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Wnt Signaling Promotes Neuronal Differentiation from Mesenchymal Stem Cells Through Activation of Tlx3

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Key Words. Wnt • Tlx3 • Mesenchymal stem cells • Differentiation • Glutamatergic neurons • Transplantation

ABSTRACT

Wnt/β-catenin signaling promotes neural differentiation by activation of the neuron-specific transcription factors, *Neurogenin1* (*Ngn1*), *NeuroD*, and *Brn3a*, in the nervous system. As neurons in cranial sensory ganglia and dorsal root ganglia transiently express *Ngn1, NeuroD*, and *Brn3a* during embryonic development, we hypothesized that Wnt proteins could instructively promote a sensory neuronal fate from mesenchymal stem cells (MSCs) directed to differentiate into neurons. Consistent with our hypothesis, Wnt1 induced expression of sensory neuron markers including *Ngn1, NeuroD*, and *Brn3a*, as well as glutamatergic markers in neurally induced MSCs in vitro and promoted engraftment of transplanted MSCs in the inner ear bearing selective loss of sensory neurons in vivo. Given the consensus function of T-cell leukemia 3 (*Tlx3*), as a glutamatergic selector gene, we postulated that the effects of canonical Wnt signaling on sensory neuron and glutamatergic marker gene expression in MSCs may be mediated by *Tlx3*. We first confirmed that Wnt1 indeed upregulates *Tlx3* expression, which can be suppressed by canonical Wnt inhibitors. Next, our chromatin immunoprecipitation assays revealed that T-cell factor 3/4, Wnt-activated DNA binding proteins, interact with a regulatory region of *Tlx3* in MSCs after neural induction. Furthermore, we demonstrated that forced expression of *Tlx3* in MSCs induced sensory and glutamatergic neuron markers after neural induction. Together, these results identify *Tlx3* as a novel target for canonical Wnt signaling that confers somatic stem cells with a sensory neuron phenotype upon neural induction. STEM CELLS *2011; 29:836–846*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) possess several unique properties that make them a particularly attractive source of cells for cell-based therapy. MSCs have the potential to self-renew and differentiate into multiple cell types [1, 2] and have been used to replace damaged cells in the nervous system using animal models of neurological disorders or traumatic brain injury [3, 4]. Recent evidence suggests that MSCs can be used not only to replace damaged neurons but also to promote endogenous neuronal cell repair or survival by releasing neurotrophic factors [5–7]. These results demonstrate multiple characteristics of MSCs that promote them as donor cells for cellular repair as well as, potential delivery vectors for therapeutic agents.

We previously demonstrated that Sonic hedgehog (Shh) and retinoic acid (RA) synergistically promote expression of sensory neuron markers, including *GATA3*, *Sox10*, glutamate receptor 4 (*GluR4*), and purinergic receptor P2X, ligand-gated ion channel 3 (*P2X3*) in MSCs directed to differentiate into neurons. However, Shh and RA failed to induce expression of the POU-domain transcription factor *Brn3a*, which is expressed in the majority of peripheral sensory neurons during

Author contributions: T.K.: conception and design, collection and/or assembly of data; data analysis and interpretation, manuscript writing; A.J.M.: conception and design, collection and/or assembly of data, data analysis and interpretation; A.S.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; K.R.K.: collection and/or assembly of data, data analysis and interpretation; R.J.C.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; J.M.M.: data analysis and interpretation, final approval of manuscript; E.F.S.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; E.H.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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development [8–10]. Surprisingly, a conditioned medium prepared from embryonic day10 (E10) mouse hindbrain/somite/otocyst induced expression of *Brn3a*, as well as, *Ngn1*, and *NeuroD* [11]. The effects of the conditioned medium appeared to be specific, as no change in the *GATA3* or *Sox10* expression level was detected. These results prompted us to postulate that a soluble protein(s), other than Shh or RA, in the conditioned medium could regulate *Brn3a*, *Ngn1*, and *NeuroD* in MSCs.

Several lines of evidence suggest that Wnt signaling plays pivotal roles in cell fate specification in the nervous systems. Wnt genes encode secreted glycoproteins that exert diverse functions during embryogenesis depending on the cellular and developmental contexts. Canonical Wnt signaling allows β-catenin to translocate to the nucleus, where it interacts with T-cell factor (TCF) family of DNA-binding proteins and regulate transcription [12–14]. Neural crest progenitor cells were shown to acquire Brn3a expression at the expense of Sox10 expression by Wnt/β-catenin signaling [15, 16]. Furthermore, Wnt signaling is essential for generation of otic progenitor cells, some of which give rise to auditory sensory neurons, at early stages of inner ear development [17, 18]. Wnt has also been shown to promote neuronal differentiation from embryonic, somatic, and neural stem cells [19–21]. On the basis of these previous studies, we hypothesized that Wnts are the soluble proteins in the conditioned medium, which promote sensory neuronal fate specification from MSCs after neural induction.

Materials and Methods

MSC Culture

MSCs were isolated from the femurs and tibias of 5- to 7-week old C57BL/6 wild-type mice (Jackson Lab, Bar Harbor, ME), maintained as described previously [11, 22]. These MSCs expressed common MSC markers, but lacked expression of hematopoietic cell markers [22]. Some of the cultured MSCs were plated on poly-D-lysine-coated culture dishes at 5×10^4 cells per centimeter square. To initiate neural differentiation, culture medium was replaced with neural induction medium containing Dulbecco's modified Eagle medium, 10 ng/ml fibroblast growth factor 2 (Peprotec, Rocky Hill, NJ), 2% B27 (Invitrogen, Carlsbad, CA), 5 μM Forskolin (Sigma, St.Louis, MO), 125 μM 3-isobutyl-1-methylxanthine (Sigma,) with 10 μM β-mercaptoethanol, and one of the following reagents: (a) recombinant human Wnt1 (1–400 ng/ml; Peprotec, Rocky Hill, NJ), (b) recombinant human Wnt3a (1–400 ng/ml; R&D Systems, Minneapolis, MN), (c) no factor (control). The cells were incubated for an additional 3 or 7 days. In function-blocking experiments, recombinant mouse Dkk1 (1–100 μg/ml; R&D Systems) or recombinant mouse sFRP2 (0.1–500 ng/ml; R&D Systems) were added to neural induction medium containing Wnt1 (100 ng/ml) prior to the start of an incubation period.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation was performed using the Chip-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacture's instructions. MSCs were incubated for 4 days in maintenance medium, followed by neural induction medium in the presence or absence of 100 ng/ml Wnt1. Cells were incubated for an additional 2 days, after which nuclear protein and genomic DNA were cross-linked by incubation with 1% formaldehyde. The nuclei were collected by Dounce homogenization and centrifugation, and sonicated to shear chromatin to an average of 200- to 800-bp fragments. Immunoprecipitations were performed with anti-TCF3/4 antibody (Millipore, 6F12-3, Billerica, MA) or the corresponding preimmune serum using Protein G magnetic beads. The isolated DNA was subjected to polymerase chain reaction (PCR) analyses using primers flanking the TCF binding sites in the *Tlx3* promoter and its 3′ noncoding region. A sample representing 0.5% of the total chromatin used for immunoprecipitation reactions was used as an input control. A primer pair for the *CyclinD1* promoter [23] was used as a positive control, whereas a primer pair for region 1 (Fig. 5D) that does not contain the TCF-binding motif in the *Tlx3* regulatory region was used as a negative control. PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining. The identity of PCR products was confirmed by cloning DNA fragments into pCR vectors (Invitrogen) and sequence analysis.

Luciferase Reporter Assays

Reporter constructs were prepared using the pGL3 vector (Promega, Madison, WI) that contains a Firefly luciferase reporter gene. A PCR fragment containing the 2,849-bp sequence lying upstream of the Tlx3 start codon was cloned into the pGL3 vector. A mutation in the Tlx3 promoter was introduced using the QuickChange Site-Directed Mutagenesis kit (Stratagene, LA Jolla, CA). For the luciferase reporter assay, MSCs were cotransfected with the Tlx3 promoter-reporter plasmid (wild-type or mutant construct) and the pGL4.74 [hRluc/TK] plasmid encoding *Renilla* luciferase (Promega). Transfected cells were grown in neural induction medium containing Wnt1 (400 ng/ml) for 3 days. Luciferase activity was measured using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized relative to the activity of *Renilla* luciferase.

Surgery and Cell Implantation

Mongolian gerbils (Charles River, Wilmington, MA) at the age of 4-month old were used in this study. The experimental protocol for this study was approved by the institutional animal care and use committee at the Indiana University School of Medicine. Surgery procedures have been previously described [24, 25]. Briefly, following anesthesia, an incision was made to expose the left posterior side of the skull. A piece of gelform (Pfizer, New York, NY) soaked with 5 μl ouabain was placed in the round window niche. The animals were allowed to recover for 1 month, during which progressive degeneration of spiral ganglion neurons takes place. Following the recovery period, the animals were anesthetized and a suspension of cultured MSCs (1×10^6 cells per microliter) in 10 μL phosphate-buffered saline (PBS) (left ear) or 10 μL PBS (right ear, control) was injected into the modiolus through a 30-gauge needle that was inserted into the bony wall of the basal turn of the cochlea.

An Alzet mini-osmotic pump (Model 2004) was used to deliver neural induction medium and Wnt1. The flow rate for infusion at 37°C was 0.25 μl/hour. Under the sterile condition, the osmotic pump was loaded with neural induction medium that contained five times higher concentrations of agents than the one used for in vitro experiments. The osmotic pump was placed in a 37°C saline bath for 12 hours, which allowed the pump to be fully functional immediately upon implantation. The infusion tip of the cannula was made by stripping the Teflon coating from 36-gauge platinum iridium wire (Cooner Wire Co., Chatsworth, CA). A 1.25-cm piece of this Teflon Tubing (0.13-mm inner diameter [ID]; 0.18-mm outer diameter [OD]) was inserted into the end of a 12-mm polyurethane tubing (0.64-mm ID; 1.10-mm OD) (Micro-Renathane, Braintree Scientific Inc., Braintree, MA) and secured with silicon rubber (Dow Corning, MDX 4-4210), leaving 6 mm as a fine infusion tip (Supporting Information Fig. 1). Seventy-two hours after the implantation, the osmotic pump was changed to a new pump containing 500 ng/ml Wnt1 and 250 ng/ml brain-derived neurotrophic factor (BDNF) under general anesthesia. The animals carried the second osmotic pump for the following 3 weeks, after which they were euthanized and their temporal bones were processed for immunohistochemistry.

RESULTS

Sensory Neuron Markers and Glutamate Receptors in Neurally Induced MSCs are Upregulated by Wnt1 in a Dose-Dependent Manner

As the Wnt receptors Frizzled (*Fz*) and low-density lipoprotein receptor-related protein 5/6 as well as key Wnt signaling components are constitutively expressed in MSCs [26], we first tested whether canonical Wnt ligands can upregulate sensory neuron markers in MSCs after neural induction. Incubation of MSCs in neural induction medium supplemented with human recombinant Wnt1 (1–400 ng/ml) for 7 days resulted in a significant upregulation of *Ngn1*, *NeuroD*, *Brn3a*, and *P2X3*, and the effects of Wnt1 were dose-dependent (Fig. 1). Wnt1 at 10 ng/ml or less had little effect on gene expression levels, whereas Wnt1 at 100 and 400 ng/ml induced robust upregulation of all sensory neuron markers examined. Recombinant Wnt3a exhibited similar but lesser effects than Wnt1 (Supporting Information Fig. 2). Interestingly, when MSCs were cultured in maintenance medium, Wnt1 did not induce any of *Ngn1*, *NeuroD*, or *Brn3a* (Supporting Information Fig. 3). We also tested the effects of Wnt1 on AMPA receptors (GluR1-4) and found that all AMPA receptors were significantly upregulated in MSCs grown in neural induction medium containing Wnt1 (Fig. 1 and Supporting Information Fig. 4A). Consistent with the RT-PCR data, Brn3a, GluR4, and VGLUT2 proteins were detected in MSCs grown in neural induction medium with Wnt1 but not in those grown in maintenance medium with Wnt1 (Fig. 2A–2F). In contrast to the sensory neuron marker genes, expression of pan-neural marker genes, such as *Tau* and *TUJ1*, was not altered by Wnt1 in neurally induced MSCs (Fig. 1). Likewise, expression of dopaminergic- (*TH*, *Nurr1*), GABAergic neuronal subtype markers (*Grik2*, *Viaat*), glial markers (glial fibrillary acidic protein [*GFAP*], Integrin beta 4 [*Itgb4*]) was unresponsive to Wnt1 (Supporting Information Fig. 4B). Flow cytometric analysis was performed to quantitatively compare expression of CD24 (pan-neural surface marker; [27]) and GluR2 between MSCs grown in the presence or absence of Wnt1 (Fig. 2G, 2H). The percentage of MSCs expressing CD24 increased dramatically after culture in neural induction medium for 7 days, but there was no significant difference in the percentage of CD24-positive cells between MSCs grown in neural induction medium only and those grown in neural induction medium containing Wnt1. In contrast, the percentage of GluR2-positive cells in neurally induced MSCs grown in the presence of Wnt1 (53.0%) was much greater than undifferentiated MSCs (2.2%) as well as neurally induced MSCs in the absence of Wnt1 (12.9%). As CD24 is expressed not only in neurons but also in granulocytes [28, 29], we tested a possible enrichment of granulocyte precursors in our culture using the hematopoietic markers, CD34, 117, and Sca1 (Supporting Information Fig. 5). The percentage of neurally induced MSCs expressing CD34, 117, or Sca1 was less than 5% and there was no difference in the percentage between cells grown with or without Wnt1, ruling out the possibility that Wnt1 promotes hematopoetic cell proliferation or differentiation.

To test whether Wnt1-induced upregulation of the sensory marker genes in MSCs can be suppressed by specific canonical Wnt inhibitors, MSCs were incubated in neural induction medium supplemented with Wnt1 (100 ng/ml) and either Dkk1 (1–100 ng/ml) or secreted frizzled-related protein 2 (sFRP2) (1-500 ng/ml) for 7 days (Fig. 3). Both Dkk1 and sFRP2 efficiently suppressed Wnt1-induced upregulation of *Ngn1*, *NeuroD*, and *Brn3a*. Furthermore, Wnt1-induced upregulation of Cyclin D1, a known Wnt target, was suppressed by

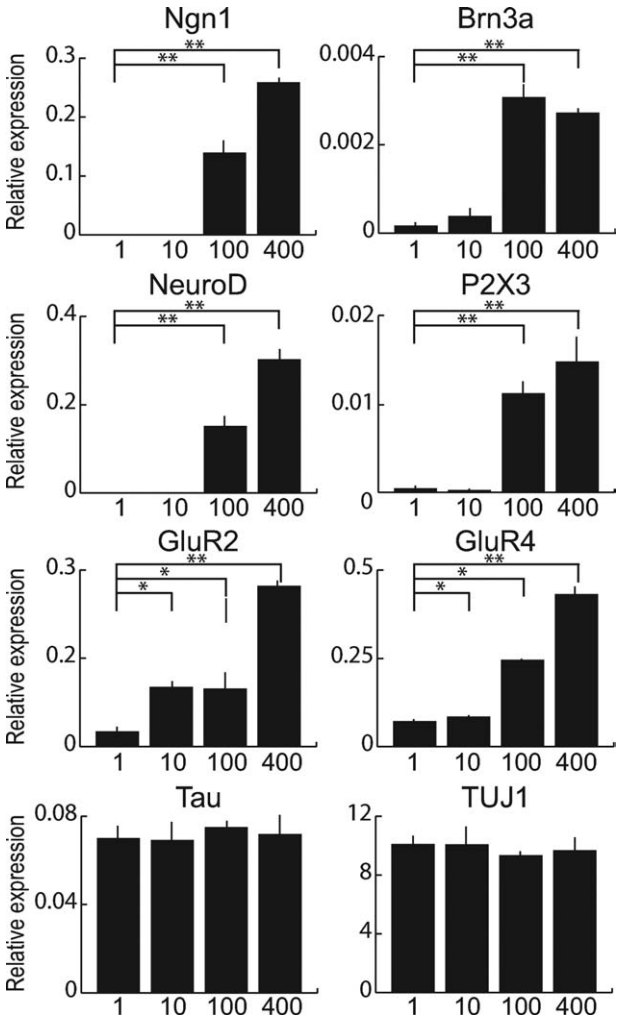

Figure 1. Wnt1 induces sensory neuron marker and AMPA receptor genes in neurally induced mesenchymal stem cells (MSCs). qRT-PCR analysis for sensory neuron markers (*Ngn1*, *NeuroD*, *Brn3a*, and *P2X3*), glutamate receptors (*GluR2* and *GluR4*), and pan-neural markers (*Tau* and *TUJ1*) in MSCs grown in neural induction medium with recombinant human Wnt1 (1–400 ng/ml) for 7 days. Values are mean ± SD. *, $p < .05$; **, $p < .001$.

Dkk1 or sFRP2 at both transcriptional and protein levels (Fig. 3 and Supporting Information Fig. 6). To test whether Dkk1 and/or sFRP2 have any toxic effects on cell viability or can silence genes nonspecifically, we examined expression of *Tau* and *TUJ1*, whose expression is not regulated by Wnt1. No significant changes in expression levels of these neural marker genes were observed in cells treated either with Dkk1 or sFRP2 even at the highest concentration used.

The canonical Wnt signaling pathway regulates β-catenin stability through Fz and LRP coreceptors. When Wnt ligands bind Fz and LRP, phosphorylation of β-catenin is inhibited, causing β-catenin stabilization, and translocation into the cellular nucleus. Using Western blot analysis, we evaluated changes in total and phosphorylated β-catenin expression in MSCs in response to Wnt1 stimulation (Fig. 4A). MSCs were cultured in neural induction medium in the presence or absence of Wnt1 for 1 or 3 days. Expression of total β-catenin was increased by Wnt1, while expression of phospho-β-catenin was decreased. Furthermore, nuclear and cytoplasmic protein fractions were extracted from neurally induced MSCs

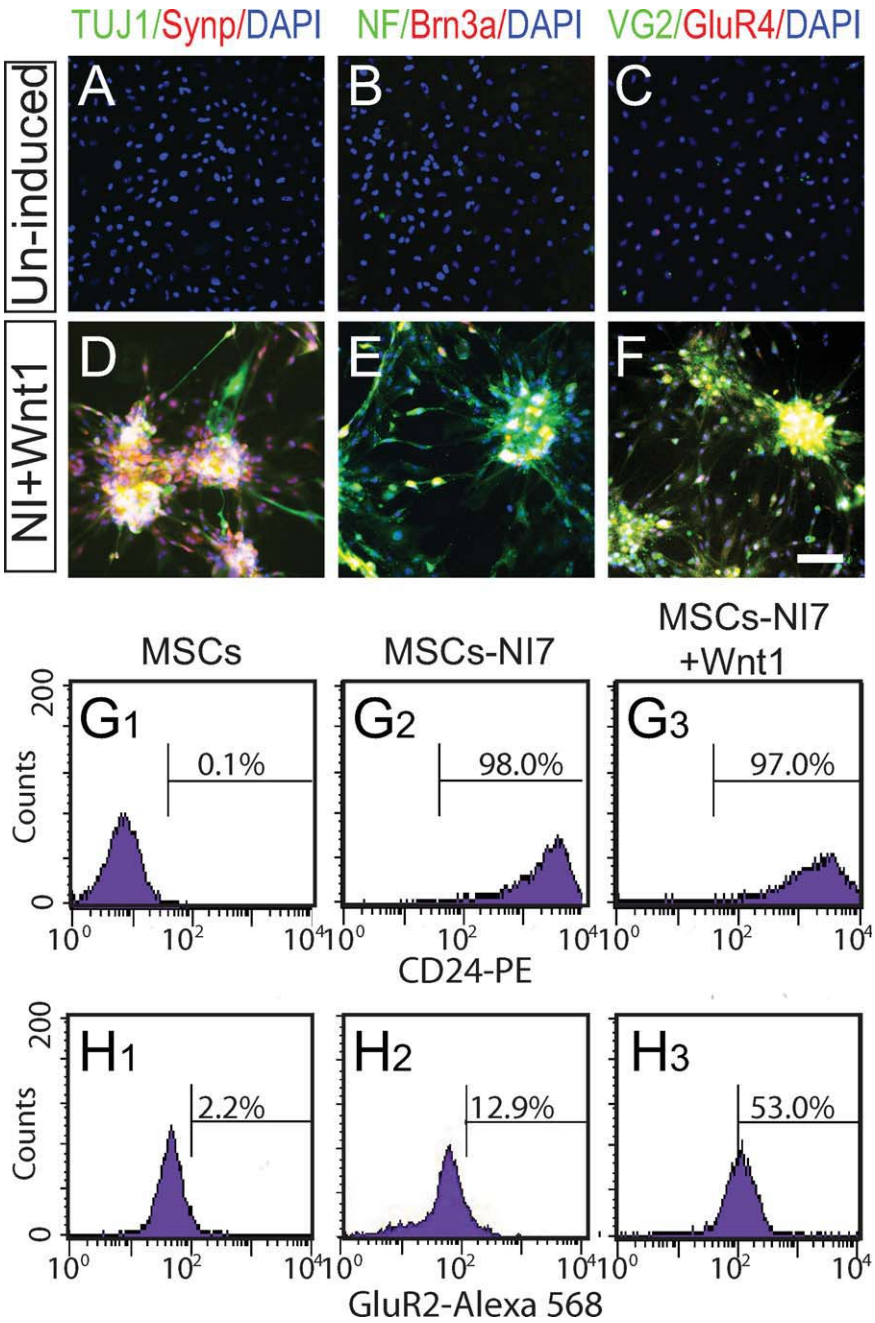

Figure 2. Sensory and glutamatergic neural markers are expressed in neurally induced MSCs in the presence of Wnt1. Immunohistochemical characterization of MSCs grown in maintenance medium **(A–C)** and neural induction medium containing Wnt1 for 7 days **(D–F)**. **(A–F):** TUJ1, NF, and VG2 immunofluorescence is shown in green. Synp, Brn3a, and GluR4 immunofluorescence is shown in red. Cells are counterstained with DAPI (blue). Scale bar = 100 μm. **(G, H):** Flow cytometry profiles of CD24-PE and GluR2-Alexa 568 fluorescence in undifferentiated MSCs and MSCs at MSCs-NI7 or MSCs-NI7+Wnt1. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; MSC, mesenchymal stem cell; VG2, VGULT2; MSCs-NI7, MSCs at neural induction day 7 without Wnt1; MSCs-NI7+Wnt1, MSCs at neural induction day 7 with Wnt1.

that had been grown in the presence or absence of Wnt1 for 3 days (Fig. 4B). Total β-catenin in the nucleus was significantly higher in MSCs grown in the presence of Wnt1 than those devoid of Wnt1. In contrast, expression of phosphorylated β-catenin in the cytoplasm was significantly lower in MSCs exposed to Wnt1 compared with those not exposed to Wnt1. These results indicate that stimulation of the canonical Wnt signaling pathway in neurally induced MSCs promotes dephosphorylation of cytoplasmic β-catenin and its translocation into the nucleus. Consistent with our qRT-PCR results (Fig. 1), an increase in the GluR4 level and no changes in the TUJ1 level were observed when MSCs were grown in the presence of neural induction medium containing Wnt1 (Fig. 4A).

Tlx3 Is Upregulated Directly by Canonical Wnt Signaling

T-cell leukemia 3 (Tlx3) is a selector transcription factor promoting an excitatory glutarmatergic neuronal phenotype over

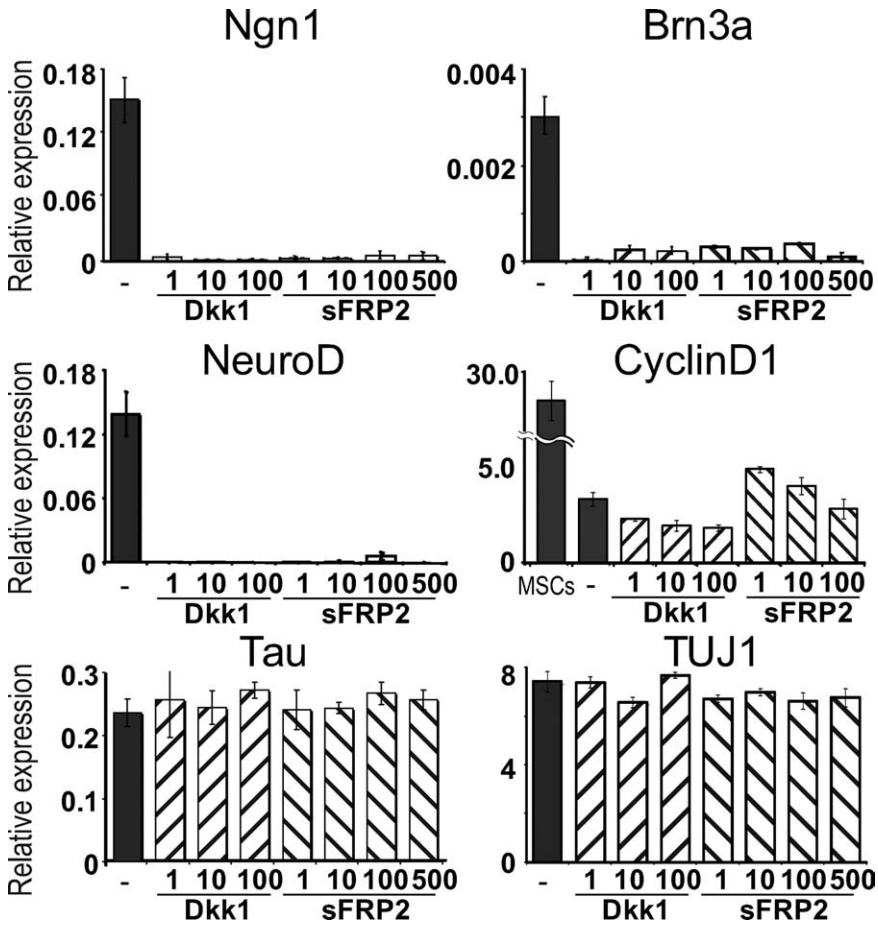

Figure 3. Wnt antagonists suppress Wnt1-induced induction of *Ngn1, NeuroD*, and *Brn3a* in MSCs. qRT-PCR for *Ngn1*, *NeuroD*, *Brn3a*, *CyclinD1*, *Tau*, and *TUJ1* in MSCs grown in neural induction medium with recombinant human Wnt1 and either Dkk1(1–100 ng/ml) or sFRP2 (1–500 ng/ml) for 7 days. Abbreviations: MSC, mesenchymal stem cell; sFRP2, secreted frizzled-related protein 2.

an inhibitory GABAergic phenotype during dorsal spinal cord development [21, 30, 31]. We recently demonstrated that forced expression of Tlx3 is sufficient to induce ectopic expression of glutamatergic neuron markers in ESC-derived neurons [32]. Moreover, Tlx3 expressing ESC-derived neurons exhibit excitatory neuronal functions, such as action potentials and excitatory postsynaptic currents. On the basis of the function of Tlx3 as a master selector gene [30] as well as the overlapping set of genes upregulated by Wnt1 and by Tlx3 (Fig. 1; [32]), we hypothesized that the effects of canonical Wnt signaling on expression of an array of sensory and glutamatergic marker genes in MSCs may be mediated, at least in part, by Tlx3. To validate this hypothesis, we first tested if Tlx3 expression in MSCs can be regulated by canonical Wnt signaling. Incubation of MSCs in neural induction medium containing Wnt1 increased the *Tlx3* mRNA level in a dose-dependent manner and the Wnt1-induced upregulation of *Tlx3* was suppressed by Dkk1 as well as by sFRP2 (Fig. 5A, 5B).

To determine whether TCF3/4, Wnt-activated DNA binding proteins, interact with the regulatory regions of *Tlx3*, we conducted chromatin immunoprecipitation assays. DNA from the immunoprecipitated complex with an anti-TCF3/4 antibody was analyzed by PCR using six primer pairs that span potential regulatory regions containing a consensus TCF-binding sequence (A/T A/T CAAA) [21] and could therefore serve as a binding site for TCF3/4. A promoter region of *Cyclin D1* was used as a positive control. Positive binding of TCF3/4 to a region approximately 540-bp upstream of the *Tlx3* start codon (region 3) was detected in neurally induced MSCs grown in the presence of Wnt1 but not in those in the absence of Wnt1 (Fig. 5C–5D). Sequence analysis of PCR products for the region 3 validated that the immunoprecipitated chromatin indeed contained sequences in this promoter region. All other *Tlx3* regulatory regions containing a consensus TCF-binding sequence exhibited a lack of binding with TCF3/4. To determine whether the TCF binding site that we have identified (region 3 in Fig. 5C) is functional, we compared the activities of the *Tlx3* promoter (nt −2,577 to +272) containing either an intact (TTTGTT) or mutated (TTTGGC) sequence (Fig. 5E). The *Tlx3* promoter was cloned into a pGL3 luciferase reporter vector and, using a site-specific mutagenesis kit, another reporter construct with a mutation in the TCF-binding motif was generated. MSCs were transfected with these reporter constructs and incubated in neural induction medium containing Wnt1 (400 ng/ml) for 4 days. Consistent with the results from chromatin immunoprecipitation assays, the *Tlx3* promoter activity was significantly reduced with a mutation in the region 3 TCF-binding site, when compared with a wild-type (Fig. 5F). Notably, the promoter activity of the *Tlx3* mutant was significantly higher than that of the negative control pGL3-basic vector lacking any promoter sequence. This could be attributed to nuclear factor Y (NFY)-

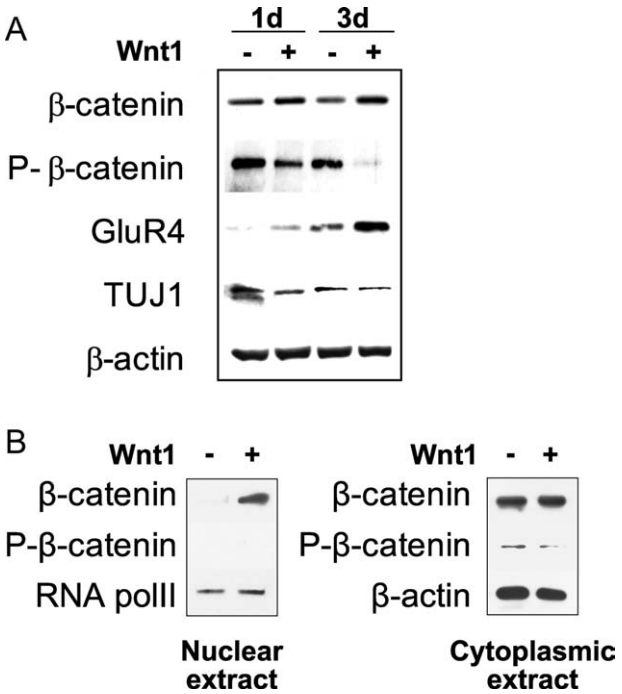

Figure 4. *β*-catenin in mesenchymal stem cells (MSCs) is dephosphorylated and translocated in response to Wnt1 after neural induction. **(A):**Western blot analysis for total *β*-catenin and P-*β*-catenin, GluR4, and TUJ1 in MSCs at 1, 3 days after neural induction with (+) or without (−) recombinant human Wnt1. *β*-actin is used as a loading control. **(B):** Expression of *β*-catenin and P-*β*-catenin in the nuclear and cytoplasmic extracts from neurally induced MSCs with Wnt1 for 3 days. RNA polII was used as an internal control for the nuclear protein fraction. Abbreviation: P-*β*-catenin, phosphorylated *β*-catenin.

dependent basal promoter activation as the mutant *Tlx3* promoter contains NFY-binding sites, which are critical for basal *Tlx3* expression [33].

Ectopic Expression of Tlx3 in MSCs Induces a Sensory Neuron Phenotype

To test whether *Tlx3* can regulate expression of those genes, which are regulated by Wnt1, MSCs, which do not constitutively express Tlx3, were transfected with a Tlx3 expression vector (pBud-eGFP-cTlx3) or a control vector (pBud-eGFP). MSCs stably expressing the *Tlx3* expression construct or control construct were subjected to neural induction. Despite dramatic changes in cell morphology, intense GFP fluorescence was maintained in virtually 100% of neurally induced MSCs, indicating that transgene expression was not silenced by neural induction (Fig. 6A). Consistent with this, high-level *Tlx3* expression was detected in MSCs expressing pBud-eGFP-cTlx3, but not in untransfected MSCs or MSCs expressing pBud-eGFP (Fig. 6B–6C).

qRT-PCR analyses were performed to evaluate changes in gene expression in neurally induced MSCs in response to ectopic *Tlx3* expression. Pan-neural markers, such as *Tau*, *TUJ1*, and *Synp*, were induced after neural induction, however, no significant differences in their expression levels were observed in the presence or absence of *Tlx3*. In contrast, expression levels of *Ngn1*, *NeuroD*, *Brn3a*, *Mash1*, and *GluR4* in *Tlx3*-expressing MSC-derived cells were significantly higher than in control MSC-derived cells at neural induction day 4 (Fig. 6D). In addition, *Brn3a*, *Mash1*, and *GluR4* expression remained elevated in cells expressing *Tlx3* at neural induction day 7, and a notable upregulation of the

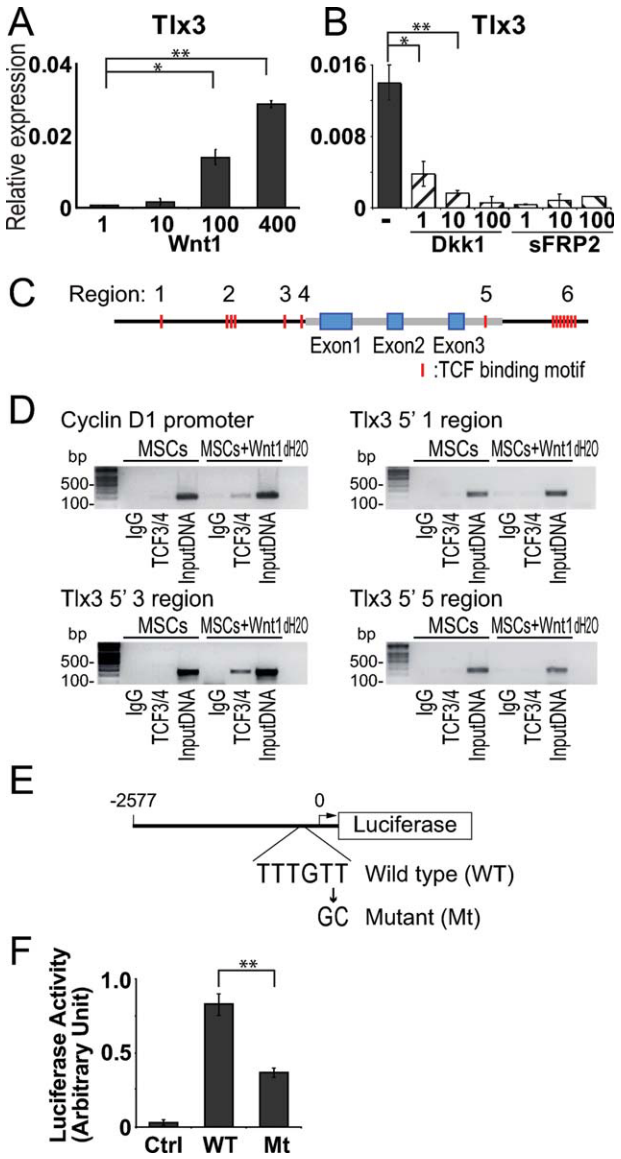

Figure 5. *Tlx3* is upregulated directly by canonical Wnt signaling. **(A, B):** qRT-PCR analysis for *Tlx3* in MSCs at day 7 of neuron induction with Wnt1 and either Dkk1 (1, 10, or 100 ng/ml) or sFRP2 (1, 10, or 100 ng/ml). **(C, D):** Chromatin immunoprecipitation with anti-TCF3/4 antibody on the resulting genomic DNA was conducted on neurally induced MSCs. Polymerase chain reaction probes to the various promoters relative to the *Tlx3* coding region are indicated in **(C)**. The red vertical lines indicate the positions of a consensus TCF-binding sequence. **(D):** The promoter region 3 exhibits binding with TCF3/4 in the presence of Wnt1, whereas other regions lack binding. Cyclin D1 promoter, a known TCF target, was used as a positive control. IgG represents the amount of DNA immunoprecipitated by normal mouse IgG (negative control). Input DNA represents a sample representing 0.5% of the total chromatin used for immunoprecipitation reactions (input control). **(E):** pLG3 luciferase reporter vector containing Tlx3 promoter (WT) and its mutant within the region 3 TCF-binding site (Mt). **(F):** Relative luciferase activity in pLG3 control vector (Ctrl), Tlx3 promoter (WT), and mutant Tlx3 promoter (mt) expressing neurally induced MSCs with Wnt1 for 3 days. *, $p < .05$; **, $p < .001$. Abbreviations: Dkk1, Dickkopf-related protein 1; MSC, mesenchymal stem cell; Mt, mutant; sFRP2, secreted frizzled-related protein 2; TCF 3/4, T-cell factor, 3/4; WT, wild type.

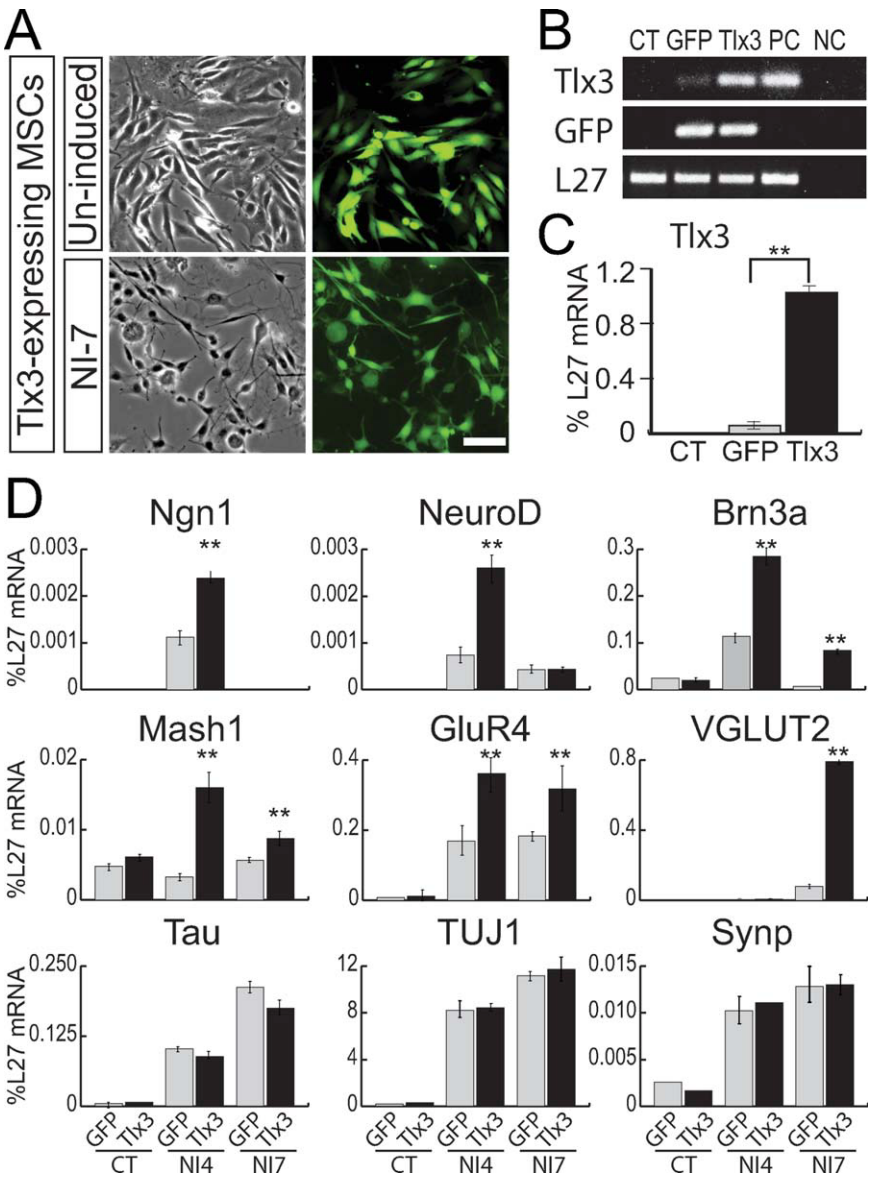

Figure 6. Forced expression of *Tlx3* results in upregulation of sensory and glutamatergic marker genes in neurally induced MSCs. **(A):** Forced expression of Tlx3 in MSCs. Virtually 100% of MSCs expressing pBud-eGFP-cTlx3 in the figure are GFP-positive and remain stably exhibiting GFP expression after neural induction. Scale bar = 100 μm. **(B, C):** RT-PCR analysis for *GFP* and *Tlx3* in neurally induced MSCs-expressing pBud-eGFP (GFP) or pBud-eGFP-cTlx3 (Tlx3). **(D):** qRT-PCR analysis for *Ngn1*, *NeuroD*, *Brn3a*, *Mash1*, *GluR4*, *VGLUT2*, *Tau*, *TUJ1*, and *Synp* in MSCs expressing the *Tlx3* expression vector (Tlx3) in comparison with those expressing the control vector (GFP). Undifferentiated MSCs (CT) or MSCs grown in neural induction medium for 4 days (NI4) or 7 days (NI7). **, $p < .001$. Abbreviations: CT, control; GFP, green fluorescent protein; MSC, mesenchymal stem cell; NI7, neural induction day 7; NI4, neural induction day 4; NC, no cDNA; PC, E10 otocyst.

glutamate transporter *VGLUT2* was observed in MSC-derived cells expressing *Tlx3* but not in those expressing the control vector. The downregulation of *Ngn1* and *NeuroD* at neural induction day 7 as compared with day 4 (Fig. 6D) is consistent with transient expression of these proneural genes in neural progenitor cells in vivo. Furthermore, the delayed upregulation of *GluR4* and *VGLUT2* in neurally induced MSCs expressing *Tlx3* coincides with that observed during embryonic neural differentiation.

Wnt1 Promotes Engraftment and Differentiation of MSCs in an Animal Model of Auditory Neuropathy

The positive effects of Wnt1 on MSC differentiation in vitro prompted us to test its effect on MSCs in vivo. We hypothesized that engraftment and neuronal differentiation would be enhanced if MSCs are implanted into the cochlea of animals bearing selective loss of auditory sensory neurons and subsequently exposed to neural induction medium and Wnt1 in situ. GFP-positive MSCs isolated from TgN(ACT-bEGFP) mice were subjected to preinduction and then implanted into eight Mongolian gerbils, deafened by ouabain [25, 34]. A modified ALZET osmotic pump was used to continuously infuse (a) neural induction medium for 3 days followed by Wnt1 and BDNF for 25 days (experimental group), (b) neural induction medium followed by BDNF alone (control one group), or (c) physiological saline for 28 days (control two group) into the implanted cochlea (Fig. 7A). After 28 days postimplantation, a very small number of MSCs were found in the cochlea of control

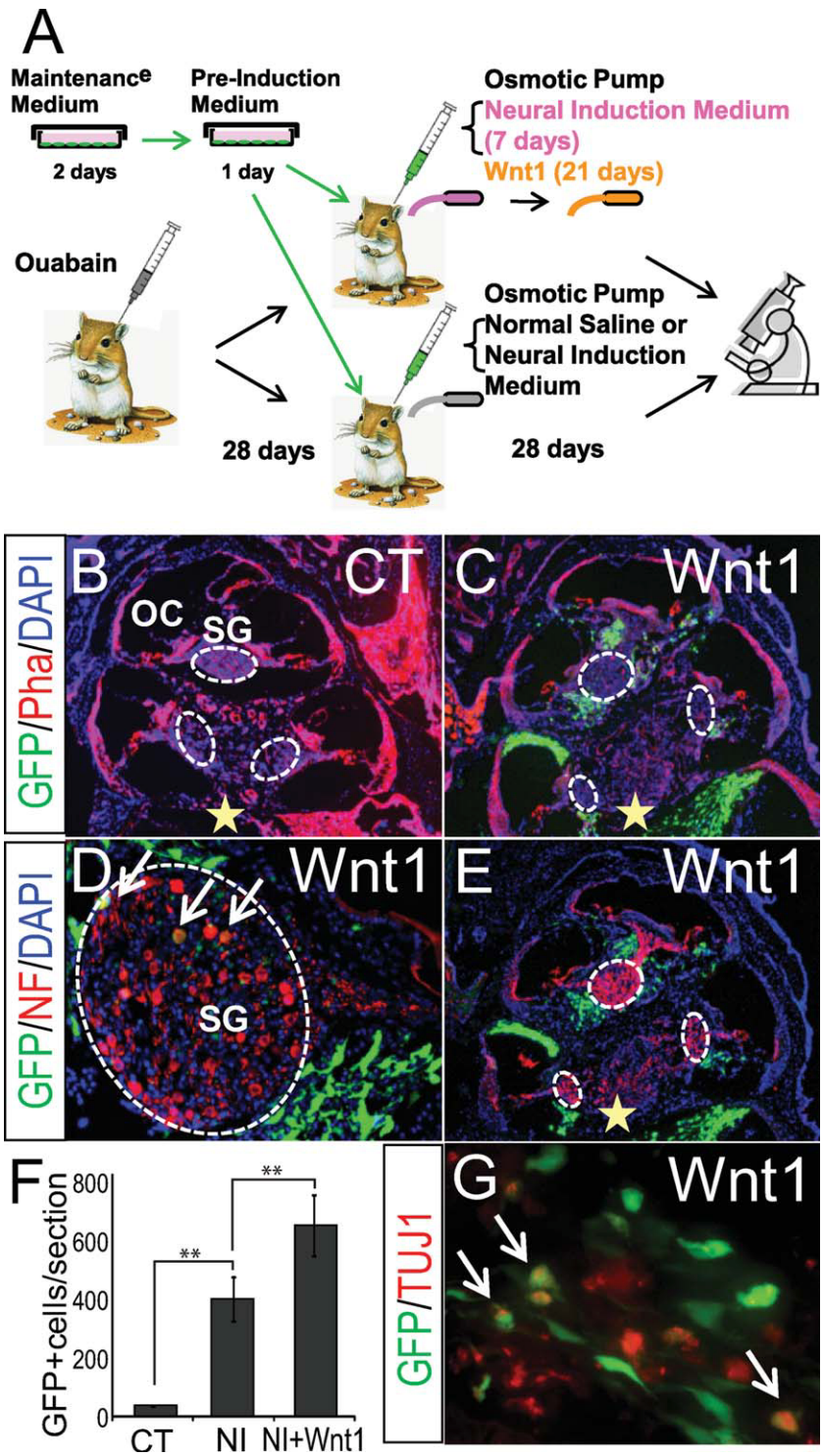

Figure 7. Wnt1 promotes engraftment and differentiation of mesenchymal stem cells (MSCs) in the gerbil cochlea with selective loss of spiral ganglion neurons. **(A):** Experimental design in in vivo experiments. **(B–G):** GFP-positive MSCs transplanted into the modiolus of the gerbil cochlea that has received physiological saline (**[B]**, control) or neural induction medium followed by Wnt1 (**C, D, E, G**). The number of average GFP-positive profiles per section for the control and NI medium and Wnt1 group (NI+Wnt1) (**F**). GFP-positive MSCs (green) are found not only in areas surrounding the SG in the cochlea but also in the sensory epithelia of the macula in the saccule. Cellular nuclei are stained with DAPI (blue). Cochlear structures are visualized with rhodamine-conjugated phalloidin (Pha, red) (**B, C**). NF and TUJ1 immunofluorescence are shown in red (**D, F, G**). Yellow star indicates the stem cell injection site. **, $p < .001$. Abbreviations: CT, control; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; NF, neurofilament; NI, neural induction medium; OC, organ of corti; SG, spiral ganglion.

animals that had received physiological saline (Fig. 7B, 7F). In contrast, a significantly greater number of GFP-positive MSC-derived cells were found in the modiolus of animals that had received neural induction medium and the number of engrafted cells was even greater in animals that had received neural induction medium followed by Wnt1 (Fig. 7C–7G). To test whether the observed difference in the number of engrafted cells between the experimental and the control 1 groups is due to the effect of Wnt1 on cell proliferation, we performed in vitro BrdU-incorporation assays (Supporting Information Fig. 7). The percentages of BrdU-positive cells in neurally induced MSCs in the presence and absence of Wnt1 after 24 hours exposure to 10 μM BrdU were 2.36% and 2.26%, respectively, and these values were much lower than the percentage of BrdU-positive cells (76%) in undifferentiated MSCs [11]. Strikingly, GFP-positive MSCs that had been injected into the basal cochlear turn were found throughout all cochlear turns and many were found in areas surrounding the spiral ganglion at 28 days postimplantation, thus indicating extensive migration of the MSCs throughout the sensorineural tissues of the cochlea. Numerous GFP-positive cells were observed areas surrounding the spiral ganglion, while only several MSCs were detected within the ganglion (Fig. 7D). Some of the GFP-positive cells exhibited spherical cell bodies with long neurites and expressed several neural markers, including neurofilament and TUJ1 (Fig. 7G).

Discussion

MSCs in the bone marrow possess several unique properties that make them a particularly attractive source of somatic stem cells for cell replacement therapy. MSCs have a high self-renewal potential, genetic stability and relative ease of collection from patients' own bone marrow. Furthermore, two other extraordinary characteristics have been recently identified, including one in our present study, which makes MSCs an excellent source of cells for autologous transplantation therapy, that is, MSCs exhibit remarkable abilities to migrate toward sites of tissue injury and have strong immunosuppressive properties [35, 36]. In this study, we sought to determine the identity of microenvironment-derived soluble proteins that promote specification of MSCs toward a sensory neuron phenotype.

We have demonstrated that Wnt1 and -3a secreted from the microenvironment surrounding the embryonic inner ear can instructively promote expression of several sensory neuron markers, including *Ngn1*, *NeuroD*, *Brn3a*, *P2X3, GluR1-4*, in MSCs that are exposed to neural induction signals (Fig. 1 and Supporting Information Fig. 2). Wnt ligands are secreted glycoproteins but are known to be highly hydrophobic and, thus, tend to adhere the plasma membrane of the secreting cells [14]. Despite this adherent property, we were able to detect both Wnt1 and Wnt3a proteins in conditioned medium prepared from the E10 mouse cervical tissues containing the otocyst, hindbrain, and somite (T. Kondo, unpublished observations). This is consistent with previous reports, in which Wnt4 secreted from COS cells exert attractive cues for responsive neurons that were located more than 300 μm from Wnt4-secreting cells [37, 38], suggesting that Wnt proteins can function as classical diffusible signaling proteins albeit for lesser spatial ranges. Although we did not measure electrophysiological properties of neurally induced MSCs used in this study, recent studies revealed that MSC-derived cells are capable of generating action potentials, as well as expressing sodium and voltage-gated calcium channels [39–41], indicating the competence of MSCs to give rise to functional neurons.

We also presented evidence that the regulation of the sensory marker genes by canonical Wnt signaling is mediated, at least in part, by the master selector gene *Tlx3*. Wnt1 was able to induce *Tlx3* expression in MSCs in a dose-dependent manner (Fig. 5A). When *Tlx3* was introduced in MSCs that do not constitutively express *Tlx3*, these cells exhibited significantly higher levels of *Ngn1*, *NeuroD*, *Brn3a*, and *GluR4* than those lacking *Tlx3* after neural induction (Fig. 6). Importantly, the upregulation of the sensory neuron marker genes in MSCs by Wnt1 or *Tlx3* was context-dependent. Both Wnt1 and *Tlx3* instructively promoted expression of the target genes only after MSCs were exposed to neural induction medium, while they had no effect on gene expression in untreated MSCs. The context-dependent regulation of gene expression in MSCs by *Tlx3* is consistent with our previous results with mouse ESCs [32]. One potential mechanism involved in this process is epigenetic transcriptional regulation mediated by Pbx3, a member of the TALE family of DNA-binding proteins. Pbx proteins are known to function as transcription cofactors and modulate DNA binding affinity and specificity of HOX proteins, such as Tlx3 [42, 43]. As most HOX proteins exhibit promiscuous DNA-binding properties despite their specific transcriptional activity, it is believed that Pbx proteins enhance target site selectivity by interacting with HOX proteins on the promoter loci of their target genes [44, 45]. The transcriptional complexes formed by Pbx and Hox proteins recruit a variety of coregulators. It is possible that some transcriptional coactivators, such as a histone acetyltransferase, might be recruited upon neural induction to the Tlx3-Pbx3 heterodimer on the *Ngn1* promoter, thereby inducing chromatin modifications and evoking transcriptional activation of *Ngn1*. Alternatively, Sox2 might be involved in the context-dependent Tlx3 actions. In the adult hippocampus, canonical Wnt signaling activates NeuroD expression in neural stem cells. The regulatory region of *NeuroD* has binding sites for TCF/LEF and Sox2, some of which form a Sox2/LEF hybrid binding motif. Wnt signaling directly control *NeuroD* transcription through TCF/LEF, but that the Sox2/LEF element serves as a dual switch either repression by Sox2 or activation by TCF/LEF [21]. Despite a lack of the Sox2/LEF hybrid binding site, a Sox2-binding site was detected in the 3′-regulatory region of *Tlx3*. As Sox2 is expressed in undifferentiated MSCs, but not after neural induction, it is possible that loss of Sox2 after neural induction might result in disinhibition of TCF-mediated transcriptional activation of *Tlx3*, thereby indirectly activating its target genes.

We have identified *Tlx3* as a direct downstream target for canonical Wnt signaling in MSCs that are grown in neural induction medium containing Wnt1. Ectopic expression of Tlx3 in MSCs upregulated the same set of genes as those which were upregulated by Wnt1 (Figs. 1, 6). An increase in nuclear β-catenin and a reduction in cytoplasmic phosphorylated β-catenin were observed concomitantly with the specific binding of TCF3/4 with the *Tlx3* promoter. Our sequence search identified a total of six sites in the *Tlx3* regulatory regions that contain consensus TCF binding motif(s). Of these putative TCF binding sites, only a region located approximately 530-bp upstream of the *Tlx3* coding region turned out to exhibit a binding with TCF3/4 and a mutation in this promoter region resulted in impaired *Tlx3* promoter activity. The identification of the functional TCF-binding site reveals that *Tlx3* is a novel Wnt target gene.

It is interesting to note that stimulation of the canonical Wnt pathway has little effect on the expression levels of *GATA3* or *Sox10*, both of which are upregulated by a combination of Shh and RA [11]. Conversely, Shh and RA failed to

induce expression of *Brn3a* and *NeuroD* in MSCs [11], which are upregulated by Wnt1 or Wnt3a (Fig. 1 and Supporting Information Fig. 2). Several lines of evidence indicate that some of the genes induced either by Wnt1 or Shh+RA in our preparations are complementary expressed in neural progenitors during development. In the dorsal spinal cord, neural crest-derived progenitors lose Sox10 expression and give rise to Brn3a-positive sensory neurons [16]. In the inner ear, NeuroD-positive progenitor cells lose NeuroD expression and differentiate into GATA3-positive auditory sensory neurons [46]. Thus, timing and balance of Wnt proteins and Shh+RA applications may be a key for instructing neurally induced MSCs to differentiate into cell populations with a specific phenotype.

Positive effects of Wnt1, when combined with neural induction medium, on engraftment of MSC-derived cells were observed in the cochlea of animals with selective loss of spiral ganglion neurons (Fig. 7). As MSCs become postmitotic after neural induction and as Wnt1 has no effect on cell growth rate of neurally induced MSCs (Supporting Information Fig. 7; [11]), the observed increase in the number of engrafted cells is most likely due to the effect of Wnt1 on cell survival. This is consistent with a recently recognized function of Wnt/β-catenin signaling for survival and maintenance of neural progenitor cells [47–50]. The fact that Wnt1-induced upregulation of CyclinD1 did not accompany an increase in cell proliferation in neurally induced MSCs seems paradoxical. However, the expression level of CyclinD1 in neurally induced MSCs in the presence of Wnt1 was only 15% of undifferentiated MSCs (Fig. 3). The extent of MSC migration was also robust, as partially differentiated MSCs implanted into the basal turn of the cochlea were found to be present in the apical turn 4 weeks after transplantation. This, however, is probably due to an innate characteristic of MSCs, rather than trophic effects of Wnt1, as cell engraftment was observed only in the basal turn of the cochlea that had received mouse ESCs (A. Matsuoka, unpublished observations). Despite the enhanced migration and engraftment, the rate of engrafted MSCs expressing pan-neural markers was low (less than 10%). This could be attributed to (a) the short-infusion period (3 days) with neural induction medium, and (b) the concentration of neural induction medium was not high enough to promote neural induction, although we used a medium that contained five times higher concentrations of reagents used for our in vitro experiments. Furthermore, Wnt1, known to exert diverse functions depending on developmental contexts, might not have promoted neuronal differentiation as neural induction was incomplete in our cell populations. Wnt proteins have been shown to promote neurogenesis, neuronal cell fate specification, axon growth, and synaptic number/transmission depending on cellular and developmental contexts [16, 38, 51, 52]. In our in vivo experimental paradigm, it would be extremely difficult to control precise timing and doses of Wnt(s) that MSCs are exposed to while undergoing neural differentiation. As Wnt signaling-mediated sensory neuron specification in MSCs is mediated by Tlx3, Tlx3-expressing cells will provide a novel strategy to conditionally generate excitatory glutamatergic neurons in vivo. Consistent with this, 50%–75% of mouse ESCs overexpressing Ngn1 were shown to differentiate into neurons with a glutamatergic phenotype in deafened guinea pig's cochleae [53]. As our results indicate that Tlx3 controls Ngn1 expression in MSCs, stimulating the Tlx3-Ngn1 pathway would likely yield stem cell-derived neurons to replace auditory neurons in the inner ear. Further investigation is required to determine the optimal conditions for neural induction from embryonic and somatic stem cells in vivo.

Conclusion

We have identified *Tlx3* as a novel target for the canonical Wnt signaling that promotes neuronal differentiation from bone marrow-derived somatic stem cells. TCF3/4, Wnt-activated DNA binding proteins, interact with a regulatory region of *Tlx3* in neurally induced MSCs and a mutation in the TCF-binding motif reduces Wnt-dependent *Tlx3* promoter activity. Thus, Wnt-induced acquisition of a sensory neuron phenotype in neurally induced MSCs appears to be mediated by Tlx3. In addition, Wnt1 promotes engraftment and survival of transplanted MSCs in the inner ear of animals bearing selective loss of sensory neurons. These results suggest that activating the Wnt-Tlx3 pathway could confer neurally competent MSCs with a sensory neuron phenotype and promote their survival.

Acknowledgments

We thank Angela Thompson, Diane Prieskorn, Leon Carter, and Zhenyun Yang for their expert technical assistance. This work was supported by National Institutes of Health grants R01 DC007390 and RC1 DC010706 (to E.H.) and the Ruth and Lynn Townsend Professorship in Communication Disorders to the University of Michigan (to J.M.M.).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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