Bone Morphogenetic Protein 2 Opposes Shh-mediated Proliferation in Cerebellar Granule Cells through a TIEG-1-based Regulation of Nmyc* [S]

Received for publication, July 2, 2007, and in revised form, October 16, 2007. Published, JBC Papers in Press, October 19, 2007, DOI 10.1074/jbc.M705414200

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Nmyc is a potent regulator of cell cycle in cerebellar granular neuron precursors (CGNPs) and has been proposed to be the main effector of Shh (Sonic hedgehog) proliferative activity. Nmyc ectopic expression is sufficient to promote cell autonomous proliferation and can lead to tumorigenesis. Bone morphogenetic protein 2 (BMP2) antagonizes Shh proliferative effect by promoting cell cycle exit and differentiation in CGNPs. Here we report that BMP2 opposes Shh mitogenic activity by blocking Nmyc expression. We have identified TIEG-1 (KLF10) as the intermediary factor that blocks Nmyc expression through the occupancy of the Sp1 sites present in its promoter. We also demonstrate that TIEG-1 ectopic expression in CGNPs induces cell cycle arrest that can lead to apoptosis but fails to promote differentiation. Moreover, TIEG-1 synergizes with BMP2 activity to terminally differentiate CGNPs and independent differentiator signals such as dibutylcAMP and prevents apoptosis in TIEG-1 arrested cells. All together, these data strongly suggest that the BMP2 pathway triggers cell cycle exit and differentiation as two separated but coordinated processes, where TIEG-1 acts as a mediator of the cell cycle arrest.

Cerebellar granular neuronal precursors (CGNPs)2 are generated within the external germinal layer during development of the cerebellar cortex. Along cerebellum development, CGNPs become postmitotic and migrate through the Purkinje cells setting forth the third layered cerebellar cortex (1–3). Clonal expansion of CGNPs is achieved by the mitogenic activity of Shh (Sonic hedgehog) signaling emanating from the Purkinje cells to the external germinal layer (4, 5). Shh transduces its signal through the Ptc (Patched) and Smo (Smoothened) receptor complex. The pathway is negatively regulated through a series of PKA, glycogen synthase kinase-3β, and casein kinase phosphorylations of the GLI/Ci transcription factors Gli2/3. This phosphorylation targets Gli2/3 to the proteasome, where it is truncated by removing its transactivation domain; it becomes a potent transcriptional repressor of the pathway. In the presence of Shh, however, PKA activity is blocked, and Gli2/3 acts as a transcriptional activator (6, 7). The deregulation of several components of the Shh pathway leads to tumorigenesis. On one hand, the receptor Ptc and SuFu (Suppressor of Fused) act as tumor suppressors, and inactivating mutations of both genes can be found in medulloblastomas (8, 9). On the other hand, Gli1 and Nmyc are potent oncogenes, and overexpression of both transcription factors can be found on glioblastomas and neuroblastomas, respectively (10–12).

Nmyc is a member of the Myc family of basic helix-loop-helix transcription factors, implicated in the regulation of cell growth, proliferation, and differentiation. In contrast to c-Myc, which is expressed in virtually all proliferating cells, Nmyc presents a more restricted pattern of expression, although both transcription factors are functionally replaceable (13–15). In the cerebellum, Nmyc is transiently expressed during development showing a pattern restricted to proliferative zones (15). Overexpression of Nmyc is associated with several neuroectodermal tumors including medulloblastoma, neuroblastoma, astrocytoma, and small cell lung carcinoma (16–19). Amplification of Nmyc can be found in ~25% of neuroblastomas, constituting a poor prognosis factor (20, 21).

Finding signals capable of terminating Shh-mediated proliferation of CGNPs would contribute not only to the knowledge on normal cerebellum development but also to the understanding of medulloblastoma formation. So far, only a few inhibitors of Shh pathway have been reported. For instance, cyclopamine, which directly impairs Smo activity, and activators of PKA such as dibutyl cAMP (DBA), forskolin, or the putative adenylate cyclase-activating peptide (22, 23). Additionally, it is very likely that the vitronectin-induced inhibition of Shh reported by our group (24) was also mediated by PKA, because a consistent elevation of cAMP-responsive element-binding protein phosphorylation was observed. We recently also reported the powerful anti-mitotic activity mediated by BMP2 in Shh-stimulated CGNPs (25). As we demonstrate in the present work, this inhibition is absolutely independent of PKA activity, constituting a novel mechanism of inhibition of the Shh pathway.

BMP2 belongs to the TGFβ family of cytokines (26, 27) and exerts its biological function promoting the heterodimerization between type I and II serine/threonine receptors, which in turn...
propagate the signal through the phosphorylation of Smad proteins. Phosphorylation of Smads causes conformational changes that allow the heteromerization of Smad1/5/8 with Smad4, the unique member common to the BMP and TGFβ pathways, acting as a transcriptional activators or repressors (for review see Refs. 26–29). There are several proteins known to be direct targets of the BMP pathway such as the Ids (inhibitor of differentiation) or the TIEG-1 (TGFβ early inducible gene-1) (30–32).

The mechanism by which BMP2 opposes Shh-induced proliferation of CGNPs remains unknown, although it has been reported that TGFβs acts over proliferating cells using multiple and coordinated mechanisms including down-regulation of c-Myc and up-regulation of the cyclin-dependent kinase inhibitor p15ink4B (33, 34). Additionally, it has been described that TIEG-1 activity promotes cell cycle arrest or apoptosis depending on the cell context (35, 36).

Here we report that Nmyc down-regulation is the critical event by which BMP2 counteracts Shh-induced proliferation of CGNPs. Furthermore, we show that TIEG-1 plays a central role on transcriptional repression of Nmyc by occupying Sp1 activator sites within Nmyc promoter. Additionally we show that overexpression of TIEG-1 promotes cell cycle arrest in CGNPs and that in the absence of coordinated differentiator signals, this arrest can lead to apoptosis. In the present work we propose a model in which BMP2 action on proliferating CGNPs can be dissected in two separated components, one anti-proliferative and another one pro-differentiative, where TIEG-1 appears to be the main effector of the anti-mitotic branch. Additional components of the BMP2 pathway appear to be required for a terminal neuronal differentiation.

EXPERIMENTAL PROCEDURES

Cerebellar Granule Cell Culture, Viral Infection, and Transfection—Cerebellar cultures and plate coating were performed using a procedure described before (25). For retroviral infection, the cells were plated in absence of Shh and incubated with the retrovirus for 2 h. Then the medium was replaced with or without Shh. After 24 h, different treatments were applied. For ordinary transfection experiments, the cells were allowed to rest for 24 h in the presence of Shh and then transfected with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. After 12 or 24 h of the transfection, fresh medium was added, and different treatments were applied. Turnover experiment were performed pulsing cells with cycloheximide for the indicated periods of time in the presence of Shh or Shh with BMP2.

RNA Preparation, Retro-Transcription, and Real Time PCR Analysis—The cells were incubated in the presence of Shh (3 μg/ml) or Shh with BMP2 (10 ng/ml) for the indicated periods of time with or without cycloheximide in 100-mm tissue culture dishes containing 8 × 10⁶ cells. Total RNA was extracted using the guanidinium thiocyanate-phenol method (37). First strand cDNA synthesis was primed with 250 ng of random primers using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real time PCR was performed in a iCyclerIQ Multicolor real time PCR detection system from Bio-Rad using the IQ SYBR GREEN Supermix from the same supplier. PCR program consisted of 1 cycle at 94 °C for 2 min plus 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 15 s. The data were then analyzed with the ΔΔCt method with Microsoft Excel. The primer sequences used were TIEG fw, 5′-CCG GAG AAG AAC CCA CAT AA-3′; TIEG rv, 5′-GAG GAA GCC ACA GCA AAG TC-3′; Nmyc fw, 5′-ATC ACT GTA CGC CCC AAG AC-3′; Nmyc rv, 5′-CCT TTG GTG GAA CGA CAC TT-3′; Gli1 fw, 5′-TTC CAT ATC AGA GCC CCA AG-3′; Gli1 rv, 5′-GAA GGG GCA GGA TAG GAG AC-3′; glyceraldehyde-3-phosphate dehydrogenase fw, 5′-CCC CCA ATG TAT CCG TG-3′; and glyceraldehyde-3-phosphate dehydrogenase rv, 5′-TAC CCC AGG ATG GAC TTT AGT-3′.

Immunoprecipitation and Immunoblotting—For Western blot analysis cell cultures were lysed after treatments in 1 × SDS loading buffer (10% glycerol, 2% SDS, 100 mM dithiothreitol, and 60 mM Tris-HCl, pH 6.8), and the DNA was disrupted by sonication. The samples were separated by SDS gel electrophoresis and transferred to nitrocellulose membranes, blocked with 8% nonfat dry milk in TTBS (150 mM NaCl, 0.05 Tween 20, and 20 mM Tris-HCl, pH 7.4), and probed with the different antibodies. The blots were developed using anti-rabbit coupled peroxidase plus the ECL system and captured with Versadoc Imaging System from Bio-Rad. The digital images were then quantified with Quantity One software (Bio-Rad).

For immunoprecipitation 6-well dishes were lysed in radio-immune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride), and after sonication, insoluble material was removed by centrifugation. The resulting supernatant was incubated overnight at 4 °C with 5 μg of monoclonal anti-NMYC antibody (BD Biosciences). Antigen-antibody complexes were collected with anti-mouse agarose beads (Amer sham Biosciences). Then beads were washed three times with Tris-buffered saline (25 mM Tris, pH 7.5, 140 mM NaCl) and boiled with 2 × SDS loading buffer. The samples were resolved with a 7% SDS-PAGE and proceeded as described above.

Antibodies and Chemicals—Monoclonal anti-Nmyc was purchased from BD Biosciences. Monoclonal antibodies anti-GFAP, anti-β-tubulin-III, anti-O4, and biotinylated anti-BrdUrd were obtained from Chemicon. Anti-GFP was purchased from Molecular Probes. Monoclonal anti-bromodeoxyuridine (BrdUrd) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Anti-actin was purchased from Santa Cruz. Antibodies against the phosphorylated Tyr-15 of cdc2 and Ser-807 of retinoblastoma were purchased from Cell Signaling Technology. For immunochemical analysis, fluorochrome-conjugated secondary antibodies anti-rabbit Alexa 488 and anti-mouse Alexa 594 were used, both of them purchased from Molecular Probes. For immunoblotting anti-mouse, anti-rabbit, and streptavidin horseradish peroxidase-conjugated were obtained from Jackson. The amino-terminal fragment of Shh was produced as a histidine fusion protein in Escherichia coli and purified using a nickel column according to manufacturer instructions (Qiagen). BMP2 was kindly provided by Genetic Institute. Cycloheximide, an inhibitor of protein synthesis, and MG-132, a reversible proteasome inhibitor, were purchased from Calbiochem.
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Retroviral Constructs and Packaging—Retroviral vector pWZL-IRES-GFP was cotransfected with gagpol plasmid and vesicular stomatitis virus G glycoprotein vector into 293 EBNA cells (Invitrogen) in the presence of G-418. One day after transfection, the medium was washed twice with PBS and replaced with fresh medium to harvest virus. The supernatants were collected every 24 h during 1 week, then pooled, and filtered through a 0.45-μm syringe filter. The supernatants were stored at 4°C for 2 months. To determine the titer of each lentiviral construct, collected supernatants were applied to a 293T cell line, and GFP-positive cells were counted after 24 h. Similar titers of each construct were applied to cerebellar granular cells.

Plasmid Constructs—TIEG-1 cDNA was kindly gifted by Thomas C. Spelsberg and subcloned into a modified version of pWPI lentiviral vector provided by Dr. Didier. TIEG-1 and its variants such as TIEG-VP16 and the mutant inactive form R440W were generated by PCR mutagenesis and subcloned into pCIG, which contains an IRES-3xNLs-GFP. Gli1 cDNA was kindly provided by Dr. Hui and was subcloned into pWZL-IRES-eGFP (Jay P. Morganstern, Millenium Pharmaceuticals, Cambridge, MA). Smoothed M2 mutant was obtained from Frederic J. de Sauvage and the dominant negative form of PKA was from Elisa Marti, and both of them were cloned into the lentiviral vector. Nmyc pWZL-IRES-eGFP was kindly provided by Dr. A. M. Kenney.

Nmyc promoter was provided by Dr. William L. Carroll and subcloned into pGL3 vector (Clontech). Directional deletions of the Nmyc promoter were performed using an Exonuclease III/S1 nuclease protocol as previously described (38). Mutant variants of the Nmyc promoter were done using standard PCR mutagenesis.

Immunocytochemistry—For BrdUrd incorporation assay, the cells were pulsed with 24 ng/ml of BrdUrd 4 h prior to fixation with 4% paraformaldehyde. The cells were then permeabilized with methanol for 5 min, washed twice with PBS, and incubated for 10 min with DNase I in a DNase buffer (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 0.1 mM CaCl₂). DNase was washed once with PBS, and the cells were incubated overnight at 4°C with primary antibody.

For deoxynucleotidyltransferase-mediated dUTP nick end labeling assay, the fixed cells were incubated for 1 h at 37°C with TdT and a biotin-labeled nucleotide, washed, and developed with streptavidin-Alexa-488.

For triple staining procedure, a previous immunocytochemistry with anti-GFAP/anti-β-tubulin-III/anti-O4 developed with anti-mouse Alexa-594 and anti-rabbit Alexa-488 was fixed with 4% paraformaldehyde, washed twice with PBS, and then incubated for 1 h with biotinylated anti-BrdUrd and developed with streptavidin-horseradish peroxidase plus DAB. All of the images were captured with a Colorview digital camera and processed with Adobe Photoshop 6.0.

Flow Cytometry of Cultured Cerebellar Granular Cells—The cells were incubated with Shh for 24 h and pulsed at different periods of time with BMP2. The cells were then washed with PBS and trypsinized in the presence of DNase. Trypsinization was stopped with 10% of fetal bovine serum. Then the cells were centrifuged and resuspended in 300 μl of PBS, and 1 ml of 4% paraformaldehyde was slowly added and incubated for 15 min. PFA was blocked with 1 vol of PBS containing 10% of FBS and 0.1% Triton-X-100 and washed again with the same buffer. Finally, RNase A and propidium iodide were added, and the samples were analyzed with a flow cytometer, and the data were plotted and quantified with WinMDI 2.9.

Nonradioactive Band Shift Assay—COS-7 cells were electroporated with FLAG-TIEG-1 pcIG, pcIG alone and incubated for 24 h. After that, the cells were trypsinized, washed with PBS, and incubated for 15 min in an hypotonic buffer (10 mM Tris, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol). Then Nonidet P-40 was added to 1% final concentration, and the cells were vortexed and centrifuged for 3 min at 13,000 rpm, the supernatant was discarded, and the pellet containing nucleus was incubated in an hypertonic buffer (20 mM Tris, pH 8.0, 1.5 mM MgCl₂, 20% glycerol, 420 mM NaCl, 1.25 mM EDTA, 1 mM dithiothreitol) under vigorous shaking at 4°C for 30 min. Then the nuclei were pelleted again for 15 min at 13,000 rpm, and the protein concentration was determined by Bradford assay.

Probes from the Nmyc promoter were amplified by PCR from the original promoter and labeled with TdT using a biotin-11-dUTP. Additional purification of the biotinylated probe was performed with an agarose gel.

Nuclear extracts were incubated for 30 min with the biotinylated probe at room temperature in a binding buffer (20 mM HEPES, pH 8.0, 50 mM KCl, 0.5 mM dithiothreitol, 50 μM EDTA, 1 mM MgCl₂, 5% glycerol) supplemented with 2 units of poly(dI-dC), 0.05% of Nonidet P-40, and 50 μM ZnCl₂. The samples were separated with a 4% acrylamide, 0.5X Tris borate-EDTA gel, prerun for 2 h. Once the gel was resolved, it was electrotransferred onto a nitrocellulose membrane with 0.5X Tris borate-EDTA for 30 min and cross-linked for 3 min with a 302-nm UV lamp. The membrane was then processed with a standard Western blot protocol with an streptavidin-peroxidase reactive.

Reporter Assay—Nmyc promoter –875/+169 cloned into the pGL3 reporter plasmid, and its deletions were transfected in 293T cells using FuGENE 6 reagent (Roche Applied Science). Transfection efficiency was normalized with a cotransfected cytomegalovirus-Renilla vector. After 36 h of transfection, the cells were lysed with a buffer containing 2.5 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, and 0.5% Nonidet P-40. The lysates were collected and measured with an Orion II Microplate Luminometer from Berthold using the reagents previously described (39). The luciferase data were normalized to the Renilla values, and the results were plotted and expressed in arbitrary units as the means and standard deviations of three experiments.

RESULTS AND DISCUSSION

BMP2 Decreases Nmyc Levels in CGNPs, Resulting in Cell Cycle Exit and Differentiation—During the development of the cerebellum, Shh activity is required for the expansion of the CGNPs, but its signal is not sustained anymore during the subsequent steps of cell cycle exit and differentiation (4, 40). The addition of Shh to CGNPs cultures maintained these cells cycling in an undifferentiated state. Otherwise cells differentiate (5). Shh signal increases proliferative markers such as BrdUrd incorporation, Tyr(P)-15-cdc2, and Ser(P)-807-Rb...
The addition of BMP2, however, overcomes Shh proliferative action (25), with the concomitant decrease of these cell cycle markers revealed by immunocytochemistry and Western blot analysis (Fig. 1, A and B). We next tested how BMP2 treatment modulates cell cycle phase distribution to discard any possible effect of BMP2 on apoptosis or anomalous cell cycle arrest within the S or G2/M phase. CGNPs treated with BMP2 were analyzed by flow cytometry at different time points, confirming that cells are progressively arrested at G1/G0 with no increase on apoptosis (Fig. 1C). These data, together with the fact that BMP2 increases differentiation markers such as tubulin β-III (25), indicated that cell cycle exit induced by BMP2 conducts to terminal neuronal differentiation rather than to apoptotic cell death.

Shh mitogenic activity acts through the up-regulation of Gli1 and Nmyc (11, 12). Therefore, we next decided to determine whether BMP2 regulated Gli1 and Nmyc mRNA levels. CGNPs cultures treated with Shh or Shh plus BMP2 were analyzed by Real time PCR with specific primers for Gli1 and Nmyc. The results showed a remarkable decrease in mRNA levels of both oncogenes 24 h after the treatment (Fig. 2A). Notably, Nmyc down-regulation was already detected as soon as 12 h after the BMP2 pulse, whereas Gli1 decrease was only evident at 24 h, thus suggesting either a more direct and/or a more efficient regulation of Nmyc transcription.

In agreement, Western blot analysis showed a significant decrease of Nmyc protein levels that could be already detected 12 h after the BMP2 addition (Fig. 2B). Together these data suggested that BMP2 antagonize Shh by down-regulating Nmyc and prompted us to ask whether Nmyc overexpression was sufficient to suppress the effect of BMP2 on Shh-stimulated CGNPs.

**Nmyc Overexpression Overcomes BMP2 Antiproliferative Effect in CGNPs**—To determine the level at which BMP2 counteracts Shh-induced proliferation, we first studied the effect of BMP2 on CGNPs cultures that were overexpressing different components of Shh, from the most upstream to the most downstream known members of the Shh pathway. Shh acts through the Ptc (Patched) and Smo (Smoothened) receptor complex that initiates a cascade of poorly understood events that give rise to the inhibition of PKA activity and thus the migration of Gli2/3 to the nucleus in its active form to activate the transcription of Gli1 and Nmyc (6). Activating mutations of Smoothened (SmoM2) or a dominant negative form of PKA (dnPKA) fully recapitulated Shh responses in vivo in the developing CNS (41, 42). Furthermore, forced expression of Nmyc or Gli1 (11, 12) is sufficient to sustain CGNP proliferation.

CGNPs cultures were infected with SmoM2, dnPKA, Gli1 and Nmyc and grown in the absence of Shh for 48 h. The cultures were then treated with BMP2 for an additional 48 h and
pulsed with BrdUrd 4 h prior to fixation. Retroviral infection of the different transgenes was monitored by GFP expression. As expected, overexpression of activating mutations of the Shh pathway such as SmoM2 and dnPKA or those corresponding to Shh target genes, such as Gli1 and Nmyc, was sufficient to induce proliferation in the absence of Shh (Fig. 3A). Interestingly, however, only Nmyc-overexpressing cells were capable to overcome the cell cycle arrest promoted by BMP2. These data strongly indicate that BMP2 opposes Shh mitogenic activity by blocking its most known downstream effector, the Nmyc protein.

To characterize whether Nmyc-expressing clones refractory to the BMP2 effect were indeed CGNPs or part of the glial component of the culture, we analyzed the growing clones using specific markers for each cellular population. Triple immunocytochemistry were performed using markers specific for oligodendrocytes (O4), astrocytes (GFAP), and neurons (tubulin β-III), combined with BrdUrd labeling (to detect proliferating cells) and with anti-GFP to detect Nmyc-positive cells. A combination of markers showed that cells BrdUrd/GFP double-labeled were negative for GFAP or O4 but positive for tubulin β-III, thus confirming that proliferating clones were indeed CGNPs (Fig. 3B).

Together these results confirmed Nmyc as a key component in the molecular mechanism used by BMP2 to antagonize Shh mitogenic activity on CGNPs. Consistently, an analogous mechanism has been proposed for the TGFβ anti-mitotic activity, acting through the down-regulation of c-Myc expression with the concomitant up-regulation of the cyclin-dependent kinase inhibitor p15ink4B (33, 34). Interestingly, these data suggest a conserved mechanism for the activity of the TGFβs/BMPs superfamily of signaling molecules that promotes cell cycle arrest on a variety of cellular contexts by down-regulating different members of the Myc family.

**BMP2 Does Not Affect Nmyc Protein Stability but Regulates Its mRNA Levels through a Mechanism Requiring Protein Synthesis**—Protein turnover is a fundamental feature in the maintenance of protein levels within the Myc family of transcription factors. Members of the Myc family, including Nmyc, are phosphorylation targets of several kinases like Ras, mitogen-activated protein kinase, or glycogen synthase kinase-3β. Such phosphorylation events are essential modulators of the protein stability and turnover (43). Therefore, because our results showed that the reduction of Nmyc expression observed after the BMP2 treatment was an important feature for its anti-proliferative activity, we next asked whether BMP2 could be affecting Nmyc protein stability.

![FIGURE 3. Nmyc ectopic expression in granular cerebellar cells overcomes BMP2 antiproliferative effect.](image-url)
To confirm or discard this possibility, we compared Nmyc protein turnover in CGNPs cultures grown in the presence of Shh or Shh with BMP2. Nmyc *de novo* expression was blocked by the addition of cycloheximide, and protein decay was measured by immunoprecipitation of Nmyc followed by Western blotting. We observed that more than half of the total Nmyc protein was already degraded 45 min after the addition of cycloheximide, and protein decay was measured by the proteasome (44) and to exclude any nonspecific deleterious effect of cycloheximide, we showed that the proteasome inhibitor MG-132 totally abolished Nmyc degradation. These data revealed the presence of one sequence that matched the TIEG-1-binding motif, immediately 5′ of the transcriptional initiation site and close to two Sp1 sites. The zinc finger domain of TIEG-1 shares a high sequence homology with Sp1, another member of the Kruppel-like family. Interestingly, it has been proposed that TIEG-1 acts as a transcriptional repressor either through the occupation of Sp1 sites or by the action of its repressor domains (46, 47), resulting in both antiproliferative and pro-apoptotic activities, depending on the cellular context (31, 36, 48, 49).

To study the possible role of TIEG-1 on proliferating CGNPs, we first characterized the time frame at which TIEG-1 expression was induced by BMP2. Proliferating CGNPs were stimulated with BMP2 for 30, 60, and 120 min and TIEG-1 expression analyzed using real time quantitative PCR. Results showed that BMP2 strongly induced TIEG-1 expression in the presence of cycloheximide (Fig. 4C), thus confirming TIEG-1 as a direct target of BMP2 activity on CGNPs.

According to our hypothesis, TIEG-1 overexpression should be enough to decrease Nmyc levels on CGNPs. To address this, CGNPs were infected with a modified version of pWPI lentiviral vector carrying TIEG-1 and 3X NLS-GFP that rendered transduction efficiencies up to 70% (Fig. 4D). As expected,
Western blot analysis of the infected CGNPs revealed that the expression of TIEG-1 was sufficient to obtain an important and consistent reduction of Nmyc protein levels in proliferating CGNPs (Fig. 4D).

To investigate whether TIEG-1 was directly regulating Nmyc transcription, we tested the ability of TIEG-1 to regulate the human Nmyc promoter on a luciferase reporter assay. A DNA fragment encompassing from −875 to +169 of the human Nmyc promoter was cloned into a pGL3 luciferase reporter vector and cotransfected in HEK-293 cells together with a Renilla expression vector used as internal control. The Nmyc promoter showed a high transcriptional activity in HEK-293 cells, and this activity was strongly repressed when cells were cotransfected with TIEG-1 (Fig. 5A). Interestingly, however, transcriptional repression was totally abolished when the inactive mutant TIEG-1 R440W was transfected (Fig. 5A). This single point mutation affects the zinc finger domain and abolishes TIEG-1 ability to bind DNA (50). These data strongly indicate that the mechanism used by TIEG-1 to repress the Nmyc promoter depends on its interaction with the DNA rather than on a mechanism based on interactions with other transcription factors.

Using the same Nmyc promoter construct as above, we next tested the ability of activated SMADs to directly regulate Nmyc promoter. As shown in supplemental Fig. S2, transfection of Smad1/5 together with the common Smad4 and with a constitutively active form of the BMP receptor ALK-3 failed to modulate Nmyc promoter activity, despite the consistent response obtained when the Id promoter was assayed. These data are in accordance with the results shown in Fig. 4B, indicating that regulation of Nmyc transcription by BMPs activity is achieved through an intermediate transcription factor rather than a direct Smad action.

**TIEG-1 Represses Nmyc Transcription through the Occupation of Sp1-binding Sites on the Nmyc Promoter**—Computer analysis of the human Nmyc promoter predicted only one TIEG-1-binding site positioned 5' to the transcription initiation point and close to two Sp1-binding sites (Fig. 5A). To investigate whether this TIEG-1-binding site was necessary and/or sufficient for the TIEG-1-mediated repression of Nmyc promoter, we performed a series of sequential unidirectional deletions of the Nmyc promoter. Six different fragments were generated, and their transcriptional activities were analyzed alone or cotransfected with TIEG-1. The results showed significant basal activity for five of those fragments (−875 to +169; −654 to +169; −445 to +169; −243 to +169; and −445 to +9), and they all preserved the ability of TIEG-1 to repress promoter activity (Fig. 5B). However, the fragment lacking the E2F sites...
(-146 to +19), failed to promote luciferase activity as previously reported (51), precluding any further analysis of TIEG-1 repressor activity in any region downstream from the E2F sites.

To circumvent this problem we cloned three separate fragments of the Nmyc promoter into a promoterless luciferase vector containing a consensus TATA box. The results showed that the unique fragment consistently repressed by TIEG-1 was the one spanning from -146 to +9 (Fig. 5C).

To further confirm that the interaction of TIEG-1 with the Nmyc promoter was located within this fragment, we next fused TIEG-1 to the VP16 transactivator domain of the herpes simplex virus. Because VP16 lacks a DNA-binding domain, the fusion TIEG-1-VP16 would stimulate transcription only through the TIEG-1 DNA-binding domain. Transcriptional activation was only observed in the -146/+9 fragment (Fig. 5D), further confirming the binding of TIEG-1 to this fragment.

We next tested whether TIEG-1 was directly binding to the -146/+9 fragment performing a nonradioactive electrophoretic mobility shift assay, using biotinylated probes of the -243/-146 and -146/+9 fragments. Nuclear extracts from COS-7 cells transfected with TIEG-1 or empty vector as a control were incubated with the probes and an electrophoretic mobility shift assay performed. The results confirmed that direct binding of TIEG-1 occurs only at the -146/+9 region (Fig. 6A).

To further delimit the specific sequence that binds to TIEG-1 within the -146/+9 fragment, partially overlapping double-stranded oligonucleotides covering the whole region were prepared, and their ability to displace TIEG-1 binding to the -146/+9 fragment was tested in an electrophoretic mobility shift assay. Interestingly, TIEG-1-specific band could be displaced with oligonucleotides containing both, Sp1- and TIEG-1-binding sites (Fig. 6B, lanes 1, 6, and 7) and with a consensus TIEG-binding site (Fig. 6B). Consistently, the maximum displacement was attained with oligonucleotide number 6, that contains both Sp1- and TIEG-binding sites.

The specificity of the displacements were further checked by using oligonucleotides where two key nucleotides of the DNA-binding motifs of Sp1 or TIEG-1 were mutated (Fig. 6C). Displacement capacity of mutated oligonucleotides was greatly reduced except for the oligonucleotide 6Tm. Oligonucleotide number 6 contains one Sp1-binding site and one TIEG-binding site, but only the mutation of the Sp1 site and not the TIEG site reduced its displacing capacity, most likely because the TIEG site is truncated in this oligonucleotide. Together these data demonstrate that TIEG-1 can bind to both the Sp1-
and TIEG-1-binding sites present on the human Nmhc promoter. Interestingly, the zinc finger domain of TIEG-1 shares a high sequence homology with Sp1, and previous studies demonstrated that TIEG-1 and Sp1 compete for the same DNA-binding site within the CD11d promoter (45). This supports an attractive conserved mechanism for transcriptional repression, because Sp1 sites have been reported to have a prominent activator role within the Nmhc promoter (52, 53).

To further test whether TIEG-1 or Sp-1 sites were the main contributors to the observed TIEG-1 mediated inhibition of the Nmhc promoter, we developed variants of the pGL3–445/H1100/Luc construct where Sp1- and TIEG-1-binding motifs were specifically mutated (TIEG-1 site GGTGTG was mutated to aaTGTG and Sp-1 site GGCGGG was mutated to GttGGG) (Fig. 6D). Mutation of TIEG-1 or a single Sp-1 DNA binding site had no effect on the ability of TIEG-1 to inhibit promoter activity. Only when both Sp-1 sites were simultaneously mutated, was the promoter activity completely lost and also the TIEG-1 repressor activity. These results confirmed that TIEG-1 blocks Nmhc promoter activity by displacing the activator factors bound to the Sp1 sites rather than acting through an inhibitory mechanism involving the TIEG-1-binding site.

**TIEG-1 Promotes Cell Cycle Arrest and Apoptosis but Not Differentiation in CGNPs**

Because BMP2 increases TIEG-1 expression yielding a down-regulation of Nmhc transcription, we next asked whether TIEG-1 expression was sufficient to mimic the full array of BMP2 responses on CGNPs (25), including cell cycle arrest and differentiation.

Strikingly, TIEG-1 expression in CGNPs resulted in a transitory cell cycle arrest initiated as soon as 12 h after transfection that was progressively declining at longer periods of time (36–48 h) (Fig. 7A). Because it has been reported that TIEG-1 possesses certain pro-apoptotic activity (36), we next performed a deoxynucleotidyltransferase-mediated dUTP nick end labeling assay to check the apoptotic activity of TIEG-1 in CGNPs. Increased apoptosis was only detected in cells expressing TIEG-1.
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TIEG-1 for long periods of time (36–48h) (Fig. 7C), confirming that the proliferation stop observed at short times was not due to apoptosis.

We next test the ability of the TIEG-1 R440W mutant, which cannot bind to the Nmyc promoter; to act as a dominant negative by competing for the same cofactors required by TIEG-1 to exert its repressor activity, thus suppressing the anti-proliferative activity of BMP2. Cells were plated in the presence of Shh (3 μg/ml) and transfected with empty vector or the R440W mutant. Two hours after transfection, CGNPs were treated with BMP2 for 24 h and pulsed with BrdUrd the last 4 h. As expected, the cells transfected with the mutant R440W were able to oppose the anti-mitotic activity of BMP2 at the tested period of time (Fig. 7B), indicating that BMP2 exerts its anti-proliferative activity mainly through TIEG-1.

BMP2 exerts concomitant anti-proliferative and differentiative actions in CGNPs, leading to differentiated neurons with very low percentages of apoptosis. Therefore, we next asked whether cell cycle arrest induced by TIEG-1 was accompanied by differentiation. Transfection of TIEG-1 resulted in no changes on tubulin β-III expression (Fig. 7D), indicating that TIEG-1 failed to promote terminal differentiation. Together these data suggested that the apoptosis induced by TIEG-1 was due to its inability to activate neuronal differentiation after the proliferation arrest, indicating the requirement of a parallel branch in BMP2 signaling independent of TIEG-1 that promotes neuronal differentiation.

To test this hypothesis, CGNPs were transfected with TIEG-1 or empty vector and then treated with vehicle, BMP2, or DBA. Interestingly, both BMP2 and DBA abolished TIEG-1 apoptosis, rendering normally differentiated neurons (Fig. 8, A and B). BMP2 and DBA promotes CGNPs differentiation through unrelated signaling cascades, BMP2 stimulates Alk3/Smad signaling cascade, and DBA is a direct activator of PKA. Additionally, neither one prevents apoptosis in nontransfected cells, indicating that it is the differentiation process by itself what prevents TIEG-1 arrested cells to undergo apoptosis.

Together, these data indicate that the anti-proliferative and pro-differentiative activities are carried out by at least two separated signals emanating from BMP2, where TIEG-1 mediates the anti-proliferative activity. Consequently, TIEG-1 and BMP2 should act in synergy or at least in an additive fashion. Accordingly, the BMP2 concentration required to promote cell cycle exit was 20 times lower in cells expressing TIEG-1 (Fig. 8C). These data are in accordance with the fact that in the presence of BMP2, cell cycle arrest is not transient, and cells are driven through a terminal differentiation.

Together our results suggest a model in which BMP2 antagonizes the Shh proliferative effect in two coordinated steps, one promoting cell cycle arrest down-regulating Nmyc through TIEG-1 and other acting through the activation of the genes responsible for the process of differentiation. A failure promoting differentiation will lead cells to apoptosis or deviate cellular signaling through parallel proliferative pathways not regulated by TIEG-1.

These data indicate the importance of promoting both mechanisms of arrest and differentiation in cancer cells, not only focusing on a reduction of mitotic index that can be easily overcome by tumor cells. It also could be of interest to analyze TIEG-1 expression in tumor samples to determine its possible relevance as a prognosis factor, as well as to determine the role of the members of the Krüppel-like factors as tumor suppressors (54).

Future work should address toward the identification and characterization of the transcription factors involved in the process of differentiation, with the aim of reconstituting the entire activity of Shh/BMP2 involved in the process of proliferation and differentiation of cerebellar granular neurons, as well as deepening in the role of the BMP pathway as tumor suppressor to find new mutations that could give rise to medulloblastoma.

Acknowledgments—We thank Elisa Marti and Deborah Burks for critical reading and style supervision of the manuscript. We also want to thank all of the researchers who sent us the cDNAs detailed under “Experimental Procedures.” Finally, we are especially grateful to the Genetic Institute, which provided all of the BMP2 used in this work.
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