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## BNP is a Novel Regulator of Embryonic Stem Cell Proliferation

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### 1. Introduction

Embryonic stem (ES) cells are derived from pre-implantation blastocysts. The blastocyst consists of an outer layer of trophoblast cells and an inner cell population called the inner cell mass (ICM). The ICM gives rise to all tissues of the body and some extraembryonic tissues, and it is from these cells that ES cells are derived (Suda et al., 1987). ES cells have two defining properties: self-renewal and pluripotency, and these make them a promising source for cell transplantation therapies (Suda et al., 1987). The precise mechanism that regulates stem cell self-renewal and pluripotency remains largely unknown. Thus investigation into the molecular and cellular mechanisms of stem cell self-renewal and pluripotency provide the necessary tools to harness the regenerative potential of ES cells for therapeutic purposes.

The two most striking features related to murine ES cell proliferation rate; (1) their unusual cell cycle structure and (2) their rapid rate of cell division (Savatier et al., 1994; Burdon et al., 2002). Murine ES cells divide with an unusually short generation time of approximately 8-10 hours (Savatier et al., 1994; Burdon et al., 2002), and have unusual cell cycle structure, consist of high proportion of cells in the S phase and, a short G1 phase. Since most cell types spend the majority of their time in G1, the short G1 phase of murine ES cells can account for their rapid rate of cell division since the length of S phase is similar within cell types.

The natriuretic peptides (NPs) are a family of three peptides: atrial NP (ANP), brain NP (BNP), and C-type NP (CNP) (Sudoh et al., 1988; Brenner et al., 1990; Sudoh et al., 1990). BNP is produced predominately in the heart (Minamino et al., 1988; Abdelalim et al., 2006a; Abdelalim et al., 2006b). The biological actions of NPs are mediated by binding to cell-surface receptors. These include NP receptor type A (NPR-A or GC-A), which is sensitive to ANP and BNP (Garbers, 1992), NP receptor type B (NPR-B), which is highly specific for CNP (Koller et al., 1991), and NP receptor type C (NPR-C), which comprises up to 95% of the total NPR population (Maack, 1992) and is known to bind all NPs with similar affinity (Levin et al. 1998). Hormone binding to NPR-A and NPR-B activates guanylyl cyclase (GC) and produces cyclic guanosine monophosphate (cGMP), the secondary messenger for a number of biological responses associated with NPs (Garber, 1992; Potter et al., 2006).

NPs are released into the circulation from cardiac cells to act as hormones to control fluid volume homeostasis and blood pressure by causing natriuresis, diuresis, vasorelaxation and inhibition of the renin-angiotensin-aldosterone system (Minamino et al., 1988; Abdelalim et

al., 2006a,b; Levin et al., 1998). In addition, cell-based studies have shown that ANP and BNP exhibit important autocrine and paracrine functions such as modulating myocyte growth, apoptosis and proliferation of smooth-muscle cells (Abell et al., 1989; Silberbach and Roberts, 2001) and cardiac myocytes (Horio et al., 2000), and suppressing cardiac-fibroblast proliferation (Redondo et al., 1998) and extracellular-matrix secretion (Redondo et al., 1998; Tsuruda et al., 2002).

BNP-transgenic mice exhibit overgrowth of the growth-plate cartilage through a cGMP-dependent mechanism (Suda et al., 1998). Furthermore, signaling through NPR-A plays a pivotal role in tumor growth (Kong et al., 2008). Although little is known about the role of NPs in pre-implantation embryo development, one study reported that NPR-B-deficient mice were sterile due to a lack of development of the reproductive system, and the majority (75%) of the NPR-B-deficient mice studied died before 100 days of age (Tamura et al., 2004). In addition, exogenous BNP can enhance clonal propagation in murine ES cells (Ogawa et al., 2004) suggesting the presence of functional NP receptors.

In this chapter, we focus on the evidence that BNP is expressed by undifferentiated murine ES cells and has a role in the regulation of proliferation and survival of murine ES cells. We also discuss the mechanisms by which BNP affects ES cells.

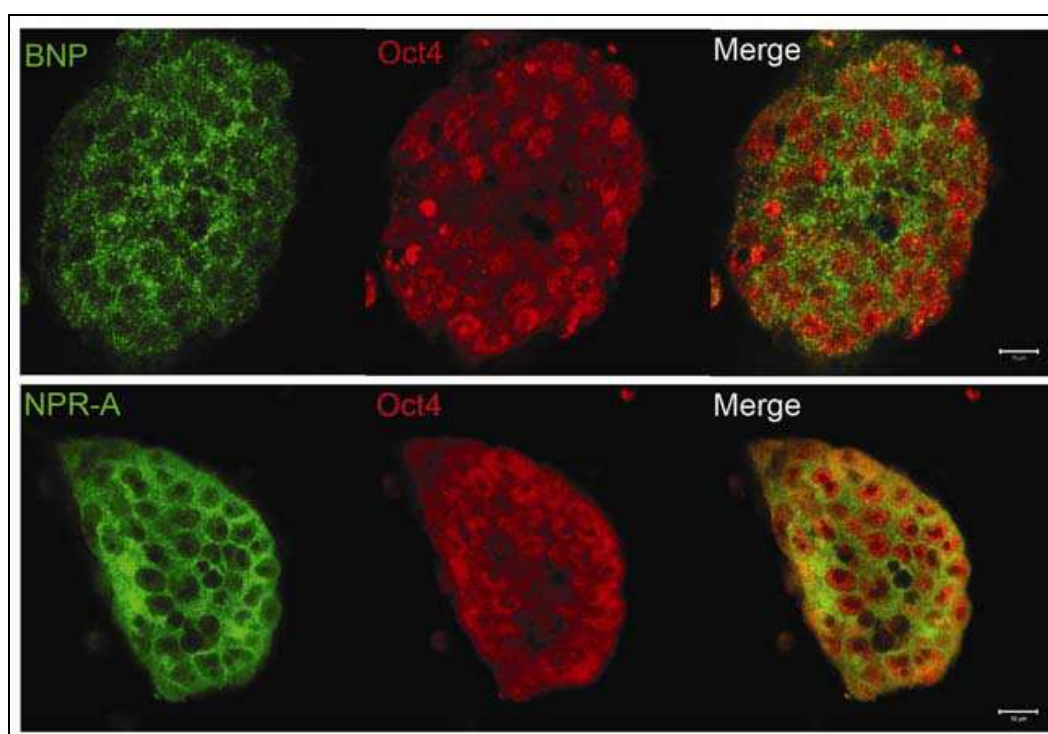


Fig. 1. Expression of BNP and NPR-A in undifferentiated ES cells. Double-immunofluorescence images of ES cells cultured in the presence of LIF, stained with antibodies against the ES-cell marker Oct4 and BNP (*upper panels*), or Oct4 and NPR-A (*lower panels*). Scale bar = 10  $\mu$ m

## 2. Expression of BNP in murine ES cells and pre-implantation embryos

### 2.1 BNP and NPR-A are expressed in undifferentiated ES cells

The pluripotent identity of ES cells is controlled by a group of transcription factors (Niwa et al., 2000; Mitsui et al., 2003; Avilion et al., 2003; Chambers et al., 2003; Loh et al., 2006). The

transcription factors Oct4, Nanog and Sox2 contribute to the hallmark characteristics of ES cells by activating target genes that encode pluripotency and self-renewal mechanisms and by repressing signaling pathways that promote differentiation (Koller et al., 1991). Also, ES cell self-renewal and pluripotency require inputs from extrinsic factors and their downstream effectors (Chambers and Smith, 2004). Self-renewal of murine ES cells under conventional culture conditions depends on the leukemia inhibitory factor (LIF). Withdrawal of LIF induces differentiation (Burdon et al., 2002). To determine whether BNP exists in undifferentiated ES cells, the BNP expression in murine ES cells was examined under self-renewal conditions (+LIF) and differentiation conditions (-LIF). Immunofluorescence analysis revealed that BNP was expressed in undifferentiated ES cells (Oct4-positive cells) cultured in the presence of LIF, and expression was down-regulated upon differentiation induced by culturing ES cells without LIF for five days. The differentiated cells, which were negative for Oct4 expression, were also negative for BNP expression (Fig. 1). Similar results were obtained by Western blotting, flow cytometry, and reverse transcription-polymerase chain reaction (RT-PCR). These findings indicate that BNP is expressed specifically in self-renewing ES cells.

2.2 BNP and its receptors are expressed in murine blastocyst

Since ES cells are derived from the ICM of pre-implantation embryos, the expression of BNP and its receptor were examined in pre-implantation embryos (Abdelalim and Tooyama, 2009). The blastocyst stage of murine pre-implantation development occurs approximately 3.5 days post-fertilization. At this stage of embryonic development the first cell differentiation step has occurred. The blastocyst is composed of the epithelial

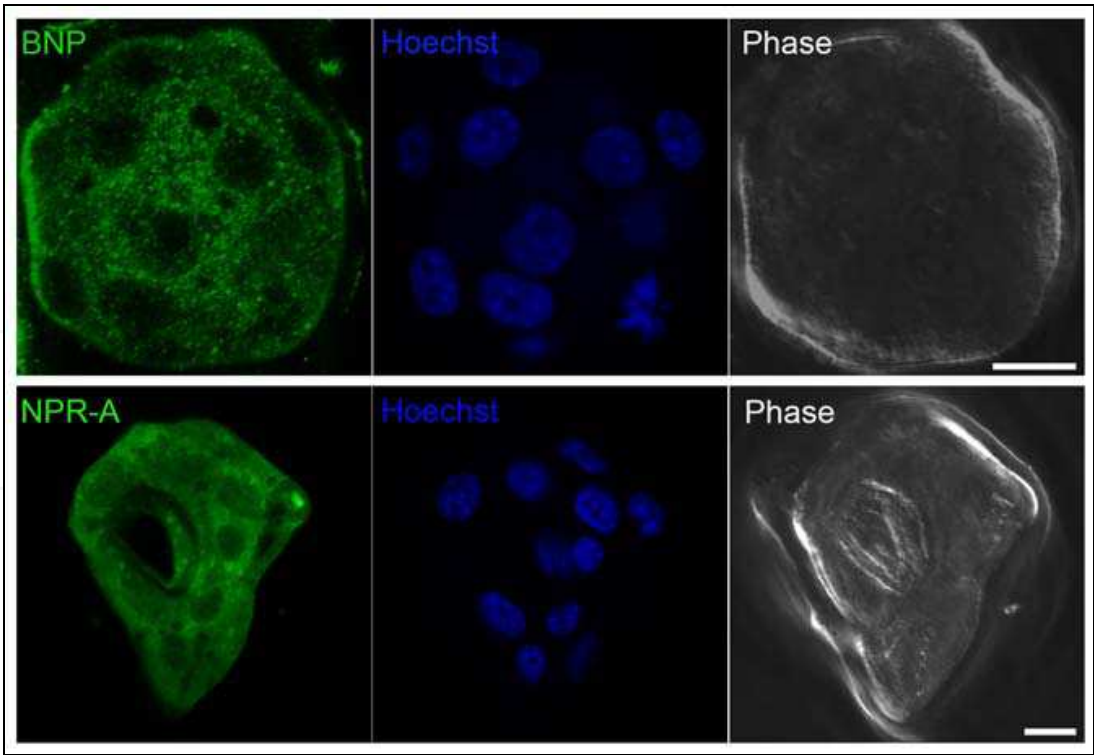


Fig. 2. Expression of BNP and NPR-A in pre-implantation embryos. Immunofluorescence images of 3.5-day-old blastocysts stained with antibodies against BNP (upper panels), or NPR-A (lower panels). Scale bar = 10  $\mu$ m



trophectoderm, which is the layer of cells that develops into the placenta, and the ICM, which consists of the pluripotent cells that give rise to the embryo. Prior to implantation, the developing embryo is dependent on signals produced by embryonic and maternal growth factors. These growth factors are known to regulate cellular proliferation and differentiation during pre-implantation development (Weil et al., 1996).

Immunofluorescence analysis showed that BNP and NPR-A were expressed in 3.5-day-old murine blastocysts (Fig. 2). The expression of BNP and NPR-A were co-localized to those of Oct4 (Abdelalim and Tooyama, 2009). Oct4 expression is required to maintain the pluripotent-cell population of the ICM and epiblast (Nichols et al., 1998). Also, Oct4 is a crucial regulator of ES-cell pluripotency, and acts as a gatekeeper to prevent ES-cell differentiation (Nichols et al., 1998). Reduction of Oct4 expression below 50% of normal levels induces differentiation of ES cells into trophoctoderm (Niwa et al., 2000). The localization of BNP and NPR-A in Oct4-positive cells in the pre-implantation embryos suggests that BNP may be involved in early embryo development.

### 3. Effect of BNP on ES cell proliferation

The use of the ribonucleic acid (RNA) interference (siRNA)-based technique to specifically knockdown BNP expression in ES cells is one approach for investigating the importance of BNP signaling in ES cells. We (Abdelalim and Tooyama, 2009) specifically knocked down BNP expression in murine ES cells using two siRNAs targeting different regions. The efficiency of BNP knockdown was examined by RT-PCR and Western blotting at 48 hours after transfection. Silencing of BNP caused a significant reduction in ES-cell number and colony size as examined at 48 hours post-transfection. Morphological examination showed no signs of differentiation (Fig. 3).

The ability of ES cells to self-renew is maintained through promotion of proliferation, and prevention of differentiation and cell death. Because it appeared that the knockdown of BNP did not promote differentiation, we were interested to know whether its knockdown would affect deoxy-ribonucleic acid (DNA) synthesis. Therefore, the proliferation, and bromodeoxyuridine (BrdU) incorporation, assays were performed to analysis the rate of proliferation. Immunofluorescence and flow-cytometry analyses showed a significant reduction in the number of BrdU-positive cells in the BNP siRNA-treated cells compared with control siRNA-treated cells. These findings suggest that knockdown of BNP signaling has a significant effect in decreasing the rate of DNA synthesis (Abdelalim and Tooyama, 2009).

The role of BNP in proliferation has been observed in other studies (Suda et al., 1998; Ogawa et al., 2004). BNP-transgenic mice exhibit marked skeletal overgrowth, and studies using in vitro organ culture of mouse tibia demonstrated that BNP increases cGMP production and activates the proliferation of growth-plate chondrocytes via GC-coupled NP receptors (Suda et al., 1998).

Undifferentiated murine ES cells express high levels of receptors specific for BNP, suggesting that BNP signaling can be transduced in ES cells via its receptors. Exogenous BNP may affect clonal proliferation of murine ES cells. Treatment of ES cells with BNP in low-density culture and in serum-free (ES cell KSR) medium for six days resulted in an increase in ES-cell propagation. Similar findings were reported by Ogawa et al. (2004). Thus, clonal propagation of murine ES cells is increased in the presence of BNP.

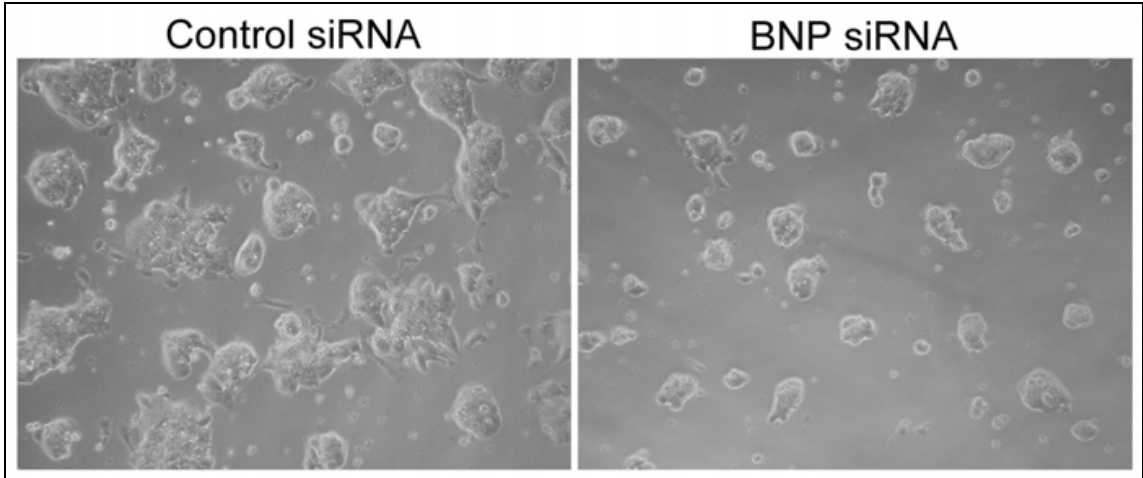


Fig. 3. BNP knockdown suppresses ES-cell proliferation. Morphologies of murine ES cells 48 hours after transfection with control siRNA or BNP siRNA

Flow-cytometric analysis of cell-cycle distribution revealed a significant reduction in the proportion of cells in S phase, and an increase in the proportion of cells in G1 and G2/M phases in ES cells treated with BNP siRNA (Fig. 5a, b). However, cell-cycle analysis showed no significant difference between the phases of the cell cycle of differentiated ES cells treated with BNP siRNA and control siRNA. These results indicate BNP function in undifferentiated ES cells but not in differentiated cells and indicate that BNP is a critical factor in sustaining viability and proliferation of murine ES cells.

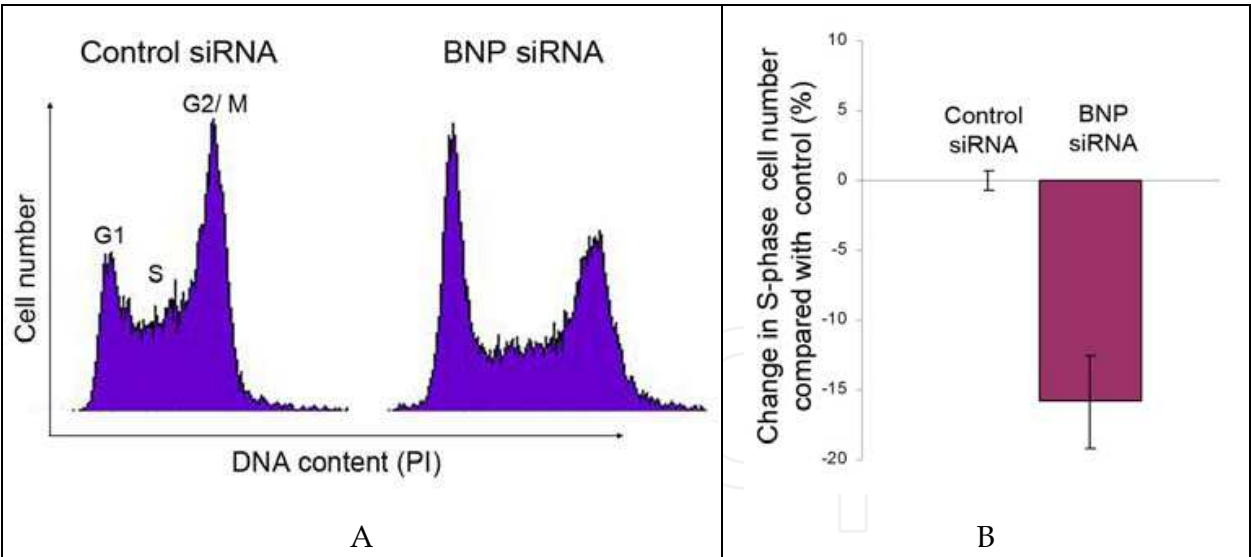


Fig. 4. Effect of BNP knockdown on ES-cell cycle. (A) Cell-cycle profile analysis of ES cells 48 hours after transfection with control siRNA or BNP siRNA. (B) Quantitative analysis of A showing the percent change of cells in S phase (n = 3). This figure is reproduced from (Abdelalim and Tooyama, 2009)

The reduction in ES-cell proliferation caused by abrogation of BNP signaling had no effect on the undifferentiated status of the ES cells as determined by morphologic examination (Fig. 3). This was confirmed by measurement of alkaline-phosphatase activity (Fig. 5), which revealed that levels of self-renewal in the control siRNA- and BNP siRNA-treated cells were

identical. Also, the expression levels of Oct4 mRNA and Nanog mRNA did not differ between control siRNA-treated cells and BNP siRNA-treated cells, suggesting that BNP signaling is not involved in the pluripotency of ES cells (Abdelalim and Tooyama, 2009).

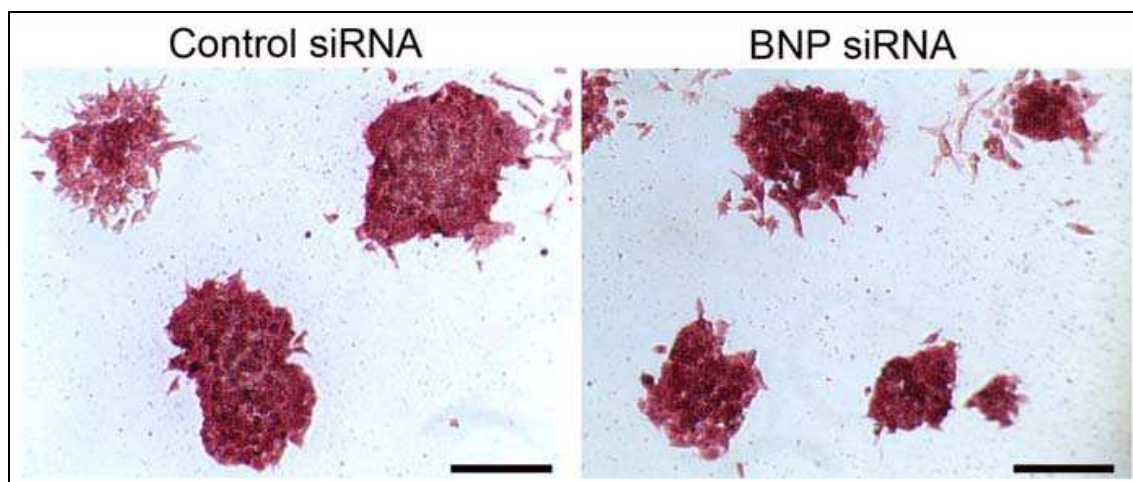


Fig. 5. Effect of BNP knockdown on alkaline-phosphatase (AP) activity. Self-renewal assay AP-staining of the ES cells treated with control siRNA or BNP siRNA four days after transfection. Scale bar = 10  $\mu$ m

#### 4. BNP signaling is mediated by GC-coupled receptors

BNP exerts its biological effects by intracellular accumulation of cGMP through the activation of particulate GCs (NPR-A and NPR-B) (Yasoda et al., 1998; Potter et al., 2006). We found that NPR-A and NPR-B are expressed in undifferentiated ES cells and pre-implantation embryos (Abdelalim and Tooyama, 2009), suggesting the involvement of particulate GCs in the signaling pathway of BNP in murine ES cells.

The intracellular levels of cGMP were measured to determine whether the cGMP pathway is involved in the effects of BNP on ES cells. Levels of cGMP were reduced significantly in ES cells treated with BNP siRNA compared with ES cells treated with the control siRNA. These findings suggest that BNP promotes ES-cell proliferation via its binding to GC-coupled NP receptors. Furthermore, the expression of NPR-B mRNA was dramatically decreased after BNP knockdown in ES cells, and no change was observed in levels of NPR-A mRNA (Abdelalim and Tooyama, 2009). These findings suggest that the reduction in cGMP levels after BNP knockdown in ES cells is a reflection of the reduced NPR-B mRNA level. Tamura et al. (2004) reported that mice lacking in NPR-B were sterile due to a lack of development of the reproductive tract, and that the majority of the NPR-B-deficient mice studied died before 100 days of age. These results indicate that the signaling pathway through NPR-B is a possible regulator for ES-cell self-renewal.

Although ANP and BNP have similar affinity for known GC-coupled NP receptors (Koller et al., 1991; Suga et al., 1992), transgenic mice overexpressing BNP exhibit skeletal overgrowth (Suda et al., 1998), while no skeletal defects have been reported in transgenic mice overexpressing ANP (Steinhilber et al., 1990). Furthermore, mice with targeted deletion of BNP (Tamura et al., 2000) exhibit a different phenotype than ANP-deficient mice (Hohn et al., 1995). Mice without BNP do not have hypertension; instead they show focal

ventricular fibrosis (Tamura et al., 2000). NPR-A-deficient mice display salt-resistant hypertension and cardiac hypertrophy without skeletal defects (Oliver et al., 1997). BNP-Tg/GC-A<sup>-/-</sup> mice continue to exhibit marked longitudinal growth of vertebrae and long bones comparable with that in BNP-Tg mice (Chusho et al., 2000). Given that ANP-transgenic mice do not display the skeletal phenotype, these reports suggest that BNP can signal through pathways independent of NPR-A. However, in cultures of embryonic-mouse tibia, BNP and CNP increased bone growth and stimulated cGMP production by signaling through NPR-B (Yasoda et al., 1998). These findings support the idea that BNP modulates ES-cell proliferation through NPR-B/cGMP-dependent mechanisms.

## 5. Effect of BNP on ES cell viability

The G1 checkpoint provides time for somatic cells to repair damaged DNA and to prevent cells with damaged DNA from entering S phase. One consequence of a checkpoint arrest is that cells with repaired DNA are less subject to apoptosis. Thus, restoration of a G1 checkpoint in ES cells predicts that these cells would be protected from cell death.

ES cells do not undergo cell-cycle arrest at the G1 and G2 checkpoints in response to DNA damage or nucleotide depletion, although they synthesize abundant quantities of transcription active p53 (Aladjem et al., 1998; Prost et al., 1998). Therefore, ES cells appear to lack the p53-dependent G1 and G2 checkpoints that characterize normal somatic cells. Several factors may account for the inability of ES cells to arrest growth at the G1-S or G2-M transitions, and how these cells maintain genome integrity in the absence of cell-cycle checkpoints remains to be clarified.

To determine the effect of BNP on ES-cell survival, apoptosis was examined by staining control siRNA-treated ES cells and BNP siRNA-treated ES cells for annexin V, a marker of apoptosis, 48 hours after transfection (Abdelalim and Tooyama, 2009). The percentage of apoptotic cells significantly increased in BNP siRNA-treated ES cells compared with control siRNA-treated cells (Fig. 6). This result suggests that endogenous BNP protects ES cells from apoptosis. However, up-regulation of gamma-aminobutyric acid type A receptors (GABA<sub>A</sub>R), a downstream target of BNP, does not cause apoptosis in ES cells (Andang et al., 2008), suggesting the involvement of other factors in apoptosis induced by BNP knockdown. The phosphoinositide 3-kinase (PI3K) signaling pathway stimulates the G1-S phase transition and is critical for the maintenance of murine ES cell self-renewal, and its inhibition induces apoptosis in ES cells (Paling et al., 2004; Storm et al., 2007). Protein analysis showed no change in the level of phospho-Akt (ser 473), a marker for PI3K, suggesting that BNP signaling does not interfere with PI3K pathway.

The transcription factor Ets-1 is required for normal survival of T cells, and Ets-1<sup>-/-</sup> T cells display a severe proliferative defect and demonstrate increased rates of spontaneous apoptosis indicating an anti-apoptotic role of Ets-1 (Bories et al., 1995; Muthusamy et al., 1995). Furthermore, Ets-1 participates in the activation of the BNP gene (Pikkarainen et al., 2003). In addition, NPR-A gene transcription and GC activity of the receptor are critically regulated by Ets-1 in target cells (Kumar et al., 2009). Knockdown of BNP in ES cells leads to a marked down-regulation in the expression of Ets-1 (Abdelalim and Tooyama, 2009). Although, no data are available on the role of Ets-1 in ES cell proliferation, the effect of BNP knockdown on Ets-1 suggest that Ets-1 may be involved in the anti-apoptotic and/or proliferative role of endogenous BNP in ES cells.



The role of Ets-1 in proliferation and apoptosis is somewhat controversial. Ets-1 expression regulates endothelial-cell proliferation during angiogenesis and is essential for normal coronary and myocardial development (Seth et al., 2005; Lie-Venema et al., 2003; Lelievre et al., 2000). These data demonstrate that BNP promotes ES-cell survival in part through regulation of Ets-1. Also, Ets-1 has an anti-apoptotic effect in smooth-muscle cells (Zhang et al., 2003). In contrast Ets-1 overexpression in human umbilical vein endothelial cells can stimulate apoptosis by modulating the expression of apoptotic genes (Teruyama et al., 2001).

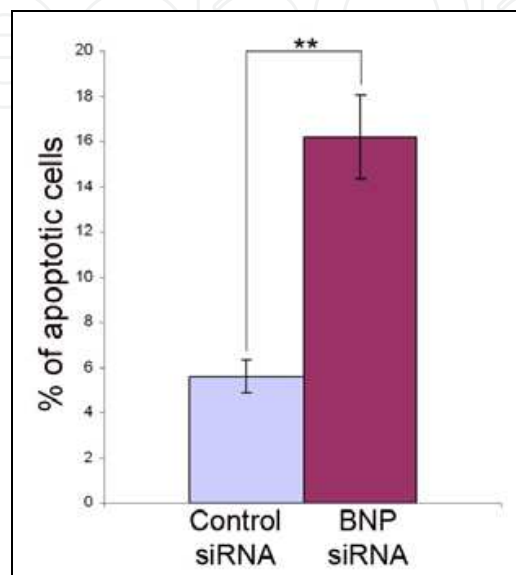


Fig. 6. Effect of BNP knockdown on survival of murine ES cells. The percentage of Annexin-V-positive cells (early apoptotic marker), as measured by flow cytometry 48 hours after transfection with control siRNA or BNP siRNA. Data represent mean  $\pm$  s.d. ( $n = 2$ );  $**P < 0.01$  (two-tailed  $t$ -test). This figure is reproduced from (Abdelalim and Tooyama, 2009)

## 6. BNP regulates GABA<sub>A</sub>R genes in ES cells

The GABA<sub>A</sub>R genes were examined to identify the genes regulated by BNP. Murine ES cells synthesize GABA and express functional GABA<sub>A</sub>Rs. Activation of GABA<sub>A</sub>R by muscimol (a GABA<sub>A</sub>R agonist) inhibits murine ES cell proliferation (Andang et al., 2008; Schwirtlich et al., 2010). The relationship between BNP and GABA<sub>A</sub>R has been previously determined; BNP suppresses GABA<sub>A</sub>R currents in retinal bipolar cells (Yu et al., 2006). Therefore, the effects of BNP knockdown on expression of GABA<sub>A</sub>R $\alpha$ 1 and GABA<sub>A</sub>R $\beta$ 3, the major subunits of GABA<sub>A</sub>Rs in ES cells (Andang et al., 2008), were examined. As expected, BNP knockdown led to up-regulation of GABA<sub>A</sub>R genes. Also, treatment of ES cells with muscimol significantly reduced the BNP expression that is associated with reductions in ES-cell proliferation, indicating a link between endogenous BNP and GABA<sub>A</sub>R signaling in the control of ES-cell proliferation (Abdelalim and Tooyama, 2009). These data suggest that endogenous BNP signaling is essential for maintaining the appropriate level of GABA<sub>A</sub>R in ES cells to promote ES-cell proliferation.

The effect of GABA<sub>A</sub>R on ES-cell proliferation is mediated by phosphorylation of H2AX in cell-cycle dependent and DNA-damage independent manners (Ichijima et al., 2005; Andang et al., 2008). In addition, the reduction of BNP levels using siRNA increases the

accumulation of  $\gamma$ -H2AX nuclear foci in ES cells.  $\gamma$ -H2AX is a critical factor in the S/G2 DNA-damage checkpoint complex (Fernandez-Capetillo et al., 2004) and for the surveillance of genome integrity (Celeste et al., 2003). Down-regulation of H2AX in ES cells using siRNA increases ES-cell proliferation (Andang et al., 2008). Of note, GABA<sub>A</sub>R may not be the only factor that mediates the BNP-induced proliferative activity. Since BNP down-regulation caused an increase in the rate of apoptosis, the accumulation of  $\gamma$ -H2AX after BNP knockdown in ES cells may be the result of activation of GABA<sub>A</sub>Rs and/or apoptosis.

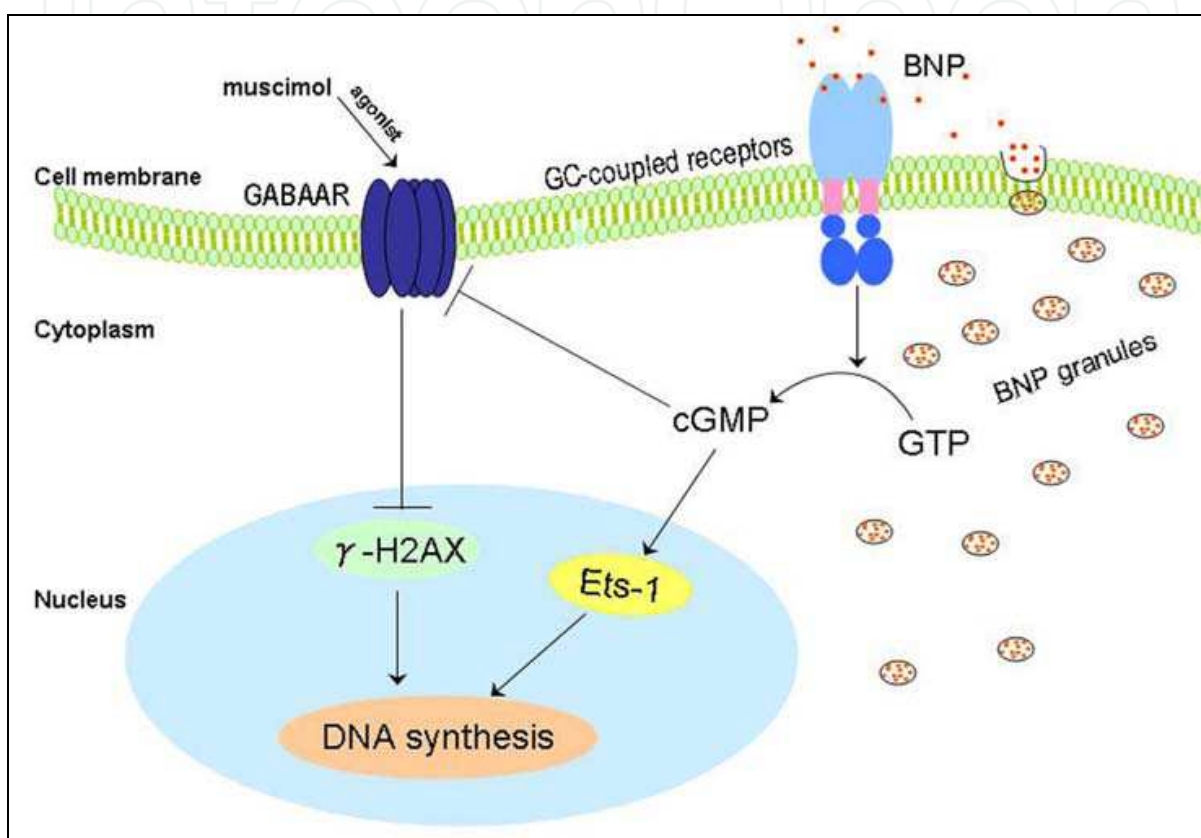


Fig. 7. Simplified schematic presentation of the role of BNP in murine ES cells. BNP and its receptors are expressed by undifferentiated ES cells. BNP activates cGMP through GC-coupled receptors, which inhibit the expression of GABA<sub>A</sub>R. Inhibition of GABA<sub>A</sub>R subsequently reduces the accumulation of  $\gamma$ -H2AX in the nucleus, increasing DNA synthesis. BNP also activates Ets-1 expression, which may contribute to survival and proliferation of ES cells

## 7. Conclusion

BNP signaling is an essential signaling pathway for the proliferation and survival of murine ES cells. BNP and its receptors are expressed by undifferentiated ES cells. Knockdown of BNP in ES cells leads to suppression of ES-cell proliferation as well as increases apoptosis. The suppression of ES-cell proliferation is due to the activation of GABA<sub>A</sub>R,  $\gamma$ -H2AX accumulation and Ets-1 inhibition. We propose two pathways that may explain the involvement of BNP in the control of proliferation of ES cells (Figure 7). In pathway 1, BNP controls the level of GABA<sub>A</sub>R, which is a negative regulator of ES-cell proliferation. In the

other pathway, BNP stimulates the transcription factor Ets-1, which may be involved in the survival and proliferation of ES cells. These findings will greatly enhance our understanding of the pathways involved in the regulation of self-renewal of ES cells. Future investigation will help delineate the mechanisms by which BNP contributes to the regulation of ES cell self-renewal.

## 8. Acknowledgments

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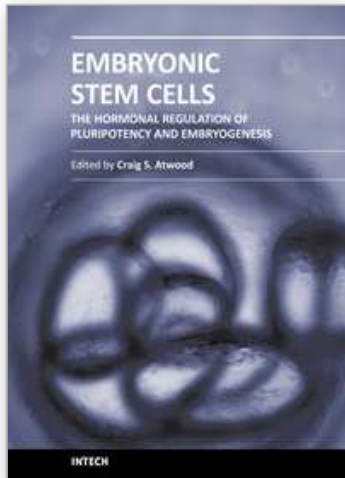
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