the transgene in the limb was apparent in cartilage, the outer layer of bone, tendon, and dermis (Fig. 1J–M). Four muscles around the radius and ulna—extensor carpi ulnaris, extensor digitorum, extensor digiti minimi, and extensor indicis—were also positive for X-gal staining (Fig. 1J–M). Muscle consists of myotubes and intramuscular connective tissue. We detected X-gal staining in cells negative for myosin heavy chain (MHC) of skeletal muscle (Fig. 1N), suggesting that *Pitx2c-lacZ/BAC* is expressed in intramuscular connective tissue rather than in myotubes (which are derived from somites). *LacZ* mRNA was asymmetrically detected in the limb of transgenic embryos with *Pitx2c-lacZ/BAC* at E11.5 and E14.5 (Fig. S2), suggesting that *Pitx2c-lacZ/BAC* is asymmetrically transcribed until later stage of limb development.

### *Endogenous Pitx2c is L-R asymmetrically expressed in the forelimb bud*

In the previous study (Marcil et al., 2003), L-R asymmetric expression of Pitx2 protein was not detected in the limb buds at E10.5 and E11.5. To examine endogenous *Pitx2c* expression in the

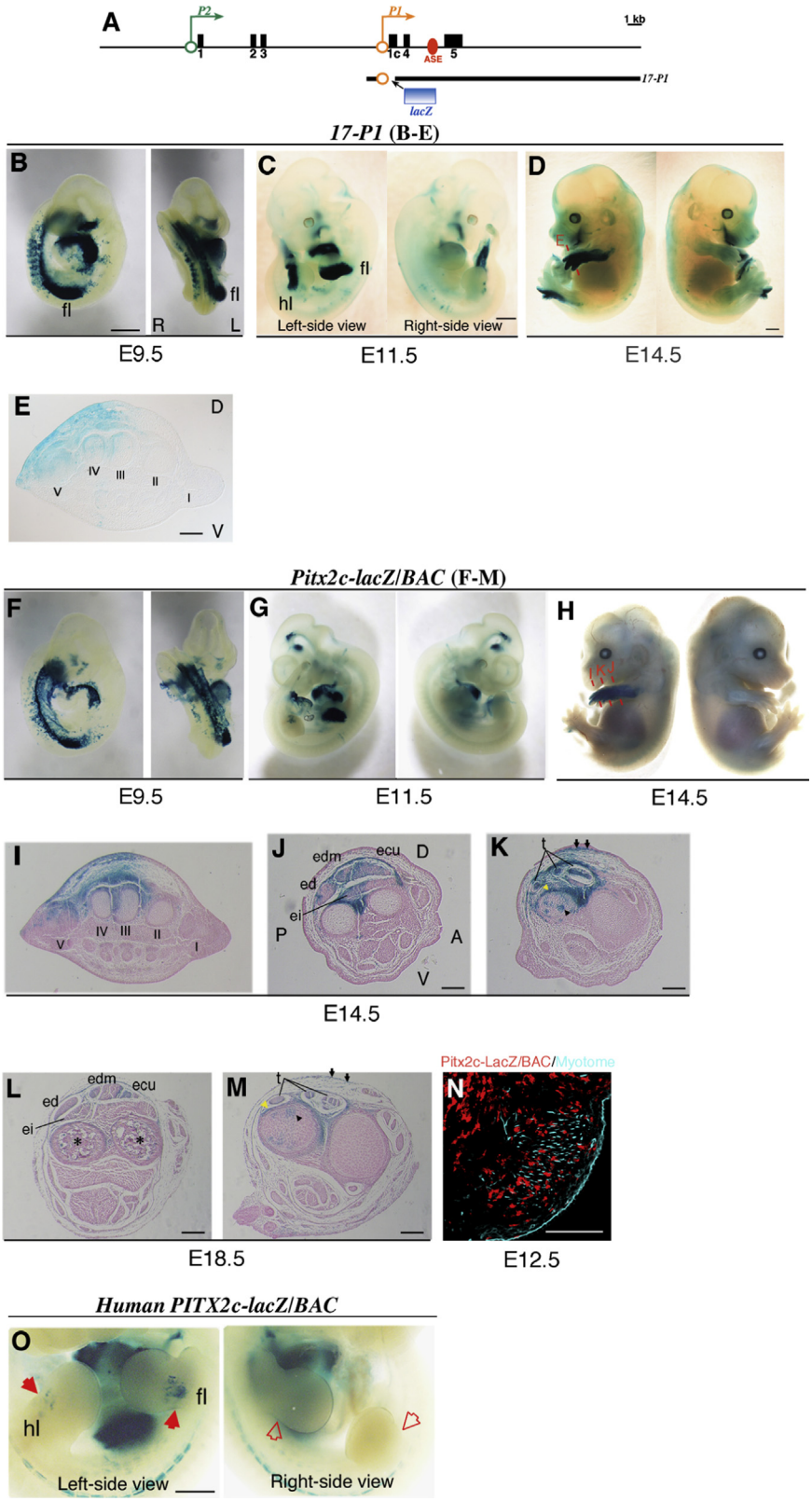


**Fig. 1.** Expression of *Pitx2c-lacZ* transgenes in the developing mouse limb. (A) The structure of the *17-P1 lacZ* construct is shown below that of the genomic region containing *Pitx2*. Exons (1, 2, 3, 1c, 4, and 5), two transcription initiation sites (*P1* and *P2*), and the enhancer for asymmetric expression (ASE) are indicated. (B–M) X-gal staining of E9.5 (B, F), E11.5 (C, G), E14.5 (D, E, H–K), and E18.5 (L, M) embryos harboring *17-P1* (B–E) or *Pitx2c-lacZ/BAC* (F–M). L-R, D-V, and A-P axes are indicated. I, II, III, IV, and V are digit numbers. Sections of the left forelimb (E, I–K) were taken at the levels indicated by the lines in (D) and (H). In both the forelimb (fl) and hindlimb (hl), *lacZ* expression is apparent only on the left side (B–D, F–H) and is confined to the dorsal region of the posterior side corresponding to digits III, IV, and V (E, I). The sections at the level of the radius and ulna (J, K) reveal *Pitx2c-lacZ/BAC* expression in several tissues, including cartilage (black arrowhead), the outer layer of bone (yellow arrowhead), tendon (t), dermis (arrows), and muscle (ecu, extensor carpi ulnaris; ed, extensor digitorum; edm, extensor digiti minimi; ei, extensor indicis). The E18.5 embryo harboring *Pitx2c-lacZ/BAC* (L, M) showed the same expression pattern as the E14.5 embryo. The staining of bone (asterisk) is background due to endogenous β-galactosidase activity (L). (N) Immunofluorescence staining of a section of the left forelimb of a *Pitx2c-lacZ/BAC* mouse embryo at E12.5 with antibodies to LacZ (red) and to skeletal muscle MHC (blue). The transgene is not expressed in myotomes. Scale bars: 200 μm. (O) X-gal staining of E11.5 embryo harboring *Human PITX2c-lacZ/BAC*. Left side–specific expression of *lacZ* is apparent in both the forelimb (fl) and hindlimb (hl), and is confined to the posterior region (arrows). Scale bars: 1000 μm (B–D, O), Scale bars: 200 μm (E, J–M).

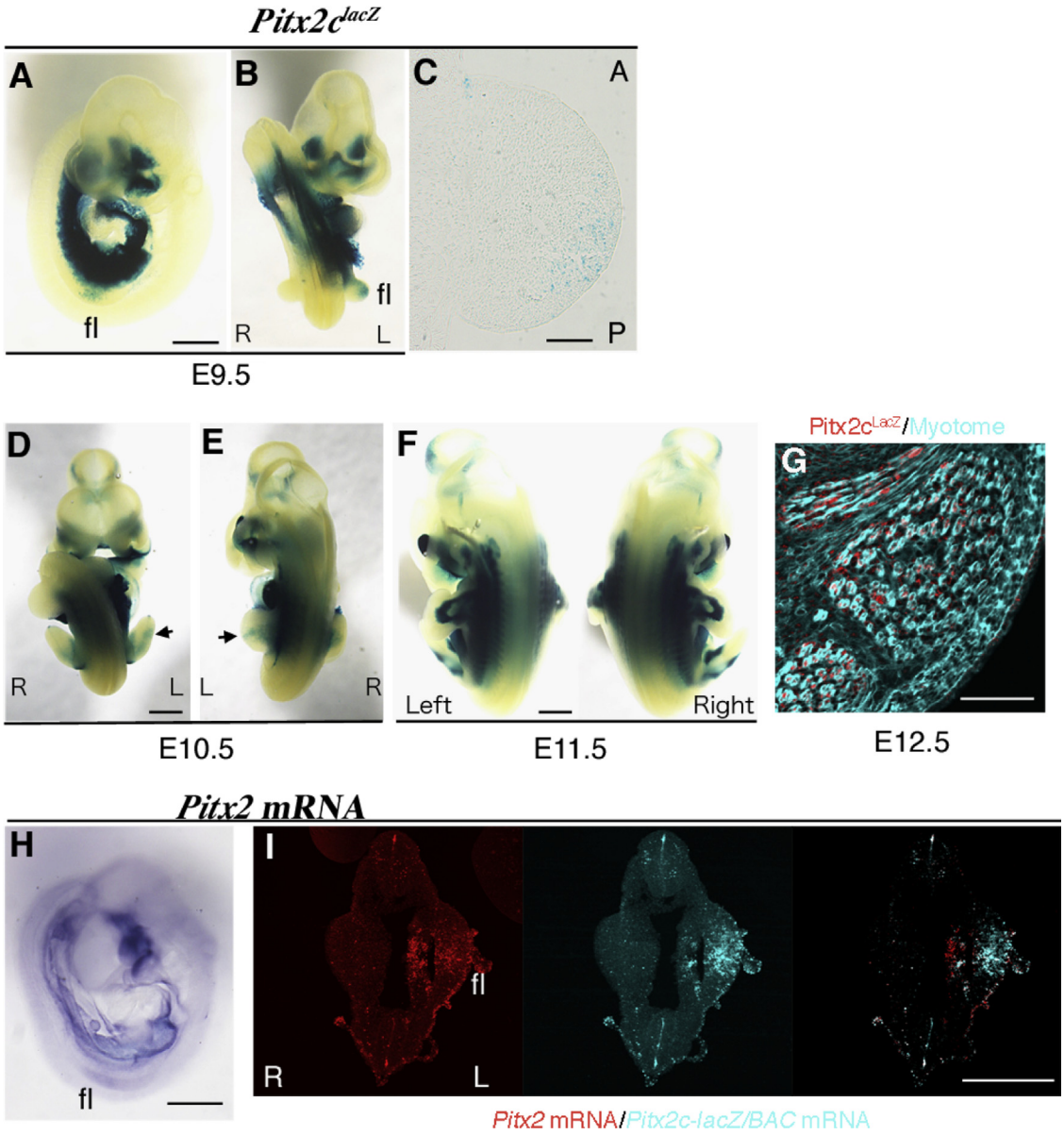


**Fig. 2.** Expression of endogenous *Pitx2c* in the developing limb. (A–F) X-gal staining of $Pitx2c^{lacZ/+}$ embryos at E9.5 (A–C), E10.5 (D, E), and E11.5 (F). Left side–specific expression of *lacZ* is apparent in the forelimb bud of the E9.5 embryo (A, B). A section of the left forelimb bud at E9.5 revealed that *lacZ* expression is confined to the posterior region (C). At E10.5, left-sided *lacZ* expression is apparent only on the dorsal side of the forelimb (arrows), whereas bilateral expression in forelimb myoblasts also begins (D, E). Only bilateral expression of *lacZ* is apparent in the limb of the E11.5 embryo (F). (G) Immunofluorescence staining of a section of the left forelimb of a $Pitx2c^{lacZ/+}$ embryo at E12.5 with antibodies to LacZ (red) and to skeletal muscle MHC (blue). Bilateral LacZ expression in the forelimb is apparent in myogenic cells. (H, I) Expression of endogenous *Pitx2* at E9.5 as revealed by whole-mount in situ hybridization (H) and fluorescence in situ hybridization (section) (I) with a probe that recognizes mRNA for all isoforms of Pitx2 (Pitx2a, Pitx2b, and Pitx2c). *Pitx2* (red fluorescence) is expressed in the left forelimb bud and is coexpressed with *LacZ* (blue fluorescence) in a *Pitx2c-lacZ/BAC* embryo (I). Scale bars: 1000 μm (A, D, F, H, I), Scale bars: 200 μm (C, G).

developing limb more precisely, we established a $Pitx2c^{lacZ}$ knock-in mouse (in which *lacZ* was introduced downstream of the *Pitx2c* promoter) by gene targeting (Fig. S1). $Pitx2c^{lacZ/+}$ mouse embryos manifested left-sided expression of the knock-in allele in primordial visceral organs as well as bilateral expression in other organs such as Rathke's pouch, the first branchial arch, and limb muscles (Fig. 2A, B, D–G). $Pitx2c^{lac/+Z}$ mice also showed L-R asymmetric *lacZ* expression in the forelimb bud at E9.5 (Fig. 2A and B), with the expression being confined to the posterior-dorsal region of the left limb bud (Fig. 2C). Such asymmetric *lacZ* expression was also apparent in the forelimb at E10.5, but it was not obvious at later stages (Fig. 2D–F). Furthermore, we detected asymmetric expression of endogenous *Pitx2* in the forelimb bud at E9.5 by in situ hybridization. *Pitx2* mRNA was thus localized to the posterior region of the left limb bud (Fig. 2H and I), as was *lacZ* expression in $Pitx2c^{lacZ/+}$ embryos. These results suggested that asymmetric expression of *17-P1* and *Pitx2c-lacZ/BAC* in the limb reflects that of endogenous *Pitx2c*. Whereas the $Pitx2c^{lacZ}$ allele recapitulates endogenous *Pitx2c* expression, both asymmetric and symmetric, *17-P1* and *Pitx2c-lacZ/BAC* appear to monitor only asymmetric expression, likely because they lack a transcriptional enhancer required for bilateral expression. To test this, we examined two additional BAC transgenes (*Pitx2c-lacZ/BAC2* and *Pitx2c-lacZ/BAC3*) that contain different genomic regions. Both transgenes manifested bilateral expression in addition to asymmetric expression in the limb (Fig. S3). However, their bilateral expression was weaker than the asymmetric expression (Fig. S3), suggesting that transcriptional regulatory elements required for bilateral expression reside in part outside of the regions contained in the three BAC clones tested. We therefore adopted *17-P1* and *Pitx2c-lacZ/BAC* for further study of asymmetric *Pitx2c* expression.

*Asymmetric expression of Pitx2c in the developing limb requires Nodal signaling*

Asymmetric expression of *Pitx2c* in primordial visceral organs is regulated by Nodal signaling (Shiratori et al., 2001, 2006). To investigate the regulation of asymmetric *Pitx2c* expression in the developing limb, we first examined the expression of *17-P1* and *Pitx2c-lacZ/BAC* in various L-R mutant mice. In *iv/iv* embryos, node cilia are immotile and fail to generate nodal flow, resulting in randomized laterality of visceral organs (Layton, 1976; Okada et al., 1999). In *inv/inv* embryos, the situs of visceral organs is reversed on the FVB background and is randomized on the B6 congenic background (Tamakoshi et al., 2006). We found that the asymmetric expression of *17-P1* in the limb was randomized both in *iv/iv* embryos and in *inv/inv* embryos on the B6 congenic background (Fig. 3A–D, Table S1). The asymmetric expression of the $Pitx2c^{lacZ}$ allele in the forelimb buds of E9.5 embryos was also randomized in *iv/iv* homozygotes (Fig. 3E and F, Table S1). These results thus suggested that left side–specific expression of *Pitx2c* in the limb is under the control of nodal flow. Cryptic and Gdf1 are cofactors essential for Nodal signaling, and left-sided expression of *Pitx2c* in LPM at E8.2 is absent in $Cryptic^{-/-}$ and $Gdf1^{-/-}$ embryos. In these mutant embryos, *17-P1* and *Pitx2c-lacZ/BAC* failed to give rise to X-gal staining in the limb at E11.5 (Fig. 3G and H, Table S1). $Pitx2c^{lacZ/+}$; $Cryptic^{-/-}$ embryos also failed to show asymmetric expression of *lacZ* in the limb bud at E9.5 (Fig. 3I, Table S1). Together, these data indicated that Nodal signaling is essential for the asymmetric expression of *Pitx2c* in the developing limb.

ASE is a Nodal-responsive enhancer of *Pitx2* that is essential and sufficient for asymmetric expression of *Pitx2*c in primordial visceral organs (Shiratori et al., 2001). Deletion of a 0.6-kb region containing ASE from *Pitx2c-lacZ/BAC* (giving rise to *Pitx2c-lacZ/BAC-ΔASE*) abolished asymmetric X-gal staining in the limb at E11.5 (Fig. S4A and D). Conversely, the construct *ASE0.9-P1*, which contains ASE and the *P1* promoter of *Pitx2c* linked to *lacZ* manifested left side-specific expression in the developing limb. However, the D-V and A-P polarities apparent with *17-P1* and *Pitx2c-lacZ/BAC* were disrupted with *ASE0.9-P1*, as evident by the presence of X-gal staining in the anterior-ventral and posterior regions of the forelimb as well as the anterior region of the hindlimb (Fig. S4A–C). These results indicated that ASE is essential for L-R asymmetric expression of *Pitx2c* in the developing limb but is insufficient for posterior-dorsal specificity. In addition to ASE, a region (region A) seemed to be required for the A-P and D-V polarities of *Pitx2c-lacZ* expression (Fig. S4A–C).

*Human PITX2c-lacZ transgene is also L-R asymmetrically expressed in the developing mouse limb*

The L-R asymmetric expression of *Pitx2* in primordial visceral organs is conserved among vertebrates. We examined whether *Human PITX2c-lacZ/BAC*, in which *lacZ* is inserted downstream of the *PITX2c* promoter, shows L-R asymmetric expression in the developing mouse limb. *Human PITX2c-lacZ/BAC* indeed showed activity similar to the mouse *Pitx2c-lacZ/BAC*. Thus, *Human PITX2c-lacZ/BAC* was also expressed in the posterior-dorsal region of the left limb at E11.5 (Fig. 1O). These results suggested that human *PITX2c* also is L-R asymmetrically expressed in the developing human limb.

*Two conserved sequences, C1 and C2, in the ASE are essential for asymmetric Pitx2c expression in the limb*

Asymmetric *Pitx2c* expression in visceral organs requires FoxH1 binding sites and an Nkx2 binding site in the ASE. The former sites are required for initiation of *Pitx2c* expression, whereas the latter is required for its maintenance (Shiratori et al., 2001). When the ASE region of *Pitx2c* was further examined, we found two sequences (C1 and C2) that are conserved between mouse and human *Pitx2* genes (Fig. S5). Deletion analysis (Fig. S5A and B) suggested that they are essential for asymmetric *Pitx2c* expression in the limb.

*Asymmetric Pitx2c expression in LPM-derived cells of the developing limb*

Mesenchymal cells of visceral organs that manifest asymmetric *Pitx2c* expression at late somite stages are derived from LPM. We examined whether *Pitx2c* expression in the developing limb occurs in LPM-derived cells. We generated *Cryptic-CreER* transgenic mouse that expresses CreER (a tamoxifen-activated form of Cre recombinase) under the control of the LPM-specific enhancer of *Cryptic* (Oki et al., 2007), to mark LPM-derived cells (Fig. S6). Treatment of pregnant mice harboring *Cryptic-CreER* and the *CAG-CAT-EGFP* or the *mTmG* reporter gene (Kawamoto et al., 2000, Muzumdar et al., 2007) with tamoxifen at E7.5 resulted in the efficient marking of LPM-derived cells with enhanced green fluorescent protein (EGFP) or membrane-targeted GFP at all subsequent embryonic stages examined. Transgenic embryos harboring *Pitx2c-lacZ/BAC*, *Cryptic-CreER* and *CAG-CAT-EGFP* were then exposed to tamoxifen at E7.5 and dissected at E10.5 or E12.5. Cells positive for both LacZ and EGFP were detected in the left forelimb at both E10.5 and E12.5 (Fig. 4A and B, Table S2), suggesting that *Pitx2c-lacZ/BAC* is expressed in LPM-derived cells of the forelimb. Some of the $LacZ^{+}$ cells are negative for EGFP, which is probably due to the limited efficiency of cell labeling by Cre-ER. Similarly, when $Pitx2c^{lacZ/+}$ embryos harboring both *Cryptic-CreER* and *mTmG* were exposed to tamoxifen at E7.5, the cells expressing *LacZ* mRNA in the left forelimb were also positive for membrane-targeted GFP at E9.5 (Fig. 4C and D). These results indicated that *Pitx2c* is asymmetrically expressed in LPM-derived mesenchymal cells of the developing limb.

To clarify whether expression of *Pitx2c-lacZ/BAC* is maintained in LPM-derived cells from E8.2, we examined the fates of *Pitx2c*-expressing cells with the use of a *Pitx2c-CreER* transgene. Embryos harboring *Pitx2c-CreER*, *CAG-CAT-EGFP* and *Pitx2c-lacZ/BAC* transgenes were exposed to tamoxifen in utero at E8.2 and harvested at E10.5 or E12.5. Only ~20% of the $EGFP^{+}$ cells in the left forelimb at either E10.5 or E12.5 were $LacZ^{+}$ (Fig. 5A–D). When the embryos were exposed to tamoxifen at E9.5, ~70% of $EGFP^{+}$ cells in the left forelimb at E12.5 were $LacZ^{-}$ (Fig. 5E and F). These results suggested that many of the cells expressing *Pitx2c-lacZ/BAC* at E8.2 or E9.5 terminate its expression as development proceeds.

*Fate of cells manifesting asymmetric Pitx2c expression in the limb bud*

To clarify the fate of *Pitx2c*-expressing cells, we crossed *Pitx2c-Cre* or *Pitx2c-CreER* mice with *Rosa26R* reporter mice, which express *lacZ* in a Cre-dependent manner (Soriano, 1999). Whereas expression of *lacZ* induced by *Pitx2c-CreER* and tamoxifen treatment at E9.5 was apparent in the posterior-dorsal region of the left forelimb at E14.5 (Fig. 5G and H), that induced by *Pitx2c-CreER* and tamoxifen treatment at E8.2 (Fig. 5I-L) or by *Pitx2c-Cre* (Fig. 5M and N) was detected in the left limb without A-P and D-V polarities. These results indicated that, whereas cells expressing *Pitx2c* in left LPM at E8.2 contribute to the left forelimb without A-P and D-V polarities, those expressing *Pitx2c* in the left forelimb bud at E9.5 contribute to various mesenchymal tissues (cartilage, outer layer of bone, tendon, dermis, and muscle connective tissue) in the posterior-dorsal region of the left forelimb.

*Pitx2c inhibits cell proliferation in the developing limb in a cell-autonomous manner*

To elucidate the role of Pitx2c produced asymmetrically in the developing limb, we examined the expression of *Pitx2c-lacZ/BAC* and

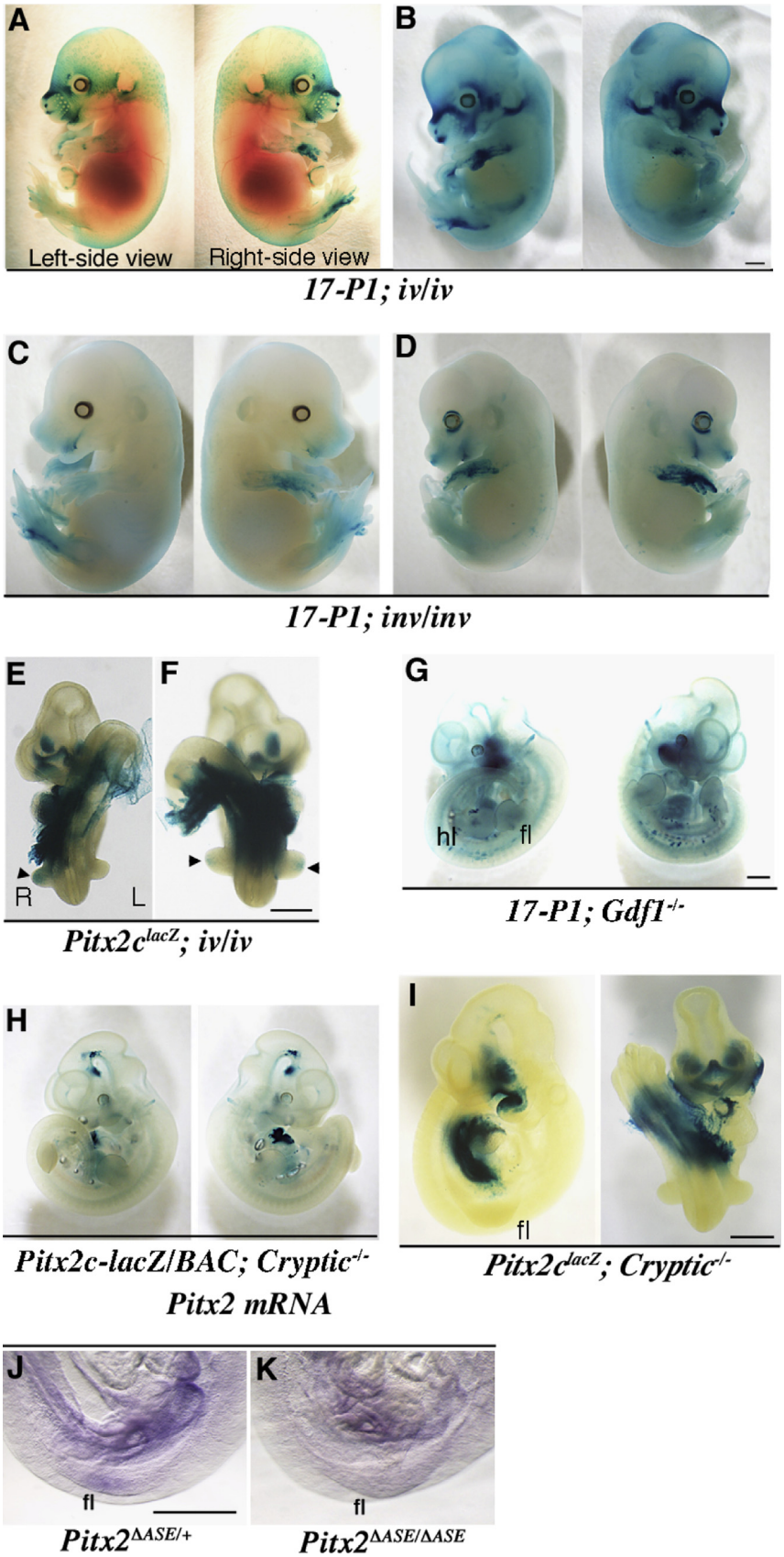


**Fig. 3.** Expression of *Pitx2c-lacZ* transgenes and endogenous *Pitx2c* in the limb of L-R mutants. (A–D) X-gal staining of *iv/iv* (A, B) and *inv/inv* (C, D) embryos harboring *17-P1* at E14.5. In *iv/iv* embryos or *inv/inv* embryos on the B6 congenic background, *lacZ* expression in the limb was randomized (right-sided (A), bilateral (B and D), or right-sided in the forelimb and left-sided in the hindlimb (C)). (E, F) X-gal staining of $Pitx2c^{lacZ/+}$; *iv/iv* embryos at E9.5. Asymmetric *lacZ* expression in the forelimb bud is randomized, with the arrowheads indicating right-sided (E) and bilateral (F) expression. Scale bars: 1000 μm. (G) X-gal staining of a $Gdf1^{-/-}$ embryo harboring *17-P1* at E11.5. Asymmetric *lacZ* expression is not apparent in left and right limbs. (H) X-gal staining of a $Cryptic^{-/-}$ embryo harboring *Pitx2c-lacZ/BAC* at E11.5. Asymmetric *lacZ* expression is absent in left and right limbs. (I) X-gal staining of a $Pitx2c^{lacZ/+}$; $Cryptic^{-/-}$ embryo at E9.5. Asymmetric *lacZ* expression is lost in the forelimb bud. (J, K) Expression of endogenous *Pitx2* was examined at E9.5 by in situ hybridization with a probe for *Pitx2*. Left-sided expression in the limb is apparent in $Pitx2^{\Delta ASE/+}$ embryo (J) but absent in $Pitx2^{\Delta ASE/\Delta ASE}$ embryo (K). Scale bars: 1000 μm.

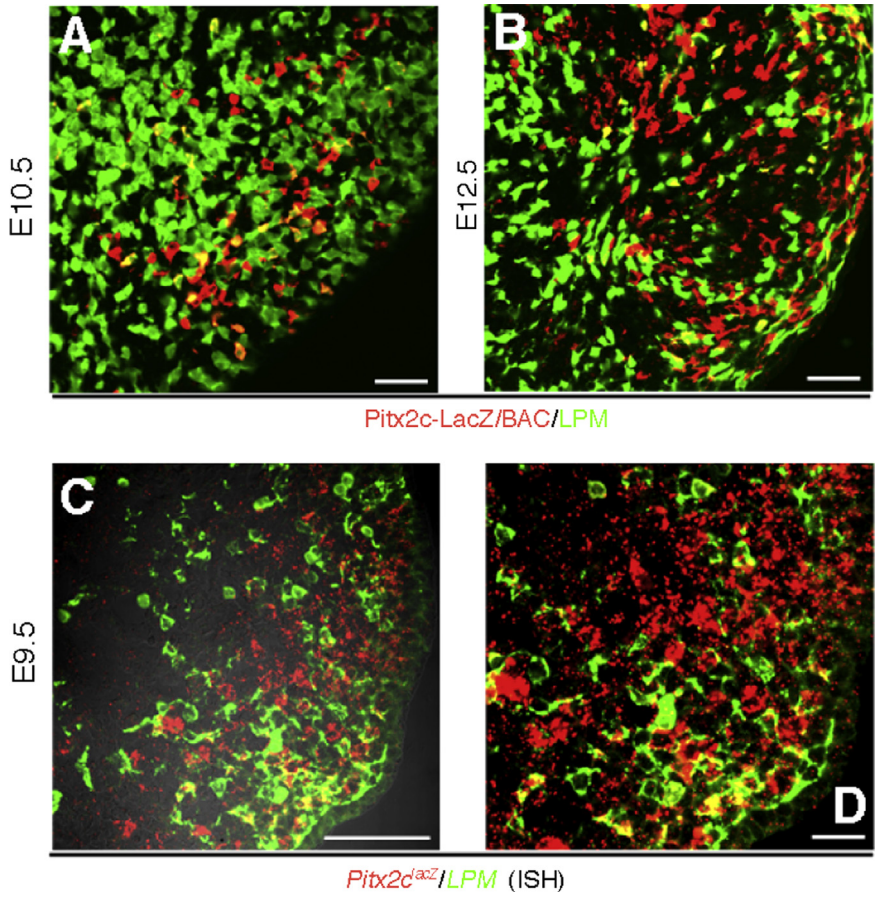


**Fig. 4.** Origin of *Pitx2c*-expressing cells in the left limb. *Pitx2c-lacZ/BAC; Cryptic-CreER; CAG-CAT-EGFP* (A, B) and *Pitx2c*$^{lacZ/+}$; *Cryptic-CreER*; *mTmG* (C, D) embryos were exposed to tamoxifen in utero at E7.5 and then collected at E10.5 (A), E12.5 (B), or E9.5 (C, D). A higher magnification image of (C) is shown in (D). Sections of the left forelimb were subjected either to immunofluorescence staining with antibodies to GFP and to LacZ (A, B) or to fluorescence in situ hybridization (ISH) with the *LacZ* probe coupled with immunofluorescence staining with an antibody to GFP (C, D). LPM-derived cells (green) are marked by the presence of EGFP (A, B) or menbrane-targeted GFP protein (C, D), with some of these cells also being found to be positive for LacZ protein or mRNA (red). Scale bars: 100 μm (A, B, D), Scale bars: 400 μm (C).

the fate of Pitx2c$^+$ cells in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos, which lack endogenous left-sided *Pitx2* expression (Shiratori et al., 2006) including that in the developing limb (Fig. 3J and K). Expression of *Pitx2c-lacZ/BAC* in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos was similar to that in the wild type (Fig. S7). However, the progeny of *Pitx2c*-expressing cells in the limb were increased in number in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos. *Pitx2c*-expressing cells were labeled at E9.5 with *Pitx2c-CreER* and *Rosa26R* transgenes, and their progeny were examined at E14.5. Unexpectedly, the number of LacZ$^+$ cells in the left forelimb was significantly higher in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos than in *Pitx2*$^{\Delta ASE/+}$ embryos, whereas the number of LacZ$^+$ cells in the heart was similar for the two genotypes (Fig. 6A–F, S). Similarly, when *Pitx2c*-expressing cells were marked at E9.5 and examined at E12.5, or were labeled at E12.5 and examined at E14.5, the number of LacZ$^+$ cells in the left forelimb was greater in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos compared with *Pitx2*$^{\Delta ASE/+}$ embryos (Fig. 6G–R, T). These results suggested that Pitx2c negatively regulates cell proliferation in the left forelimb in a cell-autonomous manner.

Finally, we examined the proliferation of *Pitx2c-lacZ/BAC*–expressing cells in the left forelimb directly by immunostaining with antibodies to phosphorylated histone H3. LacZ$^+$ cells in the extensor digiti minimi, one of the muscles that express *Pitx2c-lacZ/BAC* in the forelimb at E14.5, showed a higher proliferation rate in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos than in *Pitx2*$^{\Delta ASE/+}$ embryos both at E14.5 and at E12.5 (Fig. 6U). Again, these results indicated that Pitx2c inhibits cell proliferation in the developing left forelimb in a cell-autonomous manner.

## Discussion

We have here shown that *Pitx2c* is L-R asymmetrically expressed in the developing mouse limb. Although the limb is considered a symmetric organ, our finding suggests that L-R asymmetry, anatomic or functional, may exist in the limb.

The expression of both *17-P1* and *Pitx2c-lacZ/BAC* in the limb exhibited A-P and D-V polarities in addition to L-R asymmetry, with expression being confined to the posterior-dorsal region of the left limb. In addition to ASE, a region (region A) is required for the A-P and D-V polarities. This region contains multiple ATTA sequences, potential binding sites for Hox proteins, and two consensus binding sequences for TCF/LEF proteins. A group of *Hox* genes are expressed in the posterior region of the limb bud while *Wnt7a* is expressed on the dorsal side of developing limb, suggesting that such proteins may determine the A-P and D-V specificity of asymmetric *Pitx2c* expression in the limb.

We found that asymmetric *Pitx2c* expression in the limb depends on ASE and that two sequences (C1 and C2) located in the ASE and conserved between mouse and human *Pitx2* genes are essential for *Pitx2c* expression in the limb. Given that both C1 and C2 contain the sequence TGGGG, a potential binding site for Gli, Gli-related transcription factors may be required for *Pitx2c* expression in the limb at developmental stages later than E8.2. Thus, limb expression of *Pitx2c* is likely to receive regulatory input from the Shh signaling derived from the posterior side of limb bud.

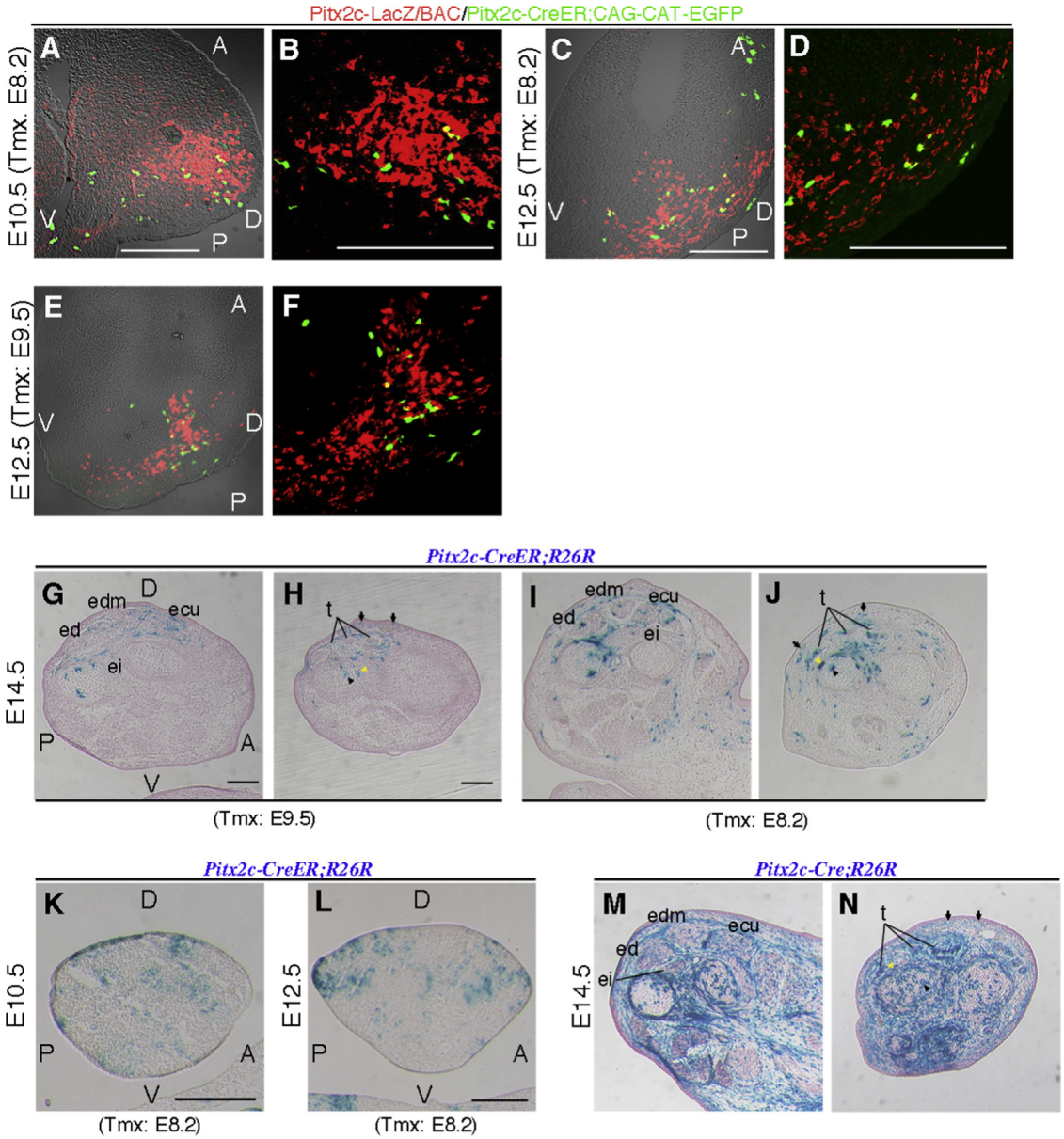


**Fig. 5.** Fate of *Pitx2c*-expressing cells in the left forelimb. (A–F) Mouse embryos harboring *Pitx2c-lacZ/BAC*, *Pitx2c-CreER*, and *CAG-CAT-EGFP* transgenes were exposed to tamoxifen (Tmx) in utero at E8.2 (A–D) or E9.5 (E, F) and harvested at E10.5 (A, B) or E12.5 (C–F). Sections of the left forelimb of the embryos were subjected to immunofluorescence staining with antibodies to LacZ and to GFP. Many EGFP$^{+}$ cells (green) do not express LacZ (red) regardless of the developmental stage for either tamoxifen treatment or embryo harvesting. (G–N) Embryos harboring *Pitx2c-CreER* and *Rosa26R* (*R26R*) transgenes and exposed to tamoxifen in utero at E9.5 (G, H) or E8.2 (I-L) as well as those harboring *Pitx2c-Cre* and *Rosa26R* transgenes (M, N) were harvested at E10.5 (K), E12.5 (L) or E14.5 (G-J, M, N). Sections of the left forelimb of the embryos were then stained with X-gal. Staining in the left forelimb either was apparent in several tissues including cartilage (black arrowheads), the outer layer of bone (yellow arrowheads), tendon (t), dermis (arrows), and muscles (ecu, ed, edm, and ei) without A-P and D-V polarities (I–N), or was limited to these tissues in the posterior-dorsal region (G, H). Scale bars: 500 μm.

*Pitx2c* is expressed in LPM-derived cells in the left forelimb. Our results suggest that LPM cells that express *Pitx2c* at E8.2 contribute to the limb without A-P and D-V polarities. After E9.5, *Pitx2c* expression is maintained only in the posterior-dorsal region of the forelimb bud whereas many of Pitx2c$^{+}$ cells gradually terminate its expression as development proceeds (Fig. 7). We also found that the proliferation rate of *Pitx2c-lacZ/BAC* expressing cells in the left forelimb of *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos was higher than that for *Pitx2*$^{\Delta ASE/+}$ embryos, suggesting that Pitx2c negatively regulates cell proliferation in a cell-autonomous manner. Such a mechanism might be expected to allow precise determination of the number of originally Pitx2c$^{+}$ cells that will contribute to the posterior-dorsal region of the left limb (Fig. 7). Pitx2 is known to regulate cell proliferation. In the developing cardiac outflow tract and pituitary gland, Pitx2 positively regulates cell proliferation by serving as a competence factor for induction of cyclin D2 gene transcription (Kioussi et al., 2002). Similarly, overexpression of Pitx2c was found to promote proliferation of skeletal muscle progenitor cells (Martinez-Fernandez et al., 2006). How then might Pitx2c negatively regulate cell proliferation in the limb bud? Given that the size of the left limb is indistinguishable between wild-type and *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice and that there is no significant difference in the percentage of proliferating cells in the extensor digiti minimi of the left limb between *Pitx2*$^{\Delta ASE/+}$ and *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice, the reduced number of Pitx2c$^{+}$ cells in the former may be compensated for by an increase in the number of

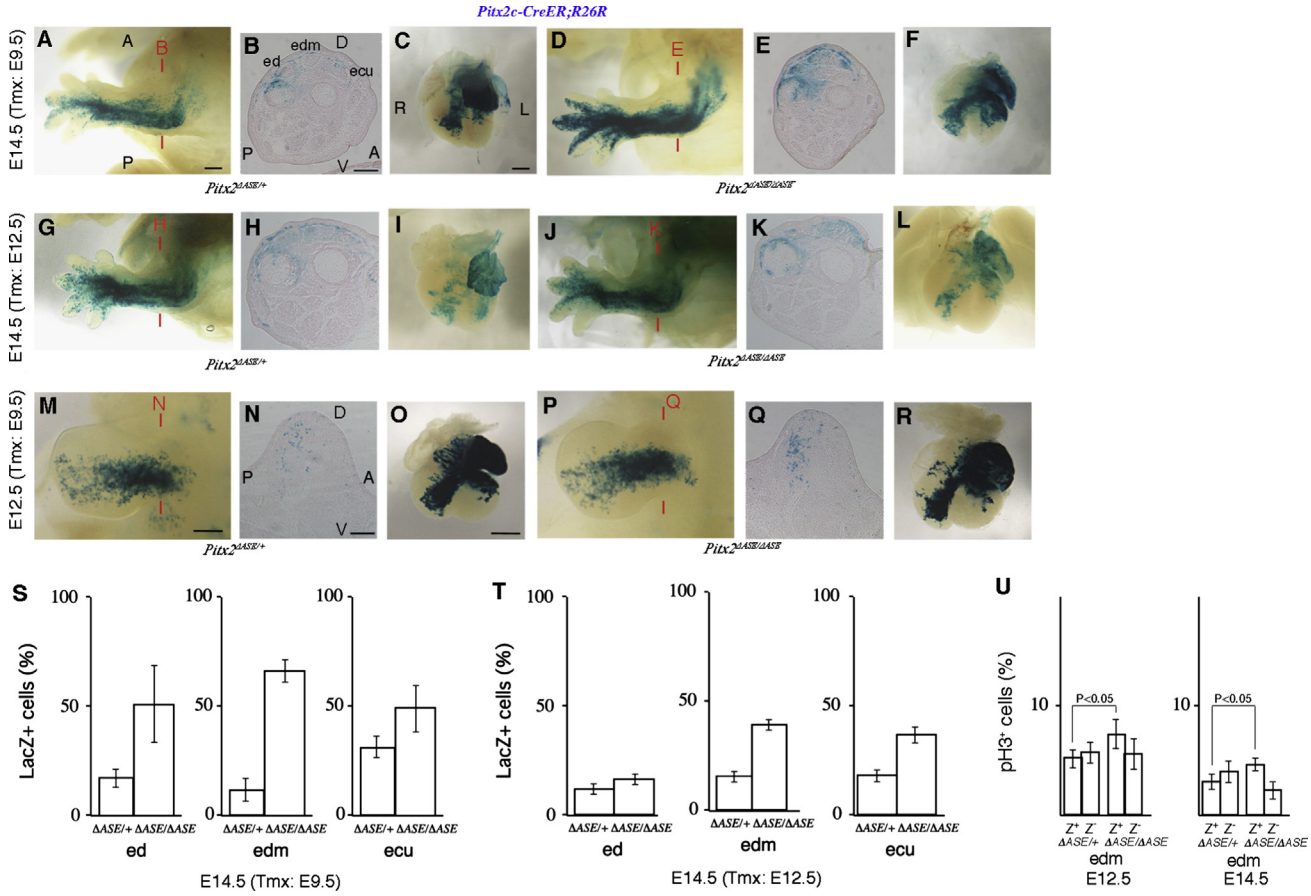


**Fig. 6.** The number of Pitx2c$^{+}$ cells in the left limb is determined by Pitx2c itself. (A–R) The left forelimb (A, D, G, J, M, P) or sections thereof (B, E, H, K, N, Q) as well as the heart (C, F, I, L, O, R) of *Pitx2*$^{\Delta ASE/+}$ (A–C, G–I, M–O) and *Pitx2*$^{\Delta ASE/\Delta ASE}$ (D–F, J–L, P–R) embryos harboring *Pitx2c-CreER* and *Rosa26R* exposed to tamoxifen in utero at E9.5 (A–F, M–R) or E12.5 (G–L) were stained with X-gal at E14.5 (A–L) or E12.5 (M–T). The sections were collected at the levels indicated in the corresponding intact embryos. (S and T) Percentage of LacZ$^{+}$ cells in muscles of the left forelimb of E14.5 embryos harboring *Pitx2c-CreER* and *Rosa26R* and exposed to tamoxifen in utero at E9.5 (S) or E12.5 (T). *Pitx2*$^{\Delta ASE/+}$ and *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos showing similar patterns of LacZ expression in the heart were evaluated. Data are means ± SEM from more than three embryos for each genotype from three independent litters, and more than 500 cells were counted per embryo (*P* value $< 0.05$). (U) Percentage of LacZ$^{+}$ (Z$^{+}$) or LacZ$^{-}$ (Z$^{-}$) cells that were positive for phosphorylated histone H3 (pH3) in the extensor digiti minimi of the left forelimb in *Pitx2*$^{\Delta ASE/+}$ and *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos harboring *Pitx2c-lacZ/BAC* at E12.5 and E14.5. Data are means ± SEM from three embryos and more than 500 cells were counted per embryo (P value $< 0.05$). ecu, extensor carpi ulnaris; ed, extensor digitorum; edm, extensor digiti minimi. Scale bars: 500 μm.

Pitx2c$^{-}$ cells without a change in the total number of Pitx2c$^{+}$ plus Pitx2c$^{-}$ cells. A cell competition mechanism (Levayer and Moreno, 2013) may thus operate between Pitx2c$^{+}$ and Pitx2c$^{-}$ cells. For example, Pitx2c may render a cell competent to respond to a proliferation-suppressing activity produced by Pitx2c$^{-}$ cells.

*Pitx1* is critical for establishing the hind limb bud. Interestingly, *Pitx1* mutants largely lose the hind limbs, but the phenotype is less severe on the left side. Furthermore, the left hindlimb becomes smaller in the double mutant, *Pitx1*$^{-/-}$; *Pitx2*$^{+/-}$ mouse (Marcil et al., 2003). Similarly the loss of *Pitx1* in the limb field is responsible for reduction of the pelvic spines in Stickleback fish (Shapiro et al., 2004) and loss of hindlimbs in Manatees (Shapiro et al., 2006), in both cases with the right limb/spine being more severely affected. These observations may be explained by asymmetric expression of *Pitx2c* in the limb bud.

We examined the patterns of axons, blood vessels, and muscles (containing tendons) in the mouse forelimb but were unable to identify any reproducible L-R differences. We also did not detect any morphological differences in the forelimb between wild-type and *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice. Given that overexpression of Pitx2a in the developing forelimb mesenchyme induced abnormal tendon positioning (Holmberg et al., 2004), Pitx2c may be responsible for generating previously unknown L-R asymmetry in the limb. Interestingly, *human PITX2c-lacZ* transgene showed L-R asymmetric expression in the mouse limb like mouse *Pitx2c-lacZ* transgene did. Furthermore, two transcriptional regulatory sequences required for asymmetric expression in the limb, the ASE and A region, are highly conserved between mouse and human *Pitx2* (Figs S2F, S7). Although it is not feasible to directly examine *PITX2c* expression in the human limb, PITX2c may have similar expression domains and function in the human limb.

Both *Pitx2c-lacZ/BAC* and *17-P1* were expressed in connective tissue of four muscles—extensor carpi ulnaris, extensor digitorum, extensor digiti minimi, and extensor indicis—that are located in the posterior-dorsal region of the limb. The limb on the left side thus contains cells that originally expressed Pitx2c in its posterior-dorsal region, whereas that on the right side does not. Of interest, all of these muscles are extensor muscles innervated by the deep branch of the radial nerve. L-R asymmetry may exist when this nerve projects to the posterior-dorsal region of the limb. It will therefore be important to search for anatomic and functional L-R asymmetry of the neural network including the deep branch of the radial nerve. Given that *Pitx2*$^{\Delta ASE/\Delta ASE}$ mice die shortly after birth, precluding study of adult animals, the generation of a mutant mouse that specifically lacks asymmetric *Pitx2c* expression in the limb bud and survives to adulthood might provide insight into the role of such expression.

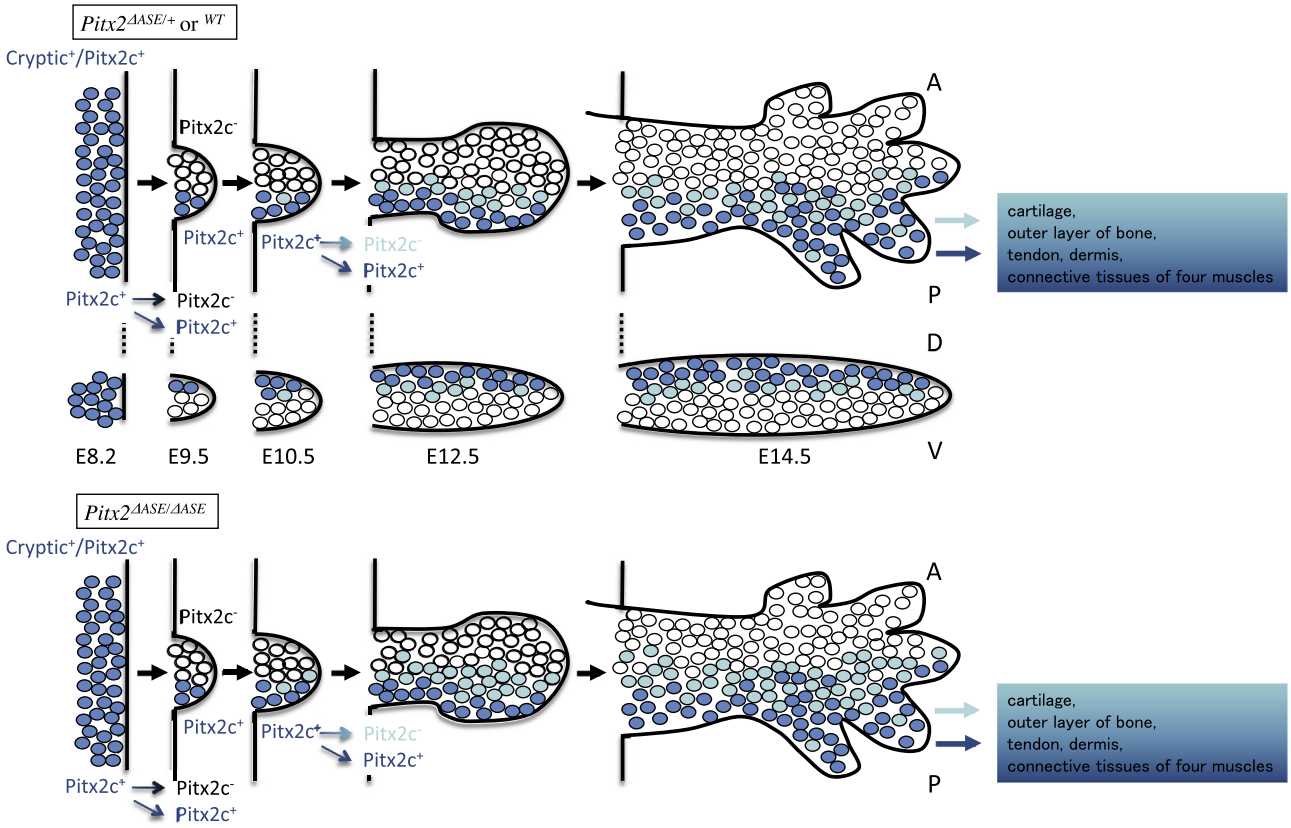


**Fig. 7.** Dynamic L-R asymmetric expression of *Pitx2c* in the developing limb. The fate of cells that express *Pitx2c* at E8.2 is summarized. Note that Pitx2c$^-$ cells at E8.2 are not included in this figure. In wild-type (WT) and *Pitx2*$^{\Delta ASE/+}$ embryos, asymmetric expression of *Pitx2c* is apparent in LPM cells at E8.2 (Cryptic$^+$/Pitx2c$^+$, dark blue circles). At E9.5, some of these Pitx2c$^+$ cells maintain *Pitx2c* expression (dark blue circle) whereas others have terminated *Pitx2c* expression (white circles). After E9.5, many of Pitx2c$^+$ cells in the developing limb gradually terminate its expression as development proceeds (light blue circles). The progeny of Pitx2c$^+$ cells in the developing limb become confined to tissues including cartilage, the outer layer of bone, tendon, dermis, and connective tissue of four muscles at E14.5 (dark blue and light blue circles). Note that Pitx2c$^+$ cells at E8.2 contribute to the limb bud without A-P and D-V polarities (represented by the distribution of all circles), although *Pitx2c* expression is maintained only in the posterior-dorsal region of the forelimb bud after E9.5 (dark blue circles). Whereas the expression pattern of a *Pitx2c* transgene in *Pitx2*$^{\Delta ASE/\Delta ASE}$ embryos is similar to that in wild-type and *Pitx2*$^{\Delta ASE/+}$ embryos, the progeny of transgene-expressing cells (dark blue and light blue circles) in the former embryos outnumber those in the latter, possibly as a result of the inhibition of cell proliferation by Pitx2c in a cell-autonomous manner.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.002.

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