

Downregulation of *LGR5* Expression Inhibits Cardiomyocyte Differentiation and Potentiates Endothelial Differentiation from Human Pluripotent Stem Cells

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SUMMARY

Understanding molecules involved in differentiation of human pluripotent stem cells (hPSCs) into cardiomyocytes and endothelial cells is important in advancing hPSCs for cell therapy and drug testing. Here, we report that *LGR5*, a leucine-rich repeat-containing G-protein-coupled receptor, plays a critical role in hPSC differentiation into cardiomyocytes and endothelial cells. *LGR5* expression was transiently upregulated during the early stage of cardiomyocyte differentiation, and knockdown of *LGR5* resulted in reduced expression of cardiomyocyte-associated markers and poor cardiac differentiation. In contrast, knockdown of *LGR5* promoted differentiation of endothelial-like cells with increased expression of endothelial cell markers and appropriate functional characteristics, including the ability to form tube-like structures and to take up acetylated low-density lipoproteins. Furthermore, knockdown of *LGR5* significantly reduced the proliferation of differentiated cells and increased the nuclear translocation of β -catenin and expression of Wnt signaling-related genes. Therefore, regulation of *LGR5* may facilitate efficient generation of cardiomyocytes or endothelial cells from hPSCs.

INTRODUCTION

Controlled and robust differentiation of cardiomyocytes and endothelial cells from human pluripotent stem cells (hPSCs) is important for their applications in regenerative medicine, disease modeling, and drug discovery (Ebert et al., 2015; Laflamme and Murry, 2011). Differentiation of these cells requires regulation of Wnt signaling in a time- and dose-dependent manner (Murry and Keller, 2008). Wnt signaling needs to be activated at the early stage and subsequently inhibited at the late stage for efficient cardiomyocyte differentiation (Kattman et al., 2011; Lian et al., 2012; Paige et al., 2010; Palpant et al., 2013, 2015), which can be achieved using small molecules (Lian et al., 2012) or growth factors (i.e., activin A and bone morphogenetic protein 4 [BMP4]) (Kattman et al., 2011; Laflamme et al., 2007). Endothelial cell differentiation from hPSCs can be induced by a brief treatment with a Wnt agonist during the first day of differentiation (Lian et al., 2014), but inhibited when the agonist is added after mesodermal commitment (Palpant et al., 2015). Given the critical role of the Wnt signaling, identifying additional molecules regulating Wnt signaling during lineage commitment may enhance efficient differentiation of cardiomyocytes and endothelial cells from hPSCs.

LGR5 is a leucine-rich repeat-containing G-protein-coupled receptor that can bind to R-spondins to potentiate Wnt signaling (Carmon et al., 2011, 2012; de Lau et al., 2011; Glinka et al., 2011). It is a stem cell marker for various

tissues (Barker et al., 2007, 2010, 2012; Chai et al., 2012; de Visser et al., 2012; Jaks et al., 2008; Sato et al., 2009), and is overexpressed in cancer stem cells and several types of tumors (Barker et al., 2009; Junttila et al., 2015; McClanahan et al., 2006; Nakata et al., 2013; Tanese et al., 2008). Since R-spondin 3 is essential for cardiac development (Cambier et al., 2014), we speculated that *LGR5* may also play a role in hPSC differentiation.

Here, we report that *LGR5* expression is transiently upregulated during the early stage of cardiomyocyte differentiation from hPSCs, and that *LGR5* promotes cardiomyocyte differentiation and inhibits endothelial cell differentiation from hPSCs.

RESULTS

LGR5 Expression Is Transiently Upregulated during the Early Stage of Cardiomyocyte Differentiation

To understand the role of *LGR5* during cardiomyocyte differentiation, we first examined its temporal expression during cardiomyocyte differentiation of H7 human embryonic stem cells (hESCs) induced by activin A and BMP4 (Figures 1A and 1B). As expected, expression of stem cell marker *OCT4* was decreased after induction, while expression of mesodermal marker *T* (Brachyury) was transiently upregulated at day 2. Subsequently, expression of mesodermal marker *MESP1* and cardiac progenitor marker *NKX2-5* was increased after day 4, and expression of cardiomyocyte

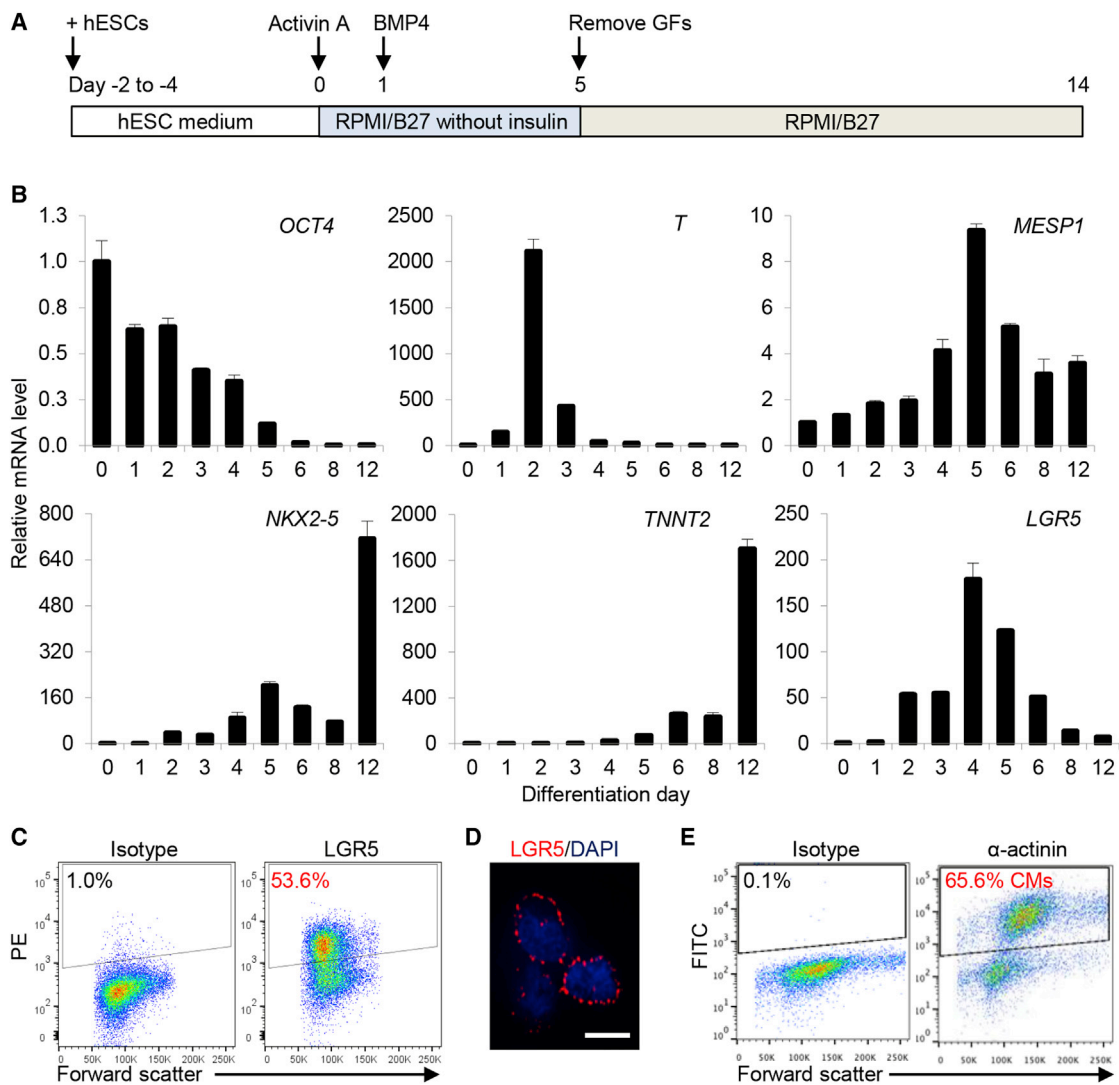


Figure 1. Transient Upregulation of *LGR5* Expression at Early Stages of Cardiomyocyte Differentiation from hPSCs

(A) Schematic of cardiomyocyte differentiation protocol using growth factors. Single cells were seeded 2–4 days before the induction with activin A (100 ng/mL) at day 0 and BMP4 (10 ng/mL) at day 1 in RPMI/B27 medium without insulin. After day 5, cells were cultured with RPMI/B27 medium without growth factors (GFs).

(B) Relative mRNA levels of genes including *LGR5* and markers for pluripotent stem cells (*OCT4*), mesendoderm (*T*), cardiac mesoderm (*MESP1*), cardiac progenitors (*NKX2-5*), and cardiomyocytes (*TNNT2*) in H7 hESCs analyzed using qRT-PCR.

(C) Flow-cytometry analysis of *LGR5* in H7 cells at day 4. Cells were stained with PE-labeled mouse anti-*LGR5* antibodies and corresponding isotype control.

(D) Detection of *LGR5* on cell surface of differentiated H7 hESCs at day 4 by immunocytochemistry. Scale bar, 20 μm.

(E) Flow-cytometry analysis of α-actinin in H7 hESCs at day 14.

n = 3 independent experiments. Data are presented as mean ± SEM. See also Figure S1.

marker *TNNT2* (cardiac troponin T) was increased after day 6. Compared with day-0 cells, ~50-fold increased *LGR5* mRNA was detected at day 2 and ~170-fold at day 4. After day 5, *LGR5* expression gradually decreased but was maintained at levels higher than that of day-0 cells. At the protein level, ~54% of the day-4 cells were positive for *LGR5* as detected by flow cytometry (Figure 1C) and *LGR5* was detected on

cell surface by immunocytochemistry (Figure 1D). Similar *LGR5* expression patterns were observed in two other hPSC lines (IMR90 induced pluripotent stem cells [iPSCs] and H9 hESCs) (Figure S1). In addition, parallel cultures of H7 hESCs, IMR90 iPSCs, and H9 hESCs at day 14 contained ~56%–66% cells that were positive for the cardiomyocyte-associated marker α-actinin (Figures 1E, S1E, and S1J).



These data show that increased *LGR5* expression occurred during mesendoderm induction (*T*) and prior to the induction of master regulators of cardiogenesis, *MESP1* and *NKX2-5*, and expression of cardiomyocyte marker *TNNT2*, suggesting that *LGR5* could play a role in specification of mesoderm and cardiovascular progenitors.

Knockdown of *LGR5* Does Not Affect Undifferentiated hPSC Growth, but Alters Anterior-Posterior Mesoderm Patterning

To examine the effect of *LGR5* knockdown on hPSC growth and differentiation, we first generated stable cell lines by targeting *LGR5* using short hairpin RNAs (shRNAs) or scrambled sequences as a control. As expected, the *LGR5* mRNA expression was significantly lower in *LGR5* shRNA cultures than in control shRNA cultures (Figure S2A). However, cell morphology, growth rate, and expression of stem cell markers were similar between control shRNA cultures and *LGR5* shRNA cultures (Figure S2). Next, the *LGR5* shRNA and control shRNA cultures were induced for cardiomyocyte differentiation. A time-course analysis showed that *LGR5* mRNA levels remained significantly lower in *LGR5* shRNA cultures than in control shRNA cultures throughout the differentiation (Figure 2C). At differentiation day 2, the morphology of *LGR5* shRNA and control shRNA cultures was similar; however, at day 5, cells from *LGR5* shRNA cultures were mostly large and flat while cells from control shRNA cultures were small and densely packed (Figures 2A and 2B). The transient expression patterns of mesendodermal markers *T* and *MIXL1* were similar in *LGR5* shRNA cultures and control shRNA cultures: the expression of *T* increased at day 1 and peaked at day 2 and the expression of *MIXL1* peaked at days 1 and 2 (Figure 2D). However, compared with control shRNA cultures, *LGR5* shRNA cultures had significantly lower levels of these mesendodermal markers (at days 1, 2, and 3 for *T* and at day 1 for *MIXL1*) (Figure 2D). These results suggest that knockdown of *LGR5* does not delay mesendodermal induction but reduces the efficiency of mesendodermal induction.

We next examined the expression of genes involved in the development of anterior and posterior mesoderm. Gene expression levels of anterior mesoderm markers *EOMES*, *GSC*, and *TBX6* were significantly lower in *LGR5* shRNA cultures than in control shRNA cultures (at day 2 for *GSC* and *TBX6*, and at days 5 and 8 for *EOMES*) (Figure 2E), whereas the expression of posterior mesoderm markers *CDX1* and *CDX4* was significantly higher in *LGR5* shRNA cultures than in control shRNA cultures at day 2 (Figure 2F). In addition, *LGR5* shRNA cultures had significantly lower levels of cardiac mesodermal markers *MESP1* and *MESP2* and endodermal markers *SOX17* and *HNF3B* than control shRNA cultures at various time points

examined (Figures 2G and 2H). These results suggest that knockdown of *LGR5* in hPSCs alters the expression of genes involved in anterior-posterior mesoderm patterning and reduces cardiac mesoderm and endoderm differentiation.

Knockdown of *LGR5* Inhibits Cardiomyocyte Differentiation from hPSCs

We next investigated the effect of *LGR5* knockdown on cardiac progenitor and cardiomyocyte differentiation of IMR90 iPSCs. At days 8 and 14 after cardiac induction, cells from *LGR5* shRNA cultures remained mostly large and flat while cells from control shRNA cultures remained densely packed (Figures 3A and 3B). At day 8, the gene expression levels of four out of five cardiac progenitor markers examined (*HAND1*, *MEF2C*, *NKX2-5*, and *TBX5* except for *ISL1*) were significantly lower in *LGR5* shRNA cultures than in control shRNA cultures (Figure 3C). While the majority of control shRNA cells started beating at days 8–10 (and persisted until day 14) (Movie S1), very few *LGR5* shRNA cells were beating (Movie S2). At day 14, cardiomyocyte purity was significantly lower in *LGR5* shRNA cultures than in control shRNA cultures. There were fewer α -actinin/*NKX2-5* double-positive cells in *LGR5* shRNA cultures than in control shRNA cultures as detected by immunocytochemistry (Figure 3D), and ~8% α -actinin⁺ cells were present in *LGR5* shRNA cultures compared with ~54% in control shRNA cultures as detected by flow cytometry (Figures 3E and 3F). In addition, mRNA levels of cardiomyocyte markers *MYH6*, *MYH7*, *MYL2*, *MYL7*, and *TNNT2* were significantly lower in *LGR5* shRNA cultures than in control shRNA cultures at day 14 (Figure 3G).

We also examined the effect of *LGR5* knockdown on cardiomyocyte differentiation from another cell line (H9 hESCs) and a different batch of IMR90 iPSCs. Knockdown of *LGR5* was observed at both the mRNA level and the protein level for both the cell lines (Figure S1): *LGR5* mRNA levels were significantly reduced in *LGR5* shRNA cultures compared with control shRNA cultures (Figures S1C and S1H), and the proportion of cells positive for *LGR5* protein was reduced to ~1% in *LGR5* shRNA cultures at day 2 compared with 3%–10% of the cells in control shRNA cultures (Figures S1B and S1G), and reduced to ~6% in *LGR5* shRNA cultures at day 5 compared with ~50% of the cells in control shRNA cultures (Figures S1D and S1I). To determine the effect of *LGR5* knockdown on cardiomyocyte differentiation, parallel cultures were maintained until day 14 and examined for purity of cardiomyocytes. Only ~4% and 1% of α -actinin⁺ cells was detected in *LGR5* shRNA cultures compared with ~57% and 56% of α -actinin⁺ cells detected in control shRNA cultures derived from IMR90 iPSCs and H9 hESCs, respectively (Figures S1E and S1J). These results further confirm that knockdown of *LGR5* inhibits cardiomyocyte differentiation.

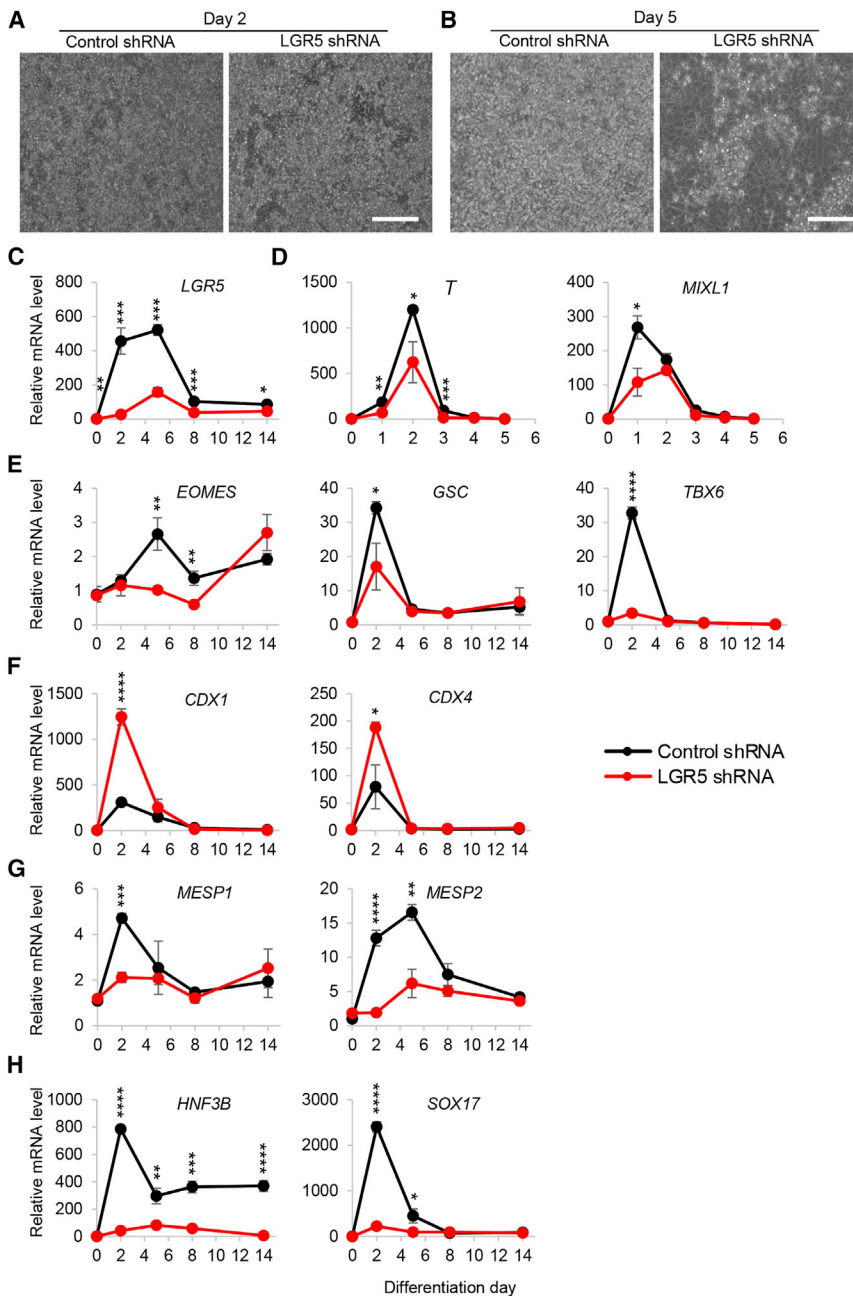


Figure 2. Knockdown of *LGR5* Alters Anterior-Posterior Mesoderm Patterning and Inhibits the Expression of Cardiac Mesodermal and Endodermal Markers at the Early Stage of Cardiomyocyte Differentiation

(A and B) IMR90 iPSC morphology of control shRNA and *LGR5* shRNA cultures at day 2 (A) and day 5 (B) of cardiomyocyte differentiation. Cells from control shRNA cultures were tightly packed but cells from *LGR5* shRNA cultures appeared as flat monolayer morphology. Scale bars, 200 μ m.

(C–H) qRT-PCR analyses of the following genes in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at differentiation days 0, 2, 5, 8 and 14: (C) *LGR5*; (D) mesendodermal markers *T* and *MIXL1*; (E) anterior mesoderm markers *EOMES*, *GSC*, and *TBX6*; (F) posterior mesoderm markers *CDX1* and *CDX4*; (G) cardiac mesodermal markers *MESP1* and *MESP2*; and (H) endodermal markers *SOX17* and *HNF3B*.

n = 3 independent experiments. Data are presented as mean \pm SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S2.

Knockdown of *LGR5* Promotes Endothelial Differentiation

At day 14, *LGR5* shRNA cultures were mostly a monolayer of cells with endothelial-like cell morphology, whereas control shRNA cultures contained beating cardiomyocytes (Figure 3B). Given this observation, we characterized endothelial cell differentiation in *LGR5* shRNA and control shRNA cultures. We examined the gene expression of *HHEX*, *TAL1*, *SOX7*, and *LMO2*, markers associated with the development of hemato-endothelial lineages which

can give rise to endothelial cells. The expression of *TAL1* and *SOX7* increased over time during cardiomyocyte differentiation from control shRNA cultures, whereas that of *HHEX* and *LMO2* did not (Figure 4A). Compared with control shRNA cultures, *LGR5* shRNA cultures had higher gene expression levels of all four hemato-endothelial markers examined at various time points (Figure 4A). We also examined the expression of endothelial cell markers during differentiation. At differentiation days 8 and 14, the relative mRNA levels of endothelial markers *CD31*, *CD34*, and

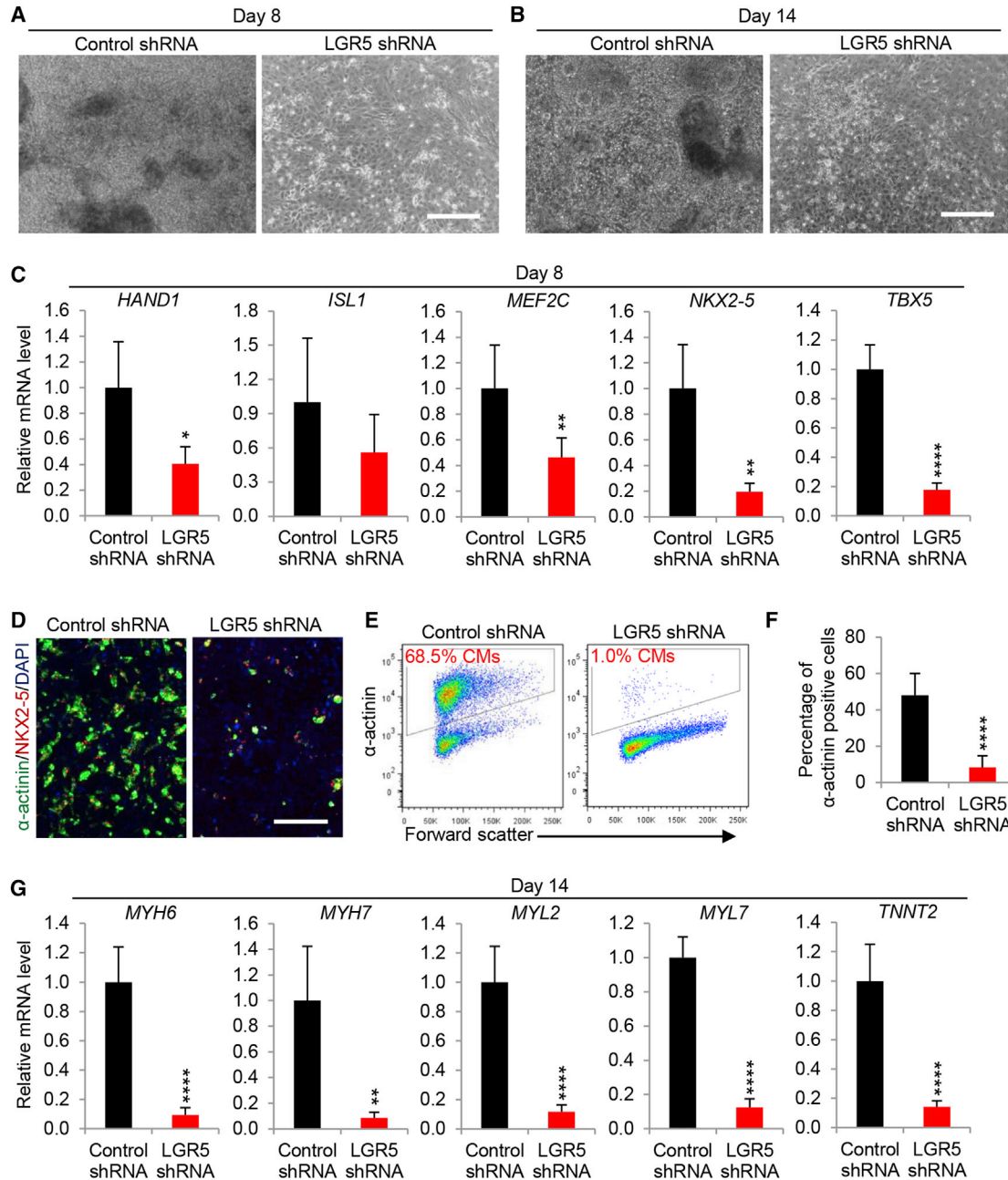


Figure 3. Knockdown of *LGR5* Inhibits Cardiomyocyte Differentiation

(A and B) Morphology of IMR90 iPSCs from control shRNA and *LGR5* shRNA cultures at day 8 (A) and day 14 (B). Cells from control shRNA cultures were tightly packed but cells from *LGR5* shRNA cultures showed flat monolayer morphology. Scale bars, 200 μ m.

(C) qRT-PCR analysis of cardiac transcription factors *HAND1*, *ISL1*, *MEF2C*, *NKX2-5*, and *TBX5* in IMR90 iPSCs at day 8.

(D) Detection of cardiomyocyte markers α -actinin and *NKX2-5* in IMR90 iPSCs at day 14 by immunocytochemistry. Scale bar, 100 μ m.

(E) Representative flow-cytometry analysis of α -actinin in IMR90 iPSCs at day 14.

(F) Summary of cardiomyocyte differentiation efficiency in IMR90 iPSCs at day 14.

(G) qRT-PCR analysis of cardiomyocyte-associated markers *MYH6*, *MYH7*, *MYL2*, *MYL7*, and *TNNT2* in IMR90 iPSCs at day 14.

n = 3–5 independent experiments. Data are presented as mean \pm SEM. *p < 0.05; **p < 0.01; ****p < 0.0001. See also Figure S1.

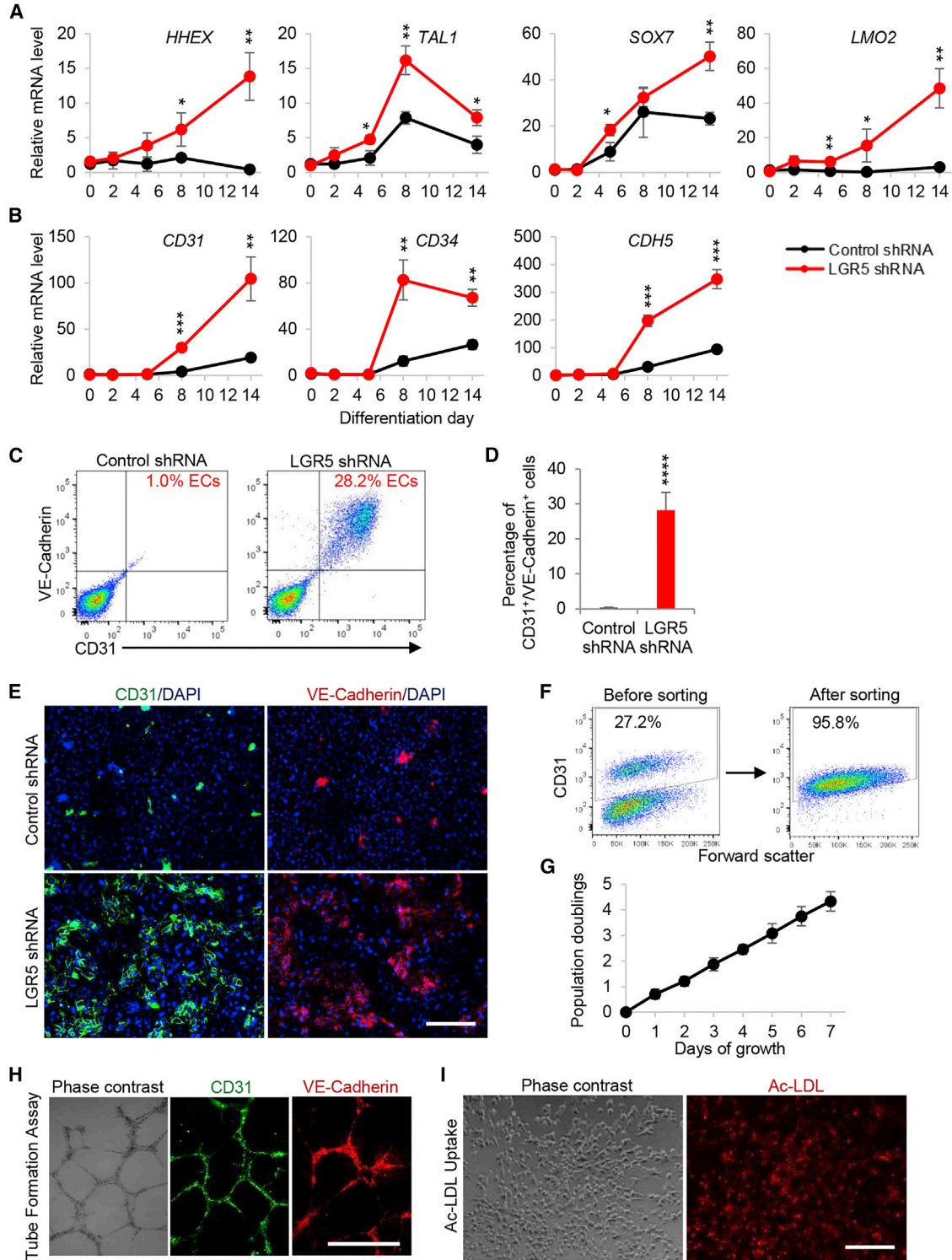


Figure 4. Knockdown of *LGR5* Potentiates Endothelial Differentiation

(A) qRT-PCR analysis of genes involved in development of hemato-endothelial lineages *HHEX*, *TAL1*, *SOX7*, and *LMO2* in IMR90 iPSCs at days 0, 2, 5, 8, and 14.
 (B) qRT-PCR analysis of endothelial cell markers *CD31*, *CD34*, and *CDH5* in IMR90 iPSCs at days 0, 2, 5, 8, and 14.
 (C) Representative flow-cytometry analysis of endothelial cell markers CD31 and VE-cadherin in IMR90 iPSCs at day 14.

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CDH5 (VE-cadherin) were higher in *LGR5* shRNA cultures than in control shRNA cultures (Figure 4B). At day 14, 28% of the cells were double positive for CD31 and VE-cadherin proteins in *LGR5* shRNA cultures, whereas only ~1%–4% of the cells were positive for these markers in control shRNA cultures (Figures 4C and 4D). These endothelial cell markers were found to be localized to cell surface in a subset of cells from *LGR5* shRNA cultures (Figures 4E and S3A). These results indicate that knockdown of *LGR5* increases the expression of markers associated with hemoto-endothelial lineages and endothelial cells.

We further examined whether the observed endothelial-like cells possessed expected functional characteristics. Following the treatment of differentiated cells at day 14 on Matrigel with vascular endothelial growth factor (VEGF) for 24 hr, cells from *LGR5* shRNA cultures formed tube-like networks (a feature of endothelial cells), whereas cells from control shRNA cultures did not (Figure S3B). Analysis of acetylated low-density lipoprotein (Ac-LDL) uptake (another feature of endothelial cells) revealed that a large proportion of the *LGR5* shRNA cultures were positive for fluorescently labeled Ac-LDL, but very few cells from control shRNA cultures were positive (Figure S3C). To further confirm these observations, we purified CD31⁺ endothelial cells (~96%) by fluorescence-activated cell sorting (FACS) from *LGR5* shRNA cultures at day 14 (Figure 4F) and then conducted cell-proliferation, tube-formation, and Ac-LDL-uptake assays. These purified CD31⁺ cells proliferated in endothelial cell medium supplemented with VEGF with a cell population doubling time of ~3 days (Figure 4G), formed tube-like networks that were also positive for VE-cadherin (Figure 4H), and showed robust Ac-LDL uptake (Figure 4I). These results support that the observed endothelial-like cells from *LGR5* shRNA cultures were bona fide endothelial cells.

Knockdown of *LGR5* Inhibits Proliferation of Differentiated hPSCs at the Early Stage

Since *LGR5* is involved in promoting cellular proliferation in other cells (Barker and Clevers, 2010; Barker et al., 2009; Nakata et al., 2013; Schepers et al., 2012; Tanese et al., 2008), we investigated whether modulation of *LGR5* expression levels affected proliferation of hPSCs

during cardiomyocyte differentiation. Both control shRNA cultures and *LGR5* shRNA cultures were subjected to cardiomyocyte differentiation and monitored for cellular proliferation. While there was a comparable number of cells in control shRNA cultures and *LGR5* shRNA cultures at the time of induction of differentiation (day 0), cell density assay showed significantly fewer cells in *LGR5* shRNA cultures than in control shRNA cultures at differentiation days 5, 8, and 14 (Figure 5A), which is consistent with the morphology of the cultures (Figures 2A, 2B, 3A, and 3B). We also examined the expression of Ki-67, an indicator for cells in active phases of the cell cycle, by flow cytometry. At differentiation day 0, the proportion of Ki-67⁺ cells was comparable between control shRNA cultures and *LGR5* shRNA cultures; >80% of the cells were positive for Ki-67 in these cultures (Figures 5B and 5C). However, at differentiation day 5, the proportion of Ki-67⁺ cells was significantly reduced in *LGR5* shRNA cultures compared with control shRNA cultures; ~79% and ~57% Ki-67⁺ cells were detected in control shRNA and *LGR5* shRNA cultures, respectively (Figures 5D and 5E). At days 8 and 14, the proportion of Ki-67⁺ cells decreased to <20% in both *LGR5* shRNA cultures and control shRNA cultures (Figure 5F). Consistent with these findings, the transcript levels of proliferation markers including *CCND1*, *MKI67*, and *PCNA* were comparable at day 0 between control shRNA cultures and *LGR5* shRNA cultures, but significantly lower at day 5 in *LGR5* shRNA cultures than in control shRNA cultures (Figure 5F). These results suggest that in the early stage of differentiation, *LGR5* plays a role in the proliferation of cardiac progenitors.

Knockdown of *LGR5* Downregulates the Expression of Canonical and Non-canonical Wnt Signaling-Related Genes

Regulation of Wnt signaling drives cardiac differentiation and development (Gessert and Kuhl, 2010). Expression of canonical and non-canonical Wnt signaling-related genes is temporally regulated during cardiomyocyte differentiation (Mazzotta et al., 2016). As expected, the relative levels of canonical Wnt target genes, *AXIN2* and *LEF1*, increased during the early stage of cardiomyocyte differentiation and peaked to 15- and 500-fold higher at differentiation day 3

(D) Summary of endothelial cell differentiation efficiency in IMR90 iPSCs at day 14.

(E) Detection of endothelial cell markers CD31 and VE-cadherin in IMR90 iPSCs at day 14 by immunocytochemistry.

(F) Enrichment of CD31⁺ cells at day 14 derived from IMR90 iPSCs by FACS.

(G) Proliferation of purified CD31⁺ cells derived from IMR90 iPSCs.

(H) Tube-forming ability of purified CD31⁺ cells derived from IMR90 iPSCs upon VEGF treatment; detection of endothelial cell markers CD31 and VE-cadherin in tube-like networks by immunocytochemistry.

(I) Ac-LDL uptake in purified CD31⁺ cells derived from IMR90 iPSCs.

n = 3 independent experiments. Data are presented as mean ± SEM. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Scale bars, 100 μm. See also Figure S3.

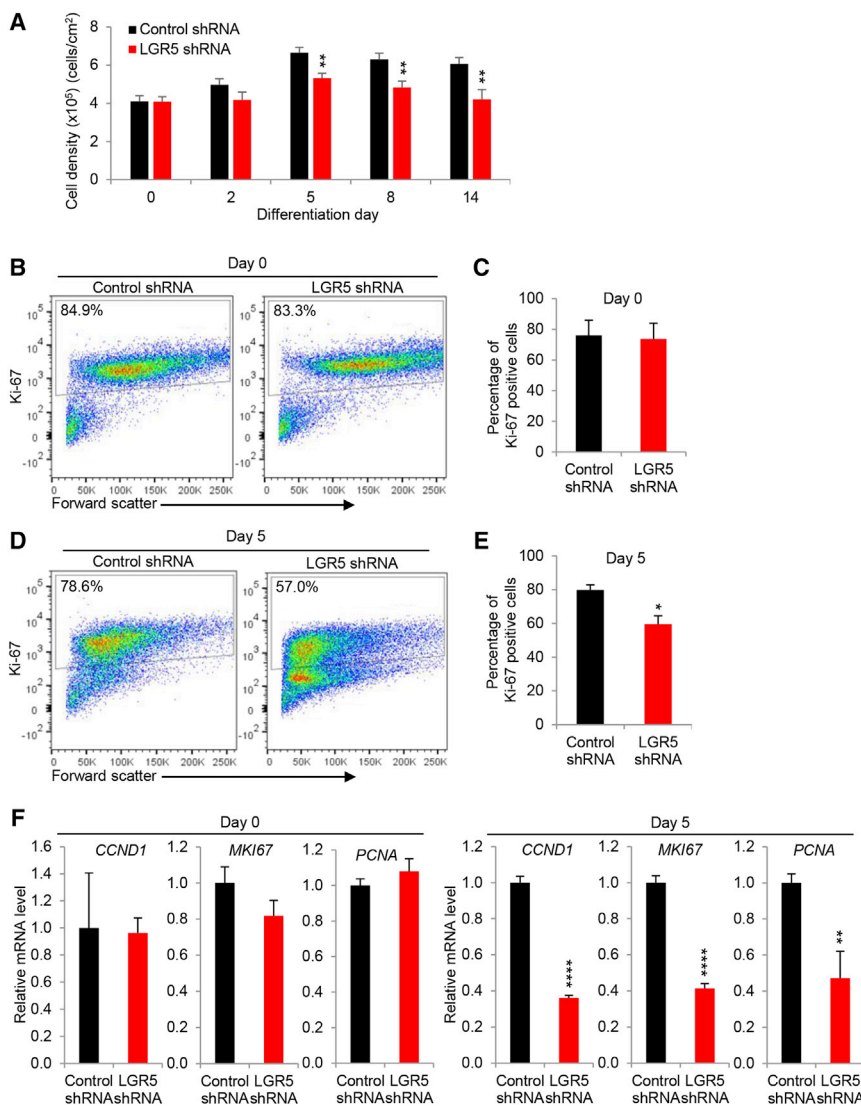


Figure 5. Knockdown of *LGR5* Inhibits Proliferation of Differentiated Pluripotent Stem Cells

(A) Cell densities of IMR90 iPSCs during cardiomyocyte differentiation.

(B) Representative flow-cytometry analysis of cell proliferation marker Ki-67 in IMR90 iPSCs at differentiation day 0.

(C) Summary of percentage of Ki-67⁺ cells in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at differentiation day 0.

(D) Representative flow-cytometry analysis of cell proliferation marker Ki-67 in IMR90 iPSCs at differentiation day 5.

(E) Summary of percentage of Ki-67⁺ cells in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at differentiation day 5.

(F) qRT-PCR analysis of proliferation genes *CCND1*, *MKI67*, and *PCNA* in IMR90 iPSCs at days 0 and 5.

n = 3 independent experiments. Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ****p < 0.0001. See also Figure S4.

compared with those at day 0, respectively (Figure S5A). Similarly, the expression of canonical Wnt genes *WNT3A* and *WNT8A* was transiently upregulated and reached to 1,800- and 6,000-fold higher at day 3 compared with those at day 0 (Figure S5B). The expression of non-canonical Wnt signaling-related gene, *WNT11*, increased and reached its highest transcript levels at days 8–12 (300-fold higher compared with those at day 0) (Figure S5C). The expression of another non-canonical Wnt signaling-related gene, *WNT5A*, peaked early from days 2 to 5 and stayed at levels higher than those at day 0 at later time points (Figure S5C).

Since *LGR5* is a receptor for R-spondins, which are potent Wnt signal regulators (Cambier et al., 2014), we investigated whether knockdown of *LGR5* affects Wnt signaling during cardiomyocyte differentiation. Compared with control shRNA cultures, *LGR5* shRNA cultures had

significantly reduced expression of the following Wnt signaling-related genes at the early stage: (1) Wnt target genes *AXIN2* and *LEF1* at days 2 or 5 (Figure 6A), (2) canonical Wnt signaling-related genes *WNT3A* and *WNT8A* at day 2 (Figure 6B), and (3) non-canonical Wnt signaling-related genes *WNT5A* and *WNT11* at days 2 and 5 or days 5 and 8 (Figure 6C).

We further investigated the effect of *LGR5* knockdown on the activation of β -catenin by analyzing protein level of active β -catenin or its unphosphorylated form at Ser-37 and Thr-41 during the early stage of cardiomyocyte differentiation. In control shRNA cultures, the proportion of cells positive for active β -catenin protein was ~55% at basal level (day 0), ~91% at differentiation day 1, and ~8% at day 4 (Figure 6D). However, the proportion of cells positive for active β -catenin in *LGR5* shRNA cultures was more than

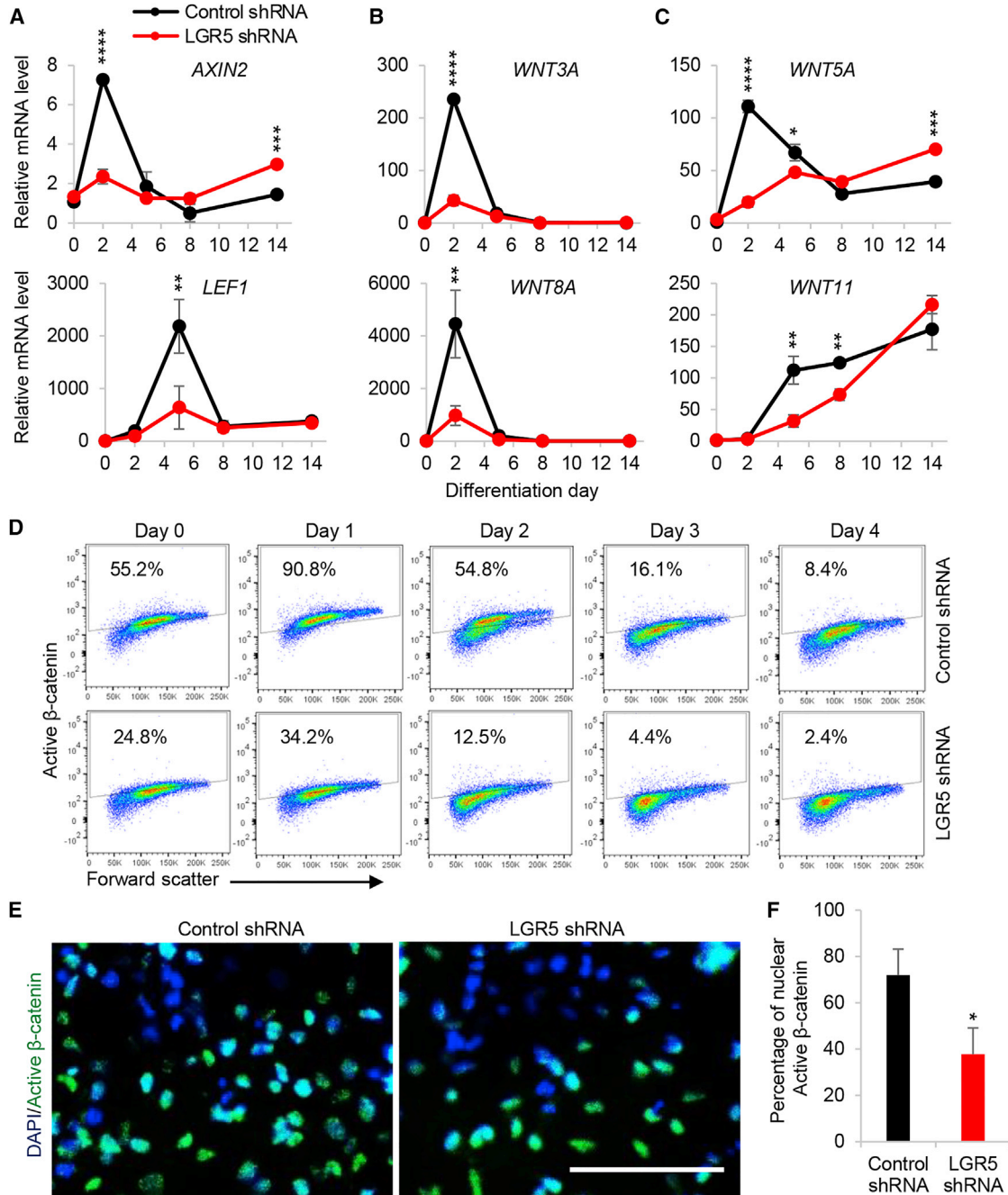


Figure 6. Knockdown of *LGR5* Inhibits the Activation of β -Catenin and the Expression of Wnt Signaling-Related Genes during Cardiomyocyte Differentiation

(A–C) qRT-PCR analysis of Wnt signaling-related genes in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at days 0, 2, 5, 8, and 14. (A) Wnt target genes *AXIN2* and *LEF1*; (B) canonical Wnt signaling-related genes *WNT3A* and *WNT8A*; and (C) non-canonical Wnt signaling-related genes *WNT5A* and *WNT11*.

(D) Flow-cytometry analysis of active β -catenin protein during cardiomyocyte differentiation in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at days 0–4.

(E) Immunocytochemistry analysis of active β -catenin protein at day 1 of cardiomyocyte differentiation in control shRNA and *LGR5* shRNA IMR90 iPSC cultures. Scale bar, 100 μ m.

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2- to 4-fold lower at all time points compared with the parallel control shRNA cultures (Figure 6D). Since nuclear translocation of active β -catenin is a hallmark for the activation of canonical Wnt pathway, we also examined the localization of active β -catenin by immunocytochemistry. We found significantly fewer cells positive for active nuclear β -catenin in *LGR5* shRNA cultures than in control shRNA cultures at day 1 (Figures 6E and 6F).

Together, these data suggest that knockdown of *LGR5* affects the expression of Wnt signaling-related genes and the activation of β -catenin during cardiomyocyte differentiation from hPSCs.

DISCUSSION

Differentiation of cardiomyocytes and endothelial cells is tightly regulated during differentiation of hPSCs, and Wnt signaling pathways are important in regulating both cardiomyocyte and endothelial cell differentiation through ligand-receptor interactions. Therefore, understanding additional molecules involved in Wnt signaling is crucial to controlling efficient cardiomyocyte and endothelial cell differentiation from hPSCs. In this study, we found that expression of *LGR5* (which encodes a cell membrane-associated regulator of Wnt signaling) was transiently upregulated during the early stage of cardiomyocyte differentiation from hPSCs. In undifferentiated cells, knockdown of *LGR5* did not affect cell growth or gene expression of stem cell markers; however, knockdown of *LGR5* reduced mesendoderm induction, altered mesoderm patterning, reduced the expression of cardiac transcription factors, and inhibited cardiomyocyte differentiation. Furthermore, knockdown of *LGR5* promoted the differentiation of hPSCs into endothelial cells with typical *in vitro* functional characteristics, including formation of tube-like structures and Ac-LDL uptake, although further confirmation in animal models is required. Knockdown of *LGR5* also inhibited cellular proliferation of early differentiated cells, and decreased the expression of Wnt signaling-related genes and nuclear-localized active β -catenin. These results suggest that *LGR5* is critical for controlling differentiation into cardiomyocytes and endothelial cells, possibly by fine-tuning Wnt signaling and regulating progenitor cell proliferation and mesoderm patterning.

LGR5 functions as a growth-promoting molecule, and has been shown to promote cellular proliferation in several

stem cell and cancer cell models (Barker and Clevers, 2010; Barker et al., 2009; Nakata et al., 2013; Schepers et al., 2012; Tanese et al., 2008). We found that knockdown of *LGR5* in hPSCs did not affect the growth and proliferation of undifferentiated cells. However, knockdown of *LGR5* significantly reduced the proliferation of differentiated cells at the early stage and resulted in poor outcome of cardiac differentiation at the late stage. It is possible that reduced proliferation of progenitors in *LGR5* knockdown cultures affects the cell density at crucial stages of cardiomyocyte differentiation, preventing the selection and expansion of cardiac progenitors and, consequently, cardiomyocyte differentiation. Thus, the effect of *LGR5* knockdown on cardiomyocyte differentiation may be mediated through a cell density-dependent mechanism. However, it is also possible that *LGR5* directly affects cell fate decisions, since knockdown of *LGR5* alters mesoderm patterning and expression of genes associated with cardiac mesoderm, hemato-endothelial lineages, and Wnt signaling.

Mesoderm patterning is an essential step in controlling progenitors to differentiate into cardiomyocytes and endothelial cells. Our results show that knockdown of *LGR5* reduces the expression of anterior mesoderm markers (*EOMES*, *GSC*, and *TBX6*) but increases the expression of posterior mesoderm markers (*CDX1* and *CDX4*) compared with control shRNA cultures. This expression pattern may contribute to decreased cardiomyocyte differentiation from anterior mesoderm-like cells (Murry and Keller, 2008) and increased endothelial cell differentiation from posterior mesoderm-like cells in *LGR5* knockdown cultures.

Knockdown of *LGR5* increased the expression of markers associated with hemato-endothelial lineages, which can differentiate into endothelial cells. These hemato-endothelial markers include: (1) *HHEX*, which encodes a transcription factor that is expressed specifically in hemato-endothelial lineages (Kubo et al., 2005; Paz et al., 2010); (2) *TAL1* (*SCL*), which encodes a transcription factor that promotes hemato-endothelial specification and suppresses cardiogenesis (Org et al., 2015; Real et al., 2012; Van Handel et al., 2012); (3) *SOX7*, which encodes a transcriptional regulator that binds and activates *CDH5*, required for the development of both hematopoietic and endothelial cells (Costa et al., 2012; Nelson et al., 2009); and (4) *LMO2*, which encodes a protein that has a crucial role in hematopoietic development (Landry et al., 2005; Meng et al., 2016; Patterson et al., 2007). Our results raise the possibility that endothelial cells in *LGR5* shRNA cultures may be

(F) Summary of percentage of cells positive for nuclear-localized active β -catenin in control shRNA and *LGR5* shRNA IMR90 iPSC cultures at day 1.

n = 3 independent experiments. Data are presented as mean \pm SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. See also Figure S5.



derived from hemato-endothelial cell lineages. Since hemato-endothelial lineages also give rise to hematopoietic cells, our results encourage future studies to examine the effect of *LGR5* knockdown on hematopoietic cell differentiation.

LGR5 is known as both a target and a regulator of Wnt signaling (de Lau et al., 2014). We found that knockdown of *LGR5* resulted in a reduction of active β -catenin (nuclear-localized β -catenin) and downregulated the expression of canonical and non-canonical Wnt signaling-related genes at the early stage of differentiation, suggesting that *LGR5* is a regulator of Wnt signaling during cardiomyocyte differentiation. Temporal regulation of Wnt signaling has been used to direct hPSC differentiation into cardiomyocytes or endothelial cells. For example, stage-specific activation and inhibition of Wnt signaling are essential for efficient cardiomyocyte differentiation (Kattman et al., 2011; Lian et al., 2012; Mazzotta et al., 2016; Paige et al., 2010; Palpant et al., 2013, 2015). Activation of Wnt signaling by small molecules can lead to high yield of endothelial cell differentiation (Lian et al., 2014). *TMEM88*, a negative regulator of Wnt/ β -catenin signaling, has been shown to regulate cardiomyocyte and endothelial cell differentiation; knockdown of *TMEM88* inhibits cardiomyocyte differentiation, but promotes endothelial cell differentiation (Palpant et al., 2013). Since Wnt signaling has a complex temporal role during cardiomyocyte and endothelial cell differentiation, the effect of *LGR5* knockdown we observed may be stage specific as well. Future studies using inducible shRNA may reveal the timing of *LGR5* expression that is critical for cardiomyocyte and endothelial cell differentiation.

Our results show remarkable changes in efficiency of cardiac and endothelial differentiation of hPSCs upon *LGR5* knockdown. Neonatal lethality was observed in *Lgr5* null mice that show ankyloglossia and gastrointestinal distension (Morita et al., 2004). The differential contribution of *LGR5* genes to human and mouse cardiomyocyte and endothelial cell differentiation remains to be further elucidated. Molecular features of early embryonic development in mice do not always match findings in humans. For example, analysis of human embryos from zygote to blastocyst demonstrates significant difference relative to the mouse in the expression of early lineage-specific genes (Niakan and Eggan, 2013). Human-mouse difference during early embryo development has also been observed in the regulation of metabolism associated with the pluripotent state (Gu et al., 2016). Naive hESCs have increased glycolytic rate compared with primed hESCs while the opposite is true in mouse-naive versus primed ESCs, possibly due to discrepancy between human and mouse in nuclear C-MYC levels in naive versus primed stem cells (Gu et al., 2016).

Conclusion

Together, these results reveal a previously unappreciated role of *LGR5* in the differentiation of cardiomyocytes and endothelial cells from hPSCs. *LGR5* expression is transiently upregulated during the early stage of cardiomyocyte differentiation from hPSCs, and although *LGR5* expression is not required for maintaining hPSCs in the undifferentiated state, knockdown of *LGR5* leads to decreased expression of key cardiac transcription factors at the early stage with eventual lack of robust beating cardiomyocytes at the late stage. Knockdown of *LGR5* also potentiates differentiation of hPSCs into endothelial-like cells. Therefore, *LGR5* is critical in cardiac and endothelial differentiation. These findings will advance our understanding of the molecular underpinnings of efficient differentiation of hPSCs into cardiovascular lineages.

EXPERIMENTAL PROCEDURES

Undifferentiated Cell Cultures and Differentiation of Cardiomyocytes and Endothelial-like Cells

Undifferentiated H7 and H9 hESCs (Thomson et al., 1998) and IMR90 iPSCs (Yu et al., 2007) were maintained as previously described (Xu et al., 2001). For cardiomyocyte differentiation, cells were induced using a growth factor-guided differentiation protocol (Jha et al., 2015). Cells were maintained as undifferentiated cells in mouse embryonic fibroblast-conditioned medium (MEF-CM) supplemented with basic fibroblast growth factor (bFGF) (8 ng/mL). Single cell suspensions were prepared using Versene and seeded at a density of 4×10^5 cells in 1 mL of MEF-CM for each well of 24-well Matrigel-coated plates. Cells were fed daily by replacing MEF-CM supplemented with bFGF (8 ng/mL) until cells reached confluence. Usually 2–3 days after seeding at day 0 of induction, medium was replaced with 1 mL of medium (RPMI 1640 with 2% B27 without insulin) supplemented with 100 ng/mL activin A. On the next day (day 1), the medium was replaced with 1 mL of RPMI 1640 medium supplemented with 2% B27 (without insulin) and 10 ng/mL BMP4, and cells were cultured without any medium change for the next 4 days. From day 5 onward, the BMP4-containing medium was replaced with 1 mL of RPMI 1640 with 2% B27 (with insulin) and the medium was changed on alternate days until day 14. Cells were observed daily under a microscope for beating cells, which typically started after day 8. For endothelial cell differentiation, mesoderm induction was accomplished by activin A and BMP4 as described for cardiomyocyte differentiation. At day 8 onward, the medium was replaced with endothelial cell growth medium consisting of M199 Medium (Lonza, #12-118F), 20% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, endothelial cell growth supplement (ECGS; Biomedical Technologies #BT-203, final concentration 50 μ g/mL), and heparin (Sigma #H3393-10KU, final concentration 100 μ g/mL) (Maciag et al., 1981; Thornton et al., 1983). The medium was changed on alternate days until day 14. Cells were pooled from multiple wells (at least 3 wells) for each biological sample and at least triplicates of biological samples were



used for each condition in assays. All cell culture reagents were purchased from Fisher Scientific unless specified.

Preparation of MEFs was approved by the Emory University Institutional Animal Care and Use Committee.

Knockdown of *LGR5*

shRNA pLKO.1 plasmid vectors expressing target-specific sequences against human *LGR5* (#11586) and non-target scrambled control (#SHC016) were obtained from the RNAi Consortium (TRC, MISSION TRC shRNA library, Sigma). shRNA-mediated gene knockdown was performed by nucleofection of plasmid vectors (for IMR90 iPSCs) or lentiviral transduction (for H9 hESCs and another batch of IMP90 iPSCs). Cells were dissociated using Versene and single cell suspension was washed once with PBS. For nucleofection, 1×10^6 cells were suspended in 100 μ L of nucleofection buffer containing 1 μ g of plasmids and nucleofected using Amaxa nucleofector II program A033. For lentiviral transduction, 1×10^6 cells were mixed with 1 MOI of viral particles and 6 μ M polybrene (Sigma). Cells were immediately collected and seeded in Matrigel-coated 6-well plates containing 1 mL of MEF-CM supplemented with 10 μ M Stemolecule Y27632, rock inhibitor (Stemgent). Cells were fed daily with MEF-CM with bFGF (10 ng/mL) for 7 days until reaching around 80%–90% confluence. The cells were then subjected to antibiotic selection for 7 days using 1 μ g/mL puromycin (the dose of puromycin was predetermined in a killing curve experiment showing that minimum puromycin concentration killing all hPSCs in 5 days was 1 μ g/mL). Stable cell lines were generated from surviving cells that were resistant to puromycin and further expanded in successive passages under puromycin selection. Efficiency of gene knockdown after three passages was examined using qRT-PCR.

Flow-Cytometry Analysis

Differentiation of cardiomyocytes was confirmed by intracellular staining of α -actinin using flow cytometry. Differentiated cultures at day 14 were harvested in 0.25% trypsin/EDTA at 37°C for 10 min and subsequently neutralized by 10% FBS in DMEM and washed with PBS. Cells were counted and 1×10^6 cells each were stained for α -actinin and isotype control along with compensation controls including unstained, ethidium monoazide bromide (EMA) only and α -actinin only. Cells were first incubated with 1 μ g/mL EMA in staining buffer (2% FBS in PBS) on ice in the dark for 15 min, pelleted, resuspended in 1 mL of PBS, and exposed to bright light on ice for 10 min in a horizontally slanted position. After washing, cells were fixed by 4% paraformaldehyde at room temperature for 15 min and permeabilized with 90% ice-cold methanol on ice for 30 min. The cells were then washed, and incubated with the blocking solution consisting of staining buffer supplemented with 20% normal goat serum at room temperature for 30 min. After blocking, cells were incubated for 20 min at room temperature with sarcomeric α -actinin primary antibodies or mouse immunoglobulin G1 isotype control in the blocking solution. Cells were washed and incubated for 15 min at room temperature in the dark with an Alexa 488 conjugated secondary antibody (Table S1). Cells were washed twice and suspended in 200 μ L of staining buffer. For staining of *LGR5* and endothelial cell surface markers CD31 and VE-cadherin (Table S1), a similar procedure was followed

except without cell permeabilization. BD FACS Canto II was used for data acquisition by adjusting voltage and compensation using appropriate excitation and detection channels; fluorescein isothiocyanate and PerCP Cy5.5 channels were used to analyze cells stained for α -actinin and EMA, respectively. Forward versus side scatter quadrants were defined, and at least 10,000 EMA negative (live) events were acquired for each sample. Dot plots were generated upon data analysis using FlowJo software.

Tube-Formation Assay

Ice-cold undiluted Matrigel (200 μ L) was used to coat each well of 48-well plates and incubated for 40 min at 37°C to allow the gel to solidify. Cultures at day 14 were dissociated using 0.25% trypsin/EDTA solution by incubating at 37°C for 5 min and further neutralized with 10% FBS in DMEM. Cell suspension was centrifuged at $200 \times g$ and cell pellets were resuspended in supplemented basal LaSR medium (DMEM Advanced F12 supplemented with GlutaMAX and L-ascorbic acid). Cells were then seeded gently at a density of 2×10^4 cells per well in 200 μ L of medium and incubated with (50 ng/mL) or without bFGF at 37°C, 5% CO₂ for 18–24 hr, after which tube formation was confirmed by microscopy (Arnaoutova and Kleinman, 2010).

Uptake of Acetylated Low-Density Lipoproteins

Cells were seeded at 10^5 cells/cm² in Matrigel-coated 96-well plates 3 days prior to the uptake assay. On the day of the assay, the medium was removed and the cells were washed twice with PBS to remove non-adherent cells. The cells were incubated with 20 μ g/mL Ac-LDL-Alexa Fluor 594 (Life Technologies, #L-35353) for 4 hr at 37°C, washed twice with PBS, and further analyzed by fluorescence microscopy for the uptake of fluorescence-labeled Ac-LDL (Niu et al., 2013; Rath et al., 2014).

Cell Sorting and Expansion of Endothelial Cells

Day-14 differentiated cells were washed in PBS and dissociated using Accutase (STEMCELL Technologies) for 10 min at 37°C. Cells were gently triturated until single cell suspension was obtained. Cells were spun down at $1,000 \times g$ for 5 min, resuspended in 0.5 mL of PBS containing APC-conjugated CD31 antibody, and incubated on ice for 30 min followed by a single 10-mL PBS wash. FACS Aria II was used to initially exclude debris by gating FSC-SSC and only single cells were further included in the analysis based on width versus height gating parameters. Isolated cells were collected in endothelial cell growth medium MV2 (PromoCell) and used to confirm functional characteristics and cell expansion ability. The purified CD31⁺ cells were expanded in the endothelial cell growth medium supplemented with 50 ng/mL VEGF (PeproTech) for one passage before being examined for cell population doublings. To further examine the proliferation of CD31⁺ cells, we seeded these purified CD31⁺ cells (2×10^4 cells/cm²) onto laminin-coated plates in the endothelial cell growth medium supplemented with 50 ng/mL VEGF (PeproTech) and counted the cells daily to evaluate cell population doublings over 7 days.

Statistical Analysis

At least three sets of independent experiments were performed for each assay, and an unpaired t test was used to compare the control



shRNA group with the *LGR5* shRNA group. Data are presented as mean \pm SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, two tables, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.07.006>.

AUTHOR CONTRIBUTIONS

R.J., conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. M.S., collection and assembly of data and final approval of manuscript. Q.W., collection and assembly of data and final approval of manuscript. C.G., collection and assembly of data and final approval of manuscript. M.K.P., collection and assembly of data and final approval of manuscript. C.X., conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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