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Hedgehog signaling pathway in the mesenchymal stem cells derived from human limbal niche

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Foundation items: National Natural Science Foundation of China (No. 81200661); Natural Science Foundation of Hubei Province (No. 2013CFB084)

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Received: 2014-06-16 Accepted: 2015-03-18

Hedgehog 信号通路在人角膜缘微环境来源的基质干细胞中表达情况的研究

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基金项目:国家自然科学基金(No. 81200661);湖北省自然科学基金(No. 2013CFB084)

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摘要

目的:探讨 Hedgehog 信号通路在人角膜缘微环境来源的基质干细胞(LNC)中的表达情况。

方法:对贴壁生长的人角膜缘来源的基质干细胞(LNC)进行分离培养及传代,对骨髓间充质干细胞系(BMMSC)进行培养传代。通过将 BMMSC 作为阳性对照,采用 Western blot 技术,免疫荧光技术,real-time PCR 技术从蛋白水平及基因水平验证 Hedgehog 信号通路成员 SHH, patched, SMO, Gli-1 在 LNC 中的表达情况。最后采用 Cell Count Kit-8 检测不同浓度 Gli 抑制剂 GANT61(空白,1,5,10,15,20,25,30μmol/L)处理 LNC 和 BMMSC48h 后的增殖抑制效应反向验证 Hedgehog 信号通路在 LNC 中的存在。

结果:Western blot 技术,免疫荧光技术,real-time PCR 技术均证明 LNC 细胞表达 Hedgehog 信号通路中的 Gli-1, patched, SMO,且 Gli 蛋白抑制剂 GANT61 可以显著抑制 LNC 细胞增殖($P<0.05$)。

结论:Hedgehog 信号通路在 LNC 细胞的增殖过程中发挥重要作用。

关键词:Hedgehog 信号通路;角膜缘细胞;骨髓间充质干细胞系

引用:王娟,李贵刚,李飞,蔡苏博,魏睿,李新宇. Hedgehog 信号通路在人角膜缘微环境来源的基质干细胞中表达情况的研究. 国际眼科杂志 2015;15(4):567-571

Abstract

• AIM: To investigate the expression of Hedgehog signaling pathway in the mesenchymal stem cells derived from human limbal niche (LNCs).

• METHODS: Culture and passage the mesenchymal stem cells derived from LNCs and bone marrow - derived mesenchymal stem cells (BMMSCs) *in vitro*. Using BMMSCs as a positive control, the expression of Hedgehog signaling pathway in LNCs was evaluated by Western blot, immunofluorescence and real - time PCR. Furthermore, different doses (1, 5, 10, 15, 20, 25, 30μmol/L) of GANT61 (the Gli inhibitor) effect on the proliferation of LNCs and BMMSCs was detected by Cell Count Kit-8.

• RESULTS: The results of Western blot, immunofluorescence and real-time PCR proved that LNCs expressed Gli - 1, patched and SMO, which are the members of Hedgehog signaling. In addition, GANT61 (the Gli inhibitor) inhibited the proliferation of LNCs significantly ($P<0.05$).

• CONCLUSION: Hedgehog signaling pathway plays an important role in the proliferation of LNCs.

• KEYWORDS: Hedgehog signaling pathway; limbal niche cells; mesenchymal stem cells from bone marrow
DOI: 10. 3980/j. issn. 1672-5123. 2015. 4. 01

Citation: Wang J, Li GG, Li F, Cai SB, Wei R, Li XY. Hedgehog signaling pathway in the mesenchymal stem cells derived from human limbal niche. *Guoji Yanke Zazhi* (*Int Eye Sci*) 2015;15(4):567-571

INTRODUCTION

Hedgehog signaling pathway plays an important role in the process of development and maintain of epigenetic structures in cells[1]. Hedgehog signaling can maintain the proliferation of normal cells, adjust the differentiation of progenitor cells and keep the steady state of adult stem cells[2,3]. The members of Hedgehog signaling pathway include Gli-1, patched, SMO and SHH. Hedgehog signaling pathway continues in mesenchymal stem cells from the bone marrow (BMMSCs) and participates in regulating cell

proliferation, differentiation and dedifferentiation[4-8]. In 2011, Xie *et al*[9] isolated a kind of microenvironment cells in human limbus named limbal niche cells (LNCs), and Li *et al*[10,11] further proved that the cells had the stem cell properties and could be differentiated into mesenchymal stem cells (MSCs), like BMMSC, suggesting that such microenvironment cells were more primitive than BMMSCs. In addition, LNCs, as a kind of microenvironment cells in human limbus, play an crucial role in proliferation, differentiation and migration of limbal stem cells[12-14] in limbal epithelial stem cell[15] deficiency (LSCD), and LSCD could lead to corneal abnormalities resulting in compromised vision and blindness. In this study, we carried out experiments to test whether or not LNCs express Hedgehog signaling pathway, which can maintain cell proliferation and adjust cell differentiation.

MATERIALS AND METHODS

Reagent Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Japan); Gli-1 antiboby (Cell Signaling, USA); patched antiboby (Santa Cruz Biotechnology, USA); GAPDH antiboby (proteintech, China); Alexafluor555 (Life technologies, USA); GANT61 (Sigma, USA); HRP-conjugated Goat Anti-Rabbit IgG (Antgene, wuhan, China); HRP-conjugated Goat Anti-Mouse IgG (Antgene, wuhan, China); CY3-coupled fluorescent secondary antibodies (Guge, wuhan, China); BMMSC (Cyagen, USA); Dulbecco's modified Eagle's medium: Nutrient Mixture F-12(1:1) (D-MEM/F-12) (Invitrogen, USA); human mesenchymal stem cell basic medium (Cyagen, USA); knock-out serum supplement (Invitrogen, USA); basic fibroblast growth factor (bFGF) (PEPROTECH, USA); leukemia inhibitory factor (LIF) (PeproTech, USA); insulin-transferrin-sodium selenite media supplement (ITS) (Sigma, USA); collagenase A (Invitrogen, USA); reverse transcription-polymerase chain reaction reagent (TOYOBO, Japan).

Methods

Cell isolation and culturing Corneoscleral rims from 18 to 60 years old donors were obtained from cornea donors at Wuhan Redcross Eyebank and managed in accordance with the Declaration of Helsinki. The limbal explants were cut into 12 pieces and digested with collagenase A at 37℃ for 24h to generate intact epithelial clusters containing the entire limbal epithelial sheet with subjacent stromal cells. Then the clusters were digested with 0.25% trypsin and 1mmol/L EDTA(T/E) at 37℃ for 10min to yield single cells. Finally, these single cells after centrifugal at 800rpm for 5min were seeded in 6-well plates on coated Matrigel in DF-12 with 10% knock-out serum containing 10ng/mL LIF, 4ng/mL bFGF, 5ug/mL insulin, 5ug/mL transferring and 5ng/mL selenium. BMMSCs were purchased from Cyagen Biosciences Inc (USA) and were cultured in human mesenchymal stem cell basic medium (Cyagen, USA) with 10% FBS. These cells were cultured at 37℃, 5% CO_2. The fourth to sixth passage cells were used in the experiment.

Flow cytometry The concentration of LNCs and BMMSCs were adjusted to be 2×10^5 cells/200uL phosphate-buffered saline (PBS). Then the cells were incubated with primary antibodies against CD90 and CD105 for 1h at room temperature[16]. Washed in PBS, the cells were carried out with CY3-coupled corresponding secondary antibodies for 1h at ratio of 1:100 to flow cytometry (FCM) (BD, USA) analysis in the dark.

Western blot Proteins were extracted with RIPA buffer supplemented with proteinase inhibitors. These two kinds of cell extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBST (phosphate-buffered saline and 0.1% Tween-20) for 1h at room temperature. The blots were then incubated at 4℃ overnight with specific primary antibodies raised against GAPDH, Gli-1, patched. After washing with PBST, the other incubation was carried out with corresponding secondary antibodies for 1h at room temperature. Immunoreactive bands were detected using an enhanced chemiluminescence kit according to the protocol.

Immunofluorescence staining Cells were seeded in the corresponding glass slides. After 24h, these cells were fixed with 4% formaldehyde for 15min, washed in PBS, and permeabilized with 0.2% Triton X-100 for 10min. Then these cells were blocked with 5% bovine serum albumin for 30min before being incubated with primary antibodies overnight at 4℃. The second day, after washing with PBS, the cells were incubated with secondary antibodies for 1h at the condition of avoiding light. The nuclei were counterstained with Hoechst before being analyzed with OLYMPUS (Japan) fluorescence microscope.

Reverse transcription and quantitative real-time PCR Cells were harvested for purification of RNA. Total RNA was extracted using Trizol reagent. Total RNA (500ng) was reverse-transcribed into cDNA and then amplified by fluorescent quantity PCR using the C1000™ Thermal Cycler CFX96™ Real-Time System. The fluorescent quantity PCR conditions were: pre-denaturation 95℃ for 3min, then 40 cycles 95℃ for 10s, 60℃ for 15s, 72℃ for 10s. The relative mRNA expression levels were normalized to GAPDH. The primers that we used were as follows: GAPDH, sense, 5'-CAGCCGCATCTTCTTGTGC-3', and antisense, 5'-GGTAACCAGGCGTCCGATA-3'; Gli-1, sense, 5'-TTCCTACCAGAGTCCCAAGT-3', and antisense, 5'-CCCTATGTGAAGCCCTATTT-3'; patched, sense, 5'-CGACAATACCCGCTACA-3', and antisense, 5'-GTGCCATAAAGGCTGAC-3'; SMO, sense, 5'-AAGAGCTGGTACGAGGACG-3', and antisense, 5'-CCACAAAGAAGCACGCATT-3'; vimentin, sense, 5'-

GACGCCATCAACACCGAGTT – 3', and antisense, 5' – CTTTGTCGTTGGTTAGCTGGT – 3'; SOX2, sense, 5' – GCCGAGTGGAAACTTTTGTCG – 3', and antisense, 5' – GGCAGCGTGTACTTATCCTTCT – 3'; POU5F, sense 5' – CTGGGTTGATCCTCGGACCT – 3', and antisense, 5' – CCATCGGAGTTGCTCTCCA – 3'; CD73, sense, 5' – GCCTGGGAGCTTACGATTTTG – 3', and antisense 5' – TAGTGCCCTGGTACTGGTCG – 3'; CD90, sense, 5' – ATCGCTCTCCTGCTAACAGTC – 3', and antisense, 5' – CTCGTACTGGATGGGTGAACT – 3'; CD105, sense, 5' – TGCACTTGGCCTACAATTCCA – 3', and antisense, 5' – AGCTGCCCACTCAAGGATCT–3'.

Cell Counting Kit – 8 Cells were digested with 0. 25% trypsin and 1 mmol/L EDTA (T/E) and seeded in 96 – well plates at the density of 4×10^4 per well. After 24h, adding the GANT61 at the different doses (0, 1, 5, 10, 15, 20, 25, 30μmol/L), these cells were cultured at 37℃, 5% CO_2 for 48h. Then, using the CCK – 8 reagent, the cells were incubated for 4h again and measured the optical density (OD) at 450nm wavelength using the all band enzyme standard instrument (Bio–tek). Cell survive rate (SR): SR = experimental group/control group average ×100%.

RESULTS

Identification of Limbal Niche Cells LNCs were identified by flow cytometry and real – time PCR (Figure 1). Flow cytometry showed that 96. 73% of LNCs expressed CD90, 92. 31% of LNCs espressed CD105, and on the other hand, 98. 61% of BMMSCs expressed CD90, 19. 59% of BMMSCs espressed CD105. The real–time PCR also showed that LNCs expressed vimentin, SOX2, POU5F, CD90, CD73.

Expression of Hedgehog Signaling Pathway in Limbal Niche Cells Western blot, immunofluorescence and real – time PCR were established in LNCs and BMMSCs at the level of protein and gene. The data showed that LNCs expressed Hedgehog signaling pathway, like BMMSCs. Gli–1 existed in cytoplasmic of LNCs and patched were expressed in cell membrane and nucleus of LNCs, especially in nucleus (Figure 2A, 2B). Western blot proved that LNCs expressed Gli–1 and patched proteins (Figure 2C). These results were consistent with Hedgehog signaling process (Figure 3). In addition, real–time PCR from the gene level proved that the expression of patched and SMO in LNCs were significantly higher than BMMSCs (Figure 2D).

GANT61 (Gli inhibitor) Inhibited the Proliferation of Limbal Niche Cells CCK8 experiment showed that GANT61 had obvious inhibition effect on proliferation of LNCs and BMMSCs (Figure 4).

DISCUSSION

Cell microenvironmentmodulation has been the focus of medical basic research. Among them, microenvironment cells are the important members of keeping the normal proliferation, differentiation, metabolism and function of

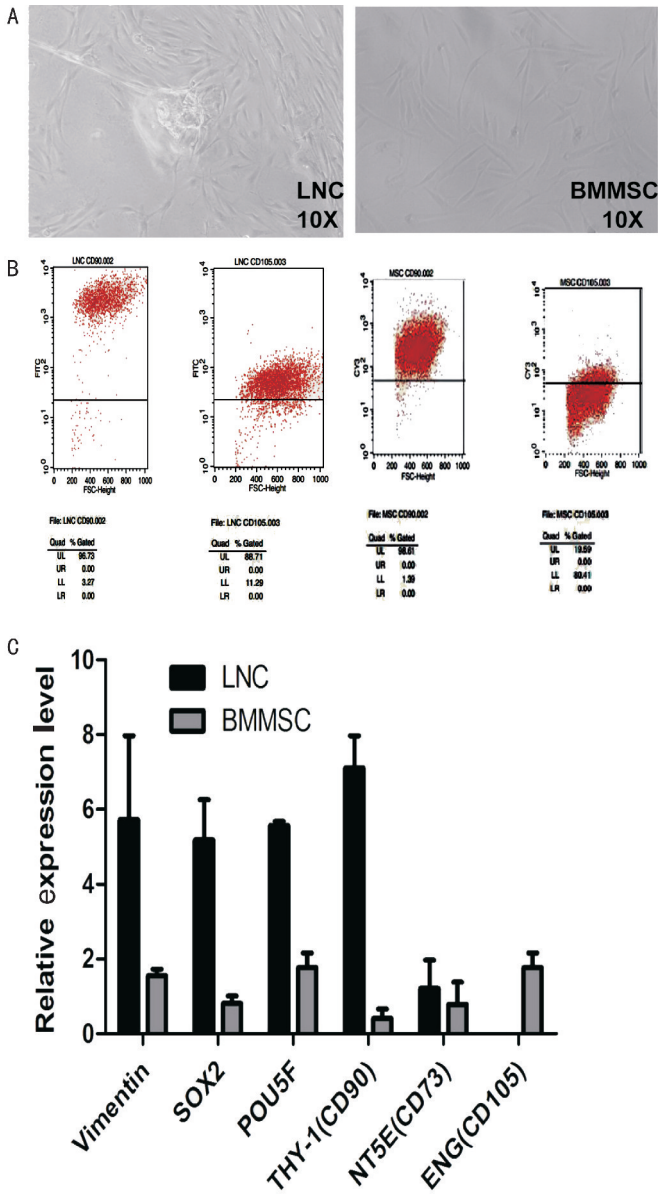

Figure 1 Identification of LNCs flow cytometry showed that the niche cells were almost a kind of cells. Flow cytometry and real–time PCR also proved the niche cells collected from cornea–scleral rims expressed some stem cell markers, for example CD90, CD105 and CD73, like BMMSCs.

cells, especially in the field of stem cell microenvironment. Microenvironment cells is the essential condition of regeneration, differentiation and migration[17]. Thus, to understand and explore the signaling pathways, which influence the proliferation and apoptosis of microenvironment cells, is extremely important. Previous research showed that LNCs[18,12] and BMMSCs are both microenvironment cells and they play a role in keeping proliferation, migration and differentiation in their respective fields.

There are three mammalian Hedgehog proteins, named sonic (SHH), India (IHH) and desert (DHH) Hedgehog. Among them, SHH is the most broadly expressed in vertebrate and its paracrine activity on adjacent cells is the most common way of pathway transduction, although SHH has also been proposed

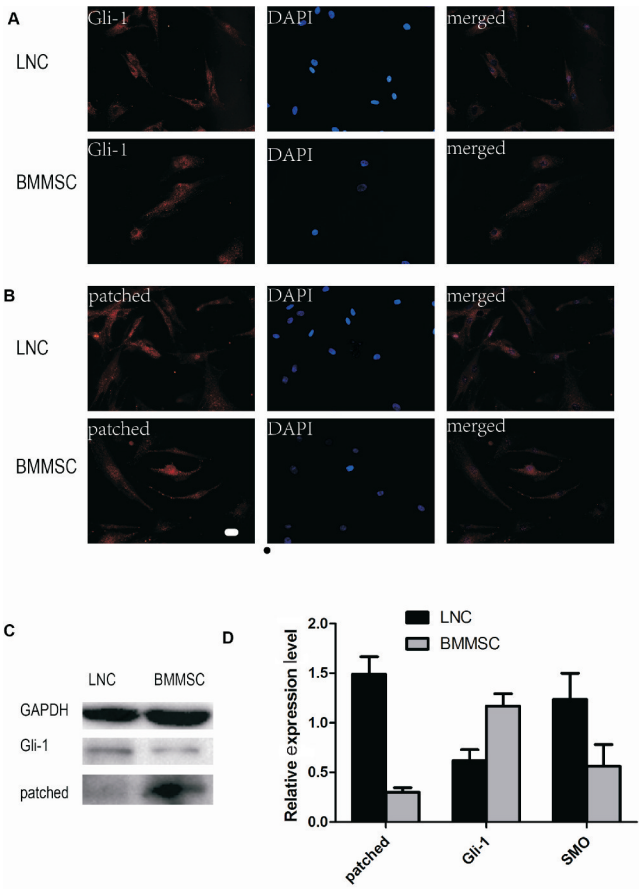

Figure 2 LNCs expressed Hedgehog signaling pathway, like BMMSCs A: Gli-1 protein positive in cytoplasm of LNCs and BMMSCs; B: Patched protein existed in cell membrane and nucleus, especially in nucleus of LNCs and BMMSCs; C: LNCs and BMMSCs both express Gli-1 and patched protein; D: At the mRNA level, the expression of patched and SMO expressed in LNCs were significantly higher in BMMSCs. Scale bar: 20μm.

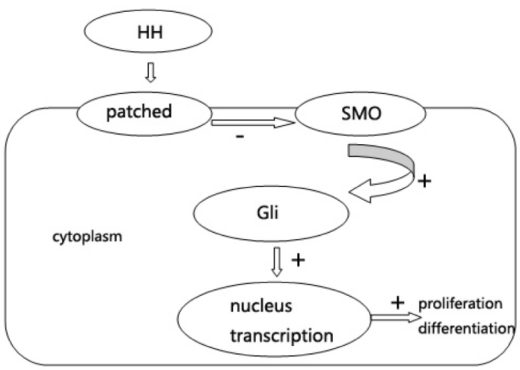

Figure 3 Hedgehog signaling pathway.

to signal in an autocrine manner. SHH signaling is initiated by the binding of the SHH ligand to its transmembrane protein, patched, which relieves the suppression of the transmembrane protein, SMO. The SMO can activate the Gli transcripition factors[19]. Hedgehog signaling functions through regulating the balance between GLI^A and GLI^R[20]. GLI^A then triggers expression of HH target gene such as Gli-1, the protein product that is a transcripitional activator and amplifies Hedgehog signaling. Then, Gli-1 gene regulated

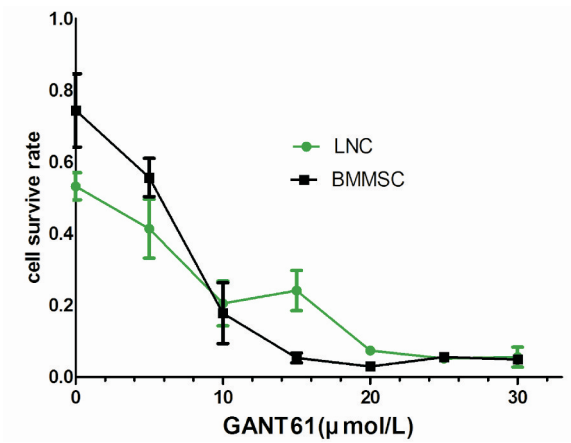

Figure 4 CCK-8 detected the inhibition effect of GANT61 (the Gli inhibitor) on proliferation of LNCs and BMMSCs.

the expression of downstream cytokines or proteins, which contribute to development, proliferation and differentiation, for example: CCND1, CCND2, FOXM1, Bcl-2, FOXA2 and so on[21]. So the Gli-1 is a crucial protein in Hedgehog signaling. Of course, the process was accused to as the canonical Hedgehog signaling pathway. In our study, the existence of Gli-1, patched, SMO had been verified in LNCs.

It is well known that Hedgehog signaling participated in mitogenic and morphogenic function during development. Recently, many researchers showed that high level of Hedgehog signaling expressed in specific populations of cells, including stem[22] and progenitor cells. Hedgehog signaling also regulates the behavior of adult stem and progenitor cells during homeostasis and repair, through many different routes to regulate cell proliferation, including maintenance of plasticity[20]. But the exact mechanism is unclear.

Established in this study, we primarily found that LNCs expressed Hedgehog signaling components including Gli-1, patched, SMO from the level of protein and gene, just like BMMSCs. In addition, our data showed that the inhibition the function of Hedgehog signaling also could suppress the proliferation of LNCs significantly.

The experiments proved the existence of Hedgehog signaling in LNCs for the first time. Improving the function of Hedgehog signaling could be a good way to promote the proliferation, differentiation and development of LNCs. Thus, LNCs can support limbal epithelial stem cells (LSCs)[12-14] better, which could help in wound healing of corneal epithelium.

ACKNOWLEDGEMENTS

The authors wish to thank Yun-Ping Lu's kind help for carrying out experiments.

REFERENCES

1 Hui CC, Angers S. Gli proteins in development and disease. *Annu Rev Cell Dev Biol* 2011;27:513-537

2 Zhou JX, Jia LW, Liu WM, Miao CL, Liu S, Cao YJ, Duan EK. Role of sonic hedgehog in maintaining a pool of proliferating stem cells in

the human fetal epidermis. *Hum Reprod* 2006; 21(7):1698-1704
3 Peng YC, Levine CM, Zahid S, Wilson EL, Joyner AL. Sonic hedgehog signals to multiple prostate stromal stem cells that replenish distinct stromal subtypes during regeneration. *Proc Natl Acad Sci U S A* 2013;110(51):20611-20616
4 Li X, Wei G, Wang X, Liu DH, Deng RD, Li H, Zhou JH, Li YW, Zeng HP, Chen DF. Targeting of the Sonic Hedgehog pathway by atractylenolides promotes chondrogenic differentiation of mesenchymal stem cells. *Biol Pharm Bull* 2012;35(8):1328-1335
5 Lin L, Shen Q, Xue T, Duan X, Fu X, Yu C. Sonic hedgehog improves redifferentiation of dedifferentiated chondrocytes for articular cartilage repair. *PLoS One* 2014;9(2):e88550
6 Ma X, Zhang X, Jia Y, Zu S, Han S, Xiao D, Sun H, Wang Y. Dexamethasone induces osteogenesis via regulation of hedgehog signalling molecules in rat mesenchymal stem cells. *Int Orthop* 2013;37(7):1399-1404
7 Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. *J Cell Biochem* 2011;112(12):3491-3501
8 Muruganandan S, Roman AA, Sinal CJ. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. *Cell Mol Life Sci* 2009;66(2):236-253
9 Xie HT, Chen SY, Li GG, Tseng SC. Isolation and expansion of human limbal stromal niche cells. Invest *Ophthalmol Vis Sci* 2012;53(1):279-286
10 Li GG, Chen SY, Xie HT, Zhu YT, Tseng SC. Angiogenesis potential of human limbal stromal niche cells. *Invest Ophthalmol Vis Sci* 2012;53(7):3357-3367
11 Li GG, Zhu YT, Xie HT, Chen SY, Tseng SC. Mesenchymal stem cells derived from human limbal niche cells. *Invest Ophthalmol Vis Sci* 2012;53(9):5686-5697
12 Xie HT, Chen SY, Li GG, Tseng SC. Limbal epithelial stem/progenitor cells attract stromal niche cells by SDF-1/CXCR4 signaling to prevent differentiation. *Stem Cells* 2011;29(11):1874-1885
13 Chen SY, Hayashida Y, Chen MY, Xie HT, Tseng SC. A new isolation method of human limbal progenitor cells by maintaining close association with their niche cells. *Tissue Eng Part C Methods* 2011;17(5):537-548
14 Li Y, Inoue T, Takamatsu F, Kobayashi T, Shiraishi A, Maeda N, Ohashi Y, Nishida K. Differences between niche cells and limbal stromal cells in maintenance of corneal limbal stem cells. *Invest Ophthalmol Vis Sci* 2014;55(3):1453-1462
15 Yoon JJ, Ismail S, Sherwin T. Limbal stem cells: central concepts of corneal epithelial homeostasis. *World J Stem Cell* 2014;6(4):391-403
16 Kim MO, Jung H, Kim SC, Park JK, Seo YK. Electromagnetic fields and nanomagnetic particles increase the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells. *Int J Mol Med* 2015. 35(1):153-160
17 Li Z, Li L. Understanding hematopoietic stem-cell microenvironments. *Trends Biochem Sci* 2006;31(10):589-595
18 Chen SY, Mahabole M, Tseng SC. Optimization of *ex vivo* expansion of limbal epithelial progenitors by maintaining native niche cells on denuded amniotic membrane. *Transl Vis Sci Technol* 2013;2(7):1
19 Borycki A, Brown AM, Emerson CP Jr. Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. *Development* 2000;127(10):2075-2087
20 Petrova R, Joyner AL. Roles for Hedgehog signaling in adult organ homeostasis and repair. *Development* 2014;141(18): 3445-3457
21 Gu HB, Li JK. Hedgehog signaling pathway in cancer. *Morden Oncology* 2011;19(4):808-811
22 Ferent J, Cochard L, Faure H, Taddei M, Hahn H, Ruat M, Traiffort E, Genetic activation of hedgehog signaling unbalances the rate of neural stem cell renewal by increasing symmetric divisions. *Stem Cell Reports* 2014;3(2):312-323