Overexpression of bone morphogenetic protein 4 in STO fibroblast feeder cells represses the proliferation of mouse embryonic stem cells *in vitro*

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Abbreviations: BMP, bone morphogenetic protein; ESC, embryonic stem cell; rAd-dnIκB, recombinant adenovirus-dominant negative IκB

Abstract

Embryonic stem cells (ESCs) can be propagated *in vitro* on feeder layers of mouse STO fibroblast cells. The STO cells secrete several cytokines that are essential for ESCs to maintain their undifferentiated state. In this study, we found significant growth inhibition of mouse ESCs (mESCs) cultured on STO cells infected with adenovirus containing a dominant-negative mutant form of IκB (rAd-dnIκB). This blockage of the NF-κB signal pathway in STO cells led to a significant decrease in [^{3}H]thymidine incorporation and colony formation of mESCs. Expression profile of cytokines secreted from the STO cells revealed an increase in the bone morphogenetic protein4 (BMP4) transcript level in the STO cells infected with adenoviral vector encoding dominant negative IκB (rAd-dnIκB). These results suggested that the NF-κB signaling pathway represses expression of BMP4 in STO feeder cells. Conditioned medium from the rAd-dnIκB-infected STO cells also significantly reduced the colony size of mESCs. Addition of BMP4 prevented colony formation of mESCs cultured in the conditioned medium. Our finding suggested that an excess of BMP4 in the conditioned medium also inhibits proliferation of mESCs.

Keywords: bone morphogenetic protein 4; culture media, conditioned; embryonic stem cells; feeder cells; NF-κB

Introduction

Several growth factors that are required for self-renewal of embryonic stem cells (ESCs) are usually provided either exogenously or by feeder cells (Smith, 2001; Ahn *et al*., 2010). Different embryonic fibroblast cell types have been tested as feeder cells for culture of undifferentiated ESCs. These cells secrete several cytokines that are essential for maintaining mouse ESCs (mESCs) in an undifferentiated state. STO, a transformed mouse fibroblast line, is commonly used as feeder cells to support mESC growth (Smith *et al*., 1988). mESCs depend on bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) to maintain their pluripotent status (Brons *et al*., 2007). Conditioned medium from STO cell culture has been studied as an economical alternative to these cytokines commonly used in mESC growth media.

BMPs are members of the transforming growth factor beta (TGF-β) family and have been implicated in embryonic development, including bone formation and repair, organogenesis, pattern formation in the early embryo, and epithelial-mesenchymal interactions (Jones *et al*., 1991; Vainio *et al*., 1993; Fainsod *et al*., 1994; De Robertis and Sasai, 1996). BMP4 is required at a very early step as an important regulator of the growth of hematopoietic stem cells (HSC), participating in the control of their proliferation, expansion and differentiation (Pearson *et al*., 2008). The major effect of BMP4 on the self-renewal of

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ESCs is accomplished by means of the inhibition of both extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways (Qi *et al.*, 2004). Human BMP4 and hematopoietic cytokines have been shown to modulate the proliferative and differentiative potentials of definitive fetal, neonatal, and adult hematopoietic progenitors (Bhatia *et al.*, 1997, 1999; Bhardwaj *et al.*, 2001; Murdoch *et al.*, 2002). ESCs cultured on embryonic feeder cells can be induced into trophoblastic differentiation by collagen IV or BMP4 (Xu *et al.*, 2002; Schenke-Layland *et al.*, 2007). Early exposure to BMP4 inhibits the neurogenic differentiation of ESCs, whereas later exposure causes induction of peripheral neuronal differentiation (Schulz *et al.*, 2004). These studies indicate that BMP4 is required for self-renewal of ESCs or for differentiation into a specific lineage if they are provided with the correct cues. BMP4 regulation is complex and precisely controlled by various transcription factors and inter- and intra- cellular signaling pathways. A recent report showed that TNF-α represses transcription of human BMP4 in lung epithelial cells through the nuclear factor κB (NF-κB) signaling pathway (Zhu *et al.*, 2007). Sequence analysis has suggested that the 5' upstream region of BMP4 contains a putative NF-κB binding site. However, few functional results are currently available regarding the regulation of BMP4 gene expression in any organ.

Here we blocked the NF-κB signaling pathway in STO feeder cells by infection with recombinant adenovirus encoding a dominant-negative mutant of IκB (rAd-dnIκB). As shown in lung epithelial cells (Zhu *et al.*, 2007), this blockade induced high expression of BMP4 in the STO cells and consequently resulted in a decreased survival of mouse ESCs in co-cultures. The addition of exogenous BMP4 to ES culture medium also inhibited colony formation from mESCs. These findings suggest that the regulated expression of BMP4 in feeder cells is important for self-renewal of mESCs *in vitro*.

Results

Inhibition of the NF-κB signaling pathway in STO cells decreases proliferation of mESCs

To investigate the cellular and molecular consequences of IκB in STO cells, we constructed an adenovirus plasmid expressing a dominant-negative mutant of IκB (rAd-dnIκB) that cannot be phosphorylated and thus abrogates the NF-κB signaling pathway and a negative control plasmid expressing *EGFP* (rAd-GFP). We infected STO feeder cells by incubation with rAd-dnIκB virus at a multiplicity of infection (MOI) values of 3, 5, and 10 and then used these feeder cells for culture of mESCs. Infection of STO cells by the rAd-dnIκB reduced the number and colony size of co-cultured mES cells as MOI increased, while the number and colony size of control STO cells infected by rAd-GFP was unaffected (Figure 1). These data suggest that inhibition of the NF-κB signaling pathway in STO cells prevents growth of mESCs. Using reverse transcription (RT)-PCR analysis with rAd-dnIκB specific primers, we confirmed that the reduced proliferation was caused by STO cells but not by mESCs themselves (Supplemental Data Figure S1).

To investigate whether inhibition of the NF-κB pathway in STO cells affected proliferation of mESCs, we performed a ^{3}H-thymidine incorporation assay. The STO cells expressing dnIκB markedly inhibited cellular proliferation of mESCs at 24 h, 48 h, and 72 h (Figure 2A). These data indicated that the

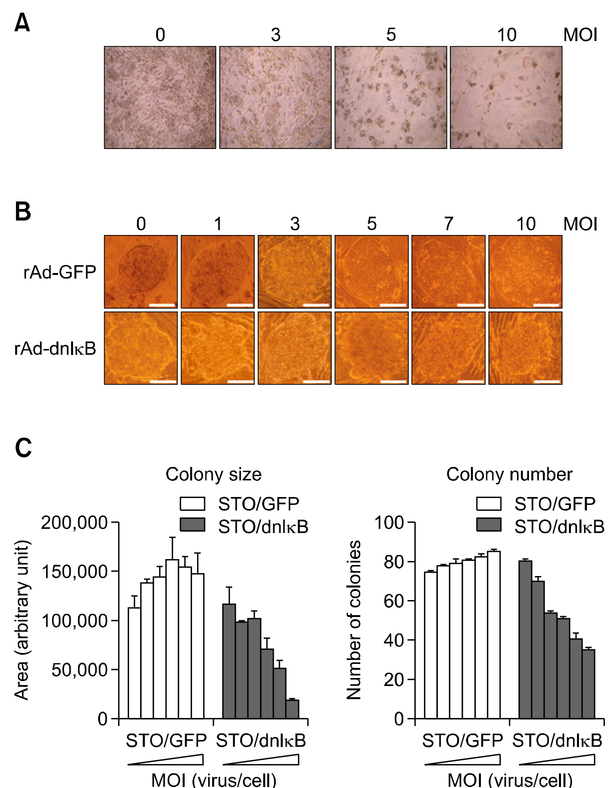

Figure 1. Inhibition of NF-κB signaling in STO feeder cells by infection with rAd-dnIκB prevents colony formation of mESCs. Photographs of representative colonies were taken on day 4 of treatment. (A) Morphological changes of mESCs cultured on STO cells infected by rAd-dnIκB at the indicated MOI. (B) The morphology of mESC colonies cultured on STO feeder cells. (C) The number and size of colonies of mESCs cultured on STO cells infected by rAd-dnIkB. The colony number was counted under a microscope, and the colony size was determined by measuring the colony area with the Image J program (NIH). All assays were performed in duplicate (scale bar, 50 μm).

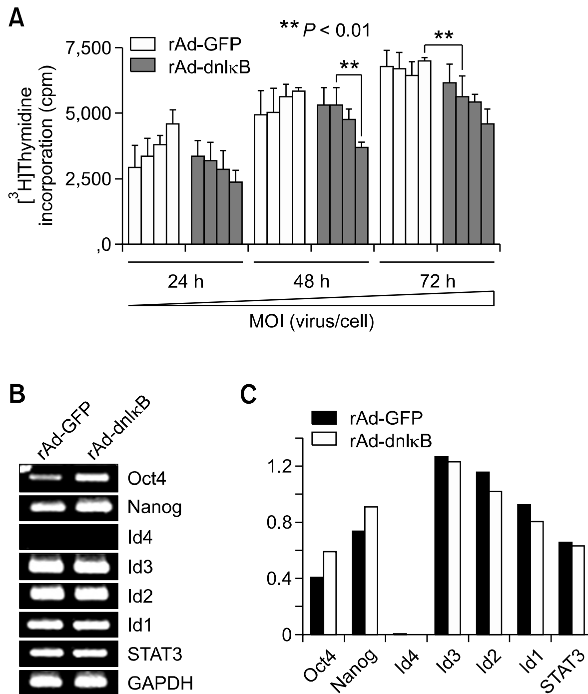

Figure 2. Proliferation of mES cells was inhibited by the STO cells infected with rAd-dnIκB. (A) The mESCs were cultured on STO cells as indicated, and proliferation of mESCs was measured by [^{3}H] thymidine incorporation assay. (B) RT-PCR analysis of the ESCs marker genes. GAPDH mRNA was measured as a control. (C) The relative density of gene expression was determined by dividing the density of each gene by that of GAPDH. **A statistically significant difference ($P < 0.01$) compared to the rAd-GFP control.

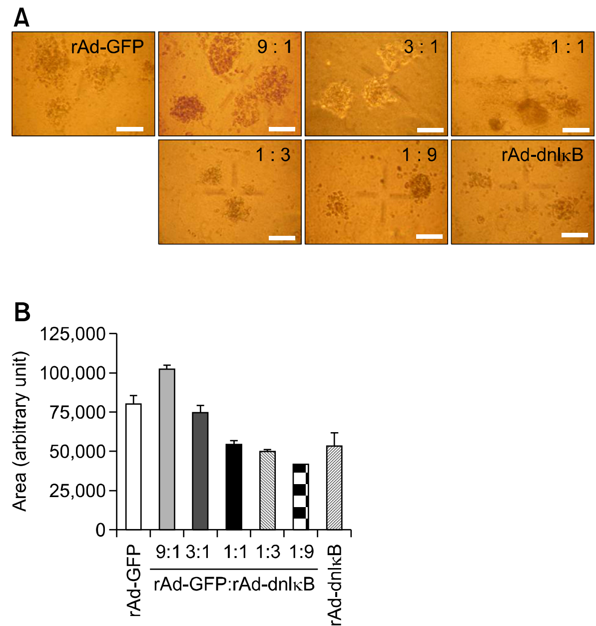

Figure 3. Effect of the conditioned media from STO cells infected with rAd-dnIκB. Conditioned medium from STO cells infected by rAd-C was mixed with that of STO cells infected by rAd-GFP at the indicated ratio (rAd-GFP only, 9:1, 3:1, 1:1, 1:3, 1:9, and rAd-dnIκB only). ESCs were cultured in the conditioned medium for three days. (A) Morphology of representative colonies and (B) comparison of colony sizes of mESCs. The colony size (> 10 colonies/sample) was determined by measuring the colony area with the Image J program (NIH). All assays were performed in duplicate (scale bar, 25 μm).

NF-κB signaling pathway was essential for STO cells to function as feeder cells in inducing self-renewal of mES cells.

We analyzed transcription of proliferation-related genes of ESCs when these cells were co-cultured with the STO cells infected by rAd-dnIκB. Figure 2B showed that mRNA levels of *Oct* 4 and *Nanog* were increased in the ESCs, whereas the expression levels of *Id* genes were unchanged.

Conditioned medium from the STO cells infected by rAd-dnIκB reduces the colony size of mESCs

To investigate whether inhibition of the NFκB signaling pathway destroyed the characteristic of STO cells to secrete ESC proliferation-stimulating factors, we analyzed colony formation of mESCs after culture with the conditioned medium collected from the rAd-dnIκB-infected STO cell cultures (Figure 3). The colony size decreased significantly as conditioned medium from rAd-dnIκB-infected STO cells was increased but remained unchanged in control medium from rAd-GFP-infected STO cells. This suggests that STO cells secrete a factor which stimulates proliferation of mESCs mediated by the NFκB signaling pathway.

Inhibition of the NFκB signal transduction pathway increases BMP4 expression in STO feeder cells

To identify factor which changed upon inhibition of the NFκB signaling pathway in STO cells, we analyzed the mRNA levels of BMP4, LIF, and Wnts genes because they are known as critical factors for maintaining the characteristics of mESCs (Hao *et al*., 2006). RT-PCR analysis detected higher expression of BMP4 in the STO cells infected by rAd-dnIκB, whereas mRNA levels of LIF and Wnts genes were not significantly different (Figure 4A). Real-time PCR confirmed level of BMP4 mRNA was increased in the rAd-dnIκB-infected STO cells (Figure 4B).

These data suggest that the enhanced level of BMP4 in STO feeder cells might inhibit the proliferation of mESCs.

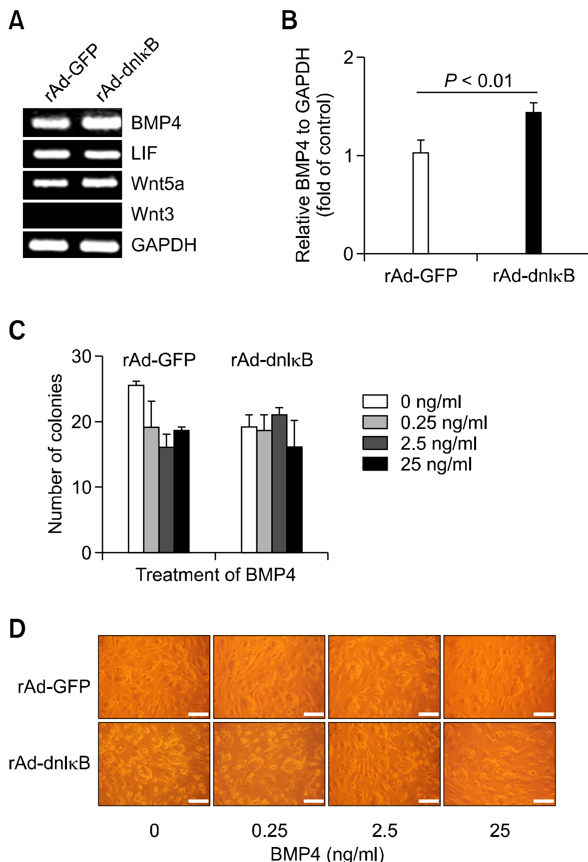

Figure 4. Overexpression of Bmp4 in STO cells infected with rAd-dnIκB inhibits mESC proliferation. (A) RT-PCR analysis of the STO feeder cells infected by rAd-GFP and rAd-dnIκB. The rAd-dnIκB infection induced overexpression of Bmp4 gene in the STO cells. GAPDH mRNA was measured as a control. (B) The relative density of genes expression was determined by dividing the density of each gene by that of GAPDH. The level of BMP4 mRNA was analyzed by using real time PCR. Results are representative of three independent experiments. (C) The number of colonies of mESCs was counted after culturing in the conditioned medium produced from STO cells plus exogenous BMP4. Addition of BMP4 resulted in a decrease in the number of colonies produced from the conditioned medium of rAd-GFP medium compared to that of rAd-dnIκB medium. (D) Morphology of mESC colonies cultured in the conditioned medium plus exogenous BMP4.

Over-production of BMP4 in STO feeder cells leads to decreased mESC proliferation

To confirm whether over-production of BMP-4 led to inhibition of mESC proliferation, we added recombinant BMP4 to the mESC culture medium. Addition of exogenous BMP4 (2.5-25 ng/ml) reduced the colony number of mESCs co-cultured with rAd-GFP-infected STO feeder cells ($P < 0.01$) to a similar level as in non-treated mESCs co-cultured with the rAd-dnIκB-infected STO cells (Figures 4C and 4D). These results indicate that BMP4 is required in appropriate amounts to maintain mESC self-renewal.

Discussion

Mammalian feeder cells continue to be broadly accepted as the method for maintaining ESC culture because these cells seem to produce some unidentified factor that makes them very effective. Extensive cell proliferation must be carefully addressed in stem cell research before theoretical possibilities of stem cells are translated into clinical applications (Park *et al.*, 2008). One active area of current stem cell research includes identification of the molecular processes underlying the uniform maintenance of proliferation, the undifferentiated state and pluripotency. Defining some of the signals important in the self-renewal of human ESCs is necessary to eliminate the need to include animal-derived materials. BMP4 has been identified as a good candidate for extensive *ex vivo* production of hESCs for therapeutic applications (Bhatia *et al.*, 1999; Hollnagel *et al.*, 1999; Ying *et al.*, 2003; Vicente Lopez *et al.*, 2011). Suppression of BMP signaling has been beneficial and not deleterious to hESC culture (Xu *et al.*, 2005). In contrast, BMP4 was not responsible for the activity of STO cells that support ES self-renewal or proliferation (Hao *et al.*, 2006). Here we more closely evaluated the ability of BMP-4 from feeder cells to maintain mESC culture. We found that a dominant-negative catalytically inactive construct (rAd-dnIκB) led to profound attenuation of NF-κB signaling, which translated into an increase of *Bmp4* expression in STO feeder cells. Similarly, down-regulation of *Bmp4* by activation of the NF-κB signaling pathway was found in other kinds of cells (Muraoka *et al.*, 2000; Zhu *et al.*, 2007). This suggests that production of BMP4 in feeder cells is possibly induced by inhibition of the NF-κB pathway.

The major effect of BMP4 on the self-renewal of ESCs is accomplished by means of the inhibition of both extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways (Qi *et al.*, 2004). Suppression of the MAPK pathway by BMP4 maintains pluripotency of ES cells by regulating gene expression of the ESC markers, *Oct4* and *Nanog*. In our study, enhanced levels of BMP4 in STO cells inhibited colony formation and increased expression of *Oct4* and *Nanog* genes of mESCs in an undifferentiated culture system, whereas *id* gene expression was unchanged in mESCs. Since a critical level of *Oct4* is known to be essential to maintain pluripotency of ESCs (Niwa *et al.*, 2000), induced expression of *Oct4* in our system may have contributed to inhibition of ESC self-renewal. Addition of exogenous BMP4 also decreased the colony number of undifferentiated mESCs in a dose-dependent manner. When CHO

cells overexpressing BMP4 were implanted in the avascular region of quail embryos, endothelial growth and capillary plexus formation were enhanced (Reese *et al*., 2004). Our data is consistent with that of a recent study showing a significant increase in the proliferation rate after addition of a small amount of BMP4 to bone marrow stem cells but a decrease to the lowest rate after adding a larger amount of BMP4 (Mazaheri *et al*., 2011). Other reports also demonstrated that high doses significantly increased apoptosis and drastically reduced cell proliferation, whereas low doses of BMP4 significantly increased cultured cell content, reduced the number of apoptotic cells, and increased the number of cycling cells (Vicente Lopez *et al*., 2011). Taken together, these results showed that excess BMP4 produced in STO cells inhibited proliferation of ESCs. Because of a smaller change in the expression of BMP4 target genes (*ids*), it is unlikely that a single signaling pathway affected by BMP4 activation is capable of entirely inhibiting self-renewal of mESCs.

Methods

STO feeder cells and mESC growth

STO feeder cells (ATCC, Toronto Ontario, Canada) were plated in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; WelGENE, Daegu, Korea), 100 U/ml penicillin, and 100 mg/ml streptomycin. Murine embryonic stem D3 cells (mESCs) were maintained on mitomycin C-pretreated STO feeder cells in DMEM supplemented with 15% FBS, 1% nonessential amino acid (Sigma, St. Louis, MO), 0.1 mM β-mercaptoethanol (Sigma), 1% penicillin and streptomycin, and 1×10^6 U LIF (Chemicon, Temecula, CA). The ESCs were passaged every three days to maintain their undifferentiated state.

Infection of STO feeder cells with adenovirus

The adenovirus vector containing dominant-negative IκB (rAd-dnIκB) or control adenovirus containing green fluorescence protein (rAd-GFP) was propagated in 293 cells and purified by CsCl density gradient. The titer of adenovirus stock was determined as plaque forming units (pfu)/ml by the limiting dilution assay. The viral preparations were dialyzed and stored at -80°C until use. Post-confluent STO cells cultured in tissue culture dishes were washed once with complete PBS and incubated with the adenovirus in DMEM (100 μl/well) at 37°C at different multiplicities of infection (MOI), that is, virus to cell ratio, as indicated. Fresh DMEM growth medium supplemented with 15% FBS was added after 1.5 h, and cells were maintained for two more days. The adenovirus was washed off with PBS, and ESCs (2×10^4 cells/ml) were seeded into these STO feeder cell dishes and cultured until assayed.

[^{3}H] Thymidine incorporation assay

ESCs (1×10^4 cells/ml) cultured on the STO feeder cells were transferred into 96-well plates and cultured for 24 h. For cell proliferation assays, [^{3}H] thymidine (Amersham Biosciences KOREA Ltd, Seoul, Korea) was added into the cell culture plates for 96 h, and cells were harvested. Thymidine incorporation was determined in a scintillation counter.

mESC culture in the STO-conditioned medium

The STO feeder cells infected by adenovirus were cultured in T-flasks containing DMEM supplemented with 10% FBS. After culture for 24 h, STO-conditioned media were prepared by collecting the supernatant from the STO cell culture medium. We centrifuged the supernatant at 1,200 rpm for 5 min followed by filtration through a Minisart filter (pore size 0.2 μm; Sigma). Filtered supernatant was used as the conditioned medium after supplementing with glucose, nonessential amino acid, β-mercaptoethanol, and antibiotics to the same concentrations as those in the ESC growth medium described above. Mouse ESCs (1×10^4 cells/ml) were seeded and cultured in Matrigel (BD BioSciences, Mountain View, CA)-coated cell culture plates at 37°C until observation by microscope. Exogenous BMP4 (R&D Systems Inc., Minneapolis, MN) was added into the mESC culture medium once a day.

RNA purification and RT-PCR

STO cells were cultured to 90-95% confluence in STO medium and then in ES cell medium for 24 h before total RNA purification using TRI reagent (Sigma). cDNA was synthesized using the Superscript II first-strand synthesis kit (Invitrogen). PCR amplification was carried out with primers specific to mouse BMP4, Wnt3, Wnt5a, STAT3, LIF, Oct4, Nanog, Id1, Id2, Id3, and Id4. RT-PCR for GAPDH was used as an internal control in each experiment.

Real time PCR

Real time PCR was performed using the StepOnePlus™ Real-time PCR System (Applied Biosystems Inc., Foster city, CA) using Eva Green dye. The mRNA expression levels of BMP4 in Ad-dnIκB cells were compared to the expression levels in Ad-GFP cells. The levels of PCR product were calculated from standard curves established for each primer pair (Livak and Schmittgen, 2001).

Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-7-06.pdf.

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