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Mammary epithelial cell interactions with fibronectin stimulate epithelial-mesenchymal transition

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Abstract

In the mammary gland, the stromal extracellular matrix (ECM) undergoes dramatic changes during development and in tumorigenesis. For example, normal adult breast tissue is largely devoid of the ECM protein fibronectin (FN) whereas high FN levels have been detected in the stroma of breast tumors. FN is an established marker for epithelial-mesenchymal transition (EMT), which occurs during development and has been linked to cancer. During EMT, epithelial cell adhesion switches from cell-cell contacts to mainly cell-ECM interactions raising the possibility that FN may have a role in promoting this transition. Using MCF-10A mammary epithelial cells, we show that exposure to exogenous FN induces an EMT response including up-regulation of the EMT markers FN, Snail, N-cadherin, vimentin, the matrix metalloprotease MMP2, α-smooth muscle actin, and phospho-Smad2 as well as acquisition of cell migratory behavior. FN-induced EMT depends on Src kinase and ERK/MAP kinase signaling but not on the immediate early gene EGR-1. FN initiates EMT under serum-free conditions; this response is partially reversed by a TGFβ neutralizing antibody suggesting that FN enhances the effect of endogenous TGFβ. EMT marker expression is up-regulated in cells on a fragment of FN containing the integrin-binding domain but not other domains. Differences in gene expression between FN and MG are maintained with addition of a sub-threshold level of TGFβ1. Together, these results show that cells interacting with FN are primed to respond to TGFβ. The ability of FN to induce EMT shows an active role for the stromal ECM in this process and supports the notion that the increased levels of FN observed in breast tumors facilitate tumorigenesis.

Keywords

fibronectin; EMT; MCF-10A cells; breast cancer; TGFβ

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Conflict of interest

The authors declare no conflicts of interest.

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Introduction

The extracellular matrix (ECM) is a key component of a cell's microenvironment and cooperates with other extracellular molecules to relay external signals into cells. Many studies have implicated the ECM in various aspects of mammary gland development and breast cancer (1-3). The laminin-rich basement membrane is critical for mammary morphogenesis and secretion of milk proteins (4-7). The stromal ECM protein fibronectin (FN) is essentially absent from normal adult breast tissue whereas increased FN mRNA and protein levels have been detected in the stroma of breast tumors (8-12). In fact, FN levels in breast tumor tissues are positively correlated with tumor malignancy and negatively correlated with the survival rate of breast cancer patients (9, 10, 13) suggesting that FN might play a role in cancer progression and/or severity. FN transmits ECM signals by binding to integrin receptors, which are heterodimeric transmembrane proteins that link the ECM with the cytoskeleton and intracellular signaling pathways (14). Like FN, β1 integrin levels are also associated with decreased survival in invasive breast cancer (13). How higher levels of FN in breast tumors contribute to tumorigenesis is not understood. In three-dimensional (3D) cell cultures on a Matrigel reconstituted basement membrane, mammary epithelial cells develop into acini similar to in vivo structures with a layer of polarized cells surrounding a hollow lumen and supported by a laminin-rich matrix (15, 16). Addition of FN to polarized, growth-arrested mammary acini stimulates cell proliferation and turns on FN expression (17) and exposure of T4-2 tumorigenic cells to anti-FN antibodies promoted a polarized acinar organization similar to that of normal breast epithelial cells in 3D culture (18). These observations suggest that FN levels might play a role during tumor formation.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose apical-basal polarity and cell-to-cell contacts and gain a mesenchymal phenotype including increased cell-to-ECM contacts and cell migration (19, 20). EMT decreases expression of epithelial marker genes such as E-cadherin and increases expression of mesenchymal marker genes such as FN, Snail, N-cadherin, vimentin, and the matrix metalloprotease MMP2. During the transition, cells go through an intermediate phase of EMT in which both epithelial and mesenchymal characteristics are present (20, 21). While TGFβ is a well-known inducer of EMT (22), the contributions of the ECM, including FN up-regulation, to this process are not understood.

We show that interactions of MCF-10A human mammary epithelial cells with FN induce an EMT response with up-regulation of EMT markers and increased cell migratory behavior. FN contributes to the development of EMT through cooperation with signals initiated by the type I TGFβ receptor. Our findings show an inductive role for FN in EMT and provide a link between changes in ECM composition and tumor progression.

Results

Fibronectin up-regulates its own expression

MCF-10A mammary epithelial cells produce FN when grown in monolayer culture on tissue culture plastic (Figure 1A); this FN is secreted into the culture medium and not assembled into a matrix (not shown). FN expression is down-regulated during acinar morphogenesis by

these cells in 3D Matrigel (MG) culture (17) or when grown on a surface coated with Matrigel (Figure 1A), indicating that MG represses FN expression. We investigated the modulation of FN expression by performing a time course analysis of FN mRNA and protein expression. FN expression was first down-regulated by growth of MCF-10A cells on MG for two days. Cells were then replated on surfaces coated with 20 μg/ml solutions of either MG or plasma FN, and expression levels were measured at increasing times after plating. Quantitative RT-PCR (qRTPCR) analyses show a time-dependent increase in FN mRNA on FN substrate but no significant increase on MG (Figure 1B). Sustained up-regulation of FN mRNA was observed at 6 hr and later but we also detected a transient increase after 2 hr. This up-regulation of FN mRNA on FN substrate was maintained for at least 4 days (Figure 1C). Levels of secreted FN increased subsequent to the initial mRNA up-regulation as detected in the culture medium of cells grown on FN for 4 or more hours (Figure 1D and not shown). Similar changes in FN mRNA up-regulation were observed when cells were replated onto a substrate of recombinant FN isolated from insect cells grown in serum-free conditions ((23), not shown). Together, these results show that MCF-10A cell interactions with FN induce up-regulation of FN mRNA and protein expression.

Fibronectin promotes an EMT response

Cells undergoing EMT lose epithelial characteristics and show changes in cell adhesion, polarity, and gene expression. FN itself is a well-known EMT marker (24), which suggests that FN-dependent FN up-regulation might be part of an EMT response. Expression of other EMT markers was measured after replating cells onto MG or FN. The fold difference between mRNA levels in cells grown on FN compared to cells on MG is shown in Table 1. MCF-10A cells show significantly increased expression of the EMT markers N-cadherin, Snail, vimentin, and matrix metalloproteinase MMP2 after 24 hr on FN relative to MG. This up-regulation is sustained for at least 4 days (Figure 2A and Supplementary Figure 1A-C). Levels of MMP3 and MMP9 mRNAs (not shown) and α-smooth muscle actin protein (Supplementary Figure 1D) were also higher on FN than on MG after 2 days.

Up-regulation of EMT marker gene expression is dependent on integrin binding to FN. Increases in FN, Snail, and N-cadherin mRNAs were comparable between cells on FN and cells plated on a ~20 kDa cell-binding domain fragment of FN (GST-$III_{9\text{-}10}$) containing the RGD sequence in III_{10} and the synergy site in III_9 (Supplementary Figure 2). Therefore, MCF-10A cell adhesion to this integrin-binding domain in the absence of other domains of FN is sufficient to induce changes in gene expression.

A sustained increase in N-cadherin mRNA was accompanied by higher N-cadherin protein levels detected on immunoblots of whole cell lysates (Figure 2B). Immunofluorescence staining of MCF-10A cells also showed higher N-cadherin in cells grown on FN (Figure 2C and Supplementary Figure 3). N-cadherin appears more peripherally localized in cells on FN than on MG. These data illustrate a significant substrate-dependent difference in EMT marker expression by MCF-10A cells and support the conclusion that cell-FN interactions contribute to development of an EMT phenotype.

EMT leads to changes in cell behavior including increased migration. To see whether exposure to FN elicits this response, MCF-10A cells were tested for migration on MG-coated Transwell filters. After 8 hr, more of the cells that had been primed by growth on FN migrated relative to cells that were primed on MG (Figure 3), indicating that in addition to marker expression, growth on FN promotes development of a migratory cell behavior.

EMT depends on ERK activation but not EGR-1 up-regulation

The early growth response-1 (EGR-1) is an immediate early gene transcription factor whose expression and activity are stimulated by ERK/MAP kinase signaling (25). This transcription factor has been shown to stimulate FN expression in melanoma cells (26) and to mediate Snail expression in HepG2 cells (27). We found that plating MCF-10A cells on FN transiently increased EGR-1 mRNA and protein levels in the whole cells and in the nuclear fraction (not shown). Also, it was previously reported that ERK2 overexpression increases levels of N-cadherin and FN in MCF-10A cells (28). To determine whether ERK and EGR-1 are required for up-regulation of FN and other EMT markers, we inhibited activation of ERK with the MAP kinase kinase (MEK) inhibitor PD98059. Phospho-ERK1/2 levels on FN were significantly reduced by the inhibitor (Figure 4A) and this treatment decreased EGR-1 mRNA levels by 65 - 70 % after 1 hr on MG or FN (Figure 4B), confirming a requirement for ERK signaling in EGR-1 up-regulation. ERK is rapidly activated in response to extracellular signals so phospho-ERK levels were monitored within 1-2 hr. Because changes in gene expression occur downstream of ERK activation, we analyzed EMT markers at 6 hr, the earliest time when changes were detected, and at 24 hr when marker up-regulation appears to peak. Neither Snail nor N-cadherin mRNA was significantly changed by PD98059 treatment for 6 hr (Figure 4C and not shown). In contrast, we observed a statistically significant increase in FN mRNA after 6 hr of PD98059 treatment on FN (Figure 4D), suggesting the ERK1/2 might be involved in reducing the level of FN production. EGR-1 siRNA treatment reduced its mRNA by 47 %, but did not cause a significant change in Snail or FN mRNA levels after 6 or 24 hrs on FN (not shown). Therefore, EGR-1 is not a critical component of the EMT pathway in MCF-10A cells on a FN substrate. In contrast, ERK activity appears to be involved in this EMT response such that inhibition of this pathway releases negative control on the level of FN expression.

Type I TGFβ receptor activity is required for FN induction of EMT markers

It is well established that TGFβ1 induces EMT in MCF-10A and other cells (22, 29-32). MCF-10A cell assay medium contains 2% serum, which includes some TGFβ1. It is possible that the effects of FN on EMT occur through cooperation between FN and TGFβ signaling pathways. Binding of TGFβ1 to its receptor stimulates phosphorylation of Smads, and our analysis of Smad2 showed an increase in phosphorylation on FN compared to MG after 1, 2, and 6 hr (Figure 5A and not shown). Treatment of cells with a type I TGFβ receptor inhibitor blocked the appearance of pSmad2 in the cells grown on MG and on FN (Figure 5A). This inhibitor also abolished the up-regulation of FN, N-cadherin, and Snail on a FN substrate detected at 6 hr (Figure 5B) and 24 hr (Figure 5C), while ubiquitin C mRNA levels were not affected by the substrate or the inhibitor (Figure 5B, C). These results show a role for TGFβ signaling through phosphorylation of Smad2 in the FN-dependent induction

of EMT. We also tested the effects of substrate on TGFβ1 expression and found no difference in its mRNA levels between MG and FN (not shown).

A role for a Src pathway in EMT

FN-integrin binding rapidly activates focal adhesion kinase (FAK) by phosphorylation on Tyr397, and this leads to recruitment of Src (33) and signaling through a FAK/Src complex (34). Enhanced phospho-FAK was observed in MCF-10A cells plated on FN for 30 minutes compared to cells on MG (Figure 6A). To determine if Src activity affects Smad, cells on either FN or MG were treated with 10 μM SU6656, a Src kinase inhibitor. pSmad2 levels decreased to about 45% of that without SU6656 (Figure 6B). Up-regulation of the EMT markers FN, Snail, and N-cadherin on a FN substrate was abolished by SU6656 treatment, while UBC mRNA levels and marker levels on MG (except Snail) did not change (Figure 6C). Therefore, Src activity is required for FN stimulation of its own expression and for induction of other EMT markers in cells on FN but not MG. Together, these data suggest that Src might have a general effect on Smad2 phosphorylation by type I TGFβ receptor, but that it has a substrate-specific role in regulating EMT marker expression on FN.

Effects of FN or MG in the absence of serum

To determine whether the substrate plays a role in EMT, we monitored Smad phosphorylation and expression of EMT markers in the absence of serum. Smad2 was activated in MCF-10A cells on FN but not on MG (Figure 7A). FN also up-regulated expression of FN, Snail, and N-cadherin mRNAs in the absence of serum (Figure 7B), indicating that the substrate promotes an EMT response. Addition of 2% serum, which contains some TGFβ, increased pSmad2 on both substrates although levels remained higher on FN (Figure 7A). pSmad2 levels were similar on MG and FN with the addition of 0.05 ng/ml TGFβ1. These results suggest that FN contributes to Smad2 activation and EMT marker induction independently of added TGFβ.

To determine whether endogenously produced TGFβ was contributing to increased pSmad2 on the FN substrate, cells grown on FN or MG in the absence of serum were treated with a TGFβ neutralizing antibody and pSmad2 levels were monitored. Untreated cells on FN had 6.2-fold higher pSmad2 levels than cells on MG (Figure 8A). Antibody-treatment reduced pSmad2 on both FN and MG. While the level in cells on MG decreased almost to zero, pSmad2 remained 3-fold higher in cells on FN than in untreated cells on MG. Addition of 0.05 ng/ml TGFβ significantly increased pSmad2 to similar levels on FN and MG (~12- and 14-fold, respectively) and also increased total Smad levels. In spite of equivalent pSmad2 levels, the difference in FN expression between FN and MG substrates was maintained (Figure 8B). Interestingly, neutralizing antibody reduced the up-regulation on both FN and MG to levels similar to those in cells without added TGFβ with >3-fold higher pSmad2 in cells on FN than in untreated cells on MG (Figure 8A). Using qRT-PCR, we confirmed that the effects of neutralizing antibody on FN expression correlate with changes in pSmad2 (Figure 8B). These results show that endogenous TGFβ and the FN substrate together are responsible for the observed increases in pSmad2.

Cooperation between FN and TGFβ in EMT

Differences in cell response on MG versus FN substrate along with results from treatment with the TGFβ neutralizing antibody show that the levels of Smad2 phosphorylation and FN expression are influenced by both FN and TGFβ. When TGFβ was present in a sufficient amount (≥ 0.05 ng/ml) and for a sufficient time (≥ 6 hr), the effects of the FN substrate were not detectable. However, when the level of TGFβ was low (such as under serum-free or 2% serum conditions), there was a significant enhancement of pSmad2 levels (Figure 7A, 9A) and expression of FN and other EMT markers in cells on FN, but not on MG (see Figure 7B). To show that interactions with the substrate contribute to the EMT response by TGFβ, we used cells grown on FN or MG for 4 hr at which time FN mRNA levels are the same on the two substrates. Culture for 4 hr with 0.05 ng/ml TGFβ1 did not change FN expression levels on MG, but it did increase FN mRNAs about 2-fold in cells on FN (Figure 9B). Therefore, cells interacting with FN are primed to respond to TGFβ such that addition of a sub-threshold level of TGFβ1, which does not stimulate cells on MG, significantly increases FN expression on a FN substrate.

Discussion

In the stroma of normal, fully developed mammary tissue, very little FN is detected. In mammary tumors, on the other hand, an increased stromal FN level correlates with the severity of the disease (9, 10). In 3D culture, FN stimulates proliferation of growth-arrested polarized mammary epithelial cells, disturbing the hollow acinar structure and promoting tumor-like behavior (17). The results presented here show that mammary epithelial cell-FN interactions induce an EMT response that includes up-regulation of FN, Snail, and other EMT markers as well as increased migratory behavior and activation of Smad2. This induction is substrate-specific, occurring on FN and on the cell-binding domain of FN but not on MG. Indeed increases in EMT marker expression and Smad2 phosphorylation occur in cells on FN under serum-free conditions and a TGFβ neutralizing antibody reduced but did not eliminate pSmad2 in cells on FN, demonstrating that substrate composition has an inductive effect on EMT. Addition of TGFβ1 enhances the effects of FN on EMT and our results suggest that interactions with FN, but not with MG, prime cells to respond to growth factor stimulation. Together our findings suggest that stromal FN alone can induce an EMT response, and that the cooperative action of stromal FN with TGFβ may promote epithelial cell changes during EMT and tumorigenesis.

Tumor formation in the breast involves changes in mammary epithelial cell interactions with the ECM. Loss of basement membrane integrity is a hallmark of cancer and occurs through changes in expression of ECM proteins or their receptors and through increased release of matrix metalloproteases (35). Basement membrane breach exposes epithelial cells to the stromal ECM, and our results support the idea that cell-stromal interactions have a synergistic role in promoting a change in cell phenotype such as EMT. Up-regulation of FN expression by cancer-associated fibroblasts (36) or transport into the tissue of FN, which can be increased in the plasma in breast cancer (37) are two ways in which stromal FN levels could be elevated. Our results showing cell stimulation by FN when levels of TGFβ are sub-

threshold indicate that an up-regulation of FN in tissue could impact the rate of tumor formation.

Stimulation of MCF-10A cells growing on FN with a low level of TGFβ1 significantly increased FN expression but did not have the same effect when cells were on MG. This observation raises the possibility that in vivo epithelial cells attached to a basement membrane respond to this growth factor differently than cells in contact with the stromal ECM. Furthermore, the response of MCF-10A cells may depend on the concentration of TGFβ in the stroma such that low levels of growth factor in a FN-rich stroma might induce EMT while these levels of TGFβ are below the threshold needed to have an effect on cells in contact with a basement membrane. Our results showing that EMT markers were up-regulated in cells on the III_{9-10} integrin-binding domain of FN suggest that intracellular signaling from FN through integrins cooperates with TGFβ1 signaling to induce FN gene expression. The possibility that TGFβ and FN cooperate through direct interactions seems less likely since TGFβ has been shown to bind to FN's III_{12-14} heparin-binding domain (38). There also may be a temporal component to the substrate effects as observed for Snail expression, which is directly downstream of Smad signaling (39). Under low TGFβ conditions at 4 hr where FN expression is equivalent on FN and MG substrates, Snail expression was significantly higher on FN (JP, unpublished observations). However, up-regulation of FN and Snail both involve Src activity suggesting that a pathway downstream of Src cooperates with a pathway initiated by pSmad2 to increase expression of EMT markers. In vivo it is possible that TGFβ1 and FN might help each other to promote the induction of EMT responses in the cells to exacerbate cancer formation.

In addition to expression of EMT markers, cell-FN interactions also enhanced cell migratory behavior, which is another important characteristic of EMT. Although cells grown on FN showed up-regulation of mesenchymal markers and increased cell motility, we did not observe a decrease in the epithelial marker E-cadherin. N-cadherin can promote cell motility in breast cancer cells regardless of E-cadherin expression (40), and others have proposed that the ratio of N-cadherin to E-cadherin determines the EMT phenotype (21). Treatment of MCF-10A cells with 5 ng/ml TGFβ1 for 2 days induced dramatic increases in FN, Snail, and N-cadherin mRNAs (63-, 35-, and 16-fold, respectively) but did not significantly change E-cadherin expression (JP, unpublished observations). Furthermore, in other reports, these cells showed modest changes at best in E-cadherin levels with extended TGFβ treatment (41-43) indicating that EMT in MCF-10A cells does not include robust changes in E-cadherin. Furthermore, decreased E-cadherin might not be a major phenotype of breast cancer development; Goyal et al. (44) reported no difference in E-cadherin expression between tumor and normal breast tissue. It is also possible that cells on FN might be in an intermediate phase of EMT in which some characteristics of both epithelial and mesenchymal phenotypes are present and up-regulation of N-cadherin without down-regulation of E-cadherin is sufficient to increase cell motility.

FN binding to integrins activates a number of intracellular pathways that synergize with signals from various growth factors (45). We have shown that together FN and TGFβ enhance the induction of an EMT response in mammary epithelial cells. We propose that increased FN in breast cancer might be both a cause and a result of tumor initiation and/or

progression. Therefore, modulating FN expression might present a new way to control tumor formation.

Materials and Methods

Cell culture

MCF-10A cells were purchased from the American Type Culture Collection and cultured as previously described (46). Cells were cultured for no more than 25 passages. When passaged, cells were grown in growth medium containing 5 % horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and Penicillin/streptomycin (Invitrogen) in DMEM/F12 (Invitrogen). For experiments, cells were plated in assay medium containing 2 % horse serum (Invitrogen), 5 ng/ml EGF (Peprotech), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin (Sigma), and Penicillin/streptomycin (Invitrogen) in DMEM/F12 (Invitrogen), unless specified otherwise. To coat the surface, tissue culture dishes were incubated with 20 μg/ml rat plasma fibronectin or 20 μg/ml Matrigel (BD Biosciences) in PBS at 4 °C overnight. Rat plasma fibronectin was purified from rat plasma (Taconic) by gelatin-Sepharose affinity chromatography as previously described (47). TGFβ1 was purchased from R&D systems.

For comparison of cell behaviors on MG and FN, MCF-10A cells were first grown on a Matrigel substrate for two days to reduce FN expression, and then replated on Matrigel or FN to be grown for various times before analysis. When an inhibitor was used, the cells grown on Matrigel for two days were resuspended in assay medium containing the inhibitor and incubated for at least ten minutes before being replated on Matrigel or FN. The following inhibitors were used: Type I TGFβ receptor inhibitor (Calbiochem), PD98059 (Enzo Lifesciences), SU6656 (Calbiochem). The TGFβ neutralizing antibody (clone 1D11, R&D Systems) was added to cells suspended in serum-free assay medium at the time of replating on Matrigel or FN.

Quantitative RT-PCR (qRT-PCR)

After various incubation times, RNA was extracted using Qiagen RNeasy mini kit following the manufacturer's instructions. cDNA was prepared with 0.5 μg RNA per 20 μl reaction volume, 0.5 mM dNTP, 2.5 μM random hexamer, 10 mM DTT (dithiothreitol), and SuperScript II Reverse Transcriptase and buffer (Invitrogen). Real-time PCR was performed using Stratagene Mx3000P following the manufacturer's instructions. GAPDH primers were used as a normalization control. To calculate mRNA fold change for a particular gene of interest, the mRNA level for each cDNA sample was normalized to the GAPDH mRNA level. The normalized value from cells on Matrigel was set to one for the indicated time point, and all other mRNA levels in the cells on FN or with other treatments were calculated relative to the Matrigel standard for the same time point. If multiple time points were being compared, the normalized value from cells on Matrigel for the shortest time point was set to one. Values from at least three experiments were averaged and standard error was calculated unless indicated otherwise.

Statistical analysis

P-value for determining statistical significance was calculated by a paired, two tailed Student's t-test and p-values less than 0.05 were considered significant.

Primer design

All primers were designed using MacVector with parameters for real-time PCR. Each primer in a pair was selected from two different exons to distinguish cDNA product from genomic DNA amplification. Products were confirmed by polyacrylamide gel electrophoresis. The primer sequences are as follows:

GAPDH: forward primer 5' TGACAACTTTGGTATCGTGGAAGG 3' reverse primer 5' AGGGATGATGTTCTGGAGAGCC 3'

FN: forward primer 5’ TGAAAGACCAGCAGAGGCATAAG 3’ reverse primer 5’ CTCATCTCCAACGGCATAATGG 3’

Snail: forward primer 5’ ATCGGAAGCCTAACTACAGCGAGC 3’ reverse primer 5’ CAGAGTCCCAGATGAGCATTGG 3’

N-cadherin: forward primer 5’ ACCAGGTTTGGAATGGGACAG 3’ reverse primer 5’ ATGTTGGGTGAAGGGGTGCTTG 3’

Vimentin: forward primer 5’ TGAAGGAGGAAATGGCTCGTC 3’ reverse primer 5’ GTTTGGAAGAGGCAGAGAAATCC 3’

MMP2: forward primer 5’ CCAACTACAACTTCTTCCCTCGC 3’ reverse primer 5’ GCAAAGGCATCATCCACTGTCTC 3’

UBC: forward primer 5’ ATTTGGGTCGCGGTTCTT 3’ reverse primer 5’ TGCCTTGACATTCTCGATGGT 3’

EGR-1: forward primer 5’ CAGCAGCACCTTCAACCCTCAG 3’ reverse primer 5’ AGTGGTTTGGCTGGGGTAACTG 3’

TGFβ1: forward primer 5’ ATTCCTGGCGATACCTCAGC 3’ reverse primer 5’ ACCCGTTGATGTCCACTTGC 3’

Type I TGFβ receptor: forward primer 5’ ATTCCTGGCGATACCTCAGC 3’ reverse primer 5’ ACCCGTTGATGTCCACTTGC 3’

In order to verify whether each primer set makes a correct product, the qRT-PCR sample was run in 6 % polyacrylamide gel, and only one band in the correct size was visual with ethidium bromide under UV illumination.

Immunoblots

Cells were lysed with mRIPA buffer (50 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 1 % Triton X-100, 10 % glycerol, 150 mM NaCl, 0.1 % SDS, 1 % DOC (deoxycholic acid), 1 mM EGTA, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 1 mM PMSF, 1 μg/ml aprotinin, and 10 ng/ml

leupeptin) to isolate total proteins from the whole cells, and a BCA assay (Thermo Scientific) was performed to determine the concentrations. To extract secreted FN, the medium from equivalent numbers of cells grown on MG or FN was incubated with gelatin-Sepharose beads and the FN was eluted by boiling the beads in electrophoresis sample buffer for 2 minutes. Equal proportions were resolved by 5 % SDS-PAGE for FN and 10 % SDS-PAGE for the other proteins, and were transferred to nitrocellulose membrane. Anti-human FN monoclonal antibody HFN 7.1 culture supernatant (1:10000, Developmental Studies Hybridoma Bank) was used to detect human FN that was secreted by the MCF-10A cells. HFN7.1 is species-specific and does not recognize mouse FN (48). Other antibodies and dilutions used include: EGR-1 (1:5000, Santa Cruz), phospho-ERK1/2 (1:5000, Sigma), total ERK1/2 (1:1000, Cell Signaling), phospho-FAK(Y397) (1:2000, Invitrogen), total FAK (1:1000, Millipore), phospho-Smad2 (1:1000, Cell Signaling), total Smad 2/3 (1:1000, Cell Signaling), N-cadherin (1:20, 13A9, gift from Keith R Johnson, University of Nebraska Medical Center), α-smooth muscle actin (1:2000, Sigma). Antibody binding was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Band intensities were quantified using ImageJ Gel analysis tool. Total band intensities were determined from multiple exposures in the linear range of exposure for each antibody. The total intensity of GAPDH was used for normalization.

Transwell cell migration assay

Cells grown on Matrigel or FN for 2 days were trypsinized and resuspended in assay medium without serum. 5×10^4 cells were plated on a transwell polycarbonate membrane with 8 μm pores (BD BioCoat™), which was placed in a 24-well dish containing assay medium with 2 % horse serum. After 8 hours, cells on top of the filter were removed with a cotton swab and the cells that had migrated to the bottom of the filter were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) and migrated cells were counted in multiple microscope fields.

Immunofluorescence

Immunofluorescence staining of cells was done as described (46). Cells grown on MG or FN-coated coverslips for 3 days were fixed, permeabilized, and stained with anti-N-cadherin (Invitrogen) antibodies in 1 % BSA/PBS followed by Alexa Fluor goat anti-mouse IgG (Molecular Probes) and DAPI (Sigma-Aldrich). Images were acquired and normalized using iVision software (BioVision technologies).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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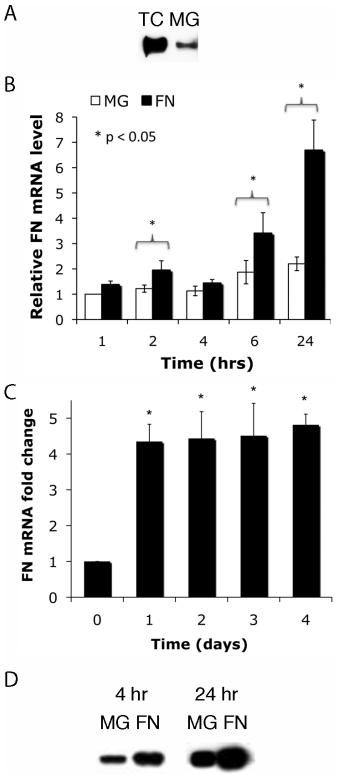

Figure 1. Up-regulation of FN expression

(A) Secreted FN protein was isolated by gelatin-Sepharose chromatography from culture media of cells grown on tissue culture plastic (TC) or on MG for 24 hr. Protein was separated by SDS-PAGE and FN was detected by immunoblotting with antihuman fibronectin HFN 7.1 antibody. (B) Mean FN mRNA levels were determined for cells on FN or on MG for the indicated times. The amount of FN mRNA detected by qRT-PCR at each time point was normalized to that of MCF-10A cells grown on MG for 1 hr. Mean +/- standard error for at least three independent experiments. * $p < 0.05$. (C) The fold difference in FN mRNA after 1, 2, 3, and 4 days in cells on FN relative to MG was determined by qRT-PCR. Time 0 RNA was isolated before plating on MG or FN. Mean +/- standard error for at least three independent experiments; * $p < 0.05$ relative to time 0. (D) Secreted FN protein was isolated and detected as in (A) at the indicated times.

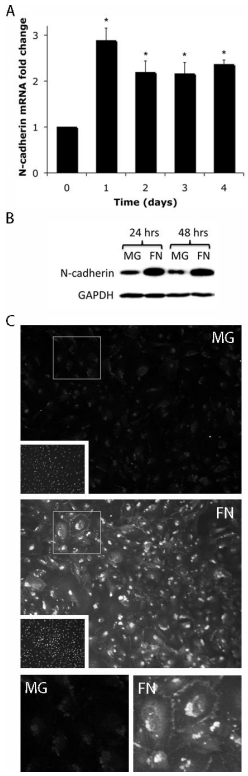

Figure 2. FN upregulates N-cadherin expression

(A) N-cadherin mRNA fold change on FN substrate relative to cells on MG was determined at the indicated days. Bars represent the mean +/- standard error for at least three independent experiments. * $p < 0.05$ relative to time 0. (B) Lysates were prepared from cells on FN or MG at the indicated times and immunoblotted with anti-N-cadherin antibodies. (C) Cells grown on MG or FN for 3 days were fixed and immunostained with anti-N-cadherin antibodies, fluorescent secondary antibodies and DAPI. Inset: DAPI. Boxed areas are shown enlarged at the bottom.

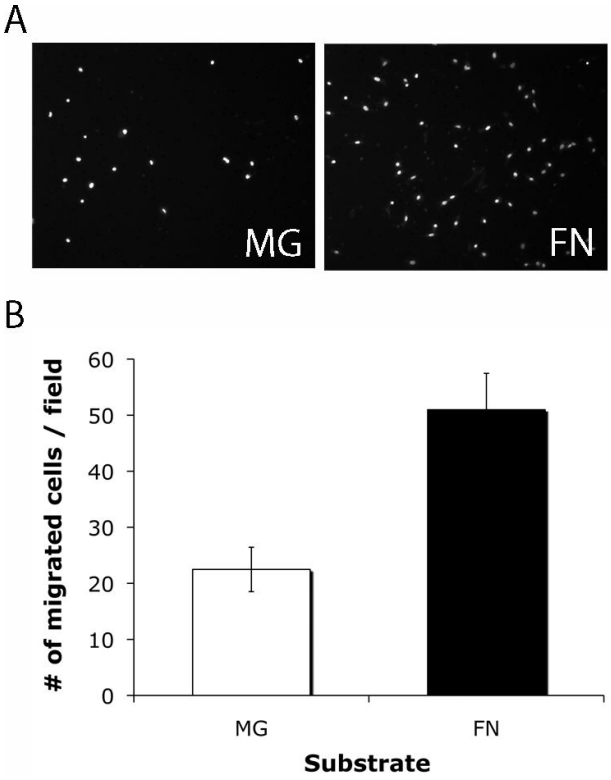

Figure 3. Cells grown on FN acquire migratory behavior

Cells were grown on MG for 2 days, and subsequently on MG or FN for 2 days before being plated on MG coated transwell membranes. After 8 hr, unmigrated cells were removed and migrated cells were stained with DAPI. (A) Representative image of a field of migrated cells previously grown on MG or FN substrate. (B) Average number of migrated cells +/- standard error for 50 (MG grown cells) or 45 (FN grown cells) fields from three independent experiments. $p < 0.001$ between cells grown on MG and FN.

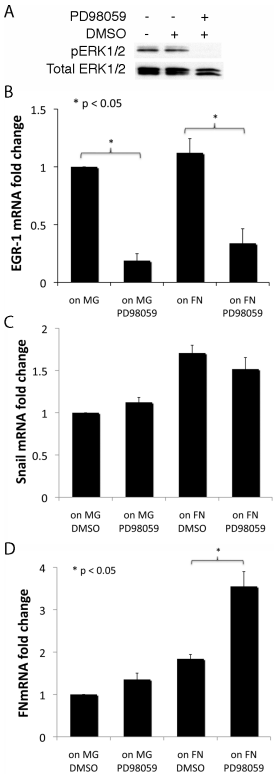

Figure 4. Effects of ERK inhibition on EGR-1, Snail, and FN expression

(A) Cells were plated on FN in the presence or absence of 20 μM MEK inhibitor PD98059 or with addition of vehicle (DMSO) for 30 min., then lysed and immunoblotted with antibodies against total ERK1/2 or phsopho-ERK1/2. (B-D) Cells were treated with 20 μM MEK inhibitor PD98059 or not on MG or FN and RNA was extracted for qRT-PCR. Mean +/- standard error for three experiments. (B) RNA isolated after 1 hr on MG or FN was used for qRT-PCR with EGR-1 primers. (C-D) RNA isolated after 6 hr was used with Snail (C) and FN primers (D).

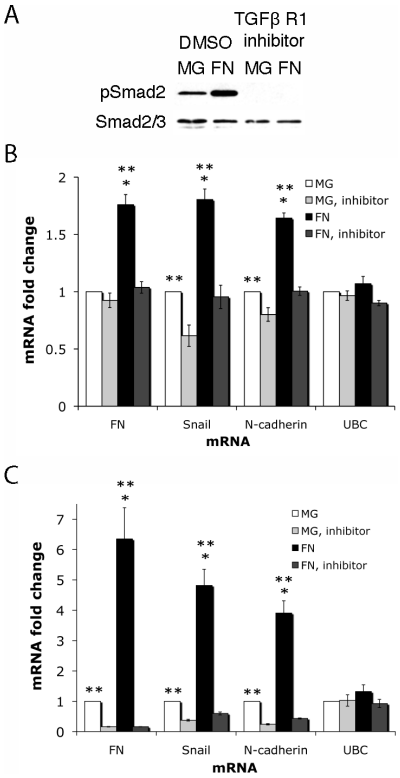

Figure 5. Type I TGFβ receptor activity is required in FN induction of EMT markers

(A) MCF-10A lysates prepared from cells grown on MG or FN without or with a type I TGFβ receptor (TGFβ R1) inhibitor for 2 hr were used in immunoblots for pSmad2 and total Smad. Results are representative of three independent experiments. (B-C) Relative mRNA levels of FN, Snail, N-cadherin, and ubiquitin C (UBC) were measured by qRT-PCR from cells grown on MG or FN for 6 hr (B) or 24 hr (C). Mean +/- standard error for at least three experiments. * $p < 0.05$ for FN/DMSO compared to MG/DMSO for FN, Snail, and N-cadherin. ** $p < 0.05$ between samples on same substrate with and without inhibitor.

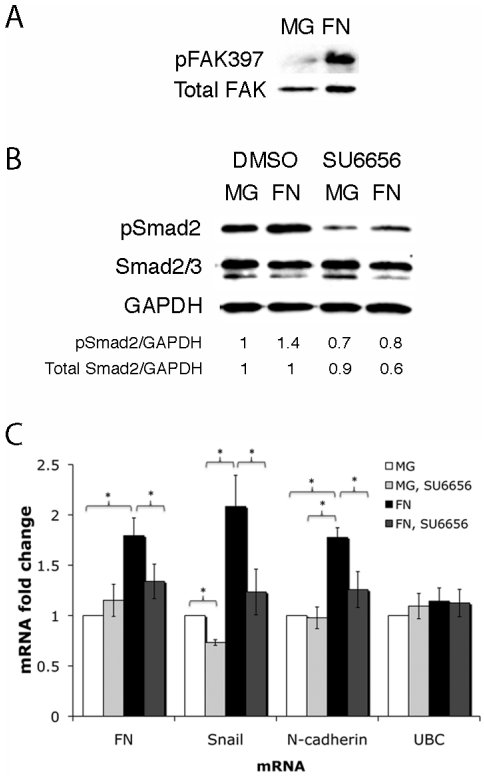

Figure 6. Inhibition of Src activity blocks EMT marker expression

(A) Tyr-397-phosphorylated FAK (pFAK397) and total FAK were detected in cell lysates prepared after growth on MG or on FN for 30 min. Image is representative of three experiments. (B) pSmad2 and total Smad2 levels detected on immunoblots after 2 hr on MG or FN with DMSO or SU6656. Band intensities of pSmad2 or total Smad2 relative to GAPDH are indicated below the blot. Data are representative of two independent experiments. (C) FN, Snail, N-cadherin mRNA levels on MG vs. FN with or without 10 μM SU6656 at 6 hr. Mean +/- standard error for three independent experiments. * $p < 0.05$ between the columns.

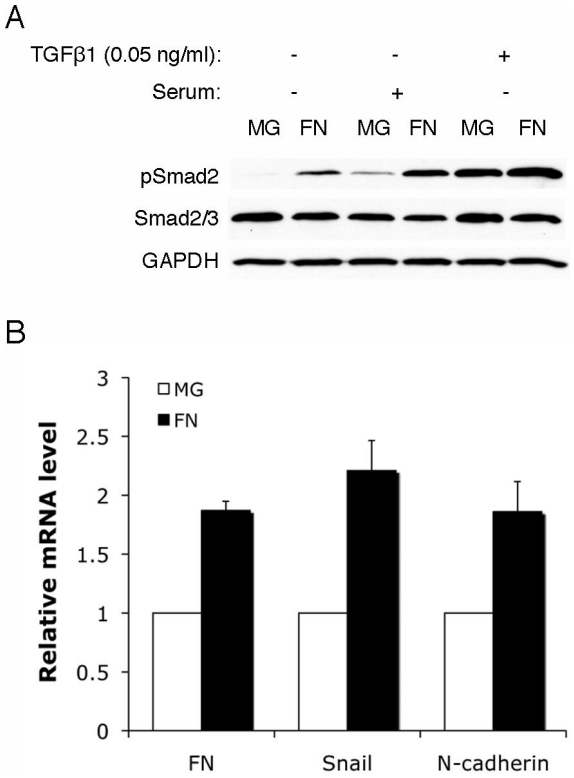

Figure 7. FN increases EMT in the absence of serum

(A) No serum, 2 % horse serum, or 0.05 ng/ml TGFβ1 without serum was added to MCF-10A cells plated on MG or FN. Cell lysates were prepared after 2 hr and used for immunoblotting with pSmad2 and total Smad antibodies. (B) qRT-PCR was used to measure mRNA levels for FN, Snail and N-cadherin in cells grown on FN or MG for 6 hr without serum. At least three independent experiments were averaged. Values on FN were normalized to levels on MG. $p < 0.05$ between cells grown on MG and FN.

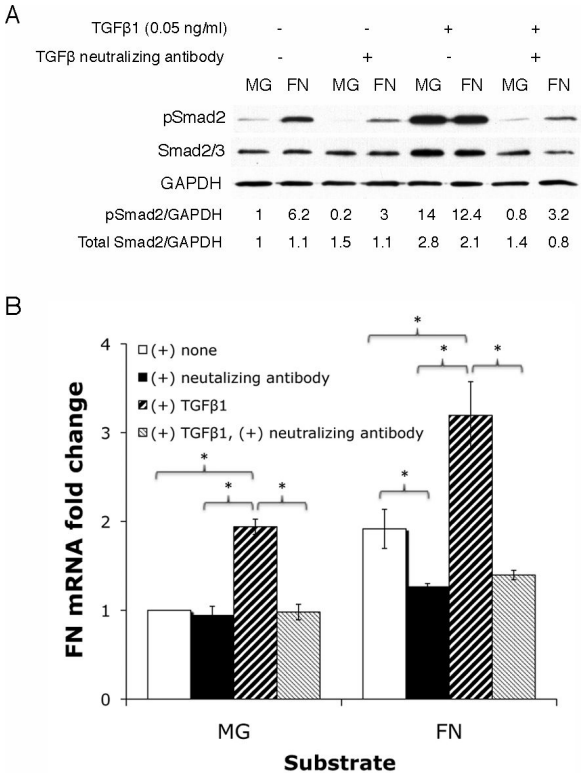

Figure 8. TGFβ neutralizing antibody reduces pSmad2 and FN mRNA up-regulation on FN

(A) 10 μg/ml of TGFβ neutralizing antibody with or without 0.05 ng/ml TGFβ1 were added to MCF-10A cells plated on MG or FN without serum. Cell lysates were prepared after 2 hr and used for immunoblotting with pSmad2, total Smad, or GAPDH antibodies. (B) qRT-PCR was used to measure mRNA levels for FN in cells grown on FN or MG for 6 hr without serum and treated as in (A). At least three independent experiments were averaged. Values were normalized to the level in untreated cells on MG. * $p < 0.05$ between the columns.

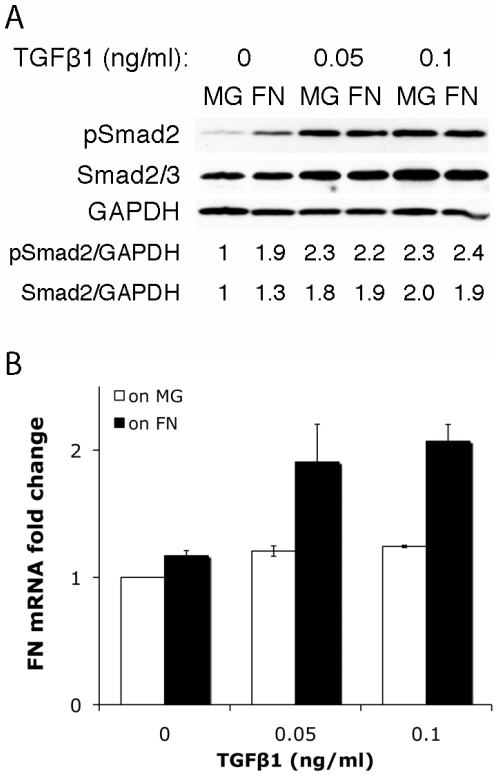

Figure 9. FN substrate and TGFβ1 cooperate to induce EMT

(A) Phospho-Smad2 and total Smad2 levels were detected in cell lysates at 2 hr after plating in medium containing 2% serum plus or minus the indicated concentrations of TGFβ1. Phospho-Smad2, total Smad2/3, and GAPDH antibodies were used in immunoblots. Band intensities of pSmad2 or total Smad2 relative to GAPDH are indicated below the blot. (B) MCF-10A cells were plated in medium containing 2% serum plus the indicated concentrations of TGFβ1. RNA was isolated at 4 hr and FN mRNA levels were measured using qRT-PCR. Mean +/- standard error for three independent experiments. The mean fold change on FN vs. MG was not statistically significant with 0.05 ng/ml TGFβ1, but significant with 0.1 ng/ml TGFβ1 ($p < 0.05$).

Table 1

Up-regulation of EMT markers

Fold change on FN relative to MG at 24 hr			
mRNA	**Avg. fold change**	**Standard deviation**	**N**
N-cadherin	2.9	0.68	6
Snail	4.2	0.99	7
Vimentin	1.6	0.15	5
MMP2	1.8	0.20	4