RB2/p130 ectopic gene expression in neuroblastoma stem cells: evidence of cell-fate restriction and induction of differentiation

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The activity of the RB2/p130 gene, which is a member of the retinoblastoma gene family, is cell-cycle-regulated and plays a key role in growth inhibition and differentiation. We used neuroblastoma cell lines as a model for studies on neural crest progenitor cell differentiation. We show that Rb2/p130 ectopic protein expression induces morphological and molecular modifications, promoting differentiation of intermediate (I) phenotype SK-N-BE(2)-C neuroblastoma cells towards a neuroblastic (N) rather than a Schwann/glia/melanocytic (S) phenotype. These modifications are stable as they persist even after treatment with an S-phenotype inducer. Rb2/p130 ectopic expression also

induces a more differentiated phenotype in N-type SH-SY-5Y cells. Further, this function appears to be independent of cell-cycle withdrawal. The data reported suggest that the Rb2/p130 protein is able to induce neuronal lineage specification and differentiation in neural crest stem and committed neuroblastoma cells, respectively. Thus, the Rb2/p130 protein seems to be required throughout the full neural maturation process.

Key words: cell cycle, neural development, retinoblastoma gene family.

INTRODUCTION

Retinoblastoma (Rb) family genes, RB, RB2/p130 and p107, play a major role in the control of the cell cycle. This function is mainly accomplished by the differential binding of Rb-family proteins (pRb) 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. pRb proteins are the substrates of different cyclin-CDK (cyclin-dependent kinase) complexes and hence undergo cyclic phosphorylation and dephosphorylation: in the hypophosphorylated active status they bind E2F family members and inhibit their function, whereas they are inactive when hyperphosphorylated [1,2].

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 cell maturation state and type [3–9].

RB-family genes are also involved in the onset of differentiation in a wide variety of cell types [2,10,11]. Several data sets indicate that pRb and pRb2/p130 play a crucial role in neurogenesis [3,4,7,12–18]. However, it is not clear whether pRb and pRb2/p130's property of inducing neuronal differentiation is merely the consequence of cell-cycle withdrawal or if it depends on the interaction with lineage-specific regulators. Indeed, interactions of Rb-family proteins with factors that play a specific role in the differentiation process of distinct lineages have been reported [1].

The differentiation of neural precursor cells into neurons or glia occurs over a protracted period and is associated with a progressive restriction in the range of fates available to individual cells. For certain classes of neural precursors, the lineage specification occurs several divisions before cell-cycle exit, whereas for others fate restriction is determined much closer to the time of cell-cycle exit or even only after they have reached a post-mitotic state. In these different occurrences, cell-cycle exit could contribute to the determination of neural lineage specification or could promote neural differentiation directly [19].

At the time of their onset, neural crest stem cells are multipotent; later they give rise to lineage-restricted progenitors, prior to their withdrawal from the cell cycle. In these cells, the exit from the cell cycle appears to contribute only to the definition of generic neural properties [19]. For these reasons neural crest stem cells could make it possible to ascertain whether Rb-family members have a peculiar role in neural differentiation, not strictly related to cell-cycle exit.

Neuroblastoma cell lines are a convenient model for studies on neural crest progenitor cells. These cell lines derive from human cancers originating from malignant neural crest cells [20]. They show various phenotypes that represent different neural populations: the neuroblastic (N) phenotype exhibits neurite-like processes and expresses opioid, muscarinic and nerve growth factor receptors as well as noradrenergic biosynthetic enzymes, whereas the substrate-adherent Schwann/glia/melanocytic (S) phenotype resembles epithelial cells and has properties of embryonic Schwann/glia/melanocytic cells of neural crest origin [21,22]. Furthermore, there is an intermediate (I) phenotype that

Abbreviations used: Rb, retinoblastoma; pRb, Rb-family protein; CDK, cyclin-dependent kinase; MOI, multiplicity of infection; CMV, cytomegalovirus; β -gal, β -galactosidase; RT-PCR, reverse transcriptase PCR; RA, all-trans retinoic acid; NF160, 160 kDa neurofilament; NSE, neuron-specific enolase; MAP-2, microtubule-associated protein-2; BrdU, bromodeoxyuridine; N phenotype, neuroblastic phenotype; S phenotype, Schwann/glia/melanocytic phenotype; I phenotype, intermediate phenotype; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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has been demonstrated to represent a multipotent embryonic precursor cell of the peripheral nervous system [23] and hence can be induced to differentiate towards the committed neuronal N phenotype or the non-neuronal S phenotype [21].

It has been reported previously that pRb2/p130 expression levels increase during neuroblastoma differentiation and that ectopic expression of the RB-family genes can induce murine neuroblastoma cells to differentiate [24,25]. In addition, evidence of a critical temporal requirement for the retinoblastoma protein family during neural determination has been reported [3,15].

Our aim was to assess the role of pRb2/p130 at different stages of neuroblastoma cell differentiation in order to clarify whether its function is required only during a specific temporal window, or if its capability of inducing neural differentiation is independent from the maturation state of the cell. In particular, we attempted to determine whether pRb2/p130 ectopic expression only induces differentiation in cells already committed towards the neuronal phenotype or if it also has a role in stem-cell fate restriction. Furthermore, we aimed to establish whether the role played by pRb2/p130 in neural stem-cell differentiation is independent from its ability to promote cell-cycle exit. To this end, we used adenoviral-mediated gene transfer to express pRb2/p130 ectopically in two different human neuroblastoma cell lines, one composed of I phenotype multipotent precursor cells of the peripheral nervous system and the other of N phenotype, neuron lineage-committed cells.

MATERIALS AND METHODS

Cell cultures

SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells were grown in monolayers and maintained at 37 °C under 5% CO₂ in RPMI 1640 containing 10% foetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin and 100 µg/ml streptomycin. To induce differentiation, 10⁻⁵ M all-*trans* retinoic acid (RA; Sigma-Aldrich, St. Louis, MO, U.S.A.) or 10⁻⁵ M bromodeoxyuridine (BrdU; Sigma-Aldrich) was added to the culture medium for the times indicated.

Adenoviral production and transduction

The recombinant Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV- β gal adenoviruses were generated as described previously [26]. Briefly, recombinant adenoviruses were constructed by co-transfecting the adenoviral shuttle plasmid pAd-CMVlink-1, either with no insertion or with the insertion of either the RB2/p130 or the *lacZ* gene behind the cytomegalovirus (CMV) promoter, and Cla I Ad5 DNA ('viral backbone') into primary HEK-293 cells, which supplied the E1 function (an adenovirus early protein) *in trans*. Homologous recombination occurred in the region of overlap, between the restricted viral DNA and the shuttle plasmids, producing recombinant Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV- β gal adenoviruses, which were purified and utilized for cell infection.

Cell cultures were transduced at different multiplicities of infection (MOIs) with Ad-CMV-Rb2/p130, Ad-CMV and Ad-CMV- β gal, and cytotoxic effects were determined by Trypan Blue exclusion. β -Galactosidase (β -gal) activity was evaluated by an *in situ* detection β -gal staining kit (Roche, Mannheim, Germany) to determine the percentage of infected cells.

RNA extraction and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from cell cultures using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's protocol. The mRNA levels of the genes

under analysis were measured by RT-PCR amplification, as reported previously [27].

Sequences for human mRNAs from the GenBank (DNASTAR, Madison, WI, U.S.A.) were used to design primer pairs for RT-PCR reactions (OLIGO 4.05 software; National Biosciences, Plymouth, MN, U.S.A.). Appropriate regions of hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA were used as controls. Each RT-PCR reaction was repeated at least three times. A semi-quantitative analysis of mRNA levels was carried out by the GEL DOC 1000 UV system (Bio-Rad, Hercules, CA, U.S.A.).

Western blotting

Cells were lysed in a buffer containing 0.1% Triton X-100 for 30 min at 4 °C. The lysates were then centrifuged for 10 min at 10000 g at 4 °C. After centrifugation, 20 µg of each sample was loaded, electrophoresed in polyacrylamide gel and electroblotted on to a nitrocellulose membrane. Primary antibodies employed to detect pRb2/p130 (1:500 dilutions) were used as described previously [26]. Primary antibodies to detect pRb/p105, p53, p27 and 70 kDa heat-shock protein (Hsp70) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and were utilized according to the manufacturer's instructions. Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and detected using the ECL system (Amersham Pharmacia Biotech, Uppsala, Sweden). Each Western blot was repeated at least three times.

RESULTS

Gene transfer and cytotoxicity

In a first set of experiments SK-N-BE(2)-C I-type and SH-SY-5Y N-type neuroblastoma cells were infected at different MOIs with Ad-CMV- β -gal viruses. Particular attention was paid to determining the MOI and the incubation time that did not cause cytotoxic effects, retained a good efficiency of infection and did not overwhelm cell machinery with high production of ectopic protein. Typically we found that about 70% of neuroblastoma cells were positive for β -gal activity when transduced with an MOI of 10 during a 72 h incubation time. Semi-quantitative RT-PCR assays and Western-blot analysis, performed 4 days after

Figure 1 Agarose-gel electrophoresis analysis of pRb2/p130, pRb/p105 (Rb), p53, p27 and HPRT RT-PCR products in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cell cultures 4 days after transduction with Ad-CMV and Ad-CMV-Rb2/p130

Table 1 mRNA and protein expression levels of the indicated genes in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells, infected with either Ad-CMV-Rb2/p130 or Ad-CMV

mRNA levels were measured using a GEL DOC instrument and normalized with respect to HPRT mRNA, chosen as the RT-PCR control. Protein levels were determined by densitometric analysis and normalized with respect to β -actin protein, the Western-blot control. Each value is the mean $\pm$ S.D. from at least three different experiments. The variations in mRNA and protein expression in Ad-CMV-Rb2/p130-infected cells compared with the control are indicated as follows: +, increased expression; -, decreased expression; n.d., not determined. MAP-2, microtubule-associated protein-2; NSE, neuron-specific enolase; NF160, 160 kDa neurofilament.

Gene	SK-N-BE(2)-C		SH-SY-5Y	
	mRNA	Protein	mRNA	Protein
RB2	+210 $\pm$ 31%	+200 $\pm$ 28%	+300 $\pm$ 35%	+340 $\pm$ 42%
RB	-12 $\pm$ 14%	-40 $\pm$ 7%	-10 $\pm$ 11%	-60 $\pm$ 12%
p53	+8 $\pm$ 14%	+11 $\pm$ 11%	+180 $\pm$ 22%	+180 $\pm$ 22%
p27	+12 $\pm$ 17%	+180 $\pm$ 12%	+220 $\pm$ 28%	+140 $\pm$ 18%
NF160	+11 $\pm$ 10%	n.d.	+210 $\pm$ 23%	n.d.
MAP-2	+220 $\pm$ 14%	n.d.	+150 $\pm$ 16%	n.d.
NSE	+170 $\pm$ 15%	n.d.	+10 $\pm$ 11%	n.d.

Figure 2 Western-blot analysis of pRb2/p130, pRb/p105, p53, p27 and 70 kDa heat-shock protein (hsp 70) proteins in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cell lysates 4 days after transduction with Ad-CMV and Ad-CMV-Rb2/p130

hsp 70, 70 kDa heat-shock protein.

infection, demonstrated that the expression of pRb2/p130 mRNA was greatly enhanced in Ad-CMV-Rb2/p130-transduced cells compared with those infected with control viruses (Ad-CMV and Ad-CMV- β -gal; Figure 1 and Table 1). In contrast, the increase in the corresponding protein (Figure 2 and Table 1) was comparable with that observed in neuroblastoma cells undergoing physiological neuronal differentiation [24,25].

Effects of enhanced pRb2/p130 expression on the cellular machinery: growth suppression and cell-cycle-related genes

It has been demonstrated that pRb2/p130 ectopic expression produces a reduction of cellular proliferation in some tumours *in vivo*, in primary tumour cells and in various established cell lines, such as glioblastomas and neuroblastomas [5,24–26,28–31]. In order to better clarify the pathway involved in pRb2/p130-induced growth suppression, we analysed the expression levels of

RB, p53 and p27, three genes that play a key role in controlling the G₁/S cell-cycle checkpoint and act through overlapping pathways with pRb2/p130 [32,33]. A role for p27 in pRb2/p130- and pRb-induced growth arrest has recently been proposed [34,35]. Moreover, it was demonstrated that p27 is a key gene in vertebrate neural cell fate determination [36]. Several authors have also shown that the RB gene is involved in neural cell development and survival through p53-dependent and -independent pathways [37].

Our analysis showed that, while no variation in p27 mRNA levels was induced in I-type SK-N-BE(2)-C cells, which express pRb2/p130 ectopically, there was a consistent increase in its expression levels in the N-type SH-SY-5Y cells infected with Ad-CMV-Rb2/p130 (Figure 1 and Table 1). However, when we shifted to protein analysis, we detected increased p27 levels in both cell lines expressing pRb2/p130 ectopically (Figure 2 and Table 1). Thus different translational or post-translational mechanisms must regulate p27 accumulation in the two cell lines.

Following Ad-CMV-Rb2/p130 infection, we did not observe variations in pRb mRNA levels, but we detected a slight decrease of pRb protein in both SK-N-BE(2)-C and SH-SY-5Y cell lines (Figures 1 and 2, and Table 1). On the other hand, p53 showed a different response to increased pRb2/p130 expression: in SK-N-BE(2)-C cells there was no accumulation of p53 mRNA and protein, whereas in the N-type SH-SY-5Y cells, which expressed pRb2/p130 ectopically, we observed a 1.8-fold increase of mRNA and protein levels with respect to Ad-CMV-infected control cells (Figures 1 and 2, Table 1).

Neuronal differentiation markers in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells

SK-N-BE(2)-C human neuroblastoma cells represent a clonal subline of the SK-N-BE(2) parental cell line and display an I phenotype [21]. Due to their capability to be induced towards both the N and S phenotypes and to self-renew by generating multipotent I progeny, SK-N-BE(2)-C cells have been described as multipotent embryonic precursor cells of the peripheral nervous system [23]. In contrast, SH-SY-5Y human neuroblastoma cells are a thrice-cloned subline of the SK-N-SH parental cell line and display an N phenotype, based on morphological observations, detection of specific markers, biochemical enzyme activity and noradrenaline uptake [21].

We thus used the SK-N-BE(2)-C and SH-SY-5Y cell lines as a model of neural/glial precursor and neuroblast-committed cells, respectively. To analyse in depth the role played by pRb2/p130 in neuroblastoma cell differentiation, a first step was to define which specific neuronal markers could allow us to study the differentiation process towards a mature neuronal phenotype in these cell lines. To this end we induced neuroblastoma cells, not infected with adenoviruses, to differentiate by RA treatment [38–41], and then performed a morphological and molecular follow-up study of the differentiation process. Morphological observations confirmed that SK-N-BE(2)-C cells acquired a neuronal phenotype, with small cell bodies and sprouting of several long neurite-like processes with varicosities along their length. SH-SY-5Y cells showed a more complex phenotype: the majority of the cells became larger and flattened, with few processes, while at later times phenotypes similar to SK-N-BE(2)-C differentiated cells appeared.

We then performed a 10-day follow-up study of three neuronal markers: intermediate 160 kDa neurofilament (NF160) [42,43], neuron-specific enolase (NSE) [44] and microtubule-associated protein-2 (MAP-2) [45,46]. The analysis of mRNA levels by semi-quantitative RT-PCR prompted us to define a specific panel

Figure 3 Variation of NF160, MAP-2 and NSE mRNA levels induced by RA treatment

SK-N-BE(2)-C (top panel) and SH-SY-5Y (bottom panel) neuroblastoma cells were treated with 10^{-5} M RA for up to 10 days, total RNA was extracted at the indicated time points and RT-PCR with primers specific for NSE, MAP-2 or NF160 mRNA were performed. The data (means $\pm$ S.D.) represent the percentage of expression with respect to control untreated cells.

of expression variations. NF160 mRNA levels showed a late increase during SK-N-BE(2)-C cell differentiation, reaching a maximum value after 10 days of treatment, and were gradually up-regulated in the neuronal-lineage-committed SH-SY-5Y cells, where they showed a peak and then a decrease (Figure 3). NSE expression levels showed a gradual early increase and reached a maximum value at day 10 of RA treatment in both SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cell lines (Figure 3). MAP-2 expression seemed to be closely related to the formation of neurite-like processes: in SK-N-BE(2)-C cells, we observed a gradual increase in its expression, with timing similar to that observed for neurite formation (Figure 3, top panel). On the other hand, in SH-SY-5Y cells, which acquired a flat phenotype

and then showed some process-bearing cells, MAP-2 mRNA levels first declined and then reached levels similar to untreated cells (Figure 3, bottom panel).

An increase in pRb2/p130 protein levels during RA-induced differentiation of LAN-5 neuroblastoma cells has already been reported [24]. We measured the variation of pRb2/p130 mRNA levels during a 10-day RA treatment, with the aim of confirming that its expression increases during the growth suppression and differentiation process. In both neuroblastoma cell lines we found that pRb2/p130 expression levels increased soon after the beginning of RA treatment, decreased to basal levels after 6–10 days and then increased again only in SK-N-BE(2)-C cells (results not shown).

Figure 4 Phase-contrast photomicrographs of living cells 4 days after Ad-CMV (A and C) and Ad-CMV-Rb2/p130 (B and D) adenoviral transduction

(A, B) SK-N-BE(2)-C neuroblastoma cells; (C, D) SH-SY-5Y neuroblastoma cells. Magnification, $\times 340$.

Induction of differentiation in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells infected with Ad-CMV-Rb2/p130 virus

Morphological and molecular analyses 4 days after viral infection

After the identification of morphological and molecular parameters of SK-N-BE(2)-C and SH-SY-5Y cell differentiation, we infected these lines with Ad-CMV-Rb2/p130 and Ad-CMV recombinant viruses to clarify the role of pRb2/p130 in different phases of neural differentiation.

Morphological observations 4 days after infection showed clearly that SK-N-BE(2)-C cells acquired a neuronal phenotype: they lost their polygonal shape and cell bodies became smaller

with several long neurite-like protruding processes. These modifications were similar to those observed during RA treatment and were pRb2/p130-specific, since no similar changes were detected in control Ad-CMV-infected cells (Figures 4A and 4B). Moreover, 4 days after infection we detected an increase of MAP-2 (2.2-fold) and NSE (1.7-fold) mRNA levels in Ad-CMV-Rb2/p130-infected cells compared with controls (Figure 5 and Table 1). The increase was consistent with MAP-2 and NSE expression patterns observed during RA-induced differentiation. The mRNA levels of NF160 did not show any increase following Ad-CMV-Rb2/p130 adenoviral infection of SK-N-BE(2)-C cells compared with controls (Figure 5 and Table 1).

Figure 5 Agarose-gel electrophoresis of NSE, MAP-2, NF160 and HPRT RT-PCR products in SK-N-BE(2)-C and SH-SY-5Y cell cultures 4 days after transduction with Ad-CMV and Ad-CMV-Rb2/p130

The ectopic expression of pRb2/p130 in SH-SY-5Y cells also gave rise to major morphological modifications resembling those observed during RA treatment: the cells acquired a neuron-like phenotype, the processes became longer and ramified, and we were not able to see the flattened cells observed during RA treatment (Figures 4C and 4D). The expression of neuronal markers also confirms that SH-SY-5Y neuroblastoma N-type cells infected with Ad-CMV-pRb2/p130 undergo major modifications: we detected an increase in both MAP-2 (1.5-fold) and NF160 (2.1-fold) neurofilament mRNA levels 4 days after Ad-CMV-Rb2/p130 infection compared with controls (Figure 5 and Table 1). In contrast, the expression of NSE remained unchanged following pRb2/p130 ectopic expression (Figure 5 and Table 1).

We also examined the correlation between cells being transduced and the number of cells exhibiting morphological changes. With this aim, we infected 100 000 SH-SY-5Y neuroblastoma cells with either Ad-Rb2/p130 or Ad-CMV- β -gal and performed a cell-proliferation assay 6 h, 3 days and 7 days after infection. We also carried out β -gal staining at the same time points. As expected, Ad-Rb2/p130-infected cells showed a reduced proliferation rate compared with cells infected with control virus, since pRb2/p130 ectopic expression inhibits cell-cycle progression [1,25]. At 6 h and 3 days after virus infection, 70 % of the cells transduced with Ad-CMV- β -gal were β -gal-positive. This value decreased to 60 % 7 days after infection. It follows that 70 % of the cells should have expressed the ectopic RB2/p130 gene following Ad-Rb2/p130 virus infection, since we utilized the same experimental conditions as for Ad-CMV- β -gal treatment. The 100 000 plated cells became 262 000 3 days after Ad-CMV- β -gal infection. It could therefore be argued that the 70 000 β -gal-positive cells observed 6 h post-infection would become 183 000 out of the 262 000 cells counted 3 days post-infection, given that 70 % of cells were β -gal-positive at the beginning of the experiment and 3 days later. At the same time the 30 000 plated cells that did not receive the β -gal virus would account for 79 000 out of the 262 000 cells counted on day 3.

We counted 150 000 cells 3 days after Ad-CMV-Rb2/p130 infection, and the 30 % uninfected cells, observed 6 h after viral infection, proliferated at the same rate as the uninfected cells detected in the β -gal viral infection. These cells should account for 79 000 out of the 150 000 cells observed 3 days post-infection. Thus the number of cells expressing the ectopic pRb2/p130 should be 71 000 (150 000–79 000). The same consideration could be made for the 7 day time point and for SK-N-BE(2)C cells. On

the basis of the above considerations we calculated the number of Rb2/p130 infected cells that exhibited morphological change. As stated above, 70 % of infected cells should have expressed the ectopic RB2 gene; 50 % of these cells showed phenotypic modifications.

Morphological and molecular analyses 10 days after viral infection

When studied 10 days after viral infection, morphological observations of the SK-N-BE(2)-C and SH-SY-5Y cell lines infected with Ad-CMV-pRb2/p130 showed clearly that approx. 40–50 % of cells were highly differentiated, showing long and ramified neurites, and that the rest showed an immature phenotype. These latter cells had not been infected by Ad-CMV-pRb2/p130 viruses and had proliferated at a high rate, thus outstripping the numbers of infected cells, which had undergone cell-cycle arrest and differentiation. As expected, the expression of neuronal molecular markers, following the significant expression increase observed 4 days after Ad-CMV-pRb2/p130 viral infection, declined to basal levels 10 days after virus treatment because of immature cells contributing to the pools of extracted mRNAs.

In order to exclude the possibility that the induction of differentiation observed in cells infected with Ad-CMV-pRb2/p130 was a non-specific consequence of growth arrest, we cultured both neuroblastoma cell lines in a proliferation-inhibiting medium, containing only 0.2 % foetal bovine serum, for 3 and 7 days. These growth conditions actually led to an inhibition of proliferation and induction of cell death, but did not induce any morphological or molecular marker modifications. The level of NSE, NF160 and MAP-2 in Ad-CMV-pRb2/p130-infected cells did not increase significantly compared with those infected with control viruses.

Lineage restriction in SK-N-BE(2)-C neuroblastoma cells infected with Ad-CMV-pRb2/p130

SK-N-BE(2)-C neuroblastoma cells are considered as neural crest stem cells, as they can be induced to differentiate experimentally along one of two lineages of neural crest development, i.e. the N and S phenotypes [21,23]. We observed that pRb2/p130 ectopic expression promoted morphological and molecular changes characteristic of the neuroblastic lineage. By contrast, we did not observe any S-type cell following Ad-CMV-Rb2/p130 infection of SK-N-BE(2)-C cells (Figures 4A and 4B). The study of expression of the 2',3'-cyclic nucleotide 3'-phosphodiesterase and vimentin genes, which are molecular markers of S-phenotype differentiation [21], confirmed that pRb2 ectopic expression did not induce Schwann/glia commitment in these cells (results not shown). These observations suggest that pRb2/p130-enhanced expression is able to promote the lineage specification of SK-N-BE(2)-C cells towards a neuroblastic fate. In order to verify whether this process is irreversible, we infected SK-N-BE(2)-C cells with Ad-CMV-Rb2/p130 and Ad-CMV recombinant viruses. Then, 4 days after viral infection, when neuronal differentiation was clearly seen in Ad-CMV-Rb2/p130-treated cells and the non-transduced cells represented only 30 % of the total, the cells were treated for 10 days with the S-phenotype inducer BrdU [47]. It has been demonstrated that BrdU induces the S phenotype and inhibits cell proliferation [47]. For this reason, SK-N-BE(2)-C cells not infected by the Ad-CMV-Rb2/p130 and Ad-CMV viruses did not proliferate during BrdU treatment, in spite of viral transduction, thus biasing the experimental results.

Following viral infection and BrdU treatment, while control cells acquired the typical S phenotype, with large, flat and

Figure 6 Phase-contrast photomicrographs of living SK-N-BE(2)-C neuroblastoma cells after adenoviral transduction and BrdU treatment

Viral infections were performed with Ad-CMV (A) and Ad-CMV-p130 (B). At 4 days after adenoviral transduction, cells were treated with 10^{-5} M BrdU for 10 days. S-phenotype cells represented in (A) are translucent and their cell edges are difficult to identify under phase-contrast microscope. Magnification, $\times 340$.

translucent cell bodies, cells expressing pRb2/p130 ectopically retained their neuron-like morphology, showing numerous elongated processes (Figure 6) and still displayed augmented NSE (1.4-fold) and NF160 (1.5-fold) expression compared with controls. Thus ectopic expression of pRb2/p130 seems to promote a restriction in the range of cell fates available to I-phenotype SK-N-BE(2)-C neuroblastoma cells.

DISCUSSION

Neuroblastoma cell lines consist of a mixed population of various phenotypes; the spontaneous interconversion between these phenotypes is called transdifferentiation [21]. Evidence has been reported demonstrating that the I cell type gives rise to committed N and S phenotypes and thus has to be considered a multipotent embryonic precursor cell of the peripheral nervous system. SK-N-BE(2)-C I-type neuroblastoma cells can be induced towards the N or S phenotype by addition of different compounds to the culture medium: RA effectively induces neuronal differentiation, whereas BrdU promotes induction towards the S phenotype [21,23,47]. Likewise, N-type SH-SY-5Y neuroblastoma cells acquire a more differentiated phenotype following RA treatment [38–41]. Therefore, neuroblastoma cell lines represent a proper model with which to study the mechanisms of neural crest cell differentiation.

These properties prompted us to use these two different neuroblastoma cell lines to investigate the effect of pRb2/p130 ectopic expression at different stages of neural differentiation. We used an adenovirus-mediated gene-delivery method to express pRb2/p130 in the I-type precursor SK-N-BE(2)-C cell line and in the neuronal-lineage-committed N-type SH-SY-5Y cell line.

The ability of pRb2/p130 ectopic expression to induce growth arrest has already been demonstrated in a variety of tumours and cell lines [5,24–26,28–31], including human neuroblastoma cell

lines [24,25]. However, the pathway responsible for this function is not yet well described. The binding of pRb2/p130 to E2F4 is well documented, but only a few targets of this complex have been identified [1,6]. In a recent paper, Howard et al. [34] showed that an inducible pRb2/p130 overexpression system produces inhibition of proliferation in a hamster glioblastoma cell line. The growth suppression is correlated with decreased cyclin A- and cyclin E-associated kinase activity and induction of p27, probably due to inhibition of p27-targeted proteolysis by cyclin E-CDK2 phosphorylation of p27 [34]. Our data could further support the hypothesis that p27 is involved in growth arrest induced by pRb2/p130. In fact, enhanced pRb2/p130 produced a consistent accumulation of p27 protein in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells (Figure 2 and Table 1). It seems that fate restriction of precursor cells and differentiation of committed neuroblasts induced by pRb2/p130 (see below) involves, at least in part, different cell-cycle regulators. In fact, pRb2/p130 ectopic expression seems to activate the p53 pathway in SH-SY-5Y neuroblastoma-committed cells and not in SK-N-BE(2)-C neuroblastoma stem cells (Figures 1 and 2, and Table 1).

To describe the effects of pRb2/p130 ectopic expression in SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells, we tried to identify a panel of specific neuronal differentiation markers, since the presence or up-regulation of different gene products, although widely described in the literature, can vary among cell lines, especially when tumour cells are used. Thus we used RA to differentiate non-infected SK-N-BE(2)-C and SH-SY-5Y neuroblastoma cells and performed a 10-day follow-up of various neuronal markers: NSE, a glycolytic enzyme specifically found in neural tissues [44], NF160, the mid-sized neurofilament [42,43], and MAP-2, a MAP that specifically stabilizes neurofilaments [45,46]. All these genes showed an increase in mRNA levels in both cell lines studied, although with different patterns, as

determined by semi-quantitative RT-PCR (Figure 3). In detail, it appears that MAP-2 and NSE, which are up-regulated during the first 2 days of RA-induced differentiation of multipotent I-type cells, are early markers of neural differentiation, whereas NF160 can be considered a late marker as it shows a delayed increase. We also followed pRb2/p130 expression during RA treatment and, as expected, we observed an increase in its mRNA levels (Figure 3).

Morphological observations and expression analysis of the above-mentioned differentiation-specific markers showed that neuronal differentiation was induced in Ad-CMV-Rb2/p130-infected neuroblastoma cells compared with corresponding control virus-infected cells. In the I-phenotype SK-N-BE(2)-C neuroblastoma cell line we observed an increase in mRNA levels of MAP-2, but not NF160, whereas in the N-phenotype SH-SY-5Y neuroblastoma cell line both cytoskeleton-associated proteins were up-regulated (Figure 5 and Table 1). The data collected during RA-induced differentiation indicate that MAP-2 up-regulation precedes NF160 mRNA increase. Consistent with these results is our observation that multipotent precursor I-type cells expressing pRb2/p130 ectopically solely show an increase in the early differentiation marker MAP-2, whereas N-type cells, which are already committed to a neuronal phenotype, present up-regulated expression of both the early MAP-2 and late NF160 markers when infected with Ad-CMV-Rb2/p130 (Figure 5 and Table 1).

With regard to NSE expression, the increase found in Ad-CMV-Rb2/p130-infected neuroblastoma cells compared with controls is in agreement with the pattern observed during RA-induced differentiation (Figure 5 and Table 1). Conversely, we were not able to detect any variation of this marker in Ad-CMV-Rb2/p130-infected SH-SY-5Y cells (Figure 5 and Table 1). These data suggest that, although clearly differentiated, these cell lines do not acquire a terminally differentiated phenotype in response to pRb2/p130 ectopic expression.

The results described raise an important issue: does cell-cycle withdrawal induced by pRb2/p130 trigger the onset of neuroblastoma cell differentiation? It has been shown that differentiation of neural precursor cells into neurons or glia can occur before cell-cycle exit, close to the final cell-cycle division or in the post-mitotic state, depending on cell type [19,48]. Several reports show that neural crest stem cells give rise to committed neural cells in a cell-cycle-independent fashion [19,23,27]. Indeed, evidence has been reported showing that both SK-N-BE(2)-C [23] and SH-SY-5Y cells [27] acquire neuron-like features in response to RA treatment, but show limited growth inhibition. Further, it has been reported that in neuroectodermal cell lines an episome-generated N-Myc antisense oligonucleotide promotes a reduction in growth rate along with an accumulation of the cells in an intermediate undifferentiated phenotype [49]. In agreement with these data, Galderisi et al. [27] demonstrated that growth arrest without induction of differentiation could be induced in SK-N-BE(2)-C cells by an antisense oligonucleotide targeted to N-Myc mRNA. However, a link between cell-cycle arrest and neuronal differentiation in SH-SY-5Y cells has been suggested [50].

We promoted cell-cycle arrest by serum starvation in both cell lines to exclude the possibility that pRb2/p130-induced neuroblastoma cell differentiation was a consequence of cell-cycle withdrawal. Neither SK-N-BE(2)-C nor SH-SY-5Y neuroblastoma cells showed morphological or molecular modifications after 3 and 7 days in 0.2% serum culture conditions. Thus increased expression of pRb2/p130 plays an active role in the induction of neural differentiation, and this function seems, at least in part, to be independent from the triggering of cell-cycle arrest. Moreover, studies on the role of E2F4, which is the major

partner of pRb2/p130 in the G₀ state of the cell cycle, yielded similar results in nerve growth factor-induced neural differentiation of PC12 cells, thus suggesting a specific, cell-cycle-independent role for the E2F4-pRb2/p130 complex [51]. These results are in agreement with observations that normal neural crest stem cells give rise to lineage-restricted progenitor cells several divisions before their cell-cycle exit [19].

An important feature of stem-cell biology is the dependence on specific signals for the restriction of developmental potential. Several mechanisms that differ in the timing of restriction and the involvement of extrinsic or intrinsic signals have been described. Many extrinsic signals regulate the expression and/or activity of proteins that direct the cell cycle [19,48]. The data reported in this paper show that increased pRb2/p130 levels restrict the repertoire of SK-N-BE(2)-C neuroblastoma precursor cell fate. In fact, they induce SK-N-BE(2)-C cells towards the N, rather than the S phenotype. An intriguing hypothesis is that pRb2/p130 is part of a transduction pathway involved in the acquisition or maintenance of neuronal specification.

Another open question is whether the restriction of cell repertoires promoted by pRb2/p130 ectopic expression in SK-N-BE(2)-C neuroblastoma precursor cells is irreversible. This seems to be the case, as cells infected with Ad-CMV-Rb2/p130 acquire a neuroblastic phenotype and are far less responsive to BrdU-induced S-phenotype differentiation compared with controls (Figure 6). Indeed, flat, translucent cells can be observed in Ad-CMV-Rb2/p130-infected cultures along with neuron-like cells. This can be ascribed to the fact that the adenovirus-mediated delivery method, although highly efficient, does not allow us to infect all the cells in the culture; hence, cells that do not carry the transgene possess a proliferation advantage and acquire the S phenotype following BrdU treatment.

In conclusion, the results reported in this paper led to the hypotheses that: (i) pRb2/p130 is able to promote stable neuronal-lineage specification in a multipotent neural stem cell and produces a more mature phenotype in cells already committed to the neuronal lineage, although its ectopic expression alone is not sufficient to determinate terminal differentiation; (ii) pRb2-induced activity appears independent from its cell-cycle-regulatory properties. The intriguing consequence of these observations is that pRb2/p130 could play an important role in the formation of the nervous system, as its function does not appear to be restricted to a temporal window during neuronal determination and differentiation, as seems to happen for pRb [3,14], but could be required during the full maturation process.

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