Cyclopamine, an Antagonist of Hedgehog (Hh) Signaling Pathway, Reduces the Hatching Rate of Parthenogenetic Murine Embryos

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Abstract

Hedgehog (Hh) pathway plays a key role in development from invertebrate to vertebrate. It is known to be involved in cell differentiation, polarity, proliferation, including the development of vertebrate limb and the establishment of flies' body plan. To investigate how the regulation of Hh pathway affects the development of parthenogenetic murine embryos, the parthenogenetically activated murine embryos were treated with either cyclopamine (Cyc), an antagonist of Hh pathway, or purmorphamine, an agonist of Hh pathway. While Cyc did not affect the blastocyst formation and its total cell number, the chemical reduced the hatching rate of embryos and the expression levels of *Fnl* mRNA. The results of the present study show the possibility that Cyc may affect the development of embryos at blastocyst stage by blocking Hh pathway and this may cause detrimental effect to the embryos at peri-, and post-implantation stages.

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INTRODUCTION

Hedgehog (Hh) signaling pathway has been widely studied after the discovery of the Hedgehog gene (Nüsslein-Volhard and Wieschaus, 1980). There has been little major change during the evolutionary process, and Hh signaling pathway is highly conserved from flies to mice (Goodrich et al., 1996). Hh signaling pathway has three Hh ligands: desert hedgehog, indian hedgehog, and sonic hedgehog (Shh), of which Shh is the most actively studied and widely expressed ligand in mammals (Goodrich et al., 1996; Petrova and Joyner, 2014). Hh signaling pathway is mediated by the G-protein-coupled like seven-pass transmembrane receptor smoothened (SMO) and the twelve-pass transmembrane receptor patched1 (PTCH1). In the absence of Hh ligand, SMO activation is inhibited by PTCH1 and leads to inhibition of downstream signaling transduction cascades (Petrov et al., 2017). Consequently, transcription factors GLI proteins undergo proteolytic processing to make GLI repressor form (GLI-R) and move into the nucleus. GLI-R occupies promoters of Hh target genes, blocking transcription of Hh target genes (Gorojankina, 2016; Wu et al., 2017). On the other hand, in the presence of Hh ligand, Hh ligand binds to PTCH1, mitigating SMO inhibition of PTCH1. It causes GLI proteins to be transformed into GLI activator form (GLI-A) through downstream cascades. GLI-A moves into the nucleus and activates Hh target genes (Yao and Chuang, 2015; Wu et al., 2017).

Hh signaling pathway is closely involved in the development of various vertebrate embryos (Ingham and McMahon, 2001) and remains important in adults (Petrova and Joyner, 2014; Kugler et al., 2015). Although Hh signaling pathway has been widely studied in post-implantation stage embryos (Franco and Yao, 2012; Tickle, 2015; Xavier et al., 2016), only a few studies have been reported about pre-implantation stage embryos. In the previous studies, it was reported that *in vitro* development of blastocyst of parthenogenetic porcine embryos were improved by Shh supplementation and cyclopamine (Cyc), an antagonist of Hh signaling pathway, inhibited the improvement by Shh supplementation (Nguyen et al., 2009; 2010). In addition to the study about parthenogenetic porcine embryos, the addition of Shh to the culture medium had similar results to the studies of *in vitro*-fertilized (IVF) goat embryo (Wang et al., 2017), and Cyc decreased early development of IVF murine zygotes (Liu et al., 2014). However, there has been no report about the relationship between the regulation of Hh signaling pathway and parthenogenetic murine embryos. Therefore, the present study was conducted to

investigate the effects of Cyc or purmorphamine (Pur), an agonist of Hh signaling pathway, on parthenogenetic murine embryos.

MATERIALS AND METHODS

1. Animals and chemicals

All animal experiments were approved under the agreement guidelines of the Institutional Animal Care and Use Committee of Seoul National University (Approval number: SNU-130123-5-5). All organic and inorganic compounds used for this study were purchased from Sigma-Aldrich Korea unless indicated otherwise.

2. Oocytes collection

Six- to eight-week old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice were superovulated by injecting 10 IU of pregnant mare's serum gonadotropin (PMSG, Daesung Microbiology Lab, Korea) and 10 IU of human chorionic gonadotropin (hCG, Daesung Microbiology Lab) at 48 h after PMSG injection. The collection of oocytes was started at 14 h after hCG injection. The oviducts were obtained from mice killed by cervical dislocation and transferred to 2 ml tissue culture medium 199 (TCM) with Earl's salts (TCM-washing) medium containing 300 IU/ml hyaluronidase. The oocytes were poured out with cumulus cells, tearing the ampullae of the oviducts using sterile needles. After about 3 to 5 min of short incubation in the TCM-washing medium containing hyaluronidase, the oocytes were washed in Hepes-buffed Chatot, Ziomek, and Bavister (HCZB) medium to denude remaining cumulus cells.

3. Parthenogenetic activation and *in vitro* culture

The denuded oocytes were parthenogenetically activated in a calcium free-CZB medium containing 10 mM SrCl_2 and 5 $\mu\text{g/ml}$ cytochalasin B for 5 h. Then, the activated oocytes were washed in HCZB medium and transferred to three groups of culture medium as described in Table 1. The parthenogenetic embryos were treated either with 5 μM Cyc for 5.5 days or with 1 μM Pur for 5.5 days. The treatment concentration of Cyc (5 μM) (Batsaikhan et al., 2014) and Pur (1 μM) (Li et al., 2008) were determined by the previous studies.

4. Nuclear labeling and total cell number count

The parthenogenetic embryos, developed to the blastocyst

Table 1. The three different culture methods

Group	Cyc (5 μ M)	Pur (1 μ M)
Control	X	X
Cyc treatment	O	X
Pur treatment	X	O

Cyc; cyclopamine, Pur; purmorphamine

Table 2. Primer sequences used for qRT-PCR

Gene	Sequence	
Gapdh	Forward	5' - AGGTCGGTGTGAACGGATTTG - 3'
	Reverse	5' - TGTAGACCATGTAGTTGAGGTCA - 3'
Fn1	Forward	5' - GATGTCCGAACAGCTATTACCA - 3'
	Reverse	5' - CCTTGCGACTTCAGCCACT - 3'
Vim	Forward	5' - CGTCCACACGCACCTACAG - 3'
	Reverse	5' - GGGGGATGAGGAATAGAGGCT - 3'
Tgf- β	Forward	5' - CTCCCGTGGCTTCTAGTGC - 3'
	Reverse	5' - GCCTTAGTTTGGACAGGATCTG - 3'
Oct4	Forward	5' - CCGTGTGAGGTGGAGTCTGGAG - 3'
	Reverse	5' - GCGATGTGAGTGATCTGCTGTAGG - 3'
Sox2	Forward	5' - GCGGAGTGGAACCTTTTGTCC - 3'
	Reverse	5' - CGGGAAGCGTGTACTTATCCTT - 3'
Bcl2	Forward	5' - GGTGGTGGAGGAACCTCTCA - 3'
	Reverse	5' - ACCTACCCAGCCTCCGTTAT - 3'

stage, were stained with Hoechst 33342. The blastocysts samples for staining were mounted on the center of slides with Hoechst 33342-including solution. Stained samples were then observed on Eclipse TE 2000-U (Nikon, Japan) with a UV excitation filter. After taking pictures of stained embryos, the total cell number was identified by counting the cell number in the pictures.

5. RNA extraction and reverse transcription

Total RNA was extracted by using TRIzol™ Reagent (Thermo Fisher Scientific, USA) for reverse transcription. TRIzol™ Reagent was added to the collected embryos to homogenize samples. Following adding chloroform and inverting for 15 sec, the samples were incubated for 5 min. Samples were then centrifuged at 12,000 x g for 15 min at 4°C. The upper aqueous phase was transferred to a new microfuge tube. Isopropanol was added to precipitate RNA, followed by staying for 10 min at room temperature and centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was removed, and the RNA pellet

was washed twice with cold 70% ethanol (EtOH). Samples were centrifuged 12,000 x g for 15 min at 4°C, and the RNA pellet was resuspended with RNase-free water. Quality of the extracted RNA was assessed by UV absorbance at 260/280 nm using Nanodrop ND-2000 (Thermo Fisher Scientific). The samples were reverse-transcribed using M-MLV Reverse Transcriptase (Mbiotech, Korea) following the manufacturer's instruction.

6. Quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed in triplicate with TB Green™ Premix Ex Taq™ II (TaKaRa, Japan) using StepOnePlus (Applied Biosystems, USA). PCR process was initiated with the initial denaturation stage at 95°C for 30 sec, followed by 40 cycles of the PCR stage at 95°C for 5 sec and 60°C for 30 sec. D-glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) served as an endogenous control and was used for normalization of the differences in individual samples. The used primer sequences were presented in Table 2.

7. Statistical analysis

All experiments in the present study were statistically analyzed using a two-tailed Student's unpaired *t*-tests (for the comparison between two groups) or a one-way ANOVA (for the comparison between three groups) by Prism software 5.0 (GraphPad Software, USA). *p* < 0.05 was defined statistical significance.

RESULTS

The effects of solvents on parthenogenetic murine embryos.

Parthenogenetic embryos were treated with KSOM medium including 0.1% EtOH or 0.05% dimethyl sulfoxide (DMSO) to investigate chemical toxicity as controls. The concentration of solvents was determined because Cyc was dissolved at 1,000 X concentration in EtOH, and Pur was dissolved at 2,000 X concentration in

DMSO, respectively. Solvent-containing KSOM medium did not show any detrimental change of the morphology of the parthenogenetic embryos (Figure 1A and 1B). The developmental rates and the total cell numbers of the embryos cultured in solvent-containing KSOM medium did not differ significantly from those of KSOM medium without any solvent (Figure 1C).

The effect of Hh signaling pathway on the hatching rate and the total cell number in parthenogenetic murine embryos

The regulation of Hh using Cyc or Pur did not affect the morphology (Figure 2A and 2B) and the blastocyst formation (Figure 2C) of the embryos. However, the hatching rate of the embryos in the Cyc treatment group was significantly decreased. Although the blastocysts in the Pur treatment group had higher total cell numbers than those of other two groups, Pur did not affect the hatching rate and the morphology of the embryos.

Figure 1. The development of control groups after 5.5 days in culture. (A) The images obtained on bright field of the blastocysts. (B) The fluorescence images obtained after Hoechst 33342-staining. (C) The rate of blastocyst formation, hatching, and total cell numbers in the blastocyst. Scale bar = 50 μ m.

Figure 2. The development of the Hh signaling regulator-treated groups after 5.5 days in culture. (A) The images obtained on bright field of the blastocysts. (B) The fluorescence images obtained after Hoechst 33342-staining. (C) The rate of blastocyst formation, hatching, and total cell numbers in the blastocyst. * $p < 0.05$, *** $p < 0.001$. Scale bar = 50 μm .

Therefore, we focused on the effect of Cyc on the development of the embryos, while Pur was excluded in the subsequent experiments.

The change of gene expression levels in the Cyc-treated embryos

To investigate the change of gene expression levels in the Cyc-treated embryos, qRT-PCR was carried out. The mRNA expression levels of *Fibronectin 1* (*Fn1*) were significantly decreased in Cyc treatment group (Figure 3A). The expression levels of *Vimentin* (*Vim*) (Figure 3B), *Tgf- β* (Figure 3D), *Oct4* (Figure 3D), *Sox2* (Figure 3E), and *Bcl2* (Figure 3F) were not different significantly.

DISCUSSION

Based on the results of the present study and the previous report (Wakayama and Yanagimachi, 2001), the early embryonic development at preimplantation stage was not affected by small amount of EtOH and DMSO *in vitro*.

While there was a precedent study in which Cyc, an antagonist of Hh signaling pathway, decreased the early embryonic development of IVF murine zygote (Liu et al., 2014), the present study shows that the Cyc did not affect the blastocyst formation of parthenogenetic murine embryos. However, Cyc decreased the hatching rate of the embryos in the present study.

Mammalian embryos are encapsulated in the zona pellucida. It is important to hatch out the zona pellucida for implantation and the following development of the embryo (Leonavicius et

Figure 3. Changes of the mRNA expression levels in the hatching-related genes; (A) *Fibronectin1* (*Fn1*), (B) *Vimentin* (*Vim*), (C) *Tgf-β*, (D) *Oct4*, (E) *Sox2*, and (F) *Bcl2*. ** $p < 0.01$.

al., 2018). In addition, the total cell number is a measurement to judge the health and viability of the preimplantation embryo (Newmark et al., 2007). Although the total cell number of the blastocysts in the present study did not differ from control group, the hatching rate was significantly lower in Cyc treatment group.

In developing murine embryos, *Fn1*, the marker of cell migration and differentiation, begins to be expressed in the inner cell mass (Wartiiovaara et al., 1979) and is mainly expressed on the basal and apical surface of the cells in the trophectoderm at late stage blastocysts. Then, *Fn1* expressed on trophectoderm interacts with uterine epithelial cells during implantation (Green et al., 2015). *Vim* and *Tgf-β*, the epithelial-mesenchymal transition (EMT)-related genes, are increased in implantation stage (Kumar et al., 2015; Bai et al., 2018). *Oct4* and *Sox2* are the markers of undifferentiated cell and expressed in inner cell mass of mammalian embryos. Decreased expression of these two genes means the loss of pluripotency (Liu et al., 2015). *Bcl2*, the anti-apoptotic gene, inhibits *Bax*, the pro-apoptotic gene (Murphy et al., 2000). Although the changes of the gene expression levels were not significant except for *Fn1*, additional experiments are needed to the role of Cyc on apoptosis and pluripotency of the embryos. The decrease of *Fn1* expression levels by Cyc might counteract hatching of the parthenogenetic murine blastocyst.

Taken together, the results of the present study show the

possibility that Cyc may affect the development of embryos at blastocyst stage by blocking Hh pathway and this may cause detrimental effect to the embryos at peri-, and post-implantation stages.

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REFERENCES

- Batsaikhan BE, Yoshikawa K, Kurita N, Iwata T, Takasu C, Kashiwara H, Shimada M. 2014. Cyclopamine decreased the expression of Sonic Hedgehog and its downstream genes in colon cancer stem cells. *Anticancer Res.* 34:6339-44.
- Bai R, Kusama K, Nakamura K, Sakurai T, Kimura K, Ideta A, Aoyagi Y, Imakawa K. 2018. Down-regulation of transcription factor OVOL2 contributes to epithelial-mesenchymal transition in a noninvasive type of trophoblast implantation to the maternal endometrium. *FASEB J.* 32:3371-3384.

- Franco HL, Yao HH. 2012. Sex and hedgehog: roles of genes in the hedgehog signaling pathway in mammalian sexual differentiation. *Chromosome Res.* 20:247-258.
- Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP. 1996. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* 10:301-312.
- Gorojankina T. 2016. Hedgehog signaling pathway: a novel model and molecular mechanisms of signal transduction. *Cell Mol Life Sci.* 73:1317-1332.
- Green CJ, Fraser ST, Day ML. 2015. Insulin-like growth factor 1 increases apical fibronectin in blastocysts to increase blastocyst attachment to endometrial epithelial cells *in vitro*. *Hum Reprod.* 30:284-298.
- Ingham PW, McMahon AP. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* 15:3059-3087.
- Kugler MC, Joyner AL, Loomis CA, Munger JS. 2015. Sonic hedgehog signaling in the lung. From development to disease. *Am J Respir Cell Mol Biol.* 52:1-13.
- Kumar V, Maurya VK, Joshi A, Meeran SM, Jha RK. 2015. Integrin beta 8 (ITGB8) regulates embryo implantation potentially via controlling the activity of TGF-B1 in mice. *Biol Reprod.* 92:109.
- Leonavicius K, Royer C, Preece C, Davies B, Biggins JS, Srinivas S. 2018. Mechanics of mouse blastocyst hatching revealed by a hydrogel-based microdeformation assay. *Proc Natl Acad Sci U S A.* 115:10375-10380.
- Li XJ, Hu BY, Jones SA, Zhang YS, Lavaute T, Du ZW, Zhang SC. 2008. Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells.* 26:886-93.
- Liu S, Bou G, Sun R, Guo S, Xue B, Wei R, Cooney AJ, Liu Z. 2015. Sox2 is the faithful marker for pluripotency in pig: evidence from embryonic studies. *Dev Dyn.* 244:619-627.
- Liu Y, Wei Z, Huang Y, Bai C, Zan L, Li G. 2014. Cyclopamine did not affect mouse oocyte maturation *in vitro* but decreased early embryonic development. *Anim Sci J.* 85:840-7.
- Migone FF, Ren Y, Cowan RG, Harman RM, Nikitin AY, Quirk SM. 2012. Dominant activation of the hedgehog signaling pathway alters development of the female reproductive tract. *Genesis.* 50:28-40.
- Murphy KM, Ranganathan V, Farnsworth ML, Kavallaris M, Lock RB. 2000. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death Differ.* 7:102-11.
- Newmark JA, Warger WC 2nd, Chang C, Herrera GE, Brooks DH, DiMarzio CA, Warner CM. 2007. Determination of the number of cells in preimplantation embryos by using noninvasive optical quadrature microscopy in conjunction with differential interference contrast microscopy. *Microsc Microanal.* 13:118-127.
- Nguyen NT, Lin DP, Yen SY, Tseng JK, Chuang JF, Chen BY, Lin TA, Chang HH, Ju JC. 2009. Sonic hedgehog promotes porcine oocyte maturation and early embryo development. *Reprod Fertil Dev.* 21:805-815.
- Nguyen NT, Lin DP, Siriboon C, Lo NW, Ju JC. 2010. Sonic Hedgehog improves *in vitro* development of porcine parthenotes and handmade cloned embryos. *Theriogenology* 74:1149-1160.
- Nüsslein-Volhard C, Wieschaus E. 1980. Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287:795-801.
- Petrov K, Wierbowski BM, Salic A. 2017. Sending and receiving hedgehog signals. *Annu Rev Cell Dev Biol.* 33:145-168.
- Petrova R, Joyner AL. 2014. Roles for Hedgehog signaling in adult organ homeostasis and repair. *Development* 141:3445-3457.
- Russell MC, Cowan RG, Harman RM, Walker AL, Quirk SM. 2007. The hedgehog signaling pathway in the mouse ovary. *Biol Reprod.* 77:226-236.
- Tickle C. 2015. How the embryo makes a limb: determination, polarity and identity. *J Anat.* 227:418-430.
- Wakayama T, Yanagimachi R. 2001. Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. *Reproduction* 122:49-60.
- Wang DC, Huang JC, Lo NW, Chen LR, Merrilod P, Ma WL, Chiang HI, Ju JC. 2017. Sonic Hedgehog promotes *in vitro* oocyte maturation and term development of embryos in Taiwan native goats. *Theriogenology* 103:52-58.
- Wartiovaara J, Leivo I, Vaheri A. 1979. Expression of the cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. *Dev Biol.* 69:247-257.
- Wu F, Zhang Y, Sun B, McMahon AP, Wang Y. 2017. Hedgehog signaling: From basic biology to cancer therapy.. *Cell Chem Biol.* 24:252-280.
- Xavier GM, Seppala M, Barrell W, Birjandi AA, Geoghegan F, Cobourne MT. 2016. Hedgehog receptor function during craniofacial development. *Dev Biol.* 415:198-215.
- Yao E, Chuang PT. 2015. Hedgehog signaling: From basic research to clinical applications. *J Formos Med Assoc.* 114:569-576.