• Basic Research •

Chordin-like 2 influences the differentiation fate of retinal pigment epithelium cells by dynamically regulating BMP pathway

Duo Li[1], *Song-Tao Yuan*[2], *Xin-Yi Xie*[1], *Han Shen*[2], *Qing-Huai Liu*[2], *Yong Yao*[1]

[1]Department of Ophthalmology, the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi 214000, Jiangsu Province, China
[2]Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu Province, China
Co-first authors: Duo Li and Song-Tao Yuan
Correspondence to: Yong Yao. Department of Ophthalmology, the Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi 214000, Jiangsu Province, China. pard1@126.com; Qing-Huai Liu. Department of Ophthalmology, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu Province, China. liuqh@njmu.edu.cn
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Abstract

● **AIM:** To explore the functions of Chordin-like 2, which is encoded by *CHRDL2*, in the process of retinal pigmented epithelium (RPE) differentiation and damage repair.

● **METHODS:** The fetal RPE cells (fRPE) was obtained from aborted fetus which obeyed medical ethics. Real-time quantitative polymerase chain reaction was used to measure expression quantity of *CHRDL2* and other functional genes expression. Knocking down and overexpression was used to analyze the functions about Chordin-like 2. Enzyme-linked immunosorbent assay (ELISA) was used to detect the secretion of bone morphogenetic proteins 4 (BMP4). Flow cytometry was used to analyze cell cycle. Cell morphology was observed by phase contrast microscope (PCM).

● **RESULTS:** In normal RPE cells, *CHRDL2* was firstly upregulated and followed a downregulation but eventually, it was expressed higher than the cells which undergone epithelial-mesenchymal transition (EMT). After knocking down *CHRDL2*, the secretion of BMP4 was decreased, RPE-related genes (*OTX2, MITF, RPE65*) were downregulated while EMT-related genes (*SNAI1, VIM*) were upregulated. However, the expression of these related genes after overexpression of *CHRDL2* had contrary results. Chordin-like 2 also regulated the cell cycle by regulating BMP pathway. When *CHRDL2* was knocked down, more fRPE cells stayed in S phase of cell cycle, while adding BMP4 reduced the proportion of the cells in S phase. However, overexpression of *CHRDL2* increased more BMP4 secretion, this effect decreased the number of cells in S phase, but exogenous BMP inhibitor also could change this effect. At last, in the process of RPE cells differentiation, adding BMP4 at early stage could intervene normal RPE differentiation. Compared with BMP4, inhibiting BMP pathway had no significant negative effect at early stage, but suppressed differentiation at late stage.

● **CONCLUSION:** BMP pathway can be activated in a correct temporal order, otherwise, the cells have incorrect differentiation orientation. And Chordin-like 2 plays a role in dynamic regulation of BMP pathway and it also regulates the differentiation of RPE cells. Therefore, this research enlightens a new direction to inhibit EMT and promote cell redifferentiation after injury.

● **KEYWORDS:** retinal pigmented epithelium differentiation; epithelial-mesenchymal transition; BMP pathway; cell proliferation

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INTRODUCTION

Epithelial-mesenchymal transition (EMT) plays many important roles in embryonic development and tissue repair. In embryonic development, reversible EMT can drive tissue growth and organ formations. However, disordered EMT in tissue repair can also cause fibrosis and even support tumor metastasis[1]. Therefore, sequential and controlled EMT is indispensable to keep normal pathophysiologic process. In retinal pigment epithelium (RPE), EMT is regarded as one of the vital pathological processes in both of neovascular and non-neovascular age-related macular degeneration (AMD) and

Table 1 Human fetal RPE medium components preparing for 500 mL medium

Components	Brand/catalog	Amount	Storage
MEM, α modification	Sigma, M-4526	500 mL	+4℃
N1 supplement	Sigma, N-6530	5 mL	+4℃
Glutamine-penicillin-streptomycin	Sigma, G-1146	5 mL	-20℃
Non-essential amino acids	Sigma, M-7145	5 mL	+4℃
Taurine	Sigma, T-0625	125 mL	-80℃
Hydrocortisone	Sigma, H-0396	10 μg	-80℃
Triiodo-thyronine	Sigma, T-5516	0.0065 μg	-80℃
Fetal bovine serum, heat inactivated	Thermo scientific, SH3007003HI	5%	-80℃

RPE: Retinal pigment epithelium; MEM: Minimum essential medium.

proliferation vitreoretinopathy (PVR)[2-3]. Fibrotic RPE cells also decrease the therapeutic effect of anti-vascular endothelial growth factor (VEGF) in wet AMD[4]. *In vitro*, sub-confluent RPE cells have two directions to differentiation, one is normal RPE cells, and another is EMT. Recently, transplantation of multiple-derived RPE cells is becoming a potential therapeutic method to treat dry AMD, but excessive EMT is a tough problem for them to differentiate into normal RPE in subretinal cavity[5-6]. Therefore, inhibiting irreversible EMT and controlling it in a proper level is an urgent affair.

Bone morphogenetic proteins (BMPs), such as BMP2, BMP4, BMP5, BMP6 and BMP7, are series of secreted cytokines which have many different functions. For example, BMP4 had inhibitory effects on EMT, and in chick optical cup, it induced RPE differentiation[7-9], but BMP4 also mediated the programed cell death in the embryo[10]. Similarly, the negative effect of BMP on cell proliferation existed in many cells, such as pulmonary artery smooth muscle cells, pancreatic α-cells and breast cancer[11-13]. Therefore, the accurate regulation of BMP pathway is crucial to correct function of BMP.

The regulation mechanism of BMP pathway is very complex. Because BMP pathway not only has many crosstalk with other signaling pathway, such as Wnt and Notch signaling pathway, but also has many endogenous inhibitors, especially a series of endogenous inhibitors of BMP pathway is a unique feature[14-15]. It has many kinds of endogenous inhibitors like Noggin, Dan family, Chordin-like 1 and Chordin-like 2[16]. In among that, Gremlin-l and Noggin already got more research compared with Chordin-like 1, and Chordin-like 2. However, the mutation of Chordin-like 1, encoded by *CHRDL1*, was founded in X-linked megalocornea, and in the research, BMP4 was downregulated after knocking down *CHRDL1*, that was an interesting appearance, besides, in developing retina of human, *CHRDL1* also had a gradual upregulation[17-18]. Therefore, in this article, we explored the functions of another strange endogenous BMP inhibitor, Chordin-like 2, which is encoded by *CHRDL2* and contains highly conserved cysteine-rich domain. It inhibits BMP signaling by binding BMPs, especially BMP2, BMP4, BMP5, BMP6, and BMP7[15]. In zebrafish embryo, Chordin-like 2 has an exact function in dorsoventral formation[19]. Besides, Chordin-like 2 also plays a role in regenerating osteoarthritic cartilage, and is expressed in uterus and colon, connective tissues, osteoblasts, epithelial cells of reproductive organs and bladder[20-21]. In RPE, the functions of Chordin-like 2 are still uncertain. Therefore, human fetal RPE cells were used to investigate its exact functions.

MATERIALS AND METHODS

Ethical Approval The cells were isolated from aborted fetus acquired from the First Affiliated Hospital of Nanjing Medical University (Jiangsu Province Hospital), and the research was registered in ClinicalTrials.gov (NCT02868424) and Chinese Clinical Trial Registry (ChiCTR-OPC-15006757). At last, Medical Ethics was signed.

Epithelial-mesenchymal Transition Model and Treatment of Fetal Retinal Pigment Epithelium Cells The common protocol was referred to isolate and culture cells[22], the components of medium were showed in Table 1, seeding density of cells was 10 000/cm^2. In normal conditions, fetal RPE (fRPE) cells will occur EMT when the cells are passaged to passage (P4). Cells were digested by trypsin and were passaged repetitively to establish the model of EMT. Exogenous TGF-β pathway inhibitor, SB431542 [Sigma, 10 μmol/L, dissolved in dimethyl-sulfoxide (DMSO)], was used to alleviate the cells which undergone EMT. Exogenous BMP pathway inhibitor, LDN193189, was used to inhibit BMP pathway.

Observation of Fetal Retinal Pigment Epithelium Phase contrast microscope (Nikon TS-100) was used to observe the morphology of cells. The appearance of cell pigments is the sign of cells which has normal functions, and they were analyzed by phase contrast microscope in bright field.

Real-time Quantitative Polymerase Chain Reaction Total RNAs in cells were extracted by TRIzol (Invitrogen Life Technologies, Shanghai, China). Revert aid first strand cDNA synthesis kit (Thermo, Shanghai, China) was used to synthesize cDNA and cDNA was amplified by FastStart Universal SYBR Green Master (Roche, Shanghai, China; Table 2). The method

of ΔΔCT was calculated to analyze target gene expression, and the results showed the mRNA transcription level of target genes. *ACTIN* was regarded as internal reference.

Small Interfering RNA/Plasmid and Transfection To knock down *CHRDL2* expression, small interfering RNA (siRNA) was transfected into cells by Lipofectamine 3000 (Invitrogen, Shanghai, China). Negative control was FAM-labeled siRNA (Invitrogen, Shanghai, China). Plasmid-*CHRDL2* and negative control plasmid (GENECHEM, Shanghai, China) were established and transfected into cells for overexpression and negative control. Transfection method was referred to the reagent protocol (Invitrogen, Shanghai, China). First, the cells were seeded to be 70%-90% confluent. At the first day after seeding, Lipofectamine 3000 was diluted in Opti-MEM medium (2 tubes)-mix well, and meanwhile, plasmid-*CHRDL2* was diluted in Opti-MEM medium and then adding P3000-reagent-mix well, after these two steps, diluted plasmid-*CHRDL2* was added into each tube of diluted Lipofectamine 3000 reagent (1:1 ration) and these mixtures were incubated for 10-15min. At last, the mixtures were added into cells.

Western Blot Conventional method was used to extract total proteins by radio immunoprecipitation assay (RIPA) and protease inhibitor. The protein samples were separated and transferred by SDS-PAGE and polyvinylidene fluoride (PVDF) membrane (Millipore, Shanghai, China). Blocker of the membranes was 5% nonfat dry milk for 1.5h. The membranes with proteins were hybridized with primary antibodies: phosphorylated-SMAD2 (BIOSS, Beijing, China 1:1000), phosphorylated-SMAD1 (Ruiying Biology, Suzhou, China 1:1000) overnight at 4°C. At last, the membranes were incubated with secondary antibodies (Servicebio, Wuhan, China) for 2h. Exposing the bands by chemiluminescence imaging system (Sysgene). β-actin was served as an internal reference.

Enzyme-linked Immunosorbent Assay The protein levels of BMP4 in culture media were qualified by ELISA kit (abcam, Shanghai) according to the manufacturer's protocol. The culture media was extracted after transfection. Standard curve was used to calculate the exact concentration of the protein. To avoid error, the number of RPE cells from each well had no significant differences, and the culture media in each well kept the same volume.

Cell Cycle Analysis Cell cycle was analyzed by flow cytometry. Cell cycle kit (Keygen Biotech, Nanjing, China) was used to detect the cell proliferation. According to the kit protocol, cells were harvested by trypsin and were fixed by 75% ethyl alcohol for 18-24h. After fixed, the cells were washed by 4°C phosphate buffer saline (PBS) and was centrifuged in 2000 rpm for 5min. The 20 μL RNase (50 μg/mL) was used to resuspend cells and digesting cells in 37°C for 30min. After adding propidium iodide (PI) and filtrating the cells in the dark environment, the samples were analyzed by flow cytometry. The results were analyzed by FlowJo_V10.

Table 2 Primer information for quantitative real-time PCR

Gene	Sequence (5'-3')	Product length (bp)
CHRDL2	AGCTGGTAAAAGGAATCTTCCAC	119
TJP1	AGCCATTCCCGAAGGAGTTG	204
PMEL	GGGCTACAAAAGGGAGCCAG	501
BEST1	TTGGGCCTTGGAAAACAGGT	101
RPE65	GCATCCTGCTGGTGGTTACA	175
MERTK	GGAAATAGCTACGCGGGGAA	170
BMP4	ATGTGGGCTGGAATGACTGG	117
SNAI1	AAGATGCACATCCGAAGCCA	237
VIM	GGACCAGCTAACCAACGACA	178
MITF	CGAGCGTCCTGTATGCAGAT	235
OTX2	CGAGGGTGCAGGTATGGTTT	227

Statistical Analysis All experiments were repeated over three times. The data were presented as mean±standard error (mean±SEM) and GraphPad Prism 6 was used to analyze the data. Differences between the data were analyzed by *t*-test, paired *t*-test, besides, the comparison of different markers was occurred between two groups was analyzed by multiple *t*-tests. $P<0.05$ was considered statistically significant.

RESULTS

Chordin-like 2 was Expressed Higher in Normal Fetal RPE Cells Than the Cells Underwent EMT Primary fRPE cells in lower passage will regain some stem cell properties and have stable proliferation capacity *in vitro*. Therefore, these cells are suitable to investigate RPE differentiation and damage repairment. Normal fRPE cells appear a cobblestone-like shape with abundant pigments and express RPE specific genes, such as *PMEL*, *TJP1*, *RPE65*, *MERTK*, and *BEST1*. Generally, in lower passage, the subconfluent cells can re-differentiate into normal morphology and proliferate stably, however, with serial passage to P3 or P4, the cells gradually have a disorder redifferentiation with a mesenchymal-like appearance, for example, EMT cells have fusiform appearance and the size of cell body become longer, besides, EMT-RPE cells also have poor proliferation until to the complete death. This process is similar as the repetitive RPE injury *in vivo*. In our research, in P2, fRPE cells still had the capacity to re-differentiate into normal RPE, but in P4, fRPE cells transdifferentiated into mesenchymal-like and lost pigments (Figure 1A). Meanwhile, *VIM*, a marker of EMT, was upregulated (Figure 1B), but the specific RPE genes were decreased (Figure 1C). Chordin-like 2 (*CHRDL2*) also was downregulated in P4 (Figure 1D). Therefore, Chordin-like 2 was expressed synchronously with normal RPE redifferentiation *in vitro*.

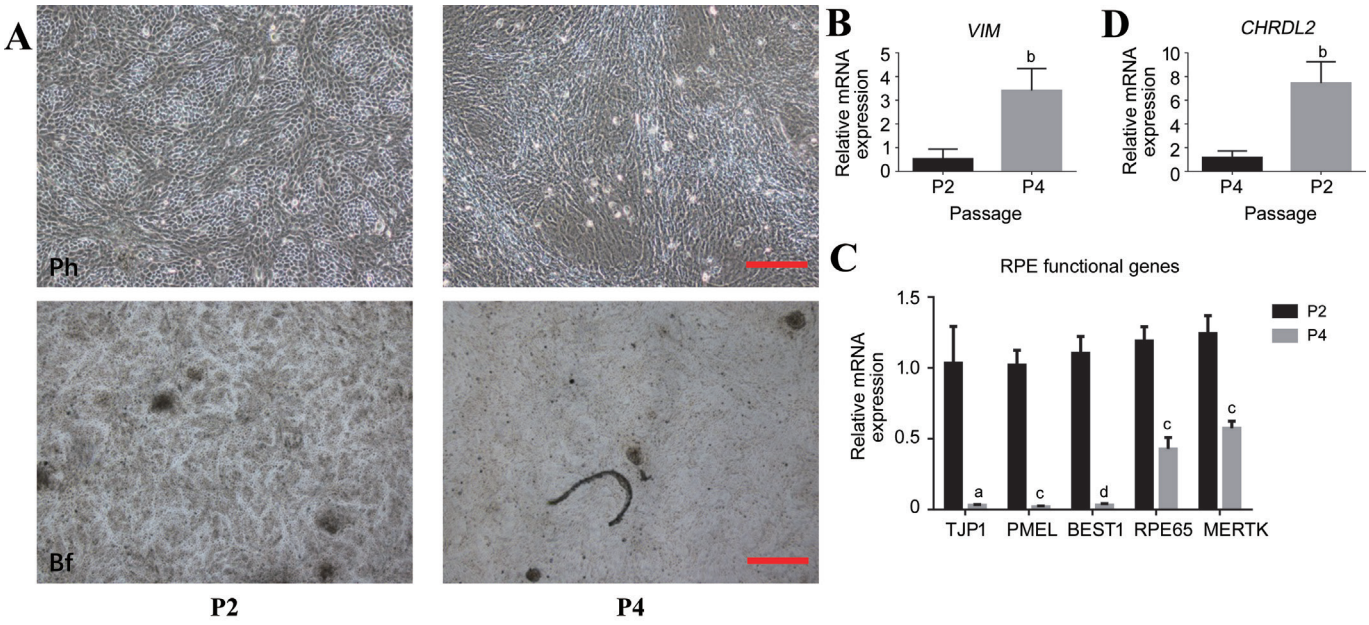

Figure 1 Chordin-like 2 was expressed higher in normal fetal RPE cells than the cells underwent EMT A: After 32d, P2 cells had a normal cobblestone-like shape and more pigments were represented in the bright-field micrographs, while P4 cells had a fibroid shape without pigment. B: Compared with the cells in P2, the cells in P4 expressed more *VIM*, a marker gene of EMT. C: The RPE-specific genes (*PMEL*, *TJP1*, *RPE65*, *MERTK*, and *BEST1*) were expressed normally in P2 cells, but in P4 cells, these genes had a lower expression. D: In P2 cells, *CHRDL2* expressed higher than EMT-RPE cells. Mean±SEM, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, $^{d}P<0.0001$. Micrograph scale bar: phase contrast (Ph): 100 μm, bright field (Bf): 500 μm. RPE: Retinal pigment epithelium; EMT: Epithelial-mesenchymal transition; EMT-RPE cells: RPE cells occurred EMT.

Chordin-like 2 had a Similar Expression Trend with BMP4 For further verifying the expression pattern of Chordin-like 2 and its relationship with BMP4 and other target genes along with the process of cell differentiation. The P4 cells which should turn into EMT were divided into two groups: Control group and SB431542 group. Cells in SB431542 group were daily treated with SB431542 (Sigma, 10 μmol/L). SB431542 is a synthetic TGF-β inhibitor, and it has been confirmed to inhibit EMT and to promote RPE differentiation[23]. After seeding, cells were harvested at 2nd, 5th, 10th, 15th, 30th day and meanwhile, the expression of *CHRDL2*, *SNAI1*, *BMP4*, *RPE65* was detected by qPCR. *SNAI1* is the key transcription factor and the trigger for EMT occurrence and *RPE65* is the marker in normal RPE cells. After treated by SB431542, the cells differentiated into RPE-like appearance over time, but in control group, the cells showed a fusiform shape and failed to redifferentiation successfully (Figure 2A). In the differentiation process, *CHRDL2*, *SNAI1*, *BMP4*, and *RPE65* had a completely different expression process in two groups. In control group, *CHRDL2* and *BMP4* had an initial higher expression than SB431542 group, but after transitorily upregulated, these two genes were downregulated and kept a lower expression, and at the same time, *RPE65* also kept a lower expression. On the contrary, although *SNAI1* had a similar expression process as *CHRDL2* and *BMP4*, their expression level was higher than SB431542 group all the time. However, in SB431542 group, *CHRDL2* had a higher final expression and *RPE65* was upregulated stably, but BMP4 kept relatively lower expression at the first five days and upregulated dramatically at the follow days. *SNAI1* had a slight upregulation and decreased after the first 5d (Figure 2B).

Knocking Down *CHRDL2* Delayed the Differentiation by Regulating BMP4 To explore the exact effects of *CHRDL2* on fRPE cells, siRNA was used to knock down *CHRDL2*. Cells were divided into 3 groups at P2: negative control group (NC group), *siCHRDL2* group and BMP+*siCHRDL2* group. BMP4 was used to investigate the effects without Chordin-like 2 regulations. After transfecting, the efficiency of transfection was detected by qPCR (Figure 3A). Four days after transfection, the morphology was observed by phase contrast microscope and had no significant difference (Figure 3B). And we collected the culture medium and analyzed the concentration of BMP4 in the medium by ELISA. Interestingly, BMP4 secretion in *siCHRDL2* group was decreased compared with NC group (Figure 3C). And in *siCHRDL2* group, the cells tended to occur EMT because of a higher expression of *SNAI1* and *VIM* and a lower expression of *MITF, OTX2* and *RPE65.* Inversely, the cells in NC group and BMP+*siCHRDL2* expressed higher RPE-related genes and lower EMT-related genes, besides, with BMP4 treatment, *VIM* was downregulated even more in BMP+*siCHRDL2* group (Figure 3D).

Overexpression of *CHRDL2* Facilitated Cell Differentiation Because of upregulating gradually along with normal differentiation of fetal RPE cells, *CHRDL2* is likely to play

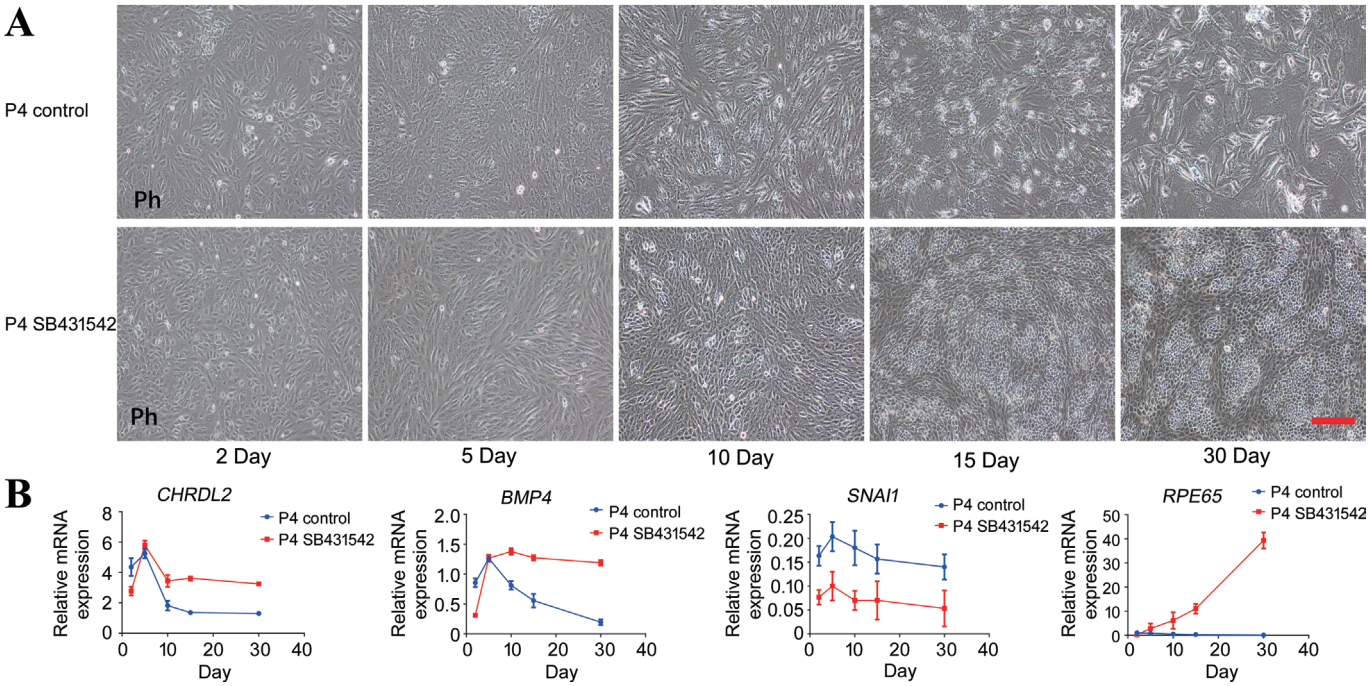

Figure 2 Chordin-like 2 had a similar expression trend with BMP4 A: The P4 cells treated by SB431542 gradually differentiated into a hexagon appearance, but in control group, the cells without any treatment transdifferentiate into mesenchymal-like cells and the capacity of cell proliferation was decreased. B: qPCR was used to evaluate the exact expression trend about *CHRDL2*, *BMP4*, *SNAI1*, and *RPE65*. The cells were harvested at 2nd, 5th, 10th, 15th and 30th day after seeding. In the SB431542 group cells, *CHRDL2*, *BMP4* had a similar expression trend at the same time, as same as *SNAI1*, but *SNAI1* had a lower expression. In the control group cells, *SNAI1* was expressed higher than SB431542 group, while *CHRDL2*, *BMP4* were downregulated after a transitory upregulation and *RPE65* kept a lower expression. Mean±SEM. Micrograph scale bar: 500 μm.

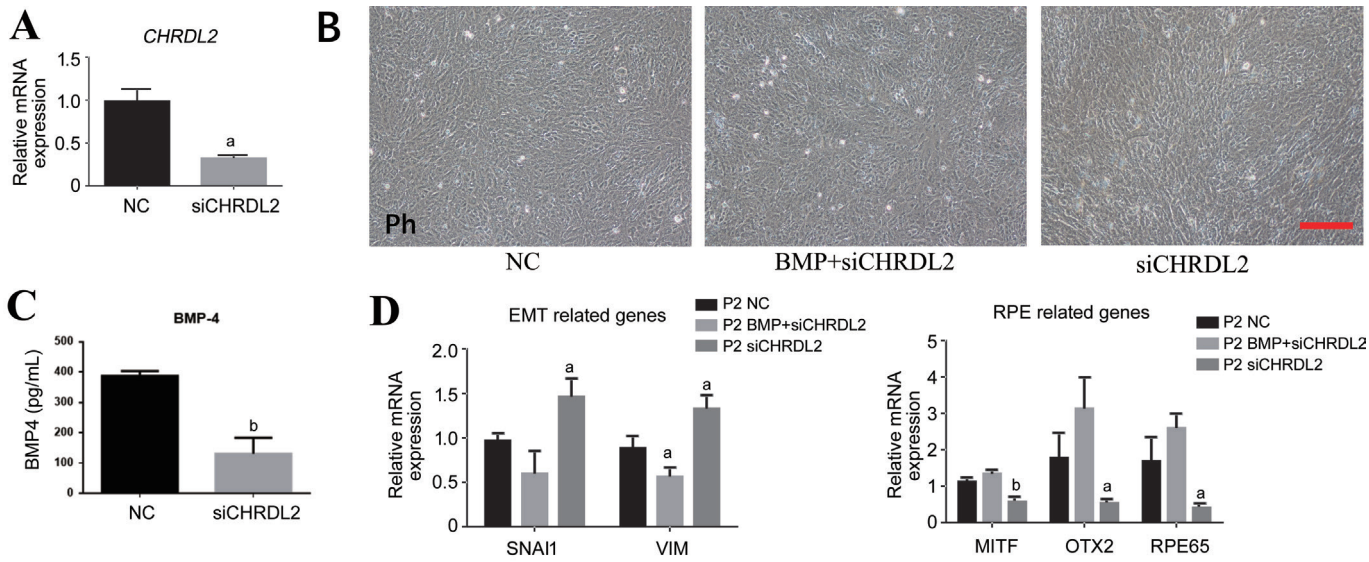

Figure 3 Knocking down *CHRDL2* delayed the differentiation by regulating BMP4 A: After the cells in passage 2 were transfected with siRNA, the expression of *CHRDL2* in the cells was lower than negative control (NC) group cells. B: The cells were divided into three groups, NC, BMP+*siCHRDL2*, *siCHRDL2*. Four days after transfection, cell morphology was checked under phase contrast microscope, the morphology of three groups had no significant difference. C: The concentration of BMP4 was measured by ELISA and when *CHRDL2* was knocked down, the secretion of BMP4 was downregulated. D: The expression of *SNAI1* and *VIM* were quantified by qPCR, in *siCHRDL2* group, the cells expressed the highest *SNAI1* and *VIM*, but in the BMP+*siCHRDL2* and negative control group, these two genes expressed lower, even in BMP+*siCHRDL2* group, *VIM* had the lowest expression. And meanwhile, the expression of *MITF*, *OTX2*, and *RPE65* is lowest in *siCHRDL2* group. Micrograph scale bar: 100 μm. Mean±SEM, $^{a}P<0.05$, $^{b}P<0.01$ *vs* control. Ph: Phase contrast.

a potential role in RPE differentiation. To upregulate the expression of *CHRDL2*, plasmid-*CHRDL2* (p*CHRDL2*) was established. Cells which should undergo EMT at P4 were divided into two groups: negative control group (NC group)

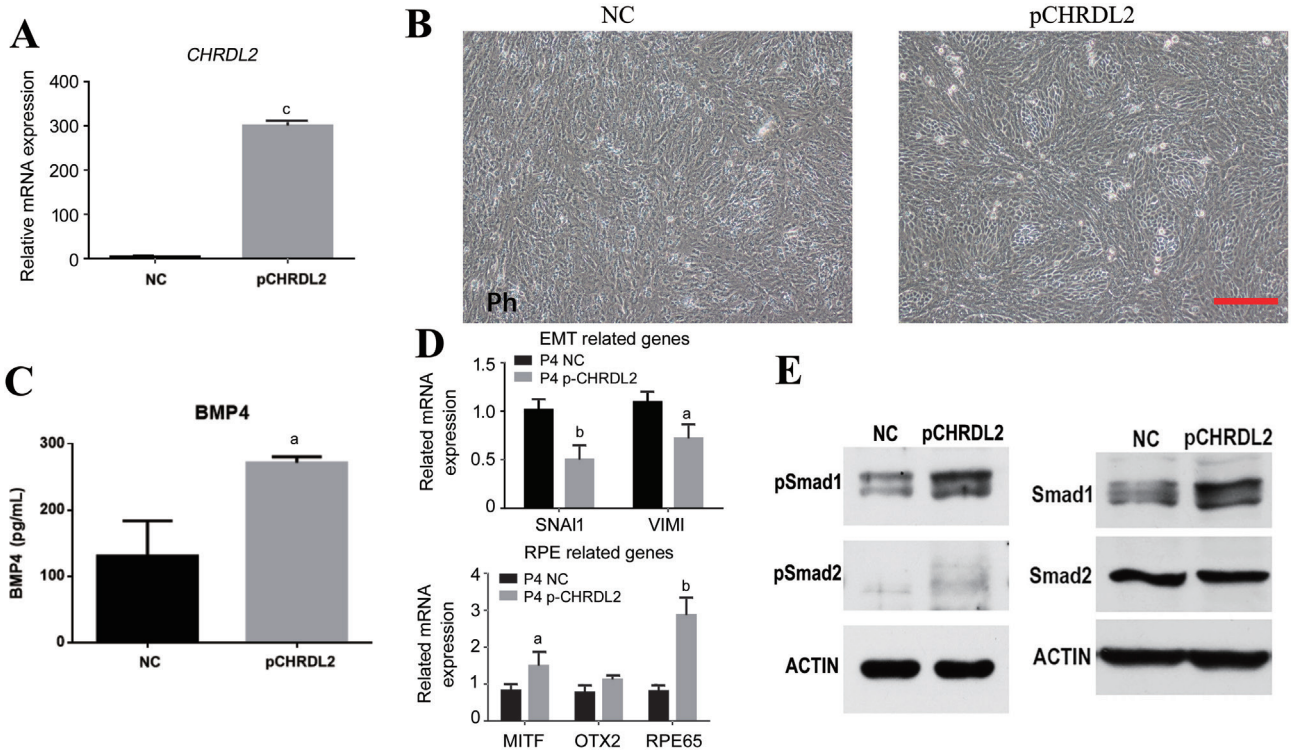

Figure 4 Overexpression of *CHRDL2* facilitated cell differentiation A: After the cells in passage 4 were transfected by plasmid-*CHRDL2*, the expression of *CHRDL2* in the cells was higher than NC group cells. B: The cells were divided into two groups, NC, p*CHRDL2*. Four days after transfection, morphology of the cells is observed by phase contrast microscope, the cells in p*CHRDL2* group started to gain more epithelial-like appearance. C: The concentration of BMP4 was measured by ELISA, when *CHRDL2* was overexpressed, the secretion of BMP4 in culture medium was increased. D: *SNAI1* and *VIM*, two EMT-related genes, were downregulated in p*CHRDL2* group. Besides, in p*CHRDL2* group, *MITF* and *RPE65* were upregulated compared with NC group, the expression of *OTX2* had no significant differences. E: In p*CHRDL2* group, p-SMAD1 was upregulated compared with NC group, and p-SMAD2 in the *pCHRDL2* group also had a little upregulation compared with NC group. Total SMAD1 expression was also increased in p*CHRDL2* group, but total SMAD2 expression had no significant difference. Actin was served as an internal reference in the Western blots. Micrograph scale bar: 100 μm. Mean±SEM, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* control group. p-SMAD1: Phosphorylated-SMAD1; p-SMAD2: Phosphorylated-SMAD2; p*CHRDL2*: Plasmid-*CHRDL2*; Ph: Phase contrast; NC: Negative control.

and p*CHRDL2* group. Four days after transfecting, the cells in two groups were observed and harvested to detect the transfection efficiency (Figure 4A). The cells showed an epithelial-like shape in the p*CHRDL2* group (Figure 4B). Meanwhile, the secretion of BMP4 in culture medium was upregulated correspondingly (Figure 4C). In p*CHRDL2* group, *RPE65* and *MITF* had higher expression than NC group, however, *SNAI1* and *VIM* was downregulated (Figure 4D). Meanwhile, about the results of Western blot, both phosphorylated-SMAD2 and especially phosphorylated-SMAD1 were upregulated in the p*CHRDL2* group, which means the BMP pathway and TGF-β pathway were activated at the same time. Besides, the expression level of total SMAD1 and SMAD2 was also analyzed by Western blot, the results showed that overexpression of *CHRDL2* could make SMAD1 upregulate, however, the expression of SMAD2 had no significant difference (Figure 4E).

Chordin-like 2 Influenced the Cell Proliferation by BMP Pathway Regulation of cell proliferation is important to tissue differentiation. In early stage of epithelial cell differentiation, rapidly and largely cell proliferation is suitable, but in anaphase, cell proliferation should get slow. In past research, BMP4 had a negative effect on cell proliferation. And according to the results we obtained, the changes of *CHRDL2* expression would change BMP secretion, therefore, to further to investigate whether this regulation could influence cell proliferation, cell cycle analysis was used. Two different experiments were done. In first experiment, the cells were divided into four groups: negative control group (NC group), BMP group (the cells were treated by BMP4 alone), *siCHRDL2* group and BMP+*siCHRDL2* group. In second experiment, the cells were also divided into four groups: NC group, LDN193189 (the cells were treated by LDN19389 alone and LDN193189 is a specific pan-inhibitor of BMP pathway), p*CHRDL2* group and LDN+p*CHRDL2* group. Cell cycle was analyzed by flow cytometry. The reason for the treatment with BMP4 and LDN193189 in *siCHRDL2* group and in p*CHRDL2* group was to explore whether change

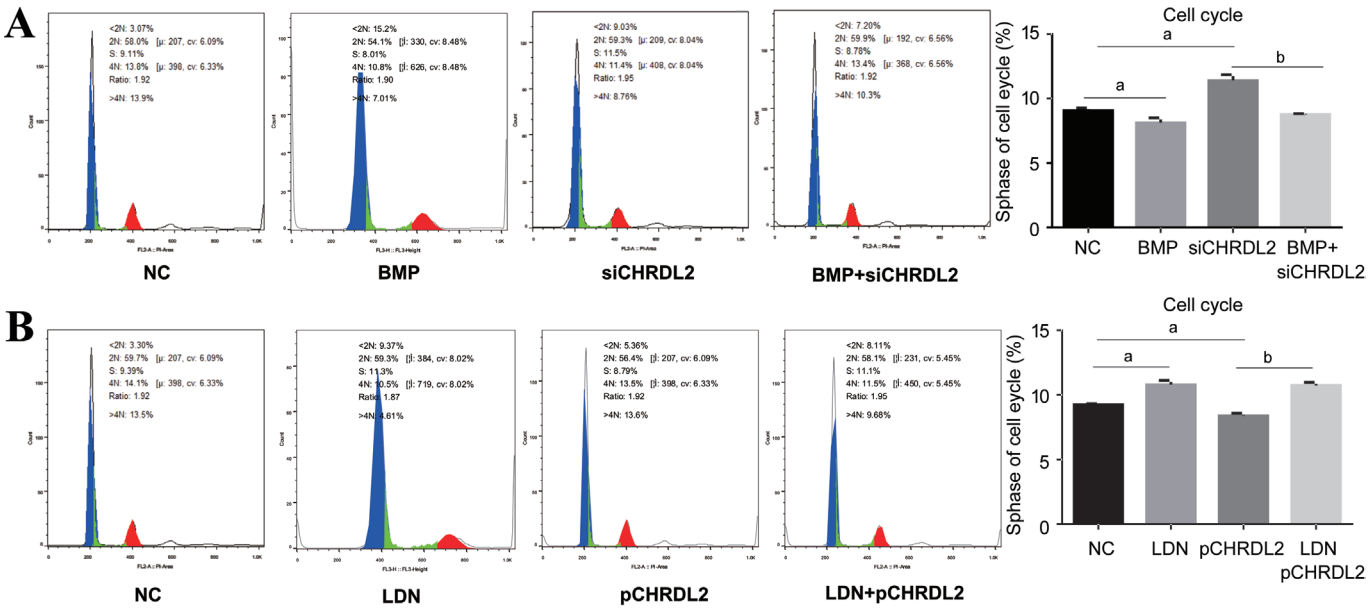

Figure 5 Chordin-like 2 influenced the cell proliferation by BMP pathway A: Cell cycle is analyzed by flow cytometry, the proportion of the cells in S phase was decreased by single BMP4 treatment compared with the cells in NC group. Compared with NC group, the cells in *siCHRDL2* had more proportion of S phase, but when BMP4 was added, the proportion of S phase was decreased. Histogram about cell cycle was shown. B: The proportion of the cells in S phase was increased by single LDN193189 treatment compared with the cells in NC group. Compared with NC group, the cells in p*CHRDL2* had less proportion of S phase, but when LDN193189 was added, the proportion of S phase was increased. Histogram about cell cycle was shown. Mean±SEM; $^{a}P<0.05$, $^{b}P<0.01$. NC: Negative control.

the activation of BMP pathway could reduce the reactions to relevant effects on cell proliferation after transfection. Cell cycle was analyzed by flow cytometry (Figure 5A). In first experiment, *siCHRDL2* group, the cells without BMP4 treating had the largest S phase proportion, but when the cells were treated by BMP4 in BMP+*siCHRDL2* group, the cells had the lowest S phase proportion. Besides, when the cells were treated by BMP4 alone, the cells also had a lower S proportion compared with NC group. On the contrary, in second experiment, overexpression of *CHRDL2* had a lowest S phase proportion, while LDN193189 could decrease the effects of overexpression and stimulate the cells into S phase. Meanwhile, single LDN193189 also could promote the number of the cells in S phase. These results demonstrated that Chordin-like 2 indeed influenced cell proliferation by regulating BMP pathway, in *siCHRDL2* group, lower BMP4 expression promoted cell division while BMP4 could intervened the effect, and in plasmid-*CHRDL2* group, higher BMP4 expression inhibited cell division but LDN193189, an inhibitor of BMP pathway, could reverse this effect.

The Correct Temporal Order of BMP Expression Required for RPE Differentiation For investigating the necessity of correct BMP pathway expression order to RPE differentiation, we divided the P3 cells into three groups: blank, LDN group and BMP4 group. We used LDN193189 (100 nmol/L, MCE) to inhibit BMP pathway to simulate early-phase BMP expression condition in normal RPE differentiation while this treatment could change the uptrend of BMP expression at later-phase. On the contrary, BMP4 was used to change the lower expression condition at early-phase. Five days after treatment, the cells in blank group and LDN group had a correct differentiation orientation and some cells started to become hexagon-like appearance, while in BMP4 group, the cells had an absolute difference. The cells in BMP4 group had a disorder differentiation. However, after 10d, LDN193189-treated cells failed to complete the differentiation, neither in BMP4-treated group (Figure 6A). Harvesting the cells and detecting the expression of *VIM*, one of EMT-markers was expressed most in LDN193189, but in other two groups, the expression of *VIM* did not have significant differences (Figure 6B). At the same time, *RPE65* had the most expression in blank group (Figure 6C). These results showed that the chaotic BMP signaling broke the order of RPE differentiation, and all these changes downregulated the expression of *RPE65* which means a wrong differentiation.

DISCUSSION

RPE is an important structure of retina. Any impairment of RPE could injure the photosensitization of retina. But fibrosis and scar formation of RPE cells often become problems after cell injury. Embryonic development of RPE cells provides an appropriate inspiration to explore the accurate mechanism of RPE repairment. Much research indicated that RPE cells differentiation depends on stable cell proliferation, integrated cell junctions, specific transcription factors expression and

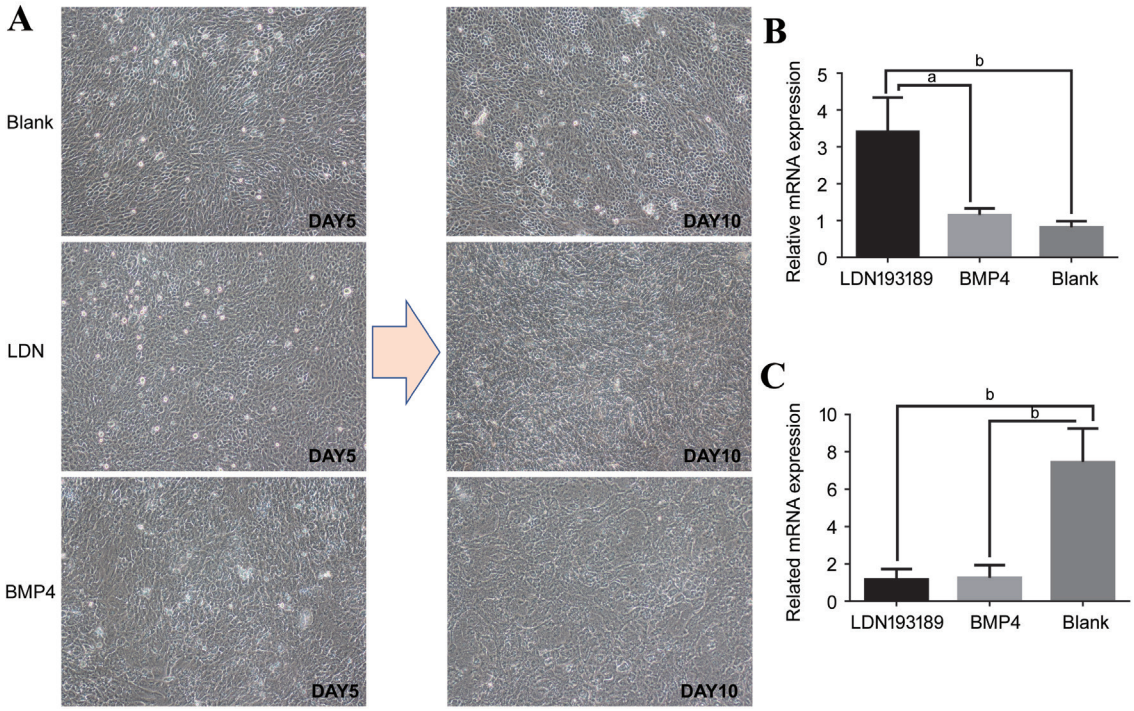

Figure 6 The correct temporal order of BMP expression was required for RPE differentiation A: The cells in P3 were divided into three groups: blank, LDN, and BMP4, and observed by phase contrast microscope. At 5d after plating, the cells in blank and LDN group showed an epithelial-like differentiation tendency, while in BMP4 the cells had a poor differentiation. At 10d, the cells in blank group continued to have a normal differentiation, while in LDN group cells were failed to complete perfect differentiation. B: The cells in LDN group had the highest expression of *VIM*. C: The cells in LDN and BMP4 group expressed lower *RPE65* while the cells in blank group expressed a higher *RPE65*. Micrograph scale bar: 100 μm. Mean±SEM, $^{a}P<0.05$, $^{b}P<0.01$ *vs* control. LDN: LDN193189 group; Ph: Phase contrast microscope.

ordered secretion of cytokines[24-25]. However, once any disorder interrupts one of these requirements, the cells will get incorrect differentiation and will undergo EMT. Many measures have been attempted to inhibit EMT, such as TGF-β inhibitor and BMP addition[25-26], *etc*. All these methods just focused on one point while ignored the dynamic changes over time. Hitherto, there still has no efficient way to inhibit EMT in injured RPE and concurrently induce them to differentiate. Therefore, with gradual cell differentiation, we showed a dynamic regulation of BMP pathway by Chordin-like 2.

BMP signaling pathway can regulate RPE differentiation and proliferation. An ordered activation of BMP pathway is essential. When sub-confluent cells are seeded *in vitro*, the cells start to proliferate, and after achieving confluent, the cells turn to differentiation. In our research, BMP4 was expressed lower at the beginning and was upregulated subsequently, and we verified the cells did not require BMP pathway at the early period in differentiation even excessive activation of BMP pathway harmfully impacted cell differentiation and proliferation. On the contrary, inhibiting BMP pathway had little effects on the cells and even had positive effect on cell proliferation at early stage, while this measure could intervene cell differentiation at later period. Therefore, BMP pathway plays different roles in the different phase of cell differentiation and exploring the regulation of BMP pathway is necessary.

BMP signaling pathway has several endogenous inhibitors, many of them have negative effects on RPE differentiation and promote EMT. Therefore, to maintain the differentiation process, some inhibitors such as Gremlin-1 are downregulated to alleviate the interruption effect on BMPs[26]. But in this research, Chordin-like 2, a novel endogenous BMP inhibitor, expressed higher strangely and had a similar expression trend with BMP4, which means it was expressed gradually along with RPE differentiation. It was an interesting performance for such BMP inhibitor, because endogenous inhibitors often play a negative role in related signaling pathway regulation. To investigate the exact functions of *CHRDL2*, cell transfection was used. According to the results, *CHRDL2* made effects on RPE differentiation and cell proliferation, these changes were determined by regulation of BMP pathway and secretion of BMP molecules. Lower *CHRDL2* expression downregulated key transcription factors (*OTX2* and *MITF*) in RPE differentiation and upregulated cell proliferation due to lower BMP4 secretion. Higher *CHRDL2* expression upregulated key transcription factor (*MITF*) and downregulated cell proliferation due to higher BMP4 expression. Besides, with higher *CHRDL2* expression, phosphorylated SMAD1 was expressed higher which means more activation of BMP

pathway. Interestingly, TGF-β was still upregulated mildly with overexpression of *CHRDL2*, that may be other pathway caused this phenomenon, such activin, because activin and TGF-β has same downstream SMAD2/3, activin has a positive effect on RPE differentiation in stem cell[24]. Total expression of SMAD1/SMAD2 also were detected, total SMAD1 was increased after overexpression of *CHRDL2*, but total SMAD2 had no significant difference. These results showed that, more *CHRDL2* also increased the expression of the important downstream molecule of BMP pathway, total SMAD1, but had no effects on the downstream molecules expression of TGF-β pathway.

The process of RPE differentiation is still not very clear, especially after impairment, but our research provides some directions. Many genes have their own expression curve and play different role in different differentiation phase. Therefore, all the key genes should express at the right time with appropriate quantity. If the cytokine was expressed at the wrong time with abnormal quantity, the so-called beneficial genes also interrupt the differentiation process and cause a wrong differentiation orientation such as BMP pathway. And Chordin-like 2 plays a role in regulating BMP pathway activation and influences RPE differentiation fate and that avoids the detrimental effects of BMP pathway on cells.

There also have some further study to explore about Chordin-like 2. First, long-term inhibition by exogenous molecule is necessary for exploring the indispensable role of Chordin-like 2. And siRNA and plasmid are the cells transient transfection, the final differentiation phenotypes and functions cannot be verified, therefore, stable transfection is still needed. BMPs contain a series of molecules such as BMP2, BMP4, BMP7, *etc.*, and every molecule not only has many common functions but also has some specific functions. However, only BMP4 was discussed in the research, other molecule should be explored. Last, fRPE cells has more ability to proliferation and differentiation after injury and Chordin-like 2 has some positive effects, but whether these effects still exist in adult cells remains to be explored.

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