EFFECTS OF LEUKEMIA INHIBITORY FACTOR ON TRANSCRIPT EXPRESSION OF PLURIPOTENT GENES IN BOVINE EMBRYONIC STEM LIKE CELLS

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ABSTRACT: Leukemia inhibitory factor (Lif) is very important for embryonic stem cell establishment. In this study, we investigated effects of Lif on transcript expression of pluripotent genes of bovine embryonic stem like cells in passage 1, passage 3 and passage 6. The results showed that Lif supplementation of medium could improve transcript expression of pluripotent gene including *nanog, oct4, sox2* and *c-myc*. Three Lif concentrations were applied for cell culture medium. We found that pluripotent gene transcripts were highest expressed in 10^3 IU/ml Lif supplemented medium, especially *nanog* and *oct4* transcript expression could be maintained until 6^{th} passage . The transcript expression was decreased in 10^4 IU/ml Lif suppmented medium, suggesting that high concentration of Lif could inhibit pluripotent gene expression. Thus, 10^3 IU/ml Lif was the most efficient concentration to improve transcript expression of pluripotent genes. There was a relationship in *nanog* expression and *c-myc* and *sox2* expression. *C-myc* and *sox2* expression were up-regulated when *nanog* was down-regulated. We also accessed *nanog* or *oct4* which were key factors for the maintenace of pluripotency and renewal of bovine embryonic stem like cell. The result demonstrated that nanog could be the key factor for pluripotency of bovine embryonic stem like cells.

Keywords: bovine embryonic stem cells, leukemia inhibitory factor (Lif), pluripotent genes, rt-pcr, transcript expression.

INTRODUCTION

Embryonic stem cells are pluripotent cells which could be derived from embryos at blastocyst stage. They have two characteristics including pluripotency and renewal. They can differentiate into more than 200 cell types in our body [3]. Embryonic stem cells express specific markers or characteristics, such as stage specific embryonic antigens, enzymatic activities including alkaline phosphatase and telomerase and pluripotent genes including *oct4, nanog, sox2, c-myc* [2]. Embryonic stem cells can differentiate *in vivo* and *in vitro* in teratomas into cells representing the three major germ layers: endoderm, mesoderm or ectoderm [8]. These pluripotent genes profile and pluripotency have been clearly characterized in mouse, human and primate embryonic stem cells. However, these characteristics have been unclear in ungulates, especially bovine. Bovine embryonic stem cell lines have not been stably established because there was no medium being suitable to maintain pluripotency and renewal of these cells [6]. One of the most important factors to maintain embryonic stem cell line is leukemia inhibitory factor. Leukemia inhibitory factor can maintain pluripotent gene expression such as *oct4, nanog, sox2, c-myc* to maintain pluripotency of embryonic stem cells. Thus, in this study, we aim to access effect of leukemia inhibitory factor on transcript expression of pluripotent genes in bovine embryonic stem like cells.

MATERIALS AND METHODS

Blastocyst production

Bovine ovaries were collected from slaughterhouse then transported to laboratory. Aspiration method was applied to collect cumulus oocyte complexes (COCs). COCs were cultured in TCM199 supplemented with 10% fetal bovine serum, 0.1 IU LF, 0.1IU FSH and 1% pen/strep in 22-24 hours. Mature oocytes were fertilized in Fert-TALP medium with sperm in 6-8 hours [5]. Sperm density was 10^6 sperm/ml. Fertilized oocytes were culture in Soffa medium [7].

Feeder layer preparation

Pregnant mouse at 12.5E was used to isolated mouse embryonic fibroblast. Fetus were collected and washed in phosphate buffer solution to discard blood. Head, arms, legs and internal organs were removed. The remained tissues were washed in phosphate buffer solution. These tissues were minced and trypsinized in trypsin-EDTA 0.25 medium to produce single cells. The single cell suspension was transferred to φ35 mm dishes containing 2 ml DMEM supplemented 10% fetal bovine serum, 1% Pen/Strep. To produce feeder layer, mouse embryonic fibroblast was incubated in 10 µg/ml mitomycin C supplemented medium in 2.5 hours. The mouse embryonic fibroblast was washed twice in DMEM supplemented with 10% fetal bovine serum, 1% Pen/Strep.

Embryonic stem like cell culture

Blastocyst embryos were denuded using 5 µg/ml pronase in 2 min and transferred into mouse embryonic fibroblast inactivated by mytomycin C 10 µg/ml. Embryonic stem like colonies were picked up and trypsinized in 0.25% trypsin-EDTA medium, then single cells were transferred into new mouse embryonic fibroblast inactivated by mytomycin C 10µg/ml. Bovine embryonic stem like cells were used to access colonic morphology, proLiferation and transcript expression of pluripotent genes.

RT-PCR and semi-quantitative analysis

Total mRNA was isolated using Trizol. RT-PCR was applied to amplify pluripotent genes including *nanog* (Forward primer: GTGTTTGGTGAACTCTCCTG, Reverse primer: GGGAATTGAAATACTTGACAG), *oct4* (Forward primer: GTTCTCTTTGGAAA GGTGTTC, Reverse primer: ACACTCGGACC ACGTCTTTC), *c-myc* (Forward primer: CGCG GTCGCCTCCTTCTCGCCCAGG, Reverse primer: GTCCGGGGAAGCGCAGGGC), *sox2* (Forward primer: CATCCACAGCAAATGAC AGC, Reverse primer: TTTCTGCAAAGCTCC TACCG), and *beta actin* (Forward primer: GG AATCCTGTGGCATCCATGAAAC, Reverse primer: AAAACGCAGCTCAGTAACAGTC CG). The RT reaction was performed in 42^{o}C in 45 min. The PCR reaction included an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 45 s, and extension at 72°C for 75 s, followed by final extension at 72°C for 5 min. Amplicons were separated through a 1% agarose gel at 100 V for 1 h. Image J software was applied to analyze one-dimensional electrophoretic gels [1].

RESULTS AND DISCUSSION

Effect of Lif on transcript expression of pluripotent genes in passage 1

Bovine embryonic stem like cells in passage 1 expressed transcript of pluripotent genes in culture medium with and without Lif (figure 1). The *nanog* expression in control group was lower than Lif supplemented groups. Embryonic stem like cells in 10^3 IU/ml Lif supplemented medium expressed *nanog* transcripts higher in 10^2 and 10^4 IU/ml Lif supplemented media. There were no differences in *oct4* transcript expression in control group and group in 10^2 and 10^4 IU/ml Lif supplemented media. However, *oct4* transcript expression was highest in embryonic stem like cells cultured in medium with 10^3 IU/ml Lif. In control group, *c-myc* transcript expression was lower than that in 10^2 and 10^4 IU/ml Lif supplemented groups. The *c-myc* transcript was highest expressed in bovine embryonic stem like cells cultured in 10^3 IU/ml Lif supplemented medium. Sox-2 transcript expression in bovine embryonic stem like cells culture in 10^3 and 10^4 IU/ml Lif supplemented medium were higher than in control and 10^2 IU/ml Lif supplemented medium.

Effect of Lif on transcript expression of pluripotent genes in passage 3

In passage 3, the transcript expression in almost bovine embryonic stem like cells groups were decreased (figure 2). The *nanog* transcript expression in control group and 10^2 and 10^4 IU/ml Lif group were lower than that in 10^3 IU/ml Lif group. There were no differences in *nanog* transcript expression between groups in passage 3 and passage 1. The bovine embryonic stem like cells cultured in 10^3 IU/ml Lif supplemented medium highest expressed *nanog*

transcript. The *oct4* transcript expression almost decreased in control group and 10^2 and 10^4 IU/ml Lif supplemented group and were lower than bovine embryonic stem like cells in passage 1. However, the *oct4* transcript was high expressed in 10^3 IU/ml Lif supplemented group. The *c-myc* and *sox-2* transcript expression of bovine embryonic stem like cells in passage 3 were lower than passage 1 but still high in 10^3 IU/ml Lif supplemented group.

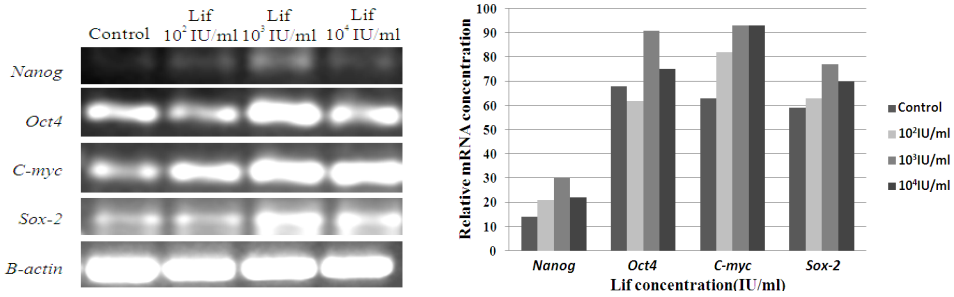

Figure 1. Effect of Lif on transcript expression of pluripotent genes of bovine embryonic stem like cellss in passage 1

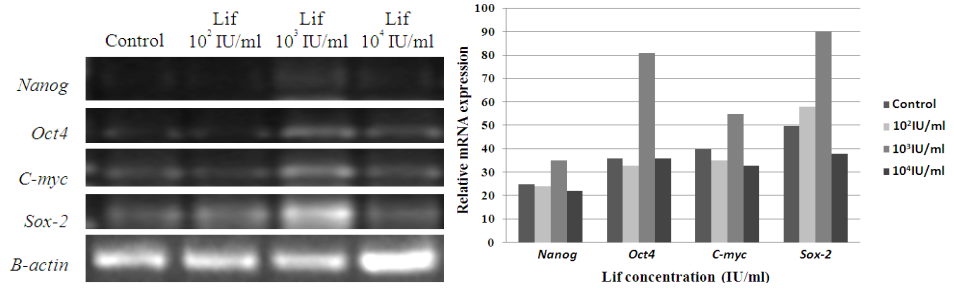

Figure 2. Effect of Lif on transcript expression of pluripotent genes of bovine embryonic stem like cellss in passage 3

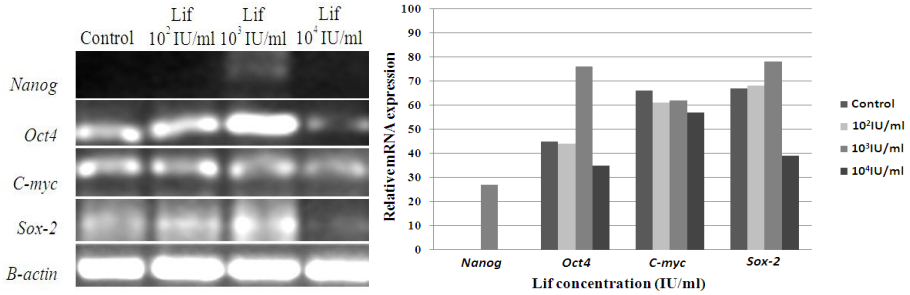

Figure 3. Effect of Lif on transcript expression of pluripotent genes of bovine embryonic stem like cellss in passage 6.

Effect of Lif on transcript expression of pluripotent genes in passage 6

The *nanog* transcript expression was observed only in bovine embryonic stem like cells cultured in 10^3 IU/ml Lif supplemented medium. There was no *nanog* transcript expression in the other groups. In passage 6, the *oct4* transcript expression in control and 10^2 IU/ml Lif supplemented groups were higher than those in 10^4 IU/ml Lif supplemented group. The *c-myc* and *sox-2* transcript expression of bovine embryonic stem like cells in passage 6 were increased higher than in passage 3. The control group and Lif supplemented groups have the same *c-myc* transcript expression. The

sox-2 transcript was low expressed in bovine embryonic stem like cells cultured in 10^4 IU/ml Lif supplemented medium.

Discussion

Medium has played very important role in embryonic stem cell establishment, especially bovine embryonic stem cell. Embryonic stem cell medium could be modified based on animal. One of the most important factors in culture medium is leukemia inhibitory factor. Lif maintains pluripotent characteristics of mouse embryonic stem cell [12], but differentiated factor in human embryonic stem cells [10]. In bovine embryonic stem cell establishment, role of Lif was still unclear. Preliminary studies showed that Lif could maintain bovine embryonic stem cells [3], but another studies demonstrated that Lif did not improve the establishment of bovine embryonic stem cell lines [11]. Thus, it is very important to detect the role of Lif.

In this study, we found that Lif clearly effected on transcript expression of pluripotent genes in bovine embryonic stem like cells. The *nanog*, *oct4*, *c-myc* and *sox-2* transcripts were highest expressed in bovine embryonic stem like cells cultured in 10^3 IU/ml Lif supplemented medium. Bovine embryonic stem cell in control group had lower transcript expression than Lif supplemented groups. In the other hand, the transcript expression of pluripotent genes was suppressed in medium supplemented with high concentration of Lif (10^4 IU/ml). Thus, high concentration is not suitable for Lif to improve pluripotent gene expression in passage 1. Almost plutipotent genes had low transcript expression in passage 3, suggesting that cell passage could effect on pluripotent gene expression and begin induce the degeneration of bovine embryonic stem like cells. However, 10^3 IU/ml Lif supplemented medium maintained high expression of *nanog*, *oct4*, *sox2* and *c-myc* in bovine embryonic stem like cells in passage 3. In passage 6, only 10^3 IU/ml Lif supplemented medium could maintain *nanog* transcript expression, in other groups *nanog* transcript could not observed. The transcript of *oct4*, *sox2* and *c-myc* were still highly expressed in medium supplemented with 10^3 IU/ml Lif. In this passage, the results also showed that *nanog* transcript of bovine embryonic stem like cells in control and 10^2 and 10^3 IU/ml Lif supplemented groups was not expressed, but *c-myc* and *sox2* transcript expression was increased and was higher than those in passage 3. It suggested that there was a relationship in *nanog* expression and *sox2* and *c-myc* expression.

Nanog and *oct4* are very important to maintain pluripotency and renewal in embryonic stem cell. *Oct4* is key factor to maintain pluripotent characteristics of mouse, rat, human and primate embryonic stem cell lines, but different from ungulates such as porcine, in which *nanog* is a key factor to pluripotency (Talbot, 2007). The key factor which maintains pluripotency of bovine embryonic stem like cells has been unclear. In passage 6, when the degenerated bovine embryonic stem like cells did not express *nanog* transcript in control group and 10^2 and 10^3 IU/ml Lif supplement groups, but the 10^3 IU/ml Lif supplemented medium could maintain *nanog* transcript. Whereas, *oct4* transcript was highly expressed in passage 1, 3, and 6, eventhough bovine embryonic stem like cells degenerated. The previous studies showed that *nanog* was a key factor for pluripotency of bovine induced pluripotent stem cell, and *nanog* improved pluripotent characteristics of bovine induced pluripotent stem cell [9]. It suggested that *nanog* could be a key factor for pluripotency of bovine embryonic stem like cells.

CONCLUSION

Leukemia inhibitory factor improved transcript expression of pluripotent genes in bovine embryonic stem like cells.

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ẢNH HƯỞNG CỦA NHÂN TỐ ỨC CHẾ BỆNH BẠCH CẦU LÊN SỰ BIỂU HIỆN CỦA MỘT SỐ GENE ĐA TIỀM NĂNG CỦA TẾ BÀO GỐC PHÔI BÒ

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TÓM TẮT

Nhấn tố ức chế bệnh bạch cầu (Lif) đóng vai trò rất quan trọng trong việc thiết lập các dòng tế bào gốc phôi, trong nghiên cứu này, chúng tôi khảo sát các tác động của Lif lên sự biểu hiện của các gene đa tiềm năng của tế bào gốc phôi bò ở mức phiên mã qua các thế hệ thứ 1, 3 và 6. Kết quả cho thấy, việc bổ sung Lif vào môi trường nuôi cấy tế bào có thể giúp tăng cường sự biểu hiện của các gene đa tiềm năng như *nanog, oct4, sox2* và *c-myc* ở mức phiên mã. Ba nồng độ Lif đã được khảo sát trong nghiên cứu này cho thấy rằng các sản phẩm phiên mã của gene đa tiềm năng được biểu hiện cao nhất ở môi trường bổ sung Lif có nồng độ 10^3 IU/ml. Đặc biệt là sự biểu hiện sản phẩm phiên mã của gene *nanog* và *oct4* có thể được duy trì tới lần cấy chuyền thứ 6. Sự biểu hiện sản phẩm phiên mã bị giảm ở môi trường nuôi cấy bổ sung 10^4 IU/ml Lif, điều đó cho thấy nồng độ Lif cao có thể ức chế sự biểu hiện của gene đa tiềm năng. Do đó, môi trường bổ sung Lif với nồng độ 10^3 IU/ml hiệu quả nhất cho việc tăng cường sự biểu hiện của các gene đa tiềm năng. Trong nghiên cứu này, chúng tôi nhận thấy, sự biểu hiện của gene *nanog* giảm thì quá trình biểu hiện của các gene *c-myc* và *sox-2* tăng. Kết quả đó chứng tỏ có mối quan hệ trong sự biểu hiện của gene *nanog* và *c-myc*, *sox-2*.

Từ khóa: gene đa tiềm năng, nhân tố ức chế bệnh bạch cầu, rt-pcr, sự biểu hiện phiên mã, tế bào gốc phôi bò.
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