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The canonical Wnt pathway directly regulates *NRSF/REST* expression in chick spinal cord

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Abstract

Neural precursor cells actively proliferate in the ventricular zone to self-renew the stem cell population, and in parallel, maintain their undifferentiated state. This progenitor pool generates postmitotic cells that migrate to the mantle layer and differentiate into mature neurons. The growth of these stem cells is strictly controlled by the canonical Wnt signaling cascade, in part mediated by the direct regulation of *Cyclin D1*, a critical regulator of cell cycle progression. Here, we report that the canonical Wnt pathway directly controls the expression of *NRSF/REST*. The Wnt-activated β-catenin/TCF complex up-regulates this gene through a conserved element found in its exon 1a, a critical result obtained by a novel in ovo transcriptional assay. Hence, our data show that the canonical Wnt signaling cascade directly regulates the *NRSF/REST* and *Cyclin D1* genes, thereby controlling the progenitor cells. In addition, we show that our in ovo transcription assay is a powerful way to analyze gene regulation in a natural in vivo context.

Keywords: *Wnt1*; *NRSF/REST*; β-catenin; Canonical Wnt pathway; Spinal cord; Progenitor cells; In ovo electroporation

Orchestrated development of the central nervous system is based on the balance between self-renewal of neural stem cells and differentiation of postmitotic cells. Neural stem cells that give rise to the various cell types are located in a distinct area of the developing nervous system, the ventricular zone (VZ). Neural precursor cells actively divide in the VZ to renew the pool of progenitor cells. In parallel, the progenitor pool generates postmitotic cells that leave the VZ and migrate to the mantle zone (MZ) to differentiate into neurons [1]. The balance between self-renewal and differentiation varies during development, yet it is strictly controlled to form correct numbers of cells with various identities and synaptic organization.

During the development of the central nervous system, Wnts and their receptors are expressed in spatially and temporally controlled manner. Genetic analyses have shown that this signaling cascade regulates the growth and patterning events of neural tissues. For example, *Wnt1* knockout mice show a significant loss of the cell population near the midbrain/hindbrain boundary [2,3]. The hippocampus is not formed in *Wnt3a* mutants [4]. *Wnt1/Wnt3a* double mutants show a reduction of brain structures in a broader area, spanning the diencephalon to the spinal cord [5]. Contrary to these loss-of-function approaches, overexpression of *Wnt* genes induces expansion and accelerated growth of neural progenitors [6]. Recent studies using the chick spinal cord as a model have shown that *Wnt* genes exert distinct activities on the proliferation of neural stem cells. In both chick and mouse spinal cords, several *Wnt* genes are expressed in similar patterns. Nonetheless, *Wnt1* and *Wnt3a* have been shown to exert mitogenic activity, whereas *Wnt3*, *Wnt4*, *Wnt7a*, and *Wnt7b* had no effects on the proliferation or differentiation of neural progenitor cells [7]. This indicates that different *Wnt* genes play distinct roles during the development of the central nervous system and that *Wnt1* and *Wnt3a* genes have potent mitogenic activities on spinal neural precursors.

β-Catenin is a central component of the canonical Wnt signaling pathway. When Wnt signaling is active, β-catenin is stabilized and translocates into the nucleus

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to interact with transcription factors of the LEF/TCF family and regulate expression of target genes. In the absence of signals, β-catenin is phosphorylated by casein kinase I and GSK3β. Phosphorylated β-catenin is ubiquitinated and degraded by proteosomes [8–10]. As expected, conditional mutation of β-catenin leads to cell loss [11]. In contrast, overexpression of activated β-catenin in the spinal cord induces expansion of neural tissues. These observations are compatible with data obtained from the loss-of-function and the gain-of-function experiments with *Wnt1* and *Wnt3a* genes. Since both Wnt1 and Wnt3a transduce signals through the canonical Wnt pathway, these observations suggest that transcriptional control mediated by the β-catenin and the LEF/TCF transcription factors is pivotal for the proliferation of neural stem cells.

As described above, Wnt signaling regulates the proliferation of the neural precursor cells, but it is not known how this signaling cascade regulates its target genes that might be involved in the control of the stem cell population. To explore further, we searched several transcription factors expressed exclusively in neural stem cells and found that the *neuron restrictive silencer factor/repressor element 1 transcription factor* (*NRSF/REST*) is the direct target of the canonical Wnt signaling cascade. NRSF/REST, a transcription factor containing zinc fingers as DNA binding domains, is a negative regulator of neuronal genes that contain the neuron restrictive silencer element (NRSE) [12,13]. In the developing spinal cord, this gene is expressed in neural precursors and turned off in postmitotic neurons [12]. Previous studies have shown that NRSF/REST represses several neuron-specific genes, such as neurotransmitter receptors, ion channels, and neural cell adhesion molecules and neurotrophins [14]. In addition, misexpression of a dominant-negative *NRSF* induced derepression of neuronal markers, thereby resulting in precocious differentiation [15].

Our data provide the first evidence of a direct connection between the Wnt signaling cascade and the *NRSF/REST*. Since *Cyclin D1*, a key regulator of cell proliferation, is known to be a direct target of the canonical Wnt pathway [16,17], our data indicate that this signaling cascade controls the neural stem cells, in part through direct regulation of the *NRSF* and *Cyclin D1* genes. Furthermore, we have developed a novel and reliable in ovo transcription assay using the in ovo electroporation technique to analyze gene regulation in living chick embryos, which enabled us to assay transcriptional control in a natural, in vivo context.

Materials and methods

cDNA cloning and expression vectors. Full-length cDNAs of mouse *Wnt1*, *β-catenin*, and dominant-negative *Lef1* were inserted into the pCAGGS expression vector [18]. Mouse full-length *Wnt1* cDNA was amplified by RT-PCR based on the published sequence. Mouse *β-catenin* and *Lef1* were gifts from Drs. H. Shibuya and S. Nakagawa, respectively. Chick *Wnt1* cDNA was a gift from Dr. M. Wassef and was used for in situ hybridization. Chick *Cyclin D1* cDNA was obtained by RT-PCR based on the published sequence. Chick *NRSF* cDNA was purchased as an EST clone (chEST 364k22) from MRC gene service.

Electroporation of the chick spinal cord. The pCAGGS constructs (dissolved in H_2O at a concentration of 5 μg/μl) were injected into the spinal cords of chick embryos at Hamburger and Hamilton (H.H.) stage 15–16. pCAGGS-*EGFP* (0.5 μg/μl) was co-electroporated to visualize the electroporated regions of the effector constructs. Five electric pulses at 17 V for 25 ms were delivered by a pair of electrodes with a BTX T-820 electroporator.

In situ hybridization and immunohistochemistry. Embryos were fixed for 2 h at 4 °C in paraformaldehyde in PBS, cryoprotected with 15% sucrose in PBS, mounted in OTC compound (Tissue-Tek), and then cryosectioned at 20 μm. Antisense RNA probes of *NRSF*, *Notch1*, *Wnt1*, and *Cyclin D1* were synthesized and labeled with DIG-RNA labeling mix (Roche) and T3, T7 polymerase (Roche), following the manufacturer's instructions. In situ hybridization was performed as described previously [19,20]. For immunohistochemistry, the sections mounted on slides were washed three times with PBS and incubated with 0.2% Triton X-100 in PBS for 30 min and 3% BSA for 30 min. Sides were then incubated with a primary antibody overnight at 4 °C. After washing three times with PBS, sides were incubated with a secondary antibody for 1 h at room temperature. Anti-βIII-tubulin (Promega) and anti-GFP (MBL) were used as primary antibodies. Alexa-488 and Alexa-568 (Molecular probes)-conjugated antibodies were used as secondary antibodies.

Reporter plasmids. Human *NRSF* genomic DNA was obtained as a BAC clone (RP11-738E22) from Children's Hospital Oakland Research Institute (CHORI). The 5′-UTR (−623 to +2769), fragment 1 (−623 to −14), fragment 2 (+31 to +1000), fragment 3 (+1039 to +1413), and fragment 4 (+1449 to +2702) were amplified by PCR with following sets of primers; sense 5′-CTGCTACCTGCCACGTCT-3′ and antisense 5′-TGGCCATAACTGTATTCTG-3′ for the 5′-UTR; sense 5′-CTGCTACCTGCCACGTCT-3′ and antisense 5′-GGGAC ACGCCCCCTCCGACG-3′ for fragment 1; sense 5′-AGCGTCCT GTGTTGGAATGT-3′ and antisense 5′-CGCCCCGGAAGTTTGCG A-3′ for fragment 2; sense 5′-GCTCGGAGCCCGACGCCT-3′ and antisense 5′-AAAGCAGCTCTTTGCAAACTC-3′ for fragment 3; and sense 5′-TTCAGTTAGGAATGCCGC-3′, and antisense 5′-ACT GGCTTAAAACCACATC-3′ for fragment 4. To construct reporter plasmids, the 5′-UTR PCR fragment was inserted upstream of the *luciferase* gene (5′-UTR-Luc). In this construct, we fused the initiation codon of the *NRSF* exon 2 to the *luciferase* gene in frame. The other PCR fragments (fragments 1–4) were inserted upstream of the HSV-TK promoter and the *luciferase* gene (1-TK-Luc to 4-TK-Luc) (Figs. 3C and D). To create a 2-TK-Luc (Mut) construct in which mutations were introduced in the Tcf binding site, QuikChange II Site-Directed Mutagensis Kit (Stratagene) was used with the following set of primers: 5′-ACTCCAGCGCCAAAGAAAAGTAGTCG-3′ and 5′-ACTT TTCTTTGGCGCTGGAGTTTCGG-3′. 3X TCF-luc and *Cyclin D1*-Luc were gifts from Drs. H. Shibuya and S. Kitazawa.

In ovo luciferase assay. The in ovo luciferase assay was carried out on chick spinal cords at H.H. stage 15–16 (Fig. 3A). For transcription assays, 1.0 μg/μl of reporter plasmid, 1.0 μg/μl of pCAGGS-effector, and 2.0 μg/μl of pCAGGS-*LacZ* as an internal control were used. pCAGGS-*EGFP* was used at 0.3 μg/μl for co-electroporation to visualize the electroporated regions. Total DNA was adjusted to 5.3 μg/μl with the empty pCAGGS vector. GFP-positive embryos were harvested 12 h after electroporation. GFP-positive regions were trimmed and washed two times with ice-cold PBS. Lysates were prepared by sonication using SONIFIER 450 (BRANSON) in the

luciferase lysis buffer. LacZ activities were measured with β-galactosidase buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgCl_2$) and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Nacalai Tesque, Japan) by Multiskan MS (Labsystems). The embryos that showed robust GFP expression were selected and subjected to measurement of LacZ activities. Samples that gave LacZ activities of more than 0.200 were used for the luciferase assay. Luciferase activities were measured in luciferase buffer (0.1 M KPO_4, 10 mM $MgCl_2$, 2.5 mM ATP, and 0.5 mM luciferin) by Luminescencer-JNR (ATTO, Japan). Relative luciferase activities were calculated from the luciferase and LacZ activities. The average values of the relative luciferase activities were obtained from five independent experiments.

Results

Expression of the NRSF, Wnt1, and Cyclin D1 genes in developing chick spinal cord

In a search of putative factors that prevent neural differentiation of actively dividing progenitor cells in the chick spinal cord, we found that *NRSF* is expressed in the VZ (Fig. 1A), as reported in the mouse [12]. Expression of *NRSF* was not observed in the MZ, where mature neurons reside (Fig. 1A).

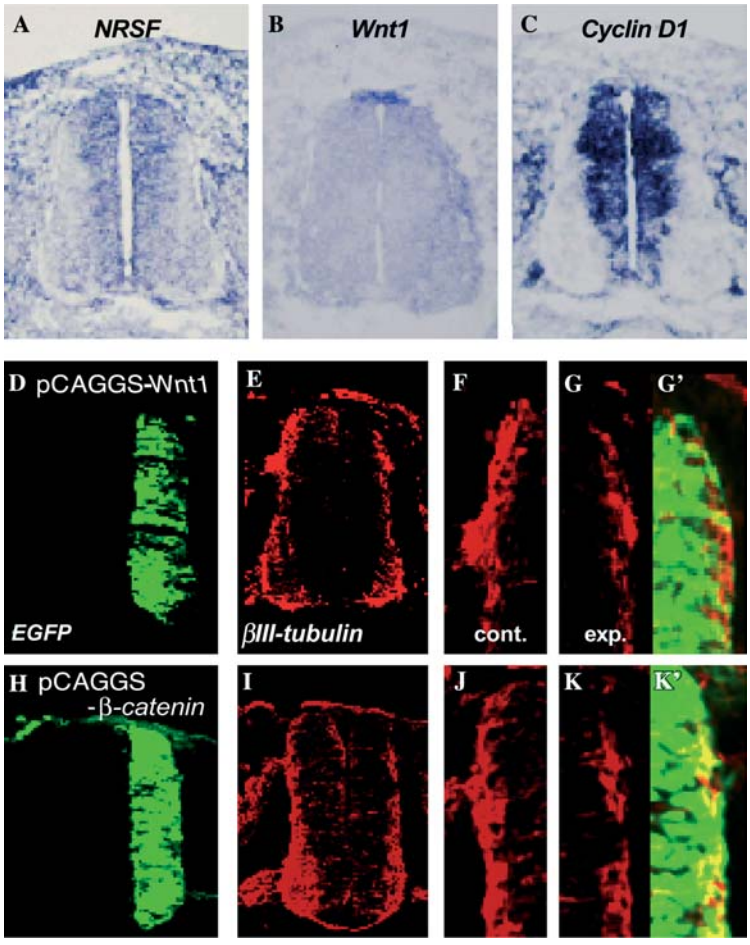

Fig. 1. Expression patterns of chick *NRSF*, *Wnt1*, and *Cyclin D1* genes in the developing spinal cord (A–C). (A) Chick *NRSF* is expressed in the ventricular zone at stage 21. (B) *Wnt1* expression is observed in the dorsal-most end of the developing spinal cord. (C) Chick *Cyclin D1* is expressed in the ventricular zone, showing a distribution similar to *NRSF*. (D) When *Wnt1* was misexpressed in the entire right half of the spinal cord along with the *EGFP* expression plasmid, robust staining of GFP proteins was observed, indicating the successful overexpression of the transgenes in the GFP-positive domain. (E) When the same section was stained with the anti-*β III-tubulin* antibody, expression of *β III-tubulin* was reduced in the electroporated side at 24 h after electroporation. (F) Distinct expression of *β III-tubulin* was observed in the mantle layer of the control side. (G) In contrast, expression of *β III-tubulin* was repressed in the experimental side. (G′) When the two images of *EGFP* and *β III-tubulin* staining were merged, some GFP-positive cells were found to be *β III-tubulin*-negative. (H) When *β-catenin* was misexpressed similarly in the right half of the spinal cord along with the *EGFP* expression plasmid, robust staining of GFP proteins was again observed. (I) In such sections, expression of *β III-tubulin* was repressed, as observed for *Wnt1* misexpression. (J) Clear expression of *β III-tubulin* was observed in the control side. (K) Repression of *β III-tubulin* was distinct in the electroporated side. (K′) By merging the two images, some *β-catenin*-positive and GFP-positive cells were found to be *β III-tubulin*-negative, indicating that activation of the *Wnt1*-mediated canonical pathway abrogates the normal differentiation processes of the spinal progenitor cells.

Previous studies have identified that dorsal–ventral growth of the spinal cord is controlled by a gradient of Wnt signaling emanating from the dorsal midline of the spinal cord. Among several *Wnts*, *Wnt1* and *Wnt3a* are expressed in the dorsal-most end of the spinal cord and have strong mitogenic activity, whereas other broadly expressed Wnts do not enhance proliferation, even when overexpressed [7]. To confirm the expression of the *Wnt1* gene, we carried out in situ hybridization and found that this gene is expressed in the roof plate of the spinal cord (Fig. 1B).

Cell cycle progression of spinal neural progenitor cells is regulated in part by the Wnt signaling cascade mediated through the β-catenin/TCF pathway. As shown previously, *Cyclin D1*, one of the critical cell cycle regulators, is the direct target of this canonical Wnt signaling cascade [16,17]. When expression of this gene was examined, we found that the chick *Cyclin D1* gene is expressed in the VZ (Fig. 1C), as reported previously [7]. These analyses on serial sections revealed that the expression domains of *NRSF* and *Cyclin D1* overlap, suggesting that the Wnt signals emanating from the dorsal-most end of the spinal cord regulate both the *Cyclin D1* and *NRSF* genes.

Activation of the Wnt signaling cascade in chick spinal cord

To analyze the effects of Wnt signaling, we misexpressed the *Wnt1* gene in the developing chick spinal cord by in ovo electroporation. In this approach, we co-electroporated an *Enhanced Green Fluorescent Protein* (*EGFP*) expression vector, pCAGGS-*EGFP*, to monitor the expression of the transgenes. In ovo electroporation was performed on the right side of the spinal cord, thereby leaving the left side normal. This unilateral misexpression enabled us to compare the induced changes in a single section.

When the *Wnt1* gene was misexpressed by pCAGGS-*Wnt1*, robust GFP expression was observed exclusively in the right side of the spinal cord when stained with the anti-GFP antibody (Fig. 1D). To assess the neural differentiation in the electroporated spinal cord, we performed an immunohistochemical analysis on the same section using an antibody raised against *β III-tubulin*, which is frequently used as a neural marker (Figs. 1E–G, a G′) [21]. In the electroporated right side, expression of *β III-tubulin* was reduced (Figs. 1E and F), whereas normal expression in the MZ was observed in the normal left side (Figs. 1E and G). At a higher magnification, a difference of *β III-tubulin* expression is more evident; in the control side, *β III-tubulin* was expressed as a distinct stripe on the outside of the developing spinal tube (Fig. 1F), whereas in the experimental side, the expression of *β III-tubulin* was sparse and thin (Fig. 1G). When the two images of the *EGFP* and *β III-tubulin* were merged, some GFP-positive green cells were found to be β III-tubulin-negative (Fig. 1G′), indicating that some populations of cells do not express *β III-tubulin*, even in the MZ.

To confirm that the canonical Wnt pathway is involved in this phenomenon, we misexpressed the *β-catenin* gene similarly. Again, robust staining of the expressed GFP was observed in the right half of the spinal cord (Fig. 1H). In this section, similar to the *Wnt1* overexpression, *β III-tubulin* expression was reduced (Fig. 1I). At a higher magnification, the distinct expression of *β III-tubulin* in the control side (Fig. 1J) and the clear reduction in the electroporated side (Fig. 1K) were observed. By merging the two images, some GFP-positive cells in the MZ were found to be *β III-tubulin*-negative, as observed with *Wnt1* overexpression (Fig. 1K′). These observations suggest that the canonical Wnt pathway controls the growth and the differentiation of the progenitor cells.

The canonical Wnt signaling pathway induces both NRSF and Cyclin D1 in the spinal cord

Next, we checked the expression of two markers, *NRSF* and *Cyclin D1*, in the spinal cord where the canonical Wnt pathway was activated. As expected, overexpression of *Wnt1* and *β-catenin* induced the expression of both the *NRSF* gene (Figs. 2B and E) and the *Cyclin D1* gene (Figs. 2C and F) in the domains where the transgenes were misexpressed, as judged by the robust GFP signals (Figs. 2A and D). These observations indicate that the *NRSF* and *Cyclin D1* genes are

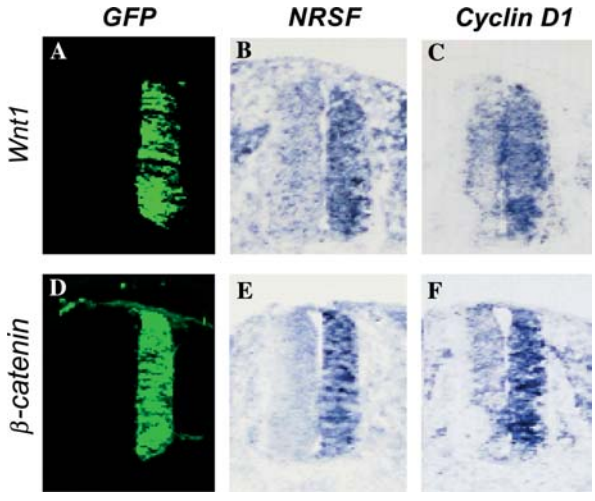

Fig. 2. Overexpression of *Wnt1* and *β-catenin* induces *NRSF* and *Cyclin D1*. (A) When *Wnt1* was overexpressed in the right side of the spinal cord along with the *EGFP* expression vector, robust GFP staining was observed. In such GFP-positive areas, *NRSF* (B) and *Cyclin D1* (C) were induced. (D) Likewise, overexpression of *β-catenin* and *EGFP* gave strong GFP signals. In such GFP-positive domains, *NRSF* (E) and *Cyclin D1* (F) were induced.

Table 1
Time courses of activation of the *NRSF* and *Cyclin D1* genes by *Wnt1* and *β-catenin*

Electroporated effector	Marker	Hours after electroporation		
		3 h	6 h	12 h
Wnt	NRSF	–[a]	+[b]	+
	Cyclin D1	–	+	+
β-Caterin	NRSF	–	+	+
	Cyclin D1	–	+	+

Wnt1 and *Cyclin D1* induced *NRSF* and *Cyclin D1* 6 h after electroporation. Activation was not detected 3 h after electroporation, but once activated, expression of *NRSF* and *Cyclin D1* was maintained even 12 h after electroporation. These data suggest that activation of *NRSF* is as fast as *Cyclin D1*, a direct target of the canonical Wnt signaling cascade.

[a] No change.

[b] Up-regulation.

the downstream targets of the canonical Wnt signaling pathway. This is compatible with previously published data that *Cyclin D1* is the direct target of this signaling cascade [16,17], although we do not know whether *NRSF* is the direct target.

Since *Cyclin D1* has been shown to be a direct target of the Wnt canonical pathway, we analyzed the time courses of the Wnt1-mediated induction of *NRSF* and *Cyclin D1* (Table 1). Three hours after *Wnt1* overexpression, no *NRSF* or *Cyclin D1* was induced. Six hours after the electroporation, both *NRSF* and *Cyclin D1* were induced. This induction was maintained even 12 h after the electroporation. Simultaneous induction of these two genes suggests that *NRSF* might be also a direct target of the Wnt canonical signaling cascade.

In ovo luciferase assay

To make a reliable system, we have developed a new in vivo assay system using living chick embryos, rather than cultured cell lines in vitro (Fig. 3A). In this system, we simultaneously electroporated four plasmids, including the luciferase reporter, effector expression plasmid, β-galactosidase plasmid, and *EGFP* expression plasmid. Twelve hours after electroporation, embryos were harvested and examined for GFP signals. Embryos expressing sufficient GFP proteins were selected and then the GFP-positive parts of the electroporated spinal cord were trimmed, harvested, and lysed for the luciferase assay and the measurement of β-galactosidase activity. Lysates that showed low β-galactosidase activities were not used in this assay for better accuracy.

To confirm that this system is suitable for the measurement of the canonical Wnt pathway, we first used a 3xTcf-luciferase reporter (3xTcf-Luc) (Fig. 3B). Without co-electroporating the appropriate effector, the luciferase activity derived from 3xTcf-Luc remained low. In contrast, when the β-catenin expression plasmid was co-electroporated, robust induction of 3xTcf-Luc was observed in a dose-dependent manner. Addition of a dominant-negative Lef1 abrogated this induction. Taken together, these observations indicate that this in ovo luciferase assay works well, giving reliable and consistent results.

NRSF as a direct target of the Wnt canonical signaling cascade

To check whether *NRSF* is a direct target of Wnt signaling, we isolated a genomic fragment that spans the 5′ UTR of the human *NRSF* gene. As shown in Fig. 3C, this region contains non-coding sequences of exons 1a, 1b, 1c, and 2, corresponding to the area between −623 and +2769. For a precise analysis, this fragment was further divided into four small pieces, areas 1–4 (Fig. 3C). The whole genomic fragment was inserted 5′ in to the *luciferase* gene (5′-UTR-Luc), and the other four pieces were inserted into a thymidine kinase (TK) promoter-luciferase construct as shown in Fig. 3D (1-Tk-Luc to 4-Tk-Luc). These five plasmids were then electroporated into the spinal cord in the absence or presence of the β-catenin expression vector.

Interestingly, the 5′-UTR-Luc was activated by β-catenin (Fig. 3E), to the same extent with 3xTcf-Luc and a *Cyclin D1* promoter-luciferase (Fig. 3E), suggesting that this β-catenin-mediated activation is comparable with those of the direct targets of Wnt signaling. To further dissect the location of the β-catenin-responsive site, we electroporated 1-Tk-Luc, 2-Tk-Luc, 3-Tk-Luc, and 4-Tk-Luc, and found that only 2-Tk-Luc was activated (Fig. 3E). This suggests that genomic fragment 2 contains the β-catenin-responsive site.

Exon 1a contains the TCF binding site and acts as a β-catenin-responsive element

Nucleotide sequences of the human and mouse *NRSF* genes corresponding to fragment 2 were compared in silico (Fig. 4A). By this sequence comparison, several conserved parts were identified, suggesting that fragment 2 contains several regulatory elements. Careful sequence inspection of these conserved elements has identified a putative TCF binding site in exon 1a (shown in red, Fig. 4A).

This putative site matches perfectly with the consensus of the TCF binding site (AACAAAG) in both human and mouse (Fig. 4B). To check whether this site acts as a β-catenin-responsive site, we introduced a mutation and constructed the same reporter plasmid (Fig. 4C). The wild type reporter was again activated by *β-catenin* in a dose-dependent manner, and this activation was severely disturbed by co-expression of

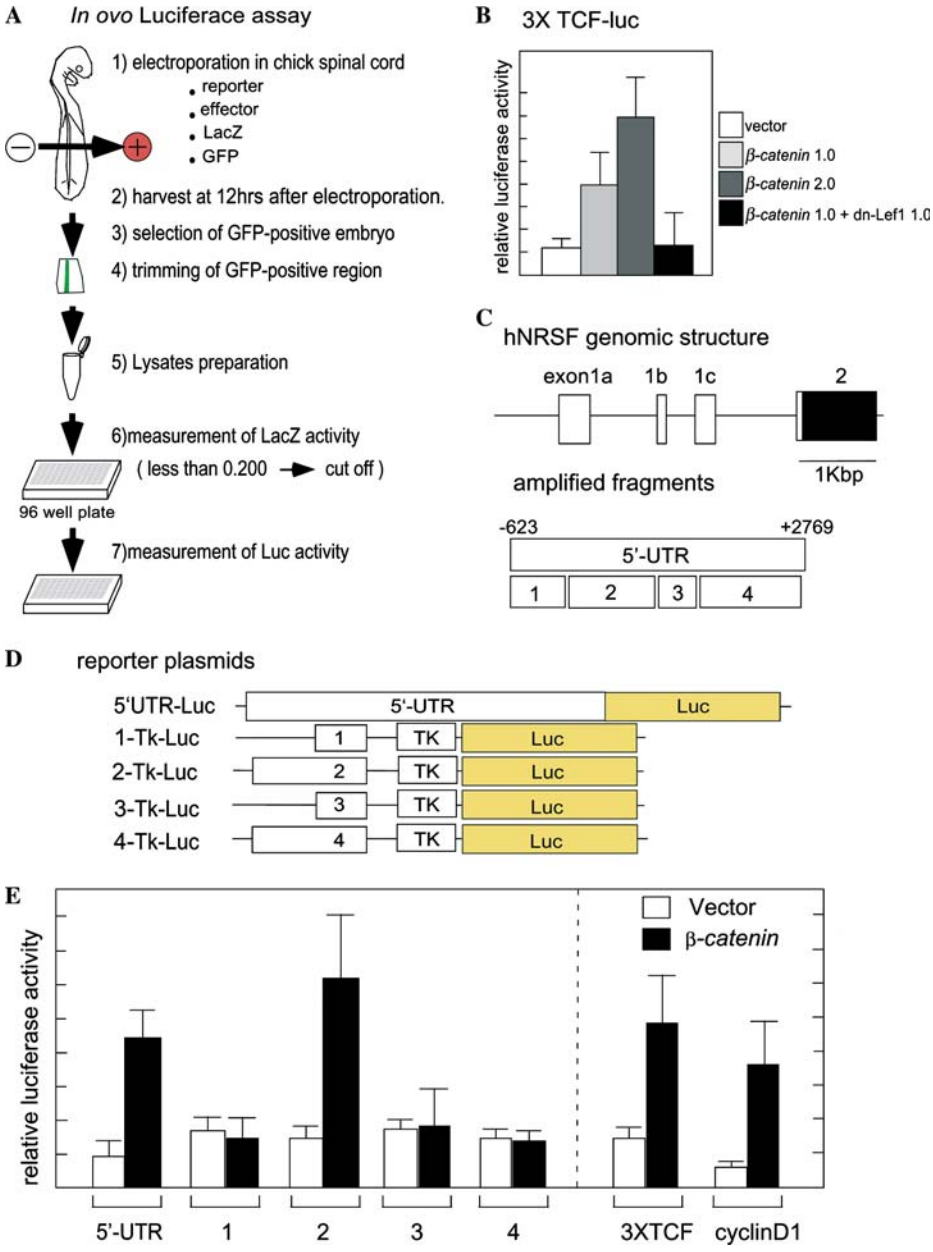

Fig. 3. In ovo transcription assay. (A) Four expression plasmids (reporter, effector, *LacZ*, and *EGFP* expression vectors) were mixed and electroporated into the chick spinal cord. Embryos were harvested and then subjected to the measurement of LacZ activity and the luciferase assay as illustrated. (B) When the 3xTCF-Luciferase reporter plasmid was electroporated along with increasing amounts of the *β-catenin* expression plasmid, activation of this reporter was observed in a dose-dependent manner. This activation was abrogated by the expression of a dominant-negative *Lef1*. (C) Schematic illustration of the human *NRSF* gene. The 5′-UTR corresponding to −623 to +2769 was amplified. This genomic fragment was further divided into four fragments (1–4). (D) Five reporter plasmids were constructed as illustrated. (E) When these reporters were electroporated along with or without the β-catenin expression plasmid, β-catenin-dependent activation was observed only for 5′UTR-Luc and 2-Tk-Luc, indicating that fragment 2 contains a β-catenin/TCF binding element. Under the same conditions, activation of 3xTCF-Luc and *Cyclin D1* promoter-Luc constructs was also observed.

the dominant-negative *Lef1*. In contrast, the mutated reporter was not activated, even under the condition that gives robust activation. This indicates that the activation of *NRSF* is dependent on the TCF binding site found in exon 1a. Hence, we conclude that *NRSF* is the direct target of the canonical Wnt signaling cascade.

Discussion

The canonical Wnt pathway and its direct target, NRSF

Neural stem cells are located in the VZ and actively proliferate to renew the pool of progenitor cells. In parallel, the progenitor pool generates postmitotic cells

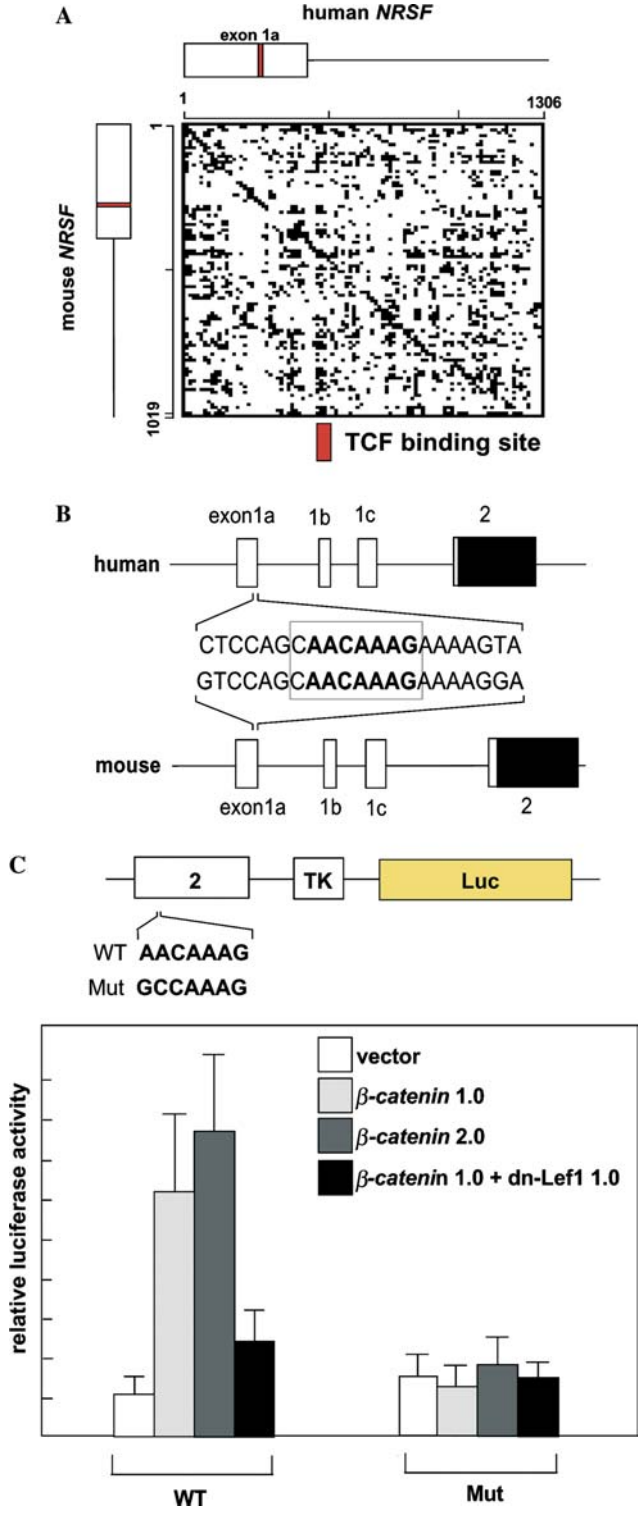

Fig. 4. (A) Dot blot analysis of human and mouse sequences corresponding to fragment 2. Putative binding sites were found in exon 1a and are shown in red. (B) Putative TCF binding sites of the human and mouse *NRSF* genes are shown. Boxed TCF binding sites were conserved perfectly in mouse and human, and match the consensus of the TCF binding motif. (C) When this TCF binding site was mutated (Mut), activation by *β-catenin* was completely abolished, whereas the wild type reporter construct (WT) showed a dose-dependent activation, which was abrogated by a dominant-negative *Lef1*.

that leave the VZ and migrate to the MZ to differentiate into neurons [1].

Several factors are known to regulate the growth of the spinal progenitor pool. *Wnt1* and *Wnt3a* are the pivotal factors controlling this process [7]. These Wnt molecules transduce their signals through the canonical Wnt pathway, in which the β-catenin and the TCF/LEF transcription factors are involved. Previous studies have revealed that *Cyclin D1*, one of the key regulators of cell cycle progression, is a direct target of this signaling cascade [16,17]. As expected, overexpression of *Wnt1* and *Wnt3a* induces expansion of the progenitor pool and a dominant-negative β-catenin exerts the opposite effects [7,11].

Wnt1 and *Wnt3a* are expressed in the dorsal side of the spinal cord; hence, they send their mitotic signals making a dorsal-high and ventral-low gradient. This asymmetric gradient results in the growth of the spinal cord, with the proliferation rate highest dorsally and the differentiation rate highest ventrally (a mitogen gradient model) [7]. This suggests that cellular proliferation and differentiation of the spinal cord are linked tightly. This further implies that these two processes might be regulated by the same Wnt1/Wnt3a-mediated canonical pathway. Since *Cyclin D1* has been shown to be a direct target, this factor is a key for this graded growth rate of the spinal progenitor cells.

In a search of putative factors that might regulate the differentiation of the progenitors, we have found that *NRSF* is expressed in a fashion very similar to *Cyclin D1*, showing the same dorsal wide and ventral narrow pattern (Figs. 1A and C), as suggested from the mitotic signals of Wnts, with a dorsal-high and ventral-low gradient. As expected, *NRSF* possesses the TCF binding site that can be directly activated by the canonical Wnt pathway (Fig. 4C). This indicates that Wnt signaling regulates both the *NRSF* and *Cyclin D1* genes. Although our data suggest that NRSF induced by the Wnt pathway might prevent premature appearance of neuronal markers in the stem cells, we do not know the mechanism that maintains the progenitor pool as multipotent stem cells. Further analyses should be performed to dissect this mechanism, in which other signaling cascades, such as Notch, operate and possibly crosstalk with the canonical Wnt signaling cascade. In this sense, it is important to note that *Caenorhabditis elegans spr-3* and *spr-4*, which encode proteins similar to the *NRSF/REST*, regulate expression of the presenilin gene, *hop-1* [22]. In addition, presenilin is involved in both the processing of Notch and the degradation of β-catenin proteins [23–25]. Hence, these data suggest that the Wnt signals might be transduced in part by the presenilin that acts in different signaling contexts.

Mutations of *β-catenin* and *Axin* genes were reported in medulloblastoma, the most common malignant brain tumor in children [26–28]. These data indicate that the activation of canonical Wnt signaling is involved in the

tumorigenesis of medulloblastoma. Furthermore, high levels of *NRSF/REST* expression were observed in medulloblastoma. Constitutively active NRSF induces de-repression of the endogenous neuronal genes and subsequently triggers apoptosis [29]. Taken together, data indicate that the direct regulation of the *NRSF* gene by the canonical Wnt pathway is involved in the processes of both neurogenesis and tumorigenesis.

In ovo transfection assay

Transcriptional controls have been analyzed thus far with cultured cell lines. This approach, however, does not reflect the natural conditions in vivo, since the signaling contexts in the cultured cell lines are different from the immature embryonic cells, and the activities of some transcription factors are dependent on the various signaling cascades. To overcome this obstacle, we developed an in ovo transcription assay using the electroporation technique.

Electroporation is now widely used in misexpression studies of genes of interest in various tissues and organs. The spinal cord is one of the best organs for electroporation, since its tube structure acts as a reservoir for the injected DNA solutions, giving a very high efficiency of transgene expression. In addition, we are able to misexpress several genes simultaneously. Taking advantage of this method, we have developed an in ovo transcription assay.

We electroporated the luciferase reporter, effector plasmid, *β-galactosidase*, and *EGFP* expression vectors. Using GFP signals derived from the *EGFP* expression plasmid, we selected embryos in which plasmids were successfully introduced at a high rate. By this selection process, we obtained reliable luciferase and LacZ activities from living embryos and thereby analyzed the precise transcriptional control in natural, in vivo conditions.

Recently, β-galactosidase reporter constructs connected with promoters and their regulatory elements have been shown to give clear *LacZ* expression in the correct tissue domains [30]. This approach has the potential to reduce the efforts to perform the same approach in transgenic mice. In addition, this study has revealed that in ovo electroporation in chick embryos serves as an efficient, reliable, and rapid experimental system for the analyses of transcriptional control.

By combining this approach and our study, the chick spinal cord model will expand our experimental designs and enable us to analyze transcriptional control in a more natural condition.

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