Supplementary Information

Supplementary Materials and Methods

Embryo Collection

Blastocysts were collected from ICR mice at 3.5-days-postcoitum (dpc) and then subjected to RT-PCR analysis and immunostaining as previously described [24].

Embryoid body (EB) formation assay

Undifferentiated ES cells were trypsinized and resuspended in ES cell culture medium without LIF (differentiation medium). Differentiation was induced by hanging drop method as a standard method of embryoid body (EB) formation. Hanging drops (one droplet contains 1000 cells) were placed on the lid of a 100-mm dish filled with phosphate-buffered saline (PBS). EBs were collected on days 3, 5, 7, 9 for RT-PCR analysis.

RNA Interference

RNA interference in murine ES cells was carried out according to the manufacturer's protocol using Lipofectamine 2000 (Invitrogen) in 6-well plates. Two pairs of siRNAs (Invitrogen) were designed for ANP (NM_008725) using the BLOCK-iT RNAi Designer software. The appropriate siRNA negative control Duplex (Cat. No. 12935-300; Invitrogen) was selected based on the percentage G/C. The ANP siRNA and control siRNA were transfected at a final concentration of 40 μM for 24 h in triplicate for each treatment. At 48 h post-transfection, ANP knockdown was confirmed by RT-PCR and Western blotting. The sequences of ANP siRNAs were as follows:

ANP siRNA1 (MSS279533): sense, 5′-AUUUGCUCCAAUAUGGCCUGGAAGC-3′; antisense, 5′- GCUUCCAGGCCAUAUUGGAGCAAAU-3′.

ANP siRNA2 (MSS279534): sense, 5′-UGGUCUAGCAGGUUCUUGAAAUCCA-3′; antisense, 5′- UGGAUUUCAAGAACCUGCUAGACCA-3′.

Supplementary Results

Expression levels of NPR-A during ES cell differentiation

NPR-A is highly expressed in undifferentiated ES cells as well as pre-implantation embryos (Supplementary Fig. 1A-C), and its expression dramatically decreases as differentiation proceeds [24]. Therefore, we examined how the expression levels of NPR-A dynamically changed during ES cell differentiation. ES cells are commonly differentiated *in vitro* by spontaneously self-assembling in hanging drop and suspension culture methods into 3-dimensional cell aggregates called embryoid bodies (EBs), which model many of the hallmarks of early embryonic development.

Murine ES cells were induced to differentiate and form EBs (Supplementary Fig. 1C). The NPR-A and pluripotency makers (Nanog and Oct4) expression patterns during EB formation (day 0 to 9) were monitored by RT-PCR (Supplementary Fig. 1D). Levels of Nanog and Oct4 genes were dramatically reduced at day 7 of differentiation. Also, NPR-A expression was found to be repressed upon ES cell differentiation. It was noted that the repression of NPR-A expression was not as dramatic as the repression of Oct4 upon differentiation (Supplementary Fig. 1D). Thus, NPR-A expression is associated with the ES cell pluripotent state and its expression decreases as cells differentiate.

NPR-A knockdown results in suppression of G1-S transition.

To monitor whether the prolonged G1 phase is associated with NPR-A knockdown or as a result of differentiation, we examined the cell cycle profile in narrow time frames during siRNA-induced NPR-A knockdown in ES cells (10, 15, 20, and 24 h) At 20 h of siRNA transfection, we observed an accumulation of cells in the G0/G1-phase in NPR-A siRNA-treated cells (Supplementary Fig. 3A-B). At this time point, NPR-A expression level was downregulated in NPR-A siRNA-treated cells as examined by RT-PCR (Supplementary Fig. 3C). However, morphological examination showed no signs of differentiation at 24 h, and NPR-A siRNA-treated cells showed differentiation phenotypes at 40 h after siRNA transfection (Supplementary Fig. 3D). Therefore, these data provide evidence that NPR-A signaling is required for progression to S-phase in ES cells.

Effect of ANP knockdown on murine ES cells

In this study we found that ANP is expressed in undifferentiated ES cells. To determine the functional role of ANP in ES cell pluripotency, we carried knockdown experiment. Two independent siRNAs targeting different regions of the ANP mRNA were used to specifically knockdown the ANP gene in ES cells. Knockdown efficiency was analyzed at 48 hours after transfection of siRNA. RT-PCR and Western blot analysis revealed a reduction in the level of ANP mRNA and ANP protein at 48 hours post-transfection in the ES cells that were transfected with ANP-targeting siRNA (ANP siRNA), compared with the ES cells that were transfected with a non-targeting siRNA (control siRNA) (Supplementary Fig. 4A-C). ANP siRNA2 gave higher level of ANP knockdown and therefore used for all subsequent experiments.

We also evaluated colony morphology and AP activity in ES cells cultured in the presence of LIF. Two days after transfection, ANP siRNA-treated cells exhibited a change in morphology with the presence of differentiating cells (Supplementary Fig. 4D). The AP-staining, which is indicative of the self-renewal property of ES cells, was markedly reduced in ES cells treated with ANP siRNA in comparison to those treated with control siRNA (Supplementary Fig. 2D). Consistent with this reduction in the number of AP positive colonies, knockdown of ANP also led to reduction in the expression of markers of pluripotency, including Nanog and Oct4 (Supplementary Fig. 4E).

The effect of ANP knockdown on ES cell pluripotency were lower than those observed in NPR-A-deficient cells due to incomplete inhibition of ANP and the presence of endogenous NPR-A.

Supplementary Discussion

It has been found that BNP has a low affinity for NPR-A with a potency approximately 10-fold lower than that of ANP [Supplementary ref. 1], which has led to speculation that may be an additional BNP-specific receptor might play a role [Supplementary ref. 2; 3]. In agreement with this, Chusho et al., [Supplementary ref. 4] suggested that it is possible that there is an as yet unidentified receptor with high affinity to BNP. Also, it has been shown that in the absence of NPR-A, testis and adrenal glands retain significant high-affinity response to BNP that can only be accounted for the presence of a novel receptor in these tissues that prefer BNP over ANP. Although the physiological significance and the biochemical component of this receptor remains to be established, its existence does reinforce the notion that ANP and BNP are likely to carry out at least some

independent action [Supplementary ref. 3].

NPR-B is another receptor that BNP is known to activate since, BNP regulates skeletal growth through NPR-B and independent of NPR-A [19; 38; Supplementary ref. 4]. In the current study, NPR-A/ ANP were shown to be involved in the maintenance of self-renewal and pluripotency of ES cells. However, knockdown of BNP in ES cells leads to suppression in proliferation without affecting on ES cell pluripotency [24]. Taken together, these findings suggest that BNP may be involved in ES cell proliferation independent of NPR-A.

Supplementary References

[1] Schulz S, Singh S, Bellet RA, Singh S, Tubb DJ, Chin H, et al. The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. Cell 1989; 58:1155 –62.

[2] Goy MF, Oliver PM, Purdy KE, Knowles JW, Fox JE, Mohler PJ, et al. Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide. Biochem J 2001; 358:379 –87.

[3] Vanderheyden M, Bartunek J, Goethals M. Brain and other natriuretic peptides: molecular aspects. Eur J Heart Fail 2004; 6: 261-268.

[4] Chusho H, Ogawa Y, Tamura N, Suda M, Yasoda A, Miyazawa T, et al. Genetic models reveal that brain natriuretic peptide can signal through different tissue-specific receptor-mediated pathways. Endocrinology 2000; 141: 3807-3813.