

Notch signaling dependent differentiation of cholangiocyte-like cells from rhesus monkey embryonic stem cells

JIN Li-Fang^{1,2}, JI Shao-Hui², YANG Ji-Feng^{2,3}, JI Wei-Zhi^{2,*}

(1. College of Life Science of Shaoxing University, Shaoxing Zhejiang 312000, China; 2. Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming Yunnan 650223, China; 3. College of Life Science of Wenzhou Medical College, Shaoxing Zhejiang 325035, China)

Abstract: Rhesus monkey embryonic stem (rES) cells have similar characteristics to human ES cells, and might be useful as a substitute model for preclinical research. Notch signaling is involved in the formation of bile ducts, which are composed of cholangiocytes. However, little is known about the role of Notch signaling in cholangiocyte commitment of ES cells. We analyzed the effect of Notch signaling on the induction of cholangiocyte-like cells from rES cells. About 80% of definitive endoderm (DE) cells were generated from rES cells after treatment with activin A. After treatment with BMP4 and FGF1 on matrigel coated wells in serum-free medium, rES-derived DE gave rise to cholangiocyte-like cells by expression of cholangiocyte specific proteins (CK7, CK18, CK19, CK20, and OV-6) and genes (GSTPi, IB4, and HNF1 β). At the same time, expression of Notch 1 and Notch 2 mRNA were detected during cell differentiation, as well as their downstream target genes such as Hes 1 and Hes 5. Inhibition of the Notch signal pathway by L-685458 resulted in decreased expression of Notch and their downstream genes. In addition, the proportion of cholangiocyte-like cells declined from ~90% to ~20%. These results suggest that Notch signaling may play a critical role in cholangiocyte development from ES cells.

Key words: Rhesus monkey; Embryonic stem cells; Cholangiocytes; Notch signaling

Notch 信号通路调控猕猴胚胎干细胞的胆管样细胞分化

金立方^{1,2}, 纪少珲², 杨纪峰^{2,3}, 季维智^{2,*}

(1. 绍兴文理学院 生命科学学院, 浙江 绍兴 312000; 2. 中国科学院昆明动物研究所 云南省动物生殖生物学重点实验室, 云南 昆明 650223; 3. 温州医学院 生命科学学院, 浙江 温州 325035)

摘要: 猕猴胚胎干细胞(rhesus monkey embryonic stem (rES))与人胚胎干细胞有相似的生物学特性, 因此是理想的临床前研究的替代模型。Notch 信号通路在胆管及胆管上皮细胞的形成中有重要的作用, 然而, 有关 Notch 信号通路在 ES 细胞的胆管分化中的作用了解甚少。该实验以 rES 为模型, 对 Notch 信号通路对 ES 细胞的胆管分化过程中的作用进行了较为系统的研究。rES 在细胞因子 Activin A 诱导作用下产生约 80%的限定性内胚层细胞。以 Matrigel 作为细胞外基质, 在含 BMP4 和 FGF1 的无血清培养体系中继续诱导 5~7 d, rES 细胞来源的限定性内胚层细胞分化产生约胆管样细胞。分化的细胞表达胆管细胞的特异性蛋白((CK7、CK18、CK19、CK20 和 OV-6)及基因(GSTPi、IB4 和 HNF1 β)。在胆管样细胞的分化过程中检测到了 Notch 1 和 Notch 2 基因及下游信号分子 hes 1 和 hes 5 的表达。用 Notch 抑制剂 L-685458 处理分化过程中的细胞可导致 Notch 1 和 Notch 2 基因及下游信号分子 hes 1 和 hes 5 的表达下降, 同时 CK19 阳性的胆管样细胞分化比率也从 90%下降至约 20%。这一研究提示 Notch 信号通路可能在 ES 细胞的胆管样分化过程中扮演重要的角色。

关键词: 猕猴; 胚胎干细胞; 胆管细胞; Notch 信号通路

中图分类号: Q813; Q959.848 **文献标志码:** A **文章编号:** 0254-5853-(2011)04-0391-05

Received date: 2011-01-21; Accepted date: 2011-06-11

Foundation items: This work was supported by research grants from Zhejiang Natural Sciences Foundation of China (Y2110911; Y2080996), and the National Key Technologies R&D Program of China (2007CB947701)

* Corresponding author (通信作者), Tel/Fax: +86-0871-5139413, E-mail: wji@mail.kiz.ac.cn

收稿日期: 2011-01-21; 接受日期: 2011-06-11

第一作者简介: 金立方 (1978-), 男, 浙江上虞人, 讲师, 博士, 研究方向为干细胞生物学; E-mail: lifangji@sohu.com

Based on their ability to proliferate and their capacity to differentiate into specific cell types, embryonic stem (ES) cells are a potential source for cell transfer therapy in hepatic diseases. Although several studies have reported on the differentiation of hepatic cells from human (Duan et al, 2007), mouse (Hamazaki et al, 2001) and monkey ES cells (Ma et al, 2008; Jin et al, 2009), it remains unclear which factors are responsible for the induction of hepatic differentiation of ES cells.

Notch proteins are cell-surface receptors activated by interactions with cell surface ligands of the Jagged/Delta family. The mammalian family of Notch receptors consists of four members: Notch 1 through 4. Binding of the ligand to the Notch receptor induces site specific cleavage resulting in the release of the Notch Intracellular Domain (NICD). The NICD translocates to the nucleus where it modulates gene expression through interaction with members of the CSL (CBF-1, Suppressor of Hairless, lag-1) family of transcription factors. Activated Notch signaling elevates the expression of specific genes, including *Hes 1* and *Hes 5*. Notch signaling has been shown to play an important role in cell-fate determination, cell survival, proliferation, and differentiation (Ma et al, 2007). The association of Notch signaling with cholangiocytes formation has been implicated in the literature (Lozier et al, 2008; Tanimizu & Miyajima, 2004); however, little is known about the mechanism of cholangiocyte commitment of ES cells.

In view of the close similarities between human and nonhuman primates, the rhesus monkey is a perfect animal model to better understand liver developmental processes in primates, and for preclinical assessment of cell transfer therapy protocols. In the present study, we analyzed the effect of Notch signaling on cholangiocyte-like cells induction from rES cells in monolayer differentiation systems.

1 Materials and Methods

1.1 Culture of rhesus monkey ES cells

The rES cell line (366.4) was obtained from Dr. James Thomson, Wisconsin National Primate Research Center. Undifferentiated rES cells were expanded on a feeder layer of mitomycin-treated mouse embryonic fibroblasts seeded on 0.1% gelatin-coated plates, and cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Fremont, CA) containing 15% FBS (Hyclone, Logan, UT), 1% nonessential amino acids (Sigma-

Aldrich, Louis, MO), 0.1 mmol/L 2-mercaptomethanol (Sigma-Aldrich, St. Louis, MO), 2 mmol/L L-glutamine (Sigma-Aldrich, Louis, MO), and 1% penicillin/streptomycin (Sigma-Aldrich, Louis, MO). Medium was changed daily, and rES colonies were manually split every four days to select for undifferentiated rES cells.

1.2 Growth factors induced cholangiocyte-like differentiation of rES cells

For definitive endoderm (DE) differentiation, the rES cell colonies were manually cut into small clumps of approximately 100 cells, plated on Matrigel-coated culture dishes, and differentiated in DMEM/F12 containing 1% FBS with 100 ng/mL human activin A (R&D systems) for 4-5 days. For cholangiocyte-like differentiation, the rES-derived DE cultures were passaged with 10 mg/mL of dispase and plated at a ratio of 1:2 on type I collagen (Sigma-Aldrich, Louis, MO) coated wells, and cultured in F12 medium supplemented with 2% knockout serum replacement (Gibco, Auckland, NZ), 100 ng/mL FGF1(R&D systems), and 20 ng/mL of BMP4(R&D systems) for 5-7 days. To study the effect of Notch signaling on cell differentiation, cells were cultured with or without 50 nmol/L Notch inhibitor (L-685458) (Bachem Bioscience, King of Prussia, PA) in F12 medium supplemented with 2% knockout serum replacement, 100 ng/mL of FGF1, and 20 ng/mL of BMP4 for 5-7 days.

1.3 Immunocytochemistry (ICC)

Cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed with PBS three times, followed by permeabilization with 0.2% Triton X-100 for 10 min and blockage with 4% goat serum for 30 min at 25 °C. Subsequently, the cells were incubated with the primary antibodies (see Tab. 1) in staining solution (PBS containing 4% goat serum) for 40 min at 37 °C, and then incubated with the appropriate Texas red, PE or FITC-conjugated secondary antibody in staining solution for 30 min at 37 °C. The negative control was incubated in the

Tab. 1 Primary antibodies

| Primary antibody | Species | Manufacturer | Dilution |
|------------------|-----------------|--------------|--------------|
| Brachyury | Rabbit PAB IgG | Santa Cruz | 1:100 |
| CK7 | Mouse MAb IgG1 | DAKO | 1:100 |
| CK18 | Mouse MAb IgG1 | Sigma | 1:200 |
| CK19 | Mouse MAb IgG1 | DAKO | 1:100 |
| CK20 | Mouse MAb IgG1 | DAKO | Ready to use |
| CXCR-4 | MouseMAbIgG1-PE | R&D | 1:20 |
| OV-6 | Mouse MAb IgG1 | sigma | 1:100 |
| SOX17 | Mouse MAb IgG3 | R&D | 10 µg/mL |

staining solution without primary antibodies.

1.4 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Calsbad, CA) according to the manufacturer's protocols. Reverse transcription was carried out with approximately 1 μ g of total RNA in 20 μ L of 1 \times master mix (1 \times reverse transcription buffer, 0.5 mol/L dNTPs, 50 pmol of oligo(dT) primer, 20 U of RNase inhibitor and 5 U of reverse transcriptase) at 42 $^{\circ}$ C for one hour. For PCR, 1 μ L of RT products was added to 1 \times PCR master mix (1 \times PCR reaction buffer, 1.5 mmol/L of $MgCl_2$, 0.5 mmol/L of dNTPs, 0.4 μ mol/L of forward primer, and 0.4 μ mol/L of reverse primer (see supplemental Tab. 2), 1.25 U of Taq DNA polymerase in a 25 μ L final volume and amplified by 25–35 cycles of PCR (95 $^{\circ}$ C 30 s; 52 – 60 $^{\circ}$ C 30 s; 72 $^{\circ}$ C 30 s) followed by a final extension at 72 $^{\circ}$ C for 5 min. All reagents in RT-PCR were purchased from Takara (Takara, Dalian, China) unless otherwise mentioned. Products of PCR were separated on a 2% agarose gel and stained with ethidium bromide. The primer sequence for IB4 is 5'-GCACGGACGAGATGTTTCAG-3'/5'-ACTTGC CAAATCCAATAGTGTAG-3'; for GSTpi is 5'-GGACGG AGACCTCACCTGTA-3'/5'-TCTTGCCTCCCTGGTTC TGG-3'; for HNF1 β is 5'-GAAACAATGAGATCACTT CCTCC-3'/5'-CTTTGTGCAATTGCCATGACTCC-3'.

1.5 Analysis

For qualitative analysis, all differentiation experiments were replicated three times, and $P \leq 0.05$ was taken as significant.

2 Results and Discussion

2.1 Efficient generation of DE from rES cells

The rES clumps containing approximately 100 cells were collected and seeded on Matrigel-coated 4-well plates, and cultured with 100 ng/mL of activin A and 1% FBS for 5 days. After one day of induction, more than 90% of cells were strongly positive for brachyury, and only a few of the brachyury positive cells were faintly costained for SOX17 (Fig. 1A). After two days of induction, the ratio of cells staining for both SOX17 and brachyury was beyond 80% (Fig. 1A). The SOX17/brachyury double positive populations are regarded as generation of DE from ES cells (D'Amour et al, 2005; Yasunaga et al, 2005). In addition, the brachyury expression level gradually declined while the SOX17 expression level gradually increased with culture, with

the peak of SOX17 expression observed at day four of differentiation (Fig. 1A), indicating that production of DE was a gradual differentiation process. On the contrary, untreated rES did not express brachyury and Sox17 (Fig. 1B).

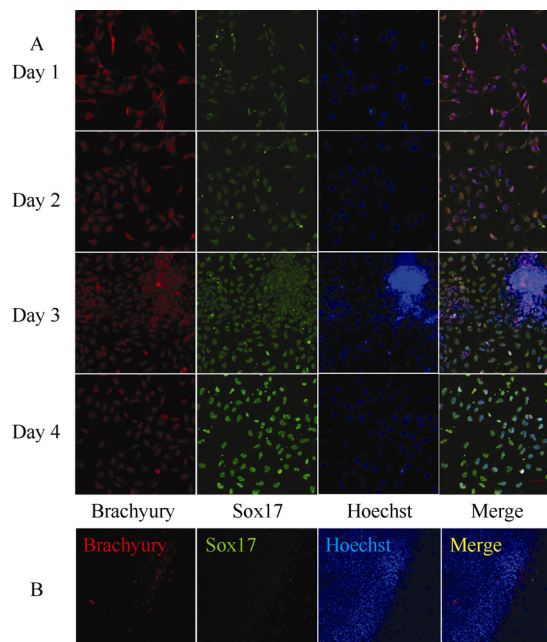


Fig. 1 Double labeling of differentiated cells by brachyury (red), SOX17 (green), Hoechst (nuclei, blue) and merged views of the same field of DE and rES cells

A) Expression of SOX17 was up-regulated and brachyury down-regulated with duration of culture in differentiating DE cells. B) Untreated rES cells were negative for brachyury and SOX17. Nuclear DNA was counterstained with Hoechst (blue), original magnification $\times 200$.

Consistent with the expression pattern of SOX17, we observed an increase in FoxA2 levels in activin A treated rES cells as differentiation proceeded (Fig. 2A),

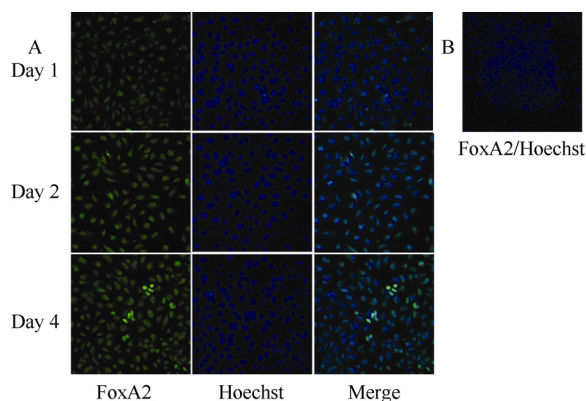


Fig. 2 FoxA2 was expressed in activin A induced rES cells A) Immunofluorescence staining positive for FoxA2 (green) and Hoechst (nuclei, blue) and merged views of the same field after 1, 2 and 4 days of differentiation. Nuclear DNA was counterstained with Hoechst (blue). B) Untreated rES cells were negative for FoxA2, original magnification $\times 200$.

and untreated rES did not express FoxA2 (Fig. 2A). Genetic studies of nonmammalian organisms support a role for FoxA2 factors in endoderm development (Rehorn et al, 1996). Thus, expression of FoxA2 in differentiated cells also indicated that DE cells were generated.

To further verify that the SOX17 positive cells were DE cells, the CXCR4 expression was examined to distinguish DE from visceral endoderm (D'Amour et al, 2005). The CXCR4 positive cells were first observed on the third day of differentiation, and the proportion of CXCR4 positive cells increased up to 80% on the fourth day of differentiation (Fig. 3). Almost 100% of CXCR4 positive cells co-expressed SOX17 (Fig. 3). As previously mentioned, SOX17 was expressed in definitive, primitive and parietal endoderm cells, while CXCR4 was expressed in the mesoderm and DE cells. Thus, the SOX17/CXCR4 double positive populations should be definitive endoderm. Furthermore, the differentiated cells did not express the visceral endoderm marker alpha fetoprotein at any point during culture (data not shown). This evidence clearly indicates that activin A efficiently generated definitive endoderm from rES under low serum conditions.

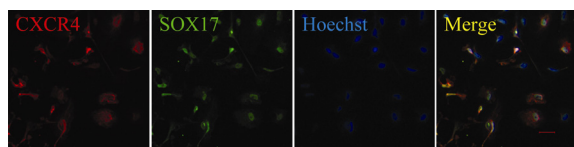


Fig. 3 Double labeling of differentiated cells by CXCR4 (red), SOX17 (green), Hoechst (nuclei, blue) and merged views of the same field at 4 days

Nuclear DNA was counterstained with Hoechst (blue), original magnification $\times 200$.

2.2 Cholangiocyte-like cells lineages differentiation of rES-derived definitive endoderm (DE) cells

Growth factors, such as FGFs, BMPs, HGF, OSM and Dex, are critical for hepatic endoderm morphogenesis and can promote hepatic differentiation (Kinoshita & Miyajima, 2002), and HGF, FGF-2 and OSM/Dex are critical for generation of hepatocytes from DE *in vitro*. In the present study, we examined the effect of BMP4 and FGF1 on hepatic fate differentiation from rES-derived DE. After 5–7 days induction, more than 90% of cultured cells expressed CK18 and CK19 (Fig. 4A), specific markers of bile duct cells. About 40% of cultured cells were stained for CK7 and CK20 (Fig. 4A), which are present in mature bile duct cells. In addition, another important protein of cholangiocyte marker OV6

was also detected in the differentiated cells (Fig. 4A). Conversely, DE cells were negative for CK7, CK20 and OV-6 (Fig. 4A). Cells were further analyzed by RT-PCR to confirm the presence of cholangiocyte-like cells in the differentiated cells. As expected, the differentiated cells expressed HNF1 β , an important transcription factor controlling bile duct cell differentiation during liver development, as well as GSTPi and IB4, which are indicative of the presence of cholangiocyte-like cells (Fig. 4B). Unexpectedly, neither AFP nor ALB were detected in induced cells at either protein or mRNA levels. Thus, expression of the CK7, CK19, CK20 and OV6 proteins as well as the HNF1 β , GSTPi and IB4 genes in cells from rES indicated that cholangiocyte-like cells were generated.

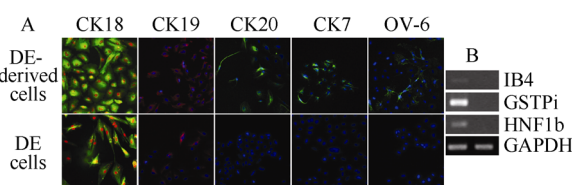


Fig. 4 Induction of cholangiocytes from rES-derived definitive endoderm by FGF1 and BMP4

A) DE-derived cells showed characteristics of cholangiocytes by expression of proteins CK18, CK19, CK7, CK20 and OV6, while DE cells were negative for CK7, CK20 and OV-6. B) RT-PCR analysis for cholangiocytes markers (IB4, GSTPi and HNF1 β) from rES-derived definitive endoderm cells (1) and rES cells or DE cells (2) Nuclear DNA was counterstained with Hoechst (blue) or PI (red), original magnification $\times 200$ or 400.

2.3 Specification of rES-derived cholangiocyte like cells was Notch dependent

The Notch signaling pathway specifies cell fate during bipotential cell fate decisions and controls various biological events (Ehebauer et al, 2006), including the expression of transcription factors for exocrine cells, formation of bile ducts, and association with human Alagille syndrome (Crosnier et al, 2000). Notch 1 and Notch 2 are specifically expressed in proliferating hepatoblasts. Deletion of Notch 2 results in cholangiocyte and bile duct defects, while deletion of Notch 1 does not result in bile duct paucity (Lozier et al, 2008). Both the Hes1 and Hes5 genes, which encode a basic helix-loop-helix protein, are downstream effectors of the Notch pathway. The Hes1-null mice formed a relatively-normal ductal plate consisting of cytokeratin- and DBA-positive cholangiocyte precursors, suggesting that the primary defect in these mice was not in the initial bipotential cell fate decision of the hepatoblasts (Kodama et al, 2004).

Given the importance of Notch activation in

cholangiocyte-like specification from rES-derived definitive endoderm, we tested the expression of Notches and downstream target genes in the differentiation cultures. Notch 1 and Notch 2 mRNA were clearly detected after five days differentiation, as well as their downstream target genes such as Hes 1 and Hes 5 (Fig. 5B, line 1). The expression of Notch genes was accompanied by the expression of Notch intracellular domain (NICD) (Fig. 5A, untreated group). These results suggested that Notch signaling was active in cholangiocyte-like differentiation from rES-derived definitive endoderm.

To further confirm the role of Notch on cholangiocyte-like specification, Notch inhibitor (L-685458) was supplemented in the differentiated medium at a concentration of 50 nM. After five days of culture, the expression of Notch genes as well as Hes 1 and Hes 5 significantly declined in the treatment group compared with the control group (Fig. 5B, line 2). As expected, the expression of NICD also declined (Fig. 5B, treatment group). In addition, the proportion of CK19 positive cells significantly decreased to ~20% (as a percentage of total number of CK19-positive cells in multiple fields of hoechst positive cells), compared with ~90% of CK19 positive cells in the control group. Thus, consistent with previous studies (Kodama et al, 2004; Tanimizu & Miyajima, 2004), our results suggested Notch was essential for cholangiocyte-like differentiation from rES-derived DE.

In this study, we established reproducible and efficient protocols for definitive endoderm and

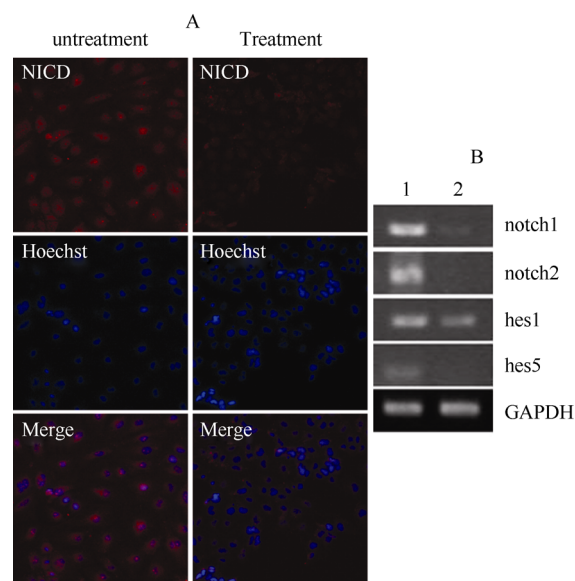


Fig. 5 Notch signaling was critical for cholangiocyte differentiation from rES-derived definitive endoderm

A) Fluorescent images of NICD in differentiated cells from rES cell-derived definitive endoderm in treatment group (with L-685458) and untreated group (without L-685458). Note the treatment group had higher NICD expression than the untreated group. Nuclear DNA was counterstained with Hoechst (blue), original magnification $\times 200$. B) RT-PCR analysis for Notches and downstream target genes were expressed in the untreated group (line 1), and their expression were absent or declined in the treatment group (line 2).

cholangiocyte-like cell generation from rES cells by monitoring lineage development at different stages. Furthermore, our results suggested that the cholangiocyte-like development of DE was Notch signaling dependent.

References:

- Crosnier C, Attie-Bitach T, Encha-Razavi F, Audollent S, Soudy F, Hadchouel M, Meunier-Rotival M, Vekemans M. 2000. JAGGED1 gene expression during human embryogenesis elucidates the wide phenotypic spectrum of Alagille syndrome [J]. *Hepatology*, **32**(3): 574-581.
- D'Amour KA, Agulnick AD, Eliazar S, Kelly OG, Kroon E, Baetge EE. 2005. Efficient differentiation of human embryonic stem cells to definitive endoderm [J]. *Nat Biotechnol*, **23**(12): 1534-1541.
- Duan Y, Catana A, Meng Y, Yamamoto N, He S, Gupta S, Gambhir SS, Zern MA. 2007. Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells *in vitro* and *in vivo* [J]. *Stem Cells*, **25**(12): 3058-3068.
- Ehebauer M, Hayward P, Arias AM. 2006. Notch, a universal arbiter of cell fate decisions [J]. *Science*, **314**(5804): 1414-1415.
- Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, Terada N. 2001. Hepatic maturation in differentiating embryonic stem cells *in vitro* [J]. *FEBS Lett*, **497**(1): 15-19.
- Jin LF, Ji SH, Guo XY, Wang XH, Ji WZ. 2009. Induction of rhesus monkey embryonic stem cells into hepatocyte-like cells by a three-step method [J]. *Zool Res*, **30**(5): 509-514 (in Chinese).
- Kinoshita T, Miyajima A. 2002. Cytokine regulation of liver development [J]. *Biochim Biophys Acta*, **1592**(3): 303-312.
- Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. 2004. The role of notch signaling in the development of intrahepatic bile ducts [J]. *Gastroenterology*, **127**(6): 1775-1786.
- Lozier J, McCright B, Gridley T. 2008. Notch signaling regulates bile duct morphogenesis in mice [J]. *PLoS One*, **3**(3): e1851, 1-6.
- Ma A, Boulton M, Zhao B, Connon C, Cai J, Albon J. 2007. A role for notch signaling in human corneal epithelial cell differentiation and proliferation [J]. *IOVS*, **48**(8): 3576-3585.
- Ma X, Duan Y, Jung CJ, Wu J, VandeVoort CA, Zern MA. 2008. The differentiation of hepatocyte-like cells from monkey embryonic stem cells [J]. *Cloning Stem Cells*, **10**(4): 485-493.
- Rehorn KP, Thelen H, Michelson AM, Reuter R. 1996. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila* [J]. *Development*, **122**(12): 4023-4031.
- Tanimizu N, Miyajima A. 2004. Notch signaling controls hepatoblast differentiation by altering the expression of liver-enriched transcription factors [J]. *J Cell Sci*, **117**(15): 3165-3174.
- Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T, Nishikawa S. 2005. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells [J]. *Nat Biotechnol*, **23**(12): 1542-1550.