

# Cell-mediated Co-action of Transforming Growth Factors: Incubation of Type $\beta$ with Normal Rat Kidney Cells Produces a Soluble Activity that Prolongs the Ruffling Response to Type $\alpha$

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**Abstract.** Intense, continuous ruffling is a characteristic of many transformed cells, but untransformed cells ruffle intensely only briefly after exposure to growth factors. We reported previously that cells of a normal rat kidney (NRK) cell line transformed by Kirsten murine sarcoma virus secrete their own ruffle-inducing agent(s) that cause sustained ruffling in either themselves or untransformed NRK cells. In the present study, we examined the roles of the transforming growth factors TGF- $\alpha$  and TGF- $\beta$  in the induction and maintenance of ruffling in untransformed NRK cells, and observed the following: TGF- $\alpha$  caused a transient epidermal growth factor (EGF)-like response, which could be blocked by prior exposure of cells to EGF or by antiserum directed against the COOH-terminus of TGF- $\alpha$ . TGF- $\beta$  caused no ruffling

and did not itself prolong TGF- $\alpha$  ruffling. A new, buffer-soluble (transferable) mediator activity produced by incubation of TGF- $\beta$  with NRK cells for 6-h extended the duration of maximal TGF- $\alpha$ -induced ruffling by several-fold. This study demonstrates that TGF- $\alpha$  alone causes an EGF-like, transient ruffling response, but neither TGF- $\alpha$  or TGF- $\beta$  alone, nor the two together, cause transformation-associated sustained ruffling. Rather, TGF- $\alpha$  acts in concert with a new, TGF- $\beta$ -dependent activity. This new activity appears to inhibit normal cellular off-regulation of TGF- $\alpha$ -induced ruffling. Inhibition of the cellular off-regulation of a growth factor response could play a key role in the unregulated growth associated with malignancy.

SEVERAL nontransforming polypeptide growth factors, including insulin (8), epidermal growth factor (EGF)<sup>1</sup> (5, 6, 9, 10), platelet-derived growth factor (19), fibroblast growth factor (see companion paper by Myrdal and Auersperg), and nerve growth factor (6), cause transient ruffling on sparsely cultured cells. In all cases, ruffling subsides within ~1 h, even in the continued presence of the factor, indicating a cellular off-regulation mechanism that limits growth factor responses. This off-regulation is at least somewhat factor specific. For example, PC12 cells previously exposed to, and therefore refractory to, EGF still ruffle when exposed to nerve growth factor (6). Similarly, normal rat kidney (NRK) cells respond to insulin after a saturating EGF exposure (unpublished observation). In a model presented previously (see companion paper) we suggested that ruffling of cultured cells may reflect a cellular mechanism by which nutrient transport molecules are inserted into the plasma

membrane from intracellular storage sites. In untransformed cells, this mechanism appears to be tightly and specifically regulated.

Numerous transformed cells (1, 12, 16), including Kirsten murine sarcoma virus-transformed NRK cells (KNRK) (14), ruffle continuously in sparse culture. We reported previously that KNRK cells secrete their own ruffling-stimulating agent(s) (companion paper), which cause ruffling in both untransformed NRK cells and in KNRK cells made quiescent by a pH increase. This ruffling does not cease for at least several hours and thus is not subject to the normal cellular off-regulation that limits the response of cells to nontransforming growth factors. Because this ruffling is similar to that caused by growth factors, except for the duration, and also because the previously described transforming growth factors TGF- $\alpha$  and TGF- $\beta$  (3, 20, 22; for review see reference 20) are secreted by KNRK cells (15), we have studied and characterized the roles of these two factors in the ruffling response. Those studies are described in this paper.

Ruffling is used here as a rapid, real-time monitor of an early cellular response to growth factors. A significant advan-

<sup>1</sup> Abbreviations used in this paper: EGF, epidermal growth factor; KNRK, Kirsten murine sarcoma virus-transformed NRK; NRK, normal rat kidney; TGF- $\alpha$ , transforming growth factor, type  $\alpha$ ; TGF- $\beta$ , transforming growth factor, type  $\beta$ .

tage of real-time monitors is that this kind of approach often permits dissection of component mechanisms that are obscured in an assay employing a longer, after-the-fact biological effect, such as growth in soft agar, thymidine uptake, or cell number increase. In particular, study of the ruffling response has permitted us to explore the phenomenon of normal cellular off-regulation of a growth factor-induced response, and the escape from this off-regulation by transforming growth factors.

## Materials and Methods

NRK cells (7) were cultured in sparse monolayers (10% fetal bovine serum, Waymouth's medium MB752/1, 37°C, 5% CO<sub>2</sub> atmosphere). On day 1 after passage, complete medium was replaced with nutrient deficient Waymouth's medium, made without glucose, amino acids, and vitamins (i.e., a buffer with the same salt composition as Waymouth's medium). After 2–4 h, live cells were examined by inverted phase microscopy using a 25× objective lens for total magnification of ~250×. We have already detailed and validated the quantification method (companion paper). In brief, ruffling was scored just before and at intervals after addition of the ruffling agent. 60–100 cells were observed per count. Intervals between counts were 3–5 min right after the agent was added and increased to 15–20 min later. In this study the following modification was employed. Ruffling was quantified by visual scan of several areas, recording numbers of cells without any marginal ruffles (*a*), with <10% of the free margin ruffled (*b*) and with ≥10% of the free margin ruffled (*c*). A weighted percentage of ruffled cells was calculated according to the equation  $(b/n + c)/(a + b + c) \times 100 = \text{percent ruffled cells}$ , with *a*, *b*, and *c* defined above and *n* a small integer. We found that *n* = 5 distinguished large responses from smaller responses without obliterating the smallest responses and that value was used in all the graphs presented below. Curves were drawn with the aid of an Apple IIe computer programmed to convert the values *a*, *b*, and *c* of individual readings into weighted percentages as just described and to plot those values against time in minutes from addition of the ruffling agent. An averaging program generated composite curves from two or more repetitions of the same experiment. In brief this program functioned as follows. After the individual curves to be averaged were selected, they were plotted onto the same graph. The computer then scanned along the horizontal (time) axis until coming to a point at which any one of the curves had an actual data point. At that time point, the computer scanned vertically and averaged the points intersected by all of the curves being considered. Alternatively, the computer scanned the horizontal axis and averaged the points along the vertical axis at every minute. Both methods produced essentially the same composite curves, but the former was much faster, so it was used to produce the composites presented here. Composite curves rather than data point averages were determined because counts were obtained in rapid sequence, and, therefore, time points at which cells were counted differed somewhat from one experiment to another, so data could not be averaged at specific time points. Individual curves were usually quite similar to the composites obtained, and individual data points never varied from the composite by >10%. In all cases, when one experimental variable was compared with another, the composite curves within a comparison were all derived from the same set of experiments. The differences obtained among the experimental variables presented here were quantitatively reproducible, observed at least five times, and never failed to occur.

EGF was obtained from Collaborative Research Inc., Lexington, MA. Rat TGF- $\alpha$  was purified from Snyder-Theillen feline sarcoma virus-transformed Fisher rat embryo cell (25) culture supernatants as previously described (13). TGF- $\beta$ , lot 784, was a generous gift of Drs. H. L. Moses and R. Tucker. Antiserum to TGF- $\alpha$  was prepared against a synthetic peptide corresponding to the COOH-terminal 17 amino acids of rat TGF- $\alpha$ . The antiserum recognizes both small and large forms of rat and human TGF- $\alpha$  and distinguishes TGF- $\alpha$  from the functionally related peptides EGF and urogastrone (11).

Total responses to factors, or combinations of factors, were quantified by measuring the area under the response curve over the period of interest with the prefactor (untreated) ruffling value used as the baseline. A Zeiss MOP-3 was used to measure areas.

## Results

### Responses to Individual Growth Factors: EGF, TGF- $\alpha$ , and TGF- $\beta$

Sparsely cultured NRK cells were equilibrated in buffer for 2 h. The addition of 2 ng/ml of EGF produced ruffling in most

cells within a few minutes (Fig. 1). Ruffling began to decline almost immediately and returned to near baseline levels within 90 min. Increasing the dose of EGF to 10 ng/ml did not significantly alter EGF-induced ruffling, but 1 ng/ml of EGF elicited a response decreased by ~50% (Fig. 1). The response to 10 ng/ml of EGF was chosen as an internal ruffling standard for further experiments. A standard curve showing the response to 10 ng/ml of EGF in the individual experiments involved is included in Figs. 3–6.

The decline in ruffling almost always followed an oscillatory course (inset, Fig. 1). The oscillatory nature of these responses complicated making quantitative comparisons, so descriptive parameters were defined. A magnitude parameter,  $R_{\max}$ , was defined as the height of the initial peak in the response curve. A duration parameter,  $D_{\max}$ , was defined as the time from addition of factor until the factor response had declined to 80% of the  $R_{\max}$  of the internal EGF standard. This decline (of 20%) occurred before the first minimum and was the most reproducible duration comparison from experiment to experiment.  $R_{\max}$  was reproducible within a few percentage points from experiment to experiment and has subsequently been developed as a rapid method to quantify growth factors (manuscript in preparation) with sensitivities as low as 0.2 ng/ml. An alternative measure of the total ruffling response to a factor was measurement of the area under the response curve. This value was also highly reproducible in repeated experiments (see below).

Treatment of cells with 2 ng/ml of TGF- $\alpha$  produced a response similar to that caused by 2 ng/ml of EGF (Fig. 2). Increasing the dose of TGF- $\alpha$  to 2.5 ng/ml increased both the intensity and the duration of ruffling somewhat as compared with the response to 10 ng/ml of EGF, but responses to 1 ng/ml of either factor were similar to each other (Fig. 3). In the experiments shown in Fig. 3,  $R_{\max}$  of 2.5 ng/ml of TGF- $\alpha$  ( $R_{\max-\alpha}$ ) was 1.16 (116% of  $R_{\max-EGF}$ ), and  $D_{\max-\alpha}$  was 1.17 (117% of  $D_{\max-EGF}$ ).

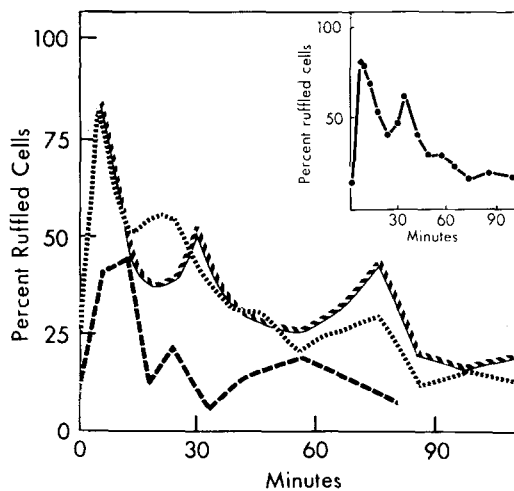


Figure 1. Ruffling on NRK cells after treatment with EGF. Sparse monolayers of NRK cells were equilibrated in buffer for 2 h and then treated with 1 (■—■), 2 (▲—▲), or 10 (●—●) ng/ml EGF (or 7.5 ng/ml, inset). The vertical axis represents a weighted percentage (see Materials and Methods) of ruffled cells. The horizontal axis represents time after addition of EGF. Curves for 2 and 10 ng/ml were each traced from a computer-drawn composite (see Materials and Methods) of two experiments. The 1 and 7.5 ng/ml curves each represent a single experiment. Data points are shown on the 7.5 ng/ml curve.

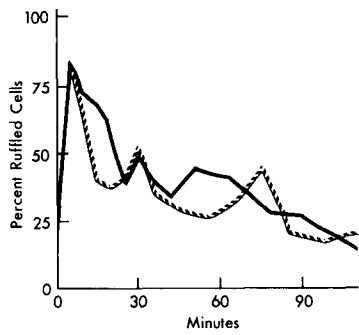


Figure 2. Comparison of ruffling caused by EGF and TGF- $\alpha$ . Cells were treated as described in Fig. 1, but with 2 ng/ml of either EGF (---) or TGF- $\alpha$  (—). Curves are each composites from two experiments.

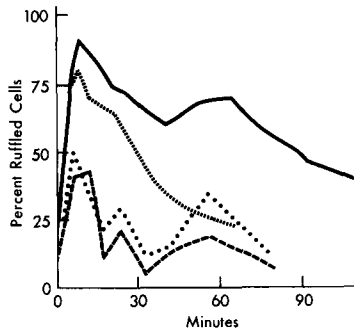


Figure 3. TGF- $\alpha$  dose differences as compared with EGF. Cells were treated as described in Fig. 1, but in a composite of two experiments with 2.5 ng/ml TGF- $\alpha$  (—) or 10 ng/ml EGF (.....) or in a separate experiment with 1 ng/ml EGF (---) or 1 ng/ml TGF- $\alpha$  (-.-.).

Cells treated with 100 ng/ml of EGF exhibited no response to a second dose of EGF and little or no response to TGF- $\alpha$ . Preincubation of TGF- $\alpha$  with rabbit IgG against a synthetic peptide corresponding to the COOH-terminal 17 amino acids of rat TGF- $\alpha$  delayed the onset of ruffling by several minutes and reduced  $R_{max}$  by 72% (to  $R_{max}$  0.28). TGF- $\beta$  (2–2.5 ng/ml) caused no ruffling and frequently reduced the baseline ruffling (before the addition of other factors) to almost nil (data not shown).

### TGF- $\alpha$ and TGF- $\beta$ Together

NRK cells were cultured and equilibrated in buffer as above and treated with 2 ng/ml of TGF- $\beta$ . At 0.5 h after the addition of TGF- $\beta$ , cells were treated with 2.5 ng/ml of TGF- $\alpha$ .  $D_{max-\alpha}$  was not increased in the presence of TGF- $\beta$ , and  $R_{max-\alpha}$  decreased by 6% (Fig. 4). In these same experiments, however, ruffling began to increase again after 2 h in the presence of both factors, until, several hours later, all cells were ruffled. Pretreatment of cells with TGF- $\beta$  for 6 h before TGF- $\alpha$  was added increased  $D_{max-\alpha}$  to nearly 500% that of untreated cells (Fig. 5).  $D_{max-\alpha}$  was not further increased by pretreatment for 13.5 h. If TGF- $\beta$  containing medium was incubated with NRK cells for  $\geq 6$  h, then transferred to fresh NRK cells for 0.5 h before addition of TGF- $\alpha$ ,  $D_{max-\alpha}$  was increased to nearly 300% that of untreated cells (Fig. 6). Under this protocol, the fresh cells were exposed to any remaining TGF- $\beta$  for only 0.5 h, which alone was not long enough to increase  $D_{max-\alpha}$  (see Fig. 4). Transfer of buffer alone preincubated with NRK cells to fresh cells, or transfer of TGF- $\beta$ -containing

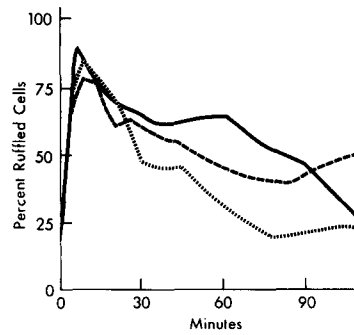


Figure 4. TGF- $\alpha$  response after a 0.5-h pretreatment with TGF- $\beta$ . Cells were equilibrated in buffer for 2 h, then treated with 2 ng/ml TGF- $\beta$  or not treated. After an additional 30 min, TGF- $\alpha$  was added to treated and untreated cells. Each curve is a composite of two experiments. Data for the composite 10 ng/ml EGF standard curve (.....) were obtained in the same experiments. (—), TGF- $\alpha$  alone, 2.5 ng/ml. (---), TGF- $\beta$ , 0.5 h, then TGF- $\alpha$ , 2.5 ng/ml.

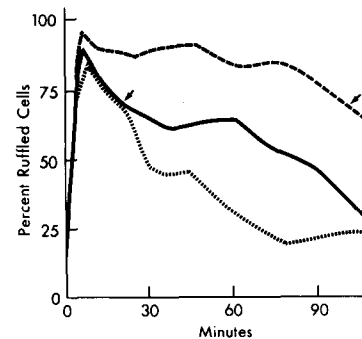


Figure 5. TGF- $\alpha$  response after 6 h of pretreatment with TGF- $\beta$ . As in Fig. 4, but with 6 h of pretreatment with TGF- $\beta$  or no pretreatment. EGF standard is included. Arrows indicate the point at which the EGF standard response has declined 20% from  $R_{max}$ . (.....), EGF, 10 ng/ml. (—), TGF- $\alpha$  alone, 2.5 ng/ml. (---), TGF- $\beta$ , 6 h, then TGF- $\alpha$ , 2.5 ng/ml.

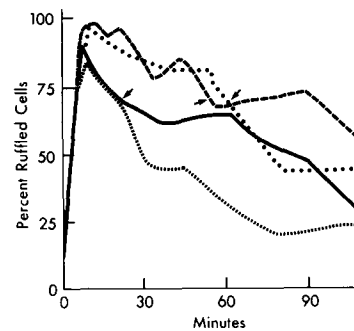


Figure 6. TGF- $\alpha$  response after transfer of TGF- $\beta$ -induced activity. NRK cells at  $\sim 35\%$  confluence were equilibrated in buffer containing 2 ng/ml of TGF- $\beta$  for 6 h (---) or 8 h (---). This TGF- $\beta$ -NRK conditioned buffer was then transferred to fresh, sparsely cultured NRK cells which had been equilibrated in buffer for 2 h. After the fresh cells had been incubated with the conditioned buffer for 30 min, TGF- $\alpha$  was added to these and to untreated cells. EGF standard is included (.....; 10 ng/ml). Each curve is a composite of two experiments. Arrows indicate the point at which the EGF standard response has declined 20% from  $R_{max}$ . (—), TGF- $\alpha$  alone, 2.5 ng/ml. (---), TGF- $\beta$ -NRK preconditioned buffer, then TGF- $\alpha$ , 2.5 ng/ml.

Table I. Quantification of Responses to TGF- $\alpha$

Pretreatment	Time interval quantified	Response	<i>n</i>	SD	% Change	P*
None	5–100 min after TGF- $\alpha$	100	4	4.2	—	—
6 h TGF- $\beta$	5–100 min after TGF- $\alpha$	135 <sup>‡</sup>	4	3.3	35	<0.001
None	5–60 min after TGF- $\alpha$	100	4	1.9	—	—
0.5 h of medium preconditioned on other NRK cells for 6–8 h with TGF- $\beta$	5–60 min after TGF- $\alpha$	124 <sup>§</sup>	4	2.5	24	<0.001

Response curves of individual experiments (as illustrated by composite curves in Figs. 5 and 6) were plotted by computer (see Materials and Methods). For each experiment, the area under the curve and above the baseline during the indicated time intervals was determined by tracing the designated area with a Zeiss MOP-3. For each designated time interval, the mean value of the TGF- $\alpha$  response with no pretreatment was set as 100.

\* Probability, by Student's *t*-test, that compared populations are the same.

‡ Relative to TGF- $\alpha$  alone for a 5–100-min interval.

§ Relative to TGF- $\alpha$  alone for a 5–60-min interval.

buffer incubated for 6 h without cells each produced slight decreases in  $R_{\max-\alpha}$  and  $D_{\max-\alpha}$ . Thus the increase in  $D_{\max-\alpha}$  was due to an activity in the buffer produced by exposure of NRK cells to TGF- $\beta$ .

The parameter  $D_{\max}$  was arbitrarily defined as a measure of the length of time that the response to a factor remained quantitatively greater than some percentage of a standard maximal response (in this case, 80% of the chosen standard  $R_{\max}$ -EGF). Clearly, then, an increase in  $D_{\max}$  could be achieved by an increase in  $R_{\max}$  or by reducing the average slope of the recovery part of the response curve. In both Figs. 5 and 6, the increase in  $D_{\max}$  appears to result from a combination of these two effects. As an alternative comparison of different treatment protocols, total responses to 2.5 ng/ml of TGF- $\alpha$  under these various conditions were quantified by measuring the area under the response curve during the relevant period. Responses to individual experiments were compared to determine the reproducibility of the response to a particular treatment and to determine whether different protocols produced statistically different responses. Table I provides these comparisons. All responses were quite reproducible (SD = 2–4% of total response). Preincubation of cells for 6 h with TGF- $\beta$  (as in Fig. 5) increased the total response to TGF- $\alpha$  during the first 100 min by 35% (with  $P < 0.001$  by Student's *t*-test). Preincubation of cells with the TGF- $\beta$  preconditioned medium (as in Fig. 6) increased the total response during the first 60 min by 24% (with  $P < 0.001$  by Student's *t*-test). This demonstrates that a 6-h exposure of NRK cells to TGF- $\beta$  significantly increased the ruffling response to TGF- $\alpha$ , whether measured as a function of duration of maximal response or as total response. Furthermore, that increase was due to a soluble activity in the medium since the preconditioned medium also produced a statistically significant increase in the TGF- $\alpha$ -induced ruffling response.

At 24 h after TGF- $\alpha$  treatment, most of the cells that had also been treated with TGF- $\beta$  were somewhat ruffled. By the ruffling measure used in the accompanying report, the combined treatment produced an extended response similar to that observed with KNRK cell-conditioned medium.

## Discussion

TGF- $\alpha$  binds to the EGF receptor (3, 23) and exhibits EGF-like biological activities. Both EGF and TGF- $\alpha$  stimulate incorporation of thymidine in cultured cells. Both factors, in the presence of TGF- $\beta$ , permit anchorage-independent growth of untransformed cells (2, 3). Both of these activities are dose

dependent. Similarly, in the experiments reported here, TGF- $\alpha$  mimics the capacity of EGF to induce transient ruffling in sparsely cultured NRK cells, in a dose range like that of previously described activities. Also, like other TGF- $\alpha$  responses, TGF- $\alpha$ -induced ruffling is probably mediated through the EGF receptor since pre-exposure to EGF blocked the TGF- $\alpha$ -induced ruffling response. At 1 or 2 ng/ml, the TGF- $\alpha$  response and the EGF response are similar within the reasonably expected accuracy of determination of concentrations and so indicate an approximate equivalence in potency. The duration of the response to 2.5 ng/ml of TGF- $\alpha$  was greater than that of the response to 10 ng/ml of EGF. The increase in  $D_{\max-\alpha}$  may be due to intrinsic differences between EGF and TGF- $\alpha$  (manuscript in preparation), or it may indicate a minor contamination of the TGF- $\alpha$  preparation, which was purified from transformed cell culture supernatants (see Materials and Methods). Such a contamination, however, probably was not another ruffling-stimulating growth factor since the TGF- $\alpha$  response was completely blocked by pretreatment with EGF.

TGF- $\beta$  is a polypeptide produced by a number of transformed and untransformed cell types (2, 18; for review see reference 20). It produces a confusing set of biological responses in cell culture assays, some of which appear to be almost opposite each other. Alone, TGF- $\beta$  is not transforming in the growth in soft agar assay (2, 3) and inhibits growth of a number of cell types in the thymidine incorporation assay (17, 24). Yet it enables either EGF or TGF- $\alpha$  to induce, reversibly, transformed characteristics in untransformed cells. Most of these properties are consistent with our observations here. TGF- $\beta$  caused no ruffling on NRK cells directly, produced a decline in baseline ruffling, and decreased  $R_{\max-\alpha}$  after short term pretreatment, in agreement with its individual lack of transforming activity and its growth inhibitory effects. But, after several hours, TGF- $\beta$ -pretreated cells exhibited an increased duration in their TGF- $\alpha$ -induced ruffling so that, together, treatment with these two factors mimics the ruffling activity (accompanying report) secreted by KNRK cells. This combined effect is analogous to the coordinated transforming activity of these two factors described elsewhere.

One means by which long term treatment of cells with TGF- $\beta$  could enhance a TGF- $\alpha$ -induced response is by increasing the responsiveness of the cells themselves. For example, Assoian et al. (4) report an apparent increase in EGF receptors on NRK cells after 6 h of TGF- $\beta$  treatment. But such a mechanism would not account for the observations in

Fig. 6 in which the TGF- $\beta$ -induced change was transferred in buffer to fresh cells. It is clear from those data that it is not TGF- $\beta$  itself that enhanced the TGF- $\alpha$  response, nor is it merely a change in the responsiveness of the cells during the 6-h TGF- $\beta$  treatment. Rather, this inhibition of off-regulation is caused by a new activity which is produced when NRK cells are incubated with TGF- $\beta$ . This activity is neither cellular TGF- $\alpha$  nor platelet-derived growth factor, since the transferred buffer did not itself cause ruffling.

Our data do not indicate whether the TGF- $\beta$ -dependent activity was a new molecular species released by the NRK cells after TGF- $\beta$  stimulation or instead was the TGF- $\beta$  molecule altered by exposure to NRK cells. But, the data do demonstrate that this newly observed activity modifies the TGF- $\alpha$ -induced ruffling response. Thus, cellular responses might be stimulated or inhibited by TGF- $\beta$  depending on the length of time of exposure to TGF- $\beta$  and on the ability of the responding cells to produce this new activity in the presence of TGF- $\beta$ .

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