BM88 Is a Dual Function Molecule Inducing Cell Cycle Exit and Neuronal Differentiation of Neuroblastoma Cells via Cyclin D1 Down-regulation and Retinoblastoma Protein Hypophosphorylation*

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Control of cell cycle progression/exit and differentiation of neuronal precursors is of paramount importance during brain development. BM88 is a neuronal protein associated with terminal neuron-generating divisions in vivo and is implicated in mechanisms underlying neuronal differentiation. Here we have used mouse neuroblastoma Neuro 2a cells as an in vitro model of neuronal differentiation to dissect the functional properties of BM88 by implementing gain- and loss-of-function approaches. We demonstrate that stably transfected cells overexpressing BM88 acquire a neuronal phenotype in the absence of external stimuli, as judged by enhanced expression of neuronal markers and neurite outgrowth-inducing signaling molecules. In addition, cell cycle measurements involving cell growth assays, BrdUrd incorporation, and fluorescence-activated cell sorting analysis revealed that the BM88-transfected cells have a prolonged G1 phase, most probably corresponding to cell cycle exit at the G0 restriction point, as compared with controls. BM88 overexpression also results in increased levels of the cell cycle regulatory protein p53, and accumulation of the hypophosphorylated form of the retinoblastoma protein leading to cell cycle arrest, with concomitant decreased levels and, in many cells, cytoplasmic localization of cyclin D1. Conversely, BM88 gene silencing using RNA interference experiments resulted in acceleration of cell proliferation accompanied by impairment of retinoic acid-induced neuronal differentiation of Neuro 2a cells. Taken together, our results suggest that BM88 plays an essential role in regulating cell cycle exit and differentiation of Neuro 2a cells toward a neuronal phenotype and further support its involvement in the proliferation/differentiation transition of neural stem/progenitor cells during embryonic development.

The formation of the nervous system is governed by a delicate balance between cell proliferation, subsequent cell cycle withdrawal, and differentiation to distinctive neuronal phenotypes (1, 2). Current observations have highlighted the existence of mechanisms coupling cell cycle exit and differentiation as well as functional cross-talk between intrinsic factors controlling these two mechanisms. A number of key factors regulating cell cycle progression have been implicated in cell fate determination and differentiation of neuronal precursors, whereas specification- and/or differentiation-inducing molecules are beginning to emerge as cell cycle regulators (3–6). However, there are still important questions regarding the timing control of the proliferation/differentiation switch that remain unanswered.

In the central nervous system, control of cell cycle progression plays an essential role in the generation of the appropriate number of neurons and the formation of functional neuronal circuits. When a neuronal progenitor is committed to undergo differentiation, it exits from the G1 phase of the cell cycle and enters into an irreversible quiescent state referred to as G0. The tumor suppressor proteins p53 and pRb6 are central regulators of this progression (7). p53, when activated, causes G1 arrest at the G0 restriction point by inducing expression of p21 and consequent inhibition of D-type cyclins and related cyclin-dependent kinases (8–10), thus preventing phosphorylation of pRb (7). Under these conditions, hypophosphorylated pRb associates with the E2F family of transcription factors, thus impairing their ability to transactivate genes required for cell cycle progression (11). As a consequence cells do not progress through the G1–to-S phase transition. Several studies have indicated that a critical event associated with cell cycle withdrawal and differentiation both in neuronal and non-neuronal cells is the cellular compartmentalization of cyclin D1, which shifts from a predominantly nuclear localization to cytoplasmic sequestration.

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6 The abbreviations used are: pRb, retinoblastoma protein; BrdUrd, 5-bromodeoxyuridine; FGF-R, fibroblast growth factor receptor; N2A, Neuro 2a cells; PBS, phosphate-buffered saline; RA, retinoic acid; siRNA, silencing RNA; RT, reverse transcription; FACS, fluorescence-activated cell sorter; TUNEL, terminal dUTP nick-end labeling; FGF, fibroblast growth factor; FGF-R, FGF receptor; m, murine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; DIOc(3), 3,3’-dihexyloxocarbocyanine; NCAM, neural cell adhesion molecule.
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(12, 13). Withdrawal from the cell cycle is accompanied by acquisition and maintenance of the neuronal phenotype, which involves a large number of genes that are expressed in a pre-determined and coordinated manner (14, 15). These include pro-neural genes, transcription factors involved in patterning, and members of the basic helix-loop-helix protein family (16–19). Several studies indicate that distinct neuronal fates are determined during the final cell cycle of neuronal progenitors, and therefore determination of the involvement of genes that act during this stage is of particular importance (1, 20, 21).

BM88 (Cend1 for cell cycle exit and neuronal differentiation 1, according to NCBI nomenclature at www.ncbi.nlm.nih.gov) has been initially identified as a protein expressed in mature neurons (22, 23). However, subsequent studies revealed that BM88 marks neuronal cells all along the different stages of the neuronal lineage; it is expressed at low levels in neuronal precursors, and its expression is distinctly up-regulated in young post-mitotic as well as in mature neurons (24, 25). Thus there is an interesting correlation between BM88 expression and the progression of progenitor cells toward neuronal differentiation in the developing embryonic brain, suggesting that BM88 may be functionally involved in this process. This is further supported by the fact that BM88 is associated in vivo with asymmetric neuron-generating cell divisions (24), while it also enhances the morphological differentiation of mouse neuroblastoma cells in vitro (26).

BM88 cloned from mammalian brain (26, 27) is an integral membrane protein composed of two 22–23-kDa polypeptide chains linked together by disulfide bridges. It is anchored to the membrane of intracellular organelles, including the outer membrane of mitochondria, the endoplasmic reticulum, and other endocytic vesicles, via a transmembrane domain so that the bulk of the protein faces toward the cytoplasm (25, 26). Of interest, the human BM88 gene is localized in chromosome 11p15.5, a region associated with overgrowth genetic disorders and several types of cancers, whereas the mouse gene is mapped at the corresponding region of the syntenic chromosome 7 (27). The recently characterized human BM88 promoter confers specific transcriptional activity in primary neurons and is activated by the transcription factor Sp1 (28). Notably, transcriptional deregulation of Sp1-dependent pathways in neurons is supposed to be at the basis of neuro-degenerative disorders (29, 30).

In this study we have sought to analyze the functional properties of BM88 using the mouse neuroblastoma Neuro 2a (N2A) cell line as an in vitro model for neuronal differentiation. We demonstrate by gain- and loss-of-function approaches that BM88 is sufficient and necessary for accomplishment of cell cycle exit and differentiation of N2A cells toward a neuronal phenotype, and we provide direct evidence that BM88 activates the p53-pRb signaling pathway that controls the balance between cell proliferation and cell cycle exit. Moreover, we demonstrate that the anti-proliferative effect of BM88 is associated with cyclin D1 down-regulation and, in many cells, with cytoplasmic cyclin D1 localization, concomitantly to pRb hypophosphorylation and subsequent withdrawal from the cell cycle at the G1/G0 transition point. Our data suggest that BM88 up-regulation upon precursor to neuron switch is functionally relevant and may form part of the underlying mechanisms operating during neurogenesis in vivo.

EXPERIMENTAL PROCEDURES

N2A Stable Transfection—For transfections, the full-length porcine BM88 cDNA was subcloned into the pcDNA3 vector (Invitrogen) at HindIII and XbaI sites. N2A cells were plated in 6-well plates at a density of 1 × 10⁵ cells/well and were allowed to reach 50% confluence. Transfections were performed for 5 h with 2 µg of plasmid pcDNA3 containing BM88 and Lipofectamine Reagent diluted in Opti-MEM medium in accordance with the manufacturer’s instructions (Invitrogen). The transfection medium was then removed, and cells were fed with complete growth medium for 48 h. Stable transfectants were selected with 1 mg/ml geneticin maintained in the growth medium for 5 weeks. For cell cloning, the cells were aliquoted into 96-well tissue culture plates in different dilutions, and stable transfectants were selected. Numerous stable cell lines expressing BM88 were isolated as determined by immunofluorescence screening using specific mouse monoclonal and rabbit polyclonal anti-BM88 antibodies (22, 25) followed by an appropriate fluorescein-conjugated secondary antibody (Molecular Probes). Two independent clones, C3C1 and C7G5, with the highest BM88 expression, were selected for further studies and were found to behave similarly in all tests performed. As controls, we used either the parental nontransfected N2A cells or cells transfected with the pcNeo vector alone (26) with no apparent differences between them.

Transient Transfections and Reporter Assays—N2A and NIH 3T3 cells were cultured in Dulbecco’s minimal essential medium containing 10% fetal calf serum and antibiotics. One day prior to transfections, cells were plated into 6-well tissue culture plates at a concentration of 1.3 × 10⁵ cells/well, and DNA was introduced by liposome-mediated transfection using 2 µg of DNA, 5 µl of Lipofectamine, and 2 µl of Plus reagent in a final volume of 1 ml of Opti-MEM (all reagents from Invitrogen). For double transfections, 2–4 µg of the coding region of the porcine BM88 cDNA (26) or 2 µg of cyclin D1 cDNA (pRc-CMV-cycD1 construct) or PC3/Tis21 cDNA (pSCT-PC3) were co-transfected with 0.2 µg of the human pRb cDNA cloned into the pCMVneoBam vector (pCMV-pRB1 construct), and all, except the BM88 cDNA, were obtained from Dr. F. Tirone, Institute of Neurobiology and Molecular Medicine, Rome, Italy (31). Transfection mixtures were replaced by serum-containing medium 5 h post-transfection, and cells were collected 48 h later and processed for Western blot analysis. For luciferase assays, 2 µg of the pWWP-luc reporter construct (provided by Dr. D. Kardassis, University of Crete Medical School, Hellas) were co-transfected with 0.2 µg of a β-galactosidase expression plasmid, pCMV/β (Clontech) (28). To correct for differences in transfection efficiencies, luciferase activity was normalized according to β-galactosidase values. All experiments were performed in duplicate at least three times, and statistical analysis was performed by the paired two-sample Student’s t test.

Adenoviral Infections—Construction of the recombinant BM88 adenovirus was performed using the Adeno-Quest system (Quantum Biotechnologies), according to the manufacturer’s instructions. Briefly, the coding region of the porcine BM88
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cDNA was inserted into the pQBI-AdCMV5-ires-GFP adenoviral vector, and recombinant adenoviral particles were produced by co-transfection of the vector and the helper virus and homologous recombination into the HEK-293A human cell line. Selection and further purification of the BM88 positive viruses were performed using the plaque assay method. The adenoviral supernatants were then used to infect N2A cells overnight. Infected cells were in turn transiently transfected with the human pRb cDNA cloned into the pCMVneoBam vector as described above.

Genotoxic Stress Assay—The chemotherapeutic agent doxorubicin (adriamycin, Sigma), known to induce genotoxic stress (32, 33), was added to NIH 3T3, wild type N2A, and N2A-BM88 subconfluent cells at a final concentration of 1.5 μM for a total period of 36 h, and cells were then fixed in 4% paraformaldehyde and processed for immunofluorescence staining.

Immunofluorescence Staining—For localization staining experiments, cells were grown on poly-L-lysine-coated cover-slips in 48-well plates and incubated for 40 min at 37 °C with the mitochondrial, endoplasmic reticulum, or Golgi markers: Mito Tracker Red CMXRos dye, DiOC6(3), or BODIPY TR ceramide (all from Molecular Probes). Live-stained cells were then fixed in 4% paraformaldehyde and incubated overnight at 4 °C with the anti-BM88 monoclonal antibody followed by a 2-h incubation with fluorescein-conjugated secondary antibody (22, 25). Other primary antibodies used for immunofluorescence single or double labeling experiments (24) include the following: mouse monoclonal anti-synaptophysin (34); mouse monoclonal anti-βIII-tubulin (TuJ1 antibody from Covance); mouse monoclonal SM1 311 (Stenberger Monoclonals, Denmark), which recognizes nonphosphorylated epitopes of high molecular weight neurofilament protein; rabbit polyclonal anti-fibroblast growth factor receptor 1 (FGF-R, Santa Cruz Biotechnology); rabbit polyclonal anti-NCAM (kind gift of Dr. G. Rougon, Institut de Biologie du Developpement, Marseille, France); mouse monoclonal anti-p21 antibody (D01, Santa Cruz Biotechnology); rabbit polyclonal anti-NCAM (kind gift of Dr. G. Rougon, Institut de Biologie du Developpement, Marseille, France); mouse monoclonal anti-p21 antibody (D01, Santa Cruz Biotechnology); mouse monoclonal anti-p53 antibody (D01, Santa Cruz Biotechnology); mouse monoclonal anti-p21 antibody (187, Santa Cruz Biotechnology); and rabbit polyclonal anti-Bax antibody (N-20, Santa Cruz Biotechnology). In situ apoptosis was analyzed by TUNEL (Roche Applied Science) as described previously (35), and cell survival was estimated by trypan blue exclusion. Quantification of the p53 immunofluorescence signal was performed in a total number of 100 cells/different condition using the Alpha Image software (Alpha Innotech Co.), as described previously (36), and the average fold increase of fluorescence signal relatively to N2A untreated cells was estimated.

Cell Cycle Measurements—DNA synthesis assays using 5′-bromodeoxyuridine (BrdUrd) were performed either by a single 4-h BrdUrd pulse (100 μM) in cells that had been maintained in culture for 2 days, followed by fixation, or by adding to cells immediately after plating repertitive doses of 100 μM BrdUrd at 2-h intervals for the duration of 18 h (cumulative labeling). BrdUrd incorporation was detected using an anti-BrdUrd monoclonal antibody (DAKO) as described previously (24). All experiments were performed in duplicate at least three times, and statistical analysis was performed by the paired two-sample Student’s t test. Image analysis was carried out using a Leica-TEC-NT/SP confocal microscope.

Western Blot Analysis—Exponentially growing cells were washed twice in cold PBS and lysed in PBS containing 0.2% SDS, 1% Triton, 1% sodium fluoride, 0.25 mM sodium orthovanadate, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Lysates were incubated for 15 min in ice, vortexed vigorously every 5 min, and clarified by centrifugation at 14,000 rpm for 15 min. Proteins were precipitated for 4 volumes of acetone at −80 °C overnight, centrifuged for 30 min, and resuspended in SDS-PAGE sample buffer. For Western blot analysis, proteins were separated by 10% SDS-PAGE (Bio-Rad) and blotted onto Protran nitrocellulose membrane (Schleicher & Schuell). Primary antibodies used were as follows: anti-BM88 monoclonal antibody (diluted 1:1000); anti-synaptophysin monoclonal antibody (Gaitanou et al. (34); 1:1000); affinity-purified polyclonal antibodies to cyclin E (1:200), cyclin B1 (1:200), cyclin D1 (1:100), and p53 (DO-1; 1:100) (all from Santa Cruz Biotechnology). Detection of pRb was performed with the G3-245 monoclonal antibody, which recognizes both the hyper- and hypophosphorylated forms of the protein (Pharmingen; diluted to a final concentration of 1 μg/ml), following protein electrophoresis in a 7.5% SDS-polyacrylamide gel to separate the two forms of pRb. Peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences, and blots were developed using either the diaminobenzidine (Sigma) chromogenic system or ECL (Amersham Biosciences), according to the manufacturer’s instructions.

FACS Analysis—N2A and N2A-BM88 cells cultured on 10-cm plates for 4 days were incubated for 10 min in PBS, 1 mM EDTA, 1% bovine serum albumin, harvested, and centrifuged for 2 min at 1200 rpm. The cell pellet was resuspended in 100 μl of PBS, 1 mM EDTA, and cells were fixed by dropwise addition of ice-cold 70% ethanol while vortexing and left overnight at −20 °C. Cells were centrifuged, washed in PBS, 1% bovine serum albumin, resuspended in 100 μl of RNase A (100 μg/ml in PBS), and incubated for 10 min at room temperature followed by staining with 700 μl of propidium iodide (50 μg/ml in PBS) for 5 min. A total of 10,000 stained cells were analyzed in a fluorescence-activated cell sorter (FACS BD Biosciences). The Modfit software (BD Biosciences) was used to determine the distribution of cells in the various cell cycle phases designated G0/G1, S, and G2/M.

BM88 Knockdown in N2A Cells Using siRNA—Two different siRNAs for BM88 (siBM88) were purchased from Dharmacon (Boulder, CO). The sequences of BM88-specific siRNAs are available upon request; the nontargeting control siRNA was also purchased from Dharmacon (catalog number D-001210-01-05). NIH 3T3 and N2A cell lines were transfected 24 h after plating with 100 nM siRNAs. Proliferation was estimated 72 h after transfection by BrdUrd incorporation after a single 3-h BrdUrd pulse. Cells were visualized using a Zeiss fluorescence photomicroscope equipped with a Leica DC300 digital camera and image analysis system (ImageJ software from National Institutes of Health). For quantification of proliferation, 1000 DAPI-positive cells were scored for BrdUrd staining from five independent experiments in each case (siCTR or siBM88 transfections). Retinoic acid (RA, 10 μM)-induced differentiation of
N2A cells (26) was initiated 24 h after siRNA transfection and was estimated 48 h later by measuring the length of 1) process outgrowth under phase-contrast optics and 2) βIII-tubulin positive processes. In both cases, 1000 cells were measured from five independent experiments, and statistical analysis was performed by the paired two-sample Student’s t test.

Quantitative RT-PCR—DNase I-treated total RNA from cells transfected with siCTR or siBM88 (100 nM) (Lipofectamine) was purified 72 h after transfection using the RNeasy mini kit (Qiagen). Quantitative RT-PCR assays were done in triplicate using the LightCycler RNA amplification kit (Roche Applied Science) on a LightCycler instrument in conjunction with gene-specific unlabeled external forward and reverse primers and pairs of differentially labeled forward internal primers (HybProbes FL and LC, emitting at 530 and 640 nm, respectively; designed and produced by TIB MOLBIOL, Syntheselabor GmbH, Berlin, Germany). Primers for glyceraldehyde-3-phosphate dehydrogenase mRNA (mGAPDH), which was used as an internal standard, and BM88 mRNA (mBM88) were as follows: mGAPDH forward, 5′-AACCTCCCTCAAGATTTGCACGCA-3′, and reverse, 5′-CTTCACTTCTACGATCTCAGCTCCT-3′; mBM88 forward, 5′-CGAGAAGCTTGAGGGCCCCAAC-3′ and reverse, 5′-CCCCACGCTATGTCCTTC-3′. Primers for murine (m) FGF-R1 forward, 5′-GGCCCAAA-3′, mBM88 forward, 5′-GGCGGGTACACTTATCCGGAC-3′, and mFGF-R1 reverse, 5′-TTTATAGAAGGGGGAGAGGACGCT-3′. Primers were as follows: mGAPDH forward, 5′-GGCGGGTAGATTTCTCAA-3′, and cyclin D1 forward (Ccnd1) were as follows: mFGF-R1 forward, 5′-GGCGGGTAGATTTCTCAA-3′, and mFGF-R1 reverse, 5′-TTTATAGAAGGGGGAGAGGACGCT-3′.

Normalized standard curves were obtained using PCR products diluted in 10 μg/ml sonicated salmon sperm DNA. Normalization of the mBM88 expression was done against mGAPDH.

**RESULTS**

*Transgene BM88 Is Appropriately Targeted in the Mitochondria and ER of N2A-BM88 Cells*—BM88 expression is elevated in the developing brain upon transition of precursor cells to post-mitotic neurons (24). This up-regulation coincides with cell cycle exit of neuronal precursors and their differentiation. To investigate whether BM88 up-regulation has functional consequences, N2A mouse neuroblastoma cells were stably transfected with the porcine bm88 cDNA (Fig. 1). The N2A cell line was chosen for the following reasons: (a) low levels of BM88 are expressed in cycling N2A cells just as in neuronal precursors *in vivo* (Fig. 1, C and E); (b) N2A cells are inherently capable of undergoing differentiation toward a neuronal phenotype given the appropriate conditions (37); (c) and others (38) have shown that BM88 expression is increased in neuroblastoma cells upon RA-driven differentiation; and (d) a marked morphological differentiation of N2A cells is observed upon BM88 overexpression in the absence of external stimuli (see Ref. 26 and this study) (Fig. 1, A and B).

Numerous stable N2A cell lines were isolated overexpressing the BM88 protein (N2A-BM88 cells) as determined by Western blot (Fig. 1, C) and immunofluorescence screening (Fig. 1, E and F) using the anti-BM88 monoclonal or polyclonal antibodies. Two of these, independent clones C3C1 and C7G5, of N2A-BM88 cells stably transfected with the BM88 CDNA, using the monoclonal anti-BM88 antibody (C) or a monoclonal anti-synaptophysin antibody (D). BM88 overexpression in N2A cells results in enhanced synaptophysin expression. E–G, double immunofluorescence labeling of control N2A (E) and N2A-BM88 cells (F and G) using polyclonal anti-BM88 (green) and monoclonal anti-synaptophysin (red) antibodies. Control N2A cells express low levels of BM88, whereas synaptophysin is nondetectable (E). Merged BM88/synaptophysin labeling in N2A-BM88 cells (G) shows that BM88 overexpression (F) leads to up-regulation of synaptophysin (G, yellow). H–J, merged micrographs of N2A-BM88 cells double-labeled for BM88 (red in H, green in I and J) and the vital dyes DIOC6(3) (green in H), BODIPY TR ceramide (red in I), and Mito Tracker Red CMXRos (red in J). BM88 is localized in the ER and mitochondria of N2A-BM88 cells (H and J), whereas minimal staining is seen in the Golgi (arrows in I). K, merged double immunofluorescence labeling of N2A-BM88 cells for BM88 (red) and the neuronal marker βIII-tubulin (green); E–K, confocal microscopic analysis. Bars in A, B, E–G, J, and K, 20 μm; Bars in H and I, 20 μm.

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**FIGURE 1.** Altered morphology and enhanced molecular neuronal phenotype of N2A-BM88 cells. A and B, N2A-BM88 cells maintained for 4 days in vitro exhibit extensive morphological differentiation with enhanced process outgrowth (B), as compared with control N2A cells (A). C and D, Western blot analysis of protein extracts from N2A cells and two independent clones, C3C1 and C7G5, of N2A-BM88 cells stably transfected with the BM88 CDNA, using the monoclonal anti-BM88 antibody (C) or a monoclonal anti-synaptophysin antibody (D). BM88 overexpression in N2A cells results in enhanced synaptophysin expression. E–G, double immunofluorescence labeling of control N2A (E) and N2A-BM88 cells (F and G) using polyclonal anti-BM88 (green) and monoclonal anti-synaptophysin (red) antibodies. Control N2A cells express low levels of BM88, whereas synaptophysin is nondetectable (E). Merged BM88/synaptophysin labeling in N2A-BM88 cells (G) shows that BM88 overexpression (F) leads to up-regulation of synaptophysin (G, yellow). H–J, merged micrographs of N2A-BM88 cells double-labeled for BM88 (red in H, green in I and J) and the vital dyes DIOC6(3) (green in H), BODIPY TR ceramide (red in I), and Mito Tracker Red CMXRos (red in J). BM88 is localized in the ER and mitochondria of N2A-BM88 cells (H and J), whereas minimal staining is seen in the Golgi (arrows in I). K, merged double immunofluorescence labeling of N2A-BM88 cells for BM88 (red) and the neuronal marker βIII-tubulin (green); E–K, confocal microscopic analysis. Bars in A, B, E–G, J, and K, 20 μm; Bars in H and I, 20 μm.
studies have demonstrated that BM88 is associated with the limiting membrane of mitochondria, endoplasmic reticulum, and small electron-lucent vesicles (25). To ensure that the transgene is properly targeted within the cell, we performed double labeling of N2A-BM88 cells with antibodies to BM88 in conjunction with several vital dyes known to mark distinct subcellular compartments. In particular, Mitotracker Red CMXRos and DiOC₆(3) were used for mitochondrial and endoplasmic reticulum (ER) labeling, whereas BODIPY TR ceramide was used for Golgi staining. Immunofluorescence labeling with the monoclonal anti-BM88 antibody in combination with the two dyes for mitochondria and the ER, followed by confocal microscopic analysis, showed clear co-localization with BM88, whereas minimal levels of co-localization were observed with the Golgi dye upon double labeling of N2A-BM88 cells (Fig. 1, H–J). These results confirmed that transgene BM88 is targeted properly within the N2A-BM88 cells.

**BM88 Overexpression in N2A-BM88 Cells Results in Acquisition of a Differentiated Neuronal Phenotype**—In a previous report we showed that BM88 overexpression in N2A cells induces a marked morphological differentiation manifested by enhanced process outgrowth in the absence of external stimuli (26) (Fig. 1, A and B), and we hypothesized that BM88 is involved in neuronal differentiation. The experiments presented here were aimed at testing this hypothesis directly. To explore whether the alterations observed in the shape of the BM88-transfected cells were suggestive of a differentiation process leading to acquisition of a neuronal phenotype, we investigated by immunocytochemistry the expression of βIII-tubulin, which is a characteristic cytoskeletal marker of differentiated neurons. Interestingly, we observed a marked increase in βIII-tubulin expression in the N2A-BM88 cells (Fig. 1K) as compared with controls, which were devoid of βIII-tubulin expression (not shown). Furthermore, we stained the N2A and N2A-BM88 cells with the SMI 311 antibody, directed at the nonphosphorylated epitopes of high molecular weight neurofilament proteins, as an additional marker of neuronal differentiation. In agreement, SMI 311 labeling was significantly induced in the BM88-transfected cells as opposed to N2A controls (Fig. 2, A and B), further confirming the enhanced differentiation state of the BM88-transfected cells toward a neuronal phenotype. We next assessed synaptic vesicle formation in N2A-BM88 cells by exploring the expression of the synaptic vesicle marker synaptophysin. A marked induction of this protein was noted both by Western blotting (Fig. 1D) and immunofluorescence labeling of N2A-BM88 cells (Fig. 1G), whereas N2A controls did not contain any detectable synaptophysin levels (Fig. 1, D and E). Equal protein loading was verified in all cases by FastRed protein staining of the blotted fractions before the immunchemical reaction (not shown) and also by immunochromedetection of α-tubulin, which is constitutively expressed in N2A-BM88 and control cells with no apparent alterations (see Fig. 4A below). These data collectively suggest that BM88 promotes neuronal differentiation in N2A-BM88 cells, possibly through cytoskeletal rearrangements but also through the synthesis of synaptic vesicle proteins.

We have previously observed that N2A-BM88 cells are more responsive to β-fibroblast growth factor than control N2A cells (39). This prompted us to check the expression of FGF receptor (FGF-R) transcripts and protein levels in these cells. Our results demonstrate a 2-fold increase of FGF-R mRNA in N2A-BM88 cells when compared with controls, as revealed by real time RT-PCR (Fig. 2F). In addition, a prominent up-regulation of FGF-R protein was noted in N2A-BM88 and control cells with no apparent alterations (see Fig. 4A below). These data collectively suggest that BM88 promotes neuronal differentiation in N2A-BM88 cells, possibly through cytoskeletal rearrangements but also through the synthesis of synaptic vesicle proteins.

Next, we investigated the expression of additional markers of neuronal differentiation. Enhanced expression of neurofilament protein, FGF-R, and NCAM in N2A-BM88 cells. A and B, merged double immunofluorescence labeling (confocal analysis) of N2A control (A) and N2A-BM88 (B) cells for BM88 (red) and nonphosphorylated neurofilament protein epitopes (NP-NF; green). C, Western blot analysis of FGF-R expression in protein extracts from N2A and N2A-BM88 cells. D and E, merged double immunofluorescence labeling (confocal analysis) of N2A control (D) and BM88 (E) cells stained for BM88 (red) and FGF-R (green). F, quantification of FGF-R mRNA levels in N2A and N2A-BM88 cells by real time RT-PCR. G and H, merged double immunofluorescence labeling (confocal analysis) of N2A control (G) and N2A-BM88 (H) cells stained for BM88 (red) and NCAM (green). Bar, 20 μm.

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FIGURE 2. Enhanced expression of neurofilament protein, FGF-R, and NCAM in N2A-BM88 cells. A and B, merged double immunofluorescence labeling (confocal analysis) of N2A control (A) and N2A-BM88 (B) cells for BM88 (red) and nonphosphorylated neurofilament protein epitopes (NP-NF; green). C, Western blot analysis of FGF-R expression in protein extracts from N2A and N2A-BM88 cells. D and E, merged double immunofluorescence labeling (confocal analysis) of N2A control (D) and N2A-BM88 (E) cells stained for BM88 (red) and FGF-R (green). F, quantification of FGF-R mRNA levels in N2A and N2A-BM88 cells by real time RT-PCR. G and H, merged double immunofluorescence labeling (confocal analysis) of N2A control (G) and N2A-BM88 (H) cells stained for BM88 (red) and NCAM (green). Bar, 20 μm.

studies have demonstrated that BM88 is associated with the limiting membrane of mitochondria, endoplasmic reticulum, and small electron-lucent vesicles (25). To ensure that the transgene is properly targeted within the cell, we performed double labeling of N2A-BM88 cells with antibodies to BM88 in conjunction with several vital dyes known to mark distinct subcellular compartments. In particular, Mitotracker Red CMXRos and DiOC₆(3) were used for mitochondrial and endoplasmic reticulum (ER) labeling, whereas BODIPY TR ceramide was used for Golgi staining. Immunofluorescence labeling with the monoclonal anti-BM88 antibody in combination with the two dyes for mitochondria and the ER, followed by confocal microscopic analysis, showed clear co-localization with BM88, whereas minimal levels of co-localization were observed with the Golgi dye upon double labeling of N2A-BM88 cells (Fig. 1, H–J). These results confirmed that transgene BM88 is targeted properly within the N2A-BM88 cells.

**BM88 Overexpression in N2A-BM88 Cells Results in Acquisition of a Differentiated Neuronal Phenotype**—In a previous report we showed that BM88 overexpression in N2A cells induces a marked morphological differentiation manifested by enhanced process outgrowth in the absence of external stimuli (26) (Fig. 1, A and B), and we hypothesized that BM88 is involved in neuronal differentiation. The experiments presented here were aimed at testing this hypothesis directly. To explore whether the alterations observed in the shape of the BM88-transfected cells were suggestive of a differentiation process leading to acquisition of a neuronal phenotype, we investigated by immunocytochemistry the expression of βIII-tubulin, which is a characteristic cytoskeletal marker of differentiated neurons. Interestingly, we observed a marked increase in βIII-tubulin expression in the N2A-BM88 cells (Fig. 1K) as compared with controls, which were devoid of βIII-tubulin expression (not shown). Furthermore, we stained the N2A and N2A-BM88 cells with the SMI 311 antibody, directed at the nonphosphorylated epitopes of high molecular weight neurofilament proteins, as an additional marker of neuronal differentiation. In agreement, SMI 311 labeling was significantly induced in the BM88-transfected cells as opposed to N2A controls (Fig. 2, A and B), further confirming the enhanced differentiation state of the BM88-transfected cells toward a neuronal phenotype. We next assessed synaptic vesicle formation in N2A-BM88 cells by exploring the expression of the synaptic vesicle marker synaptophysin. A marked induction of this protein was noted both by Western blotting (Fig. 1D) and immunofluorescence labeling of N2A-BM88 cells (Fig. 1G), whereas N2A controls did not contain any detectable synaptophysin levels (Fig. 1, D and E). Equal protein loading was verified in all cases by FastRed protein staining of the blotted fractions before the immunchemical reaction (not shown) and also by immunochromedetection of α-tubulin, which is constitutively expressed in N2A-BM88 and control cells with no apparent alterations (see Fig. 4A below). These data collectively suggest that BM88 promotes neuronal differentiation in N2A-BM88 cells, possibly through cytoskeletal rearrangements but also through the synthesis of synaptic vesicle proteins.

We have previously observed that N2A-BM88 cells are more responsive to β-fibroblast growth factor than control N2A cells (39). This prompted us to check the expression of FGF receptor (FGF-R) transcripts and protein levels in these cells. Our results demonstrate a 2-fold increase of FGF-R mRNA in N2A-BM88 cells when compared with controls, as revealed by real time RT-PCR (Fig. 2F). In addition, a prominent up-regulation of FGF-R protein was noted in N2A-BM88 and control cells with no apparent alterations (see Fig. 4A below). These data collectively suggest that BM88 promotes neuronal differentiation in N2A-BM88 cells, possibly through cytoskeletal rearrangements but also through the synthesis of synaptic vesicle proteins.
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(Fig. 2, G and H). Taken together our data illustrate that the morphological differentiation of N2A-BM88 cells is accompanied by a molecular phenotype characteristic of differentiated neurons.

Effect of BM88 on Cell Proliferation—Cell cycle progression and differentiation are opposing but tightly linked events. In particular, cell cycle G1 exit is a critical stage where cells decide either to re-enter the next cell cycle or differentiate. We therefore proceeded to carefully analyze cell proliferation in N2A-BM88 and control cells by a number of different approaches. First, we performed crystal violet growth assays in N2A-BM88 and control cells after 1–3 days in vitro. These indicated that BM88 overexpression results in at least a 50% reduction in the total cell number of N2A-BM88 transfectants at all time points tested as compared with control cells (Fig. 3A). To exclude the possibility that the fewer cells counted are because of an apoptotic effect of BM88 on N2A cells, we performed TUNEL assays in N2A-BM88 and control cells. We did not detect any TUNEL-positive cells in either population after 1–3 days in vitro, thereby demonstrating that BM88 does not induce apoptotic cell death in N2A cells. Furthermore, cell viability was equally high in N2A-BM88 and control cells as estimated by trypan blue exclusion demonstrating the absence of noticeable cell death.

Next we asked whether the smaller number of N2A-BM88 cells counted after crystal violet staining is due to a smaller percentage of cells undergoing mitosis or to an elongation of the G1 phase of the cell cycle. To this end, we introduced a number of different cell cycle measurements, including pulse incorporation of the S phase marker BrdUrd, BrdUrd cumulative labeling, and finally, analysis of the FACS profiles of N2A versus N2A-BM88 cells (Fig. 3, B–E). Parallel BrdUrd pulse labeling of BM88-transfected and control cells was performed by a single 4-h BrdUrd pulse in cells maintained for 4 days in vitro, followed by fixation. It was thus revealed that there was a 31.8 ± 7.2% decrease in the percentage of BrdUrd-positive N2A-BM88 cells entering the S phase of the cell cycle (46.0 ± 6.8% of the total cell population was BrdUrd-positive in N2A-BM88 cells versus 67.3 ± 10.6% in wild type N2A and 67.8 ± 8.2% in vector (pcNeo)-transfected N2A cells (Fig. 3, B and D)). This statistically significant reduction (p < 0.01) in the proportion of S phase cells indicates that, at least in part, the lower number of BrdUrd-positive N2A-BM88 cells as compared with controls, is because of a smaller percentage of cells re-entering the cell cycle. In addition, analysis of the cell cycle kinetics of N2A and N2A-BM88 cells by introducing the BrdUrd cumulative labeling technique over a period of 18 h, thus allowing the whole proliferative cell population to progress through the cell cycle, demonstrated that BM88 overexpression had a dual effect: (a) It decreased the growth fraction, i.e. the percentage of the whole cycling population, from 90% in N2A cells to 70% in N2A-BM88 cells. (b) It also prolonged the duration of the cell cycle as N2A-BM88 cells, by contrast to N2A, did not reach a plateau after 18 h of BrdUrd labeling (Fig. 3C). Finally, a profound effect on the distribution of the cell cycle profiles after BM88 overexpression was observed by FACS analysis of N2A and N2A-BM88 cells after 2, 4, and 6 days in culture (Fig. 3E). In both stably transfected clones, C3C1 and C7G5, and at all time points analyzed, BM88 overexpression significantly increased the percentage of cells in G1/G0 phase, whereas after 6 days in vitro a significant decrease in the proportion of cells lying in the S phase was also evident. Thus, BM88 overexpression in N2A cells induces a significant increase of the cell population in the G1/G0 phase, caused by G1 phase prolongation and induction of G0 cell cycle exit.

Effect of BM88 on Cell Cycle Regulatory Proteins: the p53 Pathway—To get an insight into the anti-proliferative signaling pathway activated by BM88, we initially investigated by Western blotting the endogenous expression levels of the tumor suppressor/cell cycle regulatory protein p53 in N2A-BM88 and N2A cells. Overexpression of BM88 resulted in increased levels of p53 protein in N2A-BM88 cells as detected by using a polyclonal anti-p53 antibody (Fig. 4A). To distinguish between p53 transcriptional activation or protein stabilization by BM88, we performed real time RT-PCR quantification of p53 mRNA in N2A and N2A-BM88 cells. In accordance to what is reported in the literature (41–43), BM88 overexpression did not result in an increase of p53 mRNA levels (data not shown) but rather induced p53 elevation through protein stabilization. To check if increased p53 levels result in activation of its known downstream growth arrest effector, the p21 promoter, we transiently transfected N2A-BM88 and control cells with the pWWP-luc construct, in which expression of the reporter gene luciferase is driven by the human p21 WAF promoter (44). A small but consistent 1.5-fold activation of the p21 promoter was observed in N2A-BM88 cells when compared with controls.

To ensure that the p53 pathway is operative in N2A and N2A-BM88 cells, we monitored p53 protein after inducing genotoxic stress in these as well as in NIH 3T3 cells known to retain an intact p53 pathway (Fig. 4, B–G). The response to genotoxic stimuli is a well studied mechanism of activation of the p53 pathway via functional p53 protein stabilization and nuclear accumulation leading to transcriptional activation of a number of downstream effector genes, including p21 and Bax which, in turn, drive the cells toward cell cycle exit and/or apoptosis, a decision that depends upon cellular context and/or environment (45). Unstressed N2A, similar to NIH 3T3 cells, express minimal levels of p53 (Fig. 4, A and F), whereas they respond to the genotoxic agent doxorubicin by raising p53 protein, which translocates to the nucleus (Fig. 4, C and G). N2A-BM88 cells, besides expressing higher basal levels of p53 protein as compared with N2A (Fig. 4, D versus B), also respond to doxorubicin by elevating even more p53, thus revealing a synergistic effect between BM88 and doxorubicin (Fig. 4E). Quantification of the fluorescence intensity revealed a 13-fold increase of p53 immunoreactivity in N2A-BM88 cells as compared with N2A, which is very similar to the observed 16-fold increase of p53 in N2A cells after treatment with doxorubicin. Finally, after genotoxic treatment of N2A-BM88 cells p53 reaches a 23-fold increase when compared with N2A (Fig. 4E). These data demonstrate that both N2A and N2A-BM88 cells retain their ability to respond to genotoxic stress by raising nuclear p53, suggesting operation in these cells of an intact p53 signaling pathway.

To further rule out the possibility that selection for mutant p53 in the cells overexpressing BM88 might have occurred, we
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A) Cell Number (x 1000)

B) % of BrdU stained N2A cells

C) % of BrdU cells

D) BM88

E) 2 days
G1+G0: 38.60
G2+M: 48.93
S: 14.44

4 days
G1+G0: 43.80
G2+M: 19.97
S: 36.23

6 days
G1+G0: 45.51
G2+M: 19.62
S: 34.87

N2A CTL
N2A-BM88 C3C1
N2A-BM88 C7G5
investigated the ability of BM88 to trigger the p53 pathway in a non-neural cell line, the NIH 3T3 cells, which have been extensively used for cell cycle studies and do not express endogenous BM88. Double immunofluorescence labeling of NIH 3T3 cells transiently transfected with the BM88 cDNA, revealed that BM88 expression is sufficient to induce p53 up-regulation and, in many cases, nuclear accumulation (Fig. 4, H and I), in a fashion comparable to p53 up-regulation caused by genotoxic stimulation of NIH 3T3 cells (Fig. 4G). Accordingly, BM88 expression resulted in elevated levels of the p53 downstream targets p21 and Bax, which, like p53 (Fig. 4F), are minimally expressed in nontransfected NIH 3T3 cells (Fig. 4, J–N). The above data demonstrate that BM88 is capable of activating the p53 signaling pathway even in a cell line of non-neural origin. However, in this case and contrary to what happens in N2A-BM88 cells (data not shown), we observed an induction of the pro-apoptotic protein Bax by BM88. This suggests that the action of BM88 is context-dependent and may lead to apoptosis in the absence of the appropriate cellular machinery driving N2A cells toward neuronal differentiation.

Effect of BM88 on Cell Cycle Regulatory Proteins: Cyclins and pRb—Next we examined the basal expression levels of select cell cycle related proteins (schematically represented in Fig. 5A). Expression data for members of the cyclin proteins in N2A-BM88 and N2A control cells are shown in Fig. 5B. There was a clear cut decrease in the expression of both cyclin D1 mRNA (50% decrease by real time RT-PCR measurements; data not shown) and protein (Fig. 5B) and a modest to moderate decrease in the expression of cyclin B1 (Fig. 5B), which through their interaction with specific cyclin-dependent kinases act as key regulators of cell cycle progression at distinct cell cycle transition points, as illustrated in Fig. 5A. The decrease in cyclins D1 and B1 was dose-dependent because higher bm88 transgene expression in clone C7G5 resulted in more efficient down-regulation of the two cyclins (Fig. 5B). No changes in the expression levels or the cellular distribution of cyclin E were observed, either by Western blotting (Fig. 5B) or immunofluorescence labeling (Fig. 5, H and I) between N2A-BM88 and N2A control cells.

It has been reported that cyclin D1 becomes predominantly cytoplasmic as primary progenitor cells, in which cyclin D1 is found in the nucleus, undergo cell cycle withdrawal and enter into a neuronal differentiation program (12). Thus it appears that nuclear cyclin D1 is associated with proliferation and cytoplasmic cyclin D1 with differentiation events. This prompted us to investigate the localization of cyclin D1 in N2A-BM88 and control cells (Fig. 5, C–G). First we estimated the percentage of cyclin D1-positive cells and found that cyclin D1 was expressed in a higher percentage of control (78 ± 5.1%, n = 3) than N2A-BM88 cells (57 ± 6%, n = 3). Of particular interest, we observed that concomitant to its down-regulation (Fig. 5D, open arrows), cyclin D1 was also localized in the cytoplasm of approximately half (46.0 ± 5.2%, n = 4) of the cyclin D1-positive N2A-BM88 cells (Fig. 5, D, white arrowheads; E, thick white arrow; and F and G, open arrowheads), thus further decreasing the N2A-BM88 cell fraction in which cyclin D1 is actively engaged in proliferation events. In some cases, the localization of cyclin D1 was primarily cytoplasmic (Fig. 5, F and G, open arrowheads), resembling its localization in post-mitotic cortical neurons (12). This was in contrast to its exclusive nuclear localization in control cells (Fig. 5, C and D, thin arrows). These findings indicate that, similar to what happens in post-mitotic neurons (12, 13), nuclear accumulation of cyclin D1, where it normally lies in its active form, is partially impaired in N2A-BM88 cells. This observation further supports the enhanced differentiation state of N2A-BM88 cells as compared with controls.

Cyclin D1 and related cyclin-dependent kinases 4 and 6 promote G0 to S-phase progression through phosphorylation of the retinoblastoma protein pRb, which constitutes an additional key regulator of the cell cycle. pRb phosphorylation/dephosphorylation determines whether a cell will re-enter the next cell cycle or, alternatively, whether it will withdraw from the cell cycle at a checkpoint in late G1 (the G1 restriction point) and remain quiescent. For post-mitotic neurons, the G1/G0 transition leading to cell cycle exit is an irreversible event because neuronal cells do not re-enter the cell cycle after a quiescent period. To check whether BM88 expression is associated with the growth-control activity of pRb, we co-expressed porcine BM88 and human pRb in NIH 3T3 cells. Co-expression was achieved either by transient co-transfection of BM88 and pRb cDNAs or by adenoviral gene transfer for BM88 using a pQBI-IREs-GFP-BM88 recombinant adenovirus followed by transfection with pRb cDNA (Fig. 5F). When pRb was expressed alone in NIH 3T3 cells, both the hypo- and hyperphosphorylated form of the protein were evident, whereas in the presence of cyclin D1 there was an apparent shift toward the hyperphosphorylated form of pRb (Fig. 5F). In contrast, co-expression of pRb and BM88 in NIH 3T3 cells resulted in a significant decrease of the hyperphosphorylated form of pRb and accumulation of its hypophosphorylated, growth inhibitory form, which is associated with cell cycle exit (Fig. 5F). As an additional control to check the fidelity of the system, we performed co-expression of pRb with PC3 protein, which is known to act as an inhibitor of cyclin D1 transcription and thus prevents pRb phosphorylation (31). Indeed, PC3 overexpression also resulted in inhibition of pRb phosphorylation (Fig. 5F).
Taken together, the above findings demonstrate that BM88 exerts its anti-proliferative effect by triggering p53 up-regulation and nuclear accumulation accompanied by cyclin D1 down-regulation and impaired nuclear localization, which in turn prevents pRb phosphorylation.

BM88 Knockdown Affects the Proliferative Capacity of N2A Cells and Impairs RA-induced Neuronal Differentiation—To figure out whether BM88 is not only sufficient but also necessary for cell cycle exit and neuronal differentiation, we performed RNA interference experiments to silence BM88 expression in N2A cells. First, different siRNAs were tested for their ability to down-regulate BM88 in NIH 3T3 cells after transient co-transfection with a BM88 expression vector. The siRNA with the highest efficiency down-regulated transgene BM88 expression in NIH 3T3 cells to non-detectable levels, as determined by immunoblotting (Fig. 6B). At the same time, actin levels, which were used as a measure of equal protein loading, remained unaffected. When the siRNA with the highest efficiency was used in N2A cells, it also reduced endogenous BM88 expression to almost nondetectable levels as judged by immunofluorescence labeling (Fig. 6A). In agreement, real-time RT-PCR analysis revealed an 87.2% reduction in BM88 mRNA levels (Fig. 6, C and D). We next examined the effect of BM88 knockdown on N2A cell proliferation. A significant enhancement of BrdUrd incorporation by 49.4% was observed 72 h after transfection with BM88-siRNA, indicative of increased proliferative capacity (Fig. 6, E and F). In addition, staining for phospho-histone H3 revealed an increased proportion of N2A cells undergoing mitosis in the BM88 knockdown cells (Fig. 6G), a finding further supported by increased mRNA levels of cyclin D1 in these cells (not shown). We then asked if BM88 silencing also influences N2A cell differentiation. N2A cells acquire a neuronal phenotype

FIGURE 4. BM88 effect on the p53 pathway. A, p53 protein expression in N2A control and the two clones, C3C1 and C7G5, of N2A-BM88 cells after 4 days in vitro. Western blot analysis of protein extracts from N2A control and N2A-BM88 cells indicates that BM88 overexpression induces the levels of the tumor suppressor protein p53. α-Tubulin was used as a quantitative marker for equal protein loading. B–E, immunofluorescence labeling for p53 protein in N2A (B and C) and stably transfected N2A-BM88 cells (D and E) without (-doxo) or with (+doxo) doxorubicin treatment. p53, which is expressed in minimal levels in untreated N2A cells (B), is up-regulated and localizes in the nucleus after doxorubicin treatment (C) or BM88 overexpression (D), whereas its levels are further increased in N2A-BM88 cells treated with doxorubicin (E). Bars in B–E, 20 μm. F–I, immunofluorescence labeling (confocal analysis) for p53 protein in NIH 3T3 cells untreated (F), following doxorubicin-induced genotoxic stress (G), or after transient transfection with BM88 cDNA (H and I). Untreated NIH 3T3 cells express basal levels of p53 (F) which is up-regulated and accumulates in the nucleus after treatment with doxorubicin (G), or after BM88 expression (H and I, open arrows). I shows merged double immunofluorescence labeling for p53 (green) and BM88 (red). J and K, merged immunofluorescence double labeling of NIH 3T3 cells, untreated (J) or transiently transfected with BM88 cDNA (K), for p21 (green) and BM88 (red). L–N, double immunofluorescence labeling (confocal analysis) of NIH 3T3 cells transiently transfected with BM88 cDNA for BM88 (green) and the p53 downstream target Bax (red). Arrows in M point to cells expressing high levels of BM88 in which Bax is up-regulated as compared with the rest of the cells in the micrograph that are BM88-negative. Bars in F–N, 20 μm.
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FIGURE 5. BM88 effect on cell cycle regulatory proteins. A, schematic diagram of the expression/activity profiles of cell cycle regulatory proteins during the different phases of the cell cycle. B, cell cycle protein expression in N2A control and the two clones, C3C1 and C7G5, of N2A-BM88 cells after 4 days in vitro. Western blot analysis of protein extracts from N2A control and N2A-BM88 cells indicates that BM88 overexpression reduces the levels of cyclins D1 and B1, which control cell cycle progression at the G1 and G2 phases of the cell cycle, respectively, although it has no effect on the levels of cyclin E. α-Tubulin was used as a quantitative marker for equal protein loading. C–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows). E–G, merged double immunofluorescence labeling of N2A control (C) and N2A-BM88 (D) cells, maintained for 4 days in vitro, using antibodies to cyclin D1 (green) and BM88 (red). Nuclear cyclin D1 localization is evident in N2A and in a fraction of N2A-BM88 cells (C and D, thin white arrows). However, in a subpopulation of N2A-BM88 cells, cyclin D1 is also localized in the cytoplasm (D, white arrowheads), whereas it is completely down-regulated in a high percentage of differentiated N2A-BM88 cells (D, wide open arrows).

DISCUSSION

During neural development, different types of neurons and glia are generated from progenitors at specific times at a sequential order allowing first for neurogenesis and then for gliogenesis. This process is to a great extent coordinated by the control of cell cycle progression and the precise timing of cell cycle exit (46, 47). Recent characterization of dual function molecules, such as the cyclin-dependent kinase inhibitor p27Kip1 or its Xenopus homologue.
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FIGURE 6. BM88 knockdown in N2A cells induces cell proliferation. A, double labeling for BM88 (top) and DAPI-stained cell nuclei (bottom) showing that siRNA targeting BM88 efficiently down-regulates endogenous BM88 expression in N2A cells, whereas siRNA control has no effect. B, immunoblot of protein extracts from NIH 3T3 cells showing the gene silencing efficiency of two different sets of siRNA targeting BM88 (siRNA-BM88 #3 and #4), when co-transfected with a BM88 expression vector in the NIH 3T3 cells. siRNA-BM88 #4 was more efficient in BM88 silencing and was used in all other experiments presented here. C and D, quantification of BM88 down-regulation in the mRNA level by real time RT-PCR. The BM88 transcripts were normalized with simultaneous quantification of GAPDH transcripts. Representative curves from three independent experiments (for both siRNA-Control and siRNA-BM88) are shown for GAPDH.

siRNA-Control experiments, whereas the three shifted on the right represent siRNA-BM88 experiments, indicative of BM88 down-regulation. Quantification (% of BM88 mRNA levels between siRNA-control and siRNA-BM88 treated cells is shown in the column graph in D, taking as 100% the expression levels of siRNA-control-treated cells (relative mRNA levels: siCTRL, 100% ± 8.3, and siBM88 12.8 ± 5.2%, p < 0.001, n = 3). E and F, BM88 knockdown in N2A cells enhances proliferation. Proliferation was measured 3 days after siRNA transfections by following BrdUrd incorporation after a single 3-h pulse (drawing at top of E). E, siRNA-BM88-transfected cells incorporate higher levels of BrdUrd as demonstrated by immunofluorescence labeling for BrdUrd (bottom) in combination with DAPI nuclei staining (bottom), whereas quantification of the data is given in F. BrdUrd incorporation (%) is as follows: siBM88, 61.8 ± 1.6%; siCTRL, 41.4 ± 3.0%, p < 0.001, n = 5). G, siRNA-BM88-transfected cells show increased numbers of phosphorylated histone H3 (pH3)-positive cells compared with siRNA-CTRL-treated cells. Phosphorylated histone H3-positive cells undergoing mitosis were identified by immunofluorescence labeling. Bars, 80 μm.

p27Xic1 (48), Geminin (49–51), the neural proliferation and differentiation control protein NPDC-1 (52), or the Hu family of neuronal RNA-binding proteins (53), has yielded intriguing insights into the functional link between the control of cell cycle progression and neuronal commitment/differentiation. Observations presented in this study imply that BM88 is a novel cell-intrinsic factor involved bi-functionally in mechanisms of cell cycle exit and induction of neuronal differentiation. In particular, BM88 knockdown by means of RNA interference indicate that BM88 is an essential component of the molecular machinery controlling cell cycle exit and differentiation in N2A cells.

BM88 Exerts Its Anti-proliferative Effect through Elongation of G1/S Progression and/or Arrest—A key cell cycle checkpoint, namely the G1 restriction point, is located at the end of G1 phase. If cells pass this point, they will almost invariably complete the cell cycle, otherwise they will become post-mitotic. BM88 overexpression in N2A cells exerts a prominent anti-proliferative effect as judged by different experimental approaches involving cell growth assays, BrdUrd incorporation, and DNA content estimation through FACS analysis. This effect may be due either to a smaller population of N2A cells undergoing mitosis or to cell cycle elongation of the BM88-transformed cells as compared with controls. Our data indicate that the BM88 anti-proliferative effect is due both to cell cycle elongation, in particular at the G1/S progression, and an arrest at the G1 phase of the cell cycle. The latter is corroborated by the fact that upon Bm88 gene silencing via RNA interference, more cells seem to undergo mitosis (Fig. 5). These findings point to a role for BM88 in controlling cell cycle exit, besides its previously indicated involvement in neuronal differentiation pathways (see Refs. 24, 26, 28, and this study). In these lines increasing evidence suggests that although select G1 phase components affect cell fate determination at this stage, the opposite is also true. Thus a number of neuronal determinants affect cells lying at G1, allowing them to take the G0 branch (54–56). For example, overexpression of the proneural genes NeuroD, Mash 1, and neurogenin 1 induces P19 embryocarcinoma cells to differentiate toward a neuronal phenotype through a mechanism that involves expression of the cyclin D1 inhibitor p27kip1 and subsequent cell cycle arrest at G1 (57). In addition, external neuronal determination signals, such as Wnt, sonic hedgehog, and retinoic acid, have been shown to regulate cell cycle exit through modulation of cyclin D1, cyclin D2, and N-myc transcription (58–61). In agreement, BM88 appears to promote cell cycle exit by affecting cyclin expression. An inverse relationship between BM88 and cyclin D1 has also been noted by another group (38) that demonstrated that suppression of cyclin D1 with antisense oligonucleotides in proliferating LAN5 neuroblastoma cells results in prominent BM88 up-regulation.

BM88 Triggers Cell Cycle Exit through the p53/Cyclin D1/pRb Signaling Pathway—The G1 phase of the cell cycle and more specifically the G1/G0 transition at the G0 restriction point, located at the end of G1 phase, is the major route through which cells exit from the cell cycle and become post-mitotic (62, 63). The most well known pathway of G1 checkpoint cell cycle control involves the anti-proliferative protein p53, and an increasing number of molecules have been shown to be involved in this signaling pathway in different systems both during normal development and in carcinogenesis (31, 64–66). Therefore, in order to investigate the mechanism by which BM88 triggers cell cycle exit, we sought to explore its participation in the p53 pathway of growth inhibition. Having a short half-life, p53 is normally maintained in low levels in unstimulated mammalian cells by continuous ubiquitination and subsequent degradation (67). When the cell is confronted with stress or another cell cycle arrest signal, p53 ubiquitination is suppressed and p53 protein becomes stabilized, accumulates in the nucleus, and activates or represses the transcription of specific target genes (68). However, as p53 is one of the most frequently mutated tumor suppressor genes in human cancers (69), resulting in loss of its nuclear accumulation and hence binding to its targets (70), we sought to ensure that p53 in our system is not mutated, and the p53 signaling pathway is active. For this reason, first we stimulated p53 induction by the chemotherapeutic agent doxorubicin (71) and compared it with the p53 increase caused by BM88 in stably transfected N2A-BM88 cells; second, we assessed the activation of the p53-mediated signaling pathway by transient BM88 expression in a cell line of non-neural origin. In both cases we noticed that BM88 is sufficient to induce up-regulation and nuclear accumulation of p53, comparable with that caused by genotoxic stress, as well as activation of p53 downstream mediators, thus providing direct evidence that p53 is not mutated in our system.

In the p53 pathway, activation by growth arrest stimuli of functional p53 or related protein family members, such as p63 and p73, results in induction of a number of transcription factors, including p21cip1/waf1, p27kip1, and PC3/tis21, which in turn inhibit cyclin/cedk action (6, 72–74). Cyclin-dependent kinases are responsible for pRb phosphorylation leading to cell cycle arrest via the p53 pathway (58–61). In agreement, BM88 appears to promote cell cycle exit by affecting cyclin expression. An
cycle progression and thus inhibition of their activity results in G1 arrest (75, 76). pRb is therefore a key molecule responsible for growth arrest and accumulation of cells in G1, in response to anti-proliferative signals. Therefore, the observed pRb de-phosphorylation caused by BM88 points to an important role of this molecule on cell cycle arrest through the p53-pRb signaling pathway. Increasing evidence suggests that transcription factors of the Sp1 family are critical for the cellular responses to p53, including activation of its downstream growth-arrest effector p21WAF/Cip1, both in neuronal (77, 78) and non-neuronal cells (44). In this context it is interesting to consider that the recently characterized BM88 promoter is also Sp1-dependent (28).

The expression as well as the intracellular localization of D-type cyclins in particular, which play a predominant role in cell cycle progression via pRb phosphorylation, is heavily dependent on the cell cycle phase (11, 79). Several studies have indicated that a critical event associated with cell cycle withdrawal and differentiation both in neuronal and non-neuronal cells is the cellular compartmentalization of cyclin D1, which shifts from a predominantly nuclear localization to cytoplasmic sequestration. In particular, Sumrejkanchanakij et al. (12) have shown that cyclin D1 becomes predominantly cytoplasmic as primary cortical progenitor cells undergo cell cycle withdrawal and terminal differentiation. In the same study it was also shown that exogenously expressed cyclin D1 sequesters in the cytoplasm of post-mitotic neurons by a mechanism inhibiting its nuclear import, although it efficiently enters the nucleus of proliferating progenitor cells. Furthermore, forced cyclin D1 expression in the nucleus of differentiated neurons resulted in apoptotic induction. In accordance, we found that BM88 reduces cyclin expression, most prominently that of cyclin D1, and even more importantly results in cyclin D1 accumulation in the cytoplasm of a large percentage of N2A cells. In addition, we found no evidence of BM88-evoked apoptosis in N2A cells. These data suggest that BM88 may form part of an important cyclin D1-related pathway, which is associated with cell cycle exit of neuronal progenitors and their survival and differentiation to post-mitotic neurons.

As noted, p53 activation, cyclin D1 inactivation, as well as pRb hypophosphorylation is a general pathway operating upon cell cycle exit both in neuronal and non-neuronal cells. In this context it is important to note that BM88 is able to exert its anti-proliferative action through pRb hypophosphorylation in a non-neural cell line, namely NIH 3T3 cells (Fig. 5) which under the influence of BM88 also up-regulate pro-apoptotic proteins (Fig. 4, L and M). This points to a possible tumor suppressor role for this protein and suggests that abrogation of the machinery involving BM88 may contribute to neuronal tumorigenesis. In these lines it is interesting to consider that the human BM88 gene is localized in a chromosomal region associated with overgrowth of genetic disorders and carcinogenesis (27).

BM88 Couples Cell Cycle Exit with Neuronal Differentiation—Gain- and loss-of-function approaches implemented in this study indicate that, concomitant to its anti-proliferative function, BM88 promotes differentiation toward a neuronal phenotype. Thus BM88 overexpression drives N2A cells to differentiate toward the neuronal pathway, whereas BM88 knockdown
BM88 Couples Cell Cycle Exit and Neuronal Differentiation

How does BM88 exert its function? As shown previously by electron microscopy (25) and here by vital dye staining, BM88 is localized at the limiting membrane of intracellular organelles, such as the mitochondria and ER. In this respect as well as in terms of its neurite outgrowth-promoting activity, BM88 resembles the anti-apoptotic protein Bcl-2 (81). In fact, we have recently observed that like Bcl-2 (82), BM88 also protects cells from C2-ceramide induced apoptosis. It will be interesting to determine whether BM88 functions via a mechanism involving calcium dynamics, as is the case with Bcl-2 (82), especially in view of emerging evidence suggesting calcium as a key regulator of proliferation/differentiation events in neuronal progenitors (83).

BM88 Is Likely to Be Involved in Terminal Neuron-generating Divisions—Another important observation that emerged from our previous in vivo study is that BM88 is associated with the dynamics of differentiative neuron-generating divisions (24). This was demonstrated by defining in real time a cohort of proliferative progenitors that exit S phase in synchrony and following their fate for 24 h. During embryonic development the progression of progenitor cells from a symmetric/proliferative pattern toward an asymmetric/neuron-generating pattern of cell divisions marks the onset of neurogenesis (84). Interestingly, recent findings suggest that lengthening of the G1 phase of the cell cycle is responsible for the onset of asymmetric/differentiative final divisions of cortical progenitors (85). These observations further suggest the involvement of cell cycle mediators in coupling the mode of progenitor cell division with differentiation. Our combined cell cycle measurement data reported here showing G1 elongation upon BM88 overexpression in N2A cells suggest an active involvement of BM88 in asymmetric/neuron-generating divisions besides cell cycle control. This scenario is further supported by our in vivo data correlating BM88 expression predominantly with the final asymmetric/neuron-generating divisions of cortical progenitors during the mid-embryonic period (24). Taken together, the above findings indicate that BM88 acts at the stage of final neuron-generating cell divisions and participates in the activation of a set of neuron differentiation genes. Experiments to identify direct BM88-interacting partners during neurogenesis are in progress in our laboratory.

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