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ROLE OF TRANSFORMING GROWTH FACTOR-α (TGF-α) IN BASAL AND HORMONE-STIMULATED GROWTH BY ESTRADIOL, PROLACTIN AND PROGESTERONE IN HUMAN AND RAT MAMMARY TUMOR CELLS: STUDIES USING TGF-α AND EGF RECEPTOR ANTIBODIES

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Summary—The biological role of transforming growth factor-α (TGF-α) in basal and hormone-stimulated proliferation of primary human and rat mammary tumor cells was studied using antibodies against TGF-α and its receptor. A monoclonal antibody, MAb-425 against human EGF receptor was added to *in vitro* soft agar, clonogenic cultures of human breast carcinoma cells under basal and estradiol(E_2)-stimulated conditions. The antibody had an antagonist effect on colony growth in 4 of 10 tumors and an agonist effect in 4 (72 and 153% of control). E_2-stimulated colony growth in 5 tumors (167% of control) and the antibody blocked E_2-stimulation in 3 of the 5. Inhibition of E_2-stimulated growth in 3 and basal growth in 4 other tumors by the EGF receptor antibody suggests that endogenously secreted TGF-α has a role as an autocrine/paracrine growth factor in constitutive and E_2-stimulated tumor cell proliferation in a majority of human tumors. A polyclonal antibody against TGF-α was used to study the role of TGF-α in E_2-, prolactin(Prl)- and progesterone(Prog)-stimulated proliferation of NMU(nitrosomethylurea)-induced rat mammary tumor cells under similar culture conditions. TGF-α, E_2, Prl and Prog stimulated colony growth equally to 176, 187, 168 and 181% of control. The antibody produced significant and similar inhibition of TGF-α and E_2-stimulated growth (95 and 83%). In contrast, inhibition of Prl- and Prog-stimulated growth by the antibody was only 24 and 37%. The TGF-α ligand antibody did not have an agonist or antagonist effect when added alone. Thus, TGF-α seems to be a major stimulatory growth factor mediating E_2-induced tumor cell proliferation in rat mammary tumors. It is less important in Prl- and Prog-induced tumor growth and not essential for basal growth in these tumors. We conclude that TGF-α is a biologically important autocrine/paracrine growth factor in primary human breast cancer cell proliferation and in E_2-induced rat mammary tumor growth.

INTRODUCTION

Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) are related stimulatory peptide growth factors that exert their mitogenic effect through a common receptor, the EGF receptor [1]. EGF stimulates proliferation of human breast cancer cells from primary human tumors [2], human breast cancer cell lines [3] and normal mammary epithelial cells [4]. Secretion of TGF-α and expression of TGF-α mRNA is seen in most human breast cancer cells [5, 6], in normal mammary tissue [7] and in experimental nitrosomethylurea(NMU)-induced rat mammary tumors [8], and is stimulated by estradiol [E_2] in hormone-responsive human breast cancer cell lines [5, 6]. Receptors for EGF are present in varying numbers in breast cancer cells (Table 1) and the EGF receptor status of primary human tumors seems to be a significant prognostic factor clinically, with a decreased survival in patients who have receptor-positive tumors [9]. These data suggest that TGF-α may have an important role as an autocrine stimulatory growth factor in breast cancer cell proliferation and in mediating the growth effects of E_2. However, the biological significance of this peptide in the proliferation of human breast cancer cells has been thrown into question by studies which showed that EGF receptor antibodies had no effect on the basal or hormone-stimulated tumor cell proliferation of several hormone-responsive breast cancer cell lines despite the secretion of TGF-α

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Table 1. Spectrum of EGF-receptor expression and response to EGF and the EGF-receptor antibody (EGF-R-Ab) in different human breast cancer cells and non-malignant mammary epithelial cells: collated from reports in the literature

Cell type	EGF-receptors	Response to EGF	Response to EGF-R-Ab	Ref.
Normal mammary epithelial cells	5×10^5	Stimulation	Inhibition	[7]
184A1N4[a]	3×10^5	Stimulation	Inhibition[b]	[4]
184A1NA-T	20×10^5	Stimulation	Inhibition[b]	[4]
MCF-7	0.03×10^5	Stimulation	Basal: unaffected E_2-stimulated: unaffected	[10]
MDA-231	3.0×10^5	No effect	No effect	[4]
MDA-468	$10–30 \times 10^5$	Inhibition	Inhibition	[4]
Human tumors	+ +	Stimulation		[2]

[a]184A1N4: human breast epithelial cell line.
[b]Inhibition of EGF-stimulated growth only.

by the cells [10]. We undertook studies with human and rat mammary cancer cells from primary tumors and used antibodies directed against TGF-α and its receptor to clarify the biological role of TGF-α in a tumor cell population which more closely represents the heterogenous nature and behavior of human tumors. This report describes these studies, which show that endogenously secreted TGF-α has a significant biological role in basal and hormone-induced proliferation of primary human and rat mammary tumor cells.

EXPERIMENTAL

Mammary tumors

Fresh human breast tumor tissue was obtained from 10 surgically excised ductal carcinomas. Experimental mammary tumors were induced in female Sprague–Dawley rats with a single injection of NMU (5 mg/100 g body wt) [11].

Soft agar clonogenic culture

Fresh human and rat mammary tumor tissue was mechanically dispersed to yield a single cell suspension as previously described [12]. The cells were plated in a bilayer soft agar clonogenic culture system using the techniques described by Hamburger and Salmon [13] and Von Hoff *et al.* [14]. The culture conditions are described in detail in an earlier publication [12]. Experiments were carried out in 35 mm^2 Petri dishes in serum free and phenol red free medium and the culture plates incubated at 37°C with 5% CO_2 and 100% humidity. BSA 0.4% was added to the lower and upper layers in the human tumor experiments. Colony (> 50 cells) number was counted on day 6 using an inverted phase microscope at × 40.

Antibodies

A IgG monoclonal, EGF receptor antibody raised against human EGF receptor from a human epidermoid carcinoma cell line, the A-431 cells [15] was used to study the role of TGF-α in human tumor experiments. The antibody was a generous gift from Dr M. Herlyn (Wistar Institute, Philadelphia, PA). A polyclonal TGF-α ligand antibody (Biotope, Washington, DC) was used in the rat mammary tumor experiments. A mouse IgG 1 immunoglobulin (Sigma, St Louis, MO) was used as the irrelevant antibody in the rat mammary tumor experiments. Synthetic human TGF-α was obtained from Biotope.

Experimental design

Human tumor experiments. The cell suspension obtained from human tumors was plated into the upper layer of the soft agar clonogenic culture in triplicate and under the following experimental conditions: control; E_2 (1 nM); EGF receptor antibody (0.5 μg/ml); and E_2 EGF receptor antibody.

Rat mammary tumor experiments

The cell suspension obtained from rat mammary tumors was plated into the upper layer of the soft agar clonogenic culture in triplicate, under the following experimental conditions control, hormone (E_2 1 nM, progesterone (Prog) 1 nM, ovine prolactin 200 ng/ml), hormone plus TGF-α antibody, hormone plus irrelevant antibody, TGF-α antibody alone (135 μg/ml), TGF-α (10 ng/ml), TGF-α plus TGF-α antibody and irrelevant antibody alone.

Rat mammary tumor indirect experiments

We observed that the TGF-α antibody produced a much smaller inhibition of prolactin (Prl)- and Prog-stimulated growth than it did of E_2-stimulated colony growth (*vida infra*). We hypothesized that this could be due to a decreased production of TGF-α in response to Prl and Prog or because TGF-α has only a minor biological role in mediating the growth induced by these two hormones, despite being secreted

upon hormonal exposure. To clarify this we carried out indirect experiments to assess TGF-α activity in conditioned medium (CM) from E_2-CM, Prl-CM and Prog-CM treated rat mammary tumor cell cultures. CM was obtained from soft agar clonogenic cultures of rat mammary tumor cells treated with E_2, Prl, Prog or control conditions as described previously [16]. Briefly, after concentration, the media were treated with dextran-coated charcoal (0.25% charcoal and 0.0025% diethylaminoethyl-dextra) and kept frozen at −70°C until used in subsequent experiments. E_2 and Prog levels were measured by specific RIA in our concentrated media [17, 18] and found to be undetectable. In these experiments, we did not measure Prl levels. However, in previous experiments where Prl-CM were identically prepared, we observed that treatment with dextran-coated charcoal reduced Prl levels below the limit of detection or to minimal amounts that are not biologically active in our system [19, 20]. The TGF-α activity present in the CM was assessed by the ability of the CM to stimulate rat mammary tumor cell colony growth in a second soft agar clonogenic culture system and the ability of a specific TGF-α antibody to block this stimulatory effect. Control-CM, E_2-CM, Prl-CM and Prog-CM were added to the soft agar cultures with and without TGF-α antibody and the colony number counted on day 6 (*vide supra*). The concentration of antibody was chosen to produce maximal inhibition of the colony stimulation induced by exogenous TGF-α (10 ng/ml).

Statistical analysis

This was done using analysis of variance according to the Student Newman–Keuls test. Comparisons of the percentage stimulation by hormone and percentage inhibition by the antibody was made by a repeated measures analysis of variance followed by linear contrasting.

RESULTS

Human tumor experiments

The growth of human tumor cells was inhibited by the EGF receptor antibody under serum free conditions in 4 of 10 tumors ($72 \pm 4\%$ of control, $P < 0.05$ in each case) (Fig. 1A), suggesting that endogenous autocrine secretion of TGF-α is involved in the basal growth of these cells. The antibody had a stimulatory, agonist effect on 4 tumors ($153 \pm 16\%$ of control, $P < 0.05$ in each case) and did not affect the growth of 2 tumors (Fig. 1A). E_2 produced a significant stimulation of tumor colony number in 5 of 10 tumors ($167 \pm 17\%$ of control, $P < 0.05$ in each case) and the EGF receptor antibody partially inhibited the stimulation produced by E_2 in 3 of the 5 tumors (Fig. 1B). The antibody by itself had a stimulatory (agonist) effect in these 3 tumors (Fig. 1A), which makes the suppression by the antibody, of the E_2-induced stimulation more significant. Tumors 9 and 10 had high basal colony numbers and did not show any further stimulation with the addition of E_2 but were significantly inhibited by the antibody. An inhibitory effect of the antibody on E_2-stimulated growth in 3 tumors and on basal growth in 4 other tumors suggests that endogenously produced TGF-α has a role in basal and hormone-stimulated tumor cell proliferation in a majority of human tumors. Additional studies with exogenous TGF-α and irrelevant antibody could not be done with the human tumors because of the small quantity of tissue and cells available which limited the number of treatment groups in each experiment.

Rat mammary tumor experiments

We were able to study the responses to hormonal stimulation with E_2, Prl, Prog and the effects of a TGF-α ligand antibody upon hormone-stimulated tumor cell proliferation more comprehensively in the rat mammary tumors due to the availability of unlimited tumor tissue and the more homogenous nature of the cell population. E_2, Prl and Prog stimulated colony number significantly to 187, 168 and 181% of control, respectively (Fig. 2). The degree of stimulation by the hormones was comparable with and not different from that produced by TGF-α (176%) (Fig. 2, $P = 0.09$). The TGF-α ligand antibody significantly inhibited hormone-stimulated colony growth by E_2, Prl and Prog. It produced maximal inhibition of E_2-induced growth, by $83 \pm 9.8\%$ ($P < 0.01$) which was not significantly different from the $94.6 \pm 1.4\%$ inhibition of TGF-α-stimulated growth ($P = 0.33$, Fig. 2). The growth stimulation by Prl and Prog was inhibited to a significantly lesser extent ($P < 0.01$) by the antibody, $24.4 \pm 8.1\%$ and $37.5 \pm 6.2\%$, respectively and was not significantly different between the 2 hormones ($P = 0.13$, Fig. 2). Basal colony formation was not altered when the TGF-α antibody was added alone (data not shown). Addition of the irrelevant antibody did not

affect tumor growth under any experimental conditions (data not shown). The indirect experiments to assess the biological TGF-α activity in the different conditioned media (C-CM, E_2-CM, Prl-CM and Prog-CM) showed that the TGF-α antibody inhibited the stimulatory action of E_2-CM, and Prog-CM to the same extent it inhibited the stimulation by exogenous TGF-α (90.7, 86.2 and 95.6%), respectively (Fig. 3). This is indirect evidence of the presence of significant amounts of TGF-α activity in these conditioned media. In contrast, the stimulatory action of Prl-CM was inhibited by only 25%. This is analogous to a bioassay that indicates there is less TGF-α present in Prl-CM compared with E_2-CM and Prog-CM, reflecting a reduced production of TGF-α by the cells in response to Prl. As in the previous experiments, the TGF-α antibody had no agonistic/antagonistic activity of its own and the irrelevant antibody did not affect colony formation under any experimental condition.

DISCUSSION

There is substantial evidence that TGF-α is secreted by human breast cancer cells and that E_2 stimulates TGF-α secretion in hormone-responsive cells [5, 10, 21]. However, the biological role of this stimulatory growth factor in basal and hormone-induced breast cancer cell proliferation remains controversial. It has been suggested that TGF-α has no significant role in the proliferation of several hormone-sensitive human breast cancer cell lines [10], while other studies have shown that TGF-α/EGF related peptides have an autocrine mitogenic effect during the first 5 days of E_2-stimulated growth of MCF-7 cells [6]. In the case of hormone independent breast cancer cell lines, the proliferation of MDA-231 cells does not seem to be influenced by EGF or the EGF receptor antibody while the MDA-468 cells which express an excess of EGF receptor are inhibited by the ligand and the antibody [4]. There is

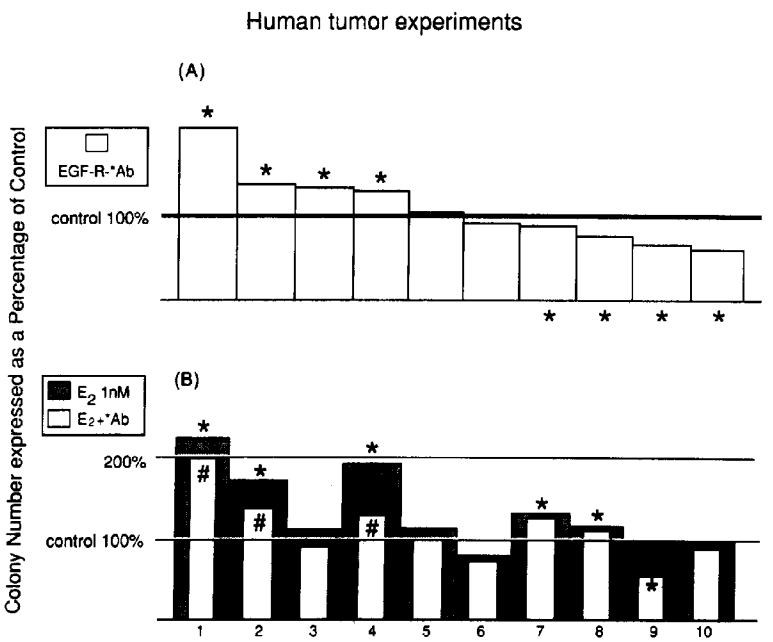

Fig. 1. Individual human tumors ($n = 10$) grown in soft agar clonogenic culture: colony number expressed as a percentage of control. The control colony number ranged from 16 to 750, median 81.5 and the overall CV between triplicates ranged from 1 to 22% (mean 7%). (A) The effect of EGF-receptor antibody (*Ab) on colony number $*P < 0.05$ compared to control. (B) The effect of estradiol (E_2, 1 nM) and E_2+*Ab on colony number. $*P < 0.05$ compared with control. The asterisks above the shaded bar indicate significant stimulation by E_2 and the asterisk inside the clear bar (tumor No. 9) denotes significant inhibition by "E_2 + Ab" in the absence of E_2 stimulation. $\#P < 0.05$ denotes significant inhibition by the *Ab of E_2-stimulated growth.

considerable variation in the expression of EGF-receptor by breast cancer cells with a variable growth response to EGF and EGF-receptor antibody (Table 1). The mechanisms responsible for this variable mitogenic effect are not clearly understood but do not seem to be due to differences in receptor affinity or structure [3]. Since primary human tumors are composed of a heterogenous tumor cell population having hormone-responsive and hormone-resistant cells, the secreted TGF-α could exert a paracrine stimulatory effect upon adjacent tumor cell populations in addition to an autocrine mitogenic action upon the same cell or cell type. We felt it was important to study primary tumors to clarify whether TGF-α has such a biological, paracrine/autocrine role in the proliferation of a mixed tumor cell population. The soft agar clonogenic culture is based on the ability of malignant stem cells from human tumors to proliferate in clones to produce colonies [13, 14]. Studies with human breast tumors have shown that such colonies have similar histologic characteristics as the original tumor [22]; and when inoculated into nude mice, form tumors that are also histologically similar to the original tumor [23]. We therefore used this culture system to study the paracrine/autocrine interactions between malignant cell populations from primary mammary tumors.

Our studies reveal that TGF-α is a major growth factor in the E_2-stimulated tumor cell proliferation of experimental rat mammary cancer. Inhibition of E_2-induced growth by 83% by the TGF-α antibody in this serum free culture system suggests that secretion of TGF-α by these cells is a major mediator of E_2 action. Though Prl and Prog stimulated tumor cell proliferation to a similar degree as E_2, the TGF-α antibody only inhibited 24 and 37% of this growth, respectively, suggesting that TGF-α does not have a major role in mediating the mitogenic action of these 2 hormones. This is probably due to less production of TGF-α by the cells in the case of Prl as the indirect assessment of biological TGF-α activity in the

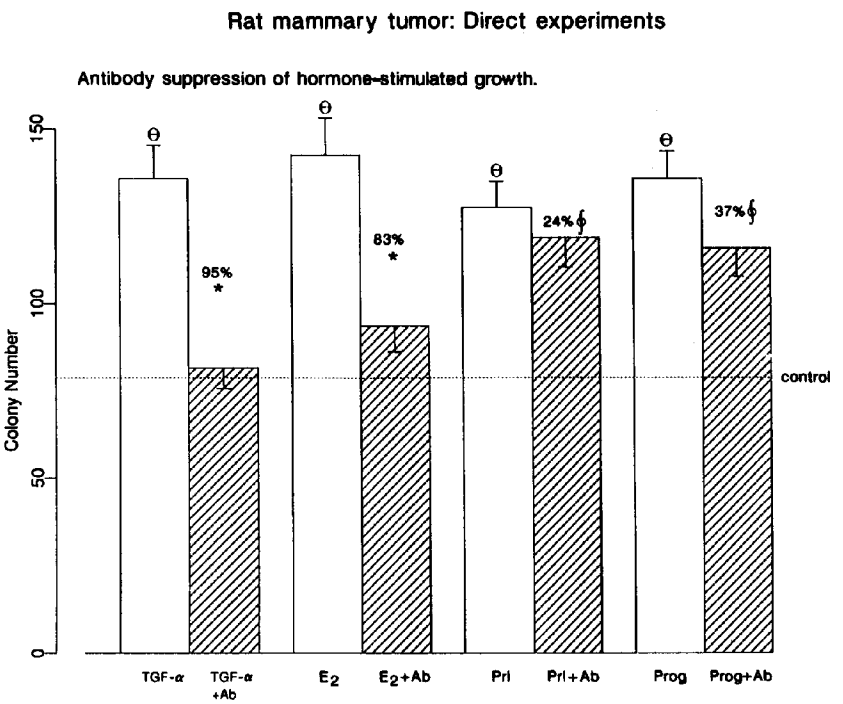

Fig. 2. The effect of TGF-α antibody (Ab) on hormone and TGF-α-induced colony growth of rat mammary tumors. The colony number is expressed as mean ± SE of 8 experiments. The dotted line is the mean basal (control) colony number and the percentage adjacent to the open bar represents the percentage inhibition by the Ab of the hormone-stimulated growth (100%). $P < 0.05$ compared with control. *$P = 0.33$ between TGF-α + Ab and E_2 + Ab, while both are significantly different from Prl + Ab and Prog + Ab, $P < 0.01$. §$P = 0.13$ between Prl + Ab and Prog + Ab.

conditioned media did demonstrate less activity in Prl-CM while Prog-CM seemed to have significant amounts of TGF-α, similar to that found in E_2-CM: 25, 86 and 91% inhibition by TGF-α-antibody of Prl-CM, Prog-CM and E_2-CM stimulation, respectively. Yet in the experiments where Prog plus the antibody are added directly to rat mammary tumor cells in culture, the TGF-α antibody blocked the mitogenic activity of Prog by only 37%. This finding suggests that though Prog induces significant TGF-α secretion, this growth factor does not seem to be a major mediator of Prog-induced tumor cell proliferation. Therefore, the mitogenic effects of Prl and Prog are probably due to growth factors or factors other than TGF-α or due to a direct action on essential cellular/nuclear enzyme systems. Overall, our data support the concept that hormones may act through several different mechanisms to induce tumor cell proliferation.

The human tumors showed a mixed response to the EGF-receptor antibody which is not unexpected given the heterogenous nature of the cell population in these tumors and the variable expression of EGF-receptor and the diverse biologic response of hormone-dependent and hormone-independent cells to the ligand. The antibody had agonist and antagonist activity in a majority of human tumors. The fact that a specific monoclonal antibody against the EGF-receptor blocked and inhibited hormone-stimulated growth and basal growth in a total of 7 of 10 tumors, provides indirect evidence that a majority of human tumors secrete a biologically active TGF-α/EGF related peptide which has an autocrine/paracrine mitogenic role in basal and hormone-stimulated tumor cell proliferation. Some caution, nevertheless, should be exerted in interpreting these results since, due to the limited number of cells available, we were unable to include a group treated with an irrelevant antibody.

We conclude that TGF-α is a significant mitogenic growth factor in both basal and E_2-stimulated proliferation in a majority of

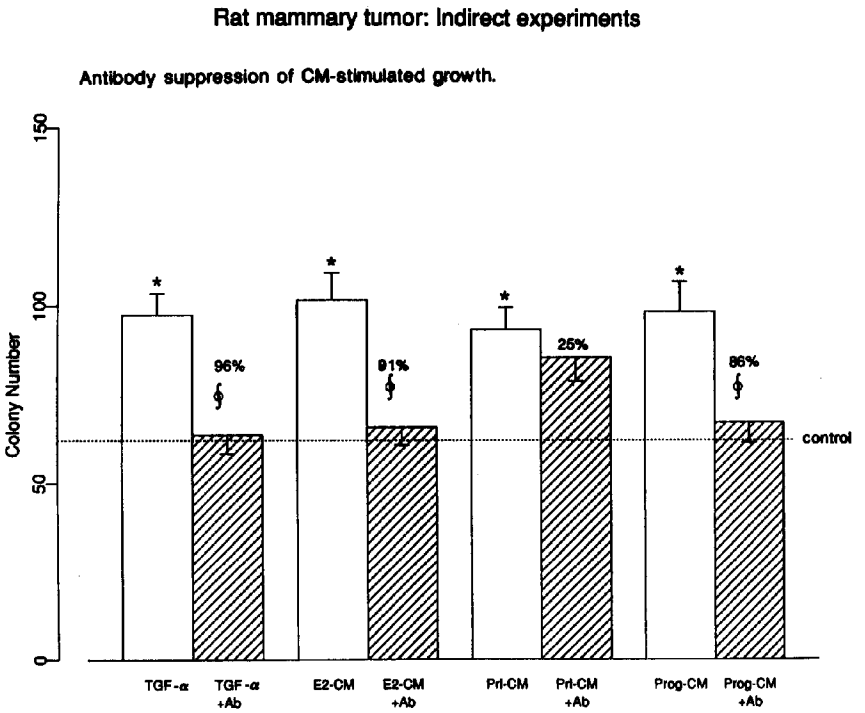

Fig. 3. Stimulation of rat mammary tumor cell colony growth by hormone treated conditioned medium (CM) and inhibition of this by the TGF-α antibody (Ab): this is a biological measure of the TGF-α activity present in conditioned medium; E_2-CM, Prl-CM and Prog-CM. *Not significantly different from one another; $P < 0.05$ compared with control. §Inhibition by Ab significantly greater than in Prl-CM + Ab, $P < 0.01$.

primary human tumors. Such a role for TGF-α in human breast cancer would conceptually support the clinical observation that the EGF-receptor status significantly influences patient prognosis and survival [9]. Similarly, it is a major mediator of E_2-stimulated growth in experimental rat mammary tumors, while Prl- and Prog-induced growth of these tumors is only partially mediated by TGF-α and basal growth seems to depend on other growth factors or mechanisms.

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