# Enhanced Motility of KGF-transfected Breast Cancer Cells

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**Abstract.** *Background: In a previous study, we reported that keratinocyte growth factor (KGF) produced a rapid increase in the motility of ER-positive breast cancer cells. Others have demonstrated that KGF treatment in rodent species produces rapid mammary ductal hyperplasia. Epithelial cells do not produce KGF; thus, in the present study, MCF-7 cells were stably transfected with a KGF-expressing vector and the motility and morphology of the transfected, non-transfected and empty vector cell lines compared. Materials and Methods: A mammalian expression vector containing a KGF cDNA was transfected into MCF-7/β cells, and two stable clones (MCF-7/β/KGF-T8 and MCF-7/β/KGF-T9) were identified. Western blotting of conditioned medium from these clones was used to confirm the expression of KGF. The motility of wild-type and KGF-transfected MCF-7 cells was compared using time-lapse videomicroscopy and a cell culture wounding model which examined cell migration over a period of 1-3 days. Results: The Western blots demonstrated that the expression of KGF in both the MCF-7/β/KGF-T8 and MCF-7/β/KGF-T9 cell lines was higher than the wild-type and MCF-7/β cell lines. The cell proliferation and migration distance was significantly greater for both KGF-transfected MCF-7 cell lines than the wild-type and MCF-7/β cell lines under the same experimental conditions. Further, changes in motile morphology were observed in both the MCF-7/β/KGF-T8 and MCF-7/β/KGF-T9 cell lines. In addition, the MCF-7/β/KGF-T8 clone was found to produce much larger tumors than both the MCF-7/β/KGF-T9 and EV clones in mouse xenografts. These results indicated that autocrine production of KGF in the KGF-transfected MCF-7 cell lines enhanced cell migration, migration-related morphology and xenograft tumor growth. Conclusion: KGF-transfected MCF-7 cells displayed a much greater motility than non-transfected cells, confirming the KGF motility enhancement effect which we previously reported. The use of KGF-transfected breast cancer cells in the xenograft model may help to study the mechanism of KGF-mediated cell motility and to identify specific KGF antagonists that may be used to prevent or impede KGF-mediated metastatic progression.*

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The metastatic dissemination of tumor cells to secondary sites has been correlated with highly motile behavior (1). It has been reported that tumor cell motility is regulated by growth factors and cytokines from stromal tissue surrounding the tumor mass (2, 3). The keratinocyte growth factor (KGF) was identified as a soluble factor in stromal tissue that enhances the growth and motility of epithelial cells (4). KGF is a member of the fibroblast growth factor family and is present in stromal tissue from the breast (4, 5). Although not produced by epithelial tissue, KGF stimulates DNA synthesis and the proliferation and migration of epithelial cells in the breast and other tissue (6).

The mammary glands of adult female animals are extremely sensitive to KGF (7). Acute systemic administration of KGF to mice and rats was shown to produce massive mammary ductal hyperplasia, an elevation of mitotic figures and, eventually, metastatic mammary carcinomas (7, 8). We previously observed that KGF produced rapid enhancement of the motility of ER-positive breast cancer cell lines, which lasted for up to 48 hours and was mediated by the Grb2-Erk1,2 pathway (9, 10). Taken together, these observations suggest that KGF-mediated stimulation of breast epithelial proliferation and migration may be an early event in the molecular cascade involved in breast cancer progression and metastasis. The present study examined the influence of KGF transfection of MCF-7 breast cancer cells on the endogenous production and release of KGF and the effect of endogenously produced KGF on the proliferation and motility of breast cancer cells.

## Materials and Methods

*Cell culture.* MCF-7 human breast cancer cells, obtained from the Michigan Cancer Foundation, U.S.A., were maintained as

monolayer cultures in RPMI 1640 media (without phenol red) supplemented with 2 mM L-glutamine, gentamicin (50 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), estradiol ($10^{-11}$ M) (all from Sigma, St. Louis, MO, USA) and bovine calf serum (Hyclone, Logan, UT, USA) (5%), as previously described (10).

*Transfection methods.* The full-length clone of human KGF cDNA was PCR-amplified using pCEV9-32 (a recombinant vector containing the full coding sequence of human KGF obtained from NIH) as template. Primers were designed using the KGF cDNA sequence (GeneBank accession no. NM002009): 5' primer (5'-TGAACACCCGGAGCACTACA-3'), 3' primer (3'-CCACTTA AAGAAATCTCCCT-5'). The PCR product was inserted into the eukaryotic expression vector PcDNA3.1/Hygro(+) (Invitrogen, San Diego, CA, USA). Prior to use for transfection, the integrity of the KGF cDNA insert was confirmed by dideoxy terminator sequencing (DNA sequencer 373A, Applied Biosystems, Foster City, CA, USA).

The pcDNA3.1/Hygro(+)KGF vector or empty vector (EV) was transfected into MCF-7 cells which had been previously stably transfected with a β-galactosidase/CMV construct containing a G418 resistance gene (11). MCF-7 cells were grown in 6-well plates until 70% confluent and transfected using Lipofectamine plus (Invitrogen, Gaithersburg, MD, USA), in accordance with the manufacturer's instructions. Lipofectamine Reagent (9 ml) and Plus Reagent (5 μl) together with 3 μg of PcDNA3.1/ Hygro (+)KGF recombinant were incubated with the cells in RPMI + 10% fetal bovine serum (FBS) overnight. Twenty-four hours after transfection, the cells were trypsinized and transferred to a 100-mm dish. These cells were then selected with 100 μg/ml of Hygromycin (Invitrogen, Gaithersburg). Individual resistant colonies were isolated with cloning rings. Eighteen separate clones were isolated and tested for cell motility in the culture wounding assay. Clones MCF-7/β/KGF-T8 (T8) and MCF-7/β/KGF-T9 (T9), which demonstrated the greatest motility, were further examined for KGF expression using an ELISA assay. Long-term cultures were maintained in media containing both Hygromycin 20 μg/ml and G418 125 μg/ml.

*KGF ELISA assay.* Cells in 80-90% confluent cultures were washed twice with phosphate-buffered saline (PBS) and then lysed in 200 μl ice-cold Reporter Gene Assay Lysis Buffer (Roche Applied Science, Indianapolis, IN, USA). The tissue lysate and cell culture medium were cleared of debris by centrifugation at 10,000 rpm for 15 minutes (4˚C), and the supernatant was collected. Total protein was determined (Bradford protein assay; Molecular Devices, Sunnyvale, CA, USA) at 595 nm.

The quantitative measurement of human KGF was accomplished using the human KGF/FGF-7 Quantikine ELISA kit (R&D systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's instructions and absorbance was measured with a microplate reader (Molecular Devices) at 450 nm with λ correction at 570 nm. All measurements were performed in duplicate. The lower limit of ELISA sensitivity was 15 pg/ml. The tissue sample concentration was calculated from the standard curve and corrected for total protein.

*Culture wounding assay.* Cell migration was evaluated using an *in vitro* wound migration assay, as described previously (12). Three days after seeding $5x10^5$ cells (MCF-7, MCF-7 EV, T8 and T9) into 60-mm culture dishes, and when the cells were approximately 80% confluent, the cultures were wounded and washed with PBS. At 24, 48 and 72 hours, cell migration was determined by measuring both the distance traveled by the cell front into the wounded area and the number of cells in the wounded area/microscopic field. Measurements were taken from 10-12 individual microscopic fields from each experiment and the data was summarized from 2-3 experiments.

*Mouse xenograft assay.* Xenograft assays were conducted as described previously (11). Nude mice were implanted with an estrogen pellet and 48 hours later were injected with $5x10^6$ cells (either MCF-7 EV, T8 or T9) in Matrigel (Fisher Scientific). The tumors were measured (3-axis measurement with digital calipers) twice weekly over a 4-week period. These animal studies were performed using an animal protocol approved by the University of Oklahoma IACUC Committee, U.S.A.

*Digital imaging.* In this study, image analysis was conducted as previously described (9). Phase contrast images of cells on an inverted microscope were recorded for time-periods of up to 4 hours using a digital camera (Olympus Corp., Model DP70-BSW-V1.2) inserted into the optics.

*Data analysis.* Multiple group comparisons were conducted using ANOVA and the Student's *t*-test for pair-wise comparisons. Group differences resulting in $p$ values of less than 0.05 were considered to be statistically significant.

## Results

*Effect on cellular KGF expression.* The effectiveness of KGF transfection on the production and release of KGF from MCF-7 cell is shown in Figure 1. The concentration of KGF in the lysates from the transfected cells was over 140 ng/ml, while in the lysates of wild-type (WT) and EV-transfected MCF-7 cells KGF was undetectable in the ELISA (Figure 1A). Similarly 2-day conditioned medium from the transfected cells was found to have a KGF concentration of 119 ng/ml, while KGF was not detectable in the media from WT and EV-transfected cells.

*Effects on cell morphology.* The effect of KGF transfection on MCF-7 cell morphology is shown in Figure 2. The morphology of EV-transfected cells (Figure 2B) was found to be identical to the WT MCF-7 cells (Figure 2A). However, a motile morphology, including pseudopodia-like membrane extensions, was observed with the KGF-transfected cells (Figure 2C).

*Effects on cell motility.* In the culture wounding assay the motility of the transfected cells was found to be enhanced, as shown in Figure 3. Cell motility, as indicated by the distance traveled by the cell into the wounded area, was the same with the WT and EV-transfected MCF-7 cells. However, cell motility was found to be significantly increased in the KGF-transfected cells on days 1-3 following culture wounding.

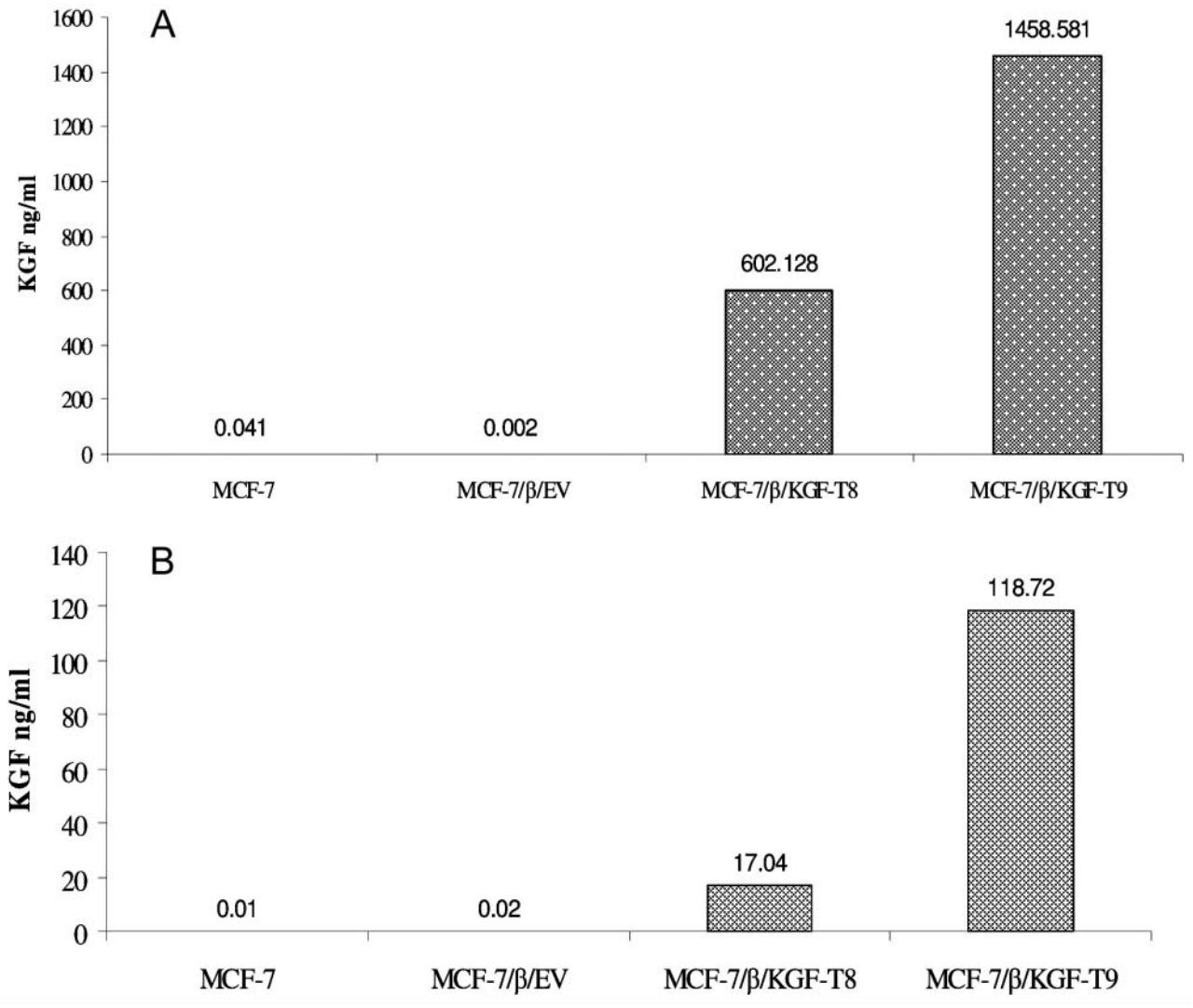


Figure 1. *Production and release of KGF from MCF-7 cells as measured by ELISA assay. Figure 1A: Concentration of KGF in cell lysates; Figure 1B: Concentration of KGF in 2-day conditioned media.*

*Effects on xenograft tumor growth.* In a mouse xenograft experiment, the KGF-transfected T8 clone was found to grow much more rapidly than the WT and EV-transfected cells (Figure 4). The xenograft tumors from the T9 clone were found to be significantly larger ($p<0.05$) than the tumors in the control groups from 12 to 22 days following cell implantation. The xenograft tumors from the T8 clone were found to be significantly larger ($p<0.001$) than the tumors in the control groups throughout the experiment. By day 30 following implantation, the mean volume of the T8 tumors was almost 4-fold greater than that of the tumors from either the T9 clone or EV-transfected MCF-7 control group.

## Discussion

KGF, a mesenchymally-derived growth factor, mediates epithelial cell proliferation and migration by binding to the KGF receptor (KGFR) in target epithelial cells. KGFR is expressed in breast epithelial cells, while KGF is expressed in the stromal cells of the breast tissue (13). The mammary glands of adult female animals are remarkably sensitive to KGF (7). KGF administration to female rats produced dramatic proliferation of the mammary glands within 5 days (7). Kitsberg and Leder (8) observed that female mice, with a constitutively up-regulated KGF transgene, developed mammary epithelial hyperplasia and eventually all animals

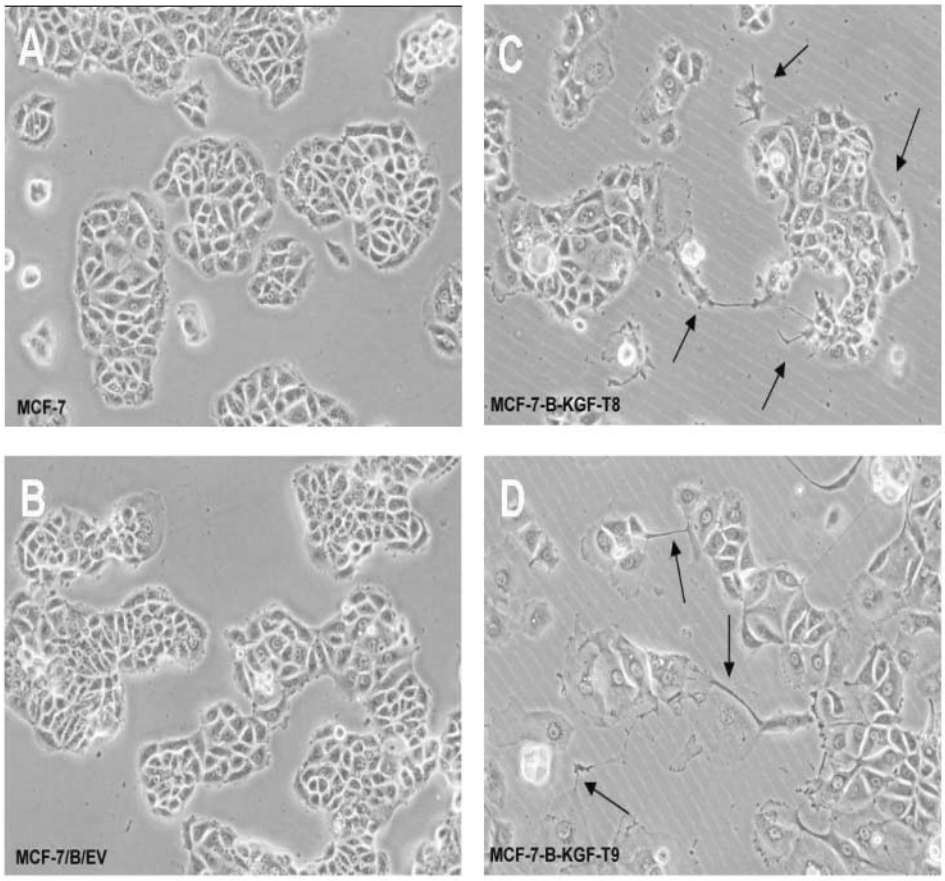


Figure 2. *Video-micrographs (200X) of MCF-7 cells. Figure 2A: Wild-type MCF-7 cells; Figure 2B: EV-transfected cells; Figure 2C: T8 cells; Figure 2D: T9 cells. The black arrows indicate the effects of KGF on cell morphology in the transfected T8 cells.*

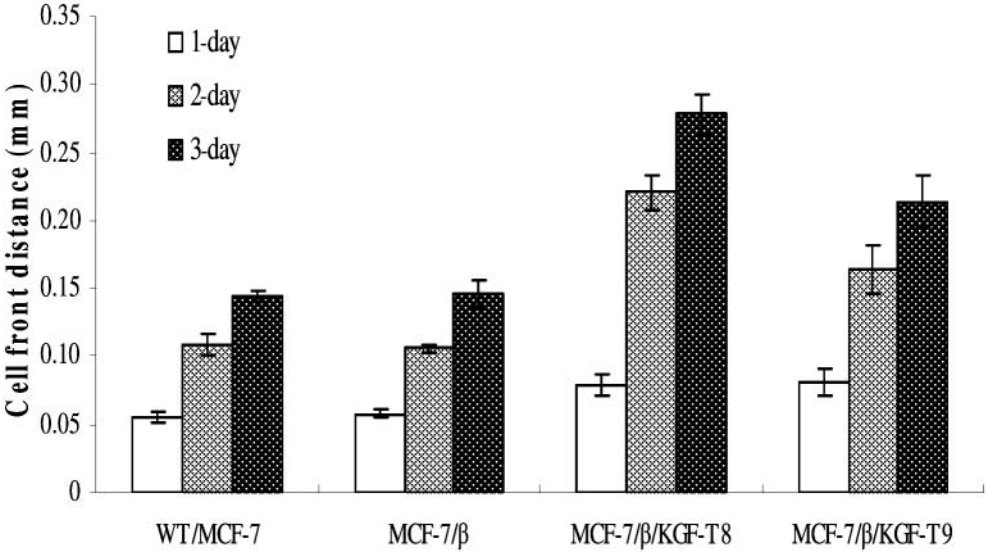


Figure 3. *Effect of KGF transfection on the migration of MCF-7 cells in wounded cultures. Each bar represents the mean distance of cell migration at the times indicated in 10-12 microscopic fields ± SEM.*

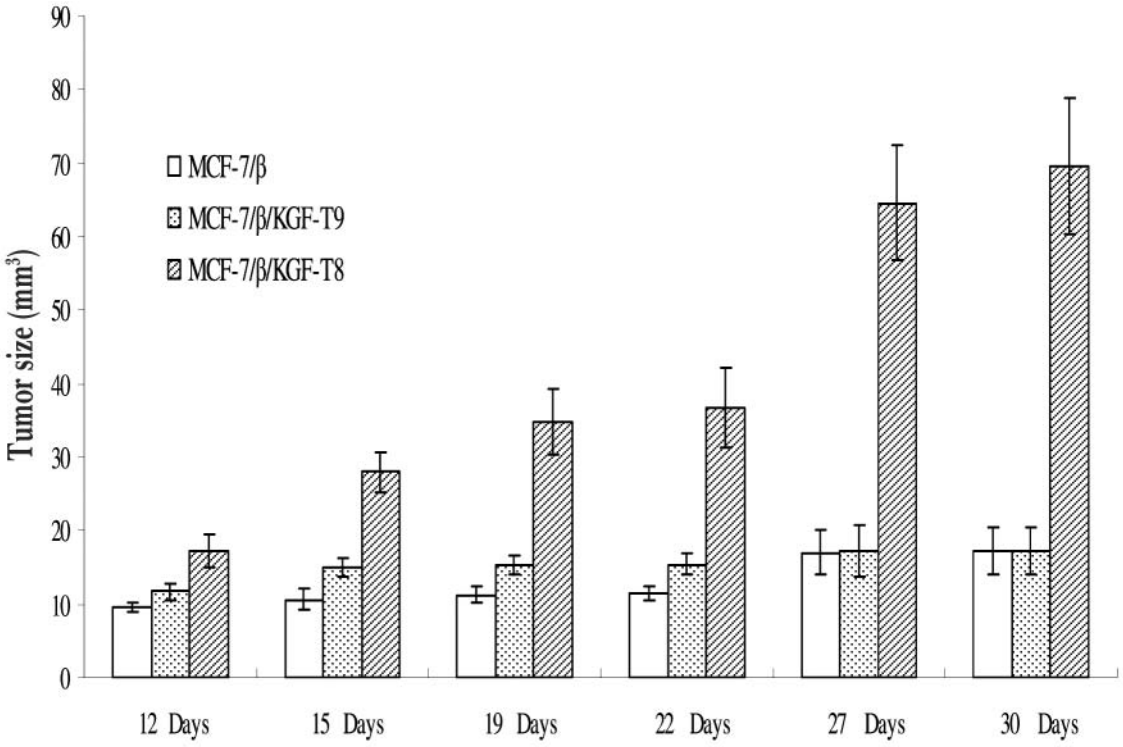


Figure 4. *Effect of KGF transfection on the growth of MCF-7 cell tumors in a mouse xenograft model. Each bar represents the mean tumor volume from 3-4 mice ± SEM.*

developed metastatic mammary carcinomas. Consistent with this concept, KGFR gene up-regulation has been observed in primary human breast cancer (14, 15), while highly malignant, metastatic breast cancer expressed relatively little KGFR (16). These observations suggest that KGF-mediated stimulation of breast epithelial proliferation and migration may be an early event in metastatic progression. We have demonstrated that KGF stimulates the proliferation and migration of ER-positive breast cancer cells due, in part, to increased expression of KGFR in these cells (9). Further, we demonstrated that, in MCF-7 cells, down-regulation of KGFR expression using a KGFR anti-sense oligonucleotide abolished KGF-mediated cell motility (17). Thus, the present study examined the influence of endogenously produced KGF on the proliferation, motility and morphology of KGF-transfected MCF-7 cells.

The study clearly demonstrated that endogenous KGF, produced and secreted by breast cancer cells, caused an alteration in the morphology of the cancer cells which is consistent with enhanced cell motility. Accordingly, enhanced migration of the cells was observed in a culture wounding assay and the KGF-transfected T8 cancer cells produced a remarkable 4-fold increase in tumor volume when implanted into nude mice. Although the T9 clone produced and secreted more KGF than the T8 clone, implantation of the T8 clone resulted in much larger tumor volumes. It was previously reported that treatment of MCF-7 cells with EGF and other growth factors, at the same range of concentration, often resulted in an inhibition of cell proliferation (18). We previously observed that EGF and IGF-1 produced maximal enhancement of MCF-7 cell invasiveness at lower media concentrations (19), which may explain the smaller volumes of the T9 tumors relative to the T8 tumors in our xenograft study.

The results of the present study demonstrated the effects of endogenously produced KGF on the growth and motility of breast cancer cells and support our previous observations concerning the effects of exogenous KGF treatment on ER-positive breast cancer cell lines (9). Further, this study supported the concept that KGF- and KGFR-mediated signal transduction could be an early event, and perhaps a critical step, in the initiation of metastatic progression of breast cancer cells. If this concept is correct, the development of therapeutic strategies to inhibit KGF production in breast stromal tissue and/or KGFR expression/activation and the associated signal transduction pathways may provide an important new approach for the prevention of breast cancer metastatic progression.

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