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

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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.

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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

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