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Oncogenically activated or ligand-stimulated *neu* kinase stimulates neurite outgrowth in PC12 cells

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Abstract Retroviral vectors pDOL/NeuN and pDOL/NeuT were used to express normal and transforming rat neu cDNAs in PC12 cells. DOL/NeuT-infected cells exhibited a high frequency of spontaneous neurite outgrowth while DOL/NeuN-infected cells showed neurite outgrowth in the presence of heregulin, a putative ligand for the neu receptor tyrosine kinase. In both cases, neurite outgrowth was preceded by phosphorylation of $p185^{neu}$ and several other cellular proteins. Thus the neu tyrosine kinase can elicit morphological and biochemical changes resembling, but distinct from, those stimulated by NGF, and heregulin stimulates neu to elicit these effects in PC12 cells.

Key words: neu/erbB2; Heregulin; Tyrosine kinase; Signal transduction; Neuronal differentiation; PC12 cell

1. Introduction

The human c-erbB2 tyrosine kinase and its rat homolog, neu, together with erbB3 [1] and erbB4 [2], are members of a family of transmembrane receptor tyrosine kinases for which the epidermal growth factor (EGF) receptor is the prototype [3]. As gene amplification and overexpression of each of these has been associated with various cancers, there is considerable interest in identifying their respective ligands and further understanding the mechanisms by which these receptors are activated. Recently, a family of peptides related to EGF have been identified as possible candidates for the neu/erbB2 ligand. The first of these was heregulin, a factor purified from medium conditioned by human mammary carcinoma cells [4], and its rat homolog, the neu differentiation factor (NDF) [5]. More recently, acetylcholine receptor-inducing activity (ARIA) [6], and glial growth factor [7] were discovered and found to be members of the heregulin family. The tissue distribution of these factors suggests that neu/erbB2 may not only be involved in regulating the growth and survival of mammary epithelial cells, but also may play a previously unappreciated role in the development and differentiation of the nervous system. It was also somewhat surprising to find that while each of these factors will promote autophosphorylation of neu/erbB2 protein in certain cells, not all cells that express neu/erbB2 bind or respond [8]. These findings call into question the role of neu/erbB2 in heregulin signalling. Recent studies in fact suggest that the erbB3 [9,10] or erbB4 proteins are necessary for heregulin-responsiveness.

In order to further examine the possible participation of neu/erbB2 and its putative ligand heregulin in neuronal activities, we have used the well established model for neuronal differentiation, the rat pheochromocytoma (PC12) cell line. We felt it was of interest to address two important questions regarding neu/erbB2 function. The first was whether the activated (transforming) neu tyrosine kinase was capable of eliciting neurite extension in a manner similar to the NGF-stimulated *trk* proto-oncogene product [11–14] or if neu/erbB2 behaved in a manner more similar to another member of the subclass I receptor tyrosine kinases, the EGF receptor. If the former were true, we then were interested in determining if PC12 cells expressing normal neu were capable of responding to heregulin and yielding a phenotype similar to that elicited by transforming neu.

2. Materials and methods

2.1. Materials

NGF (2.5S) was purchased from Boehringer-Mannheim (Indianapolis, IN). Anti-phosphotyrosine antibody was purified from the culture medium of FB2 hybridoma cells, obtained from ATCC. Anti-neu antibodies were either a rabbit polyclonal provided by Dr. Randy Schatzman, Syntax Co., Palo Alto, CA or anti c-neu Ab-3 from Oncogene Science (Uniondale, NY). The plasmids pDOL/NeuN and pDOL/NeuT were kindly provided by Dr. David Stern, Yale Medical School. Recombinant heregulin (rHRG-$\beta 1_{177-241}$) was provided by Dr. Mark Sliwkowski, Genentech, Inc.

2.2. Cell culture

PA317 retrovirus packaging cells [16] were grown in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum. PC12 cells were propagated on tissue culture plastic in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Where indicated, neurite outgrowth was tested in dishes that had been coated with collagen (rat tail, Type I) according to the supplier's (Sigma) recommended protocol.

2.3. Retrovirus-mediated transfer of NeuN and NeuT genes

Plasmids pDOL/NeuN and pDOL/NeuT [17] were transfected into PA317 retrovirus packaging cells [16] by the calcium-phosphate technique [18]. Stable-transfected packaging cells were selected in 500 μg/ml (active) antibiotic G418 (Geneticin, GIBCO). PC12 cells were seeded at 1×10^6 per 60 mm dish one day prior to infection. Virus stocks consisted of media harvested from the packaging cells, filtered through 0.4 micron filters, and supplemented with heat-inactivated horse serum (5%) and polybrene (6 μg/ml). Infections were carried out by incubating PC12 cells in virus stocks for 24 h. Cells from each 60 mm plate were then split onto three 100 mm dishes and maintained without passage for 3 to 5 weeks in medium containing G418 (400 μg/ml, active).

2.4. Immunoprecipitation and Western blot analysis

On the day prior to immunoprecipitation, the cell medium was changed to DMEM plus 0.5% FBS with or without 1 μM sodium orthovanadate. Where indicated, growth factors or TPA were added directly to the medium for a 5-min incubation. Cells were then washed in cold Tris-buffered saline (TBS) and suspended in lysis buffer consisting of TBS plus 1% NP-40, 10% glycerol, 1 mM phenyl-methylsulfonylfluoride, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 100 μM ammonium molybdate, and 1 mM sodium orthovanadate. The lysates were incubated 15 min on ice after which insoluble materials were pelleted in a microfuge (10 min). Each supernate was then incu-

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bated for 2 h on ice with 30 μl of 50% suspension of protein A-Sepharose which had been preincubated with anti-phosphotyrosine antibody. The immune complexes were pelleted, boiled in 40 μl sample buffer, fractionated by SDS-PAGE (8% acrylamide) and transferred to nitrocellulose. The blots were blocked in 3% BSA in TBS plus 0.05% Tween 20 for 1 h at room temperature and incubated overnight in antibody (either anti-PY of anti-neu). Bound antibody was detected using [^{125}I]protein A (NEN).

3. Results

3.1. Infection of PC12 cells with DOL/NeuN and DOL/NeuT viruses

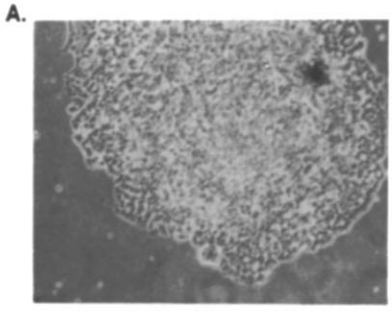
Retroviral infection of PC12 cells followed by selection in G418, yielded discrete, clonal, clusters of cells. These clones could be classified into three groups based on the morphology of their cells (Fig. 1). One type consisted of tightly packed, undifferentiated cells (Fig. 1A). A second type was similar to the first except that cells with neurite-like processes were seen at the periphery (Fig. 1B). The third type consisted of dispersed cells of which the majority displayed long neuritic processes (Fig. 1C). Clones of cells infected with the DOL/NeuN (encoding the normal neu tyrosine kinase) virus or with control constructs were indistinguishable from one another and generally appeared undifferentiated; neurite extension was rare (Fig. 1D, and data not shown). In contrast, cells in the majority of primary clones infected with DOL/NeuT virus (encoding the transforming neu protein) had neurites. These results suggested that transforming neu promoted differentiation of PC12 cells. Because of the variability noted among PC12 cells with different passage histories, we performed several infections of PC12 cells obtained from another laboratory, (Dr. M. Chao, Cornell Medical School). Again, cells infected with ZIP or DOL control vectors were indistinguishable from uninfected cells, whereas infection with DOL/NeuT virus consistently yielded populations of cells which displayed a high frequency of spontaneous neurite outgrowth (Fig. 2A).

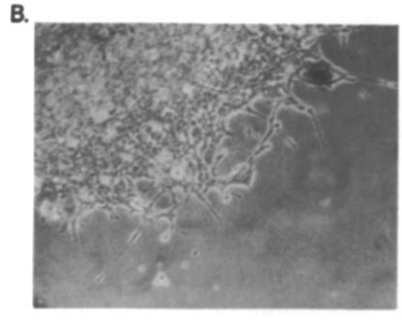
3.2. Response of DOL/NeuN- and DOL/NeuT-infected cells to NGF, dbcAMP, TPA, and sodium orthovanadate

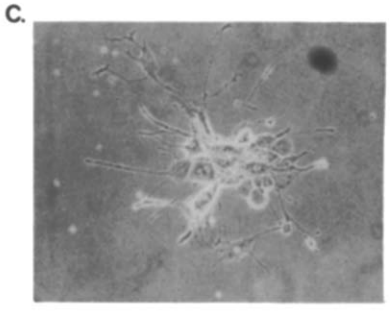
We found no evidence that infection with DOL/NeuN, DOL/NeuT, or control viruses consistently altered neurite outgrowth or cell growth rate in the presence of NGF at concentrations ranging from 0.1 to 100 ng/ml (not shown). Dibutyryl-cyclicAMP (dbcAMP) at a low concentration (40 μM) caused very little outgrowth in control or DOL/NeuN-infected populations but caused a substantial increase in the number of neurite-bearing DOL/NeuT-infected cells (Fig. 2B). Higher concentrations (0.2 mM) of dbcAMP caused significant neurite outgrowth (20–50%) in uninfected, vector-infected, and DOL/NeuN-infected cells, and almost complete (i.e. greater than 80%) neurite extension in DOL/NeuT-infected cells. After elaborating neurites in the presence of dbcAMP, many DOL/NeuT-infected cells remained viable and retained their neurites for up to two weeks in the absence of serum or NGF. Uninfected, ZIP-infected, DOL/NeuN-infected, and undifferentiated DOL/NeuT-infected PC12 all died in these conditions (not shown).

Incubation with the protein kinase C-activating phorbol ester, TPA, did not promote neurite outgrowth in uninfected PC12 cells or control vector-infected cells, but did elicit a small, but reproducible, increase in the number of neurite-bearing cells in populations of both DOL/NeuN- and DOL/NeuT-infected cells (Fig. 2C).

While testing conditions for the study of tyrosine phosphorylation, we observed that PC12 cells incubated with subtoxic levels of sodium orthovanadate (≤ 5 μM) tended to display neurite-like processes. With uninfected, vector-infected, and DOL/NeuN virus-infected PC12 cells, these processes were generally too short to be counted as neurites (arbitrarily defined







D. Number of Clones (%)

	pDOL/NeuN	pDOL/NeuT	pZIpGpA
A.	25 (67.6)	2 (5.6)	26 (70.3)
B.	11 (29.7)	14 (38.9)	10 (27.0)
C.	1 (2.7)	20 (55.6)	1 (2.7)

Fig. 1. Morphologies of cells in clones after 3 weeks of selection in G418. The cells were categorized as (A) Undifferentiated, (B) Intermediate or mixed, in which typical colonies appeared as a tight cluster of undifferentiated cells with a few cells at the edge showing neurite-like processes or (C) neurite-bearing. (D) Table showing the number and percentage of clones fitting categories A,B,C typically appearing after infection with Neu+ retroviruses or a control pZIP-based viral construct carrying an unexpressible, unrelated gene.

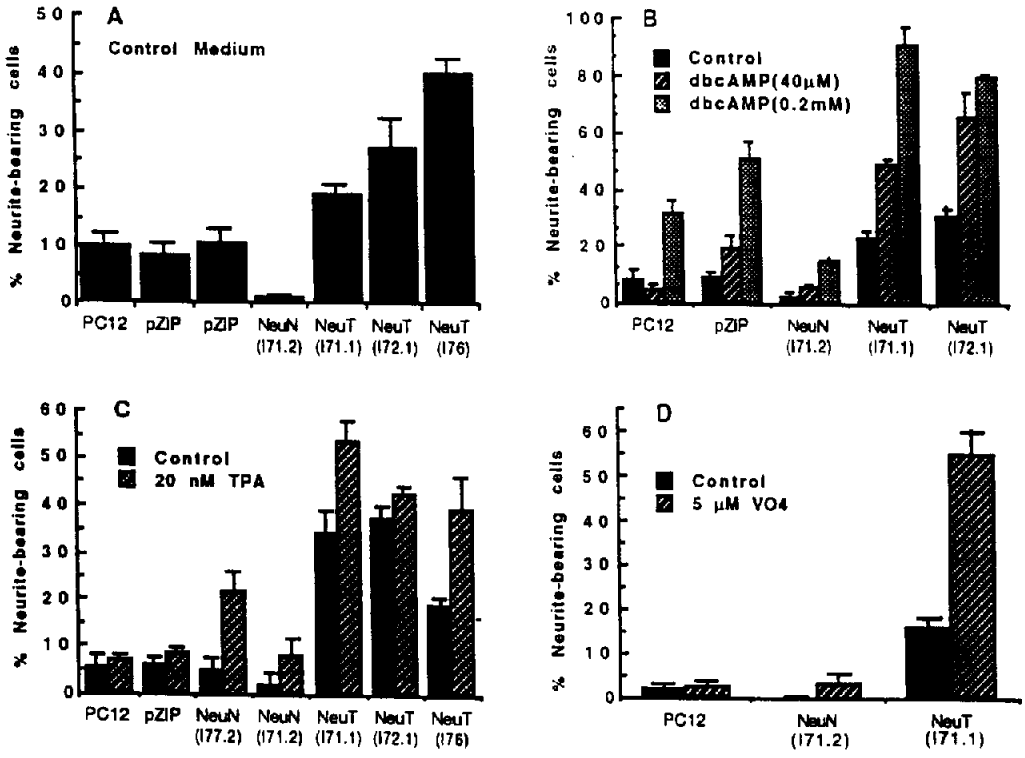


Fig. 2. Spontaneous and induced neurite outgrowth in infected and uninfected PC12 cells. Cells were plated and allowed to settle overnight in DMEM supplemented with 10% FBS and 5% horse serum. Media were then (day 0) exchanged for DMEM with 1% FBS and 0.5% HS with or without (control) dbcAMP, sodium orthovanadate, or TPA. Neurites were scored in three randomly selected fields in a phase contrast microscope. The values represent the weighted mean ± S.E. (A) Neurites scored at day 2 in control medium. Shown are the parental PC12 line, two lines independently infected with control vector ZIP, one line infected with DOL/NeuN (designated 171.2), and three lines independently infected with DOL/NeuT (171.1, 172.1 and 176). (B) Neurites scored at day 2 of incubation in the presence or absence of dbcAMP. (C) Neurites scored at day 1 of incubation in the presence or absence of 20 nM TPA. (D) Neurites scored at day 1 of incubation in the presence or absence of sodium orthovanadate.

as processes longer than two cell body diameters). In contrast, incubation of DOL/NeuT-infected cells with sodium orthovanadate resulted in a dramatic increase in the percentage of cells bearing neurites (Fig. 2D).

3.3. The effect of heregulin

The percentage of PC12 or vector control cells bearing neurites was not significantly altered during a three day incubation with recombinant heregulinβ1 (HRG), at levels up to 10 nM (Fig. 3). DOL/NeuT-infected PC12 cells also showed no morphological changes in response to HRG alone (data not shown). However, neurite outgrowth was observed in DOL/NeuN-infected PC12 cells incubated with HRG at concentrations of 1 nM and lower (Fig. 3 and data not shown). When heregulin was added together with 2 nM TPA, neurite extension was increased in PC12 cells expressing either normal neu or the transforming neu protein (Fig. 3).

3.4. Protein tyrosine phosphorylation

Anti-phosphotyrosine immunoprecipitates from DOL/NeuT-infected cells contained neu (M_r ~ 185 kDa), the phosphorylation of which was enhanced during incubation in sodium orthovanadate (Fig. 4, compare lanes 3 and 4). Similarly, treatment of DOL/NeuN-infected cells with heregulin resulted in the rapid phosphorylation of neu (Fig. 4, compare lanes 1 and 2). We also found that heregulin stimulated the phosphorylation of the same group of proteins that were detected in cells expressing the transforming neu protein (data not shown). The major proteins in anti-phosphotyrosine immunoprecipitates of orthovanadate-treated, DOL/NeuT-infected cells or heregulin-treated, DOL/NeuN-infected cells (e.g. M_r ~ 125 kDa, 95 kDa, 75 kDa, and 70 kDa) comigrated with a subset of proteins that are phosphorylated after treatment with NGF or EGF (data not shown).

4. Discussion

The biochemical events following the activation of different receptor tyrosine kinases are highly regulated and specific in PC12 cells, as evidenced by the differing effects of EGF and NGF. In the present study, we find that the expression of the transforming neu protein in PC12 cells elicits a high frequency of spontaneous neurite outgrowth and an enhanced responsiveness to agents that are known to act synergistically with NGF. Expression of the normal neu tyrosine kinase in PC12 cells correlated with an acquired responsiveness to heregulin and an increased tendency to form neurites in the presence of the protein kinase C-activator, TPA. In these respects, DOL/NeuT- and heregulin-stimulated DOL/NeuN-infected cells behaved like cells receiving NGF. Thus, the actions of neu/erbB2 and the EGF receptor are distinct in both oncogenically-activated and growth factor-stimulated context in PC12 cells.

Neurite extension from the DOL/NeuT-infected cells was enhanced when the cells were incubated with sodium orthova-

nadate, an inhibitor of tyrosine phosphatases. This was accompanied by the apparent autophosphorylation of neu and the elevated phosphorylation of several cellular proteins. These results suggest that the tyrosine kinase activity is constitutively higher in DOL/NeuT-infected cells, compared to control cells, and are consistent with the proposal that the activated neu tyrosine kinase is responsible, either directly or indirectly, for eliciting neurite outgrowth.

Some differences were observed in the characteristics of the biological responses elicited by activated neu and trk. For example, neither the NeuT- nor the NeuN-containing cells showed an enhanced sensitivity to NGF. Thus, the neu tyrosine kinase did not appear to be capable of 'priming' cells as occurs upon the prior exposure of PC12 cells to NGF or upon the expression of the v-*src* tyrosine kinase [19]. PC12 cells expressing NeuT also did not show any changes in their responsiveness to EGF, whereas NGF normally causes EGF receptor down-regulation [20]. An additional difference is that our lines of DOL/NeuT-infected PC12 cells have continued to proliferate at normal rates, while the addition of NGF to PC12 cells, as well as the expression of the v-*src* and *ras* oncogenes, cause these cells to stop proliferating [19,21,22]. The latter findings with NeuT may be related to the fact that this oncogene was first identified in cells that both proliferate and extend neurites, and it might not be expected that transforming (activated) neu would cause PC12 cells to withdrawal from the cell cycle. Taken together, these results suggest that while the NGF receptor/*trk* oncogene product and the neu/erbB-2 tyrosine kinase both elicit neurite extension in PC12 cells, they do not share identical signaling pathways in these cells.

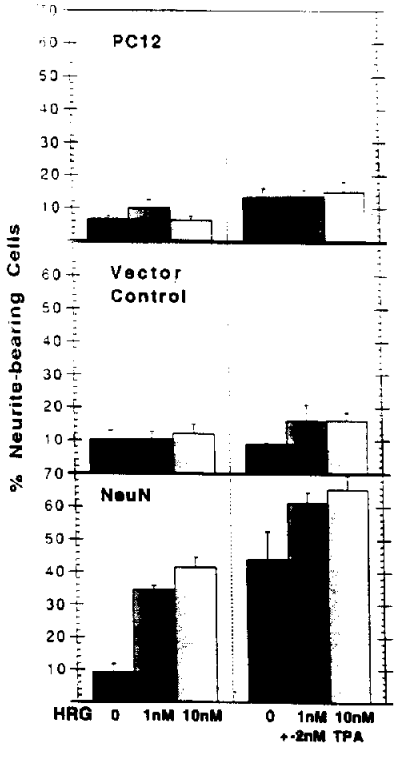


Fig. 3. Heregulin and TPA-stimulated neurite outgrowth in control and DOL/NeuN-infected PC12 cells. Plating and conditions were as described in Fig. 2. Neurites were scored at day 3 after addition of heregulin and/or TPA.

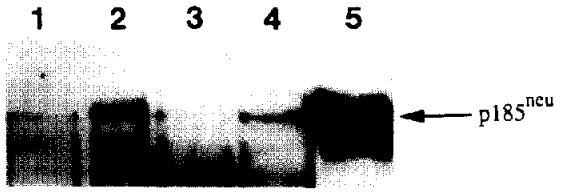


Fig. 4. Tyrosine phosphorylation in control and DOL/Neu-infected PC12 cells. Lanes 1 and 2: DOL/NeuN-infected cells (I77.2) were incubated 18 h in DMEM with 0.5% FBS and 1 μM sodium orthovanadate followed by no further treatment (lane 1) or a 5 min incubation in 10 nM heregulin (lane 2). Lanes 3 and 4: DOL/NeuT-infected cells (I71.1) were incubated 18 h in DMEM with 0.5% FBS without (lane 3) or with (lane 4) 1 μM sodium orthovanadate. Lane 5 shows p185^{neu} immunoprecipitated from an overexpressing fibroblast line, DHFR/G8 [23].

The proposal that p185neu/erbB2 is the receptor for heregulin was based on the observation that this factor binds with high affinity to breast carcinoma cells that overexpress neu/erbB-2, and that this binding stimulates phosphorylation of a protein identified as p185neu/erbB2. However, it has been proposed that unidentified cell-specific factors are required in conjunction with neu/erbB2 to form a functional heregulin receptor [2,9] or that perhaps neu/erbB2 is not the receptor at all. The present studies support a role for neu/erbB2 in mediating a heregulin response in PC12 cells. Apparently PC12 cells express all the components required for responsiveness to heregulin and these become fully functional upon the introduction of NeuN. Sliwkowski et al., have proposed that the erbB3 and neu/erbB2 proteins can act together to confer heregulin responsiveness in some cells [10] and a similar suggestion has been made for erbB4 and neu/erbB2 [24]. We are currently examining our PC12 cells for expression of the erbB3 and erbB4 gene products as a first step toward determining the possible involvement of one of these proteins in the actions of heregulin and neu/erbB2.

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References

[1] Kraus, M.H., Issing, W., Miki, T., Popescu, N.C. and Aarsonson, S.A. (1989) Proc. Natl. Acad. Sci. USA 86, 9193.
[2] Culouscou, J.-M., Plowman, G.D., Carlton, G.W., Green, J.M. and Shoyab, M. (1993) J. Biol. Chem. 268, 18407–18410.
[3] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[4] Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D. and Vandlen, R.L. (1992) Science 256, 1205–1211.
[5] Wen, D., Peles, E., Cupples, R., Suggs, S.V., Bacus, S.S., Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Ben Levy, R., Koski, R.A., Lu, H.S. and Yarden, Y. (1992) Cell 69, 559–572.
[6] Falls, D.L., Rosen, K.M., Corfas, G., Lane, W.S. and Fischbach, G.D. (1993) Cell 72, 801–815.
[7] Marchionni, M.A., Goodearl, A.D.J., Chen, M.S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Stroobant, P. and Gwynne, D. (1993) Nature 362, 312–318.
[8] Peles, E., Ben Levy, R., Tzaharr, E., Liu, N., Wen, D. and Yarden, Y. (1993) EMBO J. 12, 961–971.
[9] Carraway, K.L.I., Sliwkowski, M.X., Akita, R.W., Platko, J.V.,

Guy, P., Nuijens, A., Diamonti, A.J., Vandlen, R.L., Cantley, L.C. and Cerione, R.A. (1994) J. Biol. Chem. 269, 14303–14306.
[10] Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L. and Carraway III, K.L. (1994) J. Biol. Chem. 269, 14661–14665.
[11] Greene, L.A. and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. USA 73, 2434–2428.
[12] Halegoua, S., Armstrong, R.C. and Kremer, N.E. (1990) Curr. Top. Microbiol. Immunol. 165, 119–170.
[13] Klein, R., Jing, S., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991) Cell 65, 189–197.
[14] Kaplan, D., Hempstead, B.L., Martin-Zanca, D., Chao, M.V. and Parada, L.F. (1991) Science 252, 554–558.
[15] Chao, M.V. (1992) Cell 68, 995–997.
[16] Miller, A.D.a.B., C. (1986) Mol. Cell. Biol. 6, 2895–2902.
[17] Bargmann, C.I. and Weinberg, R.A. (1988) EMBO J. 7, 2043–2052.
[18] Chen, C.A. and Okayama, H. (1988) Biotechniques 6, 632–637.
[19] Thomas, S.M., Hayes, M., D'Arcangelo, G., Armstrong, R.C., Meyer, B.E., Zilberstein, A., Brugge, J.S. and Halegoua, S. (1991) Mol. Cell. Biol. 11, 4739–4750.
[20] Huff, K., End, D. and Guroff, G. (1981) J. Cell Biol. 88, 189–198.
[21] Brightman, M.W., Simpson, D.L., Tao-Cheng, J.-H., Bressler, J.P., Okuda, O. and Chang, L. (1990) J. Neurocytol. 19, 776–788.
[22] Rausch, R.M., Lewis, D.L., Barker, J.L. and Eiden, L.E. (1990) Cell. Mol. Neurobiol. 10, 237–255.
[23] Stern, D.F., Heffernan, P.A. and Weinberg, R.A. (1986) Mol. Cell. Biol. 6, 1729–1740.
[24] Plowman, G.D., Green, J.M., Culouscou, J.-M., Carlton, G.W., Rothwell, V.M. and Buckley, S. (1993) Nature 366, 473–475.