RB and RB2/p130 genes demonstrate both specific and overlapping functions during the early steps of in vitro neural differentiation of marrow stromal stem cells

FP Jori[1], MAB Melone[1], MA Napolitano[2], M Cipollaro[2], A Cascino[2], A Giordano[3] and U Galderisi*,[2,3]

[1] Department of Neurological Sciences, Second University of Naples, Naples, Italy
[2] Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Excellence Research Center for Cardiovascular Diseases, Second University of Naples, Naples, Italy
[3] Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, USA.
* Corresponding author: U Galderisi, Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Via Costantinopoli 16, Napoli 80138, Italy. Tel: +39-0815665886; Fax: +39-0815667547; E-mail: umberto.galderisi@unina2.it

Abstract

Marrow stromal stem cells (MSCs) are stem-like cells that are currently being tested for their potential use in cell therapy for a number of human diseases. MSCs can differentiate into both mesenchymal and nonmesenchymal lineages. In fact, in addition to bone, cartilage and fat, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes. RB and RB2/p130 genes are involved in the differentiation of several systems. For this reason, we evaluated the role of RB and RB2/p130 in the differentiation and apoptosis of MSCs under experimental conditions that allow for MSC differentiation toward the neuron-like phenotype. To this end, we ectopically expressed either RB or RB2/p130 and monitored proliferation, differentiation and apoptosis in rat primary MSC cultures induced to differentiate toward the neuron-like phenotype. Both RB and RB2/P130 decreased cell proliferation rate. In pRb-overexpressing cells, the arrest of cell growth was also observed in the presence of the HDAC-inhibitor TSA, suggesting that its antiproliferative activity does not rely upon the HDAC pathway, while the addition of TSA to pRb2/p130-overexpressing cells relieved growth inhibition. TUNEL reactions and studies on the expression of genes belonging to the Bcl-2 family showed that while RB protected differentiating MSCs from apoptosis, RB2/p130 induced an increase of apoptosis compared to controls. The effects of both RB and RB2/p130 on programmed cell death appeared to be HDAC- independent. Molecular analysis of neural differentiation markers and immunocytochemistry revealed that RB2/p130 contributes mainly to the induction of generic neural properties and RB triggers cholinergic differentiation. Moreover, the differentiation potentials of RB2/

p130 and RB appear to rely, at least in part, on the activity of HDACs.

Introduction

In addition to hematopoietic stem cells, bone marrow contains cells called marrow stromal stem cells (MSCs) that meet the criteria for stem cells of nonhematopoietic tissues. These stem cells are currently referred to as either mesenchymal stem cells, because of their ability to differentiate into mesenchymal cells (such as bone and cartilage cells, adipocytes), or marrow stromal cells, because they appear to stem from the complex array of supporting structures found in marrow.[1–3] MSCs have shown to possess great somatic plasticity since they can differentiate into nonmesenchymal lineages. In fact, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes in vitro and in vivo. MSCs are of interest because they are easily isolated from a small aspirate of bone marrow and can be expanded through as many as 50 population doublings in about 10 weeks.[3] For these reasons, the cells are currently being tested for their potential use in cell and gene therapy for a number of human diseases, such as neurological pathologies. This possibility is exciting but is far from a reality, as data on somatic plasticity are very scant and more in-depth studies are required to study the molecular pathways regulating MSC proliferation, differentiation and apoptosis. Our research was devoted to investigating the role of RB and RB2/p130 genes in MSC biology.

The retinoblastoma (Rb) family genes, RB, RB2/p130 and p107, play a major role in controlling the cell cycle. This function is mainly accomplished by the differential binding of RB family proteins to the members of the E2F family of nuclear factors, which in turn regulate the transcription of several genes involved in DNA synthesis and cell cycle progression.[4,5] Although some data indicate that pRb, pRb2/p130 and p107 are able to compensate each other, their specific binding properties suggest that each Rb family protein plays a distinct role in cell cycle regulation, depending on the cell maturation state and type.[6–10]

The Rb gene family members are also involved in the onset of differentiation in a wide variety of cell types.[5,11,12] In

particular, several data indicate that pRb and pRb2/p130 play a crucial role in neurogenesis.[7,9,10,13–19]

We evaluated the contribution of *RB* and *RB2/p130* in the differentiation and apoptosis of MSCs under experimental conditions allowing for MSC differentiation toward the neuron-like phenotype. To this end, we overexpressed either *RB* or *RB2/p130* by adenovirus transduction in rat primary MSC cultures induced to differentiate, and monitored the proliferation, differentiation and apoptosis processes.

Rb family members act by binding to the transactivation domain of E2F transcription factors and directly blocking E2F activity. In addition, they can actively repress transcription by recruiting chromatin remodeling enzymes. These enzymes fall into two classes: histone deacetylases (HDACs) and the ATP-dependent SWI/SNF complex. To establish whether the role of *RB* and *RB2/p130* in MSC biology is dependent on HDAC activity, we overexpressed such genes in differentiating MSCs in the presence of Trichostatin A (TSA), which is a specific HDAC inhibitor.[20,21] The study of the relationship between the RB gene family and HDACs is intriguing, since other research groups have already demonstrated that HDAC pathways play a role in neural cell commitment and differentiation.[20,22]

Results

In vitro neural differentiation of MSCs

Although adult rat and human MSCs can normally differentiate into mesenchymal derivative, several groups have demonstrated that MSCs can be induced to differentiate into neurons.[2,3,24,28–31] We decided to investigate the process of *in vitro* neural differentiation in more detail by defining expression patterns for genes representative of 'generic' neural differentiation, as well as genes associated with specific neurotransmission. To this end, we induced neural differentiation of MSCs as reported by Woodbury *et al*,[24,31] and monitored gene expression profiles for 5 days following neural induction.

We also analyzed the growth rate and cell death during the *in vitro* neural maturation process. As expected, cell proliferation decreased following treatment with the NIM (Neural induction medium) (Figure 1a). This observation was confirmed by BrdU incorporation; in fact, as differentiation proceeded, only a residual number of cells were positive for

BrdU, which marks cells in S phase (Figure 1b). The decrease of BrdU-positive cells was due to growth arrest in G_0/G_1 phase of cell cycle as evidenced by flow cytometry analysis

d   Flow cytometry analysis

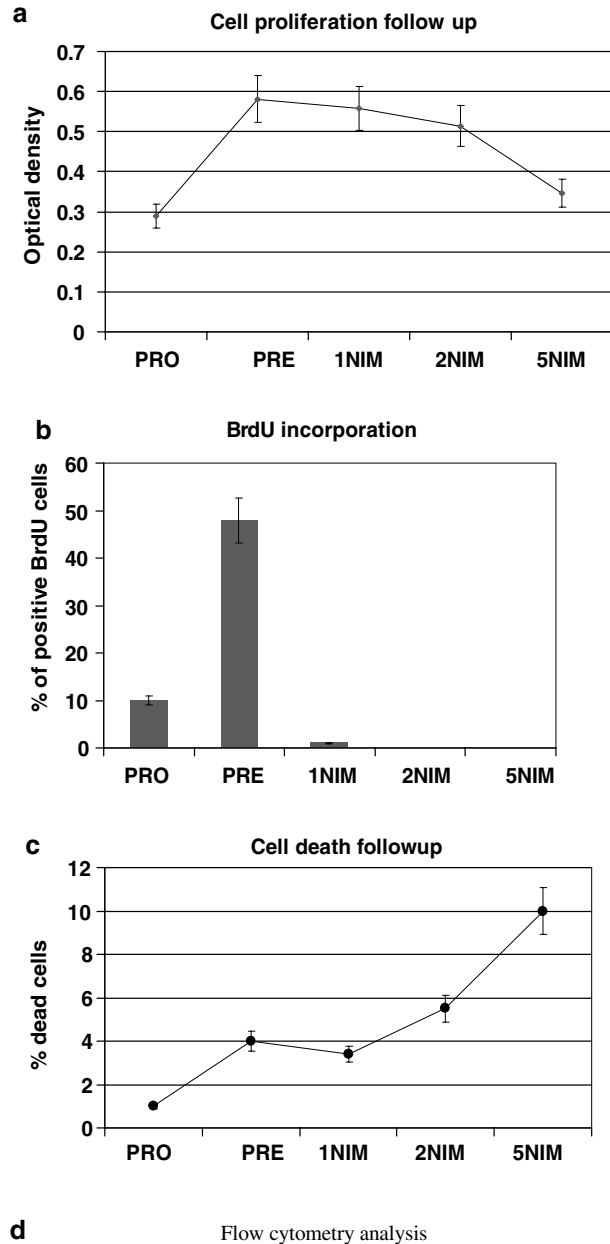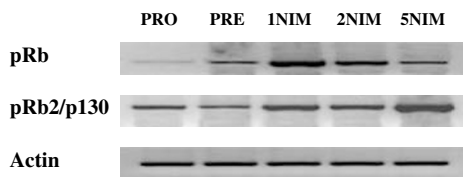|	G1	S	G2/M
PRO	*52.30*	*34.09*	*13.61*
PRE	*53.65*	*44.55*	*1.79*
1NIM	*80.88*	*10.10*	*9.02*
2NIM	*82.76*	*5.16*	*12.07*
5NIM	*82.12*	*1.08*	*16.60*

Figure 1 *In vitro* cell proliferation and cell death follow up. (**a**) Cell proliferation assay. A total of 3000 cells/well were plated in a 96-multiwell plate in PRO medium (see Materials and methods). After 24 h, cells were switched to PRE and then, after a further 24 h, cells were incubated for 1 (1NIM), 2 (2NIM) and 5 (5NIM) days in neural induction medium (NIM). Cellular proliferation was followed by spectrophotometrically measuring the dehydrogenase activity found in the cytoplasms of living cells. (**b**) BrdU incorporation assay (see Materials and methods for details). At least 2000 cells were counted in 10/12 different microscopic fields. (**c**) Cell death assay performed as described for the proliferation test, the only difference being that we measured the enzymatic activity released in culture medium by dead cells. This was normalized with respect to the total cell number. Each assay was repeated at least three times. (**d**) Flow cytometry analysis of differentiating MSCs. For each experimental condition the percentage of live cells in the different phases of cell cycle is shown

(Figure 1d). Cell growth was arrested at 1 day of NIM treatment and the cell number decreased after 5 days of incubation in NIM. The cell death profile was in agreement with cell proliferation. In fact, a cytotoxicity assay showed that in the proliferating medium (PRO) the percentage of dead cells was lower than 3%. The number of dead cells increased during the first days of differentiation and reached a peak at 5 days of NIM treatment (Figure 1c). Flow cytometry analysis suggested that the loss of cells during differentiation treatment was due to apoptosis, since the percentages of subdiploid cells (apoptotic cells) overlapped those observed in cytotoxic assays (data not shown). Generally speaking, neuron differentiation is associated with cell death. For this reason, it could be argued that MSCs progressively matured during treatment with NIM. However, at 5 days of NIM treatment, we observed the loss of some key features of differentiating neurons (see below) along with a high cell death rate. These phenomena suggest that NIM, at least under our experimental conditions, did not allow long-term neuron survival.

We subsequently analyzed the expression profile of Rb and Rb2/p130 during *in vitro* neural differentiation of MSCs. Both proteins showed a dramatic increase after 1 day incubation in NIM, which is in agreement with the onset of differentiation and the drop of proliferation rate observed. As the incubation time in NIM increased, pRb displayed a progressive reduction in expression, while pRb2/p130 showed a huge accumulation (Figure 2).

Basic helix–loop–helix (bHLH) proteins are transcription factors involved in cell fate determination and differentiation in several cell lineages. In particular, some bHLH proteins act as determination and/or differentiation factors of neuroepithelial cells.[32,33] In contrast, the Id proteins, such as Id2, inhibit cell determination and differentiation by interacting with bHLH proteins. Id proteins possess the HLH domain but lack the basic domain which is needed for DNA binding. Therefore, by interacting with bHLH proteins, they act as dominant-negative factors.[34–36]

We decided to analyze the expression profile of Id2, which counteracts the action of bHLH proteins and at the same time can be regulated by RB proteins.[34,36,37] As expected, the levels of Id2 mRNA decreased progressively as cells began to differentiate (Figure 3). However, at 5 days in NIM Id2 mRNA expression increased again, although the mRNA levels were still lower compared to those detected in proliferating cells (Figure 3).

To begin defining *in vitro* neural differentiation, we looked at the expression of genes for the Neuron-specific enolase (NSE) and neurofilament triplet proteins *NF-H*, *NF-M* and *NF-L*, which are involved in the differentiation process of neuronal cells.[38,39] NSE expression increased progressively during differentiation, as expected (Figure 3). The expression levels of NF-M and NF-L were very low in proliferating MSCs and remained low also during the differentiation process; this did not allow us to perform a semiquantitative analysis (data not shown).

We detected NF-H mRNA in proliferating MSCs; nevertheless, its expression did not increase progressively during differentiation as expected, but instead showed a bimodal expression profile (Figure 3).

At none of the analyzed time points did we observe the expression of GFAP and PLP, chosen as markers of astrocyte and oligodendrocyte maturation.[20,40] This suggests that MSCs are not able to differentiate into glial cells under our experimental conditions. We looked at the expression of several neurotransmitter markers to verify whether during the early steps of *in vitro* differentiation MSCs acquired only a generic neuronal phenotype or whether they were committed toward specific maturation pathways. At none of the time points during the differentiation follow-up did we detect the

	PRO	PRE	1NIM	2NIM	5NIM
Id2	0.65 ± 0.04	0.45 ± 0.04	0.19 ± 0.03	0.20 ± 0.02	0.41 ± 0.04
NF-H	1.05 ± 0.12	0.45 ± 0.05	1.39 ± 0.18	0.40 ± 0.08	0.36 ± 0.04
NSE	1.28 ± 0.19	2.37 ± 0.25	4.02 ± 0.26	5.65 ± 0.28	1.23 ± 0.10
TH	1.42 ± 0.20	1.80 ± 0.16	1.32 ± 0.19	0.37 ± 0.05	0.37 ± 0.09
ACHE	n.d.	n.d.	1.79 ± 0.21	1.79 ± 0.33	1.44 ± 0.18
VIP	n.d.	n.d.	0.30 ± 0.05	0.65 ± 0.09	n.d.

Figure 3 Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes during neural differentiation of MSCs. Cells were grown in PRO before being incubated for 24 h in PRE and finally grown for 1 (1NIM), 2 (2NIM) and 5 (5NIM) days in NIM. mRNA levels were measured by a GELDOC instrument and normalized with respect to HPRT, which was chosen as an internal control. Each experiment was repeated at least three times. Variations in gene expression are given as arbitrary units

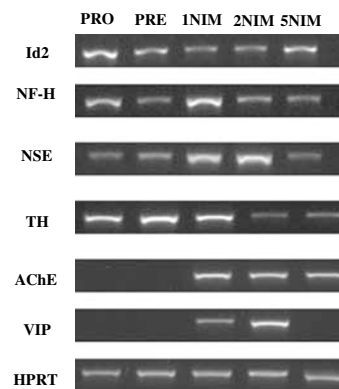|	PRO	PRE	1NIM	2NIM	5NIM
pRb	0.35 ± 0.04	0.64 ± 0.1	2.79 ± 0.28	1.64 ± 0.21	0.64 ± 0.06
pRb2/p130	2.15 ± 0.21	1.28 ± 0.15	4.55 ± 0.5	4.08 ± 0.6	7.25 ± 0.6

Figure 2 Western blot analysis of pRb and pRb2/p130 protein expression during neural differentiation of MSCs. Cells were grown in PRO before being incubated for 24 h in PRE and finally grown for 1 (1NIM), 2 (2NIM) and 5 (5NIM) days in NIM. The protein levels were measured by a GELDOC instrument and normalized with respect to β-actin, which was chosen as an internal control. Each experiment was repeated at least three times. Variations in protein expression are given as arbitrary units

expression of markers specific for dopaminergic (dopamine transporter mRNA), noradrenergic (dopamine-β-hydroxylase mRNA), serotoninergic (trypthophan hydroxylase mRNA), histaminergic (histidin decarboxylase mRNA) or GABAergic (GAD65 and GAD67 glutamic acid decarboxylase mRNAs) neurons.

On the other hand, in proliferating MSCs, we observed tyrosine hydroxylase (TH) mRNA, whose expression decreased as cells began to differentiate (Figure 3). This was associated with a progressive increase in the cholinergic marker AChe and vasoactive intestinal peptide (VIP) mRNAs (Figure 3). A combination of TH downregulation along with AChe and VIP expression is a typical feature of differentiating cholinergic neurons.[41–44]

We also performed a morphological analysis of differentiating MSCs. The molecular data were in agreement with morphological studies. We identified differentiating neurons by antineurofilament immunostaining. Cells in the PRO were negative for the expression of neurofilaments, while $28 \pm 8\%$ of differentiating cells were positive after 1 day in NIM (Figure 4a and b). These results overlapped with those regarding differentiating cholinergic neurons, which were identified by

cytochemical AChe staining (Figure 4c and d). In fact, MSCs in PRO medium were negative for AChe staining, while $26 \pm 7\%$ differentiating cells (1NIM) were strongly AChe-positive. These data suggest that MSCs differentiating into neurons acquired a cholinergic phenotype.

Adenovirus transduction caused transient increase in ectopic proteins

Given the huge increase of pRb and pRb2/p130 observed at the onset of differentiation, we decided to study whether the presence of high levels of these genes before the induction of differentiation could affect this process. We transduced proliferating MSCs with recombinant adenoviruses expressing Rb (Ad-CMV-Rb), Rb2/p130 (Ad-CMV-Rb2/p130) or with the empty vector as a control (Ad-CMV), and then we induced differentiation.

Transduction experiments were performed by making certain not to overwhelm cell machinery with nonphysiologically high and prolonged production of ectopic proteins. We chose this strategy considering that momentary increases in
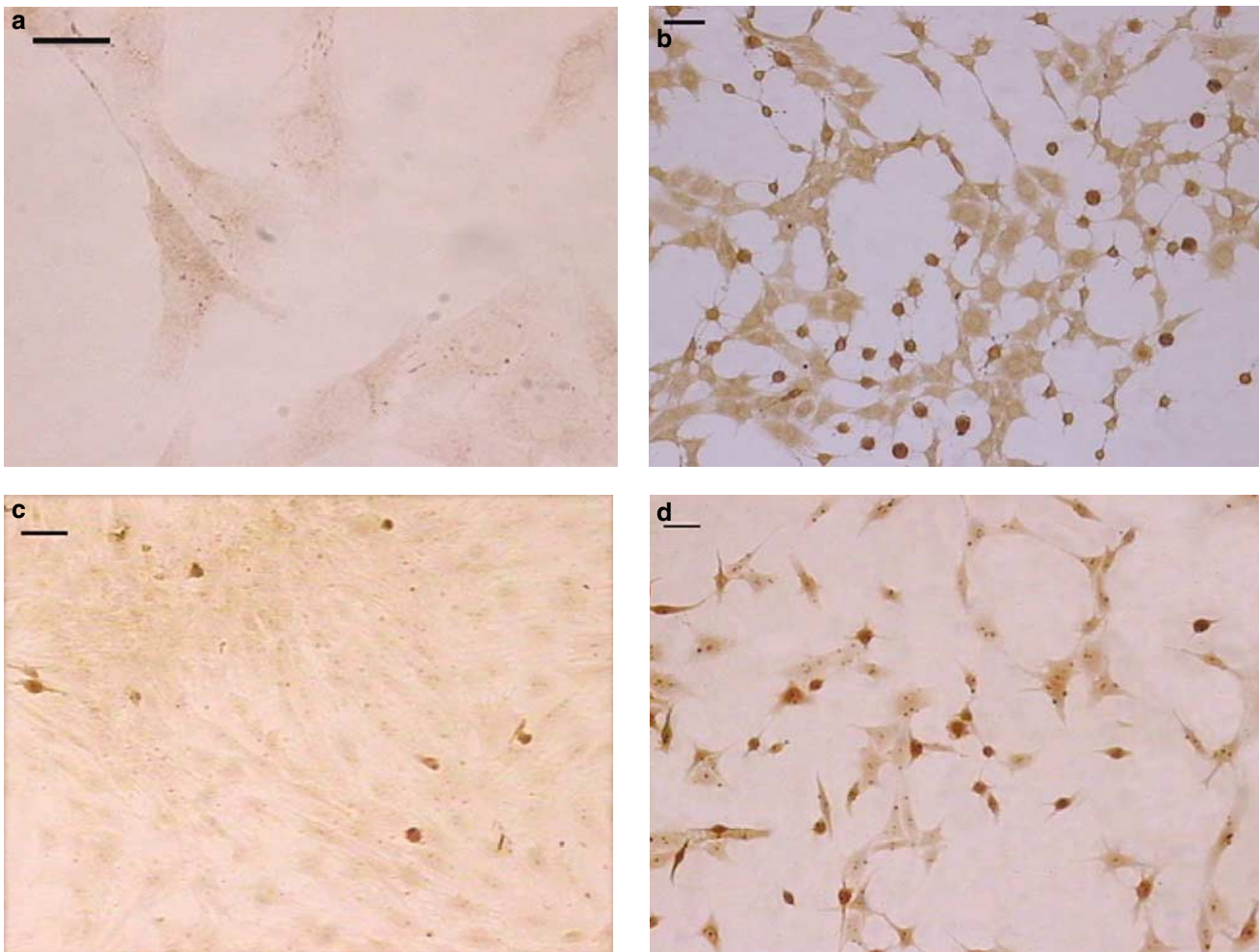

Figure 4 Morphological analysis of neural differentiation of MSCs. (**a, b**) Antineurofilament immunostaining; (**c, d**) *in situ* cytochemical detection of AChe activity. (**a, c**) cells grown in proliferative medium; (**b, d**) cells incubated 1 day in NIM. Bars in the pictures: 40 μm

the level or activity of RB family proteins greatly affect 'cell behavior'. For example, in leukocyte and adipocyte precursor cells, transient interactions of pRb with transcription factors of the C-EBP family can induce a cascade of events leading to cell differentiation.[45,46]

Western blot analysis, performed 1, 2 and 3 days after infection (PRO, PRE and 1NIM media, respectively) demonstrated that the expression of pRb2/p130 increased progressively and significantly in Ad-CMV-Rb2/p130-transduced cells compared to those infected with control viruses (Ad-CMV and Ad-CMV-β-gal) (Figure 5). On the contrary, the transduction of MSCs with Ad-CMV-Rb caused a transient increase in pRb expression at 24 h postinfection and this value fell to basal level at 48 and 72 h (Figure 5). It is worth noting that short time exposures to increased expression of such antioncogenes caused profound effects on MSC biology (see following paragraphs).

Both RB and RB2/p130 affect cell growth and apoptosis

Both RB and RB2/p130 induced a significant reduction in cell proliferation rate (data not shown). This result was in agreement with the decrease of BrdU-positive cells observed in cultures transduced with Ad-CMV-Rb and Ad-CMV-Rb2/p130 compared to controls after 1 day in NIM (Figure 6a, dark

bars). It is remarkable that the growth arrest promoted by RB is far more effective than that promoted by RB2/p130.

The effects on the apoptosis machinery were completely different. RB protected differentiating MSCs from programmed cell death, while RB2/p130 caused an increase in apoptosis compared to controls (Figure 6b, dark bars). In fact, cells transduced with Ad-CMV-Rb showed a $45\pm7\%$ decrease in the apoptotic index compared to controls, whereas cells transduced with Ad-CMV-Rb2/p130 showed a $35\pm6\%$ increase in this index (Figure 6b, dark bars).

We extended these studies by analyzing the expression of genes playing a role in apoptosis. The Bcl-2 family is the most
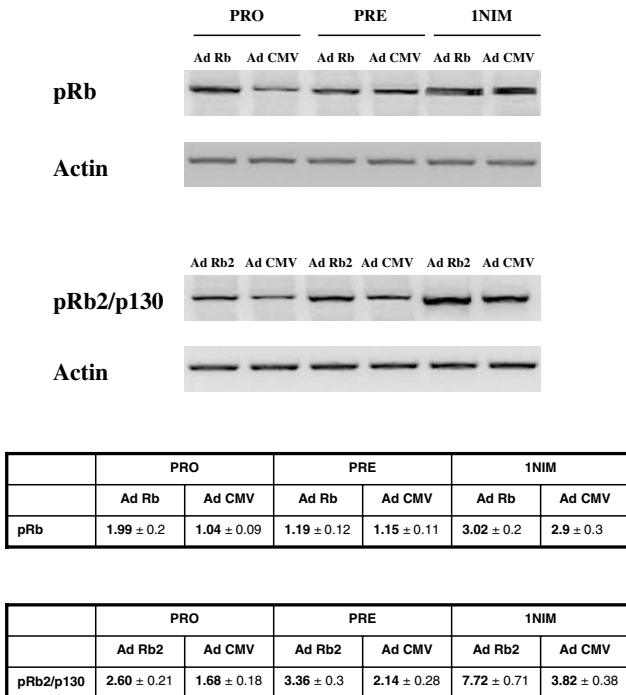
Figure 5 Western blot analysis of pRb and pRb2/p130 protein expression at the indicated time points in MSCs transduced with Ad-CMV-Rb, Ad-CMV-Rb2/p130 and Ad-CMV. Cells were transduced for 24 h with 200 MOI of adenoviruses in PRO and then incubated for a further 24 h in PRE supplemented again with adenoviruses. Lastly, cells were extensively washed in PBS and incubated for 1 day in NIM (1NIM). The protein levels were measured by a GELDOC instrument and normalized with respect to β-actin, which was chosen as an internal control. Each experiment was repeated at least three times. Variations in protein expression are given as arbitrary units
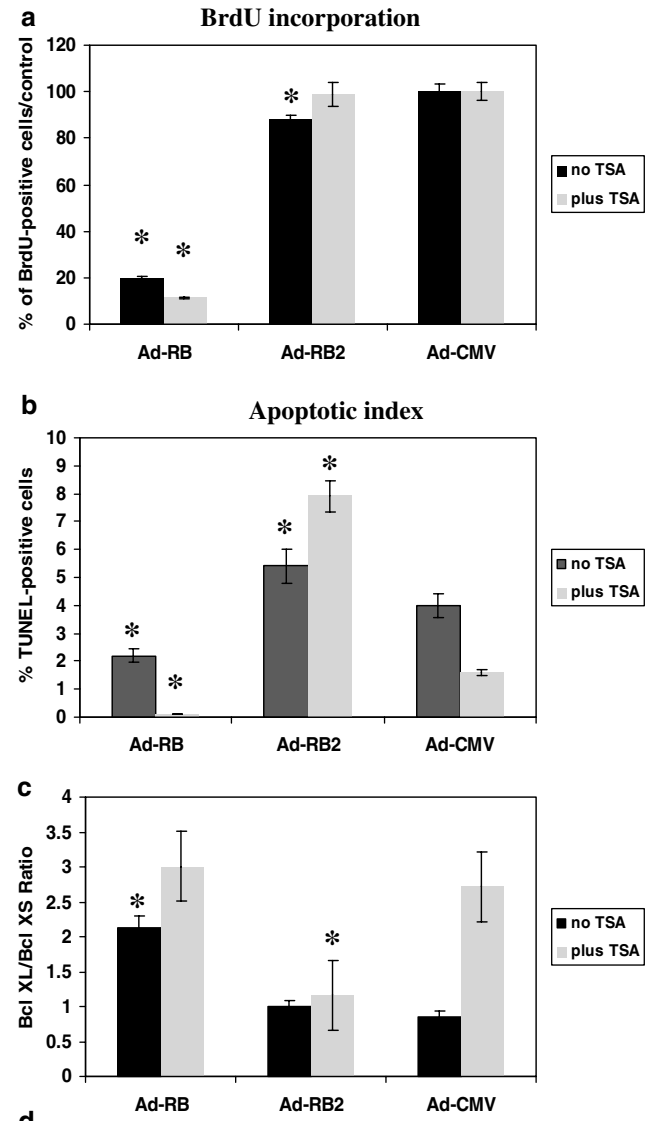
	PRO		PRE		1NIM	
	Ad Rb	Ad CMV	Ad Rb	Ad CMV	Ad Rb	Ad CMV
pRb	1.99 ± 0.2	1.04 ± 0.09	1.19 ± 0.12	1.15 ± 0.11	3.02 ± 0.2	2.9 ± 0.3

	PRO		PRE		1NIM	
	Ad Rb2	Ad CMV	Ad Rb2	Ad CMV	Ad Rb2	Ad CMV
pRb2/p130	2.60 ± 0.21	1.68 ± 0.18	3.36 ± 0.3	2.14 ± 0.28	7.72 ± 0.71	3.82 ± 0.38

	Ad-RB	Ad-RB2	Ad-CMV	Ad-RB TSA	Ad-RB2 TSA	Ad-CMV TSA
Bax	2.01 ± 0.34	4.33 ± 0.26	1.66 ± 0.18	1.1 ± 0.10	1.88 ± 0.16	1.33 ± 0.12

Figure 6 (a) BrdU incorporation, (b) TUNEL assay, (c) semiquantitative RT-PCR analysis of Bcl-X_L/Bcl-X_S ratio and (d) Bax mRNA levels in MSCs transduced with Ad-CMV-Rb, Ad-CMV-Rb2/p130 and Ad-CMV, either in the presence or in the absence of TSA. All these experiments were performed after 1 day incubation in NIM. $*P < 0.05$ respect to control virus infected cells

prominent gene group involved in cell viability, which is governed at the molecular level by a balance between proapototic and antiapoptotic signals. Bcl-2 family members that promote cell survival include Bcl-2 and the long form of Bcl-X (Bcl-X_L), whereas Bax and the short form of Bcl-X (Bcl-X_S) promote apoptosis.[47,48]

Molecular data were in agreement with the morphological analysis of the apoptotic process. Cells expressing ectopic RB showed an increase in the Bcl-X_L/ Bcl-X_S ratio compared to controls (Figure 6c, dark bars), suggesting that antiapoptotic signals overcame the proapoptotic ones. On the other hand, in cells transduced with Ad-CMV-Rb2/p130, we detected a huge increase in Bax expression, thus the balance was in favor of pro-apoptotic genes (Figure 6d).

Cell growth arrest induced by pRb2/p130, but not pRb, relies upon HDAC; the effects on programmed cell death are HDAC independent

Rb family members can repress transcription either by direct inhibition of E2F activity or by recruiting chromatin remodeling enzymes, such as HDACs.[49–51] To establish whether the role of *RB* and *RB2/p130* in MSC biology is dependent on HDAC activity, we overexpressed such genes in differentiating MSCs in the presence of TSA, which is a specific inhibitor of HDACs.[21]

The proliferation assay and the percentage of S-phase cells as determined by BrdUincorporation demonstrated that, in the presence of TSA, pRb was still able to affect cell growth (Figure 6a, light bars). This result suggests that the growth arrest induced by pRb does rely upon HDAC activity. On the contrary, cells overexpressing pRb2/p130 in the presence of TSA did not show a decrease in the percentage of BrdU-positive cells, suggesting that the effect of pRb2/p130 is mediated by HDAC activity (Figure 6a, light bars). In a previous study,[52] we reported that pRb2/p130 induces growth arrest even in the presence of TSA, based on the percentage of PCNA-positive cells. While PCNA downregulation was also confirmed at the transcriptional level (data not shown), this observation contrasts with data obtained from BrdU incorporation, which is considered a more reliable marker of proliferation.

The effects of both *RB* and *RB2/p130* on programmed cell death were not affected by HDAC inhibition. In fact, even in the presence of TSA, pRB showed an antiapoptotic activity and pRb2/p130 a proapoptotic activity. On the contrary, the inhibition of HDAC activity appeared to emphasize the effects of these genes (Figure 6b, light bars).

Molecular data were in agreement with morphological analysis of the apoptotic process (Figure 6c (light bars) and d).

RB2/p130 contributes mainly to the induction of generic neural properties, while *RB* triggers cholinergic differentiation. These differentiating activities are HDAC dependent

Ad-CMV-Rb and Ad-CMV-Rb2/p130 transduction caused an increase in cells expressing neurofilaments and AChe compared to controls (Figure 7). In cultures transduced with Ad-CMV-Rb, we found 33% of cells positive for anti-neurofilament immunostaining compared to 19% of controls ($P < 0.05$). These percentages are in agreement with those of cells positive for AChe activity: 28 *versus* 14% (Figure 7).

Cultures transduced with Ad-CMV-Rb2/p130 showed 59% of cells stained with antineurofilament Abs and 35% of cells positive for AChe activity (Figure 7).

Molecular analysis confirmed that *RB* and *RB2/p130* contribute to the neuronal maturation of MSCs. In fact, the level of NSE was strongly increased in cells overexpressing either pRb or pRb2/p130 compared to controls (Figure 8). The decrease in Id2 mRNA expression observed in Ad-CMV-Rb-transduced cells further corroborates this hypothesis (Figure 8).

The activation of the cholinergic pathway in cells over-expressing *RB* or *RB2/p130* was also demonstrated by RT-PCR analysis of AChe, TH and VIP mRNA expression (Figure 8). It is worth noting that an increased AChe expression in cells infected with Ad-CMV-Rb or Ad-CMV-Rb2/p130 did not correspond to an equivalent increase in mRNA levels (Figure 8). These data further confirm that the *AChe* gene (as well as *VIP*) exhibits a multilevel regulation of expression[53–56] and that an increase in enzymatic activity is not necessarily accompanied by an increase in mRNA level.

The low level of AChe mRNA observed in Ad-CMV-Rb2/p130-infected cells, along with the observation that not all the cells which proved positive for neurofilament staining also demonstrated AChe activity, suggest that *RB2/p130* contributes mainly to the induction of generic neural properties in differentiating MSCs. On the contrary, *RB* overexpression appeared to trigger a cholinergic differentiation pathway in MSC cultures.

The inhibition of HDAC activity by TSA treatment partially inhibited the effects of *RB* on neural commitment and differentiation of MSCs. In fact, while the percentage of cells
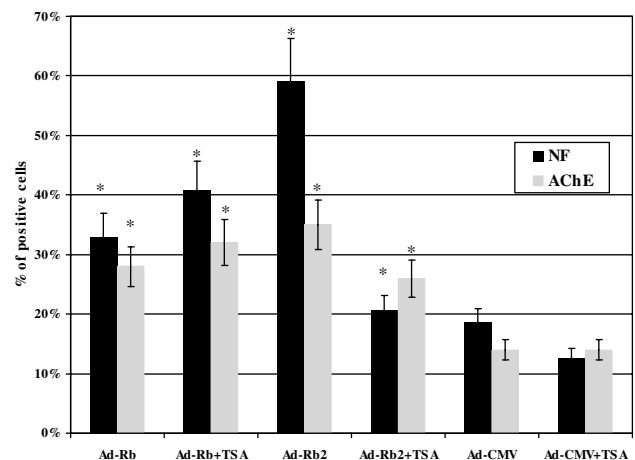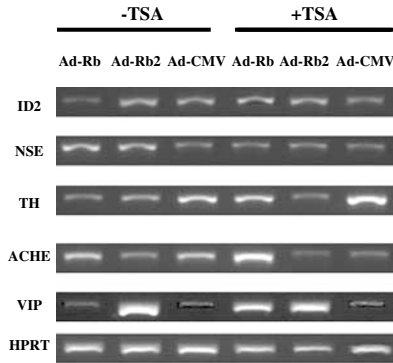

Figure 7 Neurofilament immunostaining and *in situ* AChe activity detection. The assays were carried out on MSCs transduced with Ad-CMV-Rb, Ad-CMV-Rb2/p130 and Ad-CMV, either in the presence or absence of TSA, after 1 day incubation in NIM. The antineurofilament Abs (clone NN18 from Sigma Italia, Italy) recognizes High-, Intermediate- and low-molecular weight neurofilaments. In both assays, the percentage of positive cells was determined by counting at least 1500 cells in 5/6 different microscopic fields. *$P < 0.05$ respect to control virus infected cells

Figure 8 Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes in MSCs transduced with Ad-CMV-Rb, Ad-CMV-Rb2/p130 and Ad-CMV, either in the presence or absence of TSA, after 1 day incubation in NIM. mRNA levels were measured by a GELDOC instrument and normalized with respect to HPRT, which was chosen as an internal control. Each experiment was repeated at least three times. Variations in gene expression are given as arbitrary units

committed toward a cholinergic phenotype was almost unchanged in cells overexpressing *RB* both in the presence and absence of TSA (Figure 7), the NSE mRNA level decreased significantly in cells infected with Ad-CMV-Rb in the presence of TSA compared to those transduced in the absence of the HDAC inhibitor (Figure 8). TSA treatment also inhibited the decrease in Id2 mRNA observed in cells expressing ectopic RB compared to controls (Figure 8).

The inhibition of HDACs greatly impaired the effectiveness of pRb2/p130 in inducing the neural differentiation of MSCs. The percentage of cells that proved positive for antineurofilament immunostaining and those expressing AChe activity was reduced in cells infected with Ad-CMV-Rb2/p130 in the presence of TSA compared to those transduced in the absence of the HDAC inhibitor (Figure 7). In addition, the expression of NSE was reduced in cells overexpressing pRb2/p130 in the presence of TSA compared to those not treated with TSA (Figure 8).

The observation that HDAC inhibition affects the differentiating activities of both pRb and pRb2/p130 is in agreement with the effects of TSA on cyclin kinase inhibitors (see next paragraphs).

E2F-responsive genes in pRb and pRb2/p130-overexpressing cells

In order to clarify the different effects of *RB* and *RB2/p130* genes overexpression in MSC biology, we analyzed the mRNA levels of representative E2F-responsive genes that are involved in cell proliferation (cyclin A) and apoptosis (ARF,

Table 1 Variation in the mRNA expression of the indicated E2F-responsive genes in MSCs infected with Ad-Rb or Ad-Rb2/p130 with respect to MSCs infected with control adenovirus

	−TSA		+TSA	
	Ad Rb	**Ad Rb2/p130**	**Ad Rb**	**Ad Rb2/p130**
Cyc A	(-)	(-)	(- -)	=
ARF	(-)	(- -)	=	(- -)
E2F 1	(-)	(- -)	=	(-)
APAF 1	=	=	=	=

Data were obtained by semiquantitative RT-PCR performed on RNA extracted 24 h after infection. (-): 20–40% decrease; (- -): 40–60% decrease; (=) no variation

E2F1 itself, APAF1) and whose transcription is regulated by pRb and pRb2/p130, via interaction with E2F transcription factors.[57–60] Among the time points analyzed (1, 2 and 3 days after infection, which correspond to PRO, PRE and 1NIM media, respectively), we found significative variations in mRNA expression 24 h after infection, when the levels of the Rb proteins first rose. At this time point, all the genes analyzed (except APAF1) were downregulated in cells overexpressing pRb or pRb2/p130 in the absence of TSA (Table 1). In the presence of TSA, however, we observed the downregulation of cyclin A only in cells infected with Ad-CMV-Rb, while ARF and E2F1 transcription was inhibited in cells transduced with Ad-CMV-Rb2/p130 (Table 1). These data suggest that cyclin A expression may account for the effects observed on cell proliferation in cells transduced with Ad-CMV-Rb, while apoptosis does not seem to be mediated by the E2F-responsive genes analyzed since both ARF and E2F1 are downregulated both in cells tranduced with Ad-CMV-Rb and Ad-CMV-Rb2/p130.

Molecular pathways in differentiated cells overexpressing pRb and pRb2/p130

Thereafter, we analyzed the expression levels of p53 and p27, two genes that play a key role in the control of the G_1/S checkpoint of the cell cycle and act through overlapping pathways with *RB* and *RB2/p130*.[15,36,61–63] It was also demonstrated that *p27* and *p53* are key genes in vertebrate neural cell fate determination and differentiation.[36,64–67]

These genes are mostly regulated at the protein level and are not directly dependent on Rb protein expression. For these reasons, we analyzed their expression in cells transduced with Ad-CMV-Rb and Ad-CMV-Rb2/p130 after the induction of differentiation (1NIM) by Western blot analysis.

We observed a significant increase in p27 protein in cells transduced with Ad-CMV-Rb and Ad-CMV-Rb2/p130 compared with controls (Figure 9). This suggests that the role of *RB* and *RB2/p130* genes in the neural differentiation of MSCs may also rely upon p27-mediated pathways. It has been reported that the induction of Rb proteins specifically inhibits cyclin A- and cyclin E-associated kinase activity and by doing so induces p27 levels, presumably by inhibiting p27 targeted proteolysis by cyclin-CDK phosphorylation of p27.[62] Thus, cyclin A downregulation may explain the increase in p27 protein in cells overexpressing pRb and pRb2/p130.
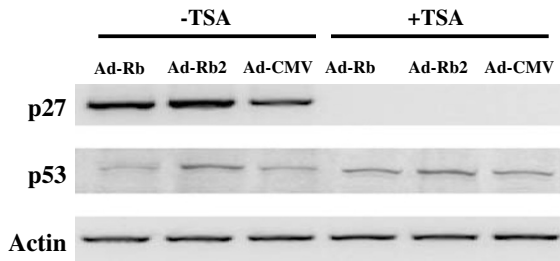
	Ad-RB	Ad-RB2	Ad-CMV	Ad-RB TSA	Ad-RB2 TSA	Ad-CMV TSA
p27	203±18	186±19	103±10	n.d.	n.d.	n.d.
p53	99±10	170±11	117±12	133±12	188±14	127±10

Figure 9 Western blot analysis of p53 and p27 protein in MSCs transduced with Ad-CMV-Rb, Ad-CMV-Rb2/p130 and Ad-CMV, either in the presence or absence of TSA, after 1 day incubation in NIM. Protein levels were measured by a GELDOC instrument and normalized with respect to β-actin, which was chosen as an internal control. Each experiment was repeated at least three times. Variations in protein expression are given as arbitrary units

The inhibition of HDAC activity had a complex effect on the regulation of the *p27* gene. The final result of the TSA treatment on MSCs seems to be the inhibition of p27 activity. We did not observe any p27 protein expression in MSCs transduced with Ad-CMV in the presence of TSA compared to those transduced in the absence of the HDAC inhibitor (Figure 9, compare Ad-CMV versus Ad-CMV + TSA).

The effects of TSA on NSE and Id2 expression in MSC differentiating cultures indicated that HDAC inhibition impairs neural differentiation. This supposition is further sustained by the observation that in TSA-treated MSCs, we observed a huge downregulation of p27 protein (Figure 9) compared to untreated cells. The ectopic expression of either *RB* or *RB2/ p130* did not revert the downregulation of p27 protein expression caused by TSA treatment (Figure 9). These data further support the hypothesis that *RB* and *RB2/p130* do play a role in the neural differentiation of MSCs depending on the HDAC pathway.

We observed an increase in p53 protein level only in cells transduced with Ad-CMV-Rb2/p130, both in the presence and absence of TSA (Figure 9). As reported above, *RB* seems to contribute to MSC growth arrest and neural differentiation to a greater extent than *RB2/p130*; thus, p53 upregulation observed in cells transduced with Ad-CMV-Rb2/p130 may be linked principally to the activation of the cell death process rather than to the inhibition of cell proliferation and to the neural differentiation process. In fact, several reports have demonstrated that p53-controlled pathways also depend upon p53 protein level. In particular, a low level of activated p53 can induce growth arrest and/or differentiation, while high p53 expression leads to programmed cell death.[68,69]

Discussion

Marrow stromal stem cell somatic plasticity

Marrow stromal cells are nonhematopoietic multipotent stem-like cells and are capable of differentiating into both mesenchymal and nonmesenchymal lineages. In fact, in addition to bone, cartilage and fat, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes *in vitro* and *in vivo*.[2,24,28,29,31,70] MSCs are of interest because they are easily isolated from a small aspirate of bone marrow and can be quickly expanded *in vitro*. For these reasons, the cells are currently being tested for their potential use in cell and gene therapy for a number of human diseases, such as neurological pathologies.

Basic studies aiming to resolve issues with regard to commitment, lineage restriction and differentiation of MSCs are of great interest. In particular, little is known about the somatic plasticity of these cells that have the potential to differentiate into neurons. To begin approaching such issues, we performed a molecular and cytochemical analysis of differentiating rat MSCs to monitor proliferation, differentiation and cell death for 5 days *in vitro* and analyzed the expression levels of certain genes playing a major role in these processes.

The *in vitro* differentiation procedure induced a neuron-like phenotype, as demonstrated by analyzing several markers of neural commitment and maturation (Figure 3).

The studies performed indicate that the progression of MSCs maturation cannot be easily followed by analyzing the mRNA expression of neurofilaments, since these genes showed a bimodal expression during *in vitro* differentiation. On the opposite, NSE and Id2 are good markers for monitoring *in vitro* neural commitment and differentiation since they showed a progressive increase (NSE) or decrease (Id2) in mRNA levels during the experimental time course (Figure 3). To better characterize the neural differentiation potential of MSCs, we checked for the expression of several neurotransmitter markers. Among the analyzed neurotransmitters, we found that differentiating MSCs expressed increasing AChe, VIP and decreasing TH mRNAs. The expression of AChe and VIP mRNAs changed progressively during the first days of MSC differentiation (Figure 3). However, these are not reliable markers for a semiquantitative analysis of MSC neuronal maturation. In fact, these genes have a complex regulation at transcriptional, post-transcriptional, translational and post-translational levels.[53–56] For this reason, the detection of their mRNAs is useful only to determine that the cholinergic pathway has been triggered.

Neurofilament immunostaining and *in situ* detection of AChe activity are very useful for qualitative analyses and further suggest that MSCs began to differentiate into cholinergic neurons (Figure 4a–d).

These molecular analyses further lend credit to the hypothesis that MSCs possess a huge somatic plasticity and are amenable to neural commitment and differentiation. Moreover, they suggest that the protocol used for neural differentiation is suitable for studies on stem cell commitment and on the early steps of neural differentiation, while it is not adequate for long-term survival of neurons *in vitro*. In fact, along with the inhibition of cell cycle, we observed a progressive increase in cell death during the incubation in NIM (Figure 1).

Role of *RB* and *RB2/p130* during *in vitro* neural differentiation of MSCs

Once we established useful molecular and morphological markers for studies on the early phases of neural commitment and differentiation of MSCs, we looked at the role played by *RB* and *RB2/p130* genes during this critical time period.

RB and *RB2/p130* genes are involved in the differentiation of several systems; thus, they could also make a key contribution in marrow stromal cell differentiation. In particular, we investigated the role of *RB* and *RB2/p130* in the differentiation and apoptosis of MSCs under experimental conditions that allow for differentiation toward the neuron-like phenotype.

We found that both proteins were highly upregulated during the critical step of neural differentiation. However, while pRb progressively decreased to basal levels at 2 and 5 days in NIM, pRb2/p130 showed a consistent accumulation (Figure 2). These data suggest that both proteins may play a role in MSCs differentiation toward a neuron-like phenotype. To gain insights into the role played by the Rb family proteins in this process, we ectopically expressed either *RB* or *RB2/p130* in rat primary MSC cultures, which were then induced to differentiate, and we monitored proliferation, differentiation and apoptosis using several technical approaches. In order to ascertain whether the effects of Rb family genes were mediated by their interaction with HDACs, the experiments were also performed in the presence of TSA, a specific inhibitor of histone deacetylases.

Both *RB* and *RB2/p130* induced a reduction in cell proliferation rate with respect to the control cells, as determined by the decrease in BrdU incorporation in cells transduced with Ad-CMV-Rb and Ad-CMV-Rb2/p130 compared to controls. These data are in agreement with the role of these genes as negative regulators of cell cycle progression.[5,71] In the presence of the HDAC-inhibitor TSA, the arrest of cell growth was still observed in pRb-overexpressing cells, but not in cells overexpressing pRb2/p130. These results suggest that the antiproliferative activity of *RB* does not rely upon the HDAC pathway, while the growth arrest induced by *RB2/P130* seems to require the activity of HDACs (Figure 6a). The analysis of cyclin A expression correlates with these observations (Table 1). Cyclin A is a downstream target of RB and is involved in RB-mediated arrest;[62,72] thus, its unaltered expression in pRb2/p130-overexpressing cells in the presence of TSA may explain why these cells do not show decreased proliferation. These data are also in agreement with our previous studies in which we demonstrated that the binding of pRb2/p130 to HDAC1 increased its ability to inhibit the transcription of the E2F-dependent promoter of cyclin A *in vivo*.[73]

Several authors have suggested that pRb and pRb2/p130 may have almost totally overlapping activities.[74] However, the data reported above and the effects of the ectopic expression of these proteins on the apoptosis machinery of differentiating MSCs confute this hypothesis. In fact, TUNEL analysis along with studies on the expression of genes belonging to the Bcl-2 family (Figure 6b–d) showed that while *RB* protected differentiating MSCs from programmed cell death, *RB2/p130* caused an increase in apoptosis compared to controls.

In this case, too, did the effects of *RB* and *RB2/p130* appear to be HDAC independent. Studies on *p53* and its downstream effector *BAX* expression may indicate that *RB2/p130* induces apoptosis through a p53-dependent pathway (Figure 9). However, we were not able to find a direct correlation between genes regulated by Rb family proteins and p53. Several reports demonstrated that pRb and pRb2/p130 regulate the transcription of different sets of E2F-responsive genes.[58,60] Thus, it was reasonable to hypothesize that the expression of the E2F-responsive genes ARF and E2F1, which are linked to the activation of the p53 pathway, may be differentially regulated in Ad-CMV-pRB2/p130-transduced cells, where we observed an increase in p53 protein expression, and in Ad-CMV-Rb-infected cells. Nevertheless, E2F1 and ARF transcription was inhibited both in cells overexpressing pRb and in cells overexpressing pRb2/p130 (Table 1). Thus, the increase in p53 protein does not seem directly associated to the pRb2/p130 regulation of E2F-dependent genes.

A further explanation may be obtained from the observation that pRb impairs E2F1 activity both by blocking its transcription and by binding and silencing its activation domain. On the opposite, pRb2/p130 can only regulate E2F1 at the transcriptional level.[56] For this reason, while the neosynthesis of E2F1 proteins is arrested, the existing E2F1 molecules could still trigger p53-associated pathways in MSCs overexpressing pRb2/p130. On the other hand, the involvement of other Rb-interacting proteins cannot be ruled out.[75]

We subsequently looked at the effects of *RB* and *RB2/p130* genes on the molecular markers of neural differentiation. Both *RB* and *RB2/p130* contributed to the induction of generic neural properties as indicated by the increase in NSE mRNA level and increased the percentage of cells immunostained with antibodies targeted against the whole class of neurofilaments. However, while in MSC cultures infected with Ad-CMV-Rb, the percentage of cells stained with antineurofilament Abs was equivalent to that of cells which proved positive for AChe activity, in those infected with Ad-CMV-Rb2/p130, the number of neurofilament-positive cells was significantly higher ($P < 0.05$) than the number of cells exhibiting detectable AChe activity (Figure 7). These results suggest that *RB2/p130* contributes principally to the induction of generic neural properties and that *RB* triggers cholinergic differentiation. HDAC activity seems to play a role in neural differentiation induced by Rb family members. In fact, the differentiation potential of *RB2/p130* is greatly affected by the inhibition of HDACs (Figures 7 and 8), and also the neural differentiation induced by *RB* ectopic expression appears to rely, at least in part, on the enzymatic activity of HDACs (Figure 8). These data are in agreement with reports showing the importance of HDAC in regulating neural and glial development.[20,22]

The data with regard to p27 expression following transduction of *RB* and *RB2/p130* suggest that, rather than having a role in RB- or RB2-mediated cell cycle arrest, this protein may contribute to their differentiating activity. In fact, despite undetectable levels of p27, the proliferation of cells overexpressing pRb is still inhibited in the presence of TSA. Thus, p27 expression seems unnecessary for pRb-dependent growth inhibition. On the opposite, p27 expression correlates with the differentiating activity of pRb and pRb2/p130. Indeed, a role for p27 in neural differentiation has been proposed.[36]

A proposed model of *RB* and *RB2/p130* in the neural differentiation pathway of MSCs

Several studies have demonstrated that a small number of proneural genes, which encode transcription factors of the bHLH family, are both necessary and sufficient to initiate the development of neuronal lineages inducing cell commitment and differentiation.[32,33] These genes could be considered at the top of the hierarchy, which decides cell fate and differentiation. Proneural genes not only determine the neuronal fate of cell progenitors but also promote the arrest of their division, and therefore they act to couple these two fundamental processes.[32,33,36] Cell cycle arrest appears to be the mechanism for insulating already specified progenitor cells from the influence of extrinsic fate-determining factors.[36,76] The regulation of the cell cycle by bHLH genes is likely to involve several 'effector genes' which can act directly on cell cycle machinery, such as the cyclin kinase inhibitors *p21*, *p27* and *p19* or the RB gene family members.[36,76] The irreversible commitment of neural progenitor to differentiation is obtained when proneural genes reach a high level of expression and/or activity. Several positive feedback mechanisms are required to increase or sustain proneural gene expression in neural precursor cells. By binding to Id2, the components of the RB family inhibit its ability to downregulate the proneural activity of bHLH proteins. In this way, RB family members can trigger a positive feedback loop that increases bHLH activity and further promotes neural differentiation.[34,36,37,77]

Substances that increase the cellular levels of cAMP are among the principal components of NIM that we used to induce the neural differentiation of MSCs.[24,29,31] Several reports have shown the induction of bHLH gene expression in response to the activation of the cAMP pathway,[78,79] suggesting that this could be one of the pathways triggered by NIM treatment to induce neural differentiation.

For these reasons, we speculate that pRb and pRb2/p130 can trigger the neural commitment and differentiation of MSCs as 'effectors' of proneural genes activated by NIM treatment. On the other hand, the overexpression of *RB* and *RB2/p130* in MSCs without NIM treatment did not induce neural differentiation *per se* (data not shown), thus suggesting that these genes act downstream of the pathways induced by NIM treatment. Once the commitment/differentiation process has been triggered, the *RB* and *RB2/p130* genes actually reinforce the maturation pathway. It should be pointed out that pRb and pRb2/p130 only share certain biochemical properties (e.g. the ability to arrest the cell cycle), while their activities on MSC survival and differentiation may have different/opposite effects. It is worth noting that pRb and pRB2/p130 can play a role in MSC biology both through HDAC-dependent (neural differentiation) and HDAC-independent (cell growth, at least for *RB*, and apoptosis) pathways.

In conclusion, in studies aiming to define a way to use MSCs for cell therapy in the treatment of neurological disorders, great attention should be paid to the role played by the *RB* gene in MSC biology, since it can contribute to cholinergic differentiation while at the same time protecting cells from programmed cell death.

Materials and Methods

Animals and MSC cultures

In accordance with protocols devised by Prockop and co-workers,[2,23] MSCs were harvested from the bone marrow of the femurs and tibias of 4- to 12-month-old rats by inserting a 21-gauge needle into the shaft of the bone and flushing it with complete α-modified Eagle's medium (αMEM) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (we indicated this medium as PRO for proliferating). Cells from one rat were plated onto two 100 mm dishes. After 24 h, nonadherent cells were discarded and adherent cells representing marrow stromal stem cells were washed twice with phosphate-buffered saline (PBS). Cells were then incubated for 5–7 days to reach confluence and extensively propagated for further experiments.

All cell culture reagents were obtained from Invitrogen Italia (Italy), unless otherwise stated.

Neural induction

Neuronal induction was performed as described by Woodbury *et al*,[24] with modifications. Briefly, prior to neuronal induction, cells were grown overnight in a pre-induction medium (PRE) composed of DMEM, 20% FBS and 10 ng/ml bFGF (Preprotech, NJ, USA). Cells were then rinsed with PBS and transferred to the neuronal induction medium (NIM) consisting of 100 μM BHA, 100 μM forskolin, 2% DMSO, 25 mM KCl, 2 mM valproic acid, 1 × B27 supplement, 10 ng/ml bFGF and 10 ng/ml PDGF (Preprotech, NJ, USA) in a base of DMEM. Cells were maintained in NIM for up to 5 days.

Cell proliferation and cell death assay

Cells were seeded at equal density in 96-multiwell plates. Cellular proliferation and cell death were determined at different time points by measuring the metabolic activity of cellular enzymes present in the cytoplasm or released in the culture media, respectively. We used the CellTiter 96 Proliferation Assay and the CytoTox 96 Cell Death Assay (both from Promega, WI, USA) according to the manufacturer's instructions.

Immunocytochemistry

We determined the percentage of neurons in MSC cultures by growing cells on glass coverslips. At different times after the chosen treatments, the coverslips were fixed with 4% paraformaldehyde for 15 min followed by three washes in PBS. The slides were then treated with 0.3% H_2O_2 in methanol for 5 min, before being washed with 1% BSA in PBS. At this point, cells were incubated with a primary antibody (clone NN18 from Sigma-Aldrich Italia, Italy) targeted against pan-neurofilaments (diluted in PBS supplemented with 1% BSA) for 90 min at room temperature (RT). Afterwards, the slides were washed three times with PBS and incubated with goat anti-mouse secondary antibodies conjugated to peroxidase (DAKO, CA, USA) for 45 min at RT. Lastly, after further washing in PBS, the slides were treated with diaminobenzidine (DAB) substrate (Roche, Germany).

For bromodeoxyuridine (BrdU) immunostaining, cells were grown on glass coverslips, incubated for 5 h with 10 μM BrdU and then treated for immunocytochemistry using the 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit II (Roche, Germany). Briefly, cells were rinsed with PBS, fixed with 70% ethanol in 50 mM glycine pH 2 at −20°C for 20 min, rinsed

again with PBS and incubated with an anti-BrdU mouse monoclonal antibody (clone BMG 6H8) containing nucleases, diluted 1 : 10 in 66 mM Tris buffer, 0.66 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. After 30 min at 37°C, the cells were washed with PBS and incubated with an anti-mouse Ig alkaline phosphatase diluted 1 : 10 in PBS for 30 min at 37°C. Lastly, color was developed by incubation with BCIP/NBT in 100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$ pH 9.5 for 20 min at RT.

Cell cycle analysis

For each assay 5×10^5 cells were collected and resuspended in 500 μl of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 μg/ml propidium iodide, RNAse A). Cells were incubated in the dark for 30 min and then analyzed. Samples were acquired on a FACSCalibur flow cytometer using the Cell Quest software (Becton Dickinson, NJ, USA) and analyzed with standard procedure using the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson, NJ, USA).

Acetylcholinesterase (Ache) cytochemistry

AChe *in situ* detection was performed according to the protocol devised by Zhang *et al.*[25] Briefly, cells were fixed with 4% paraformaldehyde for 30 min, before being rinsed with PBS. Cultures were then incubated in a fresh solution consisting of 3 mM copper sulfate, 5 mM sodium citrate, 0.5 mM potassium ferricyanide and 1.8 mM acetylthiocholine in 0.1 M phosphate buffer pH 6 for 1–2 h at 37°C. After two rinses with 0.5 mM Tris-HCl pH 7.6, the cultures were incubated for 5–10 min in an intensification solution composed of DAB substrate (Roche, Germany).

TUNEL assay and determination of apoptotic index

Cells for the TUNEL assay (*In situ* Cell Death Detection Kit from Roche, Germany) were grown on glass coverslips. They were fixed for 15 min using 4% paraformaldehyde before performing the TUNEL reaction according to the manufacturer's instructions. The cells were then observed under a fluorescence microscope. The apoptotic index was calculated by the percentage of positive TUNEL cells out of 1000 cells in five different microscope fields.

Adenoviral production and transduction

The Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV-β-gal viruses were generated as described previously.[26] Briefly, recombinant adenoviruses were constructed by cotransfecting the adenoviral shuttle plasmid pAd-CMVlink-1, with or without insertion of either the *RB2/p130* or the Lac-Z gene behind the CMV promoter, and Cla I Ad5 DNA (viral backbone) into 293 primary embryonic kidney cells, which supplied the E1 function in *trans*. Homologous recombination occurred in the overlap region between the restricted viral DNA and the shuttle plasmids, and produced recombinant Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV-β-gal adenoviruses, which were then purified and utilized for cell infection. The Ad-CMV-Rb was kindly provided by Professor J Fueyo of the University of Texas, TX, USA. To obtain enough viruses for MSC infections, recombinant adenoviruses were expanded by infecting 293 cells and then purified.

Cell cultures were transduced at different multiplicities of infection (MOI) with Ad-CMV-Rb, Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV-β-gal and cytotoxic effects were determined by trypan blue exclusion and CytoTox 96 cell death assay (Promega WI, USA). β-galactosidase activity was evaluated by an *in situ* detection β-gal staining kit (Roche, Germany) to determine the percentage of infected cells. Typically, we found that about 80% of MSCs were positive for β-galactosidase activity when transduced with 200 MOI during a 48-h incubation time.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell cultures using TRI REAGENT (Molecular Research Center Inc., OH, USA) according to the manufacturer's protocol. The mRNA levels of the genes analyzed were measured by RT-PCR amplification, as reported previously.[27]

Sequences for human mRNAs from the GeneBank (DNASTAR Inc., WI, USA) were used to design primer pairs for RT-PCR reactions (OLIGO 4.05 software, National Biosciences Inc., MN, USA). Appropriate regions of HPRT cDNA were used as controls. PCR cycles were adjusted to have linear amplification for all the targets. Each RT-PCR reaction was repeated at least three times. A semiquantitative analysis of mRNA levels was carried out by the 'GEL DOC UV SYSTEM' (Biorad Company, CA, USA).

Western blotting

Cells were lysed in a buffer containing 0.1% Triton for 30 min at 4°C. The lysates were then centrifuged for 10 min at $10\,000 \times g$ at 4°C. After centrifugation, 10–40 μg of each sample were loaded, electrophoresed in polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Primary antibodies to detect pRb, p53 and p27 were obtained from Santa Cruz Biotechnology (CA, USA). The primary antibody to detect pRb2/p130 was obtained from BD Biosciences (MA, USA), while that for β-actin (chosen as a control) was obtained from Sigma-Italia (Italy). All the Abs were used according to the manufacturers' instructions.

Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) and reacted with SuperSignal WestPico or WestFemto (Pierce, IL, USA).

Statistical analysis

Statistical significance was evaluated using analysis of variance (ANOVA) analysis followed by Student's *t*-test and Bonferroni's test.

Acknowledgements

References

1. Bianco P and Gehron Robey P (2000) Marrow stromal stem cells. J. Clin. Invest. 105: 1663–1668
2. Colter DC, Class R, DiGirolamo CM and Prockop DJ (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc. Natl. Acad. Sci. USA 97: 3213–3218
3. Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276: 71–74

4. Claudio PP, De Luca A, Howard CM, Baldi A, Firpo EJ, Koff A and Paggi MG andGiordano A (1996) Functional analysis of pRb2/p130 interaction with cyclins. Cancer Res. 56: 2003–2008

5. Stiegler P, Kasten M and Giordano A (1998) The RB family of cell cycle regulatory factors. J. Cell Biochem. Suppl. 30–31: 30–36

6. Claudio PP, Howard CM, Baldi A, De Luca A, Fu Y, Condorelli G, Sun Y, Colburn N, Calabretta B and Giordano A (1994) p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res. 54: 5556–5560

7. LeCouter JE, Kablar B, Whyte PF and Ying C andRudnicki MA (1998) Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. Development 125: 4669–4679

8. LeCouter JE, Kablar B, Hardy WR, Ying C, Megeney LA and May L andRudnicki MA (1998) Strain-dependent myeloid metaplasia, growth deficiency and shortened cell cycle in mice lacking p107. Mol. Cell Biol. 18: 7455–7465

9. Lee EY, Chang CY, Hu N, Wang CJ, Lai CC, Herrup K and Lee WH andBradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and hematopoiesis. Nature 359: 288–294

10. Lee EY, Hu N, Yuan SS, Cox LA, Bradley A and Lee WH andHerrup K (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. Genes Dev. 8: 2008–2021

11. Garriga J, Limon A, Mayol X, Rane SG, Albrecht JH, Reddy HP, Andres V and Grana X (1998) Differential regulation of the retinoblastoma family of proteins during cell proliferation and differentiation. Biochem. J. 333: 645–654

12. Lipinski MM and Jacks T (1999) The retinoblastoma gene family in differentiation and development. Oncogene 18: 7873–7882

13. Slack RS, Skerjanc IS, Lach B, Craig J, Jardine K and McBurney MW (1995) Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. J. Cell Biol. 129: 779–788

14. Slack RS, El-Bizri H, Wong J, Belliveau DJ and Miller FD (1998) A critical temporal requirement for the retinoblastoma protein family during neuronal determination. J. Cell Biol. 140: 1497–1509

15. Macleod KH, Hu Y and Jacks T (1996) Loss ofRb activates both p53-dependent and independent cell death pathways in the devoloping mouse nervous system. EMBO J. 15: 6178–6188

16. Callaghan DA, Dong L, Callaghan SM, Hou YX, Dagnino L and Slack RS (1999) Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. Dev Biol. 207: 257–270

17. Gill RM, Slack RS, Kiess M and Hamel PA (1998) Regulation of expression and activity of distinct pRb, E2F, D-type cyclin and CKI family members during differentiation of P19 cells. Exp. Cell Res. 244: 157–170

18. Nevins JR (1998) Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. 9: 585–593

19. Jori FP, Galderisi U, Piegari E, Peluso G, Cipollaro M, Cascino A, Giordano A and Melone MA (2001) RB2/p130 ectopic gene expression in neuroblastoma stem cells: evidence of cell-fate restriction and induction of differentiation. Biochem. J. 360: 569–577

20. Marin-Husstege M, Muggironi M, Liu A and Casaccia-Bonnefil P (2002) Histone deacetylase activity is necessary for oligodendrocyte lineage progression. J. Neurosci. 22: 10333–10345

21. Morrison AJ, Sardet C and Herrera RE (2002) Retinoblastoma protein transcriptional repression through histone deacetylation of a single nucleosome. Mol. Cell Biol. 22: 856–865

22. Naruse Y, Aoki T, Kojima T and Mori N (1999) Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. Proc. Natl. Acad. Sci. USA 96: 13691–13696

23. Colter DC, Sekiya I and Prockop DJ (2001) Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc. Natl. Acad. Sci. USA 98: 7841–7845

24. Woodbury D, Reynolds K and Black IB (2002) Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res. 69: 908–917

25. Zhang XJ, Yang L, Zhao Q, Caen JP, He HY, Jin QH, Guo LH, Alemany M, Zhang LY and Shi YF (2002) Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ. 9: 790–800

26. Claudio PP, Fratta L, Farina F, Howard CM, Stassi G, Numata S, Pacilio C, Davis A, M L, Volpe A, Wilson JM, Trimarco B, Giordano A and Condorelli G

(1999) Adenoviral RB2/p130 gene transfer inhibits smooth muscle cell proliferation and prevents restenosis after angioplasty. Circ. Res. 85: 1032–1039

27. Galderisi U, Di Bernardo G, Cipollaro M, Jori FP, Piegari E, Cascino A, Peluso G and Melone MA (1999) Induction of apoptosis and differentiation in neuroblastoma and astrocytoma cells by the overexpression of Bin1, a novel Myc interacting protein. J. Cell Biochem. 74: 313–322

28. Black IB and Woodbury D (2001) Adult rat and human bone marrow stromal stem cells differentiate into neurons. Blood Cells Mol. Dis. 27: 632–636

29. Deng W, Obrocka M, Fischer I and Prockop DJ (2001) In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem. Biophys. Res. Commun. 282: 148–152

30. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR (1999) Multineage potential of adult human mesenchymal stem cell. Science 284: 143–147

31. Woodbury D, Schwarz EJ, Prockop DJ and Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J. Neurosci. Res. 61: 364–370

32. Bertrand N, Castro DS and Guillemot F (2002) Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. 3: 517–530

33. Lee JE (1997) Basic helix–loop–helix genes in neural development. Curr. Opin. Neurobiol. 7: 13–20

34. Lasorella A, Iavarone A and Israel MA (1996) Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. Mol. Cell Biol. 16: 2570–2578

35. Ghil SH, Jeon YJ and Suh-Kim H (2002) Inhibition of BETA2/NeuroD by Id2. Exp. Mol. Med. 34: 367–373

36. Galderisi U, Jori FP and Giordano A (2003) Cell cycle regulation and neural differentiation. Oncogene 22: 5208–5219

37. Toma JG, El-Bizri H, Barnabe-Heider F, Aloyz R and Miller FD (2000) Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. J. Neurosci. 20: 7648–7656

38. Fliegner KH, Kaplan MP, Wood TL, Pintar JE and Liem RK (1994) Expression of the gene for the neuronal intermediate filament protein alpha-internexin coincides with the onset of neuronal differentiation in the developing rat nervous system. J. Comp. Neurol. 342: 161–173

39. Riederer BM, Porchet R and Marugg RA (1996) Differential expression and modification of neurofilament triplet proteins during cat cerebellar development. J. Comp. Neurol. 364: 704–717

40. Jori FP, Galderisi U, Piegari E, Cipollaro M, Cascino A, Peluso G, Cotrufo R, Giordano A and Melone MA (2003) EGF-responsive rat neural stem cells: Molecular follow-up of neuron and astrocyte differentiation in vitro. J. Cell Physiol. 195: 220–233

41. Hara H and Kobayashi S (1987) Vasoactive-intestinal-polypeptide (VIP)-like immunoreactive cells in the skull base of rats. A combined study using acetylcholinesterase histochemistry. Histochemistry 87: 217–221

42. Ernsberger U, Patzke H and Rohrer H (1997) The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation. Mech. Dev. 68: 115–126

43. Handler A, Lobo MD, Alonso FJ, Paino CL and Mena MA (2000) Functional implications of the noradrenergic–cholinergic switch induced by retinoic acid in NB69 neuroblastoma cells. J. Neurosci. Res. 60: 311–320

44. Wolinsky E and Patterson PH (1983) Tyrosine hydroxylase activity decreases with induction of cholinergic properties in cultured sympathetic neurons. J. Neurosci. 3: 1495–1500

45. Chen PL, Riley DJ, Chen Y and Lee WH (1996) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes Dev. 10: 2794–2804

46. Chen PL, Riley DJ, Chen-Kiang S and Lee WH (1996) Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA 93: 465–469

47. Adams JM and Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322–1326

48. Reed JC (1997) Double identity for proteins of the Bcl-2 family. Nature 387: 773–776

49. Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ and Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391: 597–601

50. Ferreira R, Magnaghi-Jaulin L, Robin P, Harel-Bellan A and Trouche D (1998) The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc. Natl. Acad. Sci. USA 95: 10493–10498

51. Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, Harbour JW and Dean DC (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101: 79–89

52. Jori FP, Napolitano MA, Melone MA, Cipollaro M, Cascino A, Giordano A and Galderisi U (2004) The role of RB and RB2/P130 genes in marrow stromal stem cells plasticity. J. Cell Physiol. 200: 201–212

53. Coleman BA and Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. J. Biol. Chem. 271: 4410–4416

54. Chew LJ, Murphy D and Carter DA (1994) Alternatively polyadenylated vasoactive intestinal peptide mRNAs are differentially regulated at the level of stability. Mol. Endocrinol. 8: 603–613

55. Li Y, Camp S and Taylor P (1993) Tissue-specific expression and alternative mRNA processing of the mammalian acetylcholinesterase gene. J. Biol. Chem. 268: 5790–5797

56. Perrier AL, Cousin X, Boschetti N, Haas R, Chatel JM, Bon S, Roberts WL, Pickett SR, Massoulie J, Rosenberry TL and Krejci E (2000) Two distinct proteins are associated with tetrameric acetylcholinesterase on the cell surface. J. Biol. Chem. 275: 34260–34265

57. Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev. 12: 2245–2262

58. Hurford RK, Cobrinik D, Lee M-H and Dyson N (1997) pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. 11: 1447–1463

59. Moroni MC, Hickman ES, Denchi EL, Caprara G, Colli E, Cecconi F, Muller H and Helin K (2001) Apaf-1 is a transcriptional target for E2F and p53. Nat. Cell Biol. 3: 552–558

60. Takahashi Y and Rayman JB andDynlact BD (2000) Analysis of promoter binding by the E2F and pRB families *in vivo*: distinct E2F proteins mediate activation and repression. Genes Dev. 14: 804–816

61. Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75: 805–816

62. Howard CM, Claudio PP, De Luca A, Stiegler P, Jori FP, Safdar NM, Caputi M, Khalili K and Giordano A (2000) Inducible pRb2/p130 expression and growth-suppressive mechanisms: evidence of a pRb2/p130, p27Kip1 and cyclin E negative feedback regulatory loop. Cancer Res. 60: 2737–2744

63. Park MS, Rosai J, Nguyen HT, Capodieci P, Cordon-Cardo C and Koff A (1999) p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. Proc. Natl. Acad. Sci. USA 96: 6382–6387

64. Almog N and Rotter V (1997) Involvement of p53 in cell differentiation and development. Biochim. Biophys. Acta. 1333: F1–F27

65. Durand B, Gao FB and Raff M (1997) Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. EMBO J. 16: 306–317

66. Erhardt JA and Pittman RN (1998) Ectopic p21(WAF1) expression induces differentiation-specific cell cycle changes in PC12 cells characteristic of nerve growth factor treatment. J. Biol. Chem. 273: 23517–23523

67. Sasaki K, Tamura S, Tachibana H, Sugita M, Gao Y, Furuyama J, Kakishita E, Sakai T, Tamaoki T and Hashimoto-Tamaoki T (2000) Expression and role of p27(kip1) in neuronal differentiation of embryonal carcinoma cells. Brain Res. Mol. Brain Res. 77: 209–221

68. Donehower LA and Bradley A (1993) The tumor suppressor p53. Biochim. Biophys. Acta. 1155: 181–205

69. Ikeda J, Tada M, Ishii N, Saya H, Tsuchiya K, Okaichi K, Mishima K, Sawamura Y, Fulci G, Liu TJ and Van Meir EG (2001) Restoration of endogenous wild-type p53 activity in a glioblastoma cell line with intrinsic temperature-sensitive p53 induces growth arrest but not apoptosis. Int. J. Cancer. 94: 35–43

70. Bianco P, Riminucci M, Gronthos S and Gehron Robey P (2001) Bone marrow stromal stem cells: nature, biology and potential applications. Stem Cells 19: 180–192

71. MacLachlan TK, Sang N and Giordano A (1995) Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit. Rev. Eukaryotic Gene Expr. 5: 127–156

72. Knudsen KE, Fribourg AF, Strobeck MW, Blanchard JM and Knudsen ES (1999) Cyclin A is a functional target of retinoblastoma tumor suppressor protein-mediated cell cycle arrest. J. Biol. Chem. 274: 27632–27641

73. Stiegler P, De Luca A, Bagella L and Giordano A (1998) The COOH-terminal region of pRb2/p130 binds to histone deacetylase 1 (HDAC1), enhancing transcriptional repression of the E2F-dependent cyclin A promoter. Cancer Res. 58: 5049–5052

74. Mulligan G and Jacks T (1998) The retinoblastoma gene family: cousins with overlapping interests. Trends Genet. 14: 223–229

75. Brehm A and Kouzarides T (1999) Retinoblastoma protein meets chromatin. TIBS 24: 142–145

76. Edlund T and Jessell TM (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell 96: 211–224

77. Iavarone A, Garg P, Lasorella A, Hsu J and Israel MA (1994) The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. 8: 1270–1284

78. Cho JH, Kwon IS, Kim S, Ghil SH, Tsai MJ, Kim YS, Lee YD and Suh-Kim H (2001) Overexpression of BETA2/NeuroD induces neurite outgrowth in F11 neuroblastoma cells. J. Neurochem. 77: 103–109

79. Shen M, Kawamoto T, Teramoto M, Makihira S, Fujimoto K, Yan W, Noshiro M and Kato Y (2001) Induction of basic helix–loop–helix protein DEC1 (BHLHB2)/Stra13/Sharp2 in response to the cyclic adenosine monophosphate pathway. Eur. J. Cell Biol. 80: 329–334