Wnt5a Contributes to the Differentiation of Human Embryonic Stem Cells into Lentoid Bodies Through the Noncanonical Wnt/JNK Signaling Pathway

Chenlu Han,1 Jinyan Li,1 Chunxiao Wang,1 Hong Ouyang,1 Xiaoyan Ding,2 Yizhi Liu,1 Shuyi Chen,1 and Lixia Luo1

1State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong, People’s Republic of China
2Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, People’s Republic of China

Correspondence: Lixia Luo, State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, Guangdong 510060, People’s Republic of China; luoxia@mail.sysu.edu.cn.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

PURPOSE. Recent work has indicated that Wnt5a has a critical role in embryonic development. We investigate whether the Wnt5a-activated noncanonical Wnt pathway is capable of promoting embryonic lens differentiation.

METHODS. A “three-stage” protocol was used to induce lens differentiation of human embryonic stem cells (hESCs) in vitro, and Wnt5a levels were modified by addition of exogenous protein and RNA interference. SP600125 was adopted to inhibit JNK cascades. The number and size of lentoid bodies obtained were measured, and quantitative RT-PCR, Western blotting, and immunofluorescence were used to detect gene and protein expression.

RESULTS. The quantity and size of lentoid bodies generated were significantly increased by addition of exogenous Wnt5a. Moreover, expression of lens-specific genes, including CRYAA, CRYAB, BFSP1, and MIP, and the lens fiber differentiation regulator PROX1 were prominently increased. We also observed activation of noncanonical Wnt signaling via upregulation of Dvl2, Rac1, and JNK. When Wnt5a-knockdown hESCs were induced to differentiate, fewer and smaller lentoid bodies resulted. In addition, expression of genes specific to lens was decreased and noncanonical Wnt/JNK pathway activity was downregulated. Accordingly, inhibition of JNK cascade suppressed the formation of lentoid bodies as well, consistent with that of Wnt5a-knockdown group.

CONCLUSIONS. Wnt5a can promote the differentiation of hESCs into lentoid bodies through the noncanonical Wnt/JNK signaling pathway, thereby contributing to the study of human lens development and moreover the underlying etiology congenital cataracts.

Keywords: Wnt5a, lentoid bodies, hESCs, Wnt/JNK, signaling pathway

Congenital cataracts, responsible for 10% to 30% of childhood blindness cases,1 are caused mainly by defects affecting lens development,2 which is a stepwise process regulated by diverse genes and signaling pathways.3 Therefore, research focused on this lens development process could provide a foundation for understanding the cause of childhood cataracts and identify appropriate treatment strategies.

It is widely recognized that Wnt glycoproteins, ligands of the seven-pass transmembrane receptors Frizzled family, regulate vertebrate embryonic development through intracellular signal transduction, such as proliferation, differentiation, survival, and apoptosis.4-12 Wnt-activated signaling can be classified into canonical Wnt/β-catenin and noncanonical Wnt pathways,9,11,12 both of which have critical roles in lens organogenesis and morphogenesis. For instance, the Wnt/β-catenin pathway is involved in cell proliferation and deciding cell fate during lens placode formation.9,11,12,14 The role of the noncanonical Wnt pathway is focused on ensuring the correct patterning of lenses,9,10,11,15 such as promoting lens cells polarity establishment and lens fiber cell cytoskeletal organization.16 Whether the noncanonical Wnt pathway involves lens cell differentiation as well has not been illustrated to date.

The secreted ligand Wnt5a is a known component of noncanonical signaling pathways,17-19 including the Wnt/planar cell polarity (PCP) and Wnt/Ca2+ pathways,18,19 the former of which regulates cell polarity, movement, and cytoskeleton through activation of Rho family GTPases and c-Jun N-terminal kinase (JNK) signaling cascades.10,20,21 Expression of Wnt5a in the lens placode has been shown to be regulated by Pax6.22 In addition, Wnt5a is maintained in lens epithelium and during the early stages of lens fiber differentiation.23-26 Recent studies also have revealed that Wnt5a has a critical role in cell differentiation. For example, it has been shown to promote formation of odontoblasts from dental papilla cells27,28 and that of osteoblasts29 and chondrocytes30 from mesenchymal stem cells. However, whether Wnt5a regulates the lens differentiation process through the noncanonical Wnt signaling pathway is unclear.
Human embryonic stem cell (hESC) serves as an excellent tool to study the mechanisms of embryonic lens development, and the methods by which they can be induced to differentiate into lens progenitor cells and lentoid bodies have been successfully established. We recapitulated early lens development in vitro using the induction protocol of Yang et al. to explore the role of Wnt5a in lens differentiation and the underlying mechanisms. For the first time to our knowledge, we demonstrated that Wnt5a promotes differentiation of underlying mechanisms. For the first time to our knowledge, we demonstrated that Wnt5a promotes differentiation of hESCs into lentoid bodies through the noncanonical Wnt/JNK signaling pathway.

**Materials and Methods**

**Cell Culture Reagents and Antibodies**

The growth factors used in our work included human Noggin (PeproTech, Inc., Rocky Hill, NJ, USA), human BMP4 (PeproTech, Inc.), human BMP7 (PeproTech, Inc.), human FGF-basic (PeproTech, Inc.), murine Wnt5a (PeproTech, Inc.), recombinant human/murine Wnt5a (R&D Systems, Minneapolis, MN, USA), and JNK Inhibitor II SP600125 (Millipore, Billerica, MA, USA).

The primary antibodies used in this study were: goat anti-β-crystallin (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-α-crystallin (1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-γ-crystallin (1:500; Santa Cruz Biotechnology, Inc.), polyclonal goat anti-Wnt5a (1:200; R&D Systems), mouse anti-phosphorylated (p)-JNK (1:200; Santa Cruz Biotechnology, Inc.), rabbit anti-SAPK/JNK (1:1000; Cell Signaling, Danvers, MA, USA), mouse anti-Rac1 (1:1000; Cell Signaling), rabbit anti-Dvl2 (1:1000; Cell Signaling), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Cell Signaling). For immunofluorescence, Alexa Fluor 488 or 568-conjugated donkey anti-rabbit, mouse, or goat secondary antibodies (1:500; Invitrogen, Carlsbad, CA, USA) were used. For Western blot analysis, the following horseradish peroxidase (HRP)-conjugated secondary antibodies were used: goat anti-mouse IgG (1:1000; Cell Signaling), goat anti-rabbit IgG (1:1000; Cell Signaling), and rabbit anti-goat IgG (1:500; Invitrogen).

**Tissue Processing**

Pregnant C57BL/6 mice were obtained from Guangdong Medical Laboratory Animal Center (Foshan, China). All animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center. The mice at embryonic days 10.5 (E10.5) and 13.5 (E13.5) were collected and fixed in 4% paraformaldehyde (Sigma-Aldrich Corp., St. Louis, MO, USA) overnight at 4°C and subsequently dehydrated through a 15% to 30% sucrose gradient. Tissue-Tek OCT compound (Sakura, Torrance, CA, USA) was used for embedding before cryosectioning.

**Differentiation of hESCs into Lentoid Bodies**

The hESC line H9 was kindly provided by Xiaoanyan Ding from the Institute of Biochemistry and Cell Biology, obtained from the National Stem Cell Bank of the WICell Research Institute. Cells were cultured on growth factor-reduced Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA)-coated plates in mTeSR1 medium (STEMCELL Technologies, Vancouver, Canada) without feeder cells. When cell density reached approximately 80% to 90%, the culture medium was changed to induce the formation of lentoid bodies according to the protocol of Yang et al. The basal culture medium was replaced with Dulbecco's modified Eagle's medium/F-12 (Gibco, Waltham, MA, USA) supplemented with 1% minimum essential medium nonessential amino acids solution (Gibco), 2 mM GlutaMAX (Gibco), 0.05% BSA (Gibco), 1× N-2 supplement (Thermo Fisher Scientific, Waltham, MA, USA), and 1× B-27 supplement (Thermo Fisher Scientific). Growth factors then were added sequentially to the medium as follows: 100 ng/mL Noggin from days 0 to 6 (stage 1), 100 ng/mL FGF2 and 20 ng/mL BMP4/BMP7 from days 7 to 18 (stage 2), and 100 ng/mL FGF2 and 20 ng/mL Wnt3a from days 19 to 35 (stage 3). The culture medium was replaced every other day. For exogenous Wnt5a addition and JNK cascade inhibition, 500 ng/mL Wnt5a were added to the medium from days 19 to 35 with or without 5 μM SP600125. Cells were photographed using the Zeiss Axio Observer Z1 microscope (Zeiss, San Diego, CA, USA). Cells at days 0, 6, 18, 19, 24, 30, and 35 were collected for further analysis.

**Immunofluorescence and Cell Proliferation Assay**

Embryonic mouse tissue cryosections on slides were incubated in citrate buffer (pH 6.0) at 95°C for 30 minutes and cooled to room temperature for antigen retrieval. They then were exposed to primary antibodies overnight at 4°C, washed three times, and incubated with Alexa Fluor-conjugated secondary antibodies for 2 hours at room temperature, before being stained with 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 5 minutes and covered with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Cells cultured on plates at days 0, 6, 18, 19, and 35 were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing with PBS-Tween (PBST), they were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich Corp.) in PBST for 10 minutes, before being blocked with 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and incubated with primary antibodies overnight at 4°C. After washing with PBST, the cells then were incubated with Alexa Fluor-conjugated secondary antibodies for 1 hour. Nuclei were counterstained with DAPI.

Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation assay. In brief, cells at day 24, which was 6 days after Wnt3a treatment with or without Wnt5a, were exposed to 10 μM BrdU (Sigma-Aldrich Corp.) for 2 hours, fixed in 4% paraformaldehyde for 10 minutes, and washed with PBST. Following incubation in 2 M HCl for 10 minutes, they were washed with PBST and incubated overnight at 4°C with a monoclonal mouse antibody against BrdU (1:50; Amersham, Little Chalfont, UK). Finally, cells were incubated with Alexa Fluor 568-conjugated donkey anti-mouse secondary antibody (1:500; Invitrogen) for 1 hour, and nuclei were counterstained with DAPI. The cells were observed using the Zeiss Axio Observer Z1. The percentage of BrdU-positive nuclei in each group was counted in at least 20 randomly selected ×20 field views.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated from cultured cells at different stages, including days 0, 6, 18, 19, 24, 30, and 35, using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. Reverse transcription was performed with a PrimeScript RT Master Mix kit (TAKARA, Kusatsu, Japan), and quantitative PCR was performed using a SYBR Premix Ex Taq kit (TAKARA) on an StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). GAPDH served as the internal control. Sequences of the primers used for qRT-PCR are listed in Supplementary Table S1.
Western Blotting

Cells at days 0, 6, 18, 19, 24, 30, and 35 under different treatment were lysed in RIPA buffer (Solarbio, Beijing, China) with 1 mM protease inhibitor cocktails (Sigma-Aldrich Corp.) for 30 minutes on ice. Total proteins in the supernatants were collected after centrifugation for 30 minutes, separated by 10% SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore). The membranes were blocked with 5% nonfat milk and incubated with primary antibodies at 4°C overnight. They then were exposed to HRP-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence system (Roche, Mannheim, Germany).

RNA Interference

Wnt5a short hairpin RNA (shRNA) plasmid sequence (Clone ID, NM_003392.3-1488s21c1) was obtained from Sigma-Aldrich Corp. and subcloned into a lentiviral plasmid. The shRNA plasmid sequences were as follows: forward, 5'-CCGGCTCCCGAGACCAGTATTATCTGGATATAAAGAGGCTTTTG-3'; reverse, 5'-AATTCTAAAATCTCACGACCCGGTATTATCTGGATATAAAGAGGCT-3'. The pLKO.1 lentiviral plasmid (Addgene, Cambridge, MA, USA) and scrambled shRNA negative-control plasmid (Addgene) were provided by Hong Ouyang from Zhongshan Ophthalmic Center, and recombinant lentiviral vectors expressing Wnt5a shRNA and scrambled shRNA were constructed. These vectors were transfected into 293 T cells along with the packaging plasmids psPAX2 and pMD2.G to generate viruses. Once 50% confluent, hESCs were exposed for 24 hours to the resulting viruses. After a period.

Statistical Methods

All values are reported as means ± SEM. A 1-way ANOVA (SPSS Statistics Version 19.0; IBM Corp., Armonk, NY, USA) and 2-tailed t-tests (Excel; Microsoft, Redmond, WA, USA) were used for comparisons among groups. P ≤ 0.05 was considered statistically significant.

RESULTS

Wnt5a Expression Pattern During Lens Development In Vivo

Wnt5a expression was detected in the inner lens vesicle at E10.5 (Fig. 1A). Subsequently, during completion of primary lens fiber cell elongation at E13.5, Wnt5a expression prominently increased in the epithelium and fiber cells (Fig. 1B). Therefore, we speculated that Wnt5a was involved in lens fiber cell differentiation in vivo. However, its function and the underlying mechanism remained unclear.

Generation of Lentoid Bodies From hESCs Using a Three-Stage Induction System

By the end of induction stage 1, cells had become more compact and round (Figs. 2A, 2B). During stage 2, cells were exposed to FG2 and BMP4/7 to stimulate the formation of lens progenitor cells. By the end of day 18, the cells appeared spindle-shaped (Fig. 2C). In stage 3, transparent three-dimensional (3D) structures began to form and gradually developed at day 35 (Fig. 2D). To characterize the hESCs-derived lentoid bodies, the expression of lens-specific proteins αA- and β-crystallin was tested at day 35 (Figs. 2E-H). Consistent with this, mRNA and protein levels of αA-, β-, and γ-crystallin were significantly increased in the lentoid bodies compared to undifferentiated hESCs (Figs. 2I, 2J).

Wnt5a Expression Reduced at the End of Lentoid Bodies Formation In Vitro

Since Wnt5a was upregulated during lens cells development, we tested whether the Wnt5a expression pattern in our differentiation system was consistent with that observed in vivo. We tested expression of Wnt5a at days 0, 6, 18, 19, 24, 30, and 35. As shown in Figure 3A, expression of Wnt5a at mRNA level was increased gradually in the initial phase, reaching its peak at day 19. However, Wnt5a expression markedly decreased at day 24 and then kept relatively low at the end of our induction period. In addition, protein level of Wnt5a also was tested in days 0, 6, 19, and 35 by Western blot (Fig. 3B) and immunofluorescent staining (Fig. 3C), which also was consistent with the mRNA level. This contrasted with our in vivo results, which indicated Wnt5a was distinctly upregulated at lens cells differentiation. Therefore, to determine further whether Wnt5a is involved in lens cell formation, we introduced exogenous Wnt5a into the cultures during stage 3 of the induction protocol.

Exogenous Wnt5a Increased the Number and Size of hESCs-Derived Lentoid Bodies

We first searched for suitable dosage of Wnt5a treatment by setting concentration gradient at 100, 200, and 500 ng/mL. As shown in Supplementary Figure S1, 100 ng/mL Wnt5a could not alter genes expression in mRNA level of lens markers CRYAA, CRYAB, CRYBB2, Bfsp1, and Bfsp2, as well as embryonic stem cell marker OCT4. However, the expressions of lens markers was significantly increased and OCT4 obviously was decreased after incubation with two higher dosages of Wnt5a, 200 and 500 ng/mL. In addition, expression of these genes was shown to be significantly promoted by treatment with 500 ng/mL Wnt5a compared to that of 200 ng/mL. As a result, we speculated that 200 ng/mL was the lowest.
dosage that could induce these lentoid bodies, so the 500 ng/mL dosage was adopted for further study.

Exposure of hESCs to 500 ng/mL Wnt5a from days 19 to 35 throughout stage 3 resulted in significantly larger and more lentoid bodies at the end of this stage (Figs. 4D, 4E). Specifically, in the presence and absence of Wnt5a, approximately 360 ± 625 and 199 ± 27 lentoid bodies/well of a 24-well plate were obtained, respectively (Fig. 4I), and mean lentoid bodies area increased from 0.023 ± 0.00064 to 0.048 ± 0.0011 mm² due to administration of Wnt5a (Fig. 4J).

We next used shRNA to establish whether downregulation of Wnt5a expression in hESCs could inhibit lentoid bodies formation. qRT-PCR revealed that Wnt5a expression in Wnt5a-knockdown hESCs was 31.5% of that in cells transduced with scrambled shRNA (Fig. 4A). Western blotting confirmed this result at the protein level (Fig. 4B). Moreover, only approximately 85 ± 8 lentoid bodies/well of a 24-well plate, with a mean area of 0.016 ± 0.0019 mm², were obtained from the Wnt5a-knockdown hESCs (Figs. 4F, 4G, 4I, 4J).

To further confirm that lentoid bodies in bright field were merely cell aggregates in size-reduced after knockdown of Wnt5a, we compared the 3D lentoid bodies in bright field and fluorescence labeled with lens specific crystallins. Our result showed that αA- and β-crystallin clearly were present in the formed lentoid bodies and co-occurred only if 3D lentoid bodies presented in bright field (Supplementary Figs. S2A–F). We also counted the numbers and areas of lentoid bodies in bright field and β-crystallin–labelled cell aggregates. There was no significantly difference in numbers and sizes between β-crystallin–labelled cell aggregates and lentoid bodies in bright field (Supplementary Figs. S2G, S2H). Thus, this confirmed that Wnt5a promoted lentoid bodies formation from hESCs, in terms of quantity and size.

**Wnt5a Promoted hESCs Differentiation Into Lentoid Bodies**

To avoid the underlying dilution by the residual cells surrounding lentoid bodies, we carefully selected lentoid bodies at day 35 for further analysis. The mRNA levels of lens markers CRYAA, CRYAB, CRYBB2, CRYGC, and BFSP1 between days 0 and 35 was significantly elevated in lentoid bodies after incubation with Wnt5a. Expression of the marker of prospective lens placode SOX2 was reduced and that of lens fiber differentiation regulator PROX1 was simultaneously and markedly increased (Fig. 5A). Correspondingly, CRYAA, CRYAB, CRYBB2, CRYGC, BFSP1/aquaporin, BFSP2/CP49, and MIP were significantly elevated in lentoid bodies after incubation with Wnt5a. Expression of the marker of prospective lens placode SOX2 was reduced and that of lens fiber differentiation regulator PROX1 was simultaneously and markedly increased (Fig. 5A). Correspondingly, CRYAA, CRYAB, CRYBB2, CRYGC, BFSP1, BFSP2, and PROX1 expression decreased and SOX2 expression increased when Wnt5a was knocked down (Fig. 6A).

**Wnt5a Enhanced the Activation of Noncanonical Wnt/JNK Pathway During Lens Differentiation Process**

To determine whether Wnt5a can activate noncanonical Wnt signaling, Western blot analysis was conducted to investigate the status of key components of this pathway. It has been shown that when stimulated by Wnts, Dishevelled (Dvl) may be phosphorylated and subsequently activate small
GTPases of the Rho/Rac family and their downstream effector JNK, which function to regulate cell shape, cytoskeletal reorganization, and cell polarity.\(^{39-41}\) We tested the status of activated JNK, Dvl, and Rac1 at days 19, 24, and 30; that is, days 1, 6, and 12 after incubation with or without Wnt5a, respectively. Compared to the control group, the ratio of p-JNK to total JNK increased at the first day of Wnt5a treatment, and kept sustained promoted as prolongation of Wnt5a addition, and finally reached the maximum value after 12 days of Wnt5a treatment (Fig. 5B). Consistent with this, the ratio of phosphorylated Dvl2 (p-Dvl2) to unphosphorylated Dvl2 (up-Dvl2) was increased (Fig. 5C) and active Rac1-GTP expression was significantly elevated as well (Fig. 5D) after 12 days of Wnt5a treatment. Moreover, Wnt5a knockdown led to reductions in the p-Dvl2/up-Dvl2, Rac1-GTP/Total Rac1, and p-JNK/JNK ratios (Figs. 6B–D), indicating that noncanonical Wnt/JNK activity was remarkably diminished because of low Wnt5a expression.

Activation of Noncanonical Wnt5a/JNK Pathway Promoted Lens Progenitor Cell Proliferation

We subsequently explored the effect of Wnt5a on cell proliferation. After approximately 6 days of Wnt5a treatment during stage 3 of the induction process, lens progenitor cells formed 3D lentoid bodies and other cells were gradually lost by detachment from the surface of the culture plate. Therefore, we tested the effect of Wnt5a during these 6 days leading up to lentoid bodies generation. After exposure to 500 ng/mL Wnt5a for 6 days, the percentage of BrdU-labeled cells obviously was elevated. The percentage of BrdU-labeled cells also significantly decreased as a result of Wnt5a knock-down (Fig. 7).

JNK Inhibitor (JNKi) Could Suppress the Differentiation From hESCs Into Lentoid Bodies

To determine whether the effect of Wnt5a depends on JNK cascades, we applied SP600125 to inhibit the phosphorylation of JNK. SP600125 (5 \(\mu\)M) was supplemented at the same time of Wnt5a treatment and the significantly suppressive effect was shown between the Wnt5a and combined Wnt5a plus JNKi groups in Figure 4C. After inhibition of JNK phosphorylation, less and smaller lentoid bodies were harvested accordingly, only approximately 143 ± 17 lentoid bodies/well of a 24-well plate and a mean area of 0.021 ± 0.00647 mm\(^2\) respectively, which confirmed that Wnt5a is capable of promoting lens cells development dependent on JNK cascades (Figs. 4H–J). Moreover, when SP600125 was added combined with Wnt5a, the proliferation of lens progenitor cell was distinctly decreased (Fig. 7).

DISCUSSION

In recent years, various studies have indicated that Wnt signaling has an important role in deciding stem cell fate. Therefore, a better understanding of how Wnt signaling...
regulates embryonic cell differentiation is crucial. Methods allowing examination of this mechanism during embryonic organ development are available, and the hESCs-lentoid bodies induction system provides an excellent experimental basis for studying early lens development. Based on the protocol of Yang et al., our previous studies also analyzed the expression dynamics of genes encoding transcription factors via genome-wide transcriptome analysis during the process of hESCs differentiation into lentoid bodies. The results showed that this induction protocol recapitulates early lens development and the three in vitro induction stages correspond to key stages of lens development in vivo, that is preplacodal ectoderm, lens placode, and lens vesicle (data not shown). In our study, we adopted the same protocol, obtaining transparent and 3D lentoid bodies (Fig. 2D) expressing crystallins (Figs. 2E–J). Focusing on the characterization of the lentoid bodies obtained and crystallins expression pattern, we found that Wnt5a promoted lentoid bodies differentiation. In addition, noncanonical Wnt/JNK pathway activation by Wnt5a was involved in this differentiation process.

**Figure 4.** Effect of Wnt5a on the number and size of hESCs-derived lentoid bodies. (A) qRT-PCR analysis showed that Wnt5a expression was similar in scrambled shRNA-transduced hESCs and nontransduced hESCs. In Wnt5a shRNA-transduced hESCs, Wnt5a expression was 31.5% of that in scrambled shRNA-transduced hESCs. This result was confirmed at protein level by Western blot analysis using GAPDH as the loading control (B). (C) Western blot analysis of p-JNK and JNK between Wnt5a incubation group (Wnt5a) and Wnt5a combined with JNKi-SP600125 group (Wnt5a þ JNKi) confirmed the downregulation of p-JNK after treatment of 5 μM SP600125. Phase-contrast microscopy was used to observe lentoid bodies in the control (D), 500 ng/mL Wnt5a (E), scrambled shRNA (F), Wnt5a shRNA (G) and Wnt5a combined JNKi (H) groups. Scale bar: 100 μm. Statistical analyses of the number (I) and size (J) of the lentoid bodies obtained were based on three independent experiments. Scale bars: means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., not significant.
Wnt signaling is initiated when secreted Wnts bind with transmembrane receptor Frizzleds and coreceptors. Once this complex is formed, Dvl is activated to transduce the signal, in turn activating either the canonical or noncanonical Wnt pathway. Wnt5a, a member of the Wnt ligand family, is capable of activating the noncanonical PCP pathway through small Rho GTPases and results in activation of downstream JNK signaling cascades. It has been reported that Wnt5a directs lens cell migration and is involved in establishing the polarity of elongating lens fiber cells. Furthermore, Wnt5a/JNK signaling contributes to the differentiation of many other cell types, including stem cell-derived cardiomyocytes.

Figure 5. Wnt5a promoted lens-specific gene expression through activation of noncanonical Wnt/JNK signaling. (A) Following treatment with 500 ng/ml Wnt5a, qRT-PCR analysis was applied to detect expression of genes encoding lens proteins (CRYAA, CRYAB, CRYBB2, CRYGC, BFSP1, BFSP2, and MIP) and lens-related transcription factors (SOX2 and PROX1) in picked lentoid bodies at day 35. (B) Activation of JNK by Western blot analysis at days 19, 24, 30, and 35. The ratio of pJNK to JNK increased at the first day after Wnt5a treatment. This ratio peaked at day 30 and then decreased. (C, D) Activation of Dvl2 and Rac1 was detected by Western blot analysis in lentoid bodies at day 30. (C) The ratio of p-Dvl2 to up-Dvl2 increased due to Wnt5a administration. (D) The ratio of Rac1-GTP (the active form) to Total Rac1 was increased by Wnt5a. Total-Rac1 served as the loading control. Scale bars: means ± SEM. *P < 0.05 (n = 5).
FIGURE 6. Knocking down Wnt5a downregulated lens-specific gene expression through suppression of noncanonical Wnt/JNK signaling. (A) QRT-PCR analysis showed that expression of genes encoding lens proteins (CRYAA, CRYAB, CRYBB2, CRYGC, BFSP1, BFSP2, and MIP) and lens-related transcription factors PROX1 were significantly reduced, and stem cell pluripotency factor SOX2 was obviously increased at day 35 in the Wnt5a shRNA group, compared to the scrambled shRNA and control groups. (B–D) Activation of Dvl2, Rac1, and JNK was tested by Western blot analysis of samples from day 30. (B) The ratio of p-Dvl2 to up-Dvl2 was decreased due to Wnt5a knockdown. (C) The ratio of Rac1-GTP to Total Rac1 was decreased at day 30 because of Wnt5a knockdown. Total Rac1 served as the loading control. (D) The ratio of p-JNK to JNK also decreased after transduction of Wnt5a shRNA. Scale bars: means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant (n = 3).
Wnt5a Promotes Differentiation of Lentoid Bodies

FIGURE 7. Wnt5a treatment before 3D lentoid bodies formation increased the number of BrdU-labeled cells. (A) Representative pictures showing BrdU labeling in the control, 500 ng/mL Wnt5a, scrambled shRNA, Wnt5a shRNA, and Wnt5a combined with JNK inhibitor groups. Scale bar: 100 μm. (B) Bar graph showing mean percentages ± SEM of BrdU-positive nuclei which were counted at least 20 randomly selected ×20 field views from three independent experiments. *P < 0.05, **P < 0.01. n.s., not significant.

The osteogenic lineage, alveolar epithelial cells, among others. However, the role of Wnt5a/JNK signaling in embryonic lens differentiation remains unclear. Therefore, we tested whether Wnt5a/JNK signaling is required for the differentiation of hESCs-derived lentoid bodies.

We initially established the spatiotemporal expression of Wnt5a in developing mice, finding it to be strongly expressed in embryonic lens, including lens vesicle, epithelium, and fiber cells. This is consistent with previous studies, in which Wnt5a has been detected throughout lens development, from lens primordium to the early lens epithelium and fiber cells. Moreover, Wnt5a has been shown to be expressed in the transitional zone of postnatal lens epithelium, which is capable of lens fiber differentiation. These results indicated that Wnt5a may have a key role in lens differentiation. We next noted that Wnt5a expression increased gradually during the first two stages of our induction protocol, which we previously found corresponded to the preplacodal ectoderm and lens placode in vivo, and thereafter decreased during the final stage, analogous to lens vesicle. Therefore, we speculated whether addition of exogenous Wnt5a during induction stage 3 could promote early lens differentiation. Indeed, compared to the original protocol of Yang et al., more and larger lentoid bodies were obtained after administration of 500 ng/mL Wnt5a (Figs. 4D, 4E, 4I, 4J). In keeping with this result, when Wnt5a was knocked down using shRNA, the number and size of the lentoid bodies decreased (Figs. 4E, 4G, 4I, 4J). These findings indicated that Wnt5a may facilitate lentoid bodies morphogenesis.

Given these notable changes, we next investigated the effect of Wnt5a at the molecular level. Expression of genes encoding lens structural proteins, intermediate filament-associated proteins, and MIP, which can be found in mature lens fibers, were remarkably upregulated after addition of exogenous Wnt5a (Fig. 5A). Moreover, since we selected lentoid bodies for these analysis, these results revealed that Wnt5a was capable to promote differentiation of lens cells in lentoid bodies. In line with this, when Wnt5a was knocked down in hESCs, expression of these genes decreased distinctly, as did that of PROX1 (Fig. 5A), which is known to be required for lens fiber morphogenesis. These results indicated that Wnt5a can promote lens-associated gene expression and lens fiber cell differentiation, consistent with previous studies concerning PROX1. The stem cell pluripotency factor SOX2 is known to be involved in the initiation of lens differentiation and specification and can be detected at the early stage of lens formation. Here, SOX2 expression obviously decreased after Wnt5a treatment, indicating a decline in cell pluripotency and, thus, a certain increase in the capability of these cells to generate mature lens fibers. However, recent studies have identified SOX2 expression in adult lens epithelium and have been shown to be required for its self-renewal, contrasting with our findings. Therefore, we speculated that the lentoid bodies obtained in our study lacked the ability for self-renewal. To conclude, our data demonstrated that Wnt5a is capable of promoting lens cell maturation in vitro.

We subsequently decided to investigate whether Wnt5a-activated Wnt/PCP signaling had a role in the effects observed. Chen et al. previously reported that Wnt/PCP signaling triggered by fibroblast growth factor (FGF), which also was applied in our induction system, promotes cytoskeletal reorganization during the transition from epithelial cells to fiber cells. Wnt/PCP signaling via Wnt5a leads to activation of Dvl and small Rho GTPases, and ultimately results in the activation of JNK. We found that phosphorylation of JNK was increased at the first day of Wnt5a treatment and reached the maximum value after 12 days of Wnt5a treatment at day 30 (Fig. 5B). Therefore, we speculated that the status of JNK cascade, as well as other core molecules in this signaling, were the highest at this time. In addition, Dvl is a core component of Wnt/PCP signaling and has a potential role in cell viability. Moreover, activated Dvl functions as a scaffold for cytoskeletal components and is involved in the stabilization of microtubules. In our study, addition of Wnt5a led to activation of downstream factor Dvl2 via phosphorylation (Fig. 5C), indicating that Dvl2 activity may act to stabilize microtubules during lens fiber differentiation. The small Rho GTPase Rac1 is known to regulate lens placodal cell elongation and also contributes to fiber cell cytoskeletal reorganization in cultured human lens epithelial cells. In our study, the activity of Rac1 was increased remarkably after addition of Wnt5a (Fig. 5D), suggesting that the latter may
promote lens differentiation by regulating cytoskeletal organization. These results were consistent with those of prior studies suggesting a critical role for Wnt/PCP signaling in cytoskeletal organization that is required for lens fiber differentiation and maturation.\textsuperscript{16} Besides, BrdU assay has shown that the Wnt5a-induced JNK cascade could promote lens epithelial cell proliferation.\textsuperscript{69} This is consistent with our present study that the JNK cascade regulated lens progenitor cells proliferation (Fig. 7).

Furthermore, the JNK cascade is required for MIP expression during differentiation of lens epithelium.\textsuperscript{70} This agrees with our observation that MIP expression increased due to upregulation of pJNK. Our finding that the activity of Dvl2, Rac1, and JNK were downregulated after knockdown of Wnt5a was consistent with a previous study demonstrating that noncanonical Wnt signaling is inactivated in Sfrp2\textsuperscript{25} Wnt5a was consistent with a previous study demonstrating that noncanonical Wnt signaling is inactivated in Sfrp2\textsuperscript{25} Wnt5a was consistent with a previous study demonstrating that noncanonical Wnt signaling is inactivated in Sfrp2\textsuperscript{25} Wnt5a was consistent with a previous study demonstrating that noncanonical Wnt signaling is inactivated in Sfrp2\textsuperscript{25} Wnt5a was consistent with a previous study demonstrating that noncanonical Wnt signaling is inactivated in Sfrp2.\textsuperscript{69} This is consistent with our present study that the JNK cascade regulated lens progenitor cell proliferation (Fig. 7).

In summary, our results showed that Wnt5a promotes lentoid bodies differentiation and that activation of noncanonical Dvl2-Rac1-JNK signaling is involved in this process. Our work provides a foundation for understanding the regulation of embryonic lens development and lens regeneration. Further studies are required to elucidate the specific molecular mechanism responsible for cytoskeletal reorganization during lens differentiation and the roles of any other Wnt/PCP signaling components.

Acknowledgments

Supported by the National Natural Science Foundation of China (Grant No. 81370994, 81770905, 81400385).

Disclosure: C. Han, None; J. Li, None; C. Wang, None; H. Ouyang, None; X. Ding, None; Y. Liu, None; S. Chen, None; L. Luo, None

References


