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Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm

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Formation of mesoderm from the pluripotent epiblast depends upon canonical Wnt/β-catenin signaling, although a precise molecular basis for this requirement has not been established. To develop a robust model of this developmental transition, we examined the role of Wnt signaling during the analogous stage of embryonic stem cell differentiation. We show that the kinetics of Wnt ligand expression and pathway activity in vitro mirror those found in vivo. Furthermore, inhibition of this endogenous Wnt signaling abrogates the functional competence of differentiating ES cells, reflected by their failure to generate Flk1$^+$ mesodermal precursors and subsequent mature mesodermal lineages. Microarray analysis at various times during early differentiation reveal that mesoderm- and endoderm-associated genes fail to be induced in the absence of Wnt signaling, indicating a lack of germ layer induction that normally occurs during gastrulation in vivo. The earliest genes displaying Wnt-dependent expression, however, were those expressed in vivo in the primitive streak. Using an inducible form of stabilized β-catenin, we find that Wnt activity, although required, does not autonomously promote primitive streak-associated gene expression in vitro. Our results suggest that Wnt signaling functions in this model system to regulate the thresholds or stability of responses to other effector pathways and demonstrate that differentiating ES cells represent a useful model system for defining complex regulatory interactions underlying primary germ layer induction.

KEY WORDS: Wnt, ES cell, Mesoderm

INTRODUCTION

Formation of the primitive streak is a hallmark of anteroposterior axis patterning in mammalian embryos. The nascent primitive streak comprises a subset of cells in the posterior epiblast adjacent to the extra-embryonic ectoderm that display a characteristic pattern of gene expression (Rossant and Tam, 2004). Subsequently, the streak extends anteriorly as cells of the prospective mesoderm and definitive endoderm ingress through the streak and migrate to appropriate locations in the embryo (Tam and Behringer, 1997). Generation of the primitive streak is regulated by multiple pathways, including Nodal, Wnt and Bmp (Rossant and Tam, 2004). These factors and their inhibitors are elaborated from diverse locations within the embryonic and extra-embryonic tissues, suggesting that integration of their graded actions may be important in regulating spatially appropriate cellular responses (Robb and Tam, 2004). In fact, regionalized cell fates in the epiblast are highly correlated with their position along the combined gradient of Nodal/Wnt/Bmp activity (Lawson et al., 1991; Lawson, 1999; Tam et al., 2003).

Among these signaling pathways, the precise mechanism of Wnt signaling in promoting specific programs of gene expression is least clear. Wnt activity is generally thought to induce target genes directly via the β-catenin-dependent conversion of TCF family transcription factors from a repressive to an active state (Eastman and Grosschedl, 1999). Alternatively, it has been proposed that Wnt signaling possesses little intrinsic capacity to activate gene expression, but rather functions in part by modifying or stabilizing the effector activity of other factors (Arias and Hayward, 2006). In mice that lack *Tcf3*, for example, embryos develop expanded and duplicated axial mesodermal structures, consistent with the suggestion that Wnt signaling during anteroposterior axis formation acts to relieve basal repression of posterior genes, allowing their further activation by secondary signals (Merrill et al., 2004).

Canonical Wnt signaling is absolutely required for primitive streak formation. Mice deficient in the canonical ligand Wnt3, in the Wnt co-receptors Lrp5/6 or in the intracellular effector of canonical Wnt signaling, β-catenin, all fail to develop a primitive streak and lack mesoderm (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999). Despite this clear role of Wnt signaling during early embryogenesis in vivo, no requirement for Wnt signaling in regulating analogous stages of ES cell differentiation has been described (Keller, 2005). However, a role for Wnt signaling in ES cell-derived germ layer induction has been suggested by studies showing that Wnt signaling can induce expression of at least one primitive streak gene and can antagonize neuronal differentiation (Arnold et al., 2000; Aubert et al., 2002).

Although embryos possess defined axes that orient the embryonic and extra-embryonic tissues, and generate gradients of morphogen activity, ES cells differentiate without discernible axes and lack tissue components such as extra-embryonic ectoderm required for gastrulation in vivo. Nonetheless, differentiating ES cells are considered to approximate the differentiating epiblast and are capable of generating derivatives of all three embryonic germ layers (Keller, 2005). Together, these properties enable examination of the mechanisms of signaling pathways during discrete developmental transitions in the absence of asymmetric embryonic structures and signaling centers (Johansson and Wiles, 1995; Kubo et al., 2004; Li et al., 2004). We therefore sought to characterize the actions of Wnt signaling in regulating features of in vitro ES cell differentiation that may be analogous to primitive streak formation in vivo.

In this study, we report that the requirement for canonical Wnt signaling during germ layer induction in vivo is maintained in the ES cell model of this developmental transition. Specifically,

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inhibition of endogenous Wnt activity completely blocks expression of primitive streak-, EMT-, endoderm- and mesoderm-associated genes, and abrogates functional development of mature mesodermal lineages. We find that a stabilized form of β-catenin is alone insufficient to promote primitive streak-associated gene expression. Instead, our results suggest that Wnt signaling may function cooperatively to regulate responsiveness to Bmp and/or Nodal signaling during germ layer induction.

MATERIALS AND METHODS

ES cell culture and differentiation

A2lox and MC50 (gift from Dr Robert Schreiber) ES cells were maintained on irradiated MEFs in IMDM supplemented with 15% FCS, NEAA (0.1 mM each), L-glutamine (2 mM), sodium pyruvate (1 mM), Pen/Strep (1000 U/ml), 2-mercaptoethanol (55 μM) and LIF (EsGro, Chemicon International; 1000 U/ml). A2lox ES cells bear a reverse tetracycline transactivator (Gossen et al., 1995) targeted to the Rosa26 locus (Zambrowicz et al., 1997) and a cassette containing a tetracycline response element, loxP-lox_{2272} sites and $neomycin_{\Delta ATG}$ resistance gene targeted to the Hprt locus.

For differentiation, ES cells were plated in suspension at 1.5×10^4 cells/ml in either serum-containing medium (SCM: IMDM with 10% FCS, NEAA, L-glutamine, NaPyruvate, Pen/Strep and 2-mercaptoethanol) or serum-free medium (SRM), where FCS was replaced with 10% Knockout Serum Replacement (Invitrogen), and supplemented where indicated with recombinant human (rh) Bmp2 (5 ng/ml), rhBmp4 (5 ng/ml), recombinant mouse (rm) Fgf8 (10 ng/ml), rhFgfb (10 ng/ml), rm cripto (100 ng/ml), rmNodal (5 ng/ml), rhTGFβ1 (5 ng/ml), rmFst (50 ng/ml), rmFz1/Fc (500 ng/ml), rmFz2/Fc (500 ng/ml), rmFz7/Fc (500 ng/ml), rmFz8/Fc (500 ng/ml), noggin/Fc (500 ng/ml), rhSfrp1 (200 ng/ml), all purchased from R&D Systems. Recombinant DKK1 was either purchased from R&D Systems or prepared by transient transfection of 293F/T cells. Except where indicated, recombinant Dkk1-His was added at 1×, defined as the concentration required to inhibit generation of Flk1-expressing cells in SCM through day 5 (~160-200 ng/ml, depending on batch; see Fig. S1A in the supplementary material).

Hematopoietic colony assays were initiated at day 6. Embryoid bodies were dissociated enzymatically using trypsin/EDTA (0.05%/0.02%) and inoculated into semisolid medium containing Scf, Epo, Il3 and Il6 (M3434; Stem Cell Technologies). Based on characteristic colony morphology and developmental kinetics, we enumerated primitive erythroid and definitive hematopoietic colonies at day 12 and day 16, respectively (Kyba et al., 2003). To analyze differentiation of cardiomyocytes, embryoid bodies were transferred at day 4 of differentiation in SCM to gelatinized plates in SRM and assayed for lineage-specific gene expression at between day 6 and day 8 (Kouskoff et al., 2005).

Generation of A2lox.sbcat, A2lox.TOPFlash and A2lox.FOPFlash ES cell lines

A2lox.sβcat

A 2363 bp PCR product was generated using the following primers: bcat-F, GACAATGGCTACTCAAGCTGACCTGA; bcat-R, CCTAAAGGACG-ATTTACAGGTCAGTATCAAA. DMSO (5% Sigma), 1 M Betaine (Sigma) and cDNA prepared from day 5 embryoid bodies was ligated into the pGEM®-T Easy Vector (Promega) to generate the plasmid Teasy-bcat. Four point mutations were introduced by serial application of Quick Change Mutagenesis (Stratagene) using the following primers to generate Teasy-sbcat: F1, ATGCTGGAATCCATGCTGGTGCCACCACCACAGCTC-CTT; R1, CACCAGCATGGATTCCAGCATCCAAGTAAGACTGCTGC; F2, GGTGCCACCGCCACAGCTCCTGCCCTGAGTG; R2, CTTGC-CACTCAGGGCAGGAGCTGTGGCGGTGG. A PCR product generated using the primers bcat-F and bcat-R and Teasy-sbcat was phosphorylated using T4PNK and ligated into the plasmid *Sma*I-digested p2lox-empty to generate p2lox.sbcat. After targeting to A2lox ES cells, A2lox.sbcat clones were evaluated for capacity to activate SUPER8XTOPFlash reporter activity, and promote cell growth and viability upon doxycycline induction (see Fig. S2 in the supplementary material).

A2lox.TOPFlash and A2lox.FOPFlash

A *Not*I/*Bam*H1 digested fragment from SUPER8×TOPFlash, a gift from Randall T. Moon (University of Washington), was blunted and ligated into blunted *Not*I-cut p2lox-empty resulting in the plasmid p2lox-TOPFlash. After targeting to A2lox ES cells, the locus consisted of TRE followed by a polyA site, a transcriptional pause, eight multimerized Tcf/Lef-binding sites, firefly luciferase and 2 polyA signals. The plasmid p2lox-FOPFlash was generated similarly.

Recombinant Dkk1-His

A PCR product generated using the primers 5′H3Dkk1 (CCAAAGCTT-CGGAGATGATGGTTGTGTG) and 3′Age1Dkk1 (GCAACCGGTGTG-TCTCTGGCAGGTGTGGA) and cDNA from day 4 embryoid bodies was digested with *Hin*dIII and *Age*I and ligated into *Hin*dIII- and *Age*I-digested pcDNA4-myc-hisA to generate a C-terminal 6His tag in frame with full length Dkk1. The resulting plasmid, pcDNA-Dkk1-his, was transfected into 293F/T cells (Invitrogen) using Ca_2PO_4 precipitation. Supernatants from transfected cells were adjusted to pH 8.0 by the addition of 1/3 volume of 1× Ni-NTA binding buffer and then purified on Ni-NTA HisBind resin (Novagen). Purified Dkk1-his was dialyzed against two changes of PBS, and was shown to consist predominantly of a closely spaced doublet (M_r=35×10^3) that was recognized by an antibody to penta-His (Qiagen) on Western analysis (Fig. S1E,F). Activity of purified Dkk1-his was confirmed by ability to inhibit SUPER8×TOPFlash reporter activity. Dkk1-his and commercially available Dkk1 were further demonstrated to display no substantial cytotoxic effects.

Immunofluorescence

For EMT analysis, cells were transferred at day 4 to plates containing gelatinized cover slips in SRM. Adherent colonies were fixed and stained directly on coverslips, non-adherent embryoid bodies were stained in solution before placement on coverslips. For analysis of neuronal differentiation, cells were washed at day 4 and resuspended in SCM, plated in SRM onto fibronectin-coated slides at day 9 and analyzed at day 13. For staining, cells were fixed in 2% formaldehyde in PBS before blocking with 1% BSA/0.5% saponin in PBS for 1 hour at room temperature. Primary and secondary antibody staining steps were performed for 1 hour at room temperature. Primary antibodies: α-E-cadherin (20 μg/ml, Zymed), α-fibronectin (2.5 μg/ml, BD Transduction Laboratories), α-βIII-tubulin (TuJ1, 5 μg/ml, R&D Systems), and α-nestin (Rat-401, 1 μg/ml, developed by S. Hockfield, obtained from DSHB, developed under auspices of NICHD, maintained by University of Iowa). Secondary antibodies: FITC $F(ab')_2$ α-rat IgG, Cy3 α-mouse IgG, Cy2 α-mouse IgG2a and Cy3 α-mouse IgG1 (3 μg/ml, Jackson ImmunoResearch Laboratories). Nuclear staining was performed using Hoechst 33342 (2 μg/ml, Molecular Probes). After staining, cells were washed, mounted on glass slides and imaged on a Nikon Eclipse E800 microscope.

Gene expression analysis

A CustomExpress Advantage 100-2187 format gene with 11 μM feature size (Affymetrix) was designed based on dynamic gene expression during 4 days of ES cell differentiation in SCM as detected using MOE430_2.0 arrays (Affymetrix). The custom array was validated by comparison with MOE430_2.0 using equivalent cRNA inputs. Biotinylated cRNA was prepared from total RNA per manufacturer's protocol. Total RNA (3 μg) was used to generate first-strand cDNA using a T7-oligo(dT) primer. After second-strand synthesis, in vitro transcription was performed using biotinylated dUTP and dCTP and hybridized to custom arrays per manufacturer's recommendation using Affymetrix GeneChip Instrument System. Data were normalized and expression values modeled using DNA-Chip Analyzer (Li and Hung, 2001; Li and Wong, 2001).

To evaluate expression of individual genes, RNA was prepared using RNeasy Kits (Qiagen) and cDNA synthesized using Superscipt III (Invitrogen). PCR analysis was performed using *Taq* Polymerase (Promega) and the following primers: *Anp* (F, TTGGCTTCCAGGCCATATTG; R, AAGAGGGCAGATCTATCGGA); *Evx1* (F, CTCTGGCCAAGGG-CAACCTAGTAG; R, CATGTAGGTGTAGAAGGCAGGGTCG); *Gapdh* (F, TGCCCCCATGTTTGTGATG; R, TGTGGTCATGAGCCCTTCC);

Gata1 (F, CATTGGCCCCTTGTGAGGCCAGAGA; R, ACCTGATGGAGCTTGAAATAGAGGC); *Gata4* (F, AAGGCAGAGAGTGTGTCAATTGTGG; R, TGGTAGTCTGGCAGTTGGCACAG); *Hand1* (F, AAGACTCTGCGCCTGGCTACCA; R, CGCCCTTTAATCCTCTTCTCGC); *Mef2c* (F, AGCAAGAACACGATGCCATC; R, GAAGGGGTGGTGGTACGGTC); *Mesp1* (F, TCCCTCATCTCCGCTCTTCAGC; R, GGTTGGAATGGTACAGTCTGGATGAG); *Mixl1* (F, AGTTGCTGGAGCTCGTCTTCCG; R, CTCTGAGAACCAGATGTGCAGACG); *Myh6* (F, GGAAGAGTGAGCGGCGCATCAAGG; R, CTGCTGGAGAGGTTATTCCTCG); *Myh7* (F, GCCAACACCAACCTGTCCAAGTTC; R, TGCAAAGGCTCCAGGTCTGAGGGC); *Myl2* (F, GCCAAGAAGCGGATAGAAGG; R, CTGTGGTTCAGGGCTCAGTC); *Myl7* (F, CAGACCTGAAGGAGACCT; R, GTCAGCGCAAACAGTTGC); *Nkx2-5* (F, CAAGTGCTCTCCTGCTTTCCCA; R, GCTCGTAGACCTGCGCCTGC); *Tal1* (F, ATTGCACACACGGGATTCTG; R, GAATTCAGGGTCTTCCTTAG); *Tbx5* (F, GGAGCCTGATTCCAAAGACA; R, TTCAGCCACAGTTCACGTTC); *Wnt3* (F, GGACTTGCAATGTCACCTCCCA; R, TGGATCCAGCCGCACAATCTAC); *Wnt8a* (F, GACCATGGGACACTTGTTAATGCTGTG; R, ACGTGAATTTGGTGGTGGTGTTACC); *Pax6* (F, CCATCTTTGCTTGGGAAATCCG; R, GCTTCATCCGAGTCTTCTCCGTTAG); *Pax7* (F, AATGGCCTGTCTCCTCAGGT; R, TCTCCTGGCTTGATGGAGTC); *Sox2* (F, GAAGGGGAGAGATTTTCAAAGAGATACAAG; R, CCAGATCTATACATGGTCCGATTCCC); *En1* (F, AAGTTCCCGGAACACAACCCTG; R, ATAGCGGTTTGCCTGGAACTCC).

Flow cytometry

Embryoid bodies were dissociated enzymatically and single cell suspensions stained for 30 minutes on ice with 1 μg/ml PE α-Flk1 (Avas12a1, eBioscience). Data were acquired on a FACS Calibur (Becton Dickinson) and analyzed using FloJo (Tree Star). Flk1 staining was determined using an isotype control.

Luciferase assays: transient transfections

A2lox or A2lox.sβcat cells were transfected with pRL-CMV (Promega) and either SUPER8×TOPFlash or SUPER8×FOPFlash reporter plasmids and distributed to plates in SRM. After 4 hours, cells were left unstimulated or activated with either LiCl (20 mM) or doxycycline (1 μg/ml) for 18 hours. Firefly luciferase counts were normalized for renilla luciferase activity and averaged between triplicate wells.

RESULTS

Canonical Wnt ligands are transiently expressed and active during ES cell differentiation

Among canonical Wnt ligands, Wnt3 and Wnt8a are expressed most highly in the pre-streak and early gastrula-stage embryo (Kemp et al., 2005). To test whether these Wnt ligands are expressed with analogous kinetics during ES cell differentiation, we measured Wnt3 and Wnt8a expression by RT-PCR during the first 5 days of differentiation (Fig. 1A). Both ligands showed gradual induction, with peak expression occurring between 2 and 3 days of differentiation, followed by subsequent decline. To determine whether expression of these Wnt ligands correlates with functional pathway activation, we evaluated activity of the Wnt-dependent SUPER8×TOPFlash reporter (Veeman et al., 2003), which contains multimerized Tcf/Lef binding sites, during the first 4 days of differentiation in serum containing medium (SCM) (Fig. 1B). Relative to pharmacological activation by LiCl, SUPER8×TOPFlash activity in undifferentiated ES cells was negligible and unaffected by addition of Dkk1, reflecting the lack of canonical Wnt pathway activity in these cells. Upon induction of differentiation in SCM, reporter activity was detected as early as day 1, increased progressively through day 4, and was completely inhibited at each time by the presence of Dkk1. As a further specificity control, activity of the SUPER8×FOPFlash reporter, harboring mutated Tcf/Lef-binding sites (Veeman et al., 2003), was found to be inactive at all times and was unaffected by Dkk1 or LiCl treatment (data not shown). These results indicate that during early serum-activated ES cell differentiation, the Wnt pathway is functionally active and the canonical ligands Wnt3 and Wnt8a are transiently expressed.

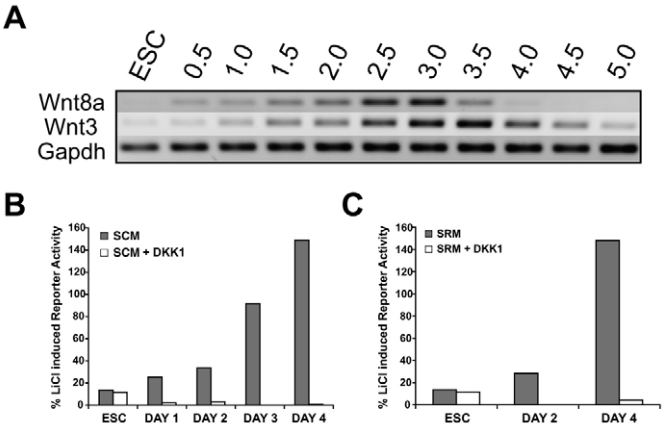

Fig. 1. Activation of canonical Wnt signaling during early ES cell differentiation. (**A**) Transient expression of Wnt8a and Wnt3 during ES cell differentiation. Semi-quantitative RT-PCR was carried out as described in the methods for *Wnt8a*, *Wnt3* and *Gapdh*. Analysis was performed on ES cells (ESC) and on ES cells differentiated in SCM for the indicated number of days. (**B**) ES cells bearing a stably integrated SUPER8×TOPFlash luciferase reporter were differentiated in SCM either in the presence or absence of Dkk1, as indicated. Luciferase activity in undifferentiated ES cells (ESC) or in ES cells differentiating for the indicated number of days in SCM or SCM with addition of Dkk1 (SCM + Dkk1) was measured. SUPER8×TOPFlash luciferase activity is presented as a percentage of the activity measured in the same conditions treated with LiCl (20 mM) for 18 hours prior to harvesting. (**C**) SUPER8×TOPFlash transfected cells in B were differentiated in serum replacement medium alone (SRM) or with the addition of Dkk1 (SRM + Dkk1) for 2 and 4 days, and luciferase activity measured as in B.

Induction of ligand expression and reporter activity may depend directly or indirectly on factors present in serum or instead be part of an intrinsic developmental program. To distinguish between these two possibilities, we evaluated SUPER8×TOPFlash reporter activity in ES cells differentiating in serum-free conditions (Fig. 1C). Similar to ES cells differentiated in SCM, Wnt reporter activity increased gradually during the first 4 days of serum-free differentiation and the canonical ligands Wnt3 and Wnt8a are both expressed at day 3 (not shown). These data suggest that Wnt activity during early ES cell differentiation is independent of extrinsic serum-derived factors and is initiated by either cell autonomous mechanisms or by intercellular signals present as cells aggregate in suspension.

Ligand-restricted temporally specific Wnt signaling is required for generation of Flk1$^+$ mesoderm

As Wnt activity is required for germ layer formation in vivo, we asked whether canonical Wnt signaling is required for the analogous processes during ES cell differentiation. To assess early mesoderm formation, we determined the frequency of cells expressing Flk1, a VEGF receptor expressed by multipotent mesoderm cells (Ema et al., 2006). In SCM alone, we find that Flk1 is expressed transiently, beginning at day 3, peaking on day 4 and declining thereafter (Fig. 2A). Addition of the Bmp2/4/7 inhibitor Noggin reduced the

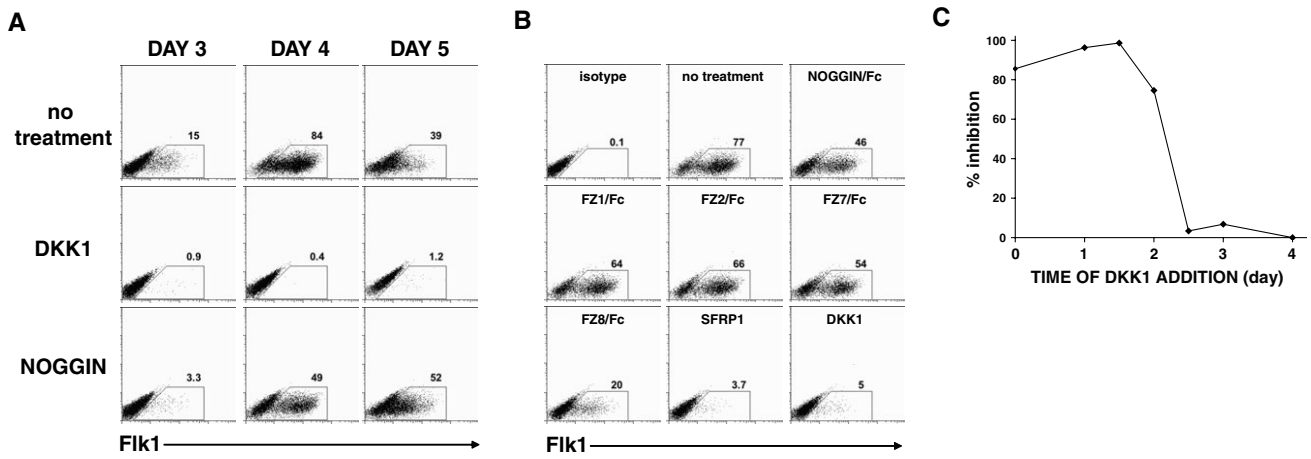

Fig. 2. Wnt signaling is required for generation of Flk1$^+$ mesoderm. (**A**) Differentiating ES cells in SCM were treated either with Dkk1 or Noggin/Fc as indicated, or left unmanipulated (no treatment). Embryoid bodies were harvested at day 3, 4 and 5, and analyzed for Flk1 expression as described in the Materials and methods. Shown are dot plots gated on live cells. Numbers represent the percentage of Flk1$^+$ within the indicated region. (**B**) ES cells were differentiated in SCM supplemented with the indicated protein, and Flk1 expression determined on day 4 as described above. (**C**) Dkk1 was added to differentiating cultures at day 0, 1.0, 1.5, 2.0, 2.5 or 3.0. The frequency of Flk1-expressing cells at day 4 was determined as described in A. The data are represented as the percentage of inhibition, measured by dividing the frequency of Flk1$^+$ cells in each Dkk1 treated culture by the frequency of Flk1$^+$ cells in parallel untreated cultures.

frequency of Flk1$^+$ cells by ~50% (Fig. 2A), as previously reported (Park et al., 2004). By contrast, addition of Dkk1 completely inhibited generation of Flk1-expressing cells throughout differentiation (Fig. 2A), suggesting a strict requirement for Wnt signaling at some point prior to mesoderm induction.

Dkk1 inhibits canonical Wnt signaling in a ligand-independent manner by binding to and inducing internalization of the Lrp/Kremen co-receptor complex (Mao et al., 2002). To determine whether the inhibitory effect of Dkk1 was restricted to a subset of Wnt ligands, we examined four soluble Frizzled receptors (Fz1, Fz2, Fz7 and Fz8) and secreted frizzled-related protein 1 (Sfrp1) for their ability to inhibit generation of Flk1$^+$ mesoderm (Fig. 2B). Addition of the Fz1/Fc, Fz2/Fc or Fz7/Fc did not substantially reduce the frequency of Flk1-expressing cells at day 4 of differentiation. By contrast, addition of Fz8/Fc and Sfrp1 caused 70% and 95% reductions, respectively, in the frequency of Flk1-expressing cells. These results suggest that Wnt-dependent Flk1 expression relies on a restricted set of Wnt ligands. Although the specificity and degeneracy of Wnt-Frizzled interactions are not well defined (Huang and Klein, 2004), the finding that Fz8 can bind XWnt8 (Hsieh et al., 1999), is consistent with our results demonstrating transient Wnt8a expression and functional inhibition by Fz8/Fc.

We next asked whether the requirement for Wnt activity during early ES cell differentiation is temporally restricted. To identify the window of time during which Wnt signaling is required for subsequent generation of Flk1$^+$ cells, we added Dkk1 at progressively later timepoints following initiation of differentiation in SCM (Fig. 2C). When Dkk1 was added at day 0, day 1.0 or day 1.5, generation of Flk1$^+$ cells by day 4 was inhibited completely. Inhibition was reduced to 80% when Dkk1 was added at day 2 and was negligible when added at day 2.5 or afterwards, indicating that generation of Flk1-expressing cells by day 4 requires Wnt signaling before day 2.5. These results, considered with the kinetics of Wnt ligand expression, suggest that Wnt signaling between day 1.5 and day 2.5 is required for generation of Flk1$^+$ mesoderm.

Canonical Wnt signaling is required for expression of genes associated with primitive streak, EMT, endoderm and mesoderm

We next sought to identify genes whose expression is Wnt dependent during ES cell differentiation. We analyzed gene expression by custom DNA microarrays, containing ~1600 probe sets, at various times of differentiation in SCM with or without addition of Dkk1 (Fig. 3). No differences in gene expression between conditions were observed before day 2 of differentiation. Beginning at day 2, significant differences emerged. Notably, the first genes inhibited by Dkk1 treatment were largely associated with the embryonic primitive streak (see Table S1 in the supplementary material). Specifically, brachyury (*T*), *Mixl1* and *Evx1* each failed to be induced in the presence of Dkk1 (Fig. 3A) and are each first expressed in the early primitive streak-stage embryo (Dush and Martin, 1992; Rivera-Perez and Magnuson, 2006; Robb et al., 2000). In addition, genes associated with subsequent events in embryogenesis were affected by Dkk1 treatment. Genes reflective of epithelial-mesenchymal transition (*Snai1*, *Fn1* and *Cdh2*) (Fig. 3B) (Barrallo-Gimeno and Nieto, 2005), mes/endoderm (*Gsc*, *Sox17*, *Foxa2*) (Fig. 3C) (Yasunaga et al., 2005) and mesoderm (*Mesp1*, *Nrp1*, *Pdgfra*) (Fig. 3D) all required Wnt signaling for their expression. Expression of the ES cell-associated genes *Zfp42* and *Socs3* was diminished with identical kinetics with or without Dkk1, indicating that Dkk1 had no effect on the earliest steps of ES cell differentiation. Together, these gene expression data suggest that Dkk1 treatment causes a global block at the ES cell equivalent of primitive streak formation. When analyzed at later timepoints (day 6-8), a reciprocal increase in expression of neuroectoderm-associated genes was detected by RT-PCR after treatment with Dkk1 from day 0-4 (Fig. 4A), consistent with the previously described role for Wnt signaling in antagonizing neural development (Aubert et al., 2002). When examined by immunofluorescence at day 13, cultures treated with Dkk1 (days 0-4) possess cells expressing neuronal βIII-tubulin, *Nestin* or *Pax6*, reflective of enhanced commitment to the neural/neuronal lineage (Fig. 4B-D).

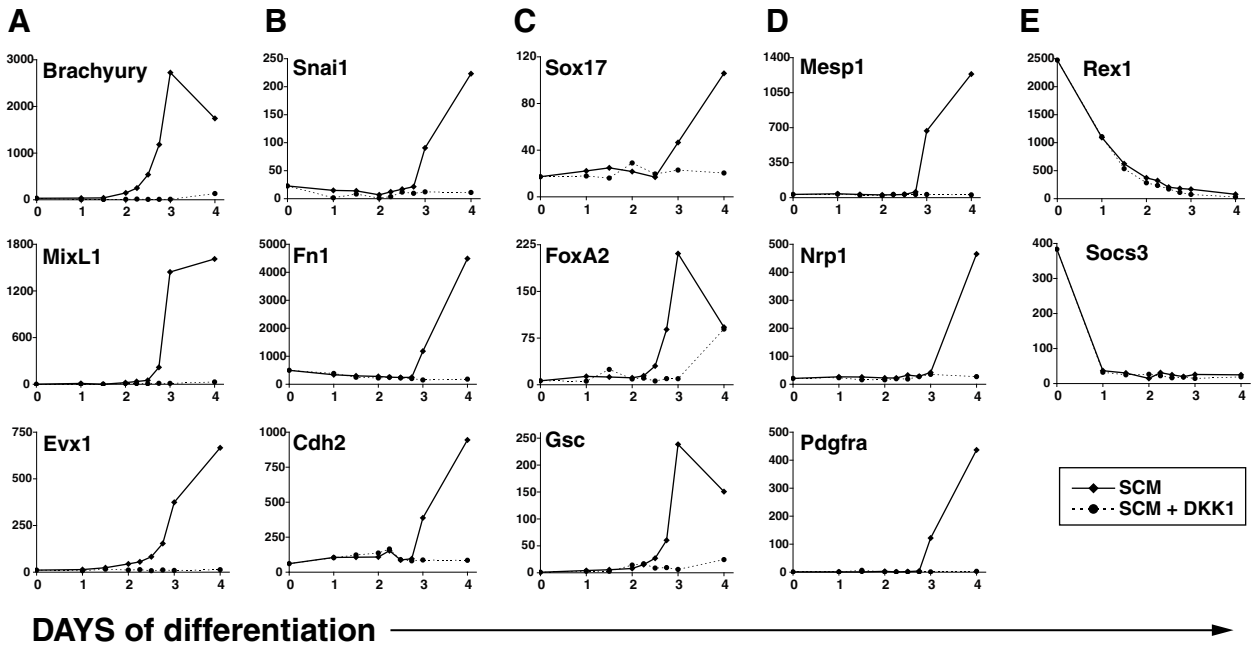

Fig. 3. Wnt-dependent expression of genes associated with primitive streak, EMT, endoderm and mesoderm. ES cells were differentiated in SCM alone or in the presence of Dkk1 (SCM + Dkk1). RNA was isolated at indicated times and analyzed using custom DNA microarrays. Normalized and modeled expression values for the indicated genes in either SCM (solid line) or SCM + Dkk1 (broken line) are expressed in arbitrary units.

Canonical Wnt signaling is required for generation of ES cell-derived mesoderm

As mesodermal genes failed to be expressed with Dkk1 treatment, we asked whether early Wnt signaling is formally required for generation of mature mesodermal lineages from ES cells. As Wnt signaling has functionally distinct roles at different points in early embryogenesis (Robb and Tam, 2004), we limited Dkk1 treatment to the first 4 days of differentiation for each long-term assay. To quantitate hematopoietic potential under different conditions, ES cells were differentiated in SCM alone or with either Dkk1 or Noggin/Fc for the first 4 days. Cells were subsequently allowed to differentiate an additional 2 days in the absence of inhibitors, and then evaluated for hematopoietic precursor frequency at day six by methylcellulose colony-forming assays (Fig. 5A). Early treatment with Noggin/Fc reduced the frequency of hematopoietic precursors at day 6, consistent with the known role for Bmp signaling in extra-embryonic mesoderm development and hematopoiesis (Snyder et al., 2004; Winnier et al., 1995). Treatment with Dkk1 for 4 days, however, completely inhibited the generation of hematopoietic precursors at day 6 (Fig. 5A), consistent with the loss of mesoderm associated gene expression described above (Fig. 3). We also examined expression of the transcription factors *Gata1* and *Tal1*, each important for hematopoietic development (Baron and Fraser, 2005). In SCM alone, expression of *Gata1* and *Tal1* (Fig. 5B) is readily detected by RT-PCR at day 6, when colony forming assays were initiated. Whereas treatment with Noggin/Fc caused a slight reduction in expression of these genes, Dkk1 treatment completely inhibited their expression (Fig. 5B). These findings are consistent with the interpretation that Wnt and Bmp signaling act either at different times or by distinct mechanisms to affect the hematopoietic competence of differentiating ES cells.

Normally, cardiomyocyte development from ES cells occurs at low frequency, as evidenced by the low but clearly detectable expression of cardio-specific genes in unmanipulated cultures (Fig. 5C). Addition of Noggin/Fc for the first 4 days of differentiation resulted in augmentation of cardiac gene expression. By contrast, Dkk1-treated cultures expressed neither cytoskeletal genes (*Myl2*, *Myl7*, *Myh6*, *Myh7*) nor transcription factors (*Gata4*, *Tbx5*, *Nkx2.5*)

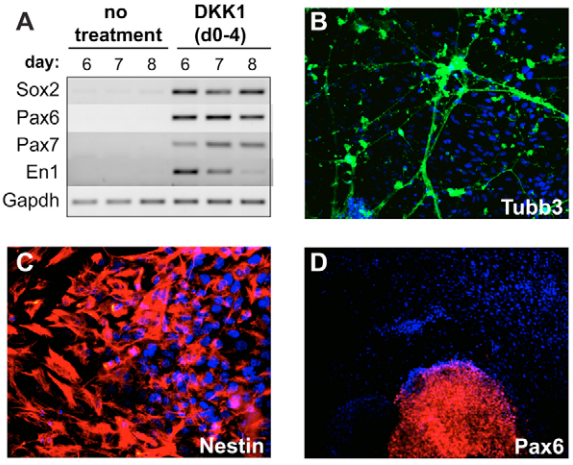

Fig. 4. Early inhibition of canonical Wnt signaling enhances long-term neuronal differentiation. (**A**) ES cells were differentiated in SCM alone or with addition of 1× Dkk1. At day 4, cells were washed and transferred to SCM without inhibitor. At days 6-8, RNA was isolated and examined for expression of indicated genes by RT-PCR. (**B-D**) Dkk1-treated cells described in A were transferred at day 9 to fibronectin-coated slides in SRM and stained for neuronal βIII-tubulin (green), nestin (red) or *Pax6* (D, red) expression at day 13. Nuclei are stained with Hoechst 33342 (blue) and images were acquired using 10× (B,D) and 20× (C) objective magnification. Untreated cells exhibited negligible staining for neuronal markers.

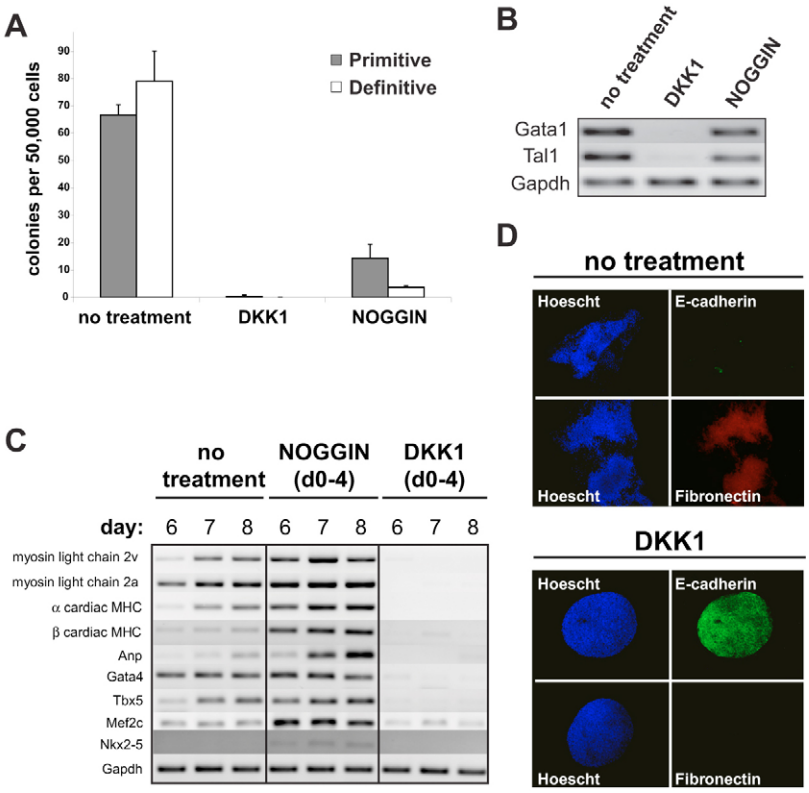

Fig. 5. Canonical Wnt signaling is required for generation of ES cell-derived mesoderm. (**A**) ES cells were differentiated in SCM alone (no treatment) or treated with either Dkk1 or Noggin/Fc for 4 days as indicated. After 4 days, cells were washed and transferred to SCM without inhibitor for an additional 2 days. On day 6, cells were assayed for hematopoietic precursor potential using methylcellulose colony-forming assays as described in the methods. (**B**) ES cells described in A were treated as indicated, harvested at day 6 and assessed for expression of the indicated genes by RT-PCR. (**C**) ES cells were differentiated as in A, except that at day 4, cells were washed and transferred to gelatinized plates in SRM without inhibitor. At days 6, 7 and 8, cells were harvested and analyzed by RT-PCR for the indicated genes. (**D**) Cells were differentiated as in C and analyzed by fluorescence microscopy for expression of E-cadherin (green) or fibronectin (red) as described in the Materials and methods. Images of representative colonies, acquired using 10× objective magnification, are shown for unmanipulated (no treatment) and Dkk1-treated conditions.

affiliated with cardiomyocytes (Fig. 5C). We therefore conclude that early Wnt signaling is required for subsequent generation of ES cell-derived cardiogenic mesoderm.

Mesoderm formation in vivo is accompanied by an epithelial to mesenchymal transition (EMT) during which cells of the epiblast acquire the capacity to migrate and undergo proper gastrulation movements (Thiery and Sleeman, 2006). As cells undergo EMT, they lose expression of the epithelial marker E-cadherin (*Cdh1*) and begin expressing the mesenchymal marker, fibronectin (*Fn1*). To determine the effect of Wnt signaling on this process, we examined expression of these markers by immunofluorescence during ES cell differentiation in the presence or absence of Dkk1 (Fig. 5D). Undifferentiated ES cells express E-cadherin, but not fibronectin (data not shown). After 5 days of differentiation, however, cells cultured in SCM uniformly express fibronectin, but not E-cadherin, indicating that these cells have undergone EMT. By contrast, cells treated with Dkk1 maintain E-cadherin expression and fail to induce fibronectin, reflecting their failure to undergo EMT (Fig. 5D). Additionally, cells differentiated in SCM possess the capacity to adhere and spread on gelatinized substrate, whereas Dkk1-treated cells fail to adhere and instead remain in suspension (Fig. 5D). Together, these results indicate that early Wnt signaling is required by cells to acquire functional competence to undergo the lineage-inducing and cell biological processes associated with gastrulation.

Stabilized β-catenin is not sufficient to induce primitive streak gene expression

To determine whether canonical Wnt signaling alone is sufficient to direct expression of primitive streak genes, we generated an ES cell line bearing a doxycycline-inducible form of a stabilized β-catenin (A2lox.sβcat) (Fig. 6). We introduced point mutations at each of the four N-terminal GSK3β phosphorylation sites of β-catenin (S33A, S37A, T41A, S45A), rendering the encoded protein refractory to basal degradation and constitutively localized to the nucleus where it can exert its function as a transactivator (Yost et al., 1996). In cells lacking the transgenic β-catenin, treatment with LiCl, but not doxycycline, induced activity of the SUPER8×TOPFlash luciferase reporter (Fig. 6A). In transgenic ES cells, however, doxycycline-induced stabilized β-catenin stimulated SUPER8×TOPFlash activity to levels equal to LiCl treatment, indicating that the mutant β-catenin is functionally active. Reporter activity was specific in all cases as the negative control SUPER8×FOPFlash reporter was unaffected by either doxycycline or LiCl treatment.

To evaluate directly the capacity of β-catenin-dependent transactivation to induce a program of primitive streak gene expression, we induced expression of the transgenic, stabilized β-catenin at day 1 of serum-free differentiation, corresponding to the time of onset of SUPER8×TOPFlash reporter activity under normal conditions. As endogenous canonical Wnt ligands are induced in these conditions (not shown), all cultures were treated with Dkk1 throughout the experiment in order to ensure the specificity of our analysis. At day 3.5 of differentiation, we examined specific gene expression by RT-PCR (Fig. 6B). Expression of brachyury was detected at low levels in untreated, serum-free conditions, as previously reported (Park et al., 2004), and was extinguished by treatment with Dkk1 (Fig. 6B), consistent with the requirement for Wnt signaling found in SCM (Fig. 3). Importantly, we find that neither brachyury nor *Evx1* expression was restored by stabilized β-catenin across a broad range of doxycycline concentrations.

Effects of soluble factors on mesoderm generation in the presence of Dkk1

As Wnt signaling does not promote primitive streak gene expression autonomously (Fig. 6), we hypothesized that it may function cooperatively to coordinate transcriptional responses to other

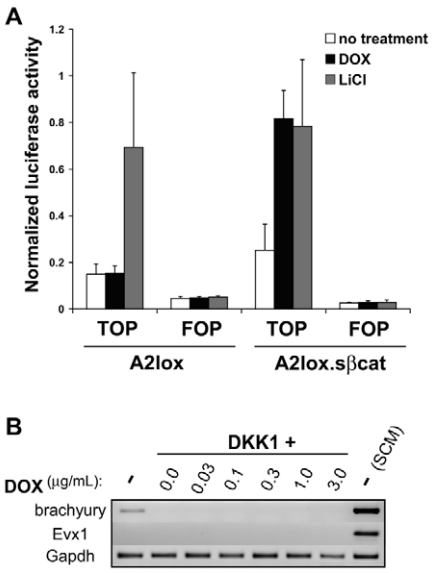

Fig. 6. Stabilized β-catenin is insufficient for induction of primitive streak-associated gene expression. (**A**) A2lox.sβcat ES cells transiently transfected with the SUPER8×TOPFlash (TOP) or SUPER8×FOPFlash (FOP) reporters were left untreated (no treatment), or treated with either 1 μg/ml doxycycline (DOX) or LiCl for 18 hours. Whole cell lysates were prepared and luciferase activity measured. Shown is the luciferase activity normalized to a co-transfected Renilla luciferase construct driven by the CMV promoter as previously described (Ranganath et al., 1998). (**B**) A2lox.sβcat ES cells were differentiated in SRM alone or in the presence of Dkk1 (+Dkk1) and of the indicated concentration of doxycycline (DOX). Dkk1 was added at the initiation of differentiation. Doxycycline was added at day 1 of differentiation. Cells were harvested at day 3.5 of differentiation and evaluated for expression of the indicated genes by RT-PCR. Expression found in ES cells differentiated in SCM without doxycycline represent positive controls.

signaling pathways. We therefore evaluated whether strong activation of other signaling pathways could by-pass the functional requirement for early Wnt signaling in later mesoderm development. For this analysis, we examined nine secreted factors that act in pathways important for primitive streak formation in vivo or are expressed during early ES cell differentiation (data not shown). In these experiments, factors were added for the first 4 days in the presence of Dkk1, at which time all cultures were washed and redistributed to longer term mesodermal differentiation assays in SCM alone (Fig. 7A-C).

When analyzed at day 4, exogenous Bmp2 or Bmp4 was able to rescue development of $Flk1^+$ cells in conditions of Wnt blockade (Fig. 7A). By contrast, TGFβ1, Nodal, Cripto, Fgf8, Fst (Fig. 7A), bFgf and activin A (not shown) were unable to restore Flk1 expression. Consistent with these results, only Bmp2 and Bmp4 restored long-term hematopoietic colony formation (Fig. 7B), and Tal1 and Gata1 expression (not shown). We further evaluated the capacity of each factor to rescue cardiogenic mesoderm development in the presence of Dkk1 (Fig. 7C). Only treatment with Bmp2, Bmp4 or cripto was capable of inducing cardiac gene expression to levels higher than control. In summary, these results show that addition of Bmp2/4 or cripto can promote development of mesodermal cell types even in the face of Wnt pathway blockade.

As Wnt signaling was required for the expression of primitive streak-associated genes (Fig. 3), we asked whether either Bmp4 or cripto were capable of inducing primitive streak genes in the presence of Dkk1. ES cells were differentiated in SCM in the presence of Dkk1 alone or Dkk1 combined with either Bmp4 or cripto (Fig. 7D). Bmp4, but not cripto, was able to restore Wnt-dependent expression of the primitive streak genes *T*, *Mixl1*, *Evx1* and *Mesp1* at days 3 and 4 of differentiation, indicating the rescue of mesoderm development by Bmp4 treatment occurred via normal developmental pathways.

Wnt and Bmp pathways act cooperatively to coordinate primitive streak-associated gene expression

As addition of Bmp4 was able to bypass the requirement for Wnt signaling during germ layer induction (Fig. 7), we asked whether these pathways act cooperatively to regulate primitive streak gene expression. To test this, we determined brachyury expression levels at day 3.5 of differentiation in serum-free conditions in the presence of Bmp and/or Dkk1 at increasing concentrations (Fig. 8A). Without Dkk1, brachyury expression increased in a dose-dependent manner in response to Bmp4 treatment. At low concentrations of Bmp4, only a small amount of Dkk1 was required to extinguish brachyury expression. By contrast, at higher concentrations of Bmp4, increasing amounts of Dkk1 were required to exert the same inhibitory effect. Importantly, Bmp4 activity was unable to overcome saturating concentrations of Dkk1, suggesting an absolute requirement for Wnt signaling in primitive streak gene expression, including brachyury (Fig. 8A), *Mixl1* and *Evx1* (not shown).

To test further whether Wnt signaling can synergize with Bmp activity, we compared brachyury expression in differentiating A2lox.sβcat cells with and without Dkk1, and treated with varying concentrations of Bmp4 and/or doxycycline (Fig. 8B). As before, Bmp4 treatment strongly induced brachyury expression and was sensitive to intermediate concentrations of Dkk1. Though induction of stabilized β-catenin displayed no effect by itself (Fig. 6B), doxycycline treatment did increase brachyury expression when combined with Bmp4 (Fig. 8B). At low dose of Bmp4, brachyury expression was extinguished by Dkk1 and not restored by doxycycline. However, at higher concentrations of Bmp4, brachyury expression was markedly increased in a β-catenin-dependent manner (Fig. 7B). These results suggest that β-catenin and Bmp4 either act directly to regulate primitive streak gene expression or indirectly by inducing other factors that direct expression of these genes. As Bmp4 has been shown to regulate Nodal activity in vivo, we measured Nodal expression under these conditions (Fig. 8B). In the absence of Bmp4, we detect a low level of Nodal expression that remains unaffected by Dkk1 or by stabilized β-catenin. However, addition of Bmp4 induced expression of Nodal, especially under conditions of enforced Wnt activity.

DISCUSSION

Although Wnt signaling is known to be required in vivo for primitive streak formation and gastrulation (Huelsken et al., 2000; Kelly et al., 2004; Liu et al., 1999), the spatiotemporal complexity of the mammalian embryo has hindered mechanistic studies of this pathway during these early developmental transitions. Thus, the purpose of this study was to test whether the requirement for Wnt signaling in vivo is conserved during analogous steps of ES cell differentiation and, in so doing, to establish an in vitro model system in which the concrete mechanisms of Wnt activity can be dissected.

In this study, we demonstrate a requirement for Wnt signaling during the earliest steps of mesendodermal differentiation of ES cells. Specifically, during ES cell differentiation, canonical Wnt signaling is required for the expression of genes associated with the

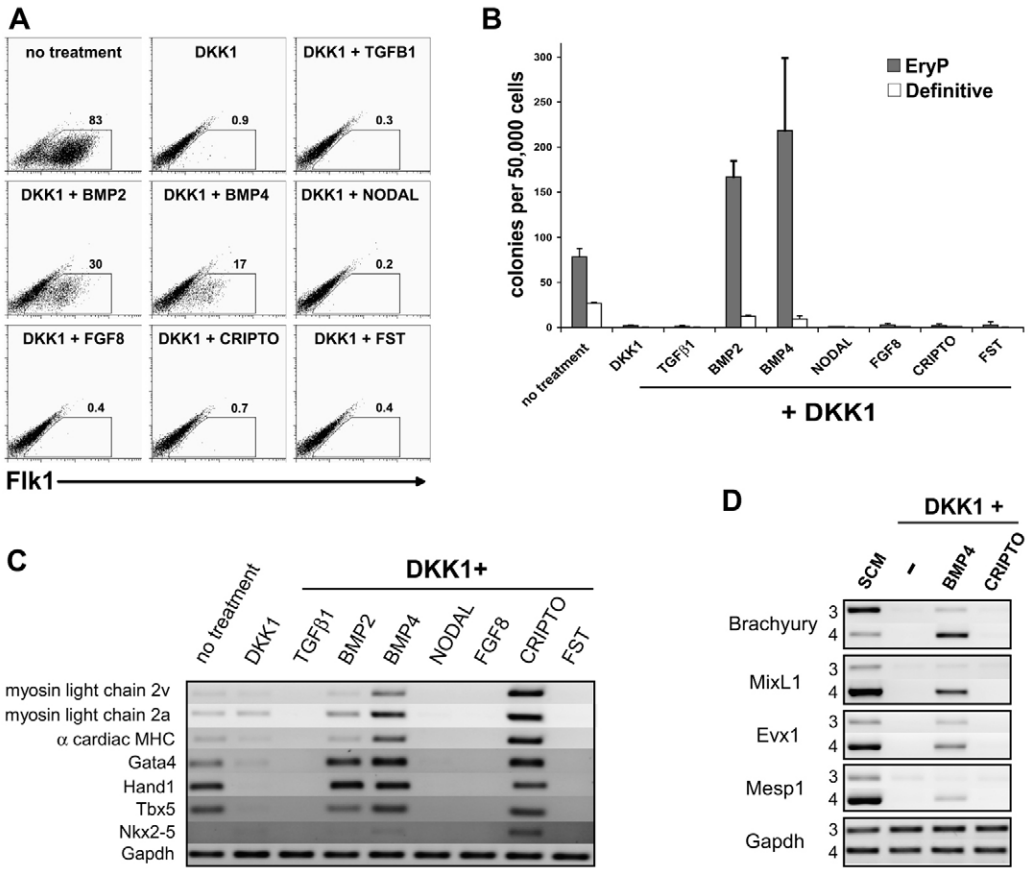

Fig. 7. Soluble factors can restore mesoderm generation in the presence of Dkk1. (**A**) ES cells were differentiated in SCM (no treatment), SCM with Dkk1 alone, or in SCM + Dkk1 and one of the following factors: TGFβ1, Bmp2, Bmp4, Nodal, Fgf8, cripto or FST. On day 4 of differentiation, cells were examined for Flk1 expression by FACS. Numbers indicate the percentage of $Flk1^+$ cells in the indicated region. (**B**) ES cells were treated as described in A, and on day 4, embryoid bodies were washed, cultured for an additional 2 days in SCM and then analyzed for hematopoeitic potential as described in Fig. 5A. (**C**) Cells were treated as described in A, embryoid bodies washed and transferred to gelatinized dishes in serum-free medium for 4 days. Cultures were analyzed at day 8 for cardiac gene expression by RT-PCR. (**D**) ES cells were differentiated in SCM alone (SCM) or SCM + Dkk1 in the absence (–) or presence of Bmp4 or cripto, as indicated. Bmp4 and cripto were added on day 1, and cells were analyzed on days 3 and 4 as indicated.

primitive streak and gastrulation in vivo, including brachyury, *Mixl1* and *Evx1*. Consistent with this finding, genes affiliated with lineages developing subsequent to gastrulation in vivo (mesoderm and endoderm), as well as genes reflective of cell biological changes occurring normally during gastrulation (EMT), all fail to be expressed in the absence of Wnt activity. Furthermore, we demonstrate formally that mature mesodermal lineages, which depend in vivo on Wnt signaling and development of the primitive streak, also fail to develop in vitro in the absence of Wnt activity. In particular, ES cells deprived of early Wnt signaling failed to generate hemogenic and cardiomyogenic mesoderm in long-term assays. Taken together, these data reflect a crucial dependence on Wnt signaling for the initiation of a global program of development during primary germ layer induction in vitro.

Using analysis of gene expression at high temporal resolution, we were able to detect the first genes whose expression was altered during ES cell differentiation by blockade of Wnt signaling. Using an in vivo approach, a recent study also identified genes whose expression was decreased in β-catenin-deficient embryos (Morkel et al., 2003). Of the nine β-catenin-dependent genes with a role in signaling or embryonic development identified by this group at E6.5, we identified eight of these in our analysis, demonstrating again the high fidelity with which differentiating ES cells recapitulate early embryogenesis. Although the in vivo approach taken by this group possesses distinct advantages, such as microdissection and analysis of different embryonic and extra-embryonic tissues, the ES cell model system allows increased temporal resolution and greater sensitivity when examining more epiblast-intrinsic roles. For example, both studies identified *Evx1* as a Wnt-dependent gene, consistent with its known expression in the primitive streak (Dush and Martin, 1992). When analyzed in vivo, *Evx1* was found to be reduced by 3.2-fold at E6.5 in β-catenin-deficient embryos. By contrast, we found *Evx1* expression to be 40-fold reduced at an equivalent time during ES cell differentiation in the absence of Wnt signaling. Given this functional fidelity and high sensitivity, studies using the ES cell model system may provide insights into the mechanisms of Wnt signaling and Wnt target gene regulation that are generalizable to the embryo in vivo.

There are divergent views of how Wnt signaling regulates gene expression. In some cases, Wnt pathway activation could act autonomously and directly to induce target gene expression through the β-catenin-dependent conversion of repressive TCF transcription factors to an active state (Logan and Nusse, 2004). In others, it has been suggested that Wnt signaling instead functions to regulate the

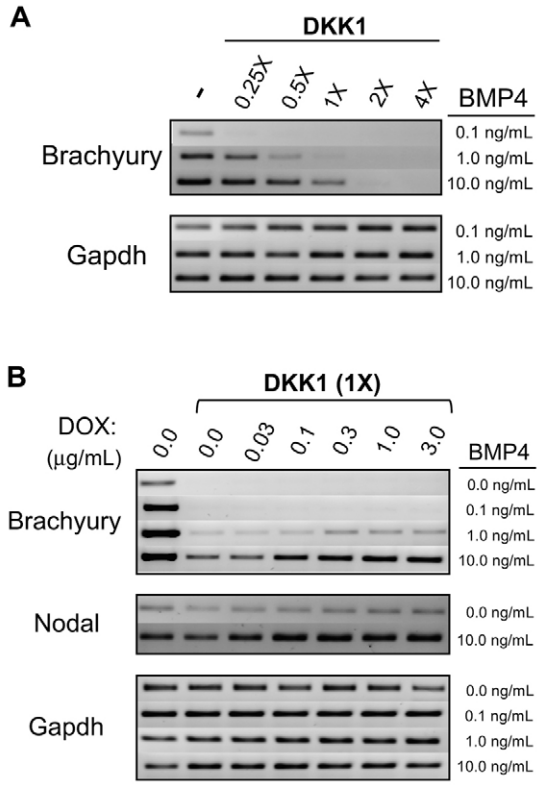

Fig. 8. Wnt and Bmp signaling act cooperatively to regulate brachyury expression. (**A**) ES cells were differentiated in SRM and the indicated concentration of Bmp4 and Dkk1. RNA was prepared at day 3.5 and analyzed by RT-PCR for brachyury and *Gapdh* expression. (**B**) A2lox.sβcat ES cells were differentiated in SRM alone or SRM plus the indicated concentration of Bmp4 and/or doxycycline. RNA was prepared at day 3.5 and analyzed by RT-PCR for brachyury, *Nodal* and *Gapdh* expression.

threshold or stability of gene transcription induced by other pathways (Arias and Hayward, 2006). Recent studies in fly and mouse, for example, suggest that Wnt signaling or β-catenin stabilization can function principally to regulate thresholds and stabilize gene expression induced secondary signals (Cox and Baylies, 2005; Lowry et al., 2005).

In our analysis, we sought to determine whether Wnt signaling acts autonomously to promote primitive streak-associated gene expression, or instead functions coordinately with other pathways to effect transcriptional responses. Using an inducible stabilized form of β-catenin, we found that Wnt activity alone is not sufficient to induce lineage-affiliated gene expression. Instead, we demonstrate that a transcriptional response to stabilized β-catenin is detectable only when other factors, such as Bmp4, are added in combination. Furthermore, the sufficiency of either β-catenin or Bmp4 to induce primitive streak-associated gene expression is absolutely dependent upon signaling by the other. These results suggest that primitive streak-associated gene expression in vitro is regulated at the molecular level by integration of at least two signaling pathways, consistent with the phenomenon in vivo whereby epiblast fate correlates with their position along the combined gradients of multiple factors. Interestingly, and in contrast to Bmp treatment, supplementing Wnt-inhibited cultures with cripto rescues cardiac gene expression without first inducing expression of PS-associated genes. This finding suggests that under some conditions, the development of mesodermal lineages in vitro may not depend on the ordered progression through developmental intermediates thought to be specifically required in vivo.

Our study does not distinguish between the direct and indirect actions of the Wnt and Bmp pathways. For example, it is possible that Bmp4 functions indirectly to influence primitive streak gene expression by first inducing *Nodal* and/or cripto (Beck et al., 2002). Consistent with this possibility, we show that Bmp signaling alone can increase *Nodal* expression and that stabilized β-catenin augments *Nodal* expression synergistically with Bmp. Further experiments beyond the scope of this study should resolve the role of *Nodal* in ES cell-derived germ layer induction. In fact, comprehensive analysis of the mechanisms underlying this process will depend on a rigorous dissection of the simultaneous actions of at least three signaling pathways (Wnt/Nodal/Bmp) and their transcriptional targets. Though the ES cell differentiation model system does not recapitulate the intricate signaling topology of the intact embryo, these data do suggest that these cells possess a functional responsiveness and requirement for Wnt signaling that mirrors that observed in vivo. As such, our findings suggest that ES cells may provide a useful and experimentally tractable model system to define complex signaling interactions at the cellular and molecular levels.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/19/3787/DC1

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