mNanog Possesses Dorsal Mesoderm-Inducing Ability by Modulating Both BMP and Activin/Nodal Signaling in Xenopus Ectodermal Cells

Aya Miyazaki¹, Kentaro Ishii¹, Satoshi Yamashita¹, Susumu Nejigane¹, Shinya Matsukawa¹, Yuzuru Ito², Yasuko Onuma², Makoto Ashashima¹,², Tatsuo Michiue¹*

¹ Department of Life Sciences (Biology), Graduate School of Arts and Sciences, the University of Tokyo, Tokyo, Japan, ² Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba City, Ibaraki, Japan

Abstract

Background: In Xenopus early embryogenesis, various genes are involved with mesoderm formation. In particular, dorsal mesoderm contains the organizer region and induces neural tissues through the inhibition of bone morphogenetic protein (BMP) signaling. In our initial study to identify novel genes necessary for maintaining the undifferentiated state, we unexpectedly revealed mesoderm-inducing activity for mNanog in Xenopus.

Methodology/Principal Findings: The present series of experiments investigated the effect of mNanog gene expression on Xenopus embryo. Ectopic expression of mNanog induced dorsal mesoderm gene activity, secondary axis formation, and weakly upregulated Activin/nodal signaling. The injection of mNanog also effectively inhibited the target genes of BMP signaling, while Xvent2 injection downregulated the dorsal mesoderm gene expression induced by mNanog injection.

Conclusions/Significance: These results suggested that mNanog expression induces dorsal mesoderm by regulating both Activin/nodal signaling and BMP signaling in Xenopus. This finding highlights the possibly novel function for mNanog in stimulating the endogenous gene network in Xenopus mesoderm formation.

Introduction

Germ layer formation is one of the most important processes in the fundamental patterning of an embryo. In Xenopus early embryogenesis, mesoderm is induced by signals secreted from endodermal tissue during the blastula stage, and nodal-related (Xnr) genes are known to play important roles in this biological process. VegT and Wnt signaling induces Hnf5/6, followed by the sequential upregulation of Xnr1/2 and Xnr4 [1–3], and consequently, various mesoderm gene activities. Activin A, a TGF-B superfamily member, was first identified as a factor that could induce both ventral and dorsal mesoderm [4]. In dorsal mesoderm, also called the Spemann-Mangold organizer, several genes including chordin (chd), noggin (nog), goosecoid (gsc), and slat-1 are expressed to induce neural tissues in the presumptive neuroectoderm [5–8]. Xenopus blastula ectodermal cells, or animal cap (AC) cells, possess multipotency and can differentiate into many types of tissues including mesoderm. However, the period for mesoderm induction in AC is limited until early gastrula. This phenomenon is known as “loss of mesodermal competence” (LMC) [9]. To identify novel factors involved with maintaining multipotency in Xenopus embryo, we first attempted to find genes involved in releasing LMC.

The first candidate gene we examined was mNanog, which encodes a homeodomain protein and is efficiently expressed in mammalian embryonic stem (ES)/induced pluripotent stem (iPS) cells [10–12]. Our preliminary experiments revealed that in the presence of Activin A treatment, mNanog injection promotes AC elongation and some mesodermal gene expression even at the late gastrula stage (data not shown). We also unexpectedly found that mNanog injection induces AC elongation without Activin A treatment and could promote the expression of dorsal mesoderm genes such as chd, gsc, and slat-1 in AC. Further experiments revealed that mNanog injection induces Activin/nodal signaling and inhibits BMP signaling. Together, these data indicated that mNanog modulates these signaling pathways to induce the dorsal mesoderm cell fate in Xenopus AC, suggesting a novel function for mNanog in embryogenesis.

Materials and Methods

Plasmids

The mNanog gene was amplified by RT-PCR with mouse cDNA (from mouse ES D3 cell line (American Type Culture Collection (ATCC))). All experiments with the mouse ES cells were approved
by the institutional ethics committee (Graduate Schools of Arts and Sciences, University of Tokyo: #19-19 and #23-10). mNanog/SK was made by inserting the amplified fragment of mNanog into the EcoRI site of pBluescriptII SK+. For injection, we inserted the EcoRI-XhoI fragment of mNanog/SK into the EcoRI-XhoI site of pCS2 to construct mNanog/CS2. AAtLA4/K/C2, Xmr2/C2, Xmnr5/C2, cmXmr1/C2, cmXmr2/C2, and Xven2/C2 were also used for microinjection [3,13–16]. For lineage tracing, we used pCS2-lacZ.

Microinjection

Microinjection was performed using a picojector (Harvard Medical Instruments). RNA for injection was synthesized with the mMESSAGE mMACHINE SP6 kit (Ambion/Applied Biosystems). Injected embryo was obtained by artificial fertilization and dejellied with 4.6% L-cysteine hydrochloride solution. Injection was performed in 5% Ficoll/1X Steinberg’s Solution (SS). Injected embryos were cultured in 0.1X SS solution.

Animal cap assay

mRNA was injected into the animal pole region of 2-cell-stage embryos. ACs were dissected at the late blastula stage (Stage 9), and then cultured to the appropriate stage with/without treatment with 10 ng/ml of Activin A. The shape of treated ACs was observed at about 12 hours after treatment. Treated AC was also assessed by the expressions of several marker genes.

RT-PCR

We synthesized cDNA with 0.3 μg of total RNA prepared from 5–10 ACs. For reverse transcription, we used Superscript III (Invitrogen), and PCR was carried out with Ex Taq DNA polymerase (Takara, Japan). Primer sets used for PCR were as follows:

**ODC:** GCCATTGTGAAGCTCTCAGTTC and TCGGGGTATTCCTTGCCAC; **Xbra:** TTCGGGTGATTCCTTGCCAC; **Goc:** CACAACATGCGAGTCTCCT and GGAGAACTGACACCAGA.

**Chd:** AACTGCGGAGCTGTGATGTT and GGCGAGATTAGTTGCTTC; **EcoR I:** CACGTGCATTCC; **Xvent2:** TTGGCTTGGAGGAGTGTGT.

**Tca:** GCACAGACGGAGCTGTGATGTT and GGCGAGATTAGTTGCTTC; **Xvent1:** AACTGCGGAGCTGTGATGTT and GGCGAGATTAGTTGCTTC.

**Ven:** CTGGAACCAG(G/T)TCTT(A/C)ACCTG and CAAAGGCAAGCCAAGC; **Xmr1:** GCAGTTAAAGATTATTTACTGCG and CAAAGGCAAGCCAAGC; **Xmr2:** ATCTGATGCCGTTCTAAGCC and GACCTTCTTATGCTTC; **Xmr3:** CTTCGCTCAGTCCAGG and GACCTTCTTATGCTTC; **Xmr5:** TCACAATCTTTCATAGGGC and GGAACCTTGAAAGGAGG; **Xvent1:** TCTTGCACTAGTCCAG and GACCTTCTTATGCTTC; **Xvent2:** TTGGCTTGGAGGAGTGTGT and TCTTGCACTAGTCCAG; **Xvent3:** GCCAGCTTGGACCAGTCTGCTG and TCTTGCACTAGTCCAG; **Xvent4:** TTGGCTTGGAGGAGTGTGT and TCTTGCACTAGTCCAG;

**mix1:** GATGAAGGGGACTGTGAGG and AATGTCTCAAGG; **mix2:** TTGGCTTGGAGGAGTGTGT and TCTTGCACTAGTCCAG.

In situ hybridization

Embryos were bleached in hydrogen peroxide–methanol before fixation in MEMFA (formaldehyde-MOPS solution) and dehydration with ethanol. Rehydrated embryos were hybridized with DIG-labeled probe for 24 h at 60°C. Embryos were then incubated with 2000x anti-DIG antibody (Roche) for 12 h, washed 5 times, and then visualized by reaction in NBT/BCIP solution (Roche).

TUNEL staining

In situ TUNEL assay for detecting apoptotic cells were carried out by previous method [17]. Briefly, fixed and bleached embryos were incubated with TdT enzyme (Invitrogen) and DIG-dUTP (Roche) for 1 day. After washing, embryos were incubated with anti-DIG antibody, washed with MAB and detected with BM-purple (Roche).

Cycloheximide (CHX) treatment

The procedure for CHX treatment was basically carried out as previously described [18]. Normal or Injected embryos were treated with 40 ng/ml of CHX in 1X Steinberg’s solution at Stage 7, and was homogenized at Stage 9.

**Xenopus Nanog** gene cloning

To clone the Xenopus homolog of Nanog gene, we carried out degenerated PCR with following primers: **U1:** CC(T/C)GA(T/C)TCA(T/C)GCACAGAATCCAGG(T/C)CC(A/G)AAA(A/A); **U2:** TC(A/T)CC(T/C)GA(T/C)TCA(T/C)GCACAGAATCCAGG(T/C)CC(A/G); **L1:** CTGGAAACGG(G/T)TCTTA(C/A)ACCTG, **L2:** CAT(T/C)CTA(A/G)TCTGAG, **L3:** TTCAT(T/C)CTA(A/G)TCTGAG, **L4:** TGCTTGGAGGAGTGTGT.

The positions of these primers are summarized in Fig. S1.

**Results**

**mNanog** injection stimulated mesoderm-inducing activity in AC

At first, we confirmed the expression of mNanog protein in Xenopus embryo. By Western blot analysis, we could detect a
protein of 40 kDa, consistent with the molecular size of the mNanog protein (Fig. 1A). Immunohistochemistry with anti-mNanog antibody showed intense mNanog reactivity in the nuclei of mNanog-injected embryos (Fig. 1B, C). Next, we examined the effects of mNanog on Xenopus early embryogenesis. 200 pg of mNanog mRNA injected into the animal pole of 4-cell embryos caused a defect in the anterior region at the late neural stage (Fig. 1D, E), although no obvious developmental delay was observed (data not shown). In 3-day-old tadpoles, head defects with small eye vesicles could be seen (Fig. 1G, Table S1). This head defect was more intense and lethality was also strikingly increased by injection with 400 pg of mNanog (Table S1), although the lethality did not manifest until the neural stage (data not shown). To examine whether the head defect occurred by apoptosis, we carried out terminal deoxynucleotidyl transferase-mediated deoxyuridine-triphosphate nick end-labeling (TUNEL) assays. mNanog injection increased the number of apoptosis-positive cells, suggesting that the head defect was due to apoptosis in the head region (Fig. 1H–J). We then observed the AC shapes. Injection of 200 pg of mNanog slightly elongated the AC in the absence of Activin A treatment (Fig. 1K, L), but less so with Activin A treatment (Fig. 1M). This elongation was dependent on the absence of Activin A treatment (Fig. 1K, L), but less so with injection with 400 pg of mNanog (Table S1), although the lethality did not manifest until the neural stage (data not shown). In 3-day-old tadpoles, head defects (Fig. 1D, E), although no obvious developmental delay was observed (data not shown). In 3-day-old tadpoles, head defects (Fig. 1D, E), although no obvious developmental delay was observed (data not shown). In 3-day-old tadpoles, head defects (Fig. 1D, E), although no obvious developmental delay was observed (data not shown). In 3-day-old tadpoles, head defects (Fig. 1D, E), although no obvious developmental delay was observed (data not shown). In 3-day-old tadpoles, head defects (Fig. 1D, E), although no obvious developmental delay was observed (data not shown).

mNanog injection promoted expression of dorsal mesodermal genes, but inhibited ventral mesodermal genes in both AC and embryos

The phenotypes of mNanog-injected embryos and their corresponding ACs suggested to us that mNanog could induce dorsal mesodermal tissues. We next performed RT-PCR analysis to examine the expression of mesodermal genes in earlier stages. When 200 pg of mNanog mRNA was injected into 2-cell embryos, the expression of dorsal mesodermal marker genes chd, gcc, and xdm-1 was increased in stage-11 ACs without Activin A treatment (Fig. 2A 1st–3rd columns; lane 3, 5, and 6). 400 pg of mNanog mRNA injection further increased these gene expressions (Fig. 2A column 1, 2, 3; lane 7). The mNanog injections only slightly enhanced the same expressions in Activin A-treated AC (Fig. 2A 1st–3rd column; lane 4, 6, 8). On the other hand, Xbra expression was not effectively induced by mNanog injection (Fig. 2A 4th column, lane 3, 5, 7), and induction of Xbra expression by Activin A treatment was clearly inhibited by mNanog injection (Fig. 2A 4th column, lane 4, 6, 8). Similar induction was observed with Xwnt8, mix, mixe, Cerberus (Cer), and Sox17a [23–27] (Fig. 2A 5th–6th columns). To assess whether the enhancement of dorsal mesodermal gene expressions was specific for mNanog function, we carried out RT-PCR with a deletion mutant of mNanog that produces a protein lacking the C-terminus domain including the W-repeat motif (mNanogΔC; Fig. 2B) [28,29]. Dorsal marker gene expression was not induced by mNanogΔCD (Fig. 2B 1st–3rd columns). Quantitative analysis of the mNanog mRNA also suggested that mNanog function in mesoderm induction requires dimerization of the mNanog protein (Fig. 2B).

To examine the effect of mNanog on endogenous mesodermal gene expressions, we performed in situ hybridization. Endogenous chd expression was observed in the dorsal lip region (Fig. 2C, black arrow), and only the control injection did not affect chd expression (Fig. 2C, white arrow). When mNanog was injected into the ventral marginal zone, ectopic chd expression was obviously induced (Fig. 2D, white arrow), suggesting that mNanog can induce chd expression in embryo, confirming the RT-PCR analysis. Xbra expression was seen around the yolk plug in normal embryo (Fig. 2E), but was specifically inhibited in the mNanog-injected area (Fig. 2F, white arrow), suggesting that mNanog negatively regulates Xbra expression. These data also indicated that mNanog affects the endogenous expression of mesodermal genes in Xenopus embryo.

To further profile the mechanism of mesoderm induction driven by mNanog, we next compared the expression of mesodermal marker genes between Activin A treatment and mNanog injection. AC from normal embryo did not express any mesodermal genes (Fig. 2G, lane 2), but following treatment with Activin A at the dose of 1–10 ng/ml chd and gcc were expressed in a dose-dependent manner (Fig. 2G, lane 6–7). Xbra was also efficiently expressed following both 1 ng/ml and 10 ng/ml Activin A treatment (Fig. 2G, lane 6–7). When mNanog was injected, gcc and chd expressions gradually increased (Fig. 2G, lane 3–5), as did Xbra expression, although the effect of mNanog injection on Xbra expression was less enhanced than that induced by Activin A treatment (Fig. 2G, 3rd column).

Several mesodermal genes including chd are induced by overexpression of canonical Wnt signaling and Xnr genes [5,30]. We therefore examined the expression of early canonical Wnt signaling target genes in our system. There was no increased expression of sia and Xnr3, known targets of canonical Wnt signaling, despite the injection of mNanog mRNA [21,31] (Fig. 2H). This result suggested that mNanog does not affect canonical Wnt signaling in the embryos stages we examined.

mNanog subsidiary utilizes Activin-nodal signaling for dorsal-mesoderm induction

Previously, it was shown that both mesoderm and endoderm formation requires activation of Activin/nodal signaling. Thus, we next examined whether the expression of Xnr genes is induced by mNanog. RT-PCR analysis indicated that Xnr1 and Xnr2 expressions were increased in a dose-dependent manner (Fig. 3A). On the other hand, expression of Xnr5/6 was not increased in mNanog-injected AC (Fig. 3B). From these results, we proposed that mesoderm induction by mNanog involves the upregulation of not Xnr5/6, but Xnr1/2. To assess whether mNanog overexpression promotes the nuclear transport of Smad2, we co-injected embryos with mNanog and Smad2GFP [32]. Without mNanog injection, GFP signal was observed in the cytoplasm of AC cells (Fig. 3C), whereas 10 pg of Xnr5 injection promoted a nuclear localization of the GFP signal (Fig. 3E). When 200 pg of mNanog was co-injected, Smad2GFP signal was occasionally observed in nuclei, although the efficiency was low (Fig. 3D). This result suggested that, at least in some cases, mNanog regulates Activin/nodal signaling through Xnr1/2.

Next, to clarify whether mesodermal gene induction was dependent on Activin signaling, coinjection experiments were performed with a truncated form of the type I Activin receptor (aALK5) [15], which acts as a dominant-negative mutant. Indeed,
Figure 1. Mesodermal tissues were induced by mNanog mRNA injection. A) Detection of exogenous mNanog protein in Xenopus embryo by Western blotting with antibodies to mNanog (upper) and alpha-tubulin (lower). Non-injected embryo control (lane 1). mNanog-injected embryo (lane 2). B–C) Subcellular localization of mNanog protein in stage-10 embryo injected with mNanog mRNA. Ectoderm from normal embryo (B) or mNanog-injected embryo (C). Dissected tissues were fixed at stage 9, and then treated with anti-mNanog antibody. The green signal indicates mNanog protein. DAPI staining was also done (blue). Scale bar; 0.02 mm. D–G) Superficial phenotype of mNanog-injected embryos. Stage-18 (D, E) and stage-38 embryos (F, G) were observed. (D, F) Uninjected embryo. (E, G) 200 pg of mNanog mRNA was microinjected into the animal pole region at the 4-cell stage. Scale bar; 0.5 mm (D) and 1 mm (F). H, I) TUNEL staining of normal (H) or mNanog-injected (200 pg; I) embryos was performed at stage 20. Apoptotic cells appear as blue dots. J) The number of apoptosis-positive cells in normal embryo (n = 14) and 200 pg of mNanog injected embryo (n = 18) was described in bar graph. Error bar indicates S.E. K–N) Comparison of AC shapes between mNanog-injected embryos with and without Activin A treatment. All ACs were dissected at stage 9 and observed at stage 18. Normal ACs (K). ACs injected with mNanog into the animal pole region (L). ACs treated with 10 ng/ml Activin A at stage 9 (M). mNanog-injected ACs treated with Activin A at stage 9 (N). Scale bar; 0.5 mm. O) Analysis of AC elongation in (K)–(N). Both the shortest and longest lengths of AC were measured, and averages of the length ratio (long/short) were expressed as bar graphs. Normal AC (n = 50), 10 ng/ml Activin A-treated AC (n = 58), AC with 200 pg of mNanog injected (n = 46), AC with 200 pg of mNanog injected and 10 ng/ml Activin A treatment (n = 47), 400 pg of mNanog injected AC (n = 52), 400 pg of mNanog injected and 10 ng/ml Activin A treatment (n = 47).
A treatment (n = 42). Error bar indicates S.E. P) RT-PCR analysis with RNA derived from stage-18 AC. Normal AC (lane 2), AC injected with 200 pg of mNanog (lane 4), or AC injected with 200 pg of mNanog and treated with Activin A (lane 5) were used. WE: whole embryo. Q–S) Secondary axis formation with mNanog injection. 400 pg of mNanog mRNA was injected into the ventral marginal zone (VMZ) at the 4-cell stage. Phenotypes were observed at stage 40. Secondary axis without head structure was observed in mNanog-injected embryo (15/30, two independent experiments). Arrow indicates a secondary axis. Scale bar; 1 mm. T, U) HE-stained histological sections of stage-40 embryo. Uninjected embryo (T). An embryo injected with 200 pg of mNanog mRNA into the VMZ (U). Arrowhead indicates notochord-like structure. Scale bar: 0.2 mm.

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**Figure 2.** mNanog possesses dorsal mesoderm-inducing activity. A) RT-PCR analysis of various marker gene expressions. Expressions of chd, gsc, and xlim-1 (dorsal mesoderm markers), Xbra (pan-mesoderm marker), Xwnt8, mix, mixer (ventral mesoderm markers), Cer, and Sox17alpha (endoderm marker) were observed. Ornithine decarboxylase (ODC) was also observed as a quantitative control. 200 pg (lane 5, 6) or 400 pg (lane 7, 8) of mNanog was injected into the AC region of 2-cell embryos. ACs were dissected at stage 9, treated with 10 ng/ml of Activin A (lane 4, 6, 8), and cultured until stage 11. Whole embryo (WE; stage 11) was also examined. B) Full-length (FL) or a deletion mutant of mNanog (deltaCD) was injected and marker gene expressions were observed. Column shows a diagram of the mNanog construct. Filled and gray boxes indicate the homeodomain (HD) and W-repeat (WR) regions, respectively. Arrow shows the position of primers for RT-PCR. Lower column shows the result. Non-injected AC (lane 1); full-length (FL) mNanog injected (lane 2); mNanog DCD injected (lane 3). The level of mNanog was also observed to check the precision of injection (4th column). C–F) The effects of mNanog injection on endogenous chd/Xbra expressions. Scale bar; 500 μm. Expressions of chd in stage-12 embryos injected with 800 pg of lacZ (C) alone or 200 pg of mNanog and 400 pg of lacZ (D) into the ventral marginal zone at the 4-cell stage. These patterns are representative of 17/17 (C) and 12/15 (D) embryos. Black arrow indicates endogenous chd expression. Expression of Xbra in stage-11 embryos injected with nothing (E) or 200 pg of mNanog and 400 pg of lacZ (F) into the ventral marginal zone at the 4-cell stage. These patterns are representative of 9/9 (E) and 8/11 (F) embryos. White arrow indicates the mNanog-injected region. Injected area was active-stained by Red-Gal (Except for (E)). D: Dorsal. V: Ventral. G) Comparison of mesodermal gene expressions between AC cells injected with several doses of mNanog (lane 3–5) and those treated with Activin A (lane 6, 7). We observed the expression of gsc (1st column), chd (2nd column), Xbra (3rd column), and ODC (4th column). H) mNanog did not induce target genes of early canonical Wnt signaling. 100 pg (lane 2), 200 pg (lane 3), or 300 pg (lane 4) of mNanog was injected into animal poles and dissected at stage 8. Similarly, 500 pg of β-catenin was injected and dissected (lane 5). Transcription of siamois (1st column) and Xnr3 (2nd column) was observed.

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tALK4 clearly suppressed Xnr1, Xnr2, gsc, and chd expressions, but not those of Xbra and xWnt8 (Fig. 3F). When tALK4 was injected, expression of Xnr1 and chd induced by mNanog was slightly suppressed, but Xnr2 and gsc expression was little changed (Fig. 3F, lane 6, 7). We further analyzed the effect of cleavage mutants of Xnr1 (cmXnr1) and Xnr2 (cmXnr2) on mesodermal gene induction by mNanog. Although cmXnr1 and/or cmXnr2 were injected, chd expression was only slightly decreased (Fig. 3G, lane 3–5). With xlim-1, coinjection with both cmXnr1 and cmXnr2 slightly reduced their respective expressions (Fig. 3G, 3rd column, lane 2, 5). Together, these results suggested that mNanog weakly modulates
mNanog modulated dorsal mesodermal marker genes by regulating BMP signaling via Xvent2

We finally examined other marker gene expressions. It is known that dorso-ventral specification in mesodermal tissue involves BMP signaling, and previous reports indicated that Xvent1 and Xvent2 facilitate BMP4 transcription, directing ventral mesodermal cell fate [16]. Our results also showed that Activin treatment induced Xvent1, Xvent2, and BMP4 expressions in AC (Fig. 4A), and when mNanog was injected, these gene expressions were obviously decreased (Fig. 4A, lane 4–6). These results suggested that dorsal mesoderm induction by mNanog is dependent on BMP signaling.

Thus, we next examined the effect of coinjecting mNanog and Xvent2. Xvent2 and 200 pg of mNanog gradually inhibited the expressions of chd, gsc, and xlan1 in a dose-dependent manner (Fig. 4B, 1st-3rd columns). BMP4 expression was detected in normal AC, and mNanog injection inhibited such expression (Fig. 4B, 4th column, lane 2). Interestingly, coinjection of mNanog with Xvent2 rescued the BMP4 expression (Fig. 4B, 4th column, lane 3–5), suggesting that mNanog suppresses Xvent2 transcription, resulting in the decreased BMP4 signaling and promotion of dorsal mesodermal gene expression.

To clarify whether mNanog function directly or indirectly affects the dorsal mesoderm gene expression, we used CHX treatment to block protein translation. Applying CHX to AC inhibited the expressions of chd, gsc, and Xv2 (Fig. 4C, lane 3, 4), and decreased the expressions of Xvent1 and Xvent2 (Fig. 4C, lane 3, 4). These data suggested that both induction of mesoderm genes and inhibition of Xvent2 expression could be indirectly regulated by mNanog.

Discussion

In this study, we showed a novel function of the Nanog gene in Xenopus embryo. In the process of LMC analysis with mNanog, we found that mNanog induces elongation of AC and expression of mesoderm marker genes. Both RT-PCR and in situ hybridization showed that mNanog effectively induces dorsal mesodermal marker genes, but not ventral mesodermal genes. This is also shown in Fig. 2G as a difference in marker gene induction between genes, but not ventral mesodermal genes. This is also shown in Fig. 4B, 4th column, lane 2). Interestingly, coinjection of mNanog with Xvent2 clearly suppressed chd, gsc, and xlan1 expression (Fig. 4B). Together with the CHX experiment, our data implicate the dorsal mesoderm-inducing activities of mNanog in the modulation of BMP signaling, possibly by indirectly regulating Xvent1/2 expression. Our results can be used to propose a model for the modulation and induction of mesoderm genes (Fig. 4D). In short, mNanog positively regulates Xv2, but it inhibits expression of BMP factors such as Xvent1/2 and BMP4, resulting in induction of chd and gsc. This function is similar to that of Tsukushi (TSK), which modulates both nodal and BMP signaling [36], suggesting that mNanog might be involved with the regulation of TSK.

Even though our experiments were conducted in an artificial system, we think they are still important in clarifying a novel mechanism involving mNanog function, as well as suggesting a novel means of endogenous mesodermal induction in Xenopus. This proposed mNanog function of mesoderm induction in itself seems opposite to its role in maintaining the undifferentiated state. However, Nanog is a possible target gene of Activin signaling [37,38], and low doses of Activin A are important in maintaining the pluripotency of ES cells in some conditions [39,40]. Although our results indicated involvement of mNanog in Activin/nodal signaling, they also suggested that mNanog contributes, at least in part, to the gene regulation mechanism around Activin/nodal signaling that underpins mesoderm formation in Xenopus. We expect that other factors involved with pluripotency, like Oct3/4 and Sox2, could also induce activity similar to that observed with mNanog, although our preliminary findings showed no mesoderm gene induction following coinjection with Sox2 or Oct61 (data not shown).

This study sought to identify the Xenopus gene homolog of mammalian Nanog by using sequences of axolotl and newt [41,42]. Although we designed six primers in homeodomain and caspase domain (Fig. S1 and M&M section) and performed seven rounds of degenerate PCR using combination of these primers, we failed to find a homolog of mammalian Nanog in Xenopus. However, we succeeded in identifying a novel function of Nanog, and we propose that mNanog modulates dorsal mesodermal marker genes by regulating BMP signaling via Xvent2.
to find any sequence identified as xNanog, although many identified were similar genes including Xvent1 (6/16) and Hoxd11 (6/16) (Fig. S1). Moreover, whole genome analysis of Xenopus tropicalis revealed no known nucleotide sequence for the XtNanog gene. Further exploration of Xenopus Nanog or another factor that substitutes for Nanog is obviously needed.

Supporting Information

Figure S1 Summary of the degenerative PCR for cloning of the Xenopus Nanog gene. Upper panel: schematic diagram of Nanog protein. CD, HD, and WR indicate the caspase domain, homeodomain, and tryptophan-rich domain, respectively. U1—2 and L1—4 indicate primer positions for the PCR. Lower panel: summary of degenerative PCR results. In Ex.6, we performed PCR with an amplified product using the U2 and L1 primers as a template. The number of obtained gene fragments is also shown. (TIF)

Table S1 The summary of phenotypes in embryos injected with mNanog into AP region. (DOCX)

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Author Contributions

Conceived and designed the experiments: TM. Performed the experiments: TM AM KI SY SN SM. Analyzed the data: TM AM YI YO SM. Contributed reagents/materials/analysis tools: TM YI YO MA SM. Wrote the paper: TM AM.

Figure 4. Dorsal mesoderm induction by mNanog was involved with inhibition of BMP signaling. A) Target genes of BMP signaling were inhibited by mNanog injection, based on the expressions of Xvent1 (1st column), Xvent2 (2nd column), BMP4 (3rd column), and ODC (4th column). 0 pg (lane 3, 4), 200 pg (lane 5), or 400 pg (lane 6) of mNanog was injected into animal poles, which were treated with 10 ng/ml of Activin A (lane 4–6) at stage 9. ACs were harvested at stage 11. B) Co-injection analysis with Xvent2 mRNA. 200 pg of mNanog (lane 2–5) and 0 pg (lane 3), 500 pg (lane 4), 1 ng (lane 5), or 2 ng (lane 6) of Xvent2 were co-injected into animal poles at the 2-cell stage. ACs were dissected at stage 9 and homogenized at stage 11 for RNA preparation. The expressions of several dorsal mesoderm genes (chd, gsc, xlim-1) and BMP4 were analyzed. C) Effect of cycloheximide (CHX) on the induction of mesoderm genes by mNanog. 0 pg (lane 1, 2) or 400 pg (lane 3, 4) of mNanog was injected into animal poles at the 2-cell stage, 0 mg/ml (lane 1, 3) or 40 mg/ml (lane 3, 4) of CHX was added. D) Model of expected mechanism of mesoderm gene induction by mNanog. “X” indicates presumptive factor(s) for regulating both Xvent1/2 and Xnr1/2 expression by mNanog.

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