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Nkx1-2 is a transcriptional repressor and is essential for the activation of Brachyury in P19 mouse embryonal carcinoma cell

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Abstract

Activation of Wnt/β-catenin signaling is crucial for the differentiation of pluripotent stem cells, namely the epiblast, embryonic stem, and embryonal carcinoma cells, into mesendoderm. However, downstream events of Wnt/β-catenin signaling that control the formation of mesendoderm are still unclear. In the present study, we used mouse P19 embryonal carcinoma cells as a model, and identified a homeodomain protein Nkx1-2 as a key regulator of mesendoderm formation. In the mouse embryo, Nkx1-2 was expressed in the primitive streak, in which the nascent mesendoderm emerges. In P19 cells, the expression of Nkx1-2 was activated by Wnt/β-catenin signaling independently of Brachyury, an evolutionary conserved early mesendoderm gene. In contrast, the expression of Nkx1-2 was both necessary and sufficient for the activation of Brachyury. Nkx1-2 acted as a transcriptional repressor to mediate the action of Wnt/β-catenin signaling to activate the Brachyury expression. We found Tcf3 as a potential target of gene repression by Nkx1-2, and the down-regulation of Tcf3 was partly required for effective activation of Brachyury by Wnt/β-catenin signaling. These results suggest that Nkx1-2 is a critical component of the gene regulatory network that operates downstream of Wnt/β-catenin signaling to regulate the formation of mesendoderm.

Keywords

Mesendoderm; Wnt/β-catenin signaling; P19 embryonal carcinoma cell; Brachyury; Primitive streak; Transcription factor

1. Introduction

The epiblast in the peri-implantation stage mammalian embryo is a group of pluripotent stem cells that are capable of giving rise to all three germ layers. The epiblast is initially a monolayer of epithelial cells. In the mouse, by the embryonic stage E6.5, cells in the future posterior side of the epiblast delaminate from the epithelial layer to form the primitive streak. The delaminated cells migrate anteriorly and laterally to give rise to mesendoderm, which is the precursor of mesoderm and definitive endoderm, whereas cells in the remaining epiblast monolayer become the ectoderm. Thus, the formation of the primitive streak marks

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Appendix A. Supplementary material Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.diff.2012.02.010.

the transition from the pluripotent state to the three germ layers (Niwa, 2007; Rossant and Tam, 2009).

The initial specification of the primitive streak involves interactions of the epiblast with the adjacent extra-embryonic tissues, specifically the extra-embryonic ectoderm and the visceral endoderm (Takaoka et al., 2007; Tam and Loebel, 2007). Several lines of experimental evidence demonstrate that activation of Wnt/β-catenin signaling is both necessary and sufficient to induce the formation of the primitive streak in the epiblast (Marikawa, 2006; Tanaka et al., 2011). For example, loss-of-function mutations of positive regulators of Wnt/β-catenin signaling, such as Wnt3, β-catenin, Lrp5/6, and porcupine, abolish primitive streak formation (Liu et al., 1999; Huelsken et al., 2000; Hsieh et al., 2003; Biechele et al., 2011). In these mutant embryos, the epiblast remains as an epithelial monolayer, and mesendoderm genes, such as Brachyury (T—Mouse Genome Informatics), are not up-regulated. On the other hand, loss-of-function of negative regulators, such as Axin1, Apc, and Tcf3 (Tcf7l1—Mouse Genome Informatics), results in expansion or duplication of the primitive streak (Zeng et al., 1997;Ishikawa et al., 2003; Merrill et al. 2004). Similar expansion or duplication of the primitive streak is also observed in embryos, in which Wnt/β-catenin signaling is ectopically activated by a Wnt ligand transgene or constitutive activation of β-catenin (Popperl et al., 1997; Kemler et al., 2004). Importantly, studies using chimeras between a wild type embryo and the β-catenin-null mutant embryonic stem (ES) cells show that the activation of Wnt/β-catenin signaling is essential in the epiblast, but not in the extra-embryonic tissues, to form the primitive streak (Huelsken et al., 2000; Barrow et al., 2007). The critical role of Wnt/β-catenin signaling in the initiation of mesendoderm formation is also evident in pluripotent stem cell lines, such as ES cell and embryonal carcinoma (EC) cell lines (Gadue et al., 2006;Lindsley et al., 2006; ten Berge et al., 2008; Sumi et al., 2008;Marikawa et al., 2009; Lyashenko et al., 2011). Mouse P19 EC cells exhibit molecular and developmental characteristics similar to the epiblast (Vidricaire et al., 1994; Niwa, 2007; Marikawa et al., 2009). We previously showed in P19 cells that knockdown of either Wnt3 or β-catenin abrogates the activation of primitive streak-specific genes, including Brachyury, in response to embryoid body formation, and that the activation of Wnt/β-catenin signaling is both necessary and sufficient to up-regulate various primitive streak-specific genes without embryoid body formation (Marikawa et al., 2009).

Despite its pivotal role, the downstream event of Wnt/β-catenin signaling, specifically how it initiates the formation of the primitive streak in the epiblast, is still unclear. The Brachyury gene is known to be a direct target of Wnt/β-catenin signaling, as its upstream promoter sequence contains the TCF/LEF-binding sites that are essential for gene activation in response to Wnt/β-catenin signaling (Yamaguchi et al., 1999b; Arnold et al., 2000). However, Brachyury-null mutant embryos form the primitive streak and appear to develop normally up to about E8.0, while at later stages they exhibit severe caudal defects (Beddington et al., 1992;Rashbass et al., 1994; Wilson and Beddington, 1997). Thus, the phenotype of the Brachyury mutation is similar to the null-mutation of Wnt3a, a Wnt ligand gene that is expressed in the caudal end at later stages (Takada et al., 1994; Yoshikawa et al., 1997), but it is clearly different from the phenotype of Wnt3- or β-catenin-mutant, both of which are essential for the initiation of the primitive streak formation (Liu et al., 1999; Huelsken et al., 2000). Therefore, Brachyury is either dispensable at early stages or operates together with other downstream targets of Wnt/β-catenin signaling to initiate mesendoderm formation in the epiblast.

In the present study, we used P19 cell line as a model of the epiblast to investigate the downstream molecular events of Wnt/β-catenin signaling and to identify the regulators that are involved in mesendoderm formation. We found that a homeodomain-containing transcription factor Nkx1-2 (also known as Sax1) was up-regulated by Wnt/β-catenin

signaling even in the absence of Brachyury activation. In contrast, the activation of Brachyury by Wnt/β-catenin signaling was abolished when Nkx1-2 was absent, whereas overexpression of Nkx1-2 was sufficient to up-regulate Brachyury. We also showed that Nkx1-2 acts as a transcriptional repressor to up-regulate Brachyury, and that one of the potential targets of Nkx1-2 was Tcf3, of which repression was partly required for maximum activation of Brachyury in response to Wnt/β-catenin signaling. Our results suggest that Nkx1-2 is a critical component of the gene regulatory network that is triggered by Wnt/β-catenin signaling to drive pluripotent cells into the mesendoderm linage.

2. Materials and methods

2.1. Cell culture

P19 EC cells (American Type Culture Collection, Manassas, VA) were cultured in MEM Alpha Medium (Invitrogen, Carlsbad, CA) with 2.5% fetal bovine serum and 7.5% calf serum. Mouse ES cells (from C57BL/6; a gift from Dr. R.C. Allsopp) were cultured in ESGRO Complete Clonal Grade Medium (Millipore, Billerica, MA) without feeder cells. BIO ([2′Z,3′E]-6-bromoindirubin-3′-oxime) was dissolved in dimethyl sulfoxide (DMSO) at 10 mM as a stock. Cell aggregates were generated by hanging drop culture, as described previously (Marikawa et al., 2009).

2.2. Microarray analysis

Total RNA was extracted from P19 cells that were treated with 2 μM BIO or vehicle control (0.02% DMSO) for 24 h, using TRI reagent (Molecular Research Center, Cincinnati, OH). cDNA probes were synthesized, using Genechip Expression 3′ Amplification One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA) and Bioarray High Yield RNA Transcript Labeling Kit (T7) (Enzo Life Sciences, Farmingdale, NY), and were hybridized to mouse genome 430 A 2.0 Array (Affymetrix), according to the manufacturer's protocol. Signals were scanned using Genechip Scanner 3000-7G (Affymetrix), and analyzed with GeneChip Operating Software (Affymetrix). Data were exported to Microsoft Excel sheet (Supplemental Materials), and probe sets with absent calls for BIO-treated samples (for the analysis of genes that are up-regulated by Wnt/β-catenin signaling) and vehicle control samples (for the analysis of genes that are down-regulated by Wnt/β-catenin signaling) were filtered out. Experiment was conducted using two independent sets of samples, presented as Experiments 1 and 2 in the Excel sheet.

2.3. Plasmids and transfection

The plasmids encoding control (non-target) shRNA, Brachyury shRNA, Tcf3 shRNA and Nkx1-2 shRNA correspond to SHC002, TRCN0000082006, TRCN0000095456 and TRCN0000084834, respectively (Sigma-Aldrich, St. Louis, MO). Nuclear GFP (nucGFP) expression construct (pEF/nuc/myc/GFP; Invitrogen), GAL4-luciferase reporter plasmid (pG5-Luc; Invitrogen), GAL4 DNA-binding domain construct (pBIND; Invitrogen), and Tcf3 expression construct (pCMV6-XL5-TCF7L1; OriGene Technologies, Rockville, MD) were obtained commercially. Expression plasmids encoding the nuclear β-galactosidase (nucβ-gal) and the constitutively active β-catenin (pt-β-catenin; Yost et al., 1996) were generous gifts from Dr. N.J. Armstrong and Dr. R.T. Moon, respectively. Expression plasmids encoding Brachyury (Bra), EnR-Bra, Nkx1-2, VP16-Nkx1-2, EnR-Nkx1-2, GAL4-Nkx1-2, GAL4-Nkx1-2Δmyc, GAL4-Nkx1-2ΔN, GAL4-Nkx1-2ΔC, and GAL4-Nkx1-2ΔH were generated, as described in Supplemental Materials. For transfection, 2×10^4 cells were seeded in each well of 24-well plates, and plasmids were transfected on the following day using Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

cDNA was synthesized from total RNA, using oligo dT(18) primer and M-MLV reverse transcriptase (Promega). Real-time PCR was performed using iCycler thermal cycler with MyiQ single color real-time PCR detection system (Bio-Rad, Hercules, CA). cDNA was amplified using iQ SYBR Green Supermix (Bio-Rad) with the following conditions: the initial denaturation at 94 ° (5 min) followed by up to 45 cycles of 94 ° (15 s), 60 ° (20 s) and 72 ° (40 s). The primer sequences for RT-PCR are shown in Supplemental Materials. Gapdh levels were used to normalize the expression levels of all other genes. All experiments were conducted using three independent sets of samples (biological replicates), and data are presented as average±standard deviation.

2.5. Mouse embryos and whole-mount in situ hybridization

B6D2F1 (C57BL/6 × DBA/2; National Cancer Institute, Frederick, MD) female mice were mated with males of the same genotype, and pregnant females were euthanized to obtain embryos. The protocol of animal handling and treatment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii. Whole-mount in situ hybridization was performed according to the standard protocol (Wilkinson and Nieto, 1993). Digoxigenin (DIG)-labeled RNA probes for Nkx1-2 and Brachyury were synthesized with T7 RNA polymerase (Promega) and DIG RNA labeling mix (Roche) from the corresponding templates. The Nkx1-2 template plasmid was generated by subcloning the DNA fragment encoding the Nkx1-2 open reading frame into pGEM-T Easy vector (Promega). The Brachyury template plasmid was a generous gift from Dr. B.G. Herrmann (Wilkinson et al., 1990).

2.6. Dual-luciferase assay

Cells were transfected with 200 ng/well of pG5-Luc (encoding the firefly luciferase) and 400 ng/well of the pBIND plasmid (encoding the GAL4 construct and the Renilla luciferase), and lysed 24 h later for reporter analyses using the Dual-Luciferase Reporter Assay System (Promega) with Gene Light 55 Luminometer (Microtech, Chiba, Japan), according to the manufacturers' instructions. All experiments were conducted using three independent sets of samples, and data are presented as average ± standard deviation.

3. Results

3.1. The expression of Nkx1-2 is activated by Wnt/β-catenin signaling independently from Brachyury

Previously, we showed that mouse P19 embryonal carcinoma cells respond to activation of Wnt/β-catenin signaling by up-regulating various primitive streak-specific genes, such as Brachyury, Wnt3, Wnt3a, Fgf8, Cdx2, and Hoxb1, within 24 h (Marikawa et al., 2009). To identify other primitive streak-specific genes that are under the control of Wnt/β-catenin signaling, we conducted genome-wide transcript analysis using the Affymetrix microarray system. To activate Wnt/β-catenin signaling, P19 cells were treated for 24 h with 2 μM BIO, a GSK3 inhibitor (Sato et al., 2004; Marikawa et al., 2009). Gene expression profiles were compared between BIO-treated and untreated P19 cells, using two independent sets of samples. To select candidate genes that are consistently activated by Wnt/β-catenin signaling, we focused on the gene probes whose hybridization signal was 3-fold or higher in BIO-treated than in untreated P19 cells in both sets of comparisons (Table S1). These genes included the previously identified downstream targets of Wnt/β-catenin signaling, such as Cdx1, T (Brachyury), Fst (Follistatin), and Axin2 (Yamaguchi et al., 1999b; Arnold et al., 2000; Prinos et al., 2001; Willert et al., 2002; Jho et al., 2002;Lickert et al., 2005), thus validating the expression profiling approach.

Brachyury is one of the known direct targets of Wnt/β-catenin signaling (Yamaguchi et al., 1999b; Arnold et al., 2000) and acts as a transcriptional activator (Conlon et al., 1996). Thus, some of the candidate genes that we selected might have been up-regulated by BIO indirectly through the activation of Brachyury. Because our main goal is to identify the genes that are activated by Wnt/β-catenin signaling independently from Brachyury, we conducted the following experiment to exclude those genes whose up-regulations by BIO were dependent on Brachyury. P19 cells were first transfected with the plasmid encoding Brachyury-specific shRNA or non-target (control) shRNA. Twenty four hours after transfection, BIO (2 μM) or vehicle only (DMSO; 0.02%) was added to the culture medium together with puromycin (10 μg/mL; to eliminate untransfected cells). After another 24 h of culture, cells were analyzed by quantitative RT-PCR (qRT-PCR) for the expression levels of the candidate genes (Fig. 1A). The knockdown of Brachyury was effective, as BIO treatment in the presence of the Brachyury shRNA was unable to up-regulate the Brachyury expression above the basal level. Nkx1-2, Sp5, Axin2, Cdx1, and Cdx2, were still robustly up-regulated by BIO even with the Brachyury knockdown (Fig. 1A), indicating that their activation was not mediated by Brachyury. In contrast, up-regulation of Fgf8 and Fst by BIO was significantly diminished by Brachyury knockdown (Fig. 1A, S1), suggesting that the activation of these genes by Wnt/β-catenin signaling was partly mediated by Brachyury. In the present study, we particularly focused on Nkx1-2, because it encodes a homeodomain-containing protein, which potentially acts a regulator of transcriptional networks, and its role in mesendoderm differentiation has not been studied before. Furthermore, no Nkx1-2 mouse knockout has been reported at this point (Mouse Genome Informatics).

First, we examined the spatial expression pattern of Nkx1-2 during early mouse development by the whole-mount in situ hybridization. While the expression of Nkx1-2 was indistinct at E6.0–E6.5 (not shown), it is clearly localized to the primitive streak by E6.5–E7.0, in a pattern similar to that of Brachyury (Fig. 1B). At E7.5–E9.0, the Nkx1-2 expression was caudally restricted, although its expression is excluded from the most caudal end of the embryo (Fig. 1B; white arrows). The expression patterns of Nkx1-2 at these stages were slightly but distinctly different from Brachyury, which was strongly expressed at the most caudal end (Fig. 1B; white arrows). These expression patterns are consistent with those described in the previous reports (Schubert et al., 1995; Tamplin et al., 2008).

To gain further insight into the relationship between Nkx1-2 and Brachyury expressions, we examined the time-course of their activation in response to Wnt/β-catenin signaling (Fig. 1C). The expression of Brachyury was essentially unchanged from the basal level after 1 h of BIO treatment, but it was significantly elevated by about 50% after 2 h of BIO treatment. In contrast, the expression of Nkx1-2 was significantly elevated from the basal level already after 1 h of BIO treatment, and it was increased by about 3-fold after 2 h of BIO treatment. Among the other genes examined, only Sp5 was significantly activated after 1 h of BIO treatment, but neither of Cdx1, Cdx2, or Fgf8 was (Fig. 1C). Thus, the timing of Nkx1-2 activation in response to BIO precedes that of Brachyury activation, which further supports that Wnt/β-catenin signaling activates Nkx1-2 independently of Brachyury.

We noticed that the expression of Nkx1-2 in P19 cells appeared to be enhanced by the knockdown of Brachyury (Fig. 1A). Also, as mentioned above, Nkx1-2 expression was weaker in the most caudal end of embryos, where Brachyury was strongly expressed (Fig. 1B). These observations raise the possibility that Brachyury may act negatively on the Nkx1-2 expression. To test this possibility, we generated P19 cells, in which Brachyury expression was stably knocked down by genomic integration of the shRNA plasmid, and examined the expression of Nkx1-2 during the development of embryoid bodies or cell aggregates. We have previously shown that P19 cell aggregates during 6 days of hanging drop culture in the presence of 1% DMSO exhibit temporal gene expression patterns that are

similar to those that take place in the primitive steak (Marikawa et al., 2009). Stable transfection of the Brachyury shRNA plasmid was sufficient to reduce the expression of Brachyury down to about 20% of the control level at Day 3 of aggregation culture, when the control cell aggregates reached the highest peak of Brachyury expression (Fig. 1C). The temporal expression pattern of Nkx1-2 in the control cell aggregates was similar to that of Brachyury, such that it steadily increased during the first 3 days of aggregation culture to reach the highest peak at Day 3, after which it declined (Fig. 1C). However, in the Brachyury knockdown aggregates, the Nkx1-2 expression was continuously up-regulated beyond 3 day of aggregation culture, and it reached the highest level at Day 5, which was about 4-fold more than the highest peak in the control aggregates at Day 3 (Fig. 1C). This suggests that Brachyury negatively regulates the expression of Nkx1-2 at later stages of aggregation culture, namely at Days 4 and 5. Importantly, the expressions of Fgf8 and Wnt3a during aggregation culture were repressed by the Brachyury knockdown (Fig. 1C). Both Fgf8 and Wnt3a are normally expressed in the primitive streak and the caudal end of the embryo, and their expressions are compromised in Brachyury mutant embryos (Rashbass et al., 1994). Thus, the shRNA-mediated knockdown of Brachyury in P19 cells was functionally effective in terms of Fgf8 and Wnt3a expression patterns.

We then tested whether Brachyury is sufficient to repress the expression of Nkx1-2. Brachyury was overexpressed in P19 cells by transient transfection of the expression plasmid encoding the mouse Brachyury (Fig. 1D). However, the overexpression of Brachyury lowered neither the basal expression of Nkx1-2 in untreated cells nor the up-regulated expression in BIO-treated cells (Fig. 1E). Thus, Brachyury is not sufficient for the repression of Nkx1-2, although it is required.

Brachyury has been shown to function as a transcriptional activator (Conlon et al., 1996). Nonetheless, the above result suggests that it may be involved in the transcriptional repression of Nkx1-2, raising the possibility that Brachyury can also act as a transcriptional repressor. To test this possibility, we generated the fusion construct EnR-Bra, in which the transcriptional repressor domain of Drosophila Engrailed (EnR) was attached to the mouse Brachyury (Fig. 1D). This fusion construct is similar to EnR-Xbra, in which EnR is attached to the Xenopus homolog of Brachyury, Xbra (Conlon et al., 1996). In Xenopus embryos, EnR-Xbra acts as a transcriptional repressor, and antagonizes the mesoderm inducing action of Xbra (Cunliffe and Smith, 1992;Conlon et al., 1996). If mouse Brachyury were to act as a transcriptional repressor in P19 cells, EnR-Bra is likely to behave similarly to the normal Brachyury. However, the overexpression of EnR-Bra significantly up-regulated the expression of Nkx1-2 (Fig. 1E), which was similar to the effect of the Brachyury knockdown (Fig. 1A), indicating that EnR-Bra antagonized the action of Brachyury. Furthermore, EnR-Bra significantly suppressed the transcriptional activations of Lhx1, Fabp7, and Tmem164 that were induced by Brachyury overexpression in P19 cells (Fig. S2), confirming that EnR-Bra acts as a transcriptional repressor to antagonize the action of Brachyury. These results suggest that the action of Brachyury as a transcriptional activator is critical for the repression of Nkx1-2.

3.2. Nkx1-2 is required for the activation of Brachyury expression by Wnt/β-catenin signaling

To examine the role of Nkx1-2 in mesendoderm induction, we knocked down its expression in P19 cells using the specific shRNA plasmid. The expression level of Nkx1-2 was reduced by about 75% in untreated cells and by about 80% in BIO-treated cells by the transfection of the Nkx1-2-specific shRNA plasmid (Fig. 2A). This knockdown was effective enough to prevent the up-regulation of Nkx1-2, as the Nkx1-2 level in BIO-treated knockdown cells was lower than the basal level in untreated control cells. We then examined the expressions of several genes that are normally expressed in the primitive streak, namely Brachyury, Sp5,

Cdx2, and Wnt3. The expressions of Brachyury, Sp5, and Cdx2 in BIO-treated cells were significantly diminished by the knockdown of Nkx1-2 (Fig. 2A), suggesting that the activation of these primitive streak-specific genes by Wnt/β-catenin signaling is dependent on Nkx1-2. The negative impact of Nkx1-2 knockdown on the Brachyury up-regulation was not due to off-target effect of the shRNA (Jackson et al., 2003), because the expression of Brachyury was rescued by exogenous Nkx1-2 in the presence of the shRNA (Fig. S3). Interestingly, Nkx1-2 knockdown did not significantly diminish up-regulation of Wnt3 by BIO (Fig. 2A). This indicates not only that Wnt3 expression is independent of Nkx1-2, but also that Nkx1-2 knockdown did not compromise the action of BIO. The latter notion was also supported by the expression pattern of Foxd3. Foxd3 is a transcription factor that is essential for the maintenance of pluripotentcy (Hanna et al., 2002), and its expression is markedly down-regulated by the activation of Wnt/β-catenin signaling in P19 cells (Marikawa et al., 2009). Here, the down-regulation of Foxd3 by BIO treatment was not affected by the Nkx1-2 knockdown (Fig. 2A), which further supports that the activation of Wnt/β-catenin signaling by BIO was not impaired by Nkx1-2 knockdown.

Lack of Brachyury activation by BIO in the absence of Nkx1-2 is of particular interest, because Brachyury is known to be a direct target of Wnt/β-catenin signaling. However, the direct activation of Brachyury by Wnt/β-catenin signaling has been demonstrated in the context of mouse ES cells (Yamaguchi et al., 1999a, b; Arnold et al., 2000). Thus, it is possible that the impact of Nkx1-2 on Brachyury expression may be different between P19 and ES cells. To test this possibility, we knocked down Nkx1-2 in mouse ES cells, and examined the transcriptional response of Brachyury to Wnt/β-catenin signaling. Both Nkx1-2 and Brachyury were up-regulated in control ES cells by BIO treatment (Fig. 2B). However, the Brachyury up-regulation was also significantly diminished by the knockdown of Nkx1-2 (Fig. 2B). These results suggest that in both P19 and ES cells, Nkx1-2 is required for the activation of Brachyury by Wnt/β-catenin signaling.

Brachyury and other primitive streak-specific genes can be up-regulated in P19 cells by cell aggregation (Vidricaire et al., 1994;Yamaguchi et al., 1999a; Marikawa et al., 2009). Thus, we examined whether Nkx1-2 is also required for the activation of the primitive streak-specific genes in cell aggregates. P19 cells were transfected with either the control or Nkx1-2 shRNA plasmid, and after 24 h they were used for cell aggregation. The gene expression levels were analyzed by qRT-PCR prior to aggregation (Day 0) and after 1 day of aggregation culture (Day 1). The Nkx1-2 level was significantly knocked down by about 80% at Day 1 (Fig. 2C). Similarly, the up-regulations of Brachyury, Sp5, Cdx2, and Fgf8 at Day 1 were significantly diminished by the knockdown of Nkx1-2 (Fig. 2C), indicating that the activation of these primitive streak-specific genes by cell aggregation is also dependent on Nkx1-2. In contrast, the activation of Wnt3 was only slightly, but not significantly, impaired by the Nkx1-2 knockdown (Fig. 2C). This result further supports that the Wnt3 expression is largely independent of Nkx1-2.

3.3. Nkx1-2 is sufficient to up-regulate Brachyury through its action as a transcriptional repressor

We have so far shown that Nkx1-2 is necessary for the activation of Brachyury. To test whether Nkx1-2 is sufficient for the activation of Brachyury, we overexpressed Nkx1-2 in P19 cells through the transfection of the plasmid construct, which drives the expression of mouse Nkx1-2 under the control of a potent and ubiquitous enhancer of the human elongation factor 1α gene (Fig. 3A). P19 cells were transfected with the Nkx1-2 expression plasmid, and analyzed after 24 h for the expression of Brachyury by qRT-PCR. The Brachyury expression level was significantly elevated by 2- to 3-fold by the overexpression of Nkx1-2 compared to the control transfection (Fig. 3B), suggesting that Nkx1-2 is sufficient to activate Brachyury expression.

We then investigated whether Nkx1-2 acts as a transcriptional activator in order to up-regulate the expression of Brachyury. Two types of fusion constructs were generated: VP16-Nkx1-2 and EnR-Nkx1-2. VP16-Nkx1-2 consisted of the transcriptional activation domain of herpes simplex virus VP16 protein (Sadowski et al., 1988; Triezenberg et al., 1988) that is attached to the N-terminus of Nkx1-2, and EnR-Nkx1-2 consisted of EnR attached to Nkx1-2 (Fig. 3A). When P19 cells were transfected, VP16-Nkx1-2 did not activate the expression of Brachyury, whereas EnR-Nkx1-2 significantly activated it by 4- to 5-fold (Fig. 3B). Thus, EnR-Nkx1-2, rather than VP16-Nkx1-2, behaved similarly to normal Nkx1-2, suggesting that Nkx1-2 acts as a transcriptional repressor to up-regulate the Brachyury expression.

To verify that Nkx1-2 is a transcriptional repressor, we generated another fusion construct GAL4-Nkx1-2, in which the DNA-binding domain of yeast GAL4 protein was attached to the N-terminus of Nkx1-2 (Fig. 3A). P19 cells were transfected with GAL4-Nkx1-2 together with the pG5-Luc plasmid, in which the firefly luciferase gene is located downstream of the GAL4-binding sites. The luciferase level was significantly lowered by GAL4-Nkx1-2, down to about 50% of the control level (Fig. 3C), indicating that the Nkx1-2 moiety contains a transcriptional repressor activity. To further determine which regions of Nkx1-2 are required for the transcriptional repression, we generated a series of deletion constructs of GAL4-Nkx1-2 (Fig. 3A). The deletions of the N-terminal domain (GAL4-Nkx1-2ΔN) or the C-terminal domain (GAL4-Nkx1-2ΔC) did not significantly affect the transcriptional repressor activity. In contrast, the deletion of the homeodomain and a part of the C-terminal domain (GAL4-Nkx1-2ΔH) significantly impaired the transcriptional repressor activity (Fig. 3C). This suggests that the region responsible for transcriptional repression resides within the homeodomain.

3.4. Repression of Tcf3 by Nkx1-2 is required for the effective activation of Brachyury by Wnt/β-catenin signaling

Nkx1-2 acts as a transcriptional repressor, but is involved in the transcriptional activation of Brachyury. Thus, it is possible that Nkx1-2 represses the expression of a transcriptional regulator that acts as a repressor of the Brachyury expression. To search for potential transcriptional mediators of the Nkx1-2 action, we re-examined the microarray data (Supplementary Materials) with the rationale that such mediators should be down-regulated by BIO treatment, which activates Nkx1-2. We selected the gene probes, whose hybridization signal for the BIO-treated cells was 50% or less compared to untreated cells in both sets of the microarray data (Table S2). From this collection, we selected known transcription factors, namely Dax1 (Nr0b1—Mouse Genome Informatics), Tcf3, and Foxd3, and examined their expression levels in response to the overexpression of Nkx1-2. Tcf3 was significantly down-regulated by the Nkx1-2 overexpression, whereas Dax1 and Foxd3 were not (Fig. 4A). Thus, Nkx1-2 was sufficient to repress the expression of Tcf3.

Tcf3 is one of the four LEF/TCF family members, which are known to act downstream of the Wnt/β-catenin signaling pathway (Arce et al., 2006). We examined the expressions of the other three members, Tcf4 (Tcf7l2—Mouse Genome Informatics), Tcf7, and Lef1. But, none of them was down-regulated by the overexpression of Nkx1-2 (Fig. 4A), indicating that Tcf3 is the only LEF/TCF family member that is repressed by Nkx1-2. In addition, the expression levels of a housekeeping gene EF1α (Eef1a1—Mouse Genome Informatics) and a pluripotency regulator Oct4 (Pou5f1—Mouse Genome Informatics) were not significantly affected by the overexpression of the Nkx1-2.

To test whether the repression of Tcf3 is required for the up-regulation of Brachyury in response to the overexpression of Nkx1-2, we co-transfected P19 cells with the Nkx1-2 expression plasmid with the Tcf3 expression plasmid. The up-regulation of Brachyury by

Nkx1-2 was slightly (by 25–30%) but significantly diminished by the overexpression of Tcf3 (Fig. 4B). In addition, we tested whether the repression of Tcf3 is required for Brachyury activation in response to Wnt/β-catenin signaling. P19 cells were co-transfected with pt-β-catenin, a constitutively active form of β-catenin (Yost et al., 1996) with or without Tcf3. The activation of Brachyury by pt-β-catenin was also significantly lowered by the overexpression of Tcf3 (Fig. 4C). In contrast, Tcf3 overexpression did not diminish the up-regulation of Nkx1-2 by pt-β-catenin, indicating that the activation of Wnt/β-catenin signaling was not impaired by Tcf3 (Fig. 4B).

We then examined whether down-regulation of Tcf3 is sufficient to activate the Brachyury expression. The expression of Tcf3 was knocked down by about 80–85% through the transfection of specific shRNA plasmid (Fig. 4D). This treatment was sufficient to significantly up-regulate Brachyury expression (Fig. 4D). Interestingly, the knockdown of Tcf3 was also sufficient to up-regulate Wnt3, raising the possibility that the positive feedback regulation between Wnt/β-catenin signaling and Wnt3 transcription (Marikawa et al., 2009) may be mediated through the down-regulation of Tcf3. In addition, the knockdown of Tcf3 restored the BIO-induced Brachyury activation that was impaired by the Nkx1-2 knockdown (Fig. 4E), further supporting that the down-regulation of Tcf3 is sufficient to activate Brachyury even in the absence of Nkx1-2. Lastly, while the overexpression of Nkx1-2 was sufficient to repress the expression of Tcf3 below the basal level (Fig. 4A), the knockdown of Nkx1-2 did not elevate the Tcf3 expression above the basal level (Fig. 4F). These results suggest that the repression of Tcf3 by elevated Nkx1-2 is responsible, at least partly, for the activation of Brachyury in response to Wnt/β-catenin signaling.

4. Discussion

Various studies have shown unequivocally that the activation of Wnt/β-catenin signaling acts as the initiation cue for the formation of the primitive streak in the epiblast (Marikawa, 2006; Tam et al., 2006; Tanaka et al., 2011). However, the downstream targets of Wnt/β-catenin signaling that are responsible for the transformation of pluripotent epiblast into mesendoderm are still unknown. The present study employed P19 cells as an in vitro epiblast model, and identified Nkx1-2 as a critical component of the gene regulatory network that comes into effect downstream of Wnt/β-catenin signaling to induce mesendoderm (Fig. 5). In this model, Wnt/β-catenin signaling first activates the expression of Nkx1-2. Five consensus recognition sequences for TCF/LEF (5′-(A/T)(A/T)CAAAG-3′) are present within the 5 kilobase upstream from the translational start codon of the Nkx1-2 gene (Supplemental Materials). However, our preliminary study did not reveal that the 5 kilobase DNA fragment was sufficient for robust transcriptional activation in response to Wnt/β-catenin signaling (data not shown). Thus, it is currently not clear whether Nkx1-2 is a direct target of Wnt/β-catenin signaling, although we showed in the present study that the activation of Nkx1-2 in response to BIO treatment occurred earlier than the other primitive streak-specific genes, such as Brachyury, Cdx1, and Cdx2, all of which are known to be directly controlled by Wnt/β-catenin signaling (Yamaguchi et al., 1999b; Arnold et al., 2000; Prinos et al., 2001; Willert et al., 2002). Nkx1-2 acts as a transcriptional repressor to down-regulate Tcf3 and likely other genes, which allows the up-regulation of mesendoderm genes, such as Brachyury. Brachyury is known to be a direct target of Wnt/β-catenin signaling (Yamaguchi et al., 1999a, b; Arnold et al., 2000). But, as demonstrated in the present study, its activation is dependent on the presence of Nkx1-2. This suggests that Nkx1-2 provides the competence to the Brachyury gene in order to respond to Wnt/β-catenin signaling. The down-regulation of Tcf3 is likely to be one of the key factors for this competence, because its overexpression diminished the activation of Brachyury in response to β-catenin and also its knockdown was sufficient to up-regulate Brachyury expression.

However, it is likely that other factors in addition to the Tcf3 repression are also involved in the competence for Brachyury activation. This is because the Brachyury was still substantially up-regulated by pt-β-catenin even when Tcf3 was overexpressed. We previously demonstrated that a pluripotency regulator Oct4 acts as a competence factor for Brachyury (Marikawa et al., 2011). However, the expression level of Oct4 was unaffected by the overexpression of Nkx1-2, suggesting that Oct4 and Nkx1-2 function as competence factors through different mechanisms.

Transcription factors that are expressed in the primitive streak, such as Nkx1-2 and Brachyury, appear to form an intricate gene regulatory network. The nature of the regulatory network may change significantly along the progression of development. For example, at early stages (E6.5–E7.0), both Brachyury and Nkx1-2 appear to be co-expressed in the primitive streak, and the expression of Brachyury is likely to be dependent on Nkx1-2, based on the present in vitro studies using P19 and ES cells. However, at later stages (E7.5–E9.0), their expression domains are segregated from each other, possibly due to repression of Nkx1-2 by Brachyury. Brachyury has been shown to act as a transcriptional activator (Conlon et al., 1996), and our present study suggests that the action of Brachyury as a transcriptional activator is responsible for the transcriptional repression of Nkx1-2. This implicates the existence of a mediator, which is turned on by Brachyury at the later stages to represses the expression of Nkx1-2 (Fig. 5). Further studies are required to determine the nature of this Brachyury-mediated repression of Nkx1-2, particularly whether it occurs in a cell-autonomous or non-cell-autonomous manner. Notably, the previous study shows that the caudal expression of Nkx1-2 is markedly diminished in the Brachyury mutant embryo at E8.5, suggesting that Brachyury is required for the activation or maintenance of Nkx1-2 expression (Schubert et al., 1995). This appears to contradict with the present study, the latter of which implicates a negative impact of Brachyury on Nkx1-2 expression. It is possible that the gene regulatory networks operating in P19 cells may be significantly different from those in the mouse embryo, although the expressions patterns of the primitive streak-specific genes in response to Wnt/β-catenin signaling are largely consistent between P19 cells and embryos (Marikawa et al., 2009). Alternatively, the diminished Nkx1-2 expression in the Brachyury mutant embryo may be due to developmental defects in the caudal region, such as increased apoptosis (Conlon and Smith, 1999), and may not be directly linked to transcriptional regulation imposed by Brachyury. Such apoptosis in response to the loss of Brachyury may be circumvented in P19 cells under the culture condition used, which may have enabled to examine the changes in gene expression pattern, namely the up-regulation of Nkx1-2. However, we also cannot exclude the possibility that behavior of aggregates made of Brachyury-knockdown cells is abnormal such that their gene expression profiles, namely enhanced Nkx1-2 expression, may not be reflecting what would happen during embryo development.

Many transcription factors, in addition to Brachyury and Nkx1-2, have been identified that are specifically expressed in the primitive streak, such as Cdx2, Eomes, Foxa2, Gbx2, Gsc, Lhx1, Snai1, and Tbx6 (Tam et al., 2001; Lickert et al., 2005; Tam and Loebel, 2007). Null mutation of each of these genes causes distinct phenotypes, some of which are due to abnormal activities of the primitive streak, such as impaired epithelial-to-mesenchymal transition or caudal truncation (Ang and Rossant, 1994; Chapman and Papaioannou, 1998; Carver et al., 2001; Hukriede et al., 2003; Chawengsaksophak et al., 2004;Arnold et al., 2008). However, none of the mutants exhibit the phenotype equivalent to the Wnt3- or β-catenin-knockout embryos, although no Nkx1-2 mouse knockout has been reported yet at this point. Regardless, it is possible that each of these genes alone cannot be responsible for mediating the action of Wnt/β-catenin signaling. Nonetheless, these transcription factors may function redundantly and cooperate with each other to control the initiation of the primitive streak formation. It is also possible that the loss of one gene may cause the up-

regulation of other genes that could compensate for the loss. These functional redundancies among multiple genes are common particularly in the mouse embryo. Further investigations using double or triple knockout embryos as well as using in vitro systems, such as ES and P19 cells that are more amenable and convenient for various experimental manipulations, should provide deeper insight into the roles of these transcription factors in the initiation of the primitive streak formation.

In the present study, we identified Tcf3 as a potential transcriptional target of Nkx1-2 to mediate the activation of Brachyury (Fig. 5). During mouse embryo development, Tcf3 protein is initially expressed throughout the epiblast, but upon gastrulation it becomes dramatically down-regulated in the primitive streak (Merrill et al., 2004). Tcf3-null mutant embryos develop expanded or duplicated axial mesoderm structures, suggesting that Tcf3 acts as a negative regulator of mesendoderm formation. Tcf3 by itself acts as a transcriptional repressor of Wnt/β-catenin signaling target genes, but this repression is removed by the association with β-catenin protein (Barker et al., 2000; Arce et al., 2006). It has been shown in mouse ES cells that loss of Tcf3 causes the same impact on self-renewal as the activation of Wnt/β-catenin signaling through a Gsk3 antagonist (Wray et al., 2011; Yi et al., 2011). Thus, down-regulation of Tcf3 by Nkx1-2 is likely to enhance Wnt/β-catenin signaling, which is possibly reflected by the elevation of Wnt3 expression, as shown in the present study (Fig. 5). Nonetheless, it is unlikely that the activation of Brachyury in response to Tcf3 knockdown is solely due to enhanced Wnt/β-catenin signaling. The present study showed that, while the knockdown of Nkx1-2 impaired the Brachyury activation in response to BIO treatment, this impairment was rescued by concomitant knockdown of Tcf3. Furthermore, while the overexpression of Tcf3 diminished the BIO-induced Brachyury activation, it did not impact the BIO-induced Nkx1-2 activation, suggesting that Tcf3 interferes with the Brachyury expression in a mechanism that is independent from the inhibition of Wnt/β-catenin signaling. Further studies are required to elucidate the mechanisms of Tcf3-mediated Brachyury repression as well as other downstream target genes that may be repressed by Nkx1-2.

Recent studies have shown that pluripotent stem cells, such as ES cells and epiblast-stem cells (EpiSCs), are comprised of heterogeneous populations that represent different states and/or stages of development (Hayashi et al., 2008; Toyooka et al., 2008; Kalmar et al., 2009; Canham et al., 2010; Han et al., 2010). EpiSCs, which are derived from the epiblast of post-implantation egg cylinder stage (Brons et al., 2007; Tesar et al., 2007), consist of at least two subpopulations, corresponding to the early and late stages of the epiblast, which differ from each other in developmental potential, gene expression profiles, and epigenetic characteristics (Han et al., 2010). Although in the present study, we used P19 cell as a model of the epiblast to investigate the regulatory mechanisms of the primitive streak formation, it is not clear whether P19 cells actually represent a particular developmental state of the epiblast. It is possible that P19 cells may represent the late epiblast, rather than the early epiblast, based on the two observations on Nkx1-2 that were made in the present study. First, we failed to detect a localized Nkx1-2 expression in early embryos at E6.0-6.5, where Brachyury is already expressed robustly in a localized manner. While the lack of detection may be a technical issue, it is also possible that Brachyury may be activated independently of Nkx1-2 in these early stages of the epiblast. Nkx1-2 may be essential to activate Brachyury only in later stages of the epiblast, which may be represented by P19 cells. Secondly, Brachyury activation by BIO was less dependent on Nkx1-2 in ES cells relative to P19 cells. Because ES cells represent earlier stages, this observation may be indicative of that Nkx1-2 is essential for Brachyury activation mainly in the late stage of the epiblast. To delineate precise roles of Nkx1-2 in Brachyury activation and the primitive streak formation, further studies are absolutely required, including knockout of the Nkx1-2 gene.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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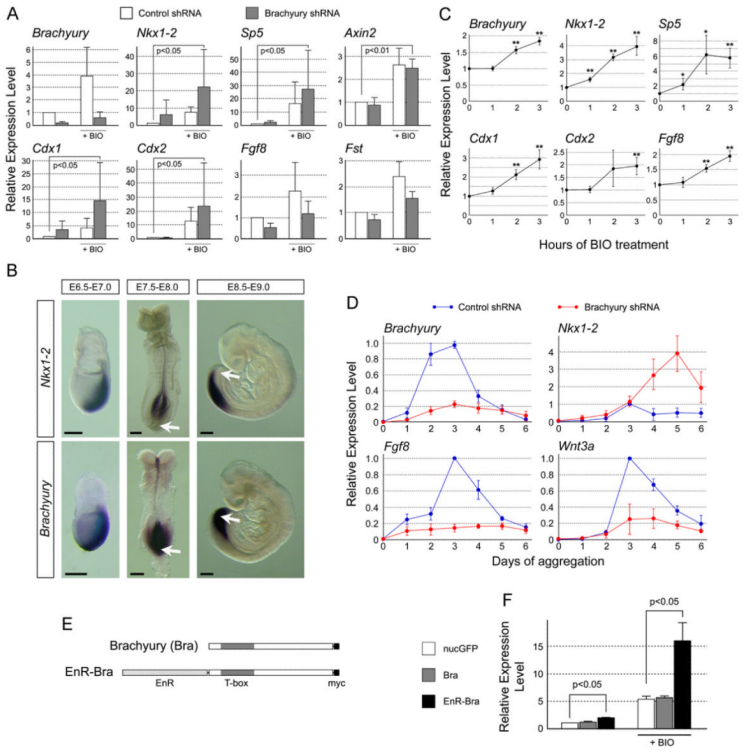

Fig. 1.

Nkx1-2 is activated by Wnt/β-catenin signaling independently of Brachyury. (A) The relative expression levels of candidate genes in Brachyury-knockdown cells in the absence or presence of BIO (2 μM), as examined by qRT-PCR. One day after transfection (400 ng shRNA plasmid/well; 24-well format), cells are cultured with puromycin (10 μg/mL) for one additional day with or without BIO, followed by RNA extraction. The data are presented as averages of three biological replicates, and error bars are standard deviations. Statistical comparisons are made by Student's *t*-test between the control shRNA without BIO treatment (i.e., basal level) and Brachyury shRNA with BIO treatment; (B) whole-mount in situ hybridization for Nkx1-2 and Brachyury in mouse embryos. White arrows point to the most caudal region, in which Nkx1-2 is underexpressed whereas Brachyury is strongly expressed. Scale bars = 200 μm; (C) gene expression time-course in response to BIO treatment, as examined by qRT-PCR. P19 cells were culture for 0, 1, 2, and 3 h in the presence of BIO (2 μM), followed by RNA extraction. The data are presented as averages of three biological replicates with standard deviations. Statistical significance of gene activation in BIO-treated cells (for 1, 2, or 3 h) relative to non-treated cells (0 h) was determined by Student's *t*-test (one asterisk: $p < 0.05$, two asterisks: $p < 0.01$); (D) temporal gene expression patterns in P19 cell aggregates that are generated from Brachyury knockdown cells. The data are presented as averages of three biological replicates with standard deviations. In each experiment, the expression level in Day 3 control aggregates is used for normalization; (E) schematic diagrams depicting the structures of Brachyury (Bra) and EnR-Bra; and (F) expression level of Nkx1-2 in response to the overexpression of nucGFP (control), Bra, or EnR-Bra by transfection of expression constructs (400 ng plasmid/well), cultured in the absence or presence of BIO (2 μM) for 24 h. The data are presented as averages and standard deviations of three biological replicates. EnR-Bra significantly up-regulates the expression of Nkx1-2 with or without BIO as examined by Student's *t*-test.

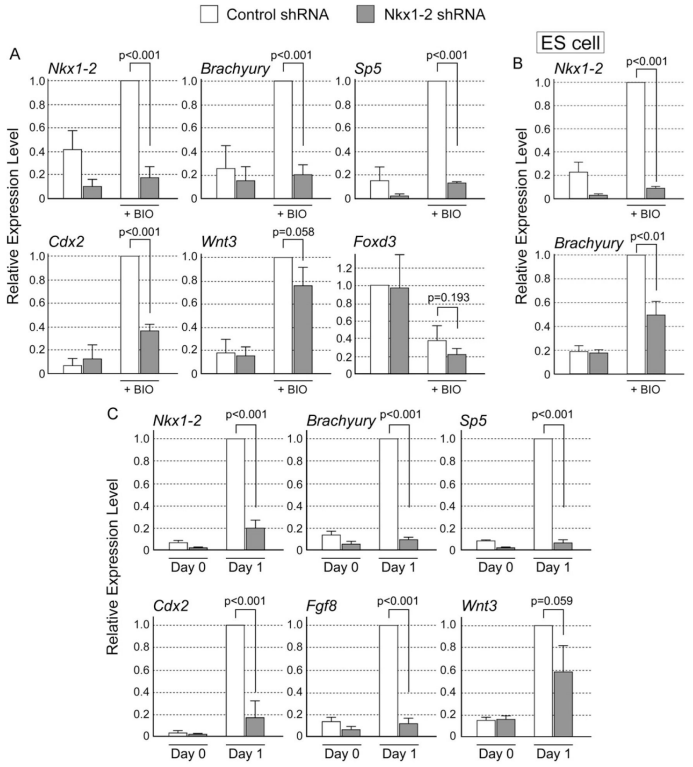

Fig. 2.

Nkx1-2 is required for the activation of Brachyury in response to Wnt/β-catenin signaling. (A) The relative expression levels of primitive streak-specific genes in Nkx1-2-knockdown cells in the absence or presence of BIO, as examined by qRT-PCR. One day after transfection (400 ng shRNA plasmid/well), cells are cultured with puromycin (10 μg/mL) for one additional day with or without BIO (2 μM), followed by RNA extraction. The data are presented as averages and standard deviations of three biological replicates. In each experiment, the expression level in control shRNA-transfected, BIO-treated cells is used for normalization, except for Foxd3, in which control shRNA-transfected, non-treated cells is used for normalization. The relative expression levels of Brachyury, Sp5, and Cdx2 in the presence of BIO are significantly diminished by the knockdown of Nkx1-2 (Student's *t*-test); (B) Brachyury activation by BIO in ES cells is also dependent on Nkx1-2. The same experimental scheme as presented in (A) is conducted for ES cells. The expression levels of Brachyury in the presence of BIO are significantly diminished by the knockdown of Nkx1-2 (Student's *t*-test); and (C) the relative expression levels of primitive streak-specific genes in P19 cell aggregates that are transiently transfected with control or Nkx1-2 shRNA plasmid. One day after transfection (800 ng shRNA plasmid/well), one group of cells are collected as Day 0 sample, and the other group of cells are aggregated by hanging drop culture for one additional day, which are collected as Day 1 sample. The expression levels of Brachyury, Sp5, Cdx2, and Fgf8 at Day 1 are significantly diminished by the knockdown of Nkx1-2 (Student's *t*-test).

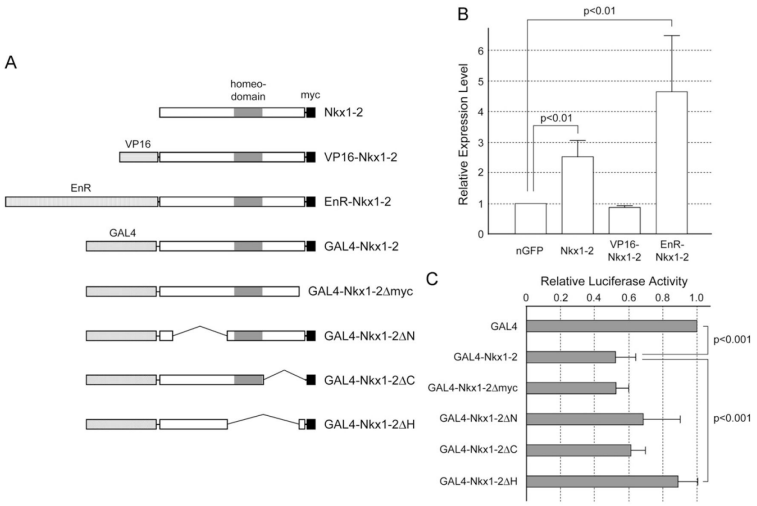

Fig. 3.

Nkx1-2 acts as a transcriptional repressor. (A) Schematic diagram depicting various Nkx1-2 constructs; (B) the relative expression levels of Brachyury in response to the overexpression of Nkx1-2 and its fusion constructs. One day after transfection (400 ng expression plasmid/well), cells are lysed for RNA extraction. Nkx1-2 and EnR-Nkx1-2 significantly up-regulate Brachyury expression (Student's *t*-test); and (C) the relative luciferase activity of the pG5-Luc reporter plasmid in response to overexpression of various GAL4-Nkx1-2 fusion constructs. GAL4-Nkx1-2 significantly represses the reporter, compared to GAL4 only control, whereas GAL4-Nkx1-2-ΔH lacks in the ability to repress pG5-Luc, compared to GAL4-Nkx1-2 (Student's *t*-test).

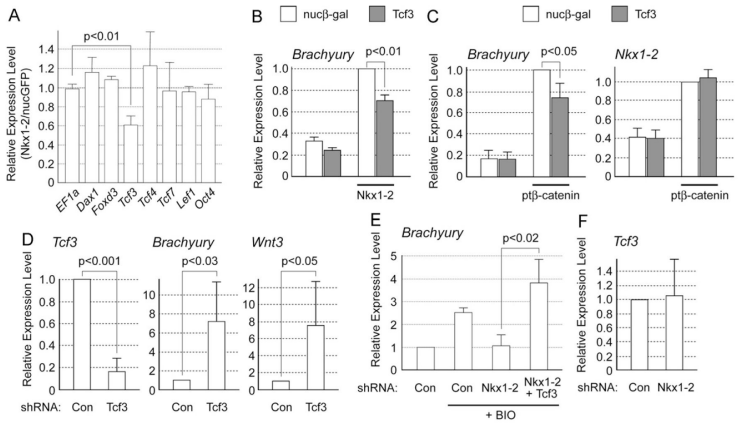

Fig. 4.

Tcf3 is repressed by Nkx1-2 overexpression. (A) Change in the relative genes expression in response to the overexpression of Nkx1-2, as examined by qRT-PCR. The values on the *y*-axis represent the ratio between control (nucGFP-transfected) and Nkx1-2-transfected cells. The Tcf3 expression level is significantly lowered by Nkx1-2 compared to control nucGFP (Student's *t*-test); (B) the relative expression levels of Brachyury in response to Nkx1-2 overexpression (400 ng/well) in the absence (transfected with 400 ng/well of the control plasmid encoding the nuclear β-galactosidase [nucβ-gal]) or presence of Tcf3 overexpression (400 ng/well). The level of Brachyury in response to Nkx1-2 overexpression is significantly lowered by Tcf3 (Student's *t*-test); (C) the relative expression levels of Brachyury and Nkx1-2 in response to pt-β-catenin (200 ng/well) in the absence or presence of Tcf3 overexpression. The level of Brachyury in response to pt-β-catenin is significantly lowered by Tcf3 (Student's *t*-test); and (D) knockdown of Tcf3 is sufficient to upregulate the Brachyury and Wnt3. The level of Tcf3 expression is significantly lowered by the transfection of shRNA plasmid against Tcf3 (400 ng/well), while the levels of Brachyury and Wnt3 expression are significantly increased (Student's *t*-test); (E) the negative impact of Nkx1-2 knockdown on the Brachyury activation is nullified by concomitant knockdown of Tcf3; and (F) knockdown of Nkx1-2 does not alter the basal expression level of Tcf3.

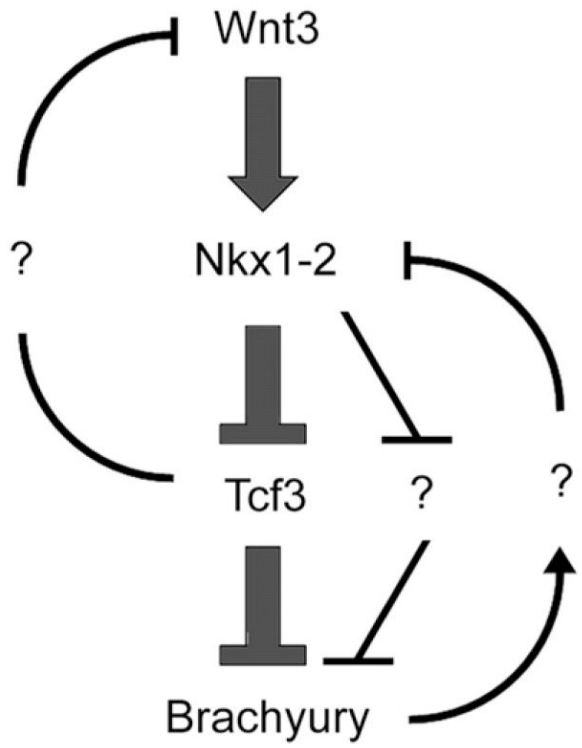

Fig. 5.
A model depicting how Wnt/β-catenin signaling initiates the gene regulatory network that involves Nkx1-2 and Brachyury.