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Role of TGF-β in EGF-induced transformation of NRK cells is sustaining high-level EGF-signaling[1]

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Abstract We have been isolating and analyzing NRK cell mutants, which fail to transform by epidermal growth factor (EGF) and transforming growth factor (TGF)-β. One such mutant, R14, can respond to the growth inhibitory signal of TGF-β to the same extent as parental NRK but fail to respond to the growth stimulatory signal of EGF. This mutant has a defect in EGF receptor (EGFR) expression. When R14 mutant expressed a high level of EGFR, however, EGF not only induced proliferation in this mutant but also induced transformation without the aid of TGF-β. These findings suggest that the major role of TGF-β in this transformation system should be to counteract the ligand-dependent down-regulation of EGFR, thereby sustaining high-level EGF-signaling.

Key words: Epidermal growth factor receptor; Transforming growth factor-β; Oncogenic transformation; NRK cell

1. Introduction

Transforming growth factor (TGF)-β is a multifunctional peptide that controls proliferation, differentiation, carcinogenesis, etc. (reviewed in [1,2]). TGF-β was originally isolated as a factor capable of inducing a reversible phenotypic transformation of rat fibroblasts [3,4]. TGF-β alone, however, does not induce transformation but rather inhibits the growth of many types of cells [2,5]. Since the TGF-β receptor genes were isolated, intracellular pathways for TGF-β signal transduction have partly been understood, and several biochemical targets for TGF-β-induced growth inhibitory signals have been identified (reviews in [6,7]).

The possible involvement of TGF-β in general oncogenic transformation has most extensively been studied with the NRK-49F (NRK) cell line, since this cell line is reversibly transformed by epidermal growth factor (EGF) in the presence of TGF-β [3]. To explore the mechanism of oncogenic transformation, we have been studying NRK and its mutants which fail to be transformed by EGF and TGF-β treatment [8–12]. One such mutant, R14, has a defect in EGF receptor (EGFR) expression but can be transformed by various oncogenes including v-*erb*B (active form of EGFR), suggesting that this mutant retains all the down-stream components necessary to transmit EGFR signal. Forced expression of the EGFR gene in this mutant not only restored its responsiveness to EGF but also induced TGF-β-independent transformation in the presence of EGF. Our study with R14 cells provides evidence that the major role of TGF-β in this transformation system is not direct modification of EGFR molecule but to retain high-level expression of EGFR and that there should be a subtle balance between such a growth-promoting signal via EGFR and growth inhibitory components of the TGF-β signal in NRK.

2. Materials and methods

2.1. Cells and mutant isolation

Cell culture and mutant isolation were done as described previously [8,9,11]. If not indicated, the final concentrations of growth factors used were 5 ng/ml for EGF and platelet-derived growth factor (PDGF), and 0.5 ng/ml for TGF-β. The lines NER-1, 14ER-1 and 14ER-2 were *egfr* cDNA (rat) transfectants isolated and cloned from NRK or R14.

2.2. Growth curve and assay for growth in soft agar, proliferation, focus formation, ^{125}I-hEGF-binding, GTPase and In-gel kinase

To determine growth curves, 100 cells were plated into a 60 mm dish. Five different fields of cells were photographed in each dish. The same fields of cells were photographed and counted every day. The experiment was done in triplicate. Assays for growth in soft agar, proliferation, focus formation and ^{125}I-hEGF-binding, GTPase, In-gel kinase were done as described previously [8,9,12].

2.3. DNA transfection and colony assay

DNA transfection was carried out as described previously [13]. Rat *egfr* cDNA was isolated from NRK pcD2-cDNA library by colony hybridization using human *egfr* cDNA as a probe. The plasmids used are pcD2neo vector, pFBJ-2neo (v-*fos*), pZipneo-erbB (v-*erb*B), SVori-8-16 (SV40 T), p3611EH (v-*raf*), pGA-FeSV (v-*fes*), pSV*erb*BVE (activated c-*erb*B-2), and pSM-FeSV (v-*fms*). The transfectants were selected in growth medium containing 400 μg/ml G418 for 2 weeks. The numbers of transformed and flat antibiotics-resistant colonies were scored separately.

2.4. Immunoprecipitation and immunoblot detection

EGFR was immunoprecipitated with an anti-EGFR antibody (Upstate Biotechnology, Lake Placid, NY, USA) from cell lysate (1 mg protein), electrophoresed on a 4–20% polyacrylamide gradient gel (Daiichi pure chemicals), blotted onto a nitrocellulose filter, and detected with an anti-EGFR or anti-phosphotyrosine antibody (Transduction Laboratories).

2.5. Analysis of the EGFR promoter

The genome DNAs were isolated from NRK and R14 mutant as described [14]. Wild-type rat *egfr* promoter was isolated from NRK genome by using Genome Walker kit (Clontech) from the *Ssp*I-digested template. The PCR primers used for the promoter region am-

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[1] GenBank accession number of rat *egfr* promoter: AB025197.

Abbreviations: TGF, transforming growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; E+T, EGF plus TGF-β; PDGF, platelet-derived growth factor

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plification were EGFR-p1 (TGA CTG CGT CGG CAA CGA CGA CGG) and AP-1 primer (Clontech) for the first cycle, and EGFR-p2 (AGA CTC GCG TCC AGG TGA CCC GTC) and AP-2 primer (Clontech) for the second cycle. EGFR-p1 and -p2 are located immediately up-stream of the translation initiation codon. The amplified fragment of about 1.5 kb was subcloned and sequenced. Based on the sequence data, a new set of primers with flanking primer-binding site for −40M13 or −21M13 primer of DYEnamic Energy Transfer forward dye primer (underlined sequences) (Amersham) was prepared for amplifying the promoter region of NRK and R14 mutant; EGFR-p3 (TGT AAA ACG ACG GCC AGT GCT CTA CAT TTC CAC AGC TTC) located at 1.5 kb up-stream of EGFR-p1, EGFR-p4 (GTT TTC CCA GTC ACG ACG TTC TCG CAG TCC CTG AGG GTC) located near EGFR-p1, EGFR-p5 (GTT TTC CCA GTC ACG ACG CAC TGC GCT GCA TTT CAG GAC C), and EGFR-p6 (TGT AAA ACG ACG GCC AGT CTC TGT GCC AGG AGT TGG CTA CC). The amplified EGFR promoter regions were directly sequenced.

3. Results

3.1. Isolation of R14 mutant

To study the molecular mechanism of oncogenic transformation, we isolated a series of NRK rat fibroblast cell mutants refractory to the transformation induced by treatment with EGF plus TGF-β (E+T) [9–11]. One such mutant, R14, was unable to form colonies in soft agar in response to E+T (Fig. 1A), while it could be transformed by all the oncogenes we tested: namely, v-*erb*B, c-*erb*B-2, v-*ras*, v-*fms*, v-*mos*, v-*src*, v-*fos*, v-*fes*, SV40 T, polyoma virus middle T antigen (PyMT) and v-*raf* (data not shown), indicating that this mutant retains all the down-stream components necessary to transmit EGFR signal. The R14 mutant grew more slowly than the parental NRK cells and was not growth-stimulated by EGF (Fig. 1B). The defect of R14 mutant was complemented by fusion with parental NRK cells, indicating that the defect of R14 is recessive (data not shown). The inability of R14 cells to respond to the mitogenic activity of EGF seems rather specific: EGF failed to induce DNA synthesis in serum-starved R14 cells, while PDGF and serum were fully active in this assay (Fig. 1C). Furthermore, when Ras and MAP-kinase in EGF-stimulated R14 cells were examined by GTPase and in-gel kinase assays, respectively, no activity was detected (data not shown). All these data suggest that the defect in R14 mutant is in EGFR itself.

3.2. R14 mutant has a defect in EGFR expression

We next estimated the amount of unoccupied EGFR on the cell surface using an EGF-binding assay. The results clearly indicated a marked reduction in the number of EGF-binding sites on R14 cell surface (data not shown). Moreover, we found that the EGFR protein and its mRNA were barely detectable in R14 cells cultured in regular growth medium (Fig. 2A,B), suggesting that the observed decrease in EGF-binding was due to the decreased level of EGFR mRNA. The time course analysis revealed that total EGFR protein is quickly degraded in NRK cells after EGF addition, while TGF-β markedly inhibited this effect (Fig. 2C for the data up to 4 h and Fig. 2B at 24 h). This can be explained by the activity of TGF-β to increase the level of EGFR mRNA (Fig. 2A). In R14 cells, the EGFR mRNA and its protein product became detectable after 24 h TGF-β treatment (Fig. 2A,B).

When treated with TGF-β, R14 mutant was able to express a low level of EGFR (Fig. 2A,B), which seems to be functional because it was able to induce proliferative response (Fig. 3B, R14/E+T). Because the sizes of mRNA and protein of EGFR and the result of Southern blot analysis of R14 cells were the same as parental NRK cells (Fig. 2A,B and data not shown), we suspected that a defect of R14 mutant could be in the promoter region of *egfr* gene that results in inefficient

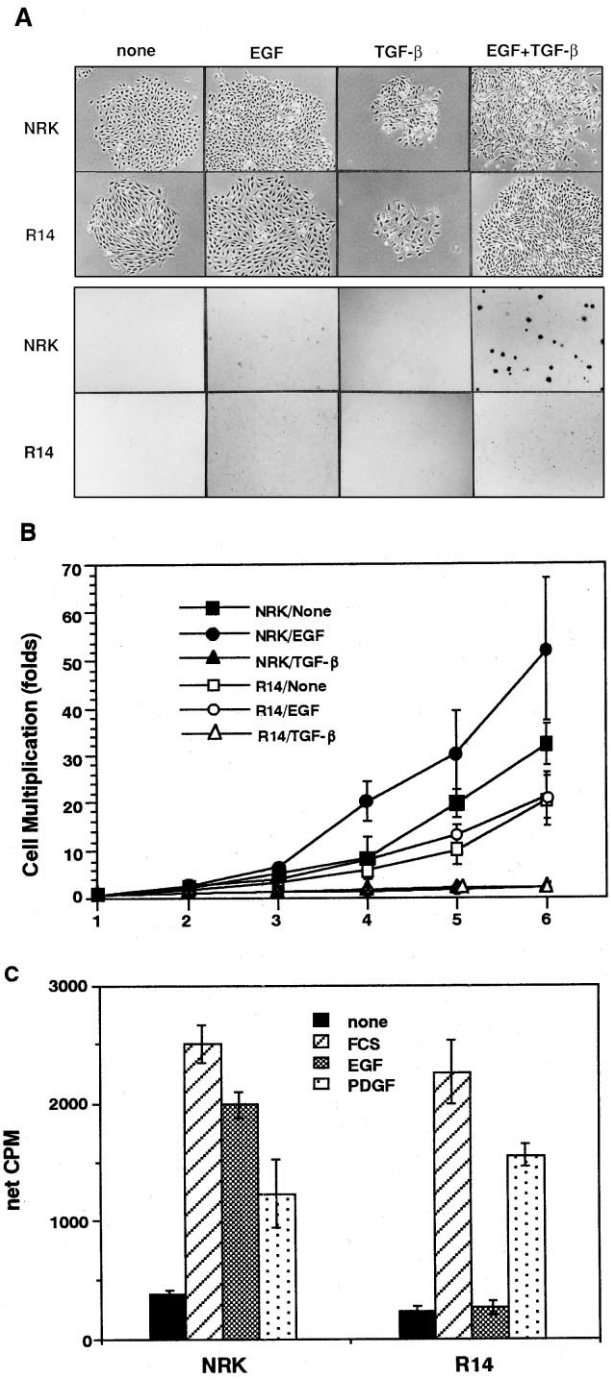

Fig. 1. Growth properties of the parental NRK line and R14 mutant. (A) The growth of NRK and R14 cells in liquid (upper panels) and soft agar (lower panels) cultures. (B) Growth curves. Increases in the number of cells are presented as the ratio to the number at day 1. Mean values from triplicate dishes are presented with S.E.M. bars. (C) Proliferative response of R14 to growth factors. Each experiment was done in triplicate and the average number of net cpm was indicated with a S.E.M. bar.

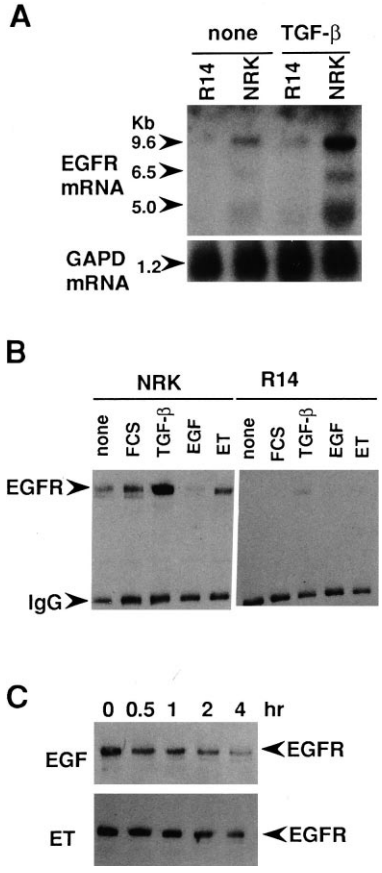

Fig. 2. Effects of EGF and TGF-β on the expression of EGFR. (A) Northern blot analysis of *egfr* mRNA in NRK and R14 cells. Cells cultured with or without TGF-β for 24 h were harvested, and poly-$(A)^+$ RNA was prepared. 5 μg of each RNA sample was subjected to Northern blot hybridization. EGFR mRNAs were detected as one major (9.6 kb) and two minor (6.5 and 5.0 kb) bands as previously reported by Petch et al. [40]. Glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA was shown as an internal standard. (B) Detection of EGFR protein in NRK and R14 cells. Cells were cultured for 24 h either in serum-free medium (none), regular growth medium (FCS), or growth medium containing EGF (EGF), TGF-β (TGF-β), or EGF plus TGF-β (E+T). The cell lysates were prepared and used to detect EGFR expression by immunoprecipitation followed by immunoblot detection. (C) Time course of EGFR down-regulation in NRK cells. Sub-confluent NRK cells were cultured in serum-free medium for 2 days and then, EGF was added to each culture. In E+T, TGF-β was added into the culture 8 h prior to the addition of EGF. After the indicated time of incubation, the cells were lysed, and EGFR was immunoprecipitated and analyzed by immunoblot detection.

mRNA transcription and hence an attenuated response to EGF. To examined the *egfr* promoters of R14 mutant, we isolated an approximately 1.5 kb promoter region up-stream of the initiation codon of *egfr* gene from both NRK and R14 cells. In human *egfr* promoter, this region contains the minimal promoter (−140 to −20 relative to the translation initiation codon) [15], p53-binding site (−265 to −239) [16], and TGF-β responsive element(s) (−919 to −860) [17]. We found the corresponding sequences to these regions in the NRK *egfr* promoter (data not shown). We directly sequenced the promoter regions and carefully compared the data from R14 and NRK. However, we could not detect any difference between the two (data not shown).

3.3. Exogenous EGFR expression rescued the R14 defect

If the reduction in EGFR is solely responsible for the phenotype of R14 mutant, then the restored expression of EGFR gene may complement its defect. When R14 cells were transfected with a rat *egfr* cDNA inserted in pcD2 expression vector, approximately 10% of the transfected colonies became responsive to EGF and transformed (Table 1). We usually obtained approximately 10% of G418-resistant colonies (0.4 mg G418/ml), which express a relatively high level of an inserted gene in pcD2 vector [8,9]. We randomly picked up 10 of these colonies and analyzed by Western blotting. All of these clones were found to express several to 10-fold higher levels of EGFR than the parental NRK cells (Fig. 4A and data not shown). Two representative clones (R14ER-1 and -2) stably expressing the transfected EGFR gene (Fig. 3A) were subjected to further characterization. Both clones had restored their ability to proliferate in response to EGF (Fig. 3B, striped bars). They had also gained the ability to form colonies in soft agar in response to EGF alone (Fig. 3C, dark bars). Unexpectedly, however, TGF-β showed rather negative effects on the EGF-induced growth stimulation as well as anchorage-independent growth in these transfectant lines (Fig. 3B, open bars; Fig. 3C, light bars). Interestingly, an EGFR transfectant line derived from the parental NRK (NER-1) showed a similar response to E+T (Fig. 3C), indicating that the unexpected results with the R14-derived transfectants cannot be attributed to some unusual property of this mutant.

3.4. Overexpression of EGFR induced TGF-β-independent transformation

NER-1 cells showed a highly transformed morphology when treated with EGF alone but less transformed morphology when treated with E+T (Fig. 4A). Such a response is not unique to this particular transfectant line, because freshly transfected NRK cells also showed a similar tendency of response to EGF and E+T when individual colonies were ex-

Table 1
Effects of EGF and TGF-β on the morphology of *egfr*-transfected cells

Cells			No. of transformed colonies/ No. of total colonies (%)
NRKneo	(E)	Expt.1	50/3450 (1.4%)
		Expt.2	42/2820 (1.5%)
	(E+T)	Expt.1	2900/2950 (98.3%)
		Expt.2	2750/2820 (97.5%)
NRK-EGFR	(E)	Expt.1	290/653 (44.4%)
		Expt.2	262/643 (40.7%)
	(E+T)	Expt.1	79/451 (17.5%)
		Expt.2	62/436 (14.1%)
R14neo	(E)	Expt.1	0/4630 (< 0.02%)
		Expt.2	0/2300 (< 0.04%)
	(E+T)	Expt.1	0/2720 (< 0.04%)
		Expt.2	0/2770 (< 0.04%)
R14-EGFR	(E)	Expt.1	20/219 (9.1%)
		Expt.2	18/173 (10.4%)
	(E+T)	Expt.1	4/186 (2.1%)
		Expt.2	8/278 (2.9%)

NRK-EGFR, R14-EGFR, NRKneo and R14neo are NRK or R14 transfectants of rat *egfr* cDNA (-EGFR) or pcD2 empty vector (neo). The transfectants were selected in G418-containing growth medium for 3 weeks and then cultured with the medium containing 5 ng EGF/ml (E) or 5 ng EGF/ml plus 0.5 ng/ml TGF-β (E+T) for 5 days, and then their morphology was examined under a microscope. None of the transfectants was transformed in regular growth medium.

amined (Table 1). Since TGF-β did not affect the viability (data not shown), the negative effects of TGF-β cannot be explained by cell death. The negative effect of TGF-β has been observed in growth with regular growth medium. The parental NRK cells grow slower with E+T than with EGF alone (Fig. 4B). This tendency is more significant in NER-1 cells (Fig. 4B). Together, these results suggest that the growth

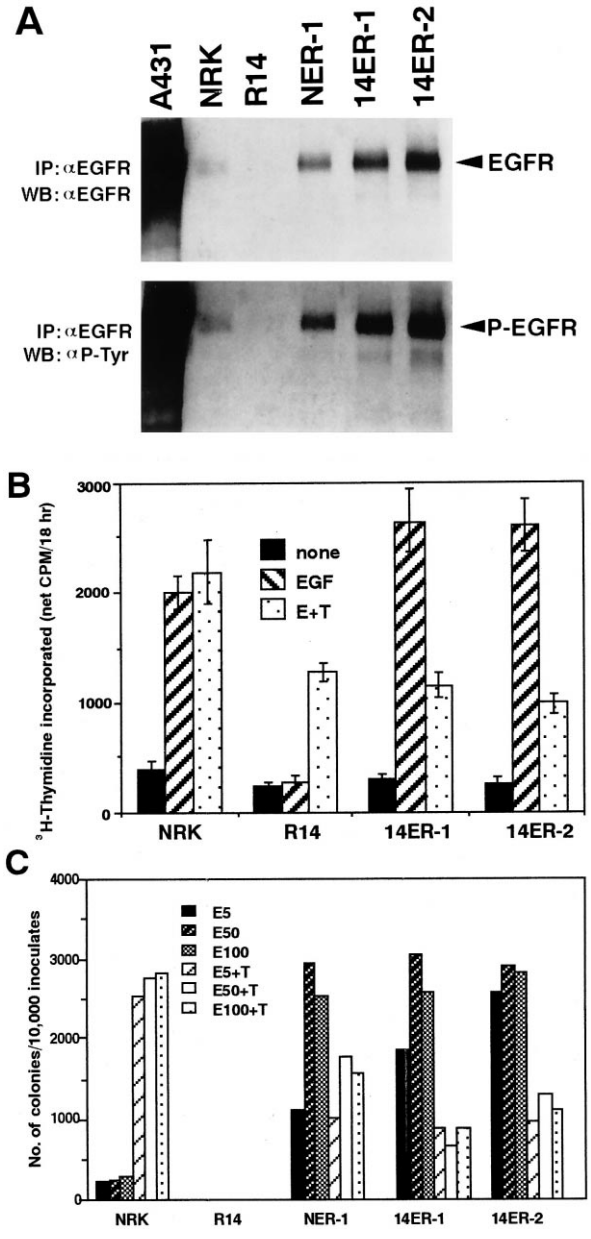

Fig. 3. Effects of *egfr* gene overexpression on the growth properties of NRK and R14 cells. (A) EGFR expression in NRK, R14 and their *egfr* transfectants. Total lysate (5 μg) of A431 cells was used as a positive control. Cells were serum-starved for 2 days and then treated with EGF for 3 min. The cell lysates were prepared, and EGFR protein was immunoprecipitated with anti-hEGFR and analyzed by immunoblot detection with anti-EGFR (upper panel) or anti-phosphotyrosine (α P-Tyr) (lower panel) antibody. (B) Proliferative response of *egfr* transfectants of R14. Mean values from triplicate experiments are presented with S.E.M. bars. (C) Growth of NRK, R14 and their *egfr* transfectants in soft agar. Cells were inoculated into semi-solid medium containing EGF at the final concentration of 5 (E5), 50 (E50) or 100 (E100) ng/ml with (+T) or without 0.5 ng/ml TGF-β. % CV is less than 3.0.

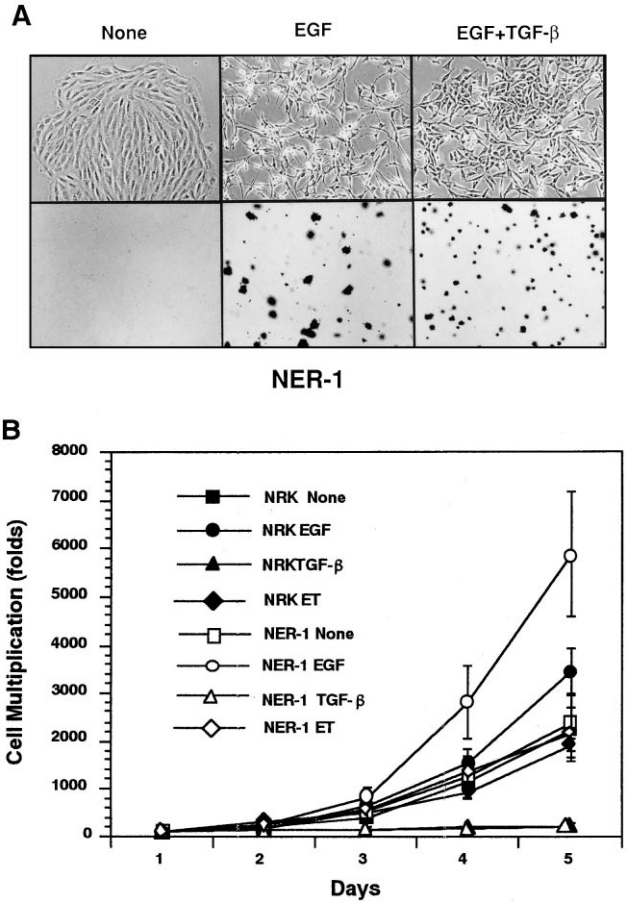

Fig. 4. Effects of TGF-β on the phenotype of *egfr* overexpressing NRK cells. (A) NER-1 cells were cultured in regular growth medium (none) or the medium containing EGF or E+T for 1 week (upper panels). NER-1 cells were inoculated into soft agar containing indicated growth factor(s) and incubated for 2 weeks (lower panels). (B) Growth curves. See Fig. 1B for detail.

and transforming properties of NRK cells regulated by E+T can be explained by a somehow deviated balance between the positive and negative components of the TGF-β signal in NRK cell line.

4. Discussion

TGF-β was originally identified as one of the factors that were secreted from transformed cells and induced malignant transformation of NRK cells when added to medium [18]. Later studies, however, demonstrated that TGF-β inhibits the growth of various types of cells [19–22] and suggest that TGF-β may be better conceived as a tumor suppressor rather than a tumor inducer [22–25]. In fact, inactivating mutations of the genes involved in the intracellular signaling for TGF-β (i.e. TGF-β receptors and Smad proteins) have been found in multiple types of human cancers [26–31]. Thus, the oncogenic stimulation of NRK by TGF-β remained to be an interesting paradox.

In 1988, Thompson et al. [32] reported that TGF-β induces the expression of EGFR in this cell line. We independently made a similar observation during this study. These findings raised the possibility that TGF-β sensitizes NRK cells to the mitogenic activity of EGF by augmenting the expression of

EGFR. On the other hand, it has been suggested that TGF-β may directly modify EGFR molecule itself in phosphorylation and alter its affinity to EGF [33,34]. Our study on R14 mutant has provided a support that TGF-β could induce cell transformation, only if the expression of EGFR can be inducible over a sufficient level. The growth of R14 mutant is, however, strongly inhibited as well as parental NRK cells when TGF-β alone is added to the medium, suggesting that the growth inhibitory signal of TGF-β is transduced through an independent cascade of the oncogenic growth stimulatory signal.

The growth inhibitory effect of TGF-β was also observed in the transformed cells culturing in liquid and semi-solid medium (Figs. 3B,C, 4B and Table 1). It is intriguing to speculate that there may be bi-directional, positive and negative cross talks between EGF- and TGF-β-signaling pathways and that growth of the cells may be regulated by the net balance of these positive and negative signals. In such system, the effects of a particular signal may become evident only when cells are placed under extreme conditions, for instance, overexpression of a single component of the pathways.

We still do not know exactly which gene is mutated in R14 cells. R14 is a recessive mutant and the only difference we found between R14 and parental NRK is in the expression of EGFR. The sequencing analysis revealed that there was no mutation in the *cis*-regulatory elements of this region in R14 mutant. So a defect should be in a certain *trans*-regulatory element for the *egfr* promoter or in a certain crucial *cis*-element for post-transcriptional regulation. Further analysis of R14 cells is needed to identify the mutation.

This study revealed that the strong EGF signal is necessary and sufficient for the transformation in this system and TGF-β plays the role of sustaining high EGFR expression. EGF-induced transformation is not specific to the NRK cells overexpressing EGFR. NIH/3T3 cells overexpressing EGFR can also be transformed in a ligand-dependent manner [35]. Overexpression of EGFR is frequently found in naturally occurring tumors [36–39]. The experimental data, including ours, clearly indicate a significant impact of EGFR overexpression on malignant transformation: EGFR overproduction would dramatically increase the chance for the cells to receive mitogenic EGF signals continuously and to accumulate mutations in other genes critical for malignant progression. Further characterization of R14 mutant may yield important insights into the regulatory mechanisms of *egfr* gene expression and may help developing a new approach to the treatment of certain types of cancer.

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