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RESEARCH LETTER

Identification of Latrophilin-2 as a Novel Cell-Surface Marker for the Cardiomyogenic Lineage and Its Functional Significance in Heart Development

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Identification of a lineage-specific marker that enables monitoring of subsets would be valuable for establishing the conditions under which pluripotent stem cells (PSCs) differentiate into cardiac progenitor cells (CPCs) and cardiomyocytes (CMCs). The demand for PSC-derived CMCs for use in studies on cardiovascular disease has increased in recent years.[1] Here, we report a new cardiac lineage marker and demonstrate its functional significance in heart development.

We established a protocol for directed PSC differentiation into CMCs after exposing cells to various combinations of cytokines for different times, based on the biology of embryonic development.[2] When mouse PSCs were stimulated with multiple cytokines, they differentiated into cardiac cells (Figure [A]). To screen surface markers of CPC during PSC differentiation, we performed microarray analysis. We aimed to discover molecules whose expression increased sequentially during cardiac lineage differentiation and exclusively in the CPC-enriched population expressing both Flk-1 (fetal liver kinase 1) and PdgfR-α (platelet-derived growth factor receptor-α).[2] Therefore, we compared gene expression profiles in 4 different cell populations: (1) undifferentiated PSCs (group 1; Flk-1$^+$ PdgfR-α$^+$ [F$^+$P$^+$], 4.9±1.1%), (2) spontaneously differentiated cells at day 4 (group 2; F$^+$P$^+$, 11.8±5.2%), (3) cells that underwent optimized cardiac differentiation at day 4 (group 3; F$^+$P$^+$, 33.0±5.8%), and (4) cells enriched for CPCs after fluorescence-activated cell sorting (group 4; F$^+$P$^+$, 90.4±4.3%; Figure [B]). In groups 1 to 4, we identified 7 genes whose expression increased continuously; among the groups, these genes showed the highest expression in group 4 (Figure [C]). Among these 7 candidate genes, we focused on the little-studied G protein–coupled receptor, latrophilin-2 (*Lphn2*), which is expressed on the cell surface and may have functional significance (Figure [D]).

To confirm that Lphn2 is necessary for cardiac development, we established *Lphn2*-knockout (KO) embryonic stem cells. Reverse transcription–polymerase chain reaction and Western blot analyses confirmed that Lphn2 expression increased during cardiac differentiation in wild-type embryonic stem cells, whereas the expression did not appear in *Lphn2*-KO embryonic stem cells (Figure [E] and [F]). During cardiac differentiation, *Lphn2*-KO cells failed to express cardiac-related genes, such as *Gata4* and *Nkx2.5*, which encode transcription factors central for heart development; *Tbx5*, a transcription factor of the primary heart field; *Isl1*, a transcription factor of the secondary heart field; and *cTnT*, a structural protein of CMCs (Figure [G]).

To verify the in vivo functional significance of Lphn2 during development, we generated *Lphn2*-KO mice (Figure [H] and [I]). All animal experiments were approved by the Institutional Animal Care and Use Committee at Seoul National University Hospital. Although *Lphn2* heterozygous (*Lphn2*$^{+/-}$) mice were alive and

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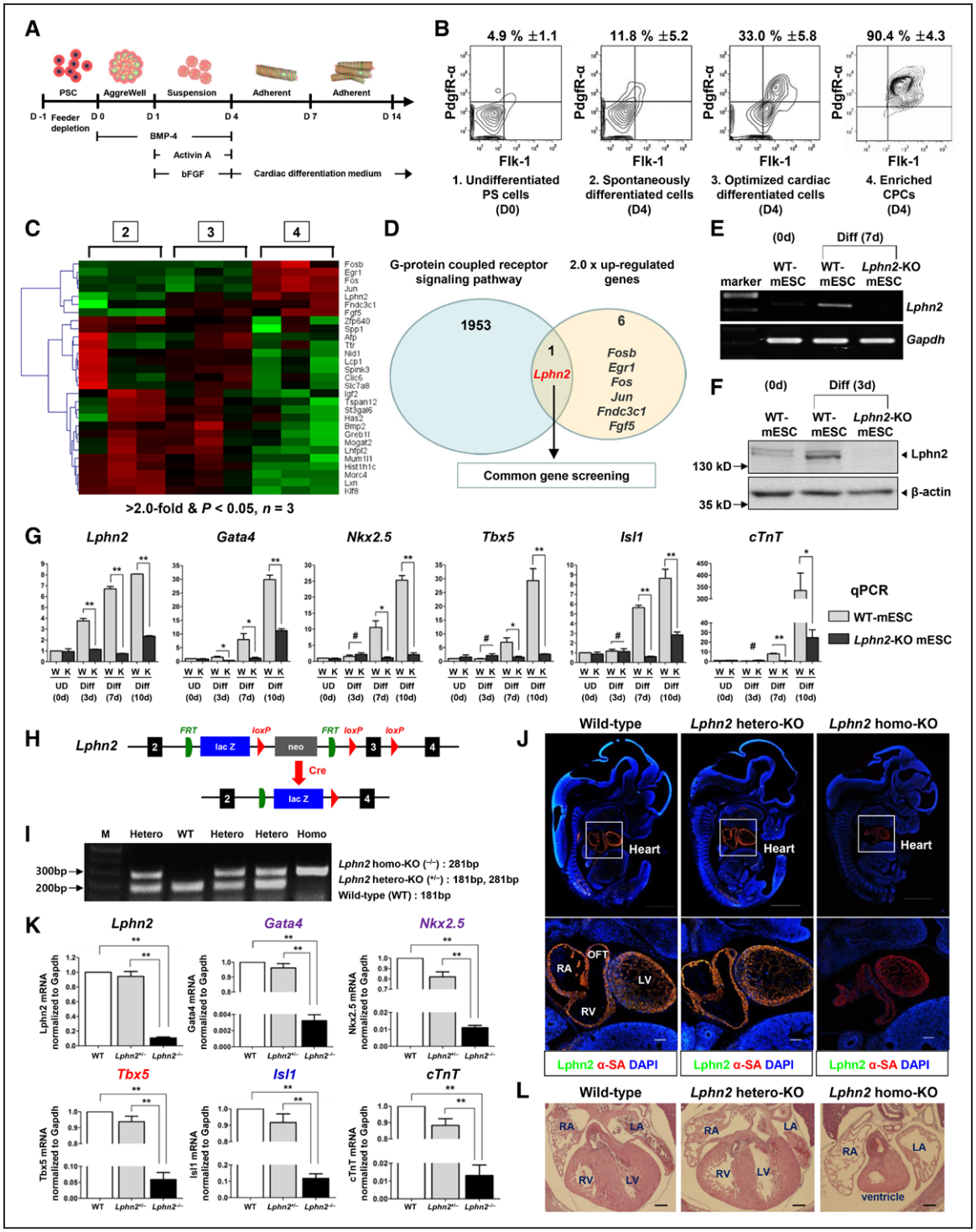

Figure. Identification of the marker expressed by cardiac lineage cells and functional significance of Lphn2 in heart development.
A, Schematic timeline of the protocol used to differentiate mouse PSCs into cardiac cells and the accompanying changes in cytokines. For the directed differentiation of PSCs to the cardiac lineage, embryoid bodies (EBs) were generated in an AggreWell plate after culturing for 1 day in EB media in the presence of BMP-4, with the subsequent addition of activin A and bFGF (FGF2) for 3 additional days. On day 4, EBs were transferred to cardiac differentiation medium containing epidermal growth factor, bFGF, cardiotrophin-1, and vascular endothelial growth factor. **B,** Flow=cytometric analysis of Flk-1^{+}PdgfR-α^{+} in undifferentiated PSCs (group 1), spontaneously differentiated cells (group 2), cells after optimized cardiac differentiation (group 3), and CPCs enriched by fluorescence-activated cell sorting (group 4) at day 4 after differentiation. Data are presented as the mean±SEM (n=3) in each group. **C,** Microarray screening to identify CPCs. Heat map compares the gene expression profiles in different cell populations. Expression fold-change signatures of populations from groups 2, 3, and 4 are shown. Red shading indicates a >2-fold increase in expression; green shading indicates a >2-fold decrease in expression (ANOVA with Benjamini and Hochberg post hoc analysis to select differentially expressed genes with false-discovery rate–adjusted P<0.05). **D,** Venn diagram of 2-fold upregulated genes and those in the G protein–coupled receptor signaling pathway as a gene ontology analysis. **E** and **F,** *Lphn2* mRNA (**E**) and protein (**F**) expression in wild-type mESCs and *Lphn2*-KO ESC-derived cardiac cells on days 3 and 7 postdifferentiation. **G,** qPCR analysis of cardiovascular lineage genes in wild-type (W) and *Lphn2*-KO (K) mESC-derived cells during cardiac differentiation. Values are shown relative to wild-type mESCs at day 0. Data are mean±SEM. **P*<0.05, ***P*<0.01, $^{\#}$*P*=NS, unpaired *t* test. n=3 biological (*Continued*)

Figure Continued. replicates. **H,** Schematic representation of the *Lphn2* KO. **I,** Representative genotyping of wild-type, heterozygous (*Lphn2*$^{+/-}$), and homozygous (*Lphn2*$^{-/-}$) KO embryos was performed by polymerase chain reaction amplification. Genomic DNA was prepared from tail biopsy samples for genotyping. **J,** Immunostaining for Lphn2 (green) and α-SA (red) in wild-type, *Lphn2*$^{+/-}$, and *Lphn2*$^{-/-}$ embryos at E11.5. Representative images of the midline by sagittal section. Blue indicates nuclear counterstain (DAPI). The white rectangle in the upper image indicates the heart magnified in the lower image. Scale bars=1000 μm (upper panels) or 100 μm (lower panels). **K,** Significant mRNA expression differences for *Lphn2* and heart development genes (*Gata4*, *Nkx2.5*, *Tbx5*, *Isl1*, and *cTnT*) in wild-type, *Lphn2*$^{+/-}$, and *Lphn2*$^{-/-}$ embryonic tissues at E12.5. Values are shown relative to the wild-type embryo. Data are mean±SEM. $^{**}P<0.01$, ANOVA test and post hoc Bonferroni test, n=3 biological replicates. **L,** A defect in heart development in the *Lphn2*$^{-/-}$ embryos. Representative hematoxylin and eosin–stained transverse sections revealing single ventricle in *Lphn2*$^{-/-}$ at E15.5. The embryos are all from the same litter. Scale bars=20 μm. α-SA indicates α-skeletal muscle actin; bFGF, basic fibroblast growth factor; BMP-4, bone morphogenetic protein-4; bp, base pair; CPC, cardiac progenitor cell; D, day; DAPI, 4′,6-diamidino-2-phenylindole; Diff, differentiation; E, embryonic day; ESC, embryonic stem cell; Flk-1, fetal liver kinase 1; Hetero, heterozygous; Homo, homozygous; KO, knockout; LA, left atrium; Lphn2, latrophilin-2; LV, left ventricle; mESC, mouse embryonic stem cell; OFT, outflow tract; PdgfR, platelet-derived growth factor receptor; PS, pluripotent stem; PSC, pluripotent stem cell; qPCR, quantitative polymerase chain reaction; RA, right atrium; RV, right ventricle; UD, undifferentiated; and WT, wild-type.

fertile, homozygous *Lphn2* (*Lphn2*$^{-/-}$) mice showed embryonic lethality. Immunofluorescent staining of E11.5 embryos of *Lphn2*$^{+/-}$ mice revealed no significant gross abnormality compared with wild-type mice embryos, whereas *Lphn2*$^{-/-}$ embryos exhibited serious defects in the development of the right ventricle, right atrium, and outflow tract (Figure [J]). Additionally, the muscle mass of the left ventricle was much smaller than those of wild-type and heterozygous embryos (Figure [J]).

Next, we investigated the mRNA expressions of regulatory genes involved in heart development during embryogenesis. Because *Lphn2*-KO cells did not express cardiac-related genes in vitro, we assessed how *Lphn2* deficiency functions in heart development. Notably, similar results were observed in the quantitative polymerase chain reaction analysis of embryonic tissues (Figure [K]). *Lphn2*$^{+/-}$ embryos were not significantly different from wild-type embryos. In contrast, *Lphn2*$^{-/-}$ embryos showed markedly reduced expression of genes encoding transcription factors that are regulators of heart development, including *Gata4*, *Nkx2.5*, *Tbx5*, and *Isl1*, as well as a cardiac structural gene, *cTnT* (Figure [K]). Also, we analyzed wild-type, *Lphn2*$^{+/-}$, and *Lphn2*$^{-/-}$ embryos to further investigate the role of Lphn2 in the late stage of heart development. Interestingly, the hearts of *Lphn2*$^{-/-}$ embryos at embryonic day 15.5, unlike those of wild-type and *Lphn2*$^{+/-}$ embryos, had a small and single ventricle, revealing the defect of cardiomyogenic development (Figure [L]).

Extensive studies on preclinical and clinical cell therapies for heart disease have used several types of cells for cardiac repair. Although CPCs have been identified using multiple markers,[3] it is very challenging to isolate PSC-derived CPCs and CMCs in vitro, because most cardiac-specific markers are intracellular molecules or transcription factors.[4,5] Thus, cell-surface markers are needed to purify CPCs and CMCs from heterogeneous cell populations during stem cell differentiation. In conclusion, we have demonstrated that Lphn2 is a unique cell-surface marker of cardiomyogenic cells. These findings provide a valuable tool for isolating CPCs from PSCs, as well as novel insights into heart development.

ARTICLE INFORMATION

Data sharing: All data and materials supporting the findings of this study are available from the corresponding author on reasonable request. Microarray results are accessible at the Gene Expression Omnibus database (National Center for Biotechnology Information; accession No., series GSE83434).

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Disclosures

None.

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