Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells.
Kranenburg, O.; Scharnhorst, V.; Eb, van der, A.J.; Zantema, A.

Published in:
Journal of Cell Biology

DOI:
10.1083/jcb.131.1.227

Published: 01/01/1995

Please check the document version of this publication:

- A submitted manuscript is the author's version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Inhibition of Cyclin-dependent Kinase Activity Triggers Neuronal Differentiation of Mouse Neuroblastoma Cells

Onno Kranenburg, Volkher Scharnhorst, Alex J. Van der Eb, and Alt Zantema

Sylvius Laboratory, Department of Molecular Carcinogenesis, Leiden University, PO Box 9503, 2300 RA Leiden, The Netherlands

Abstract. Studies on the molecular mechanisms underlying neuronal differentiation are frequently performed using cell lines established from neuroblastomas. In this study we have used mouse N1E-115 neuroblastoma cells that undergo neuronal differentiation in response to DMSO. During differentiation, cyclin-dependent kinase (cdk) activities decline and phosphorylation of the retinoblastoma gene product (pRb) is lost, leading to the appearance of a pRb-containing E2F DNA-binding complex. The loss of cdk2 activity is due to a decrease in cdk2 abundance whereas loss of cdk4 activity is caused by strong association with the cdk inhibitor (CKI) p27KiP1 and concurrent loss of cdk4 phosphorylation. Moreover, neuronal differentiation can be induced by overexpression of p27KiP1 or pRb, suggesting that inhibition of cdk activity leading to loss of pRb phosphorylation, is the major determinant for neuronal differentiation.
proliferate in normal culture medium but undergo neuronal differentiation in response to DMSO, cAMP, or serum withdrawal (Kimhy et al., 1976; reviewed by De Laat and Van der Saag, 1982; Reagan et al., 1990; Larcher et al., 1992). Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable (Kimhy et al., 1976; De Laat and Van der Saag, 1982). Thus, N1E-115 cell differentiation is an excellent in vitro model system for studying neuronal differentiation. We show that both cdk2 and cdk4 activities strongly decline during neuronal differentiation albeit via different mechanisms: the abundance of cdk2 drops to undetectable levels whereas cdk4 remains present but is inactivated due to association with p27KIP1. Moreover, overproduction of p27KIP1 or pRb efficiently induces differentiation in neuronal precursor cells, implying that down-regulation of cdk activity or overproduction of pRb is sufficient to switch from proliferation to differentiation.

Materials and Methods

Cells, Cell Culture, Transfection, and Differentiation Protocols

N1E-115 mouse neuroblastoma cells (kindly provided by Dr. R. P. de Groot) were cultured in DMEM containing 2% FCS, supplemented with 1% antibiotic-transfectant mixtures were determined by overnight exposure of the cells to calcium phosphate-DNA precipitates as described (van der Eb and Graham, 1980). The cells were then washed three times with PBS and received fresh medium. Transfection efficiencies of approximately 50% are routinely obtained using this procedure on N1E-115 cells. Expression vectors for pRb (pRcCMV-Rb) and p27KIP1 (pCMVX-p27KIP1) were kindly provided by Drs. R. Bernards (Cancer Institute, Netherlands) and T. Hunter (The Salk Institute, La Jolla, CA), respectively.

For differentiation, cells were cultured in DMEM/FCS (2%) supplemented with 1.25% DMSO. Cell cultures were then analyzed for four consecutive days.

Western Blot Analysis

Cells were washed twice with PBS, pelleted, and lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 50 mM NaCl, 0.5% NP-40, 0.1% SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml trypsin inhibitor, 0.1 mg/ml β-glycerophosphate, 0.1 mg/ml NaF, and 1 mg/ml Na3VO4). After 30-min incubation on ice, lysates were centrifuged and protein concentrations were determined with the Bio-Rad kit. Labeled cell extracts were quantified by scintillation counting. 200 μg of cell extracts were incubated with antibody-coated protein A-Sepharose beads under constant rotation. Immunocomplexes were collected by centrifugation and were washed four times with lysis buffer. Immunocomplexes, after boiling in sample buffer, were run on SDS-PAA gels. The [35S] gels were incubated in PPO-DMSO and were subsequently dried. Proteins were visualized by autoradiography. When unlabeled cell extracts were used, the presence of associating proteins was analyzed by Western blotting.

Immunokinase Assays

Cdk2 was immunoprecipitated from 50 μg cell extracts as described above. The complexes were assayed for kinase activity towards histone H1 as follows. Immunocomplexes were washed three times with lysis buffer and once with kinase buffer (20 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM DTT). Subsequently, the beads were resuspended in 50 μl kinase buffer containing 2 μg histone H1, 10 μCi [γ-32P]ATP and 20 μM ATP. The reaction mixtures were incubated at 30°C for 30 min. Reaction mixtures were boiled in sample buffer and run on SDS-PAA gels. Gels were stained with Coomassie blue, dried, and autoradiographed. Quantitation of the signals was performed by phosphorimaging. Cdk4 was immunoprecipitated from 200 μg cell extracts, prepared as described (Matsushima et al., 1994). The immunocomplexes were assayed for kinase activity towards soluble GST-Rb that was purified from bacteria. The beads were washed three times with lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM DTT). Subsequently, the beads were resuspended in 50 μl kinase buffer containing 0.5 μg GST-Rb, 10 μCi [γ-32P]ATP and 20 μM ATP. Reaction mixtures were then incubated at 30°C for 1 h. Gels were stained with Coomassie blue, dried, and autoradiographed.

E2F Gel Shift

Cells were scraped in ice-cold PBS, centrifuged, and taken up in packed cell volumes of lysis buffer (400 mM KCl, 20 mM HEPES, pH 7.6, 1.5 mM MgCl2, 0.5 mM NaF, 1 mM DTT, 1 mM PMSF, 0.1 mg/ml trypsin inhibitor, and 1 mg/ml Na3VO4). Cell suspensions were frozen and subsequently thawed on ice. After centrifugation for 20 min at 13,000 rpm, the concentrations of the cell extracts were determined and 10 μg of extract was used for binding activity to a 3P-labeled double-stranded oligonucleotide (5'-ACTAGTTTCGCGCCCTYFA-3') harboring an E2F binding site. The binding reaction was performed for 30 min at room temperature in 20 μl buffer containing 50 mM KCl, 20 mM HEPES, pH 7.6, 10 mM MgCl2, 10% glycerol, 0.5 mM DTT, 0.1% NP40, 2 μg sonicated salmon sperm DNA, 1 ng of labeled oligo, supplemented with 1 mM PMSF, and 0.1 mg/ml trypsin inhibitor. DNA-protein complexes were separated on a 4% nondenaturing PAA gel and visualized by autoradiography. For supershifts the extracts were incubated for 20 min at room temperature with 1 μg of purified IgG (anti-Rb, Ab2, or anti-PCNA, PC10; Oncogene Science, Inc., Manhasset, NY) before the binding reaction.

V8 Proteolytic Digestion

Radiolabeled proteins were excised from dried SDS-PAA gels. The slices were incubated for 10 min in the wells of the stacking gel in a buffer containing 0.1% SDS, 1 mM EDTA, 2.5 mM DTT, 0.125 M Tris, pH 6.8. The slices were subsequently overlayed with the same buffer containing 20% glycerol and 200 μg V8 protease. The gel was run for 30 min after which the power was shut off. After a 30-min incubation period the power was applied again. Normal procedures were then followed to visualize the degradation pattern.

Results

Neuronal Differentiation Is Accompanied by Changes in Cyclin and cdk Abundances, Loss of cdk2 Activity and Loss of pRb Phosphorylation

When cultured in the presence of DMSO, mouse N1E-115
neuroblastoma cells undergo neuronal differentiation. In the absence of DMSO these cells proliferate with a doubling time of $\sim 16$ h and have a relatively small and rounded appearance (Fig. 1 A). When cultured in the presence of DMSO, cell proliferation ceases (Fig. 1 B), cells flatten, enlarge, and extensive outgrowth of neurites is observed (Fig. 1 A). As neuronal differentiation requires a pRb-mediated cell cycle arrest and pRb function is inhibited by phosphorylation, down-regulation of pRb kinase activity is likely to be of major importance in the proliferation-differentiation switch. Therefore we analyzed the regulation of cyclins, cdks, and pRb during differentiation. During differentiation, the steady-state protein level of the proliferating cell nuclear antigen (PCNA) strongly declines, confirming cessation of cell proliferation (Figs. 1 B and 2). Similarly, the abundance of cyclin D1 declines, whereas cyclin D2 is undetectable and the level of cyclin D3 strongly increases (Fig. 2). The steady-state protein level of the kinase partner for D-type cyclins, cdk4, remains constant throughout the differentiation process (Fig. 2). In addition, the level of cyclin E remains constant, but the level of its kinase partner, cdk2, strongly declines (Fig. 2). Concomitant with the decline in cdk2 abundance, phosphorylation of pRb is lost: in exponentially growing cells the underphosphorylated, rapidly migrating species of pRb (Fig. 2, lower arrow) is hardly detectable, whereas two days after induction of differentiation this form of pRb is, and remains, predominant. The level of pRb does not change significantly (Fig. 2). These results are in agreement with previous studies showing the accumulation of underphosphorylated pRb and the decrease in cdc2 and cdk2 abundance in cells undergoing neuronal differentiation in vivo and in vitro (Gaetano et al., 1991; Slack et al., 1993; Lee et al., 1994; Buchkovic and Ziff, 1994; Yan and Ziff, 1995).

**Loss of pRb Phosphorylation Leads to the Appearance of a pRb/E2F Complex**

Suppression of cell growth by pRb is mediated (in part) through complex formation with E2F transcription factors, leading to their inactivation (Farnham et al., 1993; LaThangue, 1993). We analyzed whether the loss of pRb phosphorylation in response to the differentiation signal (Fig. 2) would lead to the appearance of E2F complexes containing pRb. To this end we prepared extracts of differentiating cells and assayed these extracts for the presence of complexes that could bind to an E2F consensus site. As shown in Fig. 3, one predominant complex is detected in extracts prepared from exponentially growing cells. However, simultaneously with the appearance of underphosphorylated pRb (Fig. 2, day 1), a second, more slowly migrating, E2F complex is observed. To confirm the presence of pRb in the slower migrating complex we incubated the extracts with anti-pRb or control antibodies prior to the binding reaction. Fig. 3 shows that the anti-pRb antibody, but not the control antibody (anti-PCNA), supershifts the slower migrating complex, demonstrating the presence of pRb in this complex. Antibodies directed against cyclin D3 did not affect the migration pattern of the E2F complexes (not shown). These results show that loss of pRb phosphorylation leads to complex formation between pRb and E2Fs, presumably contributing to growth arrest.

![Figure 1](image-url)  
**Figure 1.** Morphology and growth characteristics of N1E-115 cells. (A) Untreated N1E-115 cells are relatively small and have a rounded appearance. After four days of culturing in the presence of 1.25% DMSO the cells have enlarged, flattened, and extensive outgrowth of neurites is observed. (B) Proliferation of N1E-115 cells in normal and differentiation-inducing medium. Cells (100,000) were seeded in 5-cm dishes and the cells were counted for four consecutive days, using a Burker chamber. The values are means and standard deviations of three independent experiments. Bar, 100 μm.

![Figure 2](image-url)  
**Figure 2.** Western blot analysis of cyclins, cdks and pRb. Terminal differentiation of N1E-115 cells was induced by addition of DMSO and cells were harvested with 1-d intervals for four consecutive days. The abundance of the indicated proteins was analyzed using specific antibodies (see Materials and Methods). Note the difference in electrophoretic mobility between hyperphosphorylated pRb (upper arrow) and underphosphorylated pRb (lower arrow). The loss of pRb phosphorylation is concomitant with the decline in cdk2 abundance.
Inhibition of cdk2 and cdk4 Activities via Different Mechanisms

As hyperphosphorylation of pRb occurs during a burst of cyclin E-dependent cdk2 activity late in G1, cdk2 activity may be the major determinant of the pRb phosphorylation state. We therefore assayed the cdk2 kinase activity during differentiation and found that concomitant with the disappearance of hyperphosphorylated pRb and of cdk2, cdk2 activity is lost (Fig. 4).

Although the loss of cdk2 activity may suffice in explaining the loss of pRb phosphorylation, cyclin D-dependent kinases have also been shown to induce phosphorylation of pRb (Sherr, 1994). As shown in Fig. 2, the cyclin D-dependent kinase cdk4 persists, and D-type cyclins (first D1, later D3) are available throughout the differentiation process. Both cyclin D1 and cyclin D3 are capable of activating cdk4 towards pRb in vitro kinase assays and when coexpressed in the baculovirus/insect cell overexpression system (Kato et al., 1993; Matsushime et al., 1994). Therefore we assessed whether the D-type cyclins complex with and activate cdk4 during neuronal differentiation. Cdk4 and cyclin D1 were immunoprecipitated from extracts prepared from differentiating cells and the immunocomplexes were subsequently analyzed for coprecipitating partners by Western blotting. As shown in Fig. 5, decreasing amounts of cdk4 coprecipitate with cyclin D1 and vice versa, demonstrating a decline in the amount of cyclin D1- cdk4 complexes. Furthermore, increasing amounts of cyclin D3 coprecipitate with cdk4 during differentiation, reflecting the increase in cyclin D3 abundance (Figs. 2 and 5). These results demonstrate that cdk4 is complexed to a D-type cyclin throughout the differentiation process. Apart from association to a cyclin partner, the activity of cdk4 is also determined by its phosphorylation state. Phosphorylation of cdk4 by the cdk-activating kinase (CAK) is a prerequisite for its activity (Kato et al., 1994a). We therefore analyzed the phosphorylation state of cdk4 during differentiation by immunoprecipitating the protein from extracts prepared from 32P-labeled cells. As is shown in Fig. 6, phosphorylation of cdk4 is strongly diminished during differentiation (top), whereas the total amount of immunoprecipitated cdk4 does not change, as determined by subsequent Western blot analysis of the same gel (bottom). The observation that CAK-mediated cdk activation is inhibited by binding of the cdk inhibitor (CKI) p27KIP1 to cyclin-cdk complexes (Kato et al. 1994a,b) prompted us to investigate whether the loss of cdk4 phosphorylation during differentiation is accompanied by increased binding of p27KIP1. Cdk4 was immunoprecipitated from extracts prepared from 35S-labeled cells. As shown in figure 7A, a 27-kD protein associates with cdk4 in differentiated, but not in undifferentiated cells. To confirm the identity of p27 we transfected undifferentiated cells with an expression vector encoding p27KIP1 and found that the trans...
Figure 6. Phosphorylation of cdk4 during differentiation. Differentiating N1E-115 cells were labeled for 3 h with [32P]orthophosphate. Subsequently, cdk4 was immunoprecipitated and samples were analyzed by SDS-PAGE and subsequent autoradiography. The amount of phosphate incorporated into cdk4 is strongly decreased during differentiation (top). The same gel was then analyzed for the presence of cdk4 protein by Western blotting. The lower panel shows that the amount of immunoprecipitated cdk4 protein does not change during differentiation (see also Fig. 2).

Inhibition of cdk Activity Is Sufficient to Induce Neuronal Differentiation

The above mentioned experiments show that cdk2 and cdk4 activities strongly decline and that pRb is activated when neuronal differentiation is induced. These data, together with experiments demonstrating that loss of RB1 impairs neuronal differentiation (Lee et al., 1994; Williams et al., 1994), suggest that the neuronal proliferation-differentiation switch requires activation of pRb, presumably resulting from loss of cdk2 and cdk4 activities. This raises the question whether overproduction of pRb, or inhibition of cdk activity, would be sufficient to induce neuronal differentiation. We chose to address this question by transfecitng neuronal precursor cells with expression vectors for pRb or the cdk2/cdk4-inhibitor p27KIP1 (Toyoshima and Hunter, 1994), together with a lacZ expression vector as transfection marker. Analysis of DNA synthesis in the transfected (blue) cells showed that pCMV-Rb and pCMV-p27, but not the control vector, efficiently inhibited N1E-115 cell cycle progression (not shown). Four days after transfection, cells were stained for β-gal activity and the morphology of transfected (blue) cells was examined microscopically. As shown in Fig. 1, A, differentiated cells are easily distinguishable from undifferentiated cells. Fig. 8 A shows that transfection of neuronal precursor cells with expression vectors for pRb or p27KIP1, but not with the control vector, efficiently stimulated differentiation. Up to 48% of the transfected cells showed a fully differentiated phenotype as characterized by cell flattening, enlargement, and extensive outgrowth of neurites (Fig. 8, A and B). Expression of pRb was determined by Western blot analysis (Fig. 8 C) and expression of p27KIP1 was confirmed by immunoprecipitation with cdk4 (not shown, see also Fig. 7 A). The anti-pRb Western also shows that underphosphorylated forms of pRb are readily detectable in cells transfected with pRb or p27KIP1, but not in cells transfected with the control vector, demonstrating that overproduction of either pRb or p27KIP1 yields active pRb in these cell populations (Fig. 8 C). Moreover, transfection of pRb or p27KIP1 expression plasmids into N1E-115 cells greatly enhanced the expression of cyclin D3, which is, as shown in Fig. 2, confined to differentiated neurons (Fig. 8 C). These results demonstrate that overproduction of pRb, or inhibition of cdk activity by over-
terminal differentiation in cell cultures transiently transfected with the control vector (lane 1), pCMV-pRb (lane 2), or pCMV-p27KIP1 (lane 3). The cyclin D3 levels in a culture of terminally differentiated neurons is shown as a reference (lane 4). Western blot analysis for p27KIP1, (bottom) shows that underphosphorylated, active pRb (bottom arrow) accumulates in cells transfected with either pCMV-Rb (lane 2) or pCMV-p27KIP1 (lane 3) expression vectors but not in cells transfected with the control vector (lane 1). The presence of p27KIP1 was confirmed by coimmunoprecipitation with cdk4 (not shown, see also Fig. 7 A). Bar, 100 μm.

Figure 8. Induction of terminal neuronal differentiation by overexpression of pRb or inhibition of cdk activity. (A) Untreated cycling precursor cells were transfected with expression vectors for pRb or p27KIP1 in five-fold excess over a lacZ expression vector. In total, 12 μg (10-2) of plasmid DNA was transfected. After transfection, the cells were cultured for 4 d in normal growth medium. The cells were then fixed on the dishes, stained for β-gal activity and photographed. Cells transfected with the control vector retain an undifferentiated morphology (top), whereas up to 50% (see below) of the cells transfected with pCMV-Rb (bottom) or pCMV-p27KIP1 (not shown) are differentiated. (B) Microscopic analysis of percentage of differentiation in response to pRb or p27KIP1 overproduction. Cells were scored “differentiated” if the length of neurite extensions was at least the diameter of one cell body. The depicted values are means and standard deviations of four independent experiments. (C) The abundance of cyclin D3 was tested as a marker for terminal differentiation (Reagan et al., 1990). Thus far, all reports describing the regulation of cdk activity during terminal differentiation pathways show that these activities decline (Gaetano et al., 1991; Buchkovic and Ziff, 1994; Kato et al. 1994b; Kiyokawa et al., 1994; Rao et al., 1994; Kiess et al., 1995; Yan and Ziff, 1995).

Discussion

The close correlation between cell growth and the phosphorylation state of pRb was noted several years ago (Buchkovic et al., 1989; Chen et al., 1989; Mihara et al., 1989). The loss of pRb phosphorylation in non-proliferating cells correlates with loss of activity of the pRb kinases cdk2 and cdk4 (Dulic et al., 1993; Ewen et al., 1993; Koff et al., 1993; Kato et al., 1994b; Kiyokawa et al., 1994; Polyak et al., 1994b; Rao et al., 1994; Kiess et al., 1995). In the present study we have demonstrated that during neuronal differentiation both cdk2 and cdk4 activities decline. However, inactivation of these kinases was found to occur through different mechanisms. Cdk2 activity is lost simply because of a strong decline in steady-state levels of cdk2 protein, whereas cdk4 activity is regulated in a more complex way. Phosphorylation of cdk4 is lost, possibly mediated by p27KIP1. It has recently been reported that during a CAMP-induced growth arrest of murine macrophages, cdk4 activation by CAK is inhibited by association of p27KIP1 to the kinase complex (Kato et al., 1994b). Interestingly, N1E-115 cells differentiate in response to elevated CAMP levels and it has been shown that CAMP levels rise in response to DMSO treatment of these cells (Reagan et al., 1990). Thus, during DMSO-induced differentiation CAMP may mediate the induction of p27KIP1 binding to cdk4, leading to loss of cdk4 phosphorylation and activity. p27KIP1 may also directly inhibit the activity of CAK-phosphorylated cdk4 (Polyak et al., 1994a,b). In addition to an increase in intracellular CAMP levels DMSO treatment also causes an increase in intracellular Ca2+ levels (Reagan et al., 1990) and an activation of membrane-bound tyrosine kinase activity (Rubin and Earp, 1983; Srivastava, 1984; Barnekow and Gessler, 1986). These responses may well contribute to the induction of differentiation by DMSO. First, a rise in intracellular Ca2+ stimulates neuroblastoma cell differentiation (Reboulleau, 1985). Second, activation of the receptors for neurotrophins and fibroblast growth factors results in enhanced tyrosine kinase activity (revisited by Marshall, 1995). In response to sustained activation of these kinases, PC12 pheochromocytoma cells undergo neuronal differentiation (Marshall, 1995). Since the signal transduction pathways underlying NGF-induced (tyrosine kinase-dependent) and CAMP-induced differentiation function independently (Szeberenyi et al., 1990), DMSO may induce differentiation by activating distinct signalling pathways. The CAMP-dependent pathway may lead to activation of p27KIP1, whereas the tyrosine kinase-dependent pathway may generate a signal that is similar to that evoked by NGF or FGF in PC12 cells. Yet another differentiation-inducing signal for neuroblastoma cells including N1E-115 is serum withdrawal, implying that serum contains differentiation-inhibiting signalling molecules (De Laat and Van der Saag, 1982). However, serum withdrawal also leads to massive neuronal apoptotic cell death (O. Kranenburg, unpublished observation; reviewed by Johnson and Deckwerth, 1993).
Received for publication 4 May 1995 and in revised form 21 June 1995.

**References**


Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A.


Herrup. 1994. Dual roles of the retinoblastoma protein in cell cycle regula-


Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Wein-

traub. 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a


Neuronal differentiation triggered by blocking cell proliferation. Cell

Growth & Differ. 3:627-635.


versus sustained extracellular signal-regulated kinase activation. Cell. 80:

179-185.


Mihara, K., X.-R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A.


sphorylation by the human retinoblastoma gene product. Science (Wash. DC).

246:1300-1303.


Koff. 1994a. p27KIP1, a cyclin-dependent kinase inhibitor links transforming growth factor-

β and contact inhibition to cell cycle arrest. Genes & Dev. 8:9-22.


Tempst, and J. Massague. 1994b. Cloning of p27KIP1, a cyclin-dependent ki-

nase inhibitor and a potential mediator of extracellular antimitogenic sig-


vents activation of gene transcription by myogenic basic helix-loop-helix reg-


tion of angiogenisin II receptors by in vitro differentiation of murine NIE-115


Rebouche, C. P. 1986. Extracellular calcium-induced neuroblastoma cell dif-


46:920-930.

Robanus-Maandag, E. C., M. van der Valk, M. Vlaar, C. Feltkamp, J. O’Brien,

M. van Roon, N. van der Lugt, A. Berns, and H. te Riele. 1994. Develop-

mental rescue of an embryonic-lethal mutation in the retinoblastoma gene in

chimeric mice. EMBO J. 13:4261-4268.

Rubin, R. A., and H.S. Earp. 1982. Dimethyl sulfoxide stimulates tyrosine resi-
due phosphorylation of rat liver epidermal growth factor receptor. Science

(Wash. DC). 219:60-63.


Skapek, S. X., J. Rhee, D. B. Spicer, and J. B. Lasar. 1995. Inhibition of myo-
genic differentiation in proliferating myoblasts by cyclin D1-dependent ki-


1993. Regulated expression of the retinoblastoma gene in differentiating em-

bryonal carcinoma cells. Oncogene. 8:1585-1591.

Srivastava, A. K. 1985. Stimulation of tyrosine protein kinase activity by de-


Ha-ras mutan on neuronal differentiation of PC12 cells. Mol. Cell. Biol. 10:

5324-5332.


in Xenopus embryos converts ectodermal cells to a neural fate. Genes &

Dev. 8:1434-1447.

Van der Eb, A. J., and F. L. Graham. 1980. Assay of transforming activity of tu-
mor virus DNA. Method Enzymol. 65:926-939.


retinoblastoma protein regulates nuclear c-Ab1 tyrosine kinase in the cell


Weinberg, and T. Jacks. 1994. Extensive contribution of Rb-deficient cells to

adult chimeric mice with limited histopathological consequences. EMBO J.

13:4251-4259.

Yan, G.-Z., and E. B. Ziff. NGF regulates the PC12 cell cycle machinery

through specific inhibition of the cdk kinases and induction of cyclin D1. J.