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Central role of epidermal growth factor (EGF) receptor density in anchorage-independent growth of normal rat kidney cells

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Abstract Epidermal growth factor (EGF) receptor levels are known to play a central role in density dependent growth regulation of normal rat kidney (NRK) fibroblasts. Here we show that EGF receptor expression is strongly decreased when NRK cells are cultured under anchorage independent conditions, and that expression is returned to original levels upon cell re-adherence. Agents that stimulate anchorage independent growth (AIG) of NRK cells in the presence of EGF are shown to upregulate both EGF receptor promoter activity and ^{125}I-EGF binding capacity. These data show that two aspects of phenotypic transformation of NRK cells, namely density arrest and AIG, can both directly be correlated to EGF receptor levels.

Key words: Normal rat kidney fibroblast; Anchorage independent proliferation; Phenotypic transformation; Epidermal growth factor receptor expression

1. Introduction

Cell cycle progression of non-transformed fibroblasts is regulated by extracellular signals provided by polypeptide growth factors and cell adhesion. In contrast, most tumor cells can proliferate in the absence of externally added growth factors and without cell anchorage. In a number of non-transformed cell lines, polypeptide growth factors are able to induce phenotypic transformation [1], resulting in loss of density dependent growth inhibition (DDGI) and induction of anchorage independent growth (AIG). AIG is regarded as the best in vitro correlate for tumorigenic behavior of cells in vivo [2,3], but the mechanisms underlying AIG are still largely unknown. Several mechanisms have been proposed to play a role in the induction of AIG, including increased production of extracellular matrix proteins such as fibronectin [4,5], an increase in intracellular pH [6], induction of cyclin A [7] and cyclin D1 [8] expression, activation of cyclin E-cdk2 [8], activation of a connective tissue growth factor (CTGF) dependent signalling pathway [9], and modulation of protein-tyrosine phosphatase activity [10,11].

Normal rat kidney (NRK) fibroblasts have been widely used as a model system to study the role of growth factors in phenotypic transformation. When cultured in the presence of epidermal growth factor (EGF) as the only growth stimulating hormone, these immortalized cells have a normal phenotype and undergo DDGI. In the additional presence of modulating factors such as retinoic acid (RA) or transforming growth factor β (TGF-β), which by themselves are not mitogenic for these cells, density arrested NRK cells become responsive again to the growth stimulatory effect of EGF, resulting in loss of DDGI [12–15]. It has been established that the number of EGF receptors in NRK cells decreases with increasing cell density. Therefore, we have postulated that EGF treated NRK fibroblasts become density arrested as a result of a reduction in EGF receptor levels below a critical level from which they can be released by addition of such factors as RA and TGF-β that increase EGF receptor numbers [14,15].

In NRK fibroblasts, a strong parallel has been observed between the growth factor requirements for induction of DDGI and AIG [13,16]. Furthermore, mutant NRK cells have been described in which transforming deficiency is coupled to an inability to proliferate under anchorage independent conditions, suggesting that the same mechanisms may underlie these two aspects of transformation [17]. However, the role of EGF receptor densities in induction of AIG of NRK cells has not been studied directly. In the present study, we show that EGF receptor levels are strongly decreased under anchorage independent conditions and upregulated again when cells are allowed to re-adhere. Agents that induce EGF dependent AIG in NRK cells are shown to upregulate EGF receptor expression in these non-adhered cells. These data indicate that EGF receptor levels may not only control DDGI of NRK cells, but also their ability to grow under anchorage independent conditions.

2. Materials and methods

2.1. Cell cultures

2.1.1. Adherent cells. NRK cells (clone 49F) were plated at a density of 1.0×10^4 cells/cm^2, and grown to confluence in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) newborn calf serum (NCS). Confluent cells were subsequently made quiescent by incubation in serum-free medium (SF) consisting of a 1:1 mixture of DMEM and Ham's F-12 medium, supplemented with 30 nM Na_2SeO_3 and 10 μg/ml human transferrin for 3 days [13].

2.1.2. Anchorage independent growth. Tissue culture plates were coated with poly (2-hydroxyethyl-methacrylate), abbreviated as poly-HEMA, in ethanol as described [18]. One day before each experiment, confluent NRK cell cultures were trypsinized and replated at 50% confluence in order to obtain single cells in a subconfluent, proliferating culture. The next day, cells were trypsinized again and seeded in SF medium supplemented with 5 μg/ml insulin, 0.2% bovine serum albumin (BSA), and 10% (v/v) growth factor inactivated fetal calf serum (SFS medium) [19].

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2.2. MTT assay for anchorage independent growth

A total of 1.5×10^4 cells were seeded in 130 μl per well of SFS medium in 96-well tissue culture plates coated with poly-HEMA. Subsequently, growth factors to be tested were added to the cells in 20 μl binding buffer (DMEM supplemented with 0.1% BSA and 50 mM BES, pH 6.8; see [19]). After incubation for 5 days, 15 μl MTT ((3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide), 5 mg/ml) in phosphate buffered saline (PBS) was added and incubated for 4 h. The produced MTT formazan was solubilized by addition of 100 μl of SDS solution (20% (w/v) sodium dodecyl sulfate in 0.1 M HAc), and the absorbance was measured after 24 h at 570 nm, relative to the absorbance at 690 nm, using a microplate reader [18].

2.3. EGF receptor promoter activity

2.3.1. Vectors. EGF receptor gene promoter activity: Luciferase reporter gene vector pSVOALΔ5′ containing the 5′ region of the human EGF receptor gene (−1100 to −19 relative to the ATG translation site) [20] was kindly provided by Dr. G.N. Gill (University of California, San Diego, CA, USA). PDGF α-receptor gene promoter activity: Luciferase reporter gene vector (pSLA4) containing the −441 to +118 region of the human platelet derived growth factor α-receptor (PDGFαR) gene promoter (−441/+118LUC) [21]. To allow for selection of stable transfectants, a neomycin resistance encoding vector (pcDNA I/NEO, Invitrogen) was cotransfected.

2.3.2. Transfections. NRK cells were seeded at a density of 4.0×10^4 cells/cm^2 in 80 cm^2 tissue culture dishes, 1 day prior to transfection. Plasmid DNA was transfected using the calcium phosphate precipitate method [22]. Subsequently, the cells were incubated for 3 days in DMEM+10% NCS, after which 700 μg/ml geneticin was added to start the selection of the transfected cells. The surviving cells were trypsinized and cultured in the presence of geneticin (300 μg/ml) for 3 weeks.

2.3.3. Assay of luciferase activity. The stably transfected cells were cultured under the same experimental conditions as described above for the parental cells. Luciferase activity was assayed in a LKB 1250 luminometer, using the Promega luciferase assay kit. Luciferase activity was corrected for cell numbers by measuring protein concentrations according to the Lowry method using BSA as standard.

2.4. mRNA expression

EGF receptor mRNA expression was monitored semi-quantitatively by the 'primer-dropping' RT-PCR method which makes it possible to distinguish changes in gene expression of less than twofold to greater than 75-fold [23]. Optimal cycle numbers were determined as described [23]. Under these conditions competitive interference was absent and twofold changes in the abundance of PCR products were detected over a span of 4–5 cycles, making this method suitable to monitor EGF receptor mRNA expression [14].

2.5. EGF binding studies in non-adherent cells

A total of 0.5×10^6 cells were seeded in 1 ml SFS medium in 24-well tissue culture plates (adherent) or in 24-well tissue culture plates coated with poly-HEMA (non-adherent) and treated for 48 h with the indicated factors. After washing the cells three times with binding buffer, the medium was changed for 0.5 ml of binding buffer now containing in addition 4 ng/ml murine ^{125}I-EGF (a gift from Dr. T. Benraad, Department of Endocrinology, University of Nijmegen, The Netherlands). Non-specific binding was determined by a parallel treatment with a 100-fold excess of unlabelled EGF. The cells were incubated for 1 h at room temperature after which they were washed three times with PBS containing 0.1% BSA, three times with PBS and subsequently extracted with 1% (v/v) Triton X-100, prior to γ-counting [24]. ^{125}I-EGF binding was corrected for cell numbers by measuring protein concentrations according to the Lowry method using BSA as standard.

2.6. Materials

TGF-β_1 was from R&D Systems. Bradykinin (BK), lysophosphatidic acid (LPA), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), RA, transferrin, MTT, and BSA were from Sigma. Endothelin-1 (ET-1) was from Peninsula Laboratories Europe. DMEM and Ham's F-12 medium were from Gibco, NCS from Hyclone, and poly-HEMA from Aldrich-Chemie, Germany.

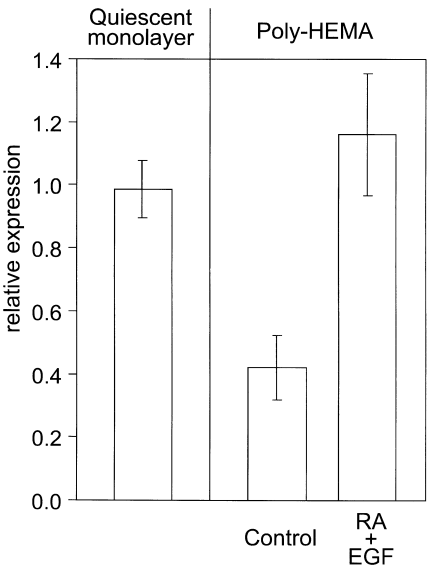

Fig. 1. Effect of cell anchorage on EGF receptor mRNA levels in NRK cells. NRK cells were cultured for 3 days on poly-HEMA coated dishes in SFS medium (poly-HEMA) in the absence (Control) or presence of 50 ng/ml RA and 5 ng/ml EGF (RA+EGF), or were cultured as monolayer cells and incubated in SF medium for 3 days (Quiescent monolayer). Indicated standard errors of the mean are based on at least five experiments.

3. Results

3.1. EGF receptor mRNA, EGF receptor promoter activity and ^{125}I-EGF binding are decreased in anchorage independent cultures of NRK cells

Fig. 1 shows EGF receptor mRNA levels, measured by the 'primer-dropping' RT-PCR method, in NRK cells that had been cultured for 3 days on poly-HEMA coated dishes. The data indicate a 2.5-fold reduction in mRNA levels in anchorage independent cells when compared to quiescent monolayer cells. When cells were grown on poly-HEMA coated dishes in the presence of EGF and RA, a combination of factors that induces AIG of NRK cells, no such reduction in EGF receptor mRNA levels was observed.

In order to study if the above reduction in mRNA levels under anchorage independent conditions is due to a repression of transcriptional activity, the activity of an EGF receptor promoter construct linked to luciferase was studied after stable transfection into NRK cells. Fig. 2 shows that basal EGF receptor promoter activity decreased in a time dependent manner after substrate release, resulting in approximately 10% of its original value after 72 h of incubation. Re-adherence of the same cells resulted in a time dependent restoration of EGF receptor promoter activity, demonstrating that the reduction in activity upon substrate release is not due to cell death. Moreover, a similar reporter construct based on the platelet derived growth factor α-receptor promoter was much less affected by cell adherence, indicating that the effects observed do not result from a general inhibition of macromolecule biosynthesis. In Fig. 3 it is shown that this decrease of EGF receptor promoter activity is parallelled by a decrease of ^{125}I-EGF binding. These data show that EGF receptor expression levels are strongly affected by cell adherence. Similar effects on EGF receptor expression have been obtained by Mansbridge et al. [25] who showed a strongly decreased bind-

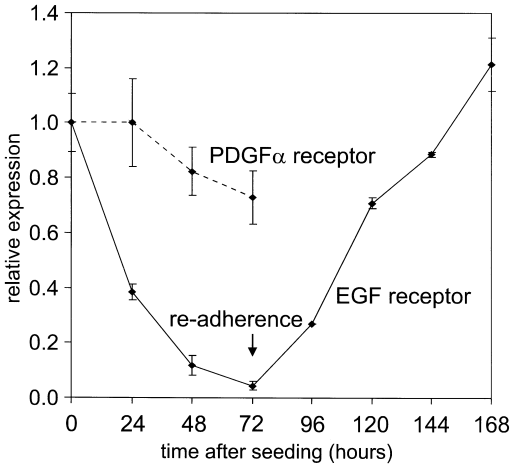

Fig. 2. Influence of anchorage independent culturing of NRK cells and subsequent re-adherence on EGF receptor promoter activity. EGF receptor (solid line) or PDGFα receptor (dashed line) promoter activity measured as a function of time after seeding the (stably transfected) cells on poly-HEMA coated dishes in SFS medium. After 3 days, the non-adherent NRK cells were allowed to re-attach in the same medium and luciferase activity was measured for another 3 days. Indicated standard errors of the mean are based on triplicate experiments.

ing of ^{125}I-EGF to A431 cells when grown in spheroids, in contrast to those of Ness et al. [26] who observed an increase in EGF receptor mRNA levels in glioma cells cultured without anchorage.

3.2. *Induction of anchorage independent growth of NRK cells is parallelled by increased EGF receptor promoter activity and increased ^{125}I-EGF binding*

Besides RA and TGF-β, several other factors have been shown to induce phenotypic transformation of NRK cells in the presence of EGF, including BK, $PGF_{2\alpha}$, ET-1, and LPA [24,27–29]. When tested on quiescent monolayer cells and density arrested cells, these various factors have been shown to enhance EGF receptor levels [14]. In order to study if these factors are also able to enhance EGF receptor expression in anchorage independent cells, we used the above cells stably transfected with the EGF receptor promoter linked to luciferase. Fig. 4 shows a comparison between the ability of these factors to induce AIG of NRK cells and their ability to enhance EGF promoter activity in anchorage independent cultures of these cells. The data presented show that ET-1, TGFβ, and $PGF_{2\alpha}$ strongly increase both EGF dependent AIG and EGF receptor promoter activity, while BK, LPA, and RA have only a minor effect in both assays. Similar results were obtained when colony formation in soft agar was measured instead of proliferation on poly-HEMA coated dishes (data not shown). Also in anchorage independent cells which were first cultured for 48 h in the absence of growth factors, EGF receptor promoter activity and AIG could still be induced upon subsequent growth factor treatment (data not shown), indicating these growth factors can up-regulate EGF receptor promoter activity even after the initial decrease observed upon loss of anchorage. ^{125}I-EGF binding studies show (Fig. 3) that particularly $PGF_{2\alpha}$ and TGF-β strongly enhance the number of EGF receptors in anchorage independent NRK cells, in parallel with their activity on EGF receptor promoter activity (Fig. 4A). RA was less active in both assays,

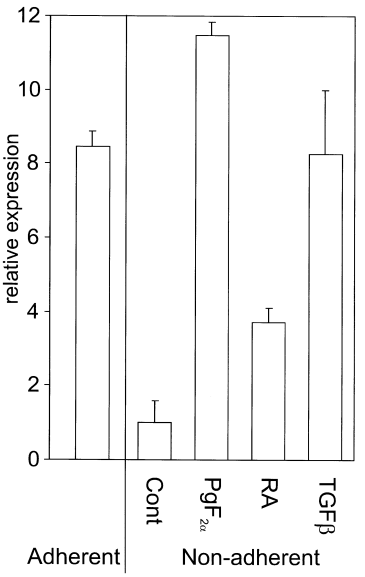

Fig. 3. Effect of anchorage independent culturing and phenotypic transformation inducing agents on EGF binding in NRK cells. NRK cells were seeded on culture dishes (adherent) or on poly-HEMA coated dishes (non-adherent), the latter in the presence of 1 μM $PGF_{2\alpha}$, 50 ng/ml RA, or 1 ng/ml TGF-β, or without additional factors (control) in SFS medium. After incubation for 2 days, ^{125}I-EGF (4 ng/ml) binding was determined and corrected for non-specific binding. Data are expressed relative to the number of cells determined by protein analysis. Indicated standard errors are based on duplicate experiments.

but clearly more potent in the binding assay than in the promoter assay. The same disconnection between the induction of EGF receptor promoter activity and AIG can be seen in the

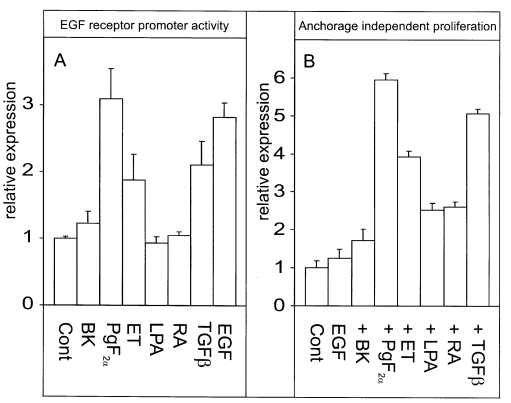

Fig. 4. Effect of phenotypic transformation inducing agents on EGF receptor promoter activity in anchorage independent NRK cells compared to their ability to induce anchorage independent proliferation. A: EGF receptor promoter activity. NRK cells were seeded on poly-HEMA coated dishes in the presence of 1 μM BK, 1 μM $PGF_{2\alpha}$, 0.1 μM ET-1, 100 μM LPA, 50 ng/ml RA, 1 ng/ml TGF-β, 5 ng/ml EGF, or in the absence of factors (Cont) in SFS medium. After incubation for 2 days, EGF receptor promoter activity was measured and corrected for cell numbers. Indicated standard errors of the mean are based on triplicate experiments. B: Anchorage independent proliferation. NRK cells were seeded on poly-HEMA coated dishes in the presence of 5 ng/ml EGF and the indicated factors, or in the complete absence of factors (Cont) in SFS medium. After incubation for 5 days, cell numbers were measured using the MTT assay. Indicated standard errors of the mean are based on quadruplicate experiments.

case of stimulation with LPA. This discrepancy will be discussed.

4. Discussion

NRK fibroblasts are unique in that their proliferation is strongly stimulated by EGF, while this growth factor is unable to induce a transformed phenotype in these cells. In our previous studies we have shown [14] that pretreatment of quiescent NRK cells with agents that enhance EGF receptor levels increases the mitogenic potential of EGF in these cells, indicating that the number of EGF receptors limits the mitogenic activity of this growth factor. Moreover, addition of such factors to confluent monolayer cultures is essential to induce phenotypic transformation of EGF treated NRK cells, by preventing them from becoming density arrested. In the present study we have shown that, similar to culturing at high cell density, also anchorage independent culturing of NRK cells results in a strong reduction of EGF receptor expression levels. As a consequence, treatment of anchorage independent cells with EGF alone is insufficient to induce these cells to proliferate. However, treatment of these cells with the modulating factors that are known to increase EGF receptor expression in monolayer cells results in anchorage independent growth of EGF treated cells. This shows that the growth factor requirements for loss of density dependent growth inhibition and anchorage independent growth of NRK cells are very similar and that in both cases EGF induced proliferation very likely relies on sufficiently high EGF receptor levels.

In this study, we have used NRK cells stably transfected with the 5′-region of the human EGF receptor gene linked to luciferase to study the effects of modulating factors on EGF receptor promoter activity. Previous studies have indicated that this promoter construct is sensitive to stimulation by EGF itself, phorbol 12-myristate 13-acetate (PMA), $(Bu)_2$cAMP, dexamethasone [20], and TGF-β [5,30]. RA has been shown to have both activating [20] and repressing [31] activity on the EGF receptor promoter, while expression studies have indicated that agents which induce PtdIns(4,5)P_2 hydrolysis in rat liver epithelial cells are able to increase EGF receptor mRNA levels in these cells [32]. We have previously shown that ET-1 and $PGF_{2\alpha}$ are both very strong inducers of PtdIns(4,5)P_2 hydrolysis in substrate attached NRK cells, while BK and LPA have only poor activity [16]. These results correlate very well with our current results on induction of EGF receptor promoter activity in anchorage independent cells.

Our present binding studies with radiolabeled EGF have indicated that RA upregulates EGF receptor mRNA and protein levels not only in monolayer NRK cells [14,29,33] but also in anchorage independent cells (Fig. 3). This increase of ^{125}I-EGF binding is parallelled by induction of AIG (Fig. 4B), but not necessarily by a comparable increase of EGF receptor promoter activity (Fig. 4A). This could be due to the fact that important RA inducible elements for positive regulation of EGF receptor expression in NRK cells are lacking in the promoter construct tested here, as indicated in earlier studies [34]. Nevertheless, in adherent NRK cells, EGF receptor promoter activity is strongly increased by RA [16]. This does not exclude the possibility, however, that important RA inducible elements for positive regulation of EGF receptor expression in non-adherent NRK cells are lacking in the promoter construct tested here. The strong increase of EGF receptor mRNA levels by RA in the presence of EGF (Fig. 1) underlines this statement. The same contrast between induction of EGF receptor promoter activity and AIG was seen when the cells were stimulated with LPA, indicating that the correlation between EGF receptor levels and EGF receptor promoter activity is only observed whit specific stimuli, in particular TGFβ and PtdIns(4,5)P_2 hydrolysing agents such as ET-1 and $PGF_{2\alpha}$.

Our present ^{125}I-EGF binding studies show that the decrease of EGF receptor mRNA levels and promoter activity is parallelled by a decrease of ^{125}I-EGF binding, while factors that induce AIG also increase ^{125}I-EGF binding. Most likely these binding studies tend to underestimate the effects of the modulating agents tested on EGF receptor expression, since anchorage independent NRK cells have a tendency to form clusters that have a limited penetration of EGF [35]. In our experience and that of others (reviewed in [15]) it is difficult, however, to quantitatively determine EGF receptor levels in NRK cells by other techniques, e.g. using immunological techniques, because of the very low receptor numbers present.

Our data show that EGF itself is able to increase EGF receptor promoter activity in NRK cells to a level which is comparable to that induced by $PGF_{2\alpha}$. In many cell types, including monolayer NRK cells [33], EGF is able to enhance EGF receptor mRNA levels [32,33,36], receptor protein levels [32], and receptor gene promoter activity [20], acting at both the transcriptional [20,33] and post-transcriptional [36] level. However, EGF by itself is able neither to induce anchorage independent proliferation of NRK cells, nor to induce proliferation of density arrested cells. This shows that an increase of EGF receptor numbers alone may not necessarily be sufficient to induce AIG of NRK cells and that additional signalling pathways may be involved. Nevertheless, the present data strongly suggest that an increase of EGF receptor numbers is a prerequisite for the induction of anchorage independent growth.

Several other mechanisms have been proposed to play a role in the induction of AIG of NRK cells. The induction of extracellular matrix proteins such as fibronectin has been shown to accompany the induction of AIG by TGF-β [4,5]. However, not all phenotypic transformation inducing factors increase fibronectin gene expression [5,10], showing that the production of extracellular matrix proteins is not obligatory for AIG. Furthermore, fibronectin is unable to replace TGFβ in inducing AIG of EGF treated NRK cells ([37] and E.J.J. van Zoelen, unpublished data). Another mechanism proposed is the modulation of protein-tyrosine phosphatase (PTPase) activity [10], since inhibition of PTPases leads to induction of AIG [10] and overexpression of the tyrosine and threonine specific cdc25 phosphatase is involved in the induction of AIG [11]. Furthermore, a CTGF dependent signalling pathway seems to be involved in TGF-β induced AIG of NRK cells [9]. Other studies show that the expression of cyclin A plays an important role in the regulation of AIG in NRK cells. The expression is suppressed when NRK cells are suspended while ectopic expression of cyclin A bypasses the adhesion requirement [7]. The regulation of cyclin A expression by adhesion is mediated at the transcriptional level [38], suggesting that proteins that are present prior to the appearance of cyclin A during cell cycle progression are targets of the adhesion signal

[39]. Unlike observations on other cell types [8,40], cyclin D1 levels or activity of the cyclin E/cdk2 complex do not appear to play a role in the induction of AIG of NRK cells [8]. It should be realized, however, that most of the studies mentioned on AIG of NRK cells have been carried out in the presence of serum containing medium with its variety of growth factors. Our previous studies on density dependent growth arrest of NRK cells and our current ones on AIG have all been carried out under growth factor defined culture conditions, to allow studies on the effects of EGF as the only growth stimulating hormone present.

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References

[1] Ozanne, B., Wheeler, T. and Kaplan, P.L. (1982) Fed. Proc. 41, 3004–3007.
[2] Shin, S.I., Freedman, V.H., Risser, R. and Pollack, R. (1975) Proc. Natl. Acad. Sci. USA 72, 4435–4439.
[3] Cifone, M.A. and Fidler, I.J. (1980) Proc. Natl. Acad. Sci. USA 77, 1039–1043.
[4] Ignotz, R.A. and Massague, J. (1986) J. Biol. Chem. 261, 4337–4345.
[5] Thompson, K.L., Assoian, R. and Rosner, M.R. (1988) J. Biol. Chem. 263, 19519–19524.
[6] Schwartz, M.A., Both, G. and Lechene, C. (1989) Proc. Natl. Acad. Sci. USA 86, 4525–4529.
[7] Guadagno, T.M., Ohtsubo, M., Roberts, J.M. and Assoian, R.K. (1993) Science 262, 1572–1575.
[8] Zhu, X., Ohtsubo, M., Böhmer, R.M., Roberts, J.M. and Assoian, R.K. (1996) J. Cell Biol. 133, 391–403.
[9] Kothapalli, D., Frazier, K.S., Welply, A., Segarini, P.R. and Grotendorst, G.R. (1997) Cell Growth Differ. 8, 61–68.
[10] Rijksen, G., Voller, M.C. and van Zoelen, E.J. (1993) J. Cell Physiol. 154, 393–401.
[11] Galaktionov, K., Lee, A.K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. and Beach, D. (1995) Science 269, 1575–1577.
[12] Anzano, M.A., Roberts, A.B., Meyers, C.A., Komoriya, A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1982) Cancer Res. 42, 4776–4778.
[13] Van Zoelen, E.J.J., Van Oostwaard, T.M. and de Laat, S.W. (1988) J. Biol. Chem. 263, 64–68.
[14] Lahaye, D.H.T.P., Camps, M.G.M., Van Erp, P.E., Peters, P.H.J. and Van Zoelen, E.J.J. (1998) J. Cell Physiol. 174, 9–17.
[15] Van Zoelen, E.J.J. (1991) Crit. Rev. Oncogen. 2, 311–333.
[16] Lahaye, D.H.T.P., Walboomers, F., Theuvenet, A.P. and Van Zoelen, E.J.J. (1999) Biochim. Biophys. Acta (in press).
[17] Kume, K., Jinno, S., Miwatani, H., Kizaka Kondoh, S., Terada, Y., Nojima, H. and Okayama, H. (1992) New Biol. 4, 504–511.
[18] Fukazawa, H., Mizuno, S. and Uehara, Y. (1995) Anal. Biochem. 228, 83–90.
[19] Van Zoelen, E.J.J., Van Oostwaard, T.M. and de Laat, S.W. (1986) J. Biol. Chem. 261, 5003–5009.
[20] Hudson, L.G., Santon, J.B. and Gill, G.N. (1989) Mol. Endocrinol. 3, 400–408.
[21] Afink, G.B., Nister, M., Stassen, B.H., Joosten, P.H., Rademakers, P.J., Bongcam Rudloff, E., van Zoelen, E.J. and Mosselman, S. (1995) Oncogene 10, 1667–1672.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[23] Wong, H., Anderson, W.D., Cheng, T. and Riabowol, K.T. (1994) Anal. Biochem. 223, 251–258.
[24] Van Zoelen, E.J.J., Peters, P.H., Afink, G.B., Van Genesen, S., De Roos, D.G., Van Rotterdam, W. and Theuvenet, A.P. (1994) Biochem. J. 298, 335–340.
[25] Mansbridge, J.N., Ausserer, W.A., Knapp, M.A. and Sutherland, R.M. (1994) J. Cell Physiol. 161, 374–382.
[26] Ness, G.O., Pedersen, P.H., Bjerkvig, R., Laerum, O.D. and Lillehaug, J.R. (1994) Exp. Cell Res. 214, 433–436.
[27] Afink, G.B., Van Alewijk, D.C.G.J., De Roos, D.G. and Van Zoelen, E.J.J. (1994) J. Cell. Biochem. 56, 480–489.
[28] Kusuhara, M., Yamaguchi, K., Kuranami, M., Suzaki, A., Ishikawa, S., Moon, H., Adachi, I., Hori, S. and Handa, S. (1992) Cancer Res. 52, 3011–3014.
[29] Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1984) Cancer Res. 44, 1635–1641.
[30] Hou, X., Johnson, A.C. and Rosner, M.R. (1994) Cell Growth Differ. 5, 801–809.
[31] Zheng, Z.-S., Polakowska, R., Johnson, A. and Goldsmith, L.A. (1992) Cell Growth Differ. 3, 225–232.
[32] Earp, H.S., Hepler, J.R., Petch, L.A., Miller, A., Berry, A.R., Harris, J., Raymond, V.W., McCune, B.K., Lee, L.W., Grisham, J.W. and Harden, T.K. (1988) J. Biol. Chem. 263, 13868–13874.
[33] Thompson, K.L. and Rosner, M.R. (1989) J. Biol. Chem. 264, 3230–3234.
[34] Maekawa, T., Imamoto, F., Merlino, G.T., Pastan, I. and Ishii, S. (1989) J. Biol. Chem. 264, 5488–5494.
[35] Mansbridge, J.N., Knuchel, R., Knapp, A.M. and Sutherland, R.M. (1992) J. Cell Physiol. 151, 433–442.
[36] Clark, A.J., Ishii, S., Richert, N., Merlino, G.T. and Pastan, I. (1985) Proc. Natl. Acad. Sci. USA 82, 8374–8378.
[37] Allen Hoffmann, B.L., Crankshaw, C.L. and Mosher, D.F. (1986) Mol. Cell Biol. 8, 4234–4242.
[38] Kramer, A., Carstens, C.P. and Fahl, W.E. (1996) J. Biol. Chem. 271, 6579–6582.
[39] Carstens, C.P., Kramer, A. and Fahl, W.E. (1996) Exp. Cell Res. 229, 86–92.
[40] Böhmer, R.M., Scharf, E. and Assoian, R.K. (1996) Mol. Biol. Cell 7, 101–111.