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ALTERED EPIDERMAL GROWTH FACTOR SIGNAL TRANSDUCTION IN ACTIVATED HA-RAS-TRANSFORMED HUMAN KERATINOCYTES

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Epidermal growth factor (EGF) can stimulate proliferation and 92 kDa gelatinase/matrix metalloproteinase (MMP-9) expression. The induction of MMP-9 is not only pathologically significant for invasion and metastasis, but also serves as a semiquantitative measure of EGF signal transduction. In order to examine the role of mutated ras p21 in EGF signal transduction, an activated Ha-ras-transformed human keratinocyte cell line was developed and characterized. Overexpression of the mutated Ha-ras p21 in these cells was demonstrated. Our results showed that EGF induced 92 kDa MMP-9 secretion was doubled in the ras-transformed keratinocytes in comparison to the parent cells. The karyotype, the expression of EGF receptor (EGFR) and transforming growth factor (TGF) alpha at the mRNA level remained unchanged. These results suggest that the presence of high levels of mutated ras p21 may be responsible for the aberrant EGF signal transduction and contributes to transformation. In addition, a reduction of TGF beta expression at mRNA level by 70% was found in the activated Ha-ras-transformed keratinocytes when compared to the parent cells.

Mutation of the ras oncogene occurs in a wide spectrum of cancers and represents one of the most prevalent genetic defects found in naturally occuring tumors (1,2). The mechanism of action of the ras oncogene in tumorigenesis remains unknown. The ras genes encode a 21 kDa protein which is associated with the inner surface of the plasma membrane where it can bind GTP and function as a "downstream" effector of various external stimuli including epidermal

Abbreviations used in this paper: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; MMP-9, 92 kDa gelatinase/Matrix metalloproteinase; RB, retinoblastoma tumor suppressor gene; DCC, deleted colon cancer gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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growth factor (EGF) (3-7). EGF receptor (EGFR) is the cellular homolog of the v-erb B oncogene and overexpression of EGFR has been associated with many epithelial cancers (8-10) including squamous cell, breast and pancreatic cancer (11). It remains possible that the presence of a large amount of mutated Ha-ras p21 results in aberrant transmission of EGF signal which then leads to the promotion and or progression of tumor formation. Transformation results from multiple events of dysregulated growth and differentiation. Human keratinocytes are an excellent system to study the regulation of growth and differentiation. Normal human keratinocytes can be induced to differentiate by calcium supplementaion (12), retinoic acid (13) and TGF beta (14). Keratinocytes respond to EGF with increased proliferation and induction of MMP-9 secretion (unpublished observation) which is not only pathologically significant in invasion and metastasis (15,16), but also serves as a convenient semiquantitative biochemical assay to assess the magnitude of signal transduction. RHEK-1 (17) is a human keratinocyte cell line which is immortalized, but not transformed, by adenovirus 12-SV40 virus. An activated Ha-ras transformed human keratinocyte cell line was developed by the introduction of the mutated Ha-ras (arginine-12) gene by electroporation into RHEK-1 cells and selection for the transformed clones. The transformed clones were isolated, characterized, and used for studying the EGF signal transduction. Several mechanisms are known to cause an increased EGF signal including overexpression of EGFR, autocrine stimulation by TGF alpha (18) and possible modulation of EGFR signal by TGF beta (19-21). The effect of the transformation with the activated Ha-ras oncogene on these mechanisms were also examined.

MATERIALS AND METHODS

Cell culture. Human keratinocyte cell line (RHEK-1) was established from primary human foreskin epidermal keratinocytes after they had been infected with Ad-12-SV40 virus. This line is immortalized but was not malignantly transformed (17). Cells were cultured in Dulbecco modified Eagle medium (DMEM) (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), hydrocortisone(5 μg/ml), L-glutamine (2 mM), HEPES (25 mM), penicillin (50 IU/ml), and streptomycin (50 μg/ml). Serum-free media (KBM, containing 0.15 mM calcium, Clonetics, San Diego, CA) was supplemented with insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), antibiotics and L-glutamine as indicated above.

Transfection of activated Ha-ras (arginine-12) into RHEK-1 by electroporation. Activated Ha-ras plasmid PHOGT1 (22) was transfected onto RHEK-1 cells by electroporation as described (23) and several independent G-418 (150 μg/ml) (GIBCO BRL,Gaithersburg, MD) resistant clones were mass propagated. Anchorage-independent growth in 0.33% agar (Difco, Detroit, MI) (23) was examined and four clones were isolated.

Immunoblot analysis of the expression of activated Ha-ras oncoprotein. Cell lysate from 1 million cells was loaded on each lane and separated on 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blotted with an antibody that would specifically recognize the mutated Ha-ras with arginine in codon 12 only, but not the c-Ha-ras oncoprotein. The 21 kDa bands were visulized by a second rabbit anti-mouse antibody, followed by ^{125}I-protein A, and autoradiography.

Cytogenetics. Cell lines were grown in DMEM with 10% FBS and 2.5 ug/ml hydrocortisone. Cells were subcultured using a 1:3 split ratio. Approximately 24-48 hours after subculturing, colcimid was added to cultures at a final concentration of 0.1 μg/ml. Thirty

minutes later, cells were removed from the culture flasks using trypsin and suspended in hypotonic solution (0.075 M KCl) for 10 minutes at room temperature. Cells were then fixed 3 times in a modified Carnoy's fixative (3 parts methanol plus 1 part acetic acid) and cells spread on glass slides. Chromosomes were Giemsa banded (24) and analyzed using standard techniques. A minimum of 25 cells were analyzed from each culture.

Induction of MMP-9 secretion by EGF. MMP-9 activity was detected by zymography (25). Cells were switched to serum-free media (KBM, Clonetics) supplemented with insulin and antibiotics with or without various concentrations of EGF for 48 hours. The culture medium was collected, centrifuged at 12,000 g, and the supernatant was used for zymogram assay. The cells were trypsinized for cell count. Gelatin (1 mg/ml) was incorporated into the SDS-polyacrylamide gel. Culture media, normalized to cell number, were loaded and the electrophoresis was carried out in the usual way except the samples were not boiled prior to electrophoresis, and the gel was cooled to 4 C during electrophoresis. After electrophoresis, the gel was washed twice in 50 mM Tris-HCl pH 7.6. and 2.5% Triton X-100 (v/v) (15 minutes) to allow renaturation of the proteinases. Gel was then rinsed four times (5 minutes) in the above buffer without Triton X-100, incubated in a buffer containing 50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, and 0.02% NaN_3 at 37 C for 24- 48 hrs depending on the activity. Following the incubation, the gel was stained with Coomassie Brilliant Blue. The enzyme activity was visualized as areas of negative staining and is semiquantitative when analyzed by densitometry.

^{125}I-EGF binding assay. ^{125}I-EGF was prepared by the chloramine T method (26). Triplicate sets of cell cultures were prepared, one for cell count, one for total binding assay, and one for non-specific binding. Cells were washed twice with DMEM containing 25 mM HEPES, pH 7.4 and 0.1% bovine serum albumin, and then incubated with various concentrations of ^{125}I-EGF for 2 hours at 4 C. The cells were then washed three times and solublized with 0.2 N NaOH at room temperature for 1 hour. Non-specific binding was determined with the same procedure, except for the addition of 100-fold excess of cold EGF. Non-specific binding has been consistently low (< 5%) and was subtracted from the total binding to yield specific binding. The data were analyzed using a Scatchard plot (27).

Northern blot analysis of TGF alpha and TGF beta. RNA was isolated by the guanidine thiocyanate-cesium chloride method. Total RNA was fractionated by 1% agarose formaldehyde gel electrophoresis and transferred to a Nytran membrane according to the directions of the manufacturer (Schleicher & Schuell). Hybridization was performed using TGF alpha probe (28), TGF beta probe, and EGFR probe (pE7 clone from American Type Culture Collection). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (29) was used as control. The probes were labeled by the random priming method (Boehringer Mannheim).

RESULTS

Selection of transformed clones and identification of the mutated Ha-ras p21. The ability to form colonies in soft agar is a characteristic of transformed cells. Parent RHEK-1 cells are not transformed and do not form colonies in soft agar. After transfection with activated Ha-ras oncogene (arginine-12) by electroportion, the transfected cells acquired the ability to form colonies in soft agar culture. This acquired property was utilized to isolate the transfected and transformed cells. Four clones (#3-6) were isolated and characterized. Tumorigenicity *in vivo* was demonstrated using athymic mice. Clone #6 cells were injected in athymic mice and tumors (xenographs) were formed. Initially, these clones were examined for their expression of mutated Ha-ras p21 using a monoclonal antibody (E.I. du Pont de Nemours & Company), which was developed against the peptide containing mutated Ha-ras with arginine in codon 12 (instead of glycine as in c-ras). This antibody which recognizes only the codon 12 arginine mutated ras, but not c-ras or other mutated

ras, was used for immunoblotting. Visualization was accomplished using rabbit anti-mouse second antibody and ^{125}I-protein A. Figure 1 shows the results with the parental and clones #3 and #6 cells. A 21 kDa band was noted in all four clones, but not in parent RHEK-1 cells. This result indicated that the mutated Ha-ras p21 (arginine-12) was indeed transferred and expressed in these clones. The parental RHEK-1 cells and all 4 clones are expected to express the endogenous c-ras to the same level. In an attempt to estimate the relative level of expression of the endogenous c-ras vs the transferred activated Ha-ras oncogene, the pan-ras antibody (Oncogene Science) which recognizes both c-ras and mutated ras p21 was used for immunoblotting. A 21 kDa band was seen in all cell types (data not shown) and the densitometry tracing demonstrated a 3-5 fold increase in the transformed clones in comparison to parental RHEK-1 cells. This increased expression is most likely a result of the expression of the exogenously transferred activated Ha-ras oncogene rather than overexpression of endogenous c-ras in these transformed clones.

Cytogenetic study. It is important to examine whether there are any visible cytogenetic changes as a result of either the process of gene transfer by electroporation or as a result of transformation by the activated Ha-ras in these transformed clones. Cells from the activated Ha-ras transformed clones #3, #4, #5, and #6 showed identical karyotypes to that seen in the parental cells, except for one minor difference in the number of copies of

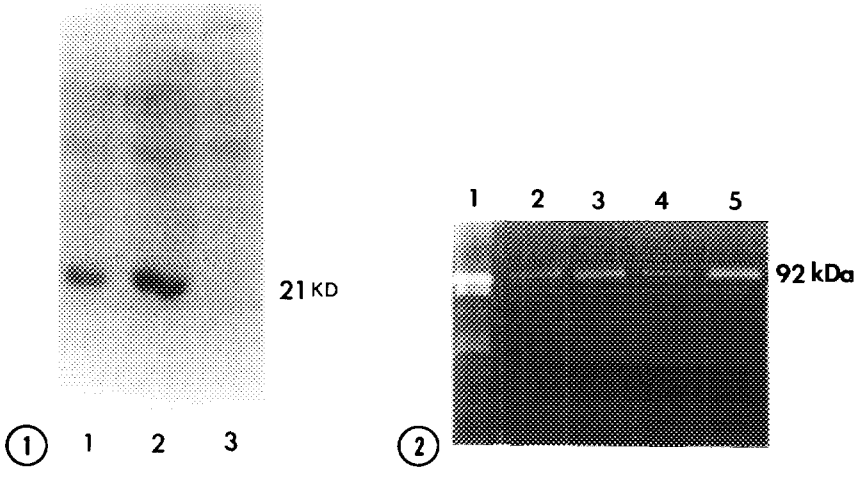

Figure 1. Immunoblot analysis demonstrating the expression of the mutated Ha-ras p21 (arginine-12). Lane 1 and lane 2: activated Ha-ras transformed clone #3 and clone #6, respectively. Lane 3: Control, RHEK-1 parent cells. Cell lysates from 1 million cells were loaded on each lane, separated by 12% SDS PAGE, and blotted with antibodies recognizing mutated ras p21 with arginine in codon 12 only, but not the normal endogenous c-ras p21 (E.I. du Pont de Nemours & Company). The 21 kd bands were visulized by a second antibody of rabbit anti-mouse, followed by ^{125}I-protein A, and autoradiography.

Figure 2. 92 kDa MMP-9 secretion analyzed by Zymography. Lane 1: standard. Lanes 2 and 3: RHEK-1 parent cells without and with EGF (20 ng/ml),respectively. Lanes 4 and 5: Activated Ha-ras transformed clone #6 without and with EGF (20 ng/ml),respectively.

chromosome 21. Ninety percent of parental cells contained three copies of chromosome 21 and 10% contained two copies of chromosome 21. All cells from clone #3 and clone #4 contained only two copies of chromosome 21. All cells from clone #5 and #6 contained three copies of chromosome 21. The significance of an extra copy of chromosome 21 is not known. It appears that activated Ha-ras induced transformation does not cause chromosomal instability or detectable cytogenetic changes. The cytogenetics were studied in detail (data not shown). Pertinent findings that are common in the parental and clones #3-#6 cells include: monosomy in chromosome 13 which carries retinoblastoma locus (RB) (30,31), monosomy for 18q21 where the deleted colon cancer (DCC) gene is located (32), and monosomy for 11p where the Ha-ras gene is located. The absence of one copy of the normal Ha-ras gene could be significant in reducing levels of normal Ha-ras p21 that compete with the mutated Ha-ras p21 (arginine-12). Thus, the cells may have been rendered more sensitive to the effects of the introduced activated Ha-ras oncogene.

EGF induced MMP-9 secretion. EGF signal transducation begins with the recognition of its specific receptor, EGFR, followed by propagation through the cytoplasm, including interaction with ras p21. The signal eventually reaches the nucleus where activation or suppresion of specific effector genes occurs. The MMP-9 represents one of the end products of the EGFR mediated signal transduction and was utilized as a semiquantitative measure of the signal. The results are shown in Fig.2. Lane 1 shows standards. Lanes 2 and 3 show MMP-9 secretion of RHEK-1 parent cells without and with EGF (20 ng/ml) stimulation respectively. Lanes 4 and 5 show the MMP-9 secretion of clone #6 without and with EGF stimulation. There is a nearly two fold (90%) increase in the EGF induced MMP-9 secretion in clones #6 compared to that of the parental cells. A comparable rise in MMP-9 secretion was demonstrated in clone #3 (data not shown).

Biochemical characterization of clone #6. Overexpression of the receptor numbers, autocrine stimulation by TGF alpha, and modulation of EGFR by TGF beta are some of the known mechanisms that alter EGFR signal transduction and all of these parameters were examined. The binding assays of RHEK-1 parent and clone #6 cells using ^{125}I-EGF are shown in Fig.3. Both the saturation curve (upper right insert in each panel) and the Scatchard plot are shown. EGFR can be saturated when the ligand concentration approaches 10 nM in both the parent and clone #6. The Scatchard plots are not linear indicating more than one binding affinitiy. The binding characteristics analyzed by the Scatchard plot are similar in the parent and clone #6, and are similar to that of A431 cells. The receptor numbers are estimated to be $2X10^5$/cell, and $1.7X10^5$/cell in RHEK-1 parent and clone #6 respectively. Flow cytometric analysis using the monoclonal antibody 528, which recognizes the binding site of EGFR, was done and showed that both cell lines were homogeneous with rather uniformed distribution in cell size and EGFR staining pattern (data not shown). Northern blot analysis was performed to examine the expression of TGF alpha and TGF beta at the RNA level for all 4 clones. The results with parental cells and clones #3 and #6 are

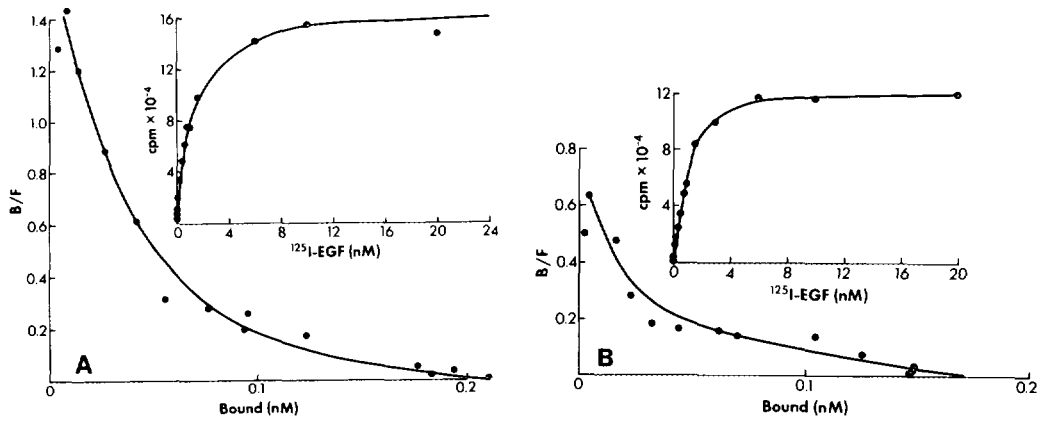

Figure 3. ^{125}I-EGF binding assays of RHEK-1 (A) and clone #6 (B). Both Scatchard plot and saturation curve are shown. The receptor number of RHEK-1 parent and clone #6 are estimated to be $2x10^5$/cell, and $1.7x\ 10^5$/cell,respectively.

shown in Fig.4. Glyceraldehyde 3 phosphate dehydrogenase cDNA (GAPDH), demonstrating a 1.6 kb band, served as an internal control for the amount of RNA loaded on each lane. TGF alpha displaying a 4.6 kb band is shown in panel A, and TGF beta displaying a 2.4 kb band is shown in panel B. Quantitative analysis using densitometry showed that the expression of TGF alpha was not significantly different from that of the parental cells. The expression of TGF beta was reduced by 70% in all 4 clones compared to parent cells. The expression of EGFR at the RNA level remained unchanged (data not shown) consistent with the comparable EGFR binding results noted above.

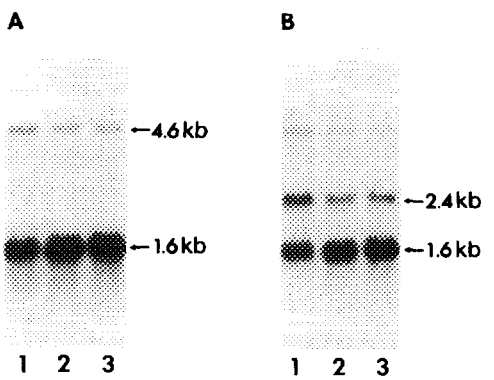

Figure 4. Northern blot analysis of TGF alpha (Fig.4A) and TGF beta (Fig.4B). Lane 1: RHEK-1 parent cell line. Lanes 2&3: Activated Ha-ras transformed clones #3 and #6, respectively. Ten μg of RNA was separated on agarose-formaldehyde gel electrophoresis, transferred to Nytran membrane, and hybridized with random primed radioactive cDNA probes. TGF alpha displays a 4.6 kb signal. TGF beta displays a 2.4 kb signal. GAPDH displaying a 1.6 kb signal serves as internal control for the amount of RNA loaded on each lane.

DISCUSSION

Mutation of ras oncogene represents one of the most common genetic defects found in solid tumors including colon and lung cancers which are refractory to current chemotherapy. The mechanism of action of mutated ras p21 in tumorigenesis is not well understood. An activated Ha-ras transformed human keratinocyte cell line was developed and characterized. We found that EGF induced 92 kDa MMP-9 secretion in the activated Ha-ras transformed keratinocytes was increased by nearly 2 fold (90%) in comparison to the parental cells. There were no detectable cytogenetic changes. The expression of EGFR and TGF alpha remained unchanged. Thus, the increased EGF signal may reflect the aberrant signal transduction secondary to the presence of a large amount of mutated Ha-ras p21. Proliferation of tumor cells assumes exponential kinetics. EGF has been shown to confer a proliferative signal. An aberrant transduction with 90% increase in a proliferative signal could result in a tremendous advantage in growth and an associated increase in the emergence of other new genetic defects during proliferation. The accumulation of crucial genetic defects can eventually lead to malignant transformation. The biological effects of the expression of mutated ras p21 may vary depending on the upstream stimuli that are normally transmitted through the ras p21 in a particular cell type.

Since all 4 clones showed decreased expression of TGF beta, it is unlikely to be incidental. It is most likely resulted from the expression of activated Ha-ras oncogene directly or indirectly. Whether this decreased expression of TGF beta contributes to the malignant transformation is not clear. TGF beta has been shown to regulate the growth and differentiation of normal human keratinocytes (14). Neither the clone #6 nor the parent RHEK-1 cells are able to respond to TGF beta 1 to undergo differentiation (unpublished data). It has been suggested that RB protein may be one of the down stream mediators of the TGF beta signal (31,33). The TGF beta signal of these clone #6 cells must be severely altered with the combination of 70% reduction in expression of TGF beta, the underlying cytogenetic defect of having only one RB gene, and expression of SV40 large T antigen (34). TGF beta has also been shown to modulate the receptors for various mitogens including EGFR (19-21). The findings of the reduced expression of TGF beta and the coexistence of increased EGF signal are consistent with our current limited knowledge about EGF and TGF beta. The significance of reduced expression of TGF beta mRNA in the EGF signal transduction in the clone #6 cells remains to be investigated.

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REFERENCES

1. Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., Van Boom, J. H., Van der Eb, A. J., and Vogelstein, B., (1987) Nature 327:929-935.
2. Bos, J. L. (1989) Cancer Res. 49:4682-4689.
3. Satoh. T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. U.S.A. 87:7926-7929.
4. Kamata T., and Feramisco, J. R. (1984) Nature 310:147-150.
5. Vogel, U. S., Dixon, A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., and Gibbs. J. B. (1988) Nature 335:90-93.
6. McCormick, F. (1989) Cell. 56:5-8.
7. Seuwen, K., and Pouyssegur, J. (1992) Adv. Cancer Res. 58:75-94.
8. Sainsbury, J. R. C., Malcolm, A. J., Appleton, D. R., Farndon, J. R., and Harris, A. L. (1985) J. Clin. Pathol. 38:1225-1228.
9. Ozanne, B., Richards, C. S. (1986) J. Pathol. 149:9-14.
10. Gullick, W. J. (1991) British Medical Bulletin 47:87-98.
11. Sandgren, E. P., Luetteke, N. C., Palmiter, R. D., Brinster, R. A., and Lee, D. C. (1990) Cell 61:1121-1135.
12. Boyce, S. T., and Ham, R. G. (1983) J. Invest. Dermatol. 81:33s-40s.
13. Smith, M.A., Parkinson, D.R., Cheson, B.D., Friedman, M.A., (1992) J Clinical Oncology 10:839-864.
14. Reiss, M., and Sartorelli, A. C. (1987) Cancer Res 47:6705-6709.
15. Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., and Shafie, S. (1990) Nature (Lond.) 284:67-68.
16. Voneda, T., Lyall, R. M., Alsina, M. M., Persons, P. E., Spada, A. P., Levitzki, A., Zilberstein, A., and Mundy, G. R. (1991) Cancer Res. 51;4430-4435.
17. Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K., and Aaronson, S. A. (1984) Science 227:1250-1252.
18. Derynck, R. (1988) Cell 54:593.
19. Massague, J. (1985) J. Cell Biol. 100:1508-1514.
20. Massague, J., Cheifetz, S., Laiho, M., Ralph, D. A., Weis, F. M. B., and Zentella. A. (1992) Cancer Surveys 12:81-103.
21. Takehara, K., Leroy, E. C., and Grotendorst, G. R. (1987) Cell 49:415-422.
22. Spandidos, D. A., and Wilkie, N. M. (1984) Nature 310:469-475.
23. Telang, N. T., Osborne, M. P., Sweterlitsch, L. A., and Narayanan, R. (1990) Cell Regulation 1:863-872.
24. Benn, P. A., and Perle, M. A. (1986) Chromosome staining and banding technique. *In* Human Cytogenetics, A Practical Approach. D. E. Rooney and B. H. Czepulkowski, editors. IRL Press, Oxford. 57-84.
25. Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J., and Werb, Z. (1986) J. Biol. Chem. 261:2814-2818.
26. Frolik, C. A., Wakefield, J. M., Smith, D. M., and Sporn, M. B. (1984) J. Biol Chem 259:10995-11000.
27. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51:660-672.
28. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., and Goeddel, D. V. (1984) Cell 38:287-297.
29. Dugaiczyk, A., Haron, J. A., Stone, E. M., Dennison, O. E., Rothblum, K. N., and Schwartz, R. J. (1983) Biochemistry 22:1605-1613.
30. Whyte P., Buchkovich, J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988) Nature 334:124-127.
31. Moses, H. L., Yang, E. Y., and Pietenpol, J. A. (1990) Cell 63:245-247.
32. Fearon, E. R., Cho, K. R., Nigro, J. M., Kern, S. E., Simons, J. W., Ruppert, J. M., Hamilton, S. R., Preisinger, A. C., Thomas, G., Kinzler, K., and Vogelstein, B. (1990) Science 247:49-56.
33. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. (1990) Cell 61:775-785.
34. Rhim, J. S., Fujita, J., Arnstein, P., and Aaronson, S. A. (1986) Science 232:385-388.