Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation

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The multilineage differentiation capacity of mouse embryonic stem (ES) cells offers a potential testing platform for gene products that mediate mammalian lineage determination and cellular specialization. Identification of such differentiation regulators is crucial to harnessing ES cells for pharmaceutical discovery and cell therapy. Here we describe the use of episomal expression technology for functional evaluation of cDNA clones during ES-cell differentiation *in vitro*. Several candidate cDNAs identified by subtractive cloning and expression profiling were introduced into ES cells in episomal expression constructs. Subsequent differentiation revealed that the Wnt antagonist Sfrp2 stimulates production of neural progenitors. The significance of this observation was substantiated by forced expression of Wnt-1 and treatment with lithium chloride, both of which inhibit neural differentiation. These findings reveal the importance of Wnt signaling in regulating ES-cell lineage diversification. More generally, this study establishes a path for rapid and direct validation of candidate genes in ES cells.

Mouse embryonic stem (ES) cells undergo unlimited self-renewal in the presence of the cytokine leukemia inhibitory factor (LIF) while retaining multilineage differentiation capacity[1]. Withdrawal of LIF and aggregation leads to differentiation of structures known as embryoid bodies[2]. Embryoid body differentiation reproduces many aspects of tissue formation during the crucial but least accessible stages of mammalian embryogenesis shortly after implantation[3]. This provides an attractive system for the discovery of genes induced during early development and for validation of function in lineage specification, commitment, and differentiation. Such investigations are crucial for the prospects of controlling human pluripotent stem cell differentiation for biomedical applications[4].

ES cells give rise to neural lineages following formation of embryoid bodies and treatment with all-*trans* retinoic acid (RA)[5–8]. The mechanism by which RA exerts this effect is obscure. We sought to identify genes that could play a role in the commitment or differentiation of the neural lineage. We generated a subtractive library enriched for genes induced in RA-treated embryoid bodies. cDNAs of interest, defined by sequence analysis, were examined for neural-restricted expression *in vitro* and in the mouse embryo. Candidates were then functionally tested by introduction into ES cells using an episomal vector system to facilitate transfection and cDNA expression. Through this screening, we identified the secreted Wnt antagonist Sfrp2 (secreted frizzled-related protein-2) as a modulator of neural differentiation from ES cells.

Results

Subtractive cDNA screen. Expression of Sry-related high mobility group (HMG)-box transcription factor Sox1 (ref. 9) was analyzed to monitor neural differentiation in embryoid bodies. Sox1 expression is activated in the neural plate and subsequently maintained specifically in dividing neural precursor cells in the mouse embryo[10]. After RA treatment, *Sox1* mRNA was strongly induced in embryoid bodies. In contrast, *Oct4* (also known as *Pou5f1*) mRNA, an essential marker for undifferentiated ES cells[11], was downregulated. Without RA treatment, *Sox1* expression was not detectable, and *Oct4* mRNA was reduced but not eliminated, reflecting nonneural differentiation with persistence of a subpopulation of undifferentiated ES cells. On the basis of these results, RNA samples from the end of day 8 were taken for cDNA preparation.

Normalized forward (+RA) and reverse (–RA) subtractive cDNA populations were generated using the PCR suppression subtractive hybridization (SSH) technique[12]. The forward subtractive cDNA was used to prepare a library potentially enriched for genes induced during neural determination. An aliquot of this library was plated, and 480 colonies containing inserts were isolated. The inserts were amplified and arrayed in duplicated sets of 96 double spots on nylon membranes. The membranes were then screened by differential hybridization with forward or reverse subtracted cDNA (Fig. 1). Clones that hybridized specifically to the forward probe were sequenced. After redundant clones had been eliminated, 96 unique sequences remained (see Supplementary Table 1 online). BLAST analyses indicated that 74 clones showed near or identical matches to known genes, 21 were expressed sequence tags (ESTs) mapping to separate UniGene clusters, and 1 showed no match. Of the known genes, no expression data are available for 23, whereas 37 (50%) have been reported in developing or adult CNS. Of the remainder, 2 genes are directly responsive to RA but not neural-restricted, and 12 (11%) have widespread expression or have been described before only in nonneural tissues.

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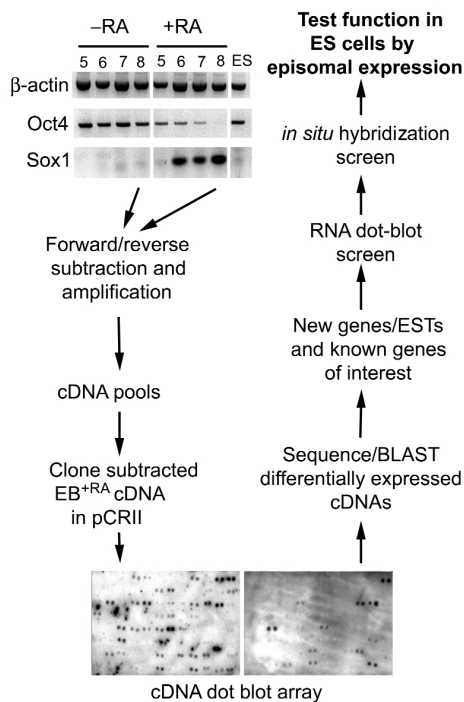**Corresponding author (Austin.smith@ed.ac.uk).*

Figure 1. Experimental overview. Total RNAs from undifferentiated ES cells and from embryoid bodies from days 5 to 8 were analyzed by RT-PCR. Subtracted cDNA from day 8 embryoid bodies was then taken through the outlined screening protocol.

layers during late gestation[25], are both of unknown function. *Sfrp2* encodes an extracellular Wnt antagonist[26]. All three genes are expressed along the length of the neural tube and in the head folds in mouse embryos at embryonic day (E) 8.5, but are absent from other embryonic lineages. Expression is maintained along the entire anteroposterior axis of the neural tube at E10.5, although regional restriction becomes evident in the brain (not shown). In transverse sections through the neural tube at E10.5, hybridization to *P311* and *Tera* mRNAs is restricted dorsally, whereas *Sfrp2* transcripts are localized medially. At this stage, *P311* transcripts are also detected in the dorsal dermamyotome, the sclerotome, and the apical ectodermal ridge, and *Sfrp2* mRNA appears beyond the CNS in two longitudinal stripes corresponding to the mesonephros, as reported earlier[15].

Functional analysis in ES-cell differentiation. Through the preceding screening steps, we focused on 11 cDNAs of potential interest. Among these, 5 (*P311*, *Tera*, *Limd1*, *LNR42*, and RIKEN cDNA 2810027019) encode unknown proteins. In addition, we considered cDNAs encoding four transcription factors (*Meis2*, *Mtsh1*, *Zic1*, *Sox11*), a splicing factor (*Sip1*), and the Wnt antagonist *Sfrp2*. Open reading frames (ORFs) were generated by PCR to test the functionality of these genes during neural specification and differentiation.

Our approach was to introduce these cDNAs in parallel into ES cells and to assay their effects on differentiation. To achieve this, we harnessed the polyoma virus DNA replication system for episomal propagation of cDNA expression constructs. Expression of polyoma virus large T protein in recipient ES cells allows extrachromosomal replication of transfected DNA vectors that contain the polyoma ori-

Confirmation of differential expression by RNA dot blot. Dot blots were prepared with RNA extracted from ES cells, embryoid bodies, neuroepithelial progenitor cells, and adult brain. We used *Sox2*-β-*geo* lineage selection[8,13] to purify neuroepithelial progenitor cells from embryoid body derivatives. Blots were hybridized with probes prepared from individual cloned inserts and normalized relative to β-actin. We eliminated a small number of false-positive clones that failed to show differential hybridization and clones that appeared to be directly induced by RA rather than specific to neural precursors. Table 1 details the genes or ESTs that exhibit high expression in neural progenitors. Several genes earlier reported to be expressed in the embryonic or fetal CNS, such as *Meis2* (ref. 14), *Sfrp2* (ref. 15), *Zic1* (ref. 16), *Sox11* (refs 17, 18), *Sox4* (ref. 19), *Opr* (ref. 20), and *mtsh1* (ref. 21), showed substantial induction during neural determination of ES cells and were downregulated in differentiated neurons and glia represented by adult brain. In addition, genes such as *Tera* and *fidgetin*[22] were identified that are expressed appreciably in ES cells and maintained in neural progenitors but downregulated during nonneural differentiation.

Expression in the developing nervous system. The likely involvement of particular genes in neural development was then assessed by whole-mount *in situ* hybridization of mouse embryos. We found that genes of hitherto uncharacterized embryonic expression, *Limd1* (ref. 23), *Tera*, RIKEN cDNA 2810027019, and the mouse homolog of *Sip1* (ref. 24), are expressed in the developing CNS. By way of example, data on three genes are presented in Figure 2. *Tera*, originally isolated from embryonal carcinoma cells, and *P311*, previously reported in adult brain regions and in neurons in striatum and superficial cortical

Table 1. RNA dot-blot expression screen[a]

	4–/4+	4–/4–	ES cells	*Sox2*+ cells	Brain
UniGene cluster Mm.22383	1.00	0.50	1.30	1.25	0.90
Tera	1.00	0.80	1.10	1.60	0.00
Fidgetin	1.00	0.60	1.10	0.90	0.00
RIKEN cDNA 2610510E10 (*mSip-1*)	1.00	0.55	0.80	0.80	0.60
FLJ20003	1.00	0.50	0.60	2.00	0.40
IDN3	1.00	0.65	0.50	1.80	0.80
UniGene cluster Mm.153458	1.00	0.35	0.50	1.60	1.50
APP-BP1	1.00	0.50	0.50	1.30	0.20
LNR42	1.00	0.50	0.50	1.30	0.20
RIKEN cDNA 1200003107	1.00	0.30	0.40	1.50	0.65
Limd1	1.00	0.25	0.40	1.50	0.10
Sox11	1.00	0.40	0.40	1.30	0.75
TCFL5	1.00	0.35	0.40	1.10	0.50
UniGene cluster Mm.43640	1.00	0.45	0.30	2.00	1.50
Sfrp2	1.00	0.40	0.30	0.95	0.10
D21	1.00	0.40	0.30	0.90	0.30
Zic1	1.00	0.40	0.20	1.50	0.90
Sox4	1.00	0.20	0.15	1.50	0.10
mtsh1	1.00	0.20	0.15	1.45	0.10
Septin-6	1.00	0.30	0.15	1.00	0.35
RIKEN cDNA 2810027019	1.00	0.25	0.10	3.00	0.30
Meis2	1.00	0.20	0.10	2.00	0.40
P311	1.00	0.20	0.10	1.30	0.30
opr	1.00	0.40	0.00	1.80	1.50
UniGene cluster Mm.203935	1.00	0.40	0.00	1.20	0.90

[a]Dot blots were probed with inserts from individual SSH clones, and the signals were quantitated by PhosphorImage analysis and normalized relative to a β-actin control. Signals are expressed as a fraction of that obtained from RA-treated embryoid bodies. RNAs are from embryoid bodies treated with RA (4–/+), embryoid bodies without RA treatment (4–/4–), ES cells, *Sox2*-selected neural precursor cells, and adult brain.
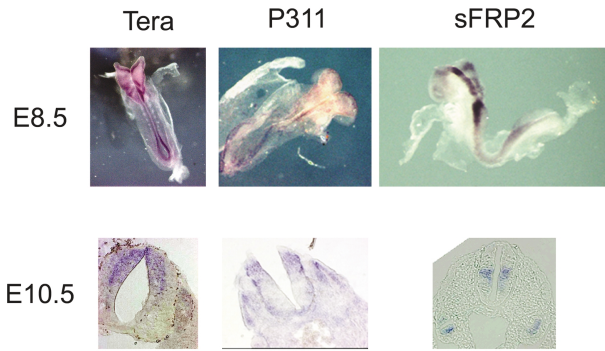
Figure 2. Expression of selected cDNAs in the embryonic CNS. Embryos at E8.5 and E10.5 were analyzed by whole-mount *in situ* hybridization using probes for the indicated mRNAs. For E10.5, transverse sections are shown through the neural tube.

gin of replication[27]. Under these conditions, 100-fold more stable transfectants are obtained than by conventional transfection protocols that rely on chromosomal integration. This process is termed supertransfection[27,28]. We have developed this method for efficient cDNA transduction and expression in ES cells[29,30]. Provided that selection is maintained, an introduced *Ori*-containing plasmid is retained extrachromosomally in several copies per cell in both undifferentiated E14/T ES cells and differentiating progeny. An additional attribute of episomal supertransfection is that the introduced cDNAs avoid silencing, position effect variegation, and other unpredictable effects on level and specificity of expression that compromise chromosomally integrated plasmid or viral vectors.

The ORFs were inserted into the pPyCAGIP bicistronic supertransfection vector[30]. After electroporation and selection in puromycin, no colonies were obtained expressing *Sip1*, indicative of a toxic effect, and only small colonies that contained mainly differentiated cells were obtained for *Meis2A*, *Sox11*, and *Zic1*, suggesting that overexpression of these factors compromises ES-cell viability or self-renewal. For the remaining seven cDNAs, large numbers of undifferentiated ES-cell colonies were obtained, comparable to those generated with an enhanced green fluorescence protein (eGFP) control vector. Colonies (~500) were pooled by trypsinization and polyclonal transfectant populations established.

The functional consequence of forced expression of these cDNAs was then examined by embryoid body differentiation with and without RA treatment. For six of the cDNAs, there were no overt differences in the differentiation profile from control transfectants. In contrast, cells expressing the Wnt antagonist Sfrp2 showed markedly enhanced neuronal differentiation in response to RA. Yet more strikingly, *Sfrp2* transfectants produced appreciable numbers of neurons without RA treatment (Fig. 3). Expression of *Sfrp2* induces the emergence of cells with extended processes that are immunoreactive for the neuronal marker class III β-tubulin (TuJ+ cells). Genes expressed in neural precursors, such as *Ngn2* (ref. 31), *Pax6* (ref. 32), and *Pax7* (ref. 33), are upregulated along with the neuronal gene *ShcC* (ref. 34). These data show that forced expression of *Sfrp2* promotes neural and neuronal gene expression and the appearance of morphologically differentiated neurons.

Induction of neural precursors by forced expression of *Sfrp2*. To obtain independent confirmation of and further insight into the effect of *Sfrp2* on neural differentiation, we took advantage of an ES-cell line engineered to monitor neural specification. In 46C ES cells, a *Sox1* reporter allele has been created by replacing the ORF of the *Sox1* gene with the coding sequence for eGFP. This GFP knockin is silent in undifferentiated ES cells but is activated early in neural specification, just as is endogenous *Sox1* (ref. 10). The kinetics and frequency of conversion of ES cells to neural precursors can therefore be monitored and quantitated by FACS analysis of GFP fluorescence.

46C ES cells do not express polyoma large T. Therefore, conventional stable integrants were generated by electroporation of linearized pPyCAG-*Sfrp*-IP. The bicistronic selection cassette permits enrichment for colonies that exhibit robust expression of the inserted cDNA. Puromycin-resistant colonies (10–15) were pooled to establish a polyclonal population that expresses *Sfrp2* (Fig. 4). Embryoid bodies were then formed from these transfectants. FACS analyses presented in Figure 4 show that *Sfrp2* expression allows development of *Sox1*-GFP+ cells without exposure to RA. The frequency of these cells is 30–40% by day 8, which is comparable to that obtained by RA treatment of the untransfected 46C cells. Furthermore, as with E14/T supertransfection, *Sfrp2* expression in 46C cells enhances the neural-inducing effect of RA such that >60% of cells are *Sox1*-GFP+ by day 8.

Wnt activity inhibits neural differentiation. Sfrp2 acts as an extracellular antagonist of Wnts by competing with transmembrane frizzled receptors[35–37]. The stimulatory effect of Sfrp2 on neural differentiation therefore implies that Wnt activity is inhibitory to the neural program. To examine this directly, we introduced a *Wnt1* expression vector into E14/T cells. The capacity of Wnt-1-overexpressing cells to produce neurons after RA treatment of embryoid bodies was compared with that of control transfectants. A greatly reduced number of TuJ+ neurons were evident (Fig. 5). The expression of neural and neuronal marker genes was also diminished in Wnt-1-expressing cells. We also investi-
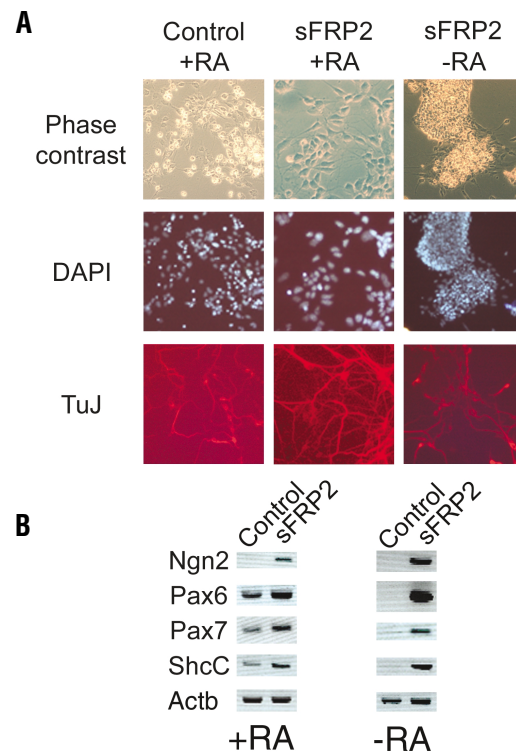

Figure 3. Effect of *Sfrp2* expression in E14/T cells during ES cell differentiation. (A,B) Embryoid bodies formed from GFP control or *Sfrp2* transfectants were stimulated from day 5 to day 8 with 10⁻⁶ M RA or vehicle (0.1% dimethyl sulfoxide, DMSO). After dissociation and plating at day 8, cells were cultured for a further four days. RNA was then extracted for RT-PCR analysis (B) or cells fixed for immunostaining with anti-class III-β tubulin (TuJ) (A). No TuJ signal was obtained from control cells without RA treatment.
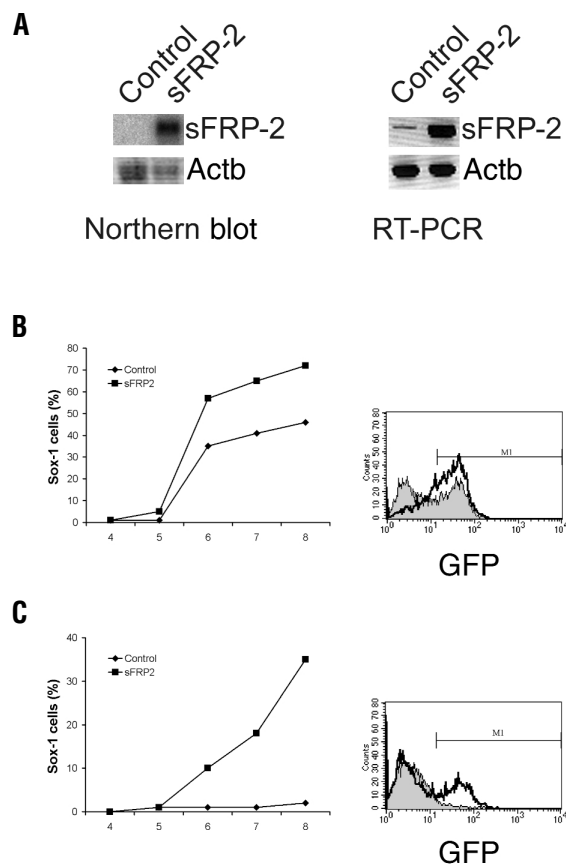
A

Northern blot   RT-PCR

B

GFP

C

GFP

Figure 4. Effect of forced expression of *Sfrp2* on differentiation of *Sox1*-GFP ES cells. (A) *Sfrp2* expression in 46C parental ES cells (control) and *Sfrp2* transfectants. *Sfrp2*-IRES-*pac* mRNA was detected by hybridization using a *pac* probe; *Sfrp2* primers for RT-PCR are within the ORF and amplify both endogenous and introduced *Sfrp2* sequences. (B, C) Proportions of *Sox1*-GFP+ cells determined by FACS in control 46C and *Sfrp2*-expressing embryoid bodies treated from day 5 to day 8 with 10^{-6} M RA (B) or vehicle (0.1% DMSO) (C). Data are representative of three independent experiments. Inserts show flow cytometry profiles at day 8 of control cells (blue) and *Sfrp2* cells (green).

gated the effect of lithium chloride, which inhibits GSK-3 and partially mimics Wnt signaling. FACS analyses showed a reduction in the number of *Sox1*+ cells generated in response to RA in the presence of lithium (Fig. 5). Collectively, these findings support the interpretation that Sfrp2 promotes neural differentiation of ES cells by blocking Wnt activity.

Discussion

By subtractive hybridization and differential screening, we isolated genes induced during neural determination of ES cells and expressed in the developing fetal CNS. Functional analysis of candidate genes then identified the Wnt antagonist Sfrp2 as a positive modulator of neural differentiation. This finding was corroborated by evidence that Wnt signaling suppresses neural development. This study thus establishes a methodology for using ES cells directly to identify genes that control pluripotent cell commitment and differentiation. A critical component in this process is efficient cDNA transduction and expression in ES cells through the use of episomal supertransfection.

Gain-of-function expression studies in ES cells are hampered by transcriptional silencing of random integration events. Retroviral expression vectors are rapidly inactivated in ES cells, and randomly integrated eukaryotic promoters also tend to be progressively

shut down, often in a mosaic fashion within clonal lines. Even where an introduced expression construct remains fully active in ES cells, it is relatively rare for transcription to be maintained ubiquitously upon differentiation. Therefore, functional assignment by conventional forced expression from integrating vectors requires time-consuming characterization of multiple clones and may still give unclear or misleading data. The use of extrachromosomal vectors overcomes these constraints by permitting the generation of stably and ubiquitously expressing transfectants at high frequency. Routine supertransfection efficiencies of 1% mean that a single selection plate of 10^5 transfected E14/T cells yields 1×10^3 colonies. With the use of puromycin selection, which rapidly eliminates nontransfected cells, the supertransfectant population is pure within 4 days and is of sufficient size ($>10^6$ cells) within 14 days to be immediately entered into differentiation assays. Therefore, episomal technology considerably reduces labor and media consumption, and increases the speed and reliability of functional expression screening in ES cells, facilitating parallel evaluation of multiple genes of interest.

The combination of subtractive cloning and functional screening identified Sfrp2 as a potent promoter of neural differentiation and, more broadly, indicates that the modulation of Wnt activity can inhibit or stimulate neural determination of ES cells. *Wnt* genes encode a large family of cysteine-rich secreted glycoproteins active in embryonic patterning and cell fate decisions. Wnt proteins induce cellular responses through binding to transmembrane receptors of the frizzled family. Secreted frizzled related proteins (Sfrps) act as extracellular antagonists by binding and sequestering Wnts. To date, five Sfrp family members have been identified in human, mouse, and chicken[26,38–41]. In different systems, Sfrp2 has been demonstrated to be a direct inhibitor of Wnt activity[35–37].

The impact of Sfrp2 on ES-cell differentiation is profound, mimicking the neural-inducing effect of RA. Conversely, forced expression of Wnt-1 drives nonneural development and suppresses neural differentiation in response to RA. Canonical Wnt signaling entails inactivation of the serine/threonine kinase GSK-3β, and subsequent accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin activates Lef/Tcf-mediated transcription[42]. RA action is mediated through the retinoid receptor proteins RAR and RXR. Intriguingly, evidence has been presented that β-catenin may interact directly with RAR in a retinoid-dependent manner and decrease activation of T-cell transcription factor (TCF)[43]. This raises the possibility that a major effect of RA in embryoid bodies could be to inhibit Wnt signaling by sequestration of β-catenin. Thus Sfrp2 and RA may act respectively as upstream and downstream antagonists of a Wnt–β-catenin 'antineural' pathway. It is noteworthy that Wnt-3a signaling through Lef1/Tcf1 has been implicated in suppression of neural differentiation in the mouse embryo[44,45] and induction of the mesodermal marker Brachyury[46].

Neural differentiation in the absence of RA treatment suggests that the effect of Sfrp2 is at the level of primary neural induction. However, Sfrp2 has been reported to promote clonogenicity and survival of glioma cells[47] and to modulate programmed cell death (PCD) in the developing chick hindbrain[48]. Therefore, Sfrp2 may also enhance survival and expansion of neural precursors.

By uncovering the induction and function of Sfrp2, this study has highlighted the importance of Wnt signaling and Wnt antagonism in directing ES-cell differentiation. Further investigations will clarify the pathways and mechanisms involved. Such studies should be expedited by the use of episomal expression technology, which may also be applied to screen candidate regulatory factors in other ES-cell differentiation protocols.
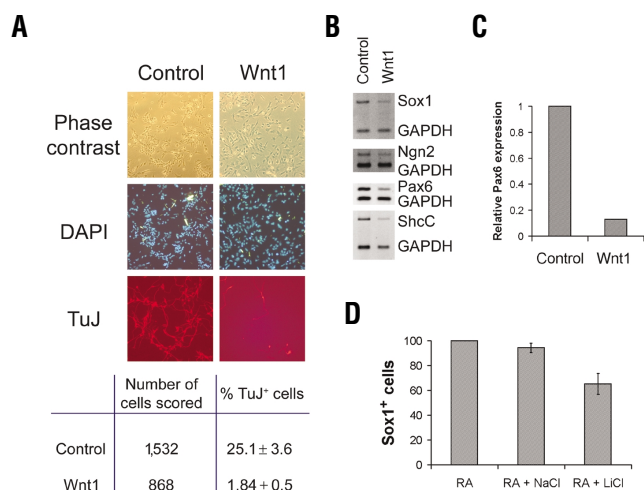
Figure 5. Wnt activity suppresses neural differentiation. (A–C) Embryoid bodies, formed from E14/T cells transfected with pPyHCAG*gfp* (control) or pPyHCAG*wnt1* (*Wnt1*), were stimulated from day 4 to day 8 with 10^{-6} M RA and then plated for three days. Cells were immunostained (A) or RNA harvested for analysis by conventional (B) or real-time (C) RT-PCR. (D) Embryoid bodies formed from 46C *Soxgfp* cells were treated from day 5 to day 6 with RA (10^{-6} M), NaCl (10 mM), and LiCl (10 mM) as indicated, and induction of GFP+ cells quantified by flow cytometry. Data are normalized relative to treatment with RA only.

Experimental protocol

ES-cell culture and differentiation. E14/T cells are derivatives of E14/Tg2a ES cells[49] that constitutively express polyoma large T from an integrated cDNA expression cassette, pMGDneo20 (ref. 27). E14/T cells retain normal ES-cell self-renewal and differentiation character. 46C cells were generated by gene knockin at the *Sox1* locus to insert the eGFP coding sequence.

ES cells were propagated without feeders in the presence of LIF[50]. Neural differentiation was induced by exposure of embryoid bodies from day 5 to day 8 to all-*trans* retinoic acid (RA, 10^{-6} M)[6]. For terminal differentiation, embryoid bodies at the end of day 8 were plated and allowed to grow out on tissue culture plastic.

For episomal supertransfection, E14/T cells (5×10^6) were electroporated with 20 µg of supercoiled plasmid at 200 V and 960 µF in a 0.4 cm cuvette (Bio-Rad, Hemel Hempstead, UK). A fraction of the cells were plated at 10^5 cells per 9 cm dish and cultured in the presence of 1.5 µg/ml puromycin for pPyCAGIP constructs or 250 µg/ml of hygromycin for pPyHCAG vectors. Resultant colonies were pooled for differentiation assays. For conventional transfection, 1×10^7 46C cells were electroporated with 100 µg of linearized plasmid DNA at 800 V and 3 µF, selected in 1 µg/ml of puromycin, and 10–15 puromycin-resistant colonies pooled and expanded for further analysis.

cDNA library construction and macroarray hybridization. Forward and reverse subtracted cDNA populations were prepared using the PCR-Select kit (Clontech, Palo Alto, CA). The amplified fragments from the forward subtraction were ligated into pCRII-topo (Invitrogen, Breda, Netherlands). Clone inserts were amplified by PCR from bacterial lysates using adapter sequences from the PCR-Select kit as primers. PCR products were spotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Subtracted probes were prepared by ^{32}P labeling of 4 µl samples of forward and reverse cDNA. Hybridization signals were quantitated using a PhosphorImager (Fuji, Japan).

RNA extraction, dot blot, and RT-PCR. Total RNA and mRNA were extracted using the RNeasy Mini-Kit and Oligotex (Qiagen, West Sussex, UK) respectively. For RNA dot blot, 100 ng of mRNA was directly spotted on Hybond-N+ membrane. For RT-PCR, 1 µg of total RNA was treated with 1 unit of DNase I for 15 min at 25°C. DNase I was inactivated with 25 mM EDTA, pH 8.0, at 65°C for 10 min and chilled on ice. First-strand random-primed cDNA was synthesized using the Superscript II Preamplification System (Gibco BRL, Paisley, UK). Primers and conditions for PCR are provided in Supplementary Table 2 online. Real-time PCR quantitation of Pax-6 transcripts was carried out in a Light Cycler (Roche, Lewes, UK).

In situ **hybridization.** Mouse embryos were dissected free from extra-embryonic membranes, fixed overnight in 4% PFA–PBS at 4°C, and dehydrated through a methanol series. Digoxygenin-labeled riboprobes were prepared by antisense and sense *in vitro* transcription from SP6 and T7 promoters adjacent to the 5′ and 3′ ends, respectively, of cDNA inserts. Embryos were rehydrated and whole-mount *in situ* hybridization carried out using an InsituPro machine (Anachem, Luton, UK).

Construction of cDNA expression vectors. Primers were designed to amplify full-length ORFs flanked by *Not*I (5′) and *Xho*I (3′) restriction sites. Sequences were amplified from E10.5 mouse embryo cDNA using Expand Long Template PCR (Roche) and cloned in pCRII-topo. After DNA sequence verification, the *Not*I-*Xho*I fragments were inserted into the expression vector pPyCAGIP. Wnt1 cDNA in the pPyHCAG vector was a gift from J. Mason, along with corresponding control GFP expression vector. pPy vectors contain polyoma *Ori* and can be propagated episomally in cells expressing polyoma T.

Note: Supplementary information is available on the Nature Biotechnology website.

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