Transforming Growth Factor β Induces Anchorage-independent Growth of NRK Fibroblasts via a Connective Tissue Growth Factor-dependent Signaling Pathway

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Abstract

Connective tissue growth factor (CTGF) is a Mr 38,000 cysteine-rich peptide, the synthesis and secretion of which are selectively induced by transforming growth factor β (TGF-β). The relationship of CTGF to TGF-β action on fibroblastic cells is not well understood. TGF-β has the unique ability to stimulate the growth of normal fibroblasts in soft agar, a property of transformed cells. We have investigated whether CTGF can substitute for TGF-β or whether CTGF action is essential for TGF-β to stimulate anchorage-independent growth (AIG) of NRK fibroblasts. Our studies demonstrate that CTGF cannot induce AIG of NRK fibroblasts. However, CTGF synthesis and action are essential for the TGF-β-induced AIG of NRK fibroblasts. Anti-CTGF antibodies specifically block TGF-β-induced AIG but have no effect on platelet-derived growth factor or epidermal growth factor-induced growth in monolayer cultures and do not cross-react with platelet-derived growth factor or TGF-β. Clones of NRK fibroblasts that express an antisense CTGF gene (NRK-ASCTGF), which blocks the expression of the endogenous CTGF gene, do not respond to TGF-β in the AIG assay. The growth and morphology of the cells (NRK-ASCTGF) in monolayer culture are unaltered from the parent NRK cell line. The addition of recombinant CTGF to the NRK-ASCTGF clones in the presence of TGF-β restores the AIG response of the cells. These studies demonstrate that the TGF-β stimulation of NRK fibroblast AIG is dependent on events induced via the synergistic action of CTGF-dependent and CTGF-independent signaling pathways.

Introduction

TGF-β is the prototypic member of a large superfamily of peptides with diverse, multifunctional activities. This family includes the different forms of TGF-β (TGF-β1-5), the “activins” and “inhibins,” and the bone morphogenetic proteins (BMP2 through BMP7; Refs. 1–4). Some of the biological functions of TGF-β include the regulation of cell proliferation and the regulation of extracellular matrix protein expression (5–8) and cell adhesion molecules (9). TGF-β is a potent growth factor that is capable of stimulating the proliferation of fibroblastic cells in an anchorage-independent fashion (10), which was the assay used during the original identification and purification of the peptide (11, 12).

The importance of cell adhesion as a specific regulator of cell proliferation is now well established (13, 14). Depriving NRK fibroblasts of a suitable adhesive surface results in cell cycle arrest of cells in the mid-G1-phase of the cell cycle (15). The addition of TGF-β enables the NRK cells to overcome this mid-G1 arrest, allowing them to proliferate under anchorage-independent conditions. The mechanism by which TGF-β causes this override of suspension-induced cell cycle arrest has not yet been elucidated.

One of the possible downstream mediators of TGF-β action that could be involved in this process is CTGF. CTGF is a cysteine-rich, heparin-binding peptide of Mr 38,000, first identified in media conditioned by human umbilical vein endothelial cells (16). CTGF is a member of a family of proteins that share a high degree of sequence homology including 38 conserved cysteine residues (17). CTGF is the only member of the CTGF family that exhibits a large and prolonged induction by TGF-β in fibroblastic cells (18). Other growth factors do not induce significant levels of CTGF gene expression. CTGF is not induced in epithelial cells or leukocytes by TGF-β. A distinct TGF-β response element has been identified in the promoter region of the CTGF gene, which appears to play a major role in the regulation of expression of this gene (19). CTGF stimulates some of the same biological responses in fibroblasts as TGF-β (20). For example, CTGF stimulates DNA synthesis and up-regulates collagen, fibronectin, and α5 integrin expression in cultured

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The abbreviations used are: TGF-β, transforming growth factor β; CTGF, connective tissue growth factor; rCTGF, recombinant CTGF; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; AIG, anchorage-independent growth; FGF, fibroblast growth factor; αFGF, acidic FGF; FBS, fetal bovine serum; NUS, NU-Serum; TCA, trichloroacetic acid.
fibroblasts. Injection of CTGF into the skin of neonatal mice induces fibroplasia similar to that observed after injection of TGF-β but not other growth factors, such as EGF or PDGF. However, CTGF does not stimulate AIG of NRK fibroblasts or inhibit the growth of mink lung epithelial cells, two biological effects that are characteristic of TGF-β (20). Collectively, these data indicate that CTGF might be a downstream mediator of at least some of the effects of TGF-β on connective tissue cell growth and matrix deposition. Thus, CTGF may be an important regulatory molecule responsible for connective tissue formation during wound repair, fibrotic disorders, and embryogenesis.

We have now investigated the role of CTGF in TGF-β-induced AIG. The results of our studies indicate that whereas CTGF alone is not sufficient to allow the AIG of NRK fibroblasts, CTGF synthesis and action are essential for the induction of AIG by TGF-β. Furthermore, our data demonstrate that TGF-β-stimulated AIG requires the activation of both CTGF-dependent and CTGF-independent signaling pathways induced by TGF-β.

Results
CTGF-specific Antibodies Inhibit TGF-β-induced AIG of NRK Fibroblasts. AIG is a unique property induced by TGF-β in NRK fibroblasts (11, 12). Using a variation of the original assay, we found that TGF-β stimulates a dose-dependent increase in DNA synthesis of NRK fibroblasts cultured on an agarose surface (Fig. 1). As we have reported previously, CTGF could not substitute for TGF-β in this assay (20). We then wanted to determine if CTGF was required for TGF-β stimulation of AIG. To accomplish this, we prepared CTGF-specific antibodies and evaluated them for the ability to block the TGF-β-induced AIG of NRK fibroblasts.

The anti-CTGF antibodies were initially characterized for any cross reactivity with PDGF or TGF-β using Western blots. Although these antibodies were able to detect up to 1 ng of CTGF, they did not exhibit any reactivity with any of the other growth factors tested (Fig. 2A). On the other hand, the anti-PDGF antibodies showed reactivity with CTGF and PDGF but not with TGF-β (Fig. 2A). We also tested the

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**Fig. 1.** TGF-β stimulates AIG of NRK fibroblasts. NRK fibroblasts were grown in monolayer, serum starved (for 24 h), and seeded into suspension culture dishes (as described in "Materials and Methods") containing TGF-β (0–20 ng/ml) or CTGF (0–20 ng/ml). Results are the average of triplicate assays; bars, SD.

**Fig. 2.** Anti-CTGF antibodies do not cross-react with PDGF/TGF-β or inhibit their activity but do inhibit TGF-β-induced AIG. In A, PDGF-BB (40 ng), TGF-β (20 ng), and CTGF (5 ng) were run on 12% SDS polyacrylamide gels, transferred to membranes, and immunoblotted with anti-CTGF and anti-PDGF antibodies as described in "Materials and Methods." In B, NRK cells were grown to confluence, starved in DMEM containing BSA (0.1 mg/ml), treated with PDGF-BB (10 ng/ml) and anti-CTGF antibodies (0–400 μg/ml) for 18 h, and labeled with [3H]thymidine for 2 h. Bars, SD. In C, Mv1Lu cells were trypsinized and seeded into 96-well dishes and treated with TGF-β (10 ng/ml) and anti-CTGF antibodies (0–400 μg/ml) for 4 days; cell number was determined based on acid phosphatase activity (22). Bars, SD. In D, NRK fibroblasts were incubated with TGF-β- and CTGF-specific antibodies (A) in increasing concentrations (from 0–400 μg/ml) in suspension cultures. Cells were also incubated with preimmune antibodies (B). Labeling and processing of all cells was carried out as described in "Materials and Methods." Results are the average of duplicate assays.
reactivity of the anti-CTGF antibodies for cross-reactivity with native PDGF and TGF-β based on neutralization of the biological activities of the peptides. The anti-CTGF antibodies did not block PDGF-induced DNA synthesis in a monolayer assay using NRK fibroblasts (Fig. 2B) or block the TGF-β-induced growth inhibition of Mv1Lu epithelial cells (Fig. 2C). This demonstrates that the anti-CTGF antibodies are not capable of neutralizing the biological activity of PDGF or TGF-β. In contrast, the anti-CTGF antibodies were able to inhibit the TGF-β-induced AIG of NRK cells in a dose-dependent fashion (Fig. 2D). The maximal inhibition ranged from 50–60% of control cultures and was observed at 500 μg/ml IgY. In contrast, the preimmune or heat-inactivated CTGF antibodies were not active as inhibitors of TGF-β-induced AIG. These data strongly indicate that CTGF is required for the TGF-β stimulation of AIG.

Induction of AIG by TGF-β Is Blocked in NRK Fibroblasts Containing an Antisense CTGF Gene. To confirm that blocking the action of CTGF prevented the ability of TGF-β to induce AIG in NRK fibroblasts, we investigated whether cells containing an antisense CTGF gene could produce CTGF after activation with TGF-β. We constructed an antisense CTGF gene that contained the intact CTGF promoter so that the expression of the antisense gene would also be induced by TGF-β and parallel the expression of the endogenous CTGF gene. This construct was composed of 827 bp of the CTGF promoter and 800 bp of the structural gene (exons 1 and 2 and a part of exon 3) in the correct orientation, a deletion of 316 bp which represented the distal portion of exon 3 to the middle of exon 4, and an inversion of the remainder of exon 4 and all of exon 5 at the 3' terminus of the gene (Fig. 3A). Stably transfected clones of NRK fibroblasts containing the antisense CTGF gene were isolated and examined for blockade of TGF-β induction of CTGF gene expression. Most of the clones did not exhibit a very high level of inhibition in the AIG assay in the presence of TGF-β, neither did they contain many copies of the antisense CTGF gene. The clone that exhibited the highest level of inhibition, also contained the highest copy number of antisense CTGF genes and was called NRK-ASCTGF. This clone was examined for its ability to express CTGF when induced by TGF-β using an immunoblot assay for CTGF.

The NRK-ASCTGF clone, unlike its wild-type NRK counterpart, did not exhibit a detectable level of CTGF protein after activation by TGF-β (Fig. 3B). Nonetheless, the NRK-ASCTGF fibroblasts exhibited a similar morphology to its parent NRK counterpart in monolayer culture (Fig. 4A). Furthermore, the response of these cells to serum stimulation of growth in a monolayer assay of [3H]thymidine incorporation was also indistinguishable from the parent NRK cell line (Fig. 4B). These results indicate that the presence of the antisense CTGF gene does not affect the response of the cells to mitogens in a monolayer assay or manifest itself as any morphological change of the cells in monolayer culture.

To determine whether the synthesis of CTGF protein was linked to AIG, we assayed the NRK and NRK-ASCTGF cells in the suspension growth assay. The results of these experiments indicate that TGF-β did not induce AIG in NRK-ASCTGF cells as it did in the wild-type NRK fibroblasts (Fig. 5A). The addition of the rCTGF restored the AIG response of these cells to a level that was comparable to the normal NRK fibroblasts activated with the same amount of TGF-β (Fig. 5A). The ability of CTGF to restore adhesion-independent growth in the NRK-ASCTGF fibroblasts is unique, because neither PDGF-BB nor aFGF were able to restore AIG (Fig. 5A). The stimulation of AIG by CTGF in the NRK-ASCTGF cells occurred in a dose-dependent fashion with 5 ng/ml CTGF (0.15 ng/ml), giving a half-maximal response, and 25–50 ng/ml (0.75–1.5 ng/ml) inducing a maximal stimulation of AIG (Fig. 5B). We also looked at the effect of CTGF on the NRK-ASCTGF clones in monolayer conditions. The fibroblasts were stimulated only marginally (about 12% of maximal) in the presence of EGF and TGF-β but showed a dose-dependent stimulation of up to 60% of maximal stimulation when CTGF was added (Fig. 5C).

We next examined the ability of CTGF to support long-term growth and colony formation in the soft agar assay. We compared the colony growth of the parent NRK cells in response to EGF alone or TGF-β and EGF with the growth of the NRK-ASCTGF clone in response to TGF-β and EGF or TGF-β, EGF, and CTGF (Fig. 6). Neither of the cell lines demonstrated colony growth in soft agar in the absence of any growth factors. The addition of EGF alone allowed some cell division to occur in the NRK cells. The addition of TGF-β and EGF to the media stimulated the growth of numerous large (>50-μm diameter) colonies. In contrast, neither EGF
alone (data not shown) nor TGF-β and EGF stimulated colony growth of the NRK-ASCTGF cell line. The addition of CTGF along with TGF-β and EGF restored colony growth of the cells to a level comparable to that seen in the parent NRK cells, which were stimulated with TGF-β and EGF. These data demonstrate that CTGF can support the sustained growth of the NRK-ASCTGF cell line in anchorage-independent conditions.

These data indicate that CTGF is an essential factor in the TGF-β signaling pathway for the induction of AIG. Furthermore, because CTGF alone is not sufficient for the induction of AIG in the absence of TGF-β, the ability of CTGF to induce AIG requires some effects of TGF-β that are induced independently of CTGF (Fig. 7).

Discussion
The unique property of TGF-β of inducing AIG of fibroblasts was the basis for the identification of the peptide (12, 21–23), yet the mechanism by which this occurs still remains unclear. Studies have suggested that extracellular matrix components and cell adhesion receptors (integrins) may play some role in this process (5). One complicating aspect of efforts to deduce the mechanism is that TGF-β, like other cytokines, appears to use a variety of intracellular signaling pathways to activate a particular response in the target cell. Thus, efforts to determine the precise pathway by which TGF-β induces a specific cellular response have proved elusive (24).

The observation that TGF-β could selectively and uniquely induce CTGF synthesis in fibroblasts (18) was the basis for our hypothesis that CTGF may be involved in a TGF-β-mediated signaling pathway. Because CTGF-specific antibodies inhibit TGF-β-induced AIG, it appears that CTGF plays an essential role in the induction of AIG by TGF-β. The inability of the NRK-ASCTGF fibroblasts to synthesize CTGF after activation by TGF-β or to grow in suspension in response to TGF-β is further proof of the essential role of CTGF in TGF-β-induced biological responses. Importantly, the addition of CTGF to the TGF-β-activated NRK-ASCTGF fibroblasts restored the AIG response of the cells. The finding that CTGF alone is unable to induce AIG but can do so in combination with TGF-β offers strong support for the hypothesis that AIG requires activation of signaling pathways induced by both TGF-β and CTGF. The products of these signaling pathways then act synergistically with one another and provide an environment that allows the AIG of the NRK fibroblasts.

Previous studies have indicated that TGF-β can stimulate DNA synthesis in various cell lines grown in monolayer culture by the induction of PDGF genes (25–27). However, our results with the NRK-ASCTGF fibroblasts grown in suspension prove that neither PDGF nor FGF had any effect in restoring AIG to these cells in the presence of TGF-β. These results indicate that PDGF, FGF, or EGF cannot substitute for CTGF in this process. Thus, whereas PDGF and EGF are required for the optimal growth of NRK fibroblasts in either monolayer or suspension (10), CTGF appears to function in a distinct manner from these growth factors.

Recent studies have suggested that another member of the CTGF gene family, Cyr61, can exhibit some synergistic effects on the stimulation of DNA synthesis with either basic FGF or PDGF in monolayer cultures of NIH/3T3 cells (28, 29). We have made similar observations with CTGF and EGF in monolayers of NRK fibroblasts (20) with one important distinction. We found that the amounts of CTGF required for activity in the AIG assay or to induce DNA synthesis in monolayer cultures in the presence of submitogenic concen-
Fig. 5. AIG of NRK-ASCTGF fibroblasts in response to various growth factors. In A, NRK fibroblasts and NRK-ASCTGF clones were treated with TGF-β and EGF, or TGF-β, EGF, and rCTGF (25 ng/ml); or aFGF (10 ng/ml); or PDGF-BB (10 ng/ml), and AIG was measured as described earlier. In B, NRK-ASCTGF fibroblasts were stimulated with TGF-β (2.5 ng/ml) and CTGF (0–100 ng/ml) in a dose-dependent manner in suspension cultures. The results are the average of triplicate assays; bars, SD. In C, NRK-ASCTGF fibroblasts were grown to confluence, serum starved for 24 h, and then stimulated with various growth factors in monolayer for 24 h before labeling with [3H]thymidine (3 h) and processing for TCA-insoluble radioactivity.

Activities of EGF are 100- to 1000-fold less than the amounts of Cyr61 reported to be necessary for synergistic stimulation of DNA synthesis with PDGF or basic FGF in the NIH/3T3 cells. We also find that Cyr61 does not substitute for CTGF in the AIG. These data indicate that CTGF has biological activities that are not shared by Cyr61 and support the possibility that CTGF could be a specific downstream mediator of TGF-β action for specific cellular responses such as AIG. The results from our studies suggest a hypothetical model for the action of TGF-β on target cells involving CTGF (Fig. 7). In this model, TGF-β activates signaling pathways and cellular responses that are both CTGF dependent and CTGF independent. Complex biological responses, such as the AIG of NRK fibroblasts, require the activation of both of these pathways, which appear to interact synergistically to induce this response.

**Materials and Methods**

### Cell Lines and Culture Conditions

All experiments were carried out with NRK 49F fibroblast cells obtained from the American Type Culture Collection (Rockville, MD). The NRK cells were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 2.5% FBS (Intergen Co., Purchase, NY), 2.5% NUS (Collaborative Biomedical Products, Bedford, MA), and 0.05% gentamicin. The antisense CTGF-containing fibroblasts (NRK-ASCTGF) were maintained in DMEM containing 2.5% FBS, 2.5% NUS, and 0.4 mg/ml G418. The mink lung epithelial cells (Mv1Lu; American Type Culture Collection) were grown in minimal Eagle’s medium containing 10% FBS and nonessential amino acids. All cells were incubated at 37°C in a 5% CO2-containing atmosphere.

### Growth Factors

Unless otherwise indicated, growth factors and other chemicals were used at the following concentrations: EGF, 5 ng/ml (Upstate Biotechnology, Lake Placid, NY); aFGF, 10 ng/ml (Bachem Fine Chemicals, Inc., Torrence, CA); PDGF-BB, 10 ng/ml (Chiron Peptide Systems, San Diego, CA); recombinant TGF-β, 2.5 ng/ml (Life Technologies, Inc.); and rCTGF, 50 ng/ml (synthesized in the laboratory; Ref. 20).

### Growth Assays

The AIG assays were performed essentially as described by Assoian et al. (30). Cells were grown initially as monolayer cultures. Cultures were trypsinized at 80% confluence and were seeded (5 × 10^5/well in a 12-well dish or 1 × 10^5/well in a 6-well dish) for use in AIG assays in NRK growth medium containing EGF (5 ng/ml).

The growth response of the cells was determined 3 days after plating on the agarose layer. Cells were labeled with 1 μCi/ml [methyl-3H]thymidine (DuPont NEN, Boston, MA) for 24 h. The cells were recovered from the wells, transferred to Eppendorf tubes, and centrifuged at 8000 rpm for 10 min. The cell pellet was then treated with 1 ml of ice-cold 5% TCA, 100 μg of BSA (as a carrier), and incubated at 4°C for 1 h. The tubes were then spun at 14,000 rpm for 10 min, and the precipitate obtained was washed twice with cold 5% TCA. DNA was solubilized in 200 μl of 0.1% SDS/0.1 M NaOH, and the incorporation of [3H]thymidine was determined using a liquid scintillation counter.

The TGF-β inhibition assay was performed with the Mv1Lu epithelial cells as described previously by Ogawa and Seyedin (31). DNA synthesis in NRK and NRK-ASCTGF cells was measured in monolayer by the [3H]thymidine incorporation assay (13). Soft agar colony growth assays were performed as described by DeLarco and Todaro (11).

### Antibody Production and Purification

IgY antibodies were prepared against recombinant human CTGF in chickens by contract service (Berkeley Antibody Company, Berkeley, CA). Laying hens were injected with CTGF emulsified with Freund's Complete Adjuvant and boosted at 7-day intervals with antigen emulsified with Freund’s Incomplete Adjuvant. Serum titers were tested by ELISA on bleeds collected every 1 to 2 weeks and reached as high as 7 × 10^6. Eggs laid by the immunized chickens were collected over a 5-week period and stored at 4–8°C prior to purification. Polyclonal chicken antibody was purified from the egg yolks using Promega's EGGstract IgY purification system (Promega Corp., Madison, WI) following the manufacturer’s directions. Briefly, lipids were precipitated from the yolks and removed by centrifugation. The supernatant was then collected and filtered through gauze; IgY was extracted by two consecutive precipitations. Pellets were resuspended in 0.01 M K_2HPO_4 (pH 7.4), 0.1 M NaCl, and 50 μg/ml gentamicin sulfate to a final concentration of 2 mg/ml. Purified IgY was then analyzed by Western blots at 10–50 μg/ml for detection of 1–33 ng/ml of CTGF.

### Preparation of Extracts and Immunoblotting

NRK fibroblasts or NRK-ASCTGF clones (grown to confluence in 100-mm dishes) were...
starved overnight in DMEM containing BSA (0.1 mg/ml) and stimulated with TGF-β for 24 h. Cells were collected by trypsinizing and centrifugation, washed three times with PBS, and resuspended in cell lysis buffer [10 mM Tris-HCl (pH 7.8) and 1% Triton X-100] at 4°C (32). The cell extracts and conditioned media were then incubated with Affi-gel heparin (Bio-Rad Laboratories) overnight. The beads were washed with 0.1 M ammonium acetate, and the bound protein eluted with 2 M ammonium acetate. The heparin-bound fractions were vacuum dried and fractionated on a 12% SDS polyacrylamide gel under nonreducing conditions (33) and electrophoretically transferred to a nitrocellulose membrane. Immunoblots were performed as described previously (34) using a chicken anti-CTGF specific antibody diluted 1:1000 in the blocking buffer.

Construction of the Antisense CTGF Plasmid and NRK-ASCTGF Cells. The entire genomic CTGF sequence was excised from a cosmid using XbaI and cloned into pBluescriptII KS plasmid (Stratagene). A plasmid containing the CTGF gene was digested with Xmal to produce three fragments. The first fragment was 1800 bp long and included the CTGF promoter and exons 1, 2, and a portion of 3. The second fragment which

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**Fig. 6.** Growth of NRK and NRK-ASCTGF fibroblasts in soft agar. NRK and NRK-ASCTGF cells were grown in soft agar in the presence of the indicated growth factors for a period of 10 days and then photographed (×40) using a phase-contrast microscope.
Cell Growth & Differentiation

CTGF Dependent and CTGF Independent Pathways of TGF-β action on Fibroblasts

TGF-β

DIRECT

SYNERGISTIC

Via CTGF

CELLULAR RESPONSE

Fig. 7. A model depicting the potential pathways of TGF-β action on target cells involving CTGF.

was 316 bp long and extended from the distal end of exon 3 to the proximal end of exon 4. The third fragment was 2100 bp long and included the remainder of exon 4 and all of exon 5. The first and third fragments were purified using low-melt agarose gels and religated. Clones that contained the 3' region of the CTGF gene in the inverse orientation were identified using PCR amplification of a 460-bp region that spans the sense-antisense junction, using primers derived from DNA segments near the end of exon 3 and the newly attached 3' untranslated region on the opposite strand. The sequence of the forward primer (AS1) was 5' -CGT-TCGTTCTGCC. The sequence of the reverse primer (AS2) was 5'-GAATT-GTGGTACGTCAG. The amplified product was analyzed by Southern blotting using a 32P-labeled XbaI fragment from the original plasmid. The desired plasmid clone (pKS-asCTGF) was grown in large scale in LB media with ampicillin, and the plasmid was isolated and purified using cesium chloride (35).

To produce the NRK-ASCTGF cells, NRK fibroblasts were grown to 70% confluence in DMEM with 1% insulin-transferrin-selenium (Collaborative Biomedical Products) and cotransfected with 1:10 ratio of pRcCMV (Invitrogen)pKS-asCTGF using Lipofectam (Life Technologies, Inc.) according to earlier described techniques (36). The pRcCMV plasmid provided neomycin resistance to the transformed cells. The cultures were transferred to media containing 0.4 mg/ml G418 after 48 h and maintained for 14 days. Separate colonies were isolated and established in new culture plates. DNA from the clones was purified by established methods (37) and screened using PCR amplification and Southern hybridization as was done for the identification of the pKS-asCTGF plasmid. Positive clones were maintained in DMEM containing 2.5% FBS, 2.5% NUS, and 0.4 mg/ml G418.

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References


