Wilms' Tumor 1 Protein and Focal Adhesion Kinase Mediate Keratinocyte Growth Factor Signaling in Breast Cancer Cells

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Abstract. *Background: Keratinocyte growth factor (KGF) has been shown to induce breast cancer metastasis in animal models. cDNA microarrays have revealed that KGF increased Wilms tumor 1 (WT1) and focal adhesion kinase (FAK) expression in breast cancer cells. The role of WT1 and FAK in KGF signaling was investigated. Materials and Methods: A cell culture wounding model was used to study the effects of WT1 and FAK down-regulation on KGF-induced proliferation and motility in breast cancer cells. Results: WT1 down-regulation inhibited KGF-mediated proliferation and motility of breast cancer cells, while FAK down-regulation inhibited proliferation, but had no significant effect on cell motility. WT1 down-regulation, but not FAK down-regulation, led to Erk1,2 inactivation. Conclusion: KGF-mediated signaling employs WT1 and FAK to regulate breast cancer cell proliferation and motility and may represent therapeutic targets for the prevention of breast cancer progression.*

Keratinocyte growth factor (KGF, also designated FGF-7), a member of the fibroblast growth factor family, was originally isolated from human embryonic lung fibroblasts (1). KGF is not produced by epithelial cells, but is produced by stromal cells (2) and acts at the KGF receptor (KGFR) found on epithelial cells. KGFR (also known as FGFR2IIIb) is a splice variant of FGFR-2 encoded by the *FGFR-2* gene (3). Thus, KGFR is a member of the fibroblast growth factor receptor (FGFR) family which are membrane-spanning tyrosine kinase receptors consisting of four known peptides whose sequences are highly conserved (4). KGF acts at the KGFR and stimulates epithelial cell

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DNA synthesis, proliferation and migration in breast and other tissue (2, 5, 6). In female mice, constitutively elevated levels of KGF have induced mammary epithelial hyperplasia and eventual development of metastatic mammary carcinomas (6). We have shown that KGF treatment up-regulated KGFR gene expression in MCF-7 human breast cancer cells (7), and induced rapid and direct motility enhancement in MCF-7 and other estrogen receptor positive breast cancer cell lines (8). Additionally, it has been demonstrated that KGF/KGFR proliferation and motility signaling is mediated by the growth factor receptor bound protein-2 (Grb2)-extracellular signal regulated kinases 1,2 (Erk1,2) pathway in MCF-7 breast cancer cells (9).

The *Wilms' Tumor 1* (*WT1*) gene was originally identified as a tumor suppressor gene responsible for Wilms' tumor (10, 11). In addition to germ-line mutations, somatic mutations of WT1, as well as loss of heterozygosity at the 11p13 locus harboring WT1 have been reported in sporadic Wilms' tumors. WT1 is a transcription factor that binds to CG- and TCC-rich sequences on promoters of target genes (12). High levels of the wild type *WT1* mRNA had been found in leukemias (13), lung (14) and breast tumors (15). Patients with high *WT1* mRNA levels in their breast tumors were found to have a lower five-year disease-free survival rate than patients whose breast tumors expressed low *WT1* mRNA levels (15). Previously we have shown that WT1 was vital in mediating the proliferation and the survival of breast cancer cells, as down-regulation of WT1 expression led to cell cycle arrest at the G1-phase and increased apoptosis in *HER2/neu*-overexpressing cells (16). Also, KGF treatment of MCF-7 cells increased the expression of WT1 mRNA by 3- to 4-fold and increased the expression of focal adhesion kinase (FAK) mRNA by 70 to 200 fold (7). Grb2 plays an important role in tyrosine kinase signaling, and its actions are known to be mediated by WT1 and FAK in some biological systems (16, 17). We have demonstrated that Grb2 is crucial to the signaling associated with KGF (9) and that KGF treatment increased Erk1,2 activity in MCF-7 breast cancer cells (9); however, whether or not Erk1,2 activation is dependent on

WT1 and/or FAK is not known. In the present study, the involvement of WT1 and FAK in the signaling pathway associated with KGF-mediated proliferation and motility of breast cancer cells was examined.

Materials and Methods

Reagents and antibodies. Human recombinant KGF was obtained from BD Pharmingen (San Diego, CA, USA). Antibodies specific for WT1 and FAK were purchased from Dako (Carpinteria, CA, USA) and BD Pharmingen, respectively. Antibodies specific for Erk1,2 and phosphorylated Erk1,2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific for Grb2 and β-actin were purchased from BD Transduction Laboratories (San Diego, CA, USA), and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), respectively. Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Cell Signaling Technology.

Oligonucleotides. The sequence of the Grb2 antisense oligonucleotide (oligo) was 5'-ATA TTT GGC GAT GGC TTC-3' as described previously (9) and of the WT1 antisense oligo was 5'-GTC GGA GCC CAT TTG CTG-3' (16). The sequence of the FAK antisense oligo was 5'-ATA ATC CAG CTT GAA CCA AG-3'. The sequence of the control oligo was 5'-GGC CTT TGA ACT CTG CTT A-3' as described previously (9, 16). All oligos were synthesized as P-ethoxy oligos (Oligos Etc., Wilsonville, OR, USA) and incorporated into liposomes as described previously (9, 16). Briefly, P-ethoxy oligo was mixed with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine in the presence of tertiary butanol, frozen in a dry ice/acetone bath, lyophilized, and stored at –20°C.

Cell culture methods and cell migration assay. The MCF-7 human breast cancer cell line was obtained from the Michigan Cancer Foundation (Detroit, MI, USA). The cells were maintained as monolayer cultures in RPMI 1640 media. Cell migration was evaluated using the *in vitro* culture wounding assay as previously described (9). MCF-7 cells grown to approximately 85% confluency were wounded once and washed with PBS. The cells were then immediately treated with 50 ng/ml KGF and 12 μM liposomal oligos. At 24, 48 and 72 h post-treatment, cell migration was determined by measuring both the distance traveled by the cell front into the wounded area (motility index) and the cell number in the wounded area (proliferation index) per microscopic field (100X). Measurements were taken from 10-12 individual microscopic fields in each experiment. The experiments were performed twice.

Western blots. The cells were lysed on ice for 30 min. The protein concentration was determined by the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal concentrations of total protein (10-30 μg) were electrophoresed on 12% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk (Bio-Rad Laboratories) for 30 min. Primary antibodies (anti-Grb2, 1:5000; anti-WT1, 1;500; anti-FAK, 1:3000; anti-Erk1,2, 1:1000; anti-phosphorylated Erk1,2, 1:5000; anti-β-actin, 1:10000) diluted in TBS-T + 1% milk were added. The
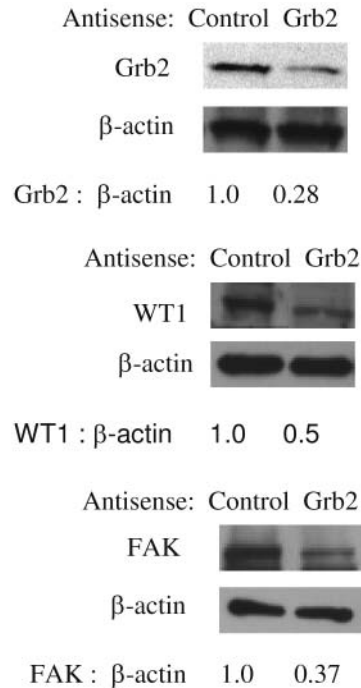

Figure 1. *Effect of down-regulation of Grb2 protein expression on FAK and WT1 levels. MCF-7 cells were treated with 50 ng/ml KGF and 12 μM liposomal oligos (Grb2 or control antisense) for 3 days. Cells were then harvested and Western blots were performed. Expression of FAK and WT1 proteins were normalized to that of β-actin.*

membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase. The protein bands were detected *via* enhanced chemiluminescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The images were scanned by an AlphaImager densitometer (Alpha Innotech Corp., San Leandro, CA, USA).

Statistical analysis. Multiple group comparisons were conducted using ANOVA and 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 of Grb2 on WT1 and FAK expression. The down-regulation of Grb2 protein expression by the addition of 12 μM liposomal Grb2 antisense oligo to the KGF-treated MCF-7 cells decreased WT1 and FAK expression by 50% and 63%, respectively (Figure 1).

Effects of WT1 and FAK down-regulation on KGF-mediated proliferation and motility. The addition of 12 μM liposomal WT1 and FAK antisense oligos to the KGF-treated MCF-7 cells decreased WT1 and FAK protein levels by 40% and 45%, respectively (Figure 2).
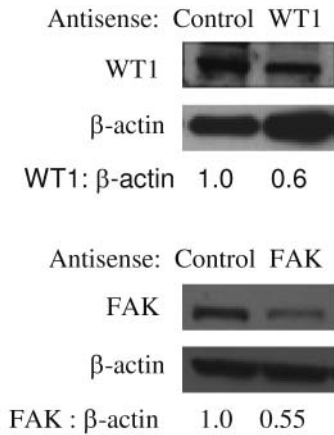
Figure 2. *Selective decrease in FAK and WT1 protein expression by liposomal antisense oligos. MCF-7 cells were treated with 50 ng/ml KGF and 12 µM liposomal oligos (WT1, FAK or control antisense) for 3 days. Cells were then harvested and Western blots were performed. Expression of FAK and WT1 proteins were normalized to that of β-actin.*

Figure 3. *Effect of WT1 and FAK on KGF-mediated signaling in MCF-7. MCF-7 cells were treated with 50 ng/ml KGF and/or 12 µM liposomal oligos (WT1, FAK or control antisense) at the beginning of the experimental period. Each bar represents the mean distance of cell migration into the wounded area from 10-12 microscopic fields ±SEM on the days indicated. *Significantly different than the untreated group. **Significantly different than the KGF-treated group.*

In the absence of KGF, liposomal WT1 and liposomal FAK antisense oligos did not affect cell proliferation (Figure 3, top panel). In the presence of KGF, down-regulation of WT1 decreased cell proliferation by 35% to 50%, and down-regulation of FAK decreased cell proliferation by 27% to 40% (Figure 3, top panel). The liposomal control oligos did not affect the proliferation of MCF-7 cells in the presence or absence of KGF (Figure 3, top panel).

The down-regulation of WT1 decreased cell motility by 40% in the presence of KGF (Figure 3, bottom panel). The down-regulation of FAK decreased KGF-induced cell motility by 15% (Figure 3, bottom panel). The liposomal control oligos did not affect cell motility (Figure 3, bottom panel).

Effect of WT1 and FAK down-regulation on KGF-induced Erk1,2 activation. The down-regulation of WT1 reduced KGF-induced phosphorylated Erk1,2 levels by 50% (Figure 4). However, the down-regulation of FAK did not inhibit, and may have increased, KGF-induced phosphorylated Erk1,2 levels (Figure 4).

Discussion

In this study, WT1 mediated the proliferative signals of KGF in breast cancer cells and additionally WT1 mediated cell motility induced by KGF. The data indicated that WT1, down-stream of Grb2, regulated Erk1,2 activity as decreased expression of WT1 led to decreased Erk1,2 phosphorylation. We have previously demonstrated that Erk1,2 activation was essential for the KGF-induced motility of breast cancer cells (9). Jomgeow *et al*. (18) have demonstrated that overexpression of
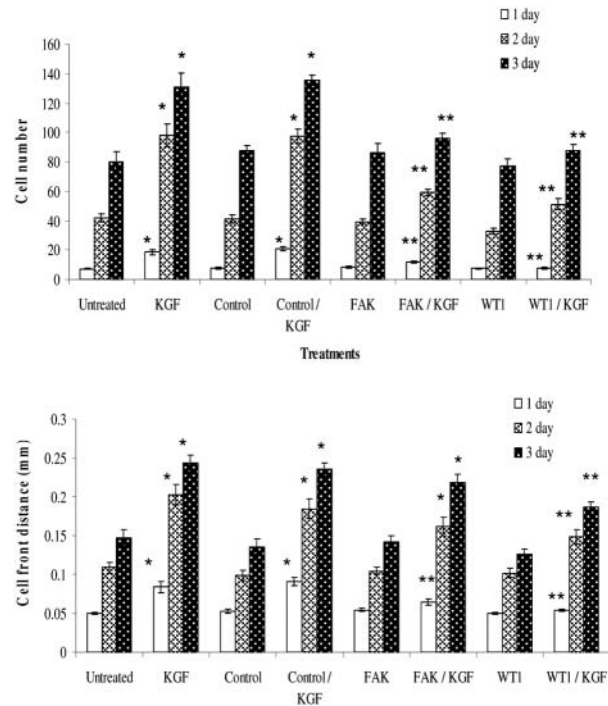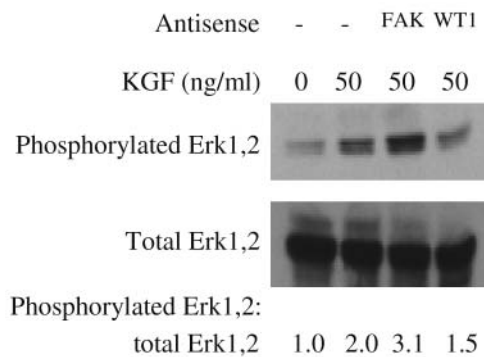

Figure 4. *Effect of down-regulation of WT1 and FAK protein on Erk1,2 activity in KGF-stimulated MCF-7 cells. MCF-7 cells were treated with 50 ng/ml KGF and 12 µM liposomal oligos (WT or FAK antisense) for 3 days. Equal amounts of protein (30 mg) were loaded for SDS-PAGE and phospho-Erk1,2 levels were determined by Western blot. Levels of phospho-Erk1,2 were normalized to those of Erk1,2.*

WT1 led to increased migration of ovarian cancer cells by modulating the expression of the cytoskeletal proteins. Interestingly, we have previously observed a diffuse distribution

of F-actin in the cytoplasm of KGF stimulated MCF-7 cells which is characteristic of cancer cell motility and invasion (19).

FAK, a non-receptor tyrosine kinase, is known to be vital to the migration and proliferation of cancer cells, and has been proposed as a cancer therapeutic target (20). Fibroblasts derived from FAK-knockout mouse embryos showed a significant decrease in cell migration compared to the cells from wild-type mice (21). Similarly, inhibition of FAK activity decreased the motility of fibroblasts and endothelial cells (22). FAK expression is required for platelet-derived growth factor and epidermal growth factor-stimulated cell motility as cells lacking FAK were refractory to the motility signals of these growth factors (23). Our data indicated that FAK was not required for KGF-induced motility, as down-regulation of FAK expression did not affect the motility of the breast cancer cells associated with KGF treatment. On the other hand, FAK mediated the KGF-induced proliferation of the breast cancer cells.

FAK has been shown to regulate cell cycle progression at the G1-phase. Exogenous expression of wild-type FAK in NIH3T3 cells accelerated G1- to S-phase transition, whereas exogenous expression of a dominant negative FAK mutant in NIH3T3 cells inhibited cell cycle progression at the G1-phase (24). FAK phosphotyrosine residues bind to various intracellular signaling molecules, which are considered to be responsible for its regulation of cell migration and proliferation. Associations of FAK with Src, the p85 subunit of phosphatidylinositol 3-kinase, and Grb7 stimulated the migration of *CHO* cells (25). However, the association of FAK with Grb2 did not stimulate cell migration; instead it stimulated cell proliferation (25). Whether FAK regulates cell migration or cell proliferation appears to be dependent on which signaling molecule it is interacting with.

There have been many reports demonstrating that FAK signaling leads to Erk1,2 activation (20, 26, 27). On the other hand, there have also been reports demonstrating that FAK signaling and Erk1,2 activation are independent events. Rosiglitazone, an activator of the PPARγ transcription factor, decreased FAK phosphorylation but had no effect on Erk1,2 phosphorylation in BEL-7404 human hepatocarcinoma cells (28). The ornithine decarboxylase inhibitor α-difluoro-methylornithine increased Erk1,2 phosphorylation, but did not affect FAK phosphorylation in MDA-MB-435 breast cancer cells (29). Melanoma chondroitin sulfate proteoglycan has been found to use independent mechanisms to activate FAK and Erk1,2 in WM1552C melanoma cells (30). Our data indicated that FAK down-regulation did not decrease, and might have increased, Erk1,2 activity. Thus it appears that FAK regulation of Erk1,2 activity is dependent on the initiated cell signals.

In summary, we show for the first time that KGF signaling is transduced *via* WT1 and FAK, which are down-stream of Grb2, to promote the motility and the proliferation of breast cancer cells. KGF-induced breast cancer cell proliferation and motility, which is mediated predominantly by the Erk1,2 pathway, is mainly associated with WT1 signaling. These results suggest that WT1 and FAK may be important therapeutic targets for the prevention of cancer progression.

Acknowledgements

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