PIERCE1 is critical for specification of left-right asymmetry in mice

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The specification of left-right asymmetry of the visceral organs is precisely regulated. The earliest breakage of left-right symmetry occurs as the result of leftward flow generated by asymmetric beating of nodal cilia, which eventually induces asymmetric Nodal/Lefty/Pitx2 expression on the left side of the lateral plate mesoderm. PIERCE1 has been identified as a p53 target gene involved in the DNA damage response. In this study, we found that Pierce1-null mice exhibit severe laterality defects, including situs inversus totalis and heterotaxy with randomized situs and left and right isomerisms. The spectrum of laterality defects was closely correlated with randomized expression of Nodal and its downstream genes, Lefty1/2 and Pitx2. The phenotype of Pierce1-null mice most closely resembled that of mutant mice with impaired ciliogenesis and/or ciliary motility of the node. We also found the loss of asymmetric expression of Cerl2, the earliest flow-responding gene in the node of Pierce1-null embryos. The results suggest that Pierce1-null embryos have defects in generating a symmetry breaking signal including leftward nodal flow. This is the first report implicating a role for PIERCE1 in the symmetry-breaking step of left-right asymmetry specification.

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The left-right (LR) asymmetric pattern of the visceral organs, including the lung, heart, stomach, and spleen, is conserved across mammalian species. Mice have a lung with four right lobes and one left lobe, a left-pointing heart, and a left-sided stomach and spleen. The normal specification of LR asymmetry of the visceral organs is called situs solitus (SS). In contrast, situs inversus totalis (SI) is a malformation in which the specification of LR asymmetry of the visceral organs is completely reversed. Loss of LR specification also results in heterotaxy with aberrant positioning of visceral organs and isomerism of normally asymmetric organs1–3.

Because sonic hedgehog (SHH) and NODAL were first identified as left-right determinants in chicks approximately 20 years ago4, numerous other genes have been shown to be involved in the four steps required for specification of LR asymmetry5,6. The first step requires symmetry breaking, which is mediated by leftward flow in the node that results from the posterior tilt of the rotation axis of nodal cilia7,8. The absence or immotility of nodal cilia in the iv/iv (inversus viscerum) mutant strain is caused by a missense mutation on the iv gene, which encodes dynein axonal heavy chain 11 (DNAHC11), an active component of a nodal cilium17,18.

The second step of LR asymmetry specification involves the transfer of asymmetric signal(s) to the left side of the lateral plate mesoderm (LPM). According to two cilia model, non-motile polycystin-2 containing cilia of the perinodal cells (crown cells) recognizes nodal flow generated by motile cilia at the node and initiates an asymmetric calcium signal at the left border of the node19. The absence or immotility of nodal cilia has been reported in several mutant strains of mice that exhibit abnormal LR asymmetry9–13. One of them is iv/iv (inversus viscerum) mutant mice, which display diverse patterns of laterality, including SS, SI, and left and right isomerisms14–16. Immotility of nodal cilia in the iv/iv mutant strain is caused by a missense mutation on the iv gene, which encodes dynein axonal heavy chain 11 (DNAHC11), an active component of a nodal cilium17,18.

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Scientists have found that PIERCE1 is an important regulator of LR specification. This is the first report to identify PIERCE1 as one of the key regulators of LR asymmetry specification.

**Results and Discussion**

**PIERCE1** is a null allele. We previously reported that PIERCE1 is a downstream target of p53 and may play an important role in maintaining genomic integrity against genotoxic stresses; however, the in vivo role of PIERCE1 has yet to be elucidated. To investigate the function of PIERCE1 in vivo, PIERCE1-deficient mice were generated using an embryonic stem (ES) cell clone harboring a gene-trap cassette in intron 2 of the PIERCE1 locus. Mice homozygous for the gene-trapped allele (PIERCE1+/−) were obtained by intercrossing heterozygotes. Whereas PIERCE1 expression is prominent in several tissues, including the brain, lung, kidney, and testis, PIERCE1 transcripts were undetectable in these tissues in PIERCE1+/− mice by reverse transcriptase (RT)–PCR. This indicates that PIERCE1 is a null allele of PIERCE1.

PIERCE1 deficiency results in randomization of body situs. Of 169 offspring obtained from intercrosses between PIERCE1+/− mice, 52 mice (31%) were wild-type (WT), 95 mice (56%) were PIERCE1−/−, and 22 mice (13%) were PIERCE1−/− (Supplementary Table S1), which deviates significantly from the expected Mendelian ratio (Chi square \( P = 0.0013 \)). Because fetal lethality was not evident, these results indicate that PIERCE1−/− mice are partially embryonic lethal. The surviving PIERCE1−/− mice appeared to be grossly normal, viable, and fertile. Upon autopsy, however, we found striking alterations in PIERCE1−/− mice (Fig. 1A–D). Among 78 PIERCE1−/− adults examined, 44 mice (59%) showed SS and 32 (41%) showed SI. SI mutants also exhibited complete mirror-image reversal of position of the heart, stomach, spleen (Fig. 1A), and kidney (Fig. 1D) along the LR axis. The lobation patterns of the lungs (Fig. 1B) and liver (Fig. 1C) were reversed as well. This phenotype demonstrates that PIERCE1 deficiency results in partial embryonic lethality associated with heterotaxia.

PIERCE1 deficiency results in partial embryonic lethality associated with heterotaxia. As the number of PIERCE1−/− mice generated from heterozygous intercrosses was much less than the expected Mendelian ratio (Supplementary Table S1), we examined the possibility that PIERCE1−/− mice were embryonic lethal. Most of the E13.5 and E14.5 PIERCE1−/− embryos exhibited grossly normal appearance and were recovered at the expected Mendelian ratio (Chi square \( P = 0.5246 \)), but 10 out of 30 PIERCE1−/− embryos at E14.5 were found dead (Supplementary Table S1). Interestingly, PIERCE1−/− embryos at E13.5 and E14.5 exhibited a wide spectrum of LR asymmetry defects, including SI and left and right isomerisms (Fig. 1E). SS embryos had normal lungs with one left lobe and four right lobes, whereas the lungs of SI embryos had a mirror image of the normal lung pattern. Most of the dead mutant embryos exhibited bilateral uni-lobed lungs (left pulmonary isomerism, LPI; Fig. 1E) or bilateral tetra-lobed lungs (right pulmonary isomerism, RPI; Fig. 1E) with defects, such as persistent truncus arteriosus in great arteries (Supplementary Fig. S2A). To analyze LR asymmetry defects in detail, 20 E13.5 PIERCE1−/− embryos were examined using micro-computed tomography (μCT). PIERCE1−/− embryos displayed SS (9/20), SI (2/20), and various forms of heterotaxy (9/20), including LPI and RPI, with associated cardiac anomalies (Supplementary Table S2). While the apex of the heart pointed leftward in all wild-type embryos, the cardiac apex in PIERCE1−/− mice pointed to the left (12/20), the right (dextrocardia, 4/20), or the midline (mesocardia, 4/20) (Supplementary Table S2 and Supplementary Fig. S3). Most embryos with SI or heterotaxy had various cardiac anomalies, such as interventricular septal defects, hypoplastic heart defects, mitral valve defects, atrioventricular canal, and dilated atria. Laterality defects of the lung lobation pattern were also observed in mutant embryos (Supplementary Table S2 and Supplementary Fig. S3). Among nine mutant embryos with heterotaxy, three had bilateral tetra-lobed lungs (RPI), three had bilateral...
uni-lobed lungs (LPI), and one had a normal lung lobation pattern without a postcaval lobe (Supplementary Table S2 and Supplementary Fig. S3). We found that the inferior vena cava was reversed (to the left side) in four heterotaxy mutants, as well as in all SI mutants (Supplementary Table S2). Reverse positioning of the stomach (to the right side of the abdomen) was found in some heterotaxy mutants as well as SI mutants (Supplementary Table S2 and Supplementary Fig. S3). Taken together, these data indicate that the partial embryonic lethality observed in Pierce1−/− mice is closely associated with heterotaxy.

**Randomized expression of the asymmetric genes, Nodal, Lefty1/2, and Pitx2 in Pierce1−/− embryos.** The specification of LR asymmetry is determined by the expression of Nodal and its downstream genes, Lefty1, Lefty2, and Pitx2, in the LPM during early embryonic stages. Nodal expression is detected in the perinodal cells and on the left side of the LPM, Lefty1 is expressed on the left side of the midline, and Lefty2 and Pitx2 are found in the left LPM of wild-type mouse embryos. These asymmetric gene expression patterns were observed in both wild-type and Pierce1−/− embryos (Fig. 2). Although there was no noticeable difference in Nodal expression in the perinodal cells in Pierce1−/− embryos (n = 11; Supplementary Fig. S4), Nodal expression

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**Figure 1. Diverse laterality phenotypes in Pierce1−/− mice.** (A) The SI Pierce1−/− mice show dextrocardia and right-sided spleens. Arrow and arrowhead indicate the heart apex and the spleen, respectively. (B,C) Mirror-imaged inversion of the lobulation patterns of the lung (B) and the liver (C) in SI Pierce1−/− mice. The normal lung is bilaterally asymmetric with one left lobe (LL) and four right lobes (superior [SL], middle [ML], inferior [IL], and postcaval [PL] lobes). SI embryos exhibit a mirror image of the normal lung pattern. (D) Reversed rostral-caudal arrangement of the kidneys in SI Pierce1−/− mice. Arrows indicate the positions of right and left kidneys. (E) Lung lobation patterns (ventral views) of E14.5 Pierce1−/− embryos with SS, SI, bilateral uni-lobed lungs (LPI), and bilateral tetra-lobed lungs (RPI). Approximately half of the Pierce1−/− embryos had LPI or RPI. Red and blue lines indicate postcaval (PL) and middle (ML) lobes, respectively. IL, inferior lobe.
in the LPM was randomized in Pierce1−/− embryos: normal (left side, 4/9), inverted (right side, 1/9), bilateral (both sides, 1/9), or absent (neither side, 3/9) (Fig. 2A). Left1 expression was down-regulated in the midline and Left2 expression was detected on the left side (7/18), right side (4/18), both sides (4/18), and neither side (3/18) of the LPM in Pierce1−/− embryos (Fig. 2B). Likewise, Pitx2 expression was also randomized: on the left (6/22), right (8/22), both (6/22), and neither (2/22) side of the LPM (Fig. 2C). Left- and right-sided expression of the asymmetric genes in Pierce1−/− mice may represent SS and SI, respectively. Conversely, bilaterally present or absent expression of these genes in Pierce1−/− embryos may correspond to left or right isomerisms, respectively.

The randomized expression of asymmetric genes suggests that PIERCE1 functions during the initial step(s) of LR specification in the node. The expression of Pierce1 in E8.0 embryos was determined by whole-mount in situ hybridization using a Pierce1 riboprobe. We observed relatively strong expression of Pierce1 in the node area (Supplementary Fig. S3), suggesting that PIERCE1 functions at the node for LR specification. Based on our results, PIERCE1 is likely involved in the earliest step(s) of LR asymmetry determination, such as symmetry breaking at the node.

Among numerous mutant mouse models that manifest laterality defects (Supplementary Table S3), only a few of them (Ar13b−/−, Dnahc5−/−, iv/iv, Noto−/−, Rfx3−/−, and Zic3−/−) exhibit the full spectrum of laterality phenotypes shown in Pierce1−/− mice. Randomized expression of Nodal in the LPM of Ar13b−/−, iv/iv, Noto−/−, and Zic3−/− embryos and biased bilateral expression in Rfx3−/− embryos have been reported.36–38, Interestingly, all of these mutants have defects in either ciliogenesis and/or ciliary motility: 1) short cilia (Ar13b−/−, Noto−/−, Rfx3−/−, and Zic3−/−)36,37,40,41, 2) disorganized alignment of cilia (Dnahc5−/−), and 3) rigid and immotile cilia (iv/iv)36, ARL13B is a small regulatory GTPase involved in ciliogenesis40. NOTO is an essential regulator of multiple genes involved in ciliogenesis and ciliary motility, including Dnahc11 (iv), Dnahc5, and Nphp3 via the Foxj1 and Rfx3 transcription factors36.

To investigate if ciliogenesis is affected in Pierce1−/− embryos, the nodes of E7.5 embryos were examined with scanning electron microscopy (SEM). We found no malformations of monolalia development, such as duplication, bifurcation, partial bifurcation, bulging, or disorganized alignment in the nodal cells of E7.5 Pierce1−/− embryos (Fig. 3). Next, we examined if expression of Dnahc11 (iv) and Noto is affected in Pierce1−/− embryos (n = 5 for Dnahc11; n = 13 for Noto). Expression of both genes was detected at the node of E8.0 Pierce1−/− embryos, comparable to the levels detected in wild-type controls (Supplementary Fig. S6). As the earliest responding gene to leftward flow in the node5,5, Cerl2 is bilaterally expressed in the perinodal cells at the early headfold stage when leftward nodal flow is locally generated. The local flow is sufficient to induce down-regulation of Cerl2 on the left-side at the late headfold stage44. We observed right-side dominant expression of Cerl2 in the node of WT and Pierce1−/− embryos, while bilateral expression of Cerl2 was detected in Pierce1−/− embryos (Fig. 4). These results strongly support that Pierce1 plays a critical role in generating a symmetry breaking signal including leftward nodal flow.

In this study, we uncovered the role of PIERCE1 in the regulation of LR asymmetry. Pierce1−/− mice at mid-gestational periods were recovered in the expected Mendelian ratio and exhibited a wide spectrum of
laterality defects, including isomerisms. In contrast, the mice presented at postnatal periods deviated significantly from the expected Mendelian ratio and only exhibited SS and SI without isomerisms. These results indicate that the mice with isomerisms die in utero due to various cardiovascular malformations associated with isomerisms. The laterality phenotypes of Pierce1−/− mice suggest that PIERCE1 plays a pivotal role in determining the LR axis at the symmetry-breaking stage. The phenotype of Pierce1−/− mice most closely resembles that of mutant mice with impaired ciliogenesis and/or ciliary motility of the node. Especially, morphologically normal nodal cilia and bilateral expression of Cerl2 in the node of Pierce1−/− embryos suggest that Pierce1−/− has defects in generating a symmetry breaking signal including leftward nodal flow.

Even if the expression difference of Cerl2 existed between the left and right sides of the node in Pierce1−/− embryos, the difference was very minor compared with that in controls. Thus, Nodal activity was expected to be suppressed by Cerl2 in the both side of the node. However, we observed randomized expression of Nodal, Lefty2, and Pitx2 in the LPM (Fig. 2). Possible interpretation of this discrepancy is that the amount of bilaterally expressed Cerl2 in Pierce1−/− embryos is around threshold level to suppress NODAL activity, and thus the level of Cerl2 in some Pierce1−/− embryos is not sufficient to suppress NODAL activity in one or both side of the node. Nodal expression in the LPM is initially activated by transported NODAL protein from the node through the NODAL-responsive asymmetric enhancer of the Nodal gene, and then it is regulated through positive and negative feedback loops by NODAL itself and LEFTY2, respectively [25, 26]. In Pierce1−/− embryos, incomplete suppression of NODAL activity could initiate positive feedback loop for Nodal expression in the LPM. This discrepancy is also observed in Arl13b−/− embryos [34]. Five out of six Arl13b−/− embryos with 4–5 somites have bilateral expression of Cerl2, while Nodal expression is detected on the left side (5/19), right side (3/19), or both sides (11/19) [34].

Notable expression of Pierce1 is detected in adult tissues including the brain, lung, kidney and testis as well as in the node of E8.0 embryos (Supplementary Fig. S5) [32]. In fact, only distinct cells in these tissues have potential to generate motile cilia and flagella, implying that a dedicated genetic program controls gene expression for formation of motile cilia and flagella [32]. Previously we showed that Pierce1 knockdown compromises the transcriptional activation of several p53 target genes upon UVC irradiation [33]. In addition, the hemagglutinin (HA)-tagged PIERCE1 protein was localized in the nucleus as well as in the cytoplasm (Supplementary Fig. S7). Based on these lines of evidence, we speculate that PIERCE1 contributes to the transcriptional control of genes involved in ciliogenesis and/or ciliary motility in the node although any functionally-defined protein domain of PIERCE1 has not been defined yet.

Further investigation regarding the biochemical, molecular, and genetic mechanisms by which PIERCE1 interacts with known factors involved in LR specification would elucidate the precise role of PIERCE1 during LR asymmetry specification.

Methods

Generation of mice carrying targeted mutations in the Pierce1 gene. A Pierce1-deficient mouse model was generated using a gene-trapped mouse ES cell clone (Cell Line ID: OST3440, Lexicon Genetics, Inc.). The male chimeras were bred with C57BL/6J females, and germline F1 mice were bred with mice on a C57BL/6J or FVB/N background. In this study, most of the adult mice and embryos had mixed genetic backgrounds. The targeted allele was confirmed by PCR analysis. A three-primer PCR strategy was conducted to genotype mice and embryos using the following primers: 5′-CGAAGGCCAATTAGTGAAGTCAAGC-3′ as a common primer (C), 5′-CCAGAGAACAGGACTAAGAAGCACG-3′ as a wild-type-specific primer (WT), and 5′-ATAAACCCCTCTTGAGTGTGACGACG-3′ for the gene-trap allele-specific primer (GT) (Supplementary Fig. S1A). To detect Pierce1 transcripts, complementary DNA (cDNA) samples were prepared using total RNAs from the brain, lung, kidney, and testis of control and Pierce1−/− mice as previously described [35], and RT-PCR was conducted using primer pairs specific for Pierce1 (5′-CCAGTAACCAACCTACGGA-3′, 3′-CAAGCAGAACTCCATAGTGTCG-5′) and Pierce1 (5′-CACCCATGTTTTCTTGCTCTG-3′, 3′-TTTTGTTTGTTTGTGTTTG-5′) for the gene-trap allele, respectively.
and 5′-AGTGGGTGATGTGATTGTCA-3′) and Gapdh (5′-ATCACTGCCACTCAGAAGAC-3′ and 5′-CACCACCTTCTTGATGTCATC-3′). All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Yonsei University (approval reference number, 2007-0004). All mice were maintained in a specific pathogen-free facility at the Laboratory Animal Research Center at Yonsei University.

### Whole-mount in situ hybridization

To study the expression patterns of Cerl2, Dnahc11, Lefty1/2, Nodal, Noto, Pierce1, and Pitx2 in E8.0 ~ E8.5 embryos, whole-mount in situ hybridization was performed as previously described35. Antisense RNA probes were produced with template DNAs of Cerl234, Dnahc1139, Lefty1/246, Nodal45,

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**Figure 4. Loss of asymmetric expression of Cerl2 in the node of Pierce1−/− embryos.** (A–H) The expression patterns of Cerl2 in the node of control and Pierce1−/− embryos with 3–5 somites were examined by whole-mount in situ hybridization using the Cerl2 antisense riboprobe. (A) In control embryos, the Cerl2 expression on the left-side of the node is down-regulated. (B–H) The right-side dominant expression of Cerl2 is not clearly observed in Pierce1−/− embryos (n = 7). All images are rear views. The orientation of the embryos is indicated by R (right) and L (left).
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Author Contributions
Y.H.S., I.-J.B., Y.J.L. and Y.S.G. performed experiments and analyzed data, Y.H.K. analyzed data, and Y.H.S., S.P.O., Y.J.L. and H.-W.L. designed the study, analyzed data, and wrote the manuscript.

Additional Information
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