The Expression of *Wnt5a* Gene Throughout Definitive Endoderm Induction Process in Induced Pluripotent Stem Cells

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Abstract

Background: Induced pluripotent stem cells (iPSCs) have the ability to proliferate indefinitely and differentiate into three germ layers of ectoderm, mesoderm, and endoderm. Definitive induction is the first and the most delicate stage of differentiation of various iPSC-derived organs. It has been found that the Wnt signaling pathway implicates in embryogenesis, organogenesis, and cell communication.

Objectives: In the present study, we aimed to investigate the expression pattern of the *Wnt5a* gene as an indicator of non-canonical Wnt signaling activity during definitive endoderm induction of iPSCs.

Methods: Human iPSCs (RSCB0042) were acquired from Royan stem cell bank of Royan Institute (Tehran, Iran). The iPSCs were cultured on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (MEF), and iPSC colonies were collected for embryoid body (EB) generation by suspension culture method. Then endoderm induction step was performed using a series of small molecules. The quantitative real-time PCR was used to assess the mRNA expression of *wnt5a*, *Nanog*, *OCT4*, *SOX17*, and *FOXA2* genes.

Results: The production of efficient EBs confirmed by a decrease in *Nanog* and *Oct4* gene expression and the success of DE (definite endoderm) induction step was confirmed by a high expression level of DE specific genes, *Sox17*, and *FoxA2*. A significant upregulation of *Wnt5a* in EB samples and a minor decrease at day 4 was observed. However, the differentiation process followed by an incremental fashion in *Wnt5a* mRNA expression starting from day 4 of differentiation among the samples of days 6 and 8 (DE stage).

Conclusions: Our results suggest that *Wnt5a* is more activated at the later steps of endoderm induction rather than the early steps, which may be due to the stimulation of canonical Wnt signaling. Finding the expression level of *Wnt5a* could rise insights for developing more efficient differentiation induction protocols.

Keywords: Non-Canonical Wnt Signaling, Differentiation, Definitive Endoderm, Induced Pluripotent Stem Cells, Gene Expression

1. Background

The reprogramming techniques of somatic cells by the introduction of pluripotency transcription factors led to the generation of induced pluripotent stem cells (iPSCs). Like embryonic stem cells, iPSCs have the ability to proliferate indefinitely and differentiate into three germ layers of ectoderm, mesoderm, and endoderm (1). Definitive endoderm cells can be differentiated into internal organs such as lungs, liver, pancreas, and thyroid (2). Therefore, definitive induction is the first and the most delicate stage of differentiation of various iPSC-derived organs. There are diverse signaling pathways involved in definitive endoderm generation, including TGFβ, SHH, and Wnt pathways (3, 4).

Wnt signaling pathway comprises two different classes of canonical and non-canonical Wnt pathways. Both are controlled by a set of glycoprotein ligands, which are recognized by their cognate cell surface receptors, Frizzled family, and coreceptors of low-density-lipoprotein-related protein5/6 (LRP5/6). The attachment of Wnt ligands to frizzled receptors leads to the activation of Dishevelled (Dvl) protein (5, 6). In canonical Wnt signaling, β-catenin plays the central role, which is under constant degradation by a destructive complex, consist of, glycogen synthase kinase-3β (GSK-3β), adenomatous polyposis coli (APC), casein-kinase 1α (CK1α), and Axin (6, 7). By induction of canonical Wnt signaling by specific ligands such as Wnt3a, β-catenin is

translocated into the nucleus and activates Tcf/Lef transcription factors to initiate the expression of target genes ([8], [9]). Non-canonical Wnt/planar cell polarity (PCP) signaling is induced by different sets of ligands, such as *Wnt4* and *Wnt5a* ([10], [11]). The signal transduction cascade in Wnt/PCP pathway is initiated independent of β-catenin, from Dvl to secondary effectors, c-Jun N-terminal kinase (JNK), and the small GTPases of Rho and Rac, which lead to cytoskeleton rearrangement and activation of AP1 transcription factors to alter gene expression ([12]).

Wnt signaling pathway has been established to be implicated in embryogenesis, organogenesis, and cell communication. However, canonical Wnt signaling is responsible for cell hemostasis and proliferation whereas, Wnt/PCP is in favor of differentiation of stem cells into various types of cell lineages such as adipocytes, osteocytes, and cardiac cells ([6], [13]-[17]).

2. Objectives

This study was designed to investigate the expression pattern of the *Wnt5a* gene as an indicator of non-canonical Wnt signaling activity during definitive endoderm induction of iPSCs.

3. Methods

3.1. Cell Culture

The commercially available Human iPSCs (RSCB0042) were purchased from Royan stem cell bank (Royan Institute, Tehran, Iran). Briefly, iPSCs were cultured on a feeder layer of mitomycin (M7949, Sigma, Germany) inactivated mouse embryonic fibroblasts (MEF) incubated at 37°C, 5% CO_2 and humidity of 80 - 90% ([18]). Dulbecco modified eagle medium (DMEM)/Ham's F12 (Gibco, Life Technologies, USA) was used as the basic medium for iPSC culture supplemented with 20% knockout serum replacement (KOSR) (10828020, Gibco), 5 μg/mL selenium, insulin-transferrin-selenium (ITS) (I1884, Gibco), 100 Unit/mL penicillin, 100 μg/mL streptomycin (P4333, Sigma-Aldrich), 0.1 mM non-essential amino acids (M7145, Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (15433, Merck) and 12 ng/mL basic fibroblast growth factor (bFGF) (F0291, Sigma-Aldrich). The culture media was exchanged every other day, until iPSC colonies covered at least 70% of the culture flask surface. Then iPSC colonies were collected for embryoid body (EB) generation by suspension culture method ([19]). Subsequently, iPSC colonies were detached from culture flask by cell scraper and resuspended in ultra-low attachment plates (SPL, Korea) with DMEM/ F12 medium supplemented

with 20% fetal bovine serum (FBS) (Gibco), 100 Unit/mL penicillin, and 100 μg/mL streptomycin for three days. Then, EBs were collected and cultured in six-well cell culture plates (SPL) coated with 2% gelatin (G1890, Sigma, Germany) and kept for 3 days at 37°C, 5% CO_2, and humidity of 80 - 90%. The endoderm induction step was performed using a series of small molecules based on our previous researches ([4]). Therefore, the previous medium is removed and EBs were cultured in RPMI1640 medium (21875034, Gibco) containing 30 ng/mL of Activin A (H4666, Sigma) and 3 μM CHIR99021 (SML1046, Sigma) without serum supplement. Further CHIR99021 was removed from the medium after 24 hours with a gradual increase in serum supplement in the medium. Therefore, the next day, the medium was replaced with a fresh one containing 0.2% KOSR and 30 ng/mL of Activin A. After 48 hours, the medium was removed with the replacement of 2% KOSR containing medium, which was continued for three days. Cell samples were harvested for RNA extraction at days 0 (iPSC), 3 (EB), 4, 5, 6, and 8 (definitive endoderm).

3.2. Quantitative Real-time PCR

Collected cell samples were washed by Phosphate buffered saline (PBS), and then total RNA was extracted by RnaSol mRNA extraction kit (Alphabio, USA) according to the manufacturer's instruction. In order to prepare complementary DNA (cDNA), PrimeScript First Strand cDNA Synthesis Kit (6110A, Takara, Japan) was used with 500 ng of total RNA. The quantitative real-time PCR was performed by StepOnePlus instrument (Applied Biosystem, USA) and SYBR® Premix EX TaqTM II kit (RR820A, Takara, Japan). The unique forward and reverse primers for *Wnt5a* mRNA (sequences and melting temperatures are presented in Table 1) were uniquely designed by AlleleID software (version 7.5). Amplification was performed in 10 μL aliquots with 10 pM of each primer. *GAPDH* gene was used as an internal control for expression normalization. All reactions were done in triplicate.

3.3. Statistical Analysis

Real-time data were analyzed by LinRegPCR software (version 2017.1) to identify the highly efficient reactions (95%105% and 0.99). Statistical analysis and fold changes were calculated by GenEX software (v.7.0.2.164). The Fold changes ratio was quantified against iPSC. One-way ANOVA was used for the comparison between cell samples with Tukey-Kramer's for post hoc test. The P-value of less than 0.05 was considered statistically significant.

Table 1. The Sequence of the Primers Used in This Study

Gene Name	Sequence (5' to 3')	Tm	Product Length (BP)
Wnt family member 5A (WNT5A)	F- GGATGGCTGGAAGTGCAATG	59.54	112
	R- TTCATACCTAGCGACCACCA	58.14	
GAPDH	F- GGACTCATGACCACAGTCCA	60	119
	R- CCAGTAGAGGCAGGGATGAT		

4. Results

4.1. Differentiation Induction

The success of the differentiation process was verified by the assessment of specific marker genes. Initially, the pluripotency characteristics of iPSCs were confirmed by the observation of pluripotency marker genes, *Nanog*, and *Oct4* genes expression. Also, EBs were efficiently produced from fully grown iPSC colonies and showed a decrease in *Nanog* and *Oct4* gene expression (0.175 ± 0.043; $P < 0.001$ and 0.213 ± 00.053; $P < 0.001$ respectively). The significant downregulation of *Nanog* and *Oct4* genes during the rest of the differentiation induction was the indicator of successful escape from pluripotency (Figure 1A). The success of DE induction step was confirmed by the assessment of DE specific genes, including *Sox17* and *FoxA2*. Both genes showed elevated expression levels compared to iPSC (9.937 ± 0.464; $P < 0.001$ and 209.615 ± 1.08; $P < 0.001$ respectively) (Figure 1B).

4.2. Wnt5a Expression

As shown in Figure 2, the expression profile of *Wnt5a* mRNA represents non-canonical Wnt signaling during the differentiation induction process. We observed a significant upregulation of *Wnt5a* in EB samples (5.033 ± 1.55; $P < 0.001$). However, a minor decrease was observed on day 4 (3.715 ± 0.279; $P < 0.001$). The differentiation process followed by an incremental fashion in *Wnt5a* mRNA expression started from day 4 of differentiation (4.508 ± 0.314; $P < 0.001$) among the samples of days 6 and 8 (DE stage) (6.9751 ± 1.216; $P < 0.001$ and 7.709 ± 0.926 $P < 0.001$).

5. Discussion

The evolutionary conserved non-canonical Wnt signaling is involved in development, cell proliferation, and differentiation in coordination with canonical Wnt signaling (12, 20). Wnt/PCP is a type of non-canonical Wnt signaling, which is activated by the *Wnt5a* ligand is a well-known antagonist of the canonical pathway (10, 21-23). Canonical Wnt signaling is mostly involved in cell proliferation and stemness maintenance and only the early stages of differentiation induction (13, 23). Therefore, Wnt/PCP should be activated after the initial stage of endoderm induction. In this study, we demonstrate the expression of the *Wnt5a* gene is decreased at the early endoderm induction stage, which is consistent with the introduction of canonical Wnt signaling inducer, namely chir99021. However, during the rest of the endoderm induction process, *Wnt5a* expression ramped up. Therefore, the specific stage of *Wnt5a* expression, as non-canonical Wnt signaling, has a decisive role during definitive endoderm induction and differentiation toward different lineages such as the pancreas (3). Baksh et al. have shown that *Wnt5a* can suppress the canonical Wnt signaling pathway, which can promote the osteogenic differentiation of MSCs (20) since canonical Wnt signaling functions only at the early differentiation induction as suggested by Paige et al. (24). In the same way, Vijayaragavan et al. reported that the non-canonical Wnt signaling is required for the embryonic stem cells to escape from pluripotency (25). Moreover, *Wnt5a* was shown to promote the cardiac cells, dental papilla cells, chondrocyte, adipocyte, and osteogenic differentiation (15-17, 26, 27). Therefore, it can be postulated that Wnt/PCP is activated at the later steps of endoderm differentiation and positively promotes the differentiation process. The study of *Wnt5a* expression may provide new insights into developing more effective differentiation protocols considering the fact that definitive endoderm induction is the initial step for the generation of many internal organs and is the most delicate step throughout the differentiation induction.

5.1. Conclusion

Non-canonical Wnt signaling contributes to developmental pathways and differentiation. We studied the expression profile of the *Wnt5a* gene, during endoderm induction and suggest that *Wnt5a* is more activated at the later steps of endoderm induction rather than the early step, which may be due to the stimulation of canonical Wnt signaling. Understanding the expression pattern of *Wnt5a* may provide insights into developing more efficient differentiation induction protocols.
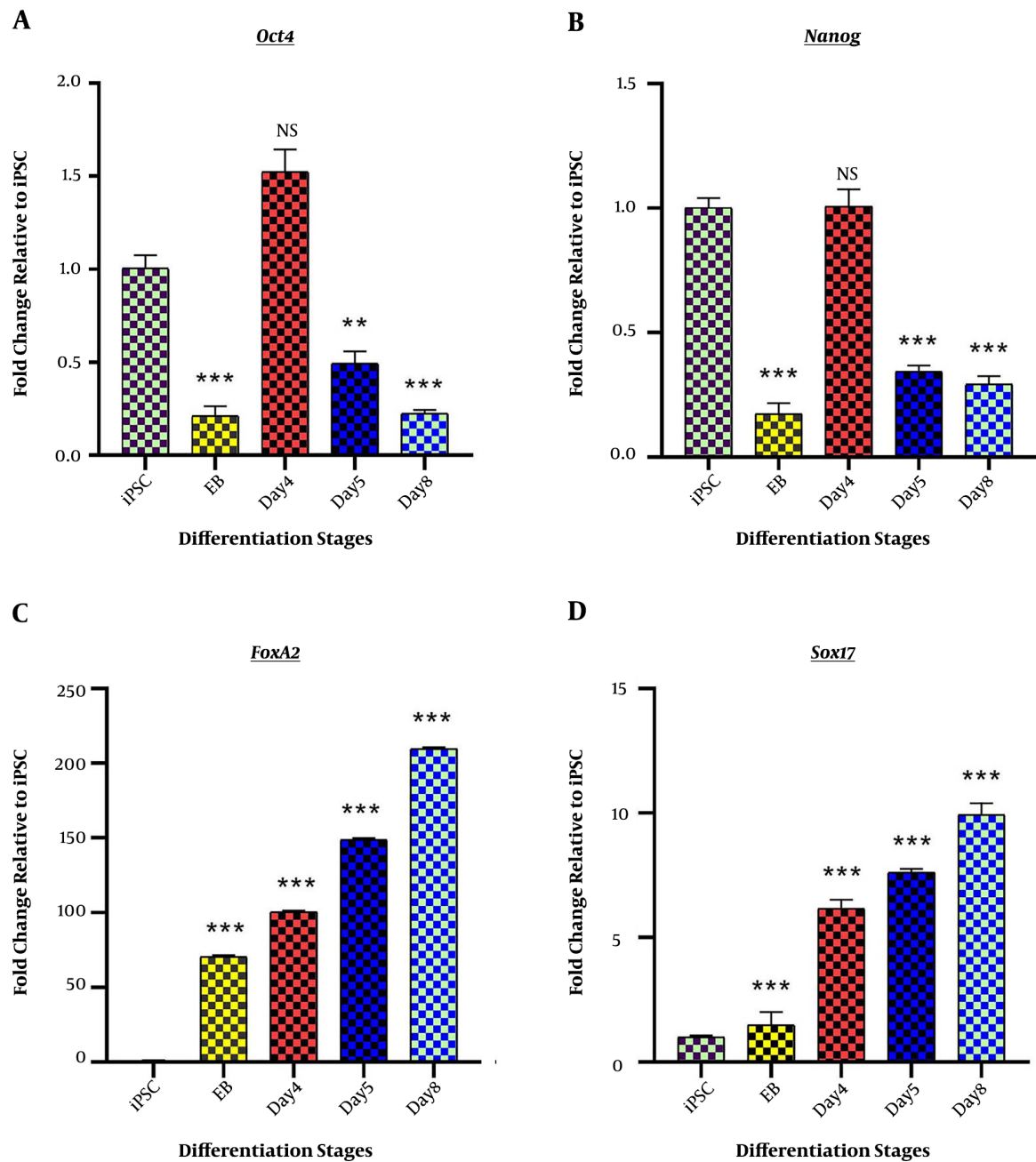
Figure 1. Stage-specific differentiation marker genes; Fold change ratio of mRNA expression is presented on "Y" axis, while differentiation stages, including iPSCs, Embryoid body (EB), day 4 of differentiation (day 4), day 5 of differentiation (day 5), and endoderm (day 8) are depicted on "X" axis. (A) The expression profile of pluripotency-specific genes, *Oct4* and *Nanog*, represents the successful scape form stemness at day 8 (endoderm stage). (B) The expression profile of endoderm marker genes, *Sox17* and *FoxA2*, represents the completion of endoderm induction. *P < 0.05; ** P < 0.01; *** P < 0.001; NS: not-significant.
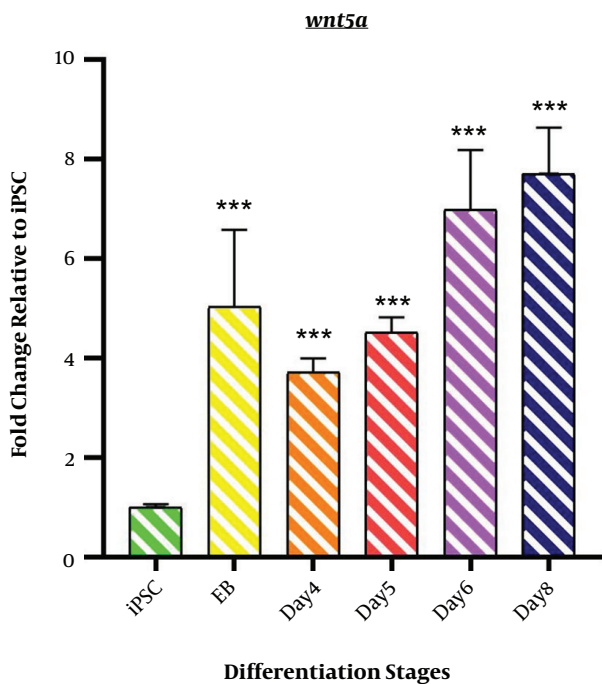
wnt5a

Figure 2. Expression profile of *Wnt5a* gene; The expression pattern of *Wnt5a* is presented by the fold change compared to the iPSCs on vertical axis at different stages of differentiation induction on horizontal axis, including iPSCs, Embryoid body (EB), day 4 of differentiation (day 4), day 5 of differentiation (day 5), day 6 of differentiation (day 6), and endoderm (day 8). *P < 0.05; ** P < 0.01; *** P < 0.001; NS: not-significant.

Footnotes

Authors' Contribution: Study concept and design: N. A.; analysis and interpretation of data: Sh. L., and L. K.; drafting the manuscript: Sh. L.; critical revision of the manuscript for important intellectual content: N. A., and S. Gh.; statistical analysis and performing experiments: Sh. L.

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