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PRINCIPAL INVESTIGATOR: Rachel B. Hazen, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, New York 10029

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Rachel B. Hazen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai School of Medicine
New York, New York 10029

E-Mail: rachel.hazan@mssm.edu

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The intracellular signaling events that cause tumor cells to become metastatic are not well understood. N-cadherin and FGF-2 synergistically increase migration, invasion and secretion of extracellular proteases in breast tumor cells. Here, we define a metastatic signaling cascade activated by N-cadherin and FGF-2. In the presence of N-cadherin, FGF-2 caused sustained activation of the MAPK-ERK pathway, leading to MMP-9 gene transcription and cellular invasion. N-cadherin prevented the FGF receptor (FGFR) from undergoing ligand-induced internalization, resulting in increased FGFR stability. Association of FGFR with N-cadherin was mediated by the first two Ig-like domains of FGFR. These results suggest that protection of the FGFR from ligand-induced downregulation by N-cadherin enhances receptor signaling and provides a mechanism by which tumor cells can acquire metastatic properties

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Introduction

It had been hypothesized that one of the factors contributing to the escape of nascently metastasizing tumor cells from the primary tumor mass is reduced tumor cell adhesion caused by the loss of the cell adhesion molecule, E-cadherin (Takeichi, 1993). However, the finding that a related adhesion molecule, N-cadherin, is upregulated in many invasive cancer cell lines (Hazan et al., 1997; Tran et al., 1999) and aggressive tumors (Li et al., 2001; Tomita et al., 2000), has forced a reevaluation of this view. A simple reduction in tumor adhesive strength may not be the only critical determinant in the acquisition of invasive cellular behavior. Rather, a shift in the adhesive specificity of tumor cells from E- to N-cadherin-mediated adhesion would allow tumor cells to associate with the surrounding stroma (Hazan et al., 1997) and vasculature, both of which express N-cadherin, thereby facilitating the detachment and migration of cells away from the primary tumor (Voura et al., 1998).

It was found that expression of N-cadherin by tumor cells has additional consequences on cellular behavior other than a simple change in cellular adhesive specificity. For example, N-cadherin induced an invasive cellular morphology in squamous tumor cells (Islam et al., 1996) and stimulated the migration, invasion (Hazan et al., 2000; Nieman et al., 1999) and metastasis of breast cancer cells (Hazan et al., 2000). The effects of N-cadherin expression on tumor cells were exacerbated by FGF-2, suggesting that N-cadherin and FGFR synergize to generate signals that can alter the invasive behavior of tumor cells (Hazan et al., 2000). This possibility was further strengthened by experiments demonstrating that secretion of the matrix metalloprotease, MMP-9, was dramatically elevated upon FGF-2 treatment of N-cadherin expressing tumor cells (Hazan et al., 2000). The mechanism whereby N-cadherin cooperates with the FGFR to stimulate an invasive response remained unidentified. We postulate that the synergy between N-cadherin and FGFR might transduce specific signals that lead to metastasis. Here we show that N-cadherin and FGFR cooperate to activate an intracellular signaling cascade which results in tumor invasion. N-cadherin associates with the FGFR and this interaction is mediated by the extracellular first two Ig-like domains on the FGFR. As a consequence of this interaction, the FGFR is not down regulated by FGF-2, causing FGFR accumulation at the cell surface, sustained MAPK-ERK activation, increased MMP-9 expression, resulting in tumor cell invasiveness.

Progress Report

N-cadherin causes invasiveness in the chicken CAM assay

We tested the ability of N-cadherin to affect invasion of MCF-7 cells in the chicken chorioallantoic membrane(CAM) assay. This experiment was performed in collaboration with Dr. Liliana Ossowski according to a well-established protocol (Ossowski, 1988). Cell monolayers were labeled with 2μci/ml ^{125}I-IudR, washed of free label, and 3X10^5 cells were inoculated into the wounded CAM and incubated for 24 hours at 37°C. CAMs were rinsed of non-invading cells and processed as described to detect invasion of radiolabeled cells (Ossowski, 1988). We found that MCF-7 cells when transfected with N-cadherin were 3-4 times more invasive than control untransfected cells. Thus, N-cadherin is shown to stimulate cellular invasion and migration in vitro in Bowden chambers and in vivo in the CAM assay and nude mice (Ossowski, 1988). Based on these findings we chose to explore the mechanism by which N-cadherin promotes metastasis of MCF-7 breast tumor cells.

N-cadherin increases the interaction of MCF-7 cells with stromal cell lines

We tested the ability of N-cadherin to confer interaction of MCF-7 cells with HS578N stromal cells using the method that we published (Hazan et al., 2000). We found that two independent N-cadherin transfected MCF-7 cell lines (N-cad-15) and (N-cad-17) were able to robustly adhere to stromal cells as compared to parental MCF-7 cells (Fig. 1).

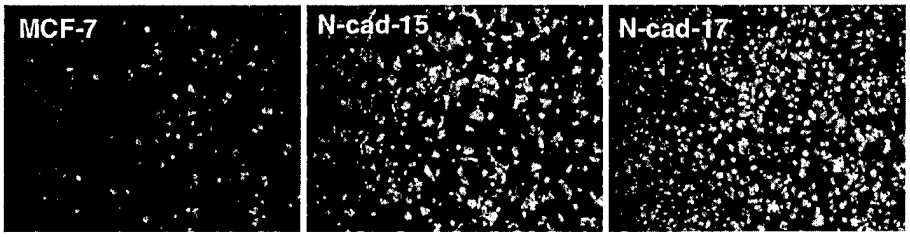

Figure. 1. Adhesion of N-cadherin transfected cells to the stroma. Control MCF-7 cells (left panel), MCF-7 transfected with N-cadherin,N-cad-15 and N-cad-17 (middle and right panels) were labeled with the fluorescent dye,diO and allowed to adhere to a monolayer of unlabelled HS578N breast stromal cells for a 16 hr period. The non-adhesive cells were removed by washing with PBS and bound cells were fixed and visualized by fluorescent microscopy.

N-cadherin and FGFR form a specific signaling complex

FGF-2 stimulated the expression of MMP-9 in MCF-7 breast cancer cells only when these cells were transfected with N-cadherin (Hazan et al., 2000). These results suggested the possibility that the FGFR and N-cadherin form a specific signaling complex at the cell surface which eventually results in the activation of MMP-9 gene transcription. We therefore sought to determine whether other growth factors could stimulate MMP-9 secretion in N-cadherin transfected MCF-7 (MCF-7-N-cad) cells. While FGF-2 elicited a striking MMP-9 response in MCF-7-N-cad cells (Fig. 2, top panel, lane 2), Insulin, EGF, HGF and PDGF (Fig. 3, top panel, lanes 3-6, respectively) did not stimulate MMP expression despite the ability of each of these growth factors to stimulate signaling in MCF-7 cells. Control MCF-7 cells exhibited only low MMP levels in response to any of the growth factors tested (not shown).

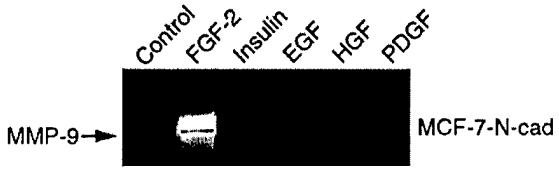

Figure 2. N-cadherin stimulated MMP-9 secretion is FGF-2 specific. MCF-7-N-cad cells were treated for 18 hr with the indicated growth factors at 50 ng/ml and MMP-9 activity was assessed by zymmography. FGF-2 stimulated robust MMP-9 secretion only in MCF-7-N-cad cells and not in control MCF-7-neo cells (not shown)

Sustained MAPK activation leads to MMP-9 gene expression and invasiveness

In light of studies showing a connection between MMP gene transcription and the MAPK-ERK pathway (Westermarck and Kahari, 1999), we examined whether FGF-2-stimulated expression of MMP-9 in N-cadherin expressing cells was accompanied by changes in MAPK activity and whether MAPK phosphorylation was differentially activated by FGF-2 in the presence and absence of N-cadherin. We compared the levels of phosphorylated ERK (P-MAPK) in N-cadherin-expressing MCF-7 cells (MCF-7-N-cad; Fig. 3Aa, c) to those in control MCF-7 cells (MCF-7-neo; Fig. 3Ab, d) in response to increasing concentrations of FGF-2 at two time points, 10 min (Fig 3A, a-b) and 18 hr (Fig. 3A, c-d). As a control, the total levels of ERK (T-MAPK) were also determined for each condition (Fig 3A, right panels). As little as 10 ng/ml FGF-2 stimulated a marked increase in P-MAPK in MCF-7-N-cad cells after 10 min of treatment (Fig 3A a, lane 3). In contrast, control cells required a higher concentration of FGF-2 (50 ng/ml) and still elicited a relatively weaker P-MAPK signal (Fig 3Ab, lane 4). Moreover, while MAPK phosphorylation declined after 18 hr to background levels in control MCF-7-neo cells (Fig 3Ad, lane 4), it persisted over this period in MCF-7-N-cad cells (Fig 3Ac, lanes 3-4). Changes in MAPK activation in response to FGF-2 were due to increased ERK phosphorylation and not to alterations in the total pool of MAPK (T-MAPK) in both cell lines (Fig 3Aa-d, lanes 5-8).

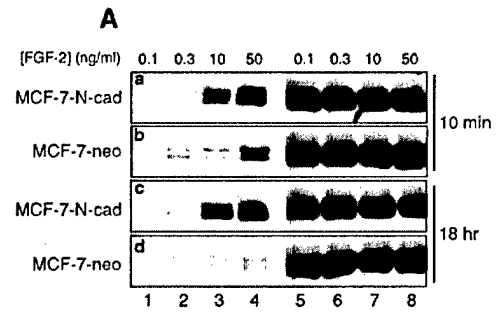

Figure 3 FGF-2 activation of MAPK is enhanced and sustained in the presence of N-cadherin. (A) MCF-7-N-cad (a and c) or MCF-7-neo (b and d) were treated with increasing concentrations of FGF-2 for 10 min (a and b) or 18 hr (c and d) in the presence of 5 μg/ml Heparin. Cell lysates were by immunoblotting using anti-phospho-ERK1/2 antibodies (left panels) or total MAPK (right panels).

We would therefore expect that blockade of either MAPK-ERK or MMP-9 by specific inhibitors should prevent basement-membrane invasion of N-cadherin-expressing MCF-7 cells in response to FGF-2. FGF-2 stimulated the invasion of MCF-7-N-cad cells through Matrigel-coated (Fig. 4 B) as well as across uncoated filters (Fig. 4F and J) relative to cells untreated with growth factor (Fig.4A and E). Treatment of MCF-7-N-cad cells with inhibitors of either MEK1 (40 μM PD 98059; Fig. 4) or MMP-9 (2 μM GM 6001; Fig. 4D) resulted in fewer cells crossing Matrigel-coated filters in response to FGF-2. Interestingly, the inhibition of MEK1 (Fig. 4G) or MMP-9 (Fig.4H) had no effect on the FGF-2 stimulated migration of MCF-7-N-cad cells through uncoated filters.

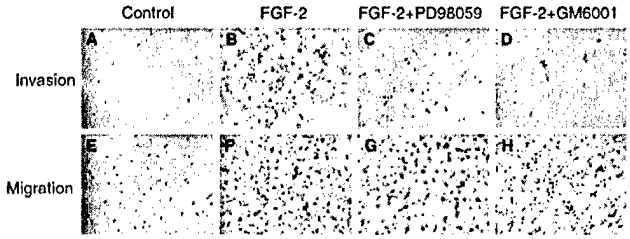

Figure 4. FGF-2 stimulated invasion of MCF-7-N-cad cells is mediated by MAPK-ERK-MMP-9 activation. MCF-7-N-cad cells were untreated (A and E) or treated for 18 hr with 50 ng/ml FGF-2 and 5 μg/ml Heparin (B and F) in the presence of either 40 μM of PD98059 (C and G), 2μM GM6001 (D and H). Cells were assayed for their ability to migrate through 8 μm porous filters, coated with 10 μg Matrigel (invasion; 5A-D) or left uncoated (migration; 5E-H) towards a chemottractant for a 8 hr period. Cells that did not migrate were removed from the upper side to the filters and the migrating cells on the reverse side were stained and photographed.

These results demonstrate that upstream activation of MMP-9 by the MAPK-ERK pathway is tightly associated with the invasive behavior of N-cadherin expressing cells in response to FGF-2. These observations also reveal that invasion and migration are distinct cellular processes, both activated by FGF-2 in the presence of N-cadherin, yet transduced by separate signaling pathways.

N-cadherin protects the FGF receptor from ligand-induced downregulation

To begin elucidate the mechanism underlying the persistent stimulation of MAPK-ERK by FGF-2 in the presence of N-cadherin, we sought to determine whether N-cadherin affects the steady state levels of FGFR-1 after FGF-2 treatment. A Flag-tagged FGFR-1 construct was transiently expressed in L-fibroblast cells (L), which express no known cadherins, or in L-cells which have been stably transfected with N-cadherin (LN), or a non-adhesive N-cadherin mutant (NW2A) in which a critical residue for adhesive activity (Trp-2) was converted to alanine (Tamura et al., 1998). Transfected cells were stimulated for 18 hr with saturating amounts of FGF-2 and the total levels of tagged FGFR were assessed by immunoblotting (Fig. 5A). Similarly, MCF-7 cells expressing either empty vector (MCF-7-neo) or N-cadherin (MCF-7-N-cad) as well as HEK 293T cells, which express endogenously N-cadherin, were transfected with tagged FGFR and subjected to the same analysis. Incubation of L-cells with FGF-2 resulted in the down-regulation of FGFR in L-cells (Fig. 5A, lanes 1-2), consistent with expected ligand-

induced receptor degradation (Sorokin et al., 1994). In contrast, FGF-2 did not cause the down-regulation of FGFR in LN cells, resulting in higher expression levels of FGFR beyond those found in untreated cells (Fig. 5A, lanes 3-4). The non-adhesive mutant N-cadherin NW2A was also able to support FGFR stability as much as the wild-type N-cadherin (Fig. 5A, lanes 5-6), thus suggesting that the adhesive activity of N-cadherin (Tamura et al., 1998), does not contribute to enhancing FGFR expression. The protective effect of N-cadherin was also observed in MCF-7 cells. Increases in FGFR-Flag expression were observed in MCF-7-N-cad cells (Fig. 5A, lanes 7-8), but not in MCF-7-neo cells following FGF-2 treatment (Fig. 5A, lanes 9-10). Finally, FGF-2 treatment of HEK 293T cells, which express endogenous N-cadherin, also led to increases in FGFR-Flag expression (Fig. 5A, lanes 11-12).

N-cadherin did not prevent ligand-induced downregulation of other growth factor receptors such as EGFR, PDGFR or VEGFR. LN cells were transiently transfected with expression vectors for these receptors (Fig. 5B, lanes 1, 3, and 5, respectively). Treatment of cells with cognate growth factors at 50 ng/ml for 18 hr resulted in complete downregulation of each receptor (Fig. 5B, lanes 2, 4, and 6). Thus N-cadherin interferes only with the ligand-induced downregulation of FGFR-1.

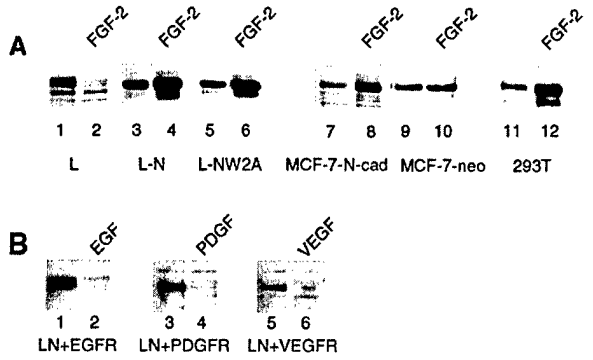

Figure 5. N-cadherin prevents FGF-2 induced FGFR downregulation but not that of other receptors (A) Mouse L-cell fibroblasts (L, lanes 1-2) or L-cells stably transfected with N-cadherin (LN, lanes 3-4) or a non-adhesive N-cadherin mutant (LNW2A, lanes 5-6) were transiently transfected with FGFR-Flag and then treated for 18 hr with or without 100 ng/ml FGF-2 in the presence of 5 μg/ml Heparin. MCF-7-N-cad cells (lanes 7-8), MCF-7-neo (lanes 9-10), or 293T cells (lanes 11-12) were treated similarly. Cells were harvested and FGFR levels were determined by immunoblotting of cell extracts using anti-Flag antibodies. (B) LN cells expressing EGFR, PDGFR, VEGFR were treated without and with ligand for 18hr and the levels of each receptor were determined in cell lysates.

N-cadherin attenuates the FGF-2-induced internalization of the FGF receptor

We next examined the effect of N-cadherin on ligand-induced internalization of FGFR-1. L or LN cells, transfected with FGFR-1, were incubated with saturating amounts of FGF-2 on ice to allow ligand binding in the absence of internalization. Cells were washed of excess FGF-2 and internalization was initiated by incubation at 37^{0}C for various time points. At each time point, remaining surface-bound FGF-2 was stripped from the cell surface, TCA-precipitated, electrophoresed and immunoblotted with an antibody to FGF-2 (Fig. 6A). As shown by densitometric analysis of the immunoblots in Fig. 6A, the kinetics of FGF-2 internalization were much more rapid in L-cells than in LN cells. Fifty percent of FGF-2 was internalized by 7 min in L-cells while LN cells required 30-40 min to internalize 50% of surface-bound FGF-2. FGF-2 was maximally internalized by L-cells (90%) by ~20-30 min, whereas in LN cells, it was only internalized by 50% by ~30-40 min (Fig. 6A).

To determine whether the reduced internalization of FGFR-1 resulted in increased receptor stability, the half-life of FGFR-1 was measured in L and LN cells transiently expressing FGFR-1 after FGF-2 stimulation. Serum-starved cells were treated with 20 μg/ml cycloheximide together with FGF-2 over a 9-hr period and the levels of FGFR-1 were determined by immunoblotting at each indicated time point. As shown in Fig. 6B, the half-life of FGFR-1 in L-cells is ~2 hrs after FGF-2 treatment while in LN cells, the half-life of FGFR-1 is extended to ~6 hrs after FGF-2 stimulation.

To determine whether changes in FGFR protein levels in the presence of N-cadherin result in increased cell surface expression, we performed FACS analysis of endogenous FGFR-1

in L, LN cells and in MCF-7 cells with or without N-cadherin, using an antibody to the extracellular domain of the FGFR-1 (Fig. 6C). FGF-2 caused reduction in cell surface expression of FGFR in L cells and MCF-7 neo cells by a mean value of 30% ±8 and 23% ±9 respectively (Fig. 6C, top left and right panels, respectively). In contrast, the levels of FGFR in N-cadherin expressing cells, LN and MCF-7-N-cad cells (Fig. 6C, bottom left and right panels), were increased by a mean of 50% ±10 and 32% ±10 respectively. These results suggest that N-cadherin prevents the down-regulation of FGF receptors, leading to sustained and enhanced FGFR expression at the cell surface.

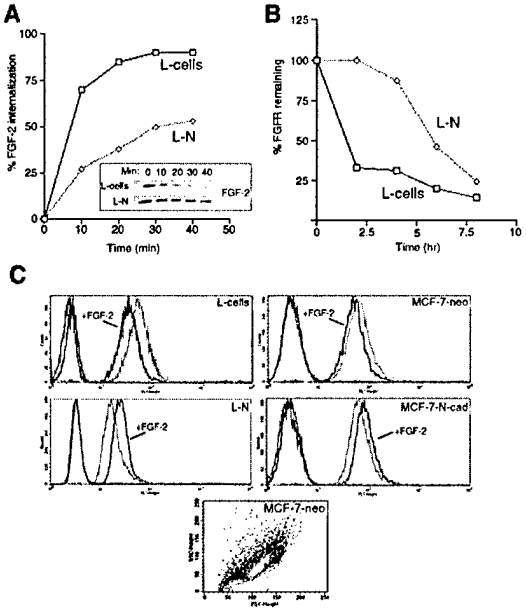

Figure 6. N-cadherin attenuates FGF-2-induced FGFR-1 internalization and degradation. A. Rate of internalization of FGF-2 in L-cells (squares) versus LN cells (diamonds) was determined by cell surface stripping of bound FGF-2 at various times points after saturation with FGF-2. (Inset) Immunoblots showing levels of cell surface stripped FGF-2 at each indicated time point. Graph represent the scanned data from the Inset. **B.** FGF-2 stimulated FGFR-1 degradation in L and LN cells was monitored by cycloheximide chase from 1-9 hrs. FGFR-1 contents in cell lysates was analyzed by immunoblotting and the data scanned and plotted in a graph. **C.** Cell surface expression of FGFR-1 in L and LN was determined before and after treatment with FGF-2 for 18 hrs using FACS analysis. The histogram plots were gated on the window shown on the scatter plot at the bottom of the figure.

In Task 3 of our original grant we proposed to construct chimeric N/E cadherin constructs to evaluate the domains that are required for the effects of invasiveness and metastasis by N-cadherin. Since E-cadherin does not increase invasiveness, then replacement of the active domain in N-cadherin with the analogous one in E-cadherin should render the chimeric molecule inactive for induction of metastasis, migration and invasion. However, we found a simpler and more effective approach to identify the active domain of N-cadherin for invasion. We explored the association of the FGFR with truncated constructs of N-cadherin.

An extracellular complex between N-cadherin and the FGF receptor

To examine the basis for the cooperation between FGFR and N-cadherin in sustaining receptor stability and MAPK-ERK signaling, we examined whether these two proteins form a physical complex. We examined the association of N-cadherin with FGFR-1 in 293T cells, which express endogenous N-cadherin, that were transiently transfected with Flag-tagged FGFR-1. FGFR-Flag immunoreactivity was observed in immunoprecipitates obtained with N-cadherin antisera (Fig.7A, lane 3) but was not found in those with pre-immune sera (Fig. 7A, lane 1). N-cadherin immunoprecipitates from Vector-transfected 293T cells did not show any FGFR-Flag immunoreactivity (Fig.7A, lane 2). We examined whether FGFR and N-cadherin interact via

their extracellular or intracellular moieties. Flag-tagged FGFR-1 extracellular or intracellular domains were transfected into 293T cells and analyzed for their ability to co-precipitate with N-cadherin. The extracellular FGFR-Flag (FGFR-ECD) co-precipitated with N-cadherin with high efficacy (Fig. 7A, lane 4). In contrast, the FGFR-Flag intracellular (FGFR-ICD) was not found in N-cadherin immunoprecipitates (Fig. 7A, lane 5). We mapped the region of N-cadherin that interacts with FGFR in a similar fashion. We found that FGFR co-precipitated only with N-cad-ECD (Fig. 7B, top panel, lane 1) but not with N-cad-ICD (Fig.7B, top panel, lane 2). As a control, ß-catenin, which binds to the intracellular domain of N-cadherin was found complexed to N-cad-ICD (Fig. 7B, bottom panel, lane 2) and not to N-cad-ECD as expected (Fig. 7B, bottom panel, lane 1).

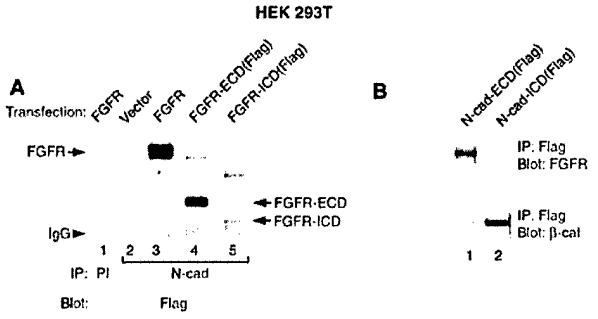

Figure 7 . N-cadherin and the FGFR interact exclusively via the extracellular domain. (A) Transfected Flag-tagged FGFR (lane 3) or FGFR-ECD (lane 4) but not FGFR-ICD (lane 5) co-precipitate with endogenous N-cadherin in HEK 293T. No co-immunoprecipiation was seen with preimmune sera (lane1) or with vector transfected cells (lane2). (B) Similarly, transfected Flag-tagged N-cad-ECD (lane 1) but not N-cad-ICD (lane2) interacts with the FGFR in HEK 293T. As control, N-cad-ICD (lane 2) but not N-cad-ECD (lane 1) co-precipitated well with ß-catenin

Reportable Outcomes

The tasks from the original grant proposal are listed below with comments pertaining to each one.

Task 1.1. Completed.
Task 1.2. Not necessary because the chicken CAM assay was successful using human N-cadherin.
Task 1.3 Completed.
Task 1.4. Completed.
Task 1.5. Completed (Hazan et al., 2000).
Task 1.6. Completed (ref).
Task 1.7. Completed (see Fig. 1 above).

Task 2.1. Completed (Hazan et al., 2000).
Task 2.2. Completed.
Task 2.3. Completed (last year's progress report).
Tasks 2.4-3.5. Since we found that N-cadherin and not E-cadherin co-expression with the FGFR-1 is directly associated with the metastastic behavior of MCF-7 breast tumor cells, we focused our efforts in characterizing the domains on N-cadherin that associate with the FGFR-1. We found an association of the extracellular domain of N-cadherin with the FGFR and not the intracellular domain. This new information has prompted a shift in our research, making the completion of this and subsequent tasks not as important for the understanding of the molecular basis of the N-cadherin induced metastasis. We have delineated the molecular basis for the N-cadherin-based tumor metastasis. This important research has resulted in a major publication in *Cancer Cell* (see below). We will however use this information in the future to build chimeric E/N-cadherin molecules after we characterize the domains of interaction of these molecules with

the FGFR-1. Such information will focus our approach with a more clear idea of expected results and outcomes.

Key Research Accomplishments

-Shown that N-cadherin and FGF-2 synergize to stimulate a robust and sustained phosphorylation/activation of the MAPK/ERK kinase.
-Shown that N-cadherin potentiates the effect of FGF-2 but not of EGF or Insulin in producing ERK1/2 phosphorylation
-Shown that MAPK/ERK activation is responsible for MMP-9 expression and gene transcription
-Shown that N-cadherin stabilizes FGFR expression preventing receptor downregulation and internalization by FGF-2 resulting in chronic expression of FGFR at the cell surface.
-Shown that N-cadherin and the FGFR interact with each other through their extracellular domain.
-Shown that the FGFR interacts with the FGFR through Ig domains 1 and 2 and that this interaction does not involve the HAV motif located within the FGFR Ig domain 2.

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A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor

Kimita Suyama,[2] Irina Shapiro,[2] Mitchell Guttman, and Rachel B. Hazan[1]

Derald H. Ruttenberg Cancer Center, The Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029
[1]Correspondence: rachel.hazan@mssm.edu
[2]These authors contributed equally to this work.

Summary

The intracellular signaling events causing tumor cells to become metastatic are not well understood. N-cadherin and FGF-2 synergistically increase migration, invasion, and secretion of extracellular proteases in breast tumor cells. Here, we define a metastatic signaling cascade activated by N-cadherin and FGF-2. In the presence of N-cadherin, FGF-2 caused sustained activation of the MAPK-ERK pathway, leading to MMP-9 gene transcription and cellular invasion. N-cadherin prevented the FGF receptor (FGFR) from undergoing ligand-induced internalization, resulting in increased FGFR-1 stability. Association of FGFR-1 with N-cadherin was mediated by the first two Ig-like domains of FGFR-1. These results suggest that protection of the FGFR-1 from ligand-induced downregulation by N-cadherin enhances receptor signaling and provides a mechanism by which tumor cells can acquire metastatic properties.

Introduction

Aberrant cellular growth is a primary cause in the development of malignant tumors, but additional events take place which enable tumor cells to invade tissue barriers and metastasize to distant sites. These events include detachment of cells from the primary tumor, the crossing of tissue boundaries, entrance and exit from the circulatory system, the infiltration of distant organs, and the formation of metastatic implants (Liotta et al., 1991).

It has been hypothesized that one of the factors contributing to the escape of nascently metastasizing tumor cells from the primary tumor mass is reduced tumor cell adhesion caused by the loss of the cell adhesion molecule, E-cadherin (for review, see Takeichi, 1993). However, a related adhesion molecule, N-cadherin, is upregulated in invasive cancer cell lines (Hazan et al., 1997; Tran et al., 1999) and tumors (Li et al., 2001; Tomita et al., 2000), and has effects on cellular behavior that are beyond a simple change in adhesive specificity. N-cadherin induced an invasive morphology in squamous tumor cells (Islam et al., 1996) and stimulated migration, invasion (Hazan et al., 2000; Nieman et al., 1999), and metastasis of breast cancer cells (Hazan et al., 2000). The effects of N-cadherin were exacerbated by FGF-2, suggesting that N-cadherin and FGFR-1 synergize to generate signals which alter invasive behavior (Hazan et al., 2000). This possibility was strengthened by the demonstration that secretion of the matrix metalloprotease, MMP-9, was elevated upon FGF-2 treatment of N-cadherin-expressing tumor cells (Hazan et al., 2000).

Similarities between tumor invasion and physiologic responses, such as neurite outgrowth, have been noted (Taguchi et al., 2000). The synergistic activity of FGFR and N-cadherin was shown to generate intracellular signals that promote neuronal growth (Williams et al., 1994; Doherty et al., 2000). The functional cooperativity between the FGFR with N-cadherin in neurite outgrowth was postulated to involve the HAV motif on FGFR and the fourth extracellular domain (EC4) on N-cadherin, based on the inhibitory effect of peptides derived from the HAV domain as well as function-blocking anti-EC4 antibodies on neuronal growth (Williams et al., 2001). The findings from these neurite outgrowth studies were consistent with those of tumor invasion, which showed that the N-cadherin EC4 domain was required for the migration of cells stimulated by N-cadherin (Kim et al., 2000).

Studies have linked the activation of the MAPK-ERK pathway to the transcription of matrix metalloproteases (Westermarck and Kahari, 1999), which are critical mediators of invasive growth (Chang and Werb, 2001; Stetler-Stevenson et al., 1993). MAP kinases are known to play a pivotal role in a diversity of cellular responses, including cell proliferation, differentiation,

SIGNIFICANCE

For tumor cells to metastasize to distant sites, they must detach from the primary tumor. It is thought that reduced cell adhesion, caused by the loss of E-cadherin, initiates tumor invasiveness. However, a related adhesion molecule, N-cadherin, is upregulated in invasive tumors and has consequences on cellular behavior other than a simple change in cellular adhesive specificity. N-cadherin interacts with the FGFR-1 and attenuates its ligand-induced downregulation, resulting in sustained MAPK-ERK activation, MMP-9 gene transcription, and cellular invasiveness. Solving the molecular details of the N-cadherin-FGFR interaction may provide valuable approaches for controlling tumor metastasis.

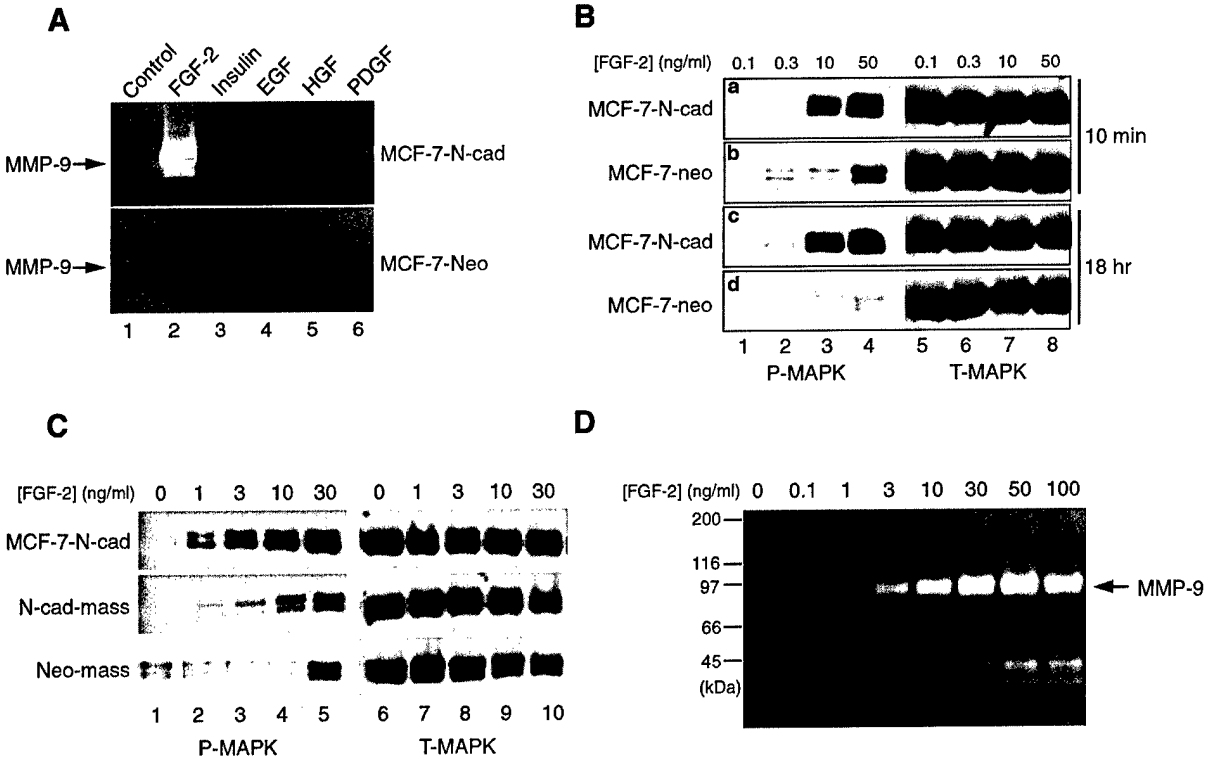

Figure 1. FGF-2-mediated expression of MMP-9 and sustained ERK phosphorylation is dependent on N-cadherin

A: MCF-7-N-cad cells (top) or MCF-7-neo cells (bottom) were treated with the indicated growth factors at 50 ng/ml, and MMP-9 secretion was assessed by zymmography.

B: MCF-7-N-cad (**a** and **c**) or MCF-7-neo (**b** and **d**) were treated with the indicated concentrations of FGF-2, including 5 μg/ml heparin, for 10 min (**a** and **b**) or 18 hr (**c** and **d**). Lysates were electrophoresed and probed with antibodies to phospho-ERK1/2 (P-MAPK, left) or total MAPK (T-MAPK, right).

C: Clonal MCF-7-N-cad cells (top), an aclonal population of stable N-cadherin transfectants (N-cad-mass, middle), or an aclonal population of stable vector transfectants (Neo-mass, bottom) were treated for 10 min with the indicated concentrations of FGF-2 and processed as above.

D: MCF-7-N-cad cells were treated with the indicated concentrations of FGF-2 for 18 hr and MMP-9 production was assessed by zymmography.

senescence, migration, and invasion (Chang and Karin, 2001). One current belief is that differences in the magnitude and/or duration of MAPK-ERK signaling determine signal specificity, thus creating a repertoire of responses (Schaeffer and Weber, 1999). We postulated that the synergy between N-cadherin and FGFR-1 might alter the duration of the MAPK-ERK signal, thus leading to metastasis.

We show that N-cadherin and FGFR-1 cooperate to activate a signaling cascade that results in tumor invasion. N-cadherin associates with the FGFR-1 via the extracellular first two Ig-like domains on the FGFR-1. As a consequence of this interaction, FGFR-1 is not efficiently internalized by FGF-2, causing sustained cell surface expression of FGFR-1, leading to persistent MAPK-ERK activation, MMP-9 expression, and tumor cell invasion.

Results

N-cadherin and FGFR-1 initiate metastatic signals

FGF-2 stimulated the expression of MMP-9 in MCF-7 breast cancer cells when the cells were transfected with N-cadherin (Hazan et al., 2000). We determined whether other growth factors could stimulate MMP-9 secretion in N-cadherin transfected MCF-7 (MCF-7-N-cad) cells. While FGF-2 elicited an MMP-9 response in MCF-7-N-cad cells (Figure 1A, top panel, lane 2), other growth factors, such as insulin, EGF, HGF, and PDGF (Figure 1A, top panel, lanes 3–6, respectively), did not stimulate MMP expression, despite the ability of each of these growth factors to elicit signaling in these cells (see Figure 2A). Control MCF-7 cells (MCF-7-neo) exhibited only low MMP levels in response to any of the growth factors tested (Figure 1A, bottom panel, lanes 1–6).

Sustained MAPK activation leads to MMP-9 gene expression and invasiveness

The transcription of MMP genes depends on MAPK-ERK activity (Westermarck and Kahari, 1999). We examined whether FGF-2 induction of MMP-9 expression in N-cadherin-expressing cells was accompanied by changes in MAPK-ERK activity. The levels of phosphorylated ERK (P-MAPK) in N-cadherin-expressing MCF-7 cells (MCF-7-N-cad; Figures 1Ba and 1Bc) were compared to those in control MCF-7 cells (MCF-7-neo; Figures 1Bb and 1Bd) in response to increasing concentrations of FGF-2 at two time points, 10 min (Figures 1Ba and 1Bb) and 18 hr (Figures 1Bc and 1Bd). As a control, the total levels of ERK (T-MAPK) were determined (Figure 1B, right panels). As little as 10 ng/ml FGF-2 stimulated an increase in P-ERK in MCF-7-N-cad cells after 10 min of treatment (Figure 1Ba, lane 3). Control cells required a higher concentration of FGF-2 (50 ng/ml) and still

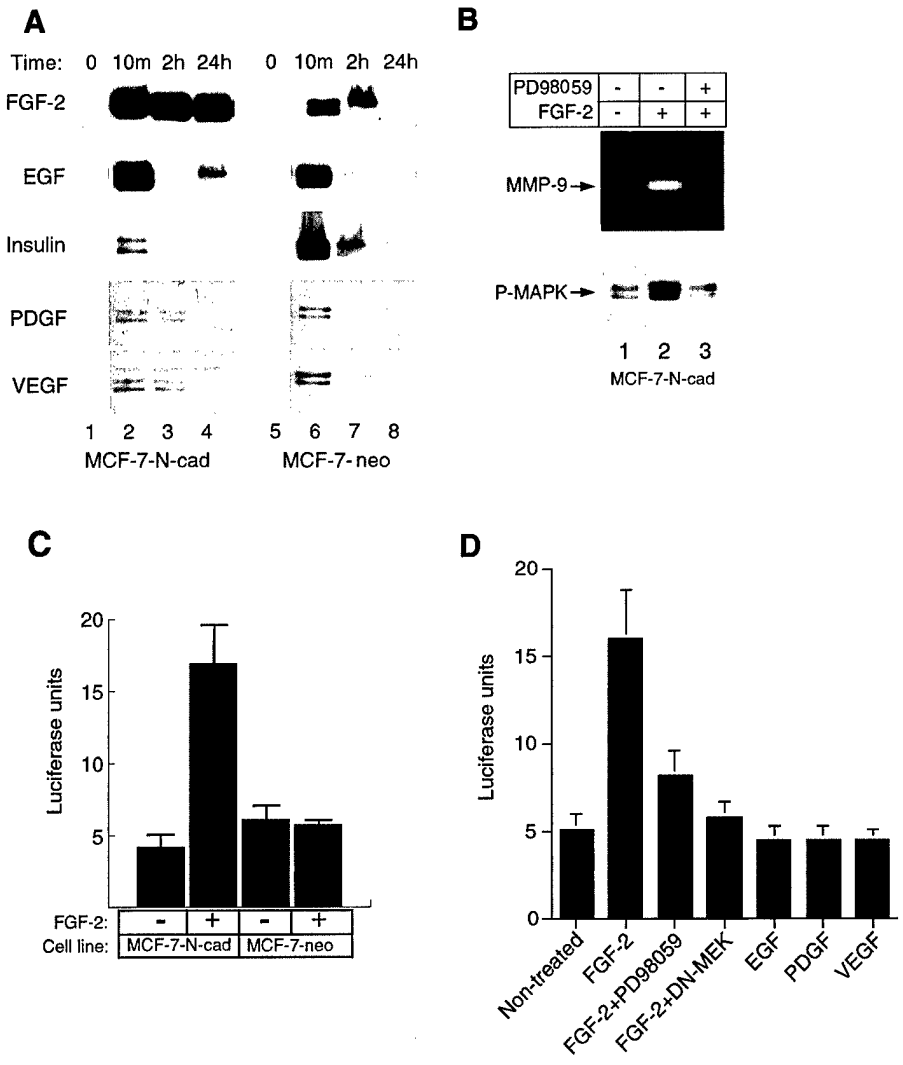

Figure 2. FGF-2, but not other growth factors, synergizes with N-cadherin to promote sustained MAPK-ERK phosphorylation which leads to MMP-9 gene transcription

A: MCF-7-N-cad cells (left) or MCF-7-neo cells (right) were left untreated (lanes 1 and 5) or treated with the indicated growth factors at 50 ng/ml and were lysed at 10 min (lanes 2 and 6), 2 hr (lanes 3 and 7), or 24 hr (lanes 4, and 8). ERK phosphorylation was assayed by immunoblotting.

B: MCF-7-N-cad cells were treated for 18 hr with (lane 3) or without 40 μM PD98059 (lanes 1–2) in the absence (lane 1) or presence (lanes 2–3) of 50 ng/ml FGF-2. MMP-9 activity was assessed by zymmography (top), and ERK phosphorylation was determined (bottom).

C: MCF-7-N-cad or MCF-7-neo cells were treated for 18 hr with or without FGF-2, and the MMP-9 promoter activity was determined using a luciferase reporter construct.

D: MCF-7-N-cad cells were treated with the indicated agents and MMP-9 promoter activity was determined by luciferase assay.

elicited a weaker P-ERK signal (Figure 1Bb, lane 4). MAPK phosphorylation declined after 18 hr to background levels in control MCF-7-neo cells (Figure 1Bd, lane 4), but it persisted in MCF-7-N-cad cells (Figure 1Bc, lanes 3–4). Changes in MAPK activation in response to FGF-2 were not due to alterations in the total pool of MAPK (T-MAPK) in both cell lines (Figures 1Ba–1Bd, lanes 5–8).

N-cadherin sensitized cells to low levels of FGF-2 (1 ng/ml) (Figure 1C, top panel, lane 2). An aclonal population of N-cadherin-transfected MCF-7 cells (N-cad-mass), expressing 10-fold less N-cadherin than MCF-7-N-cad clonal cells (Hazan et al., 2000), was also sensitized to FGF-2 (3–10 ng/ml; Figure 1C, middle panel, lanes 3–4). Control aclonal cultures (Neo-mass) required a higher concentration of FGF-2 (30 ng/ml) to elicit detectable ERK phosphorylation after 10 min of treatment with FGF-2 (Figure 1C, lower panel, lane 5). Total pools of MAPK were not affected by FGF-2 treatment (Figure 1C, lanes 6–10). The rise in MAPK phosphorylation in MCF-7-N-cad cells was accompanied by an increase in MMP-9 secretion in MCF-7-N-cad cells in response to FGF-2 (Figure 1D; compare to Figure 1C, top left panel).

The discrepancy between FGF-2 and other growth factors in inducing MMP-9 expression in N-cadherin-expressing MCF-7 cells was due to differences in the duration of MAPK-ERK activation. FGF-2 caused a persistent phosphorylation of MAPK in MCF-7-N-cad cells starting at 10 min and lasting over 24 hr (Figure 2A, lanes 2–4), while in control cells, FGF-2-induced ERK phosphorylation was lost after only 2 hr treatment with growth factor (Figure 2A, lanes 6–8). In contrast, EGF, Insulin, PDGF, and VEGF stimulated short-term ERK phosphorylation that was of similar duration in both MCF-7-N-cad (Figure 2A, lanes 2–4) and control cells (Figure 2A, lanes 6–8).

We confirmed the involvement of the MAPK-ERK pathway in the regulation of MMP-9 expression using the MEK1 inhibitor, PD98059. MCF-7-N-cad cells were treated with FGF-2 for 18 hr in the presence or absence of 40 μM PD98059. Treatment of MCF-7-N-cad cells with PD98059 blocked the phosphorylation of MAPK-ERK (Figure 2B, lower panel, lane 3), and inhibited MMP-9 production in response to FGF-2 (Figure 2B, top panel, lane 3). Inhibition of another FGF-2-responsive pathway, PLCγ, by U73122 (Wu et al., 2000) did not reduce ERK phosphorylation or MMP-9 expression induced by FGF-2 (not shown).

Increases in MMP-9 levels stimulated by FGF-2 in N-cadherin-expressing MCF-7 cells were due to MMP-9 gene

transcription. We found a 4-fold increase in MMP-9 promoter activity in FGF-2-treated MCF-7-N-cad cells, but not in FGF-2-treated control cells (Figure 2C). Inhibition of ERK by a dominant negative MEK (DN-MEK) or PD 98059 reversed the increase in MMP-9 promoter activation by FGF-2 (Figure 2D). Other growth factors such as EGF, PDGF, or VEGF did not activate the MMP-9 promoter (Figure 2D).

We would expect that activation of the MAPK-ERK pathway by an activated form of MEK1 in control MCF-7 cells would bypass the requirement for N-cadherin and FGFR-1 to stimulate invasion. Retroviral infection of a constitutively active MEK1 (MEKEL) (Habelhah et al., 2001) in MCF-7 cells increased MEK1 levels (Figure 3A, lane 2) and increased MAPK-ERK phosphorylation (Figure 3A, lane 4) as compared to noninfected MCF-7 cells (Figure 3A, lanes 1 and 3). This also led to increased MMP-9 production (Figure 3A, lanes 5–6) as well as enhanced invasion of MCF-7 cells through Matrigel (Figure 3B, right panel), relative to controls (Figure 3B, left panel).

We tested whether blockade of MAPK-ERK or MMP-9 would suppress the invasion of MCF-7-N-cad cells in response to FGF-2 (Figures 3C–3E). FGF-2 stimulated the invasion of MCF-7-N-cad cells through Matrigel-coated (Figure 3Cb) and uncoated filters (Figure 3Cf) relative to untreated cells (Figures 3Ca and 3Ce). Inhibitors of MEK1 (40 μM PD98059; Figure 3Cc) or MMP-9 (2 μM GM6001; Figure 3Cd) caused fewer cells to cross the Matrigel barrier in response to FGF-2. An inactive GM6001 analog did not block the invasion or migration of FGF-2 treated-MCF-7-N-cad cells (Figures 3D and 3E). Interestingly, the inhibition of MEK1 (Figure 3Cg) or MMP-9 (Figure 3Ch) had no effect on the FGF-2-stimulated migration of MCF-7-N-cad cells through uncoated filters.

Endogenous N-cadherin and FGFR-1 expression is associated with sustained MAPK-ERK activation by FGF-2

We compared the kinetics of MAPK-ERK phosphorylation in several tumor cell lines as a function of N-cadherin expression and FGF-2 stimulation. The T47D and ZR-75-1 breast cancer cell lines, the prostate DU145 and LNCap cell lines, and the melanoma cell line 888, which express E-cadherin (Figure 4A, top panel, lanes 1–5) but not N-cadherin (Figure 4A, middle panel, lanes 1–5), were compared to the breast MDA-MB-134, bladder Tsu.Pr-1, and melanoma MeWo tumor cell lines, which express N-cadherin (Figure 4A, middle panel, lanes 6–8) but not E-cadherin (Figure 4A, top panel, lanes 6–8). Most of these cell lines, except for LNCap, contained the FGFR-1 (Figure 4A, bottom panel, lanes 2–8) or FGFR-2 (Figure 4A, bottom panel, lane 1), which was expressed either as one or three protein bands. This could be due to alternative splicing of FGFR-1, often found in tumors (Luqmani et al., 1995), or possibly to degradation. FGF-2 stimulation of E-cadherin-expressing tumor cell lines T47D, ZR-75-1, DU145, LNCap, and 888 resulted in transient phosphorylation of MAPK-ERK after 10 min (Figure 4B, lanes 1–2), which declined after 18 hr (Figure 4B, lane 3). N-cadherin-expressing cell lines, MDA-MB-134, TSU.Pr-1, and MeWo exhibited ERK phosphorylation (Figure 4B, lane 2) that was sustained over 18 hr stimulation with FGF-2 (Figure 4B, lane 3). The total levels of ERK were unaltered by FGF-2 treatment (not shown).

N-cadherin protects the FGF receptor from ligand-induced downregulation

We determined whether N-cadherin affects the steady state levels of FGFR-1 after FGF-2 treatment. A Flag-tagged FGFR-1 construct was transiently expressed in L cells (L), which express no known cadherins, N-cadherin (LN), or a nonadhesive N-cadherin mutant (L-NW2A) (Tamura et al., 1998). Cells were stimulated for 18 hr with FGF-2 and the levels of tagged FGFR-1 were assessed by immunoblotting (Figure 4C). MCF-7 cells expressing either vector (MCF-7-neo) or N-cadherin (MCF-7-N-cad), as well as HEK 293T cells, which express endogenous N-cadherin, were also transfected with tagged FGFR-1 and subjected to the same analysis (Figure 4C).

Incubation of L cells with FGF-2 resulted in downregulation of FGFR-1 (Figure 4C, lanes 1–2), consistent with ligand-induced receptor degradation (Sorokin et al., 1994). In contrast, FGF-2 did not downregulate FGFR-1 in LN cells, resulting in higher levels of FGFR-1 (Figure 4C, lanes 3–4). The mutant N-cadherin NW2A also supported FGFR-1 stability as much as the wild-type N-cadherin (Figure 4C, lanes 5–6), suggesting that the adhesive activity of N-cadherin does not contribute to this effect. Increases in FGFR-1-Flag expression were also observed in MCF-7-N-cad cells (Figure 4C, lanes 7–8), but not in MCF-7-neo cells, following FGF-2 treatment (Figure 4C, lanes 9–10). Finally, FGF-2 treatment of HEK 293T cells also led to increases in FGFR-1-Flag (Figure 4C, lanes 11–12). The endogenous levels of α-tubulin were found to be unchanged by FGF-2 treatment of all the cell lines tested (not shown).

N-cadherin did not prevent ligand-induced downregulation of other growth factor receptors such as EGFR, PDGFR, or VEGFR. LN cells were transiently transfected with expression vectors for these receptors (Figure 4D, lanes 1, 3, and 5, respectively). Treatment of cells with cognate growth factors at 50 ng/ml for 18 hr resulted in complete downregulation of each receptor (Figure 4D, lanes 2, 4, and 6). Thus, N-cadherin interferes only with the ligand-induced downregulation of FGFR-1.

We examined the effect of N-cadherin on ligand-induced internalization of FGFR-1. L or LN cells, transfected with FGFR-1, were incubated with saturating amounts of FGF-2 on ice to allow ligand binding in the absence of internalization. Cells were washed of excess FGF-2 and internalization was initiated by incubation at 37°C for various time points. At each time point, remaining surface-bound FGF-2 was stripped from the cell surface, TCA-precipitated, electrophoresed, and immunoblotted with an antibody to FGF-2 (Figure 5A). As shown by densitometric analysis of the immunoblots in Figure 5A, the kinetics of FGF-2 internalization were much more rapid in L cells than in LN cells. While 50% of FGF-2 was internalized by 7 min in L cells, it required a much longer period (30–40 min) for LN cells to internalize FGF-2 to the same extent. Moreover, while L cells cleared almost all of the FGF-2 (90%) from the cell surface by ~20–30 min, LN cells still kept ~50% of their FGF-2 on the cell surface by 30–40 min after the start of internalization (Figure 5A).

To determine whether the reduced internalization of FGFR-1 resulted in increased receptor stability, the half-life of FGFR-1 was measured in L and LN cells transiently expressing FGFR-1 after FGF-2 stimulation. Serum-starved cells were treated with 20 μg/ml cycloheximide together with FGF-2 over a 9 hr period, and the levels of FGFR-1 were determined by immunoblotting at each indicated time point. As shown in Figure 5B, the half-

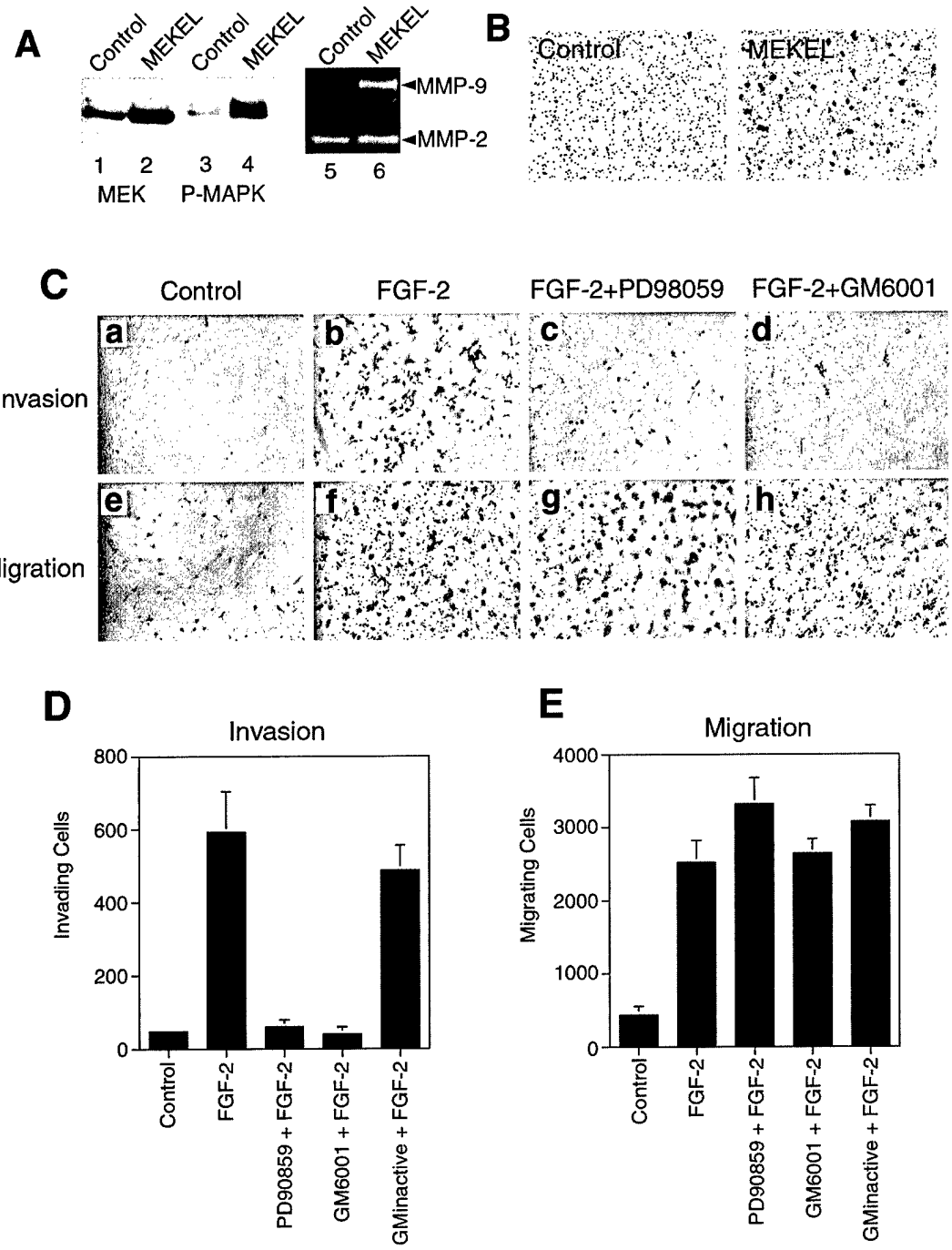

Figure 3. FGF-2 cooperates with N-cadherin in a MAPK-ERK and MMP-9 dependent fashion to promote invasion but not migration

A: In the absence of FGF-2 or N-cadherin (control MCF-7 cells), retroviral infection of a constitutively active MEK1 (MEKEL) caused increased levels of MEK1 (lane 2) and P-ERK (lane 4) relative to uninfected cells (lanes 1 and 3). MEKEL also caused an increase in MMP-9 expression (lane 6) as shown by zymmography relative to controls (lane 5).

B: Increased invasion as a result of MEKEL (right panel) relative to control invasion (left panel).

C: MCF-7-N-cad cells were untreated (**a** and **e**) or treated for 18 hr with 50 ng/ml FGF-2 and 5 μg/ml heparin (**b** and **f**) in the presence of either 40 μM of PD98059 (**c** and **g**), 2 μM GM6001 (**d** and **h**), or 2 μM of an inactive GM6001 analog (**D** and **E**). Cells were assayed for their ability to migrate through 8 μm porous filters, coated with Matrigel (invasion; **a–d**) or left uncoated (migration; **e–h**), toward a chemottractant for a 8 hr period.

(**D** and **E**) Quantification of invasion and migration assay. Cells were counted in triplicate wells and in five identical experiments. The bars in **D** and **E** represent mean ± SD.

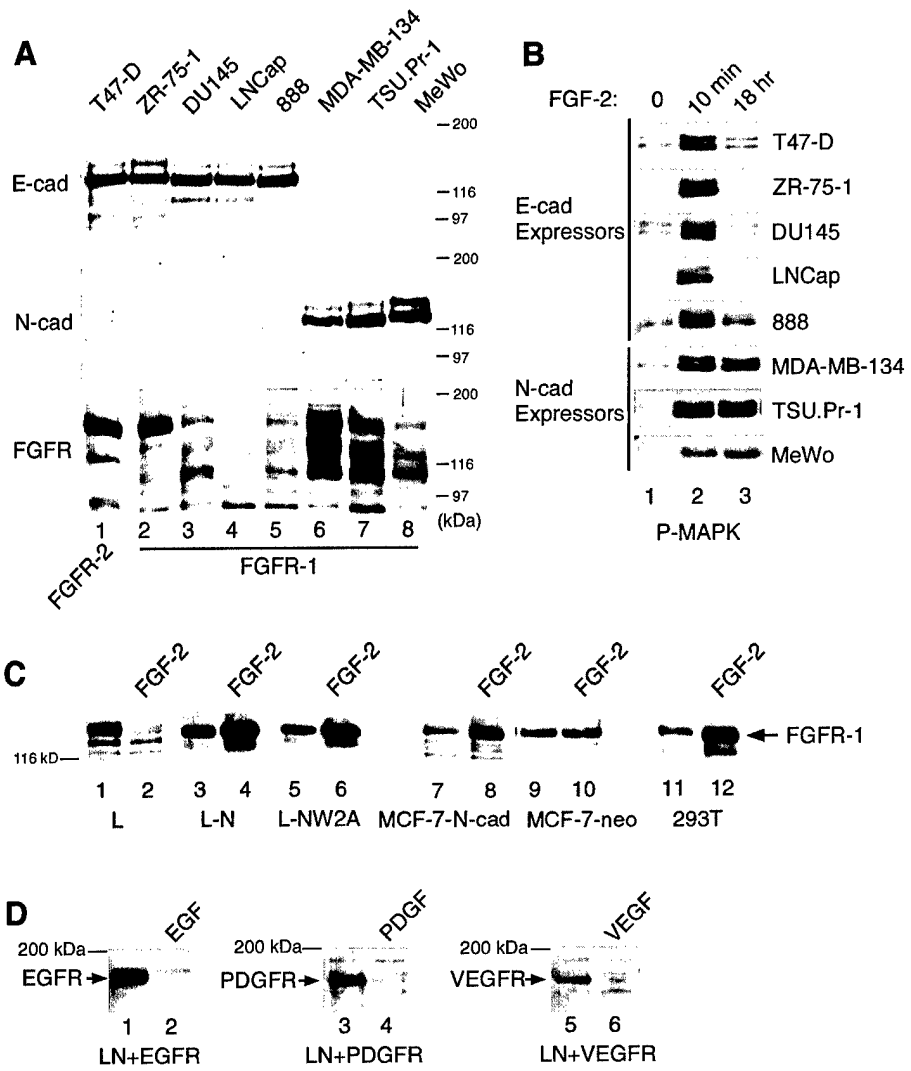

Figure 4. Sustained ERK activation and FGFR-1 stabilization by FGF-2 correlates with N-cadherin expression

A: The breast T47D, ZR-75-1, MDA-MB-134, the prostate DU145, LNCap, the bladder TSU.Pr-1, and melanoma 888 and MeWo tumor cell lines were evaluated by immunoblot for the expression of E-cadherin (top panel), N-cadherin (middle panel), and FGFR-1 or 2 (bottom panel). FGFR-1 was present in most cell lines as either one or three protein bands.

B: ERK phosphorylation was sustained only in the N-cadherin-positive MDA-MB-134, TSU.Pr-1, and MeWo cell lines over 18 hr stimulation with FGF-2 (lane 3). The E-cadherin-expressing cell lines (T47D, ZR-75-1, DU145, LNCap, and 888) exhibited only a transient ERK activation after FGF-2 treatment. In these cells, ERK phosphorylation was reduced after 18 hr (lane 3) relative to 10 min (lane 2) of treatment with FGF-2. Total MAPK levels were determined in each condition and found unchanged by FGF-2 treatment (not shown).

C: Mouse L cell fibroblasts (L, lanes 1–2) or L cells stably transfected with N-cadherin (LN, lanes 3–4) or a nonadhesive N-cadherin mutant (LNW2A, lanes 5–6) were transiently transfected with FGFR-1-Flag and then treated for 18 hr with or without 100 ng/ml FGF-2. MCF-7-N-cad cells (lanes 7–8), MCF-7-neo cells (lanes 9–10), or 293T cells (lanes 11–12) were treated similarly. Cells were harvested and FGFR-1 levels were determined by immunoblotting of cell extracts using anti-Flag antibodies.

D: EGFR, PDGFRα, or VEGFR expression constructs were transiently expressed in LN cells and treated (lanes 2, 4, and 6) with cognate growth factors or left untreated (lanes 1, 3, and 5). Levels of receptors before and after treatment with ligand were evaluated by immunoblotting with specific antibodies. As loading controls, α-tubulin levels were assessed in each sample from **C** and **D** and found to be unaltered by ligand treatment (not shown). Molecular weigths (kDa) are indicated.

life of FGFR-1 in L cells is ~2 hr after FGF-2 treatment, while in LN cells, the half-life of FGFR-1 is extended to ~6 hr after FGF-2 stimulation.

We performed FACS analysis of endogenous FGFR-1 in L cells, LN cells, and MCF-7 cells with or without N-cadherin, using an antibody to the extracellular domain of the FGFR-1 (Figure 5C). FGF-2 caused reduction in cell surface expression of FGFR-1 in L cells and MCF-7-neo cells by a mean value of 30% ±8 and 23% ±9, respectively (Figure 5C, top left and right panels, respectively). In contrast, the levels of FGFR-1 in N-cadherin-expressing cells and LN and MCF-7-N-cad cells (Figure 5C, bottom left and right panels) were increased by a mean of 50% ±10 and 32% ±10 respectively. We therefore conclude that N-cadherin prevents the FGFR-1 from undergoing internalization, thus resulting in increased receptor expression at the cell surface and enhanced downstream signaling.

N-cadherin and the FGF receptor form a complex

To examine how N-cadherin promotes FGFR-1 stability, we determined whether the two proteins form a physical complex. We examined the association of endogenous N-cadherin with FGFR-1 in 293T cells that transiently expressed Flag-tagged FGFR-1. FGFR-1-Flag immunoreactivity was observed in immunoprecipitates with N-cadherin antisera (Figure 6A, lane 3), but not in those with preimmune sera (Figure 6A, lane1). N-cadherin immunoprecipitates from vector-transfected 293T cells did not show any FGFR-1-Flag immunoreactivity (Figure 6A, lane 2). We also examined the interaction of N-cadherin with FGFR-2 in HEK 293T and found similar coprecipitation of N-cadherin with FGFR-2 as with FGFR-1 (not shown).

The association of N-cadherin with FGFR-1 was similarly tested in N-cadherin-expressing L cells and found to be greater in FGF-2-treated than untreated cells (Figure 6B, lanes 1–2). FGF-2 remained bound to the N-cadherin-associated FGFR-1 as indicated by the reactivity of an anti-FGF-2 antibody with N-cadherin immunoprecipitates (Figure 6C, lanes 1–2). The increase in FGFR-1 expression in FGF-2-treated LN cells is associated with higher levels of phosphorylated FGFR-1 (Figure 6D, lanes 1–2), suggesting that N-cadherin stabilizes both FGFR-1 expression and function.

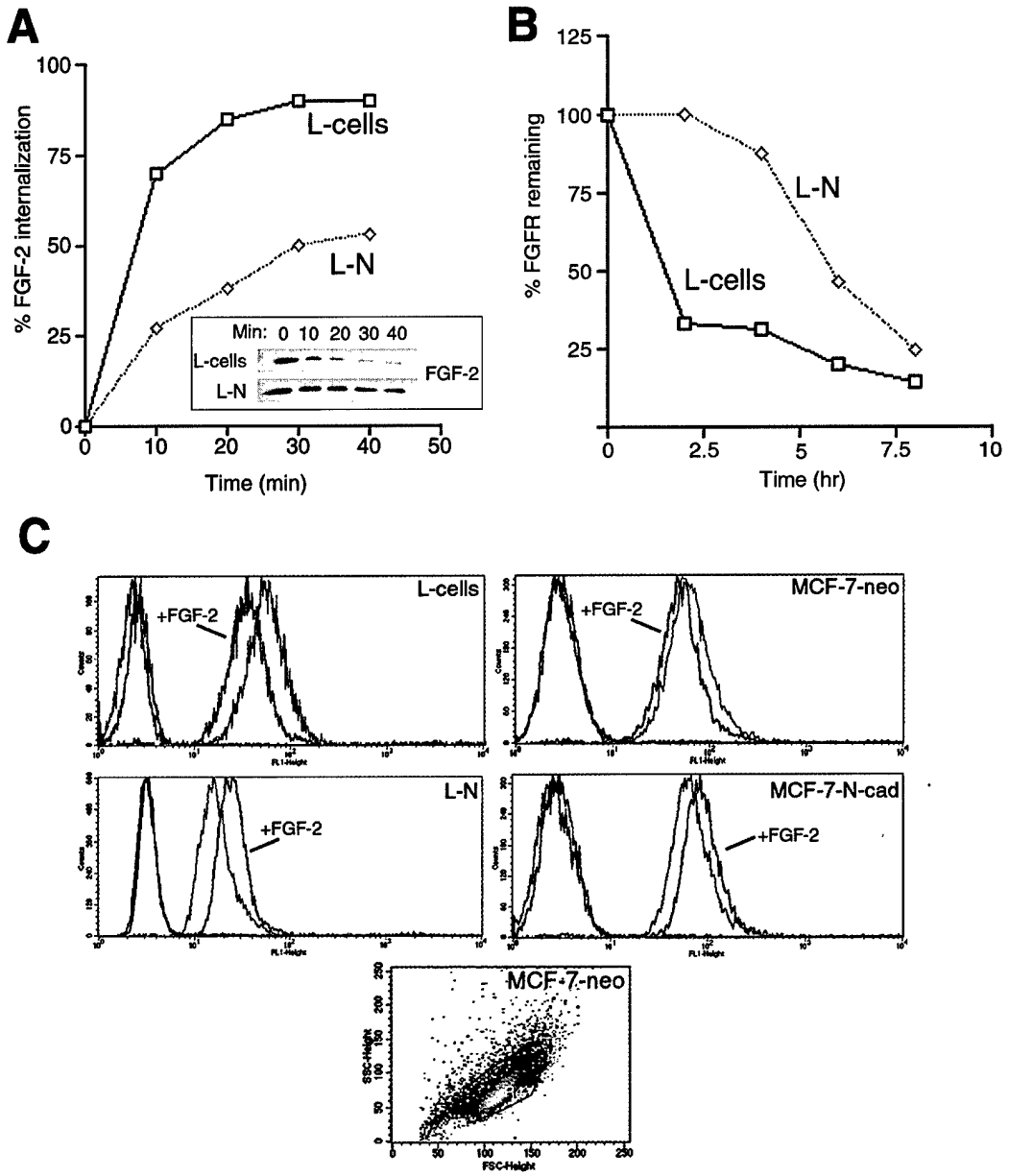

Figure 5. N-cadherin attenuates FGF-2-induced FGFR-1 internalization and degradation and results in chronic FGFR-1 expression at the cell surface

A: Rate of internalization of FGF-2 in L cells (squares) versus LN cells (diamonds) as determined by cell surface stripping of bound FGF-2 at various time points after treatment (see Experimental Procedures). (Inset) Immunoblots of cell surface stripped FGF-2 at each time point. Blots were scanned and FGF-2 levels were quantified by densitometry and values expressed as the percent of FGF-2 before transfer of cells to 37°C.

B: The effect of N-cadherin on FGF-2-induced degradation of FGFR-1 was measured by treatment of L and LN cells transfected with FGFR-1 with 20 μg/ml cycloheximide and 100 n/ml FGF-2 including 5 μg/ml heparin for 1 to 9 hr. Cell lysates were harvested and analyzed for FGFR-1 contents by immunoblotting. Data were normalized to the values of endogenous α-tubulin and expressed as the percent of the levels of FGFR-1 before addition of FGF-2 (time 0).

C: Endogenous expression of cell surface-associated FGFR-1 was analyzed by flow cytometry for the binding of an anti-FGFR-1 antibody recognizing the extracellular domain of FGFR-1. As a negative control, same cells were similarly analyzed for reactivity with preimmune antibody. The histogram plots were gated on the window shown on the scatter plot at the bottom of the figure.

N-cadherin did not interact with other receptor tyrosine kinases. Receptor-specific immunoprecipitates from LN cells expressing FGFR-1 were compared to those expressing the PDGFR or VEGFR (Figure 6E). While N-cadherin is found at high levels in FGFR-1 immunoprecipitates (Figure 6E, lane 2), it fails to precipitate with either the PDGFR or VEGFR (Figure 6E, lanes 4 and 6; respectively), despite efficient pull down of these receptors by their respective antibodies (Figure 6E, lanes 3 and 5; respectively).

It remained important to examine whether the interaction of N-cadherin with FGFR-1 occurs in vivo in tumor cell lines which endogenously express these molecules. FGFR-1 immunopre-

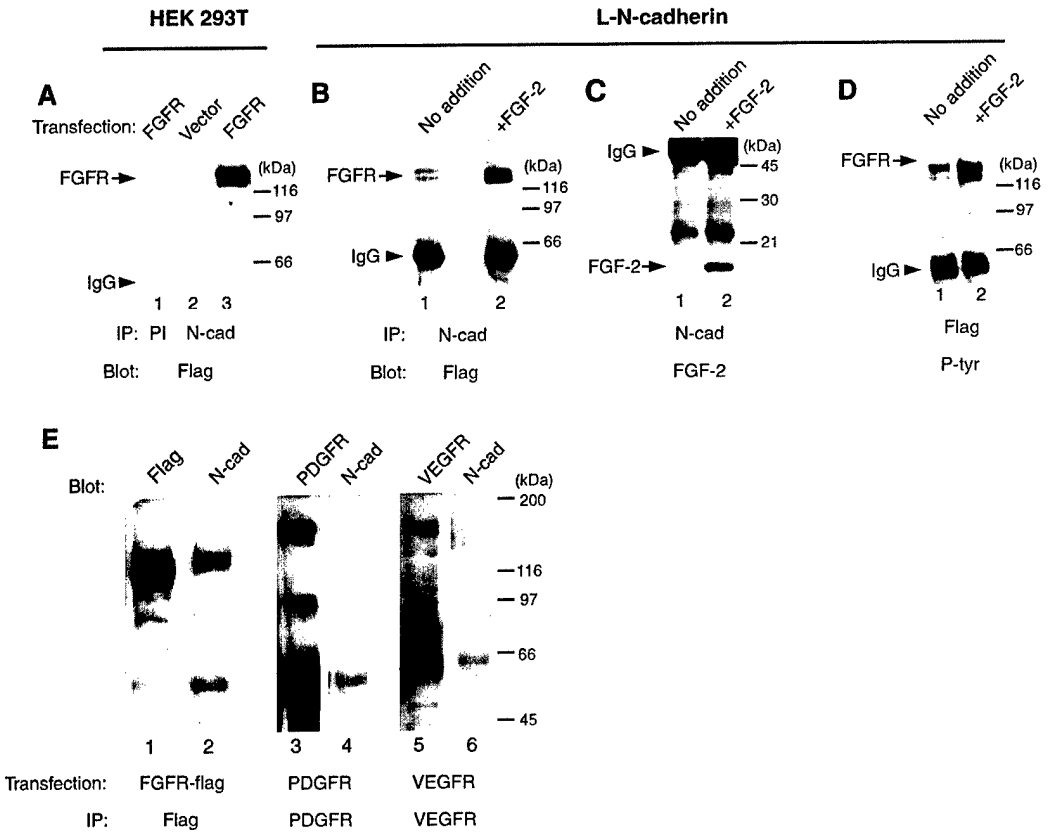

Figure 6. The FGFR-1 and N-cadherin form a complex that is increased upon FGF-2 treatment

A: 293T cells were transfected with full length FGFR-1-Flag (lanes 1 and 3) or an empty vector (lane 2). Cells were lysed, N-cadherin was immunoprecipitated, and the immunoprecipitates were electrophoresed and probed with an anti-Flag antibody (lanes 1–3). Control immunoprecipitates were carried out with preimmune serum (lane 1).

B: N-cadherin-expressing L cells (LN) were transfected with FGFR-1-Flag, serum-starved for 4 hr, and then treated for 18 hr with 100 ng/ml FGF-2 and 5 μg/ml heparin (lane 2) or untreated (lane 1) and N-cadherin immunoprecipitates were probed with anti-Flag antibodies.

C: Immunoprecipitates were performed as in **B** and were probed with anti-FGF-2 antibodies.

D: Immunoprecipitates were performed using an anti-Flag antibody to pull down tagged FGFR-1 from LN cells and were probed with anti-phosphotyrosine antibodies.

E: The FGFR-1-Flag, PDGFR, or VEGFR constructs were transfected into LN cells, immunoprecipitated with specific receptor antibodies, immunoblotted with anti-N-cadherin antibodies (lanes 2, 4, and 6), stripped, and reprobed with anti-receptor antibodies (lanes 1, 3, and 5). Molecular weights are indicated.

cipitates from the tumor cells that express N-cadherin; MDA-MB-134, TSU.Pr-1, and MeWo exhibited strong reactivity with N-cadherin (Figure 7A, top panel, lanes 2, 4, and 6). In contrast, FGFR-1 immunoprecipitates from cells lacking N-cadherin, such as ZR-75-1, DU145, or 888, did not show any background reactivity with N-cadherin (see Supplemental Data at http://www.cancercell.org/cgi/content/full/2/4/301/DC1). FGFR-1 was efficiently precipitated in all cell lines (Figure 7A, bottom panel, lanes 2, 4, and 6). Control IgGs did not immunoprecipitate the FGFR-1 or N-cadherin in any of the cell lines tested (Figure 7A, lanes 1, 3, and 5).

We tested whether endogenous complex formation between N-cadherin and the FGFR-1 is associated with invasive capacity and MMP-9 production. The N-cadherin-expressing cell line, TSU.Pr-1, exhibited an increase in Matrigel invasion after FGF-2 stimulation, which was reduced by treatment with the MEK1 inhibitor PD98059 (Figure 7B, left panel). Accordingly, FGF-2 also caused an increase in MMP-9 expression (Figure 7C, lanes 4–5), which was reversed by PD98059 (Figure 7C, lane 6). MMP-2 expression was unaffected by the same treatment (Figure 7C, lanes 4–6). Thus, the results with the TSU.Pr-1 cells are consistent with our findings with MCF-7 breast cancer cells.

A similar analysis of the N-cadherin-expressing MeWo cells shows that they invade Matrigel at high rate in the absence of FGF-2 and do not display enhanced invasion in response to FGF-2 (Figure 7B, right panel). Accordingly, these cells secrete high amounts of MMP-2 in the absence or presence of FGF-2 (Figure 7C, lanes 7–8). PD98059 did not reduce the invasion of MeWo cells (Figure 7B), although it weakened the MMP2 production of these cells (Figure 7C, lane 9). These results are in agreement with published data, showing that MeWo cell invasion depends on the p38-MAPK pathway (Denkert et al., 2002). We speculate that the N-cadherin/FGFR-1/ERK pathway is not the predominant pathway for the invasion of these cells.

We found that the MDA-MB-134 breast carcinoma cell line did not exhibit any quantifiable invasiveness in Matrigel before or after FGF-2 treatment (not shown). This is consistent with the lack of MMP-9 induction by FGF-2 (Figure 7C, lanes 1–2).

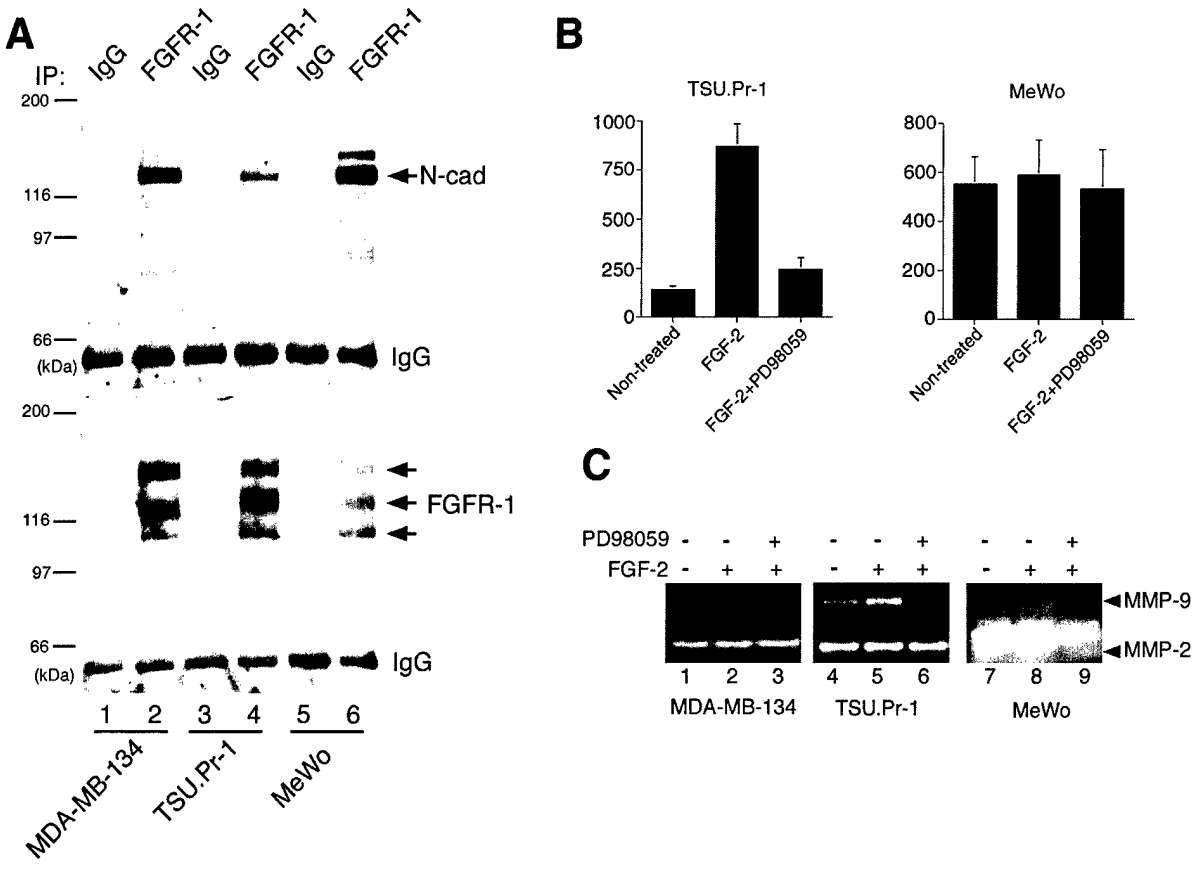

Figure 7. Endogenous complex formation between N-cadherin and FGFR-1 can be associated with increased invasion of tumor cell lines

A: 3 mg lysate from N-cadherin expressing breast (MDA-MB-134), bladder (Tsu.Pr-1), or melanoma (MeWo) cell lines were immunoprecipitated with anti-FGFR-1 rabbit antibodies (lanes 2, 4, and 6) or with normal rabbit IgG (lanes 1, 3, and 5). Immunoprecipitates were electrophoresed and probed with anti-N-cadherin (top panel) or stripped and probed with anti-FGFR-1 (bottom panel).

B: Matrigel invasion of TSU.Pr-1 and MeWo cells in response to FGF-2 with or without PD98059.

C: Zymmogenic activity of MDA-MB-134 (lanes 1–3), TSU.Pr-1 (lanes 4–6), or MeWo cells (lanes 7–9) in response to 18 hr treatment with 100 ng/ml FGF-2 in the absence or presence of 40 μM PD98059.

This suggests that although MDA-MB-134 cells express N-cadherin and FGFR-1 and exhibit persistent MAPK-ERK activation by FGF-2, they may be deficient in signaling pathways that converge with MAPK-ERK, or in downstream effectors of ERK, to produce the invasive response.

An extracellular complex between N-cadherin and the FGF receptor

We examined whether N-cadherin and the FGFR-1 interact via their extracellular or intracellular moieties. Flag-tagged FGFR-1 extracellular or intracellular domains were transfected into 293T cells and analyzed for their ability to coprecipitate with endogenous N-cadherin. The extracellular FGFR-1-Flag (FGFR-ECD) coprecipitated with N-cadherin with high efficacy (Figure 8A, lane 1). In contrast, the FGFR-1-Flag intracellular domain (FGFR-ICD) was not found in N-cadherin immunoprecipitates (Figure 8A, lane 2). All cells expressed the transfected FGFR-1 constructs at similar levels and all reactions contained similar amounts of immunoprecipitated N-cadherin (not shown).

We mapped the region of N-cadherin that interacts with FGFR-1 in a similar fashion. We cotransfected Flag-tagged extracellular or intracellular N-cadherin domains with untagged FGFR-1 in 293T cells and isolated the resulting complexes by immunoprecipitation with anti-Flag antibodies. FGFR-1 coprecipitated only with N-cad-ECD (Figure 8B, top panel, lane 1) but not with N-cad-ICD (Figure 8B, top panel, lane 2). β-catenin, which binds to the intracellular domain of N-cadherin, was found complexed to N-cad-ICD as expected (Figure 8B, bottom panel, lane 2). N-cad-ECD did not coprecipitate with β-catenin (Figure 8B, bottom panel, lane 1).

To determine which sites on the extracellular domain of FGFR-1 interact with N-cadherin, we constructed deletions of each of the three extracellular Ig-like domains of FGFR-1 (Figure 8C). Since the HAV motif within the second Ig-like domain (D2) of FGFR-1 has been postulated to be required for the interaction of FGFR-1 with N-cadherin (Doherty and Walsh, 1996; Williams et al., 1994), we tested the effect of HAV mutagenesis, replacing Ala 146 with Glu, on the interaction of FGFR-1 with N-cadherin. Mutating HAV to HEV did not prevent the coprecipitation of the FGFR-ECD with N-cadherin in 293T cells (Figure 8D, lane 3

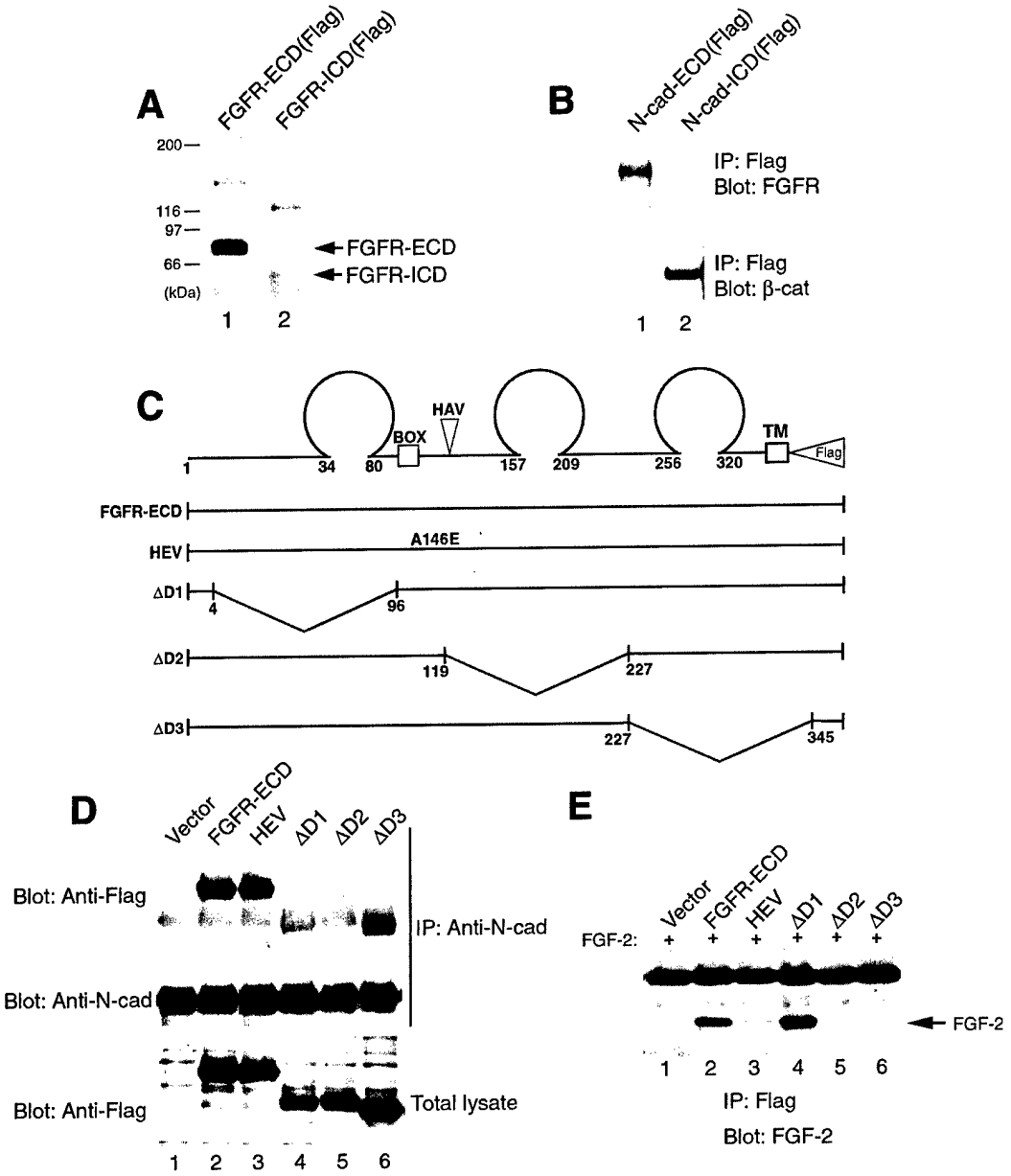

Figure 8. FGFR-1 interacts with N-cadherin extracellularly, through the Ig domains 1 and 2 of the FGFR-1 extracellular domain

A: An FGFR-1-Flag extracellular domain construct (FGFR-ECD; lane 1) or an FGFR-1-Flag intracellular domain construct (FGFR-ICD; lane 2) was transfected into 293T cells and analyzed for its ability to coprecipitate with endogenous N-cadherin.

B: 293T cells were cotransfected with N-cad-ECD-Flag (lane 1) or N-cad-ICD-Flag (lane 2) and an untagged FGFR-1 construct. Flag immunoprecipitates were electrophoresed and probed with anti-FGFR-1 antibodies (top) or β-catenin (bottom) antibodies.

C: Schematic diagram of the FGFR-ECD Flag deletion constructs. Residues are numbered beginning with the first residue of the mature protein minus the signal sequence.

D: Each FGFR-ECD Flag deletion construct was transfected into 293T cells and analyzed for its ability to coprecipitate with the endogenous N-cadherin in these cells. N-cadherin immunoprecipitates were probed with anti-Flag (top panel) or with anti-N-cadherin (middle panel). Starting lysates were probed with anti-Flag to verify expression levels.

E: FGFR-ECD Flag deletion constructs were analyzed for their ability to bind FGF-2 after transfection into 293T cells. Detergent extracts from cells treated or not treated with 100 ng/ml FGF-2 in the absence of heparin for 18 hr were immunoprecipitated with anti-Flag antibodies and probed with an anti-FGF-2 antibody.

compared to lane 2). As expected, the HAV-mutated FGFR-1 did not bind FGF-2 (Plotnikov et al., 2000) (Figure 8E, lane 3). Thus, the HAV motif in FGFR-1 is not critical for the interaction of FGFR-1 with N-cadherin. In contrast, deletion of the first FGFR-1 Ig-like domain (D1) abolished the coprecipitation of FGFR-ECD-Flag with N-cadherin (Figure 8D, lane 4). Deletion of domain 2 (D2) also inhibited the coprecipitation of FGFR-ECD with N-cadherin (Figure 8D, lane 5), but deletion of domain

3 (D3) did not prevent FGFR-ECD from associating with N-cadherin (Figure 8D, lane 6), suggesting that D3 is not involved in the recognition of N-cadherin by FGFR-1. Thus, the interaction of FGFR-1 with N-cadherin requires both the D1 and D2 domains.

To control for the correct folding of the various FGFR-ECD deletion constructs, we tested their ability to bind ligand after transfection into 293T cells (Figure 8E). ΔD1, as expected, bound FGF-2 (Figure 8E, lane 4) with even greater efficacy than the FGFR-ECD (Figure 8E, lane 2), while ΔD2 or ΔD3 were incapable of ligand binding (Figure 8E, lanes 5 and 6), consistent with the requirement of both of these domains for ligand interaction (Plotnikov et al., 2000). All of these constructs were efficiently transported to the cell surface after transfection, as determined by anti-Flag immunofluorescence (not shown).

Discussion

We have shown that N-cadherin can promote invasiveness of tumor cells by a mechanism involving more than a change in cellular adhesion. A cascade of signaling events activated by FGF-2 and dependent on N-cadherin results in expression of extracellular proteases as well as cellular invasion. N-cadherin associates with the FGFR-1 at the cell surface and attenuates ligand-induced receptor downregulation, leading to increased FGFR-1 stability, persistent expression of FGFR-1 at the cell surface, sustained MAPK-ERK activation, MMP-9 gene expression, and tumor cell invasion.

The difference between FGF-2 and other growth factors in inducing MMP-9 expression lies in the ability of FGF-2 to activate sustained MAPK in the presence of N-cadherin. These results are consistent with evidence suggesting that the time course of signaling by ligand-activated receptors determines both amplitude and specificity of the output (Marshall, 1995; Simon, 2000). For instance, PC12 cells proliferate when treated with EGF and differentiate in response to NGF. Both events require MAPK phosphorylation, but with differential kinetics. Namely, while EGF causes a transient activation of ERK2, NGF stimulates sustained ERK2 phosphorylation (Marshall, 1995). Similarly, differential activation of MAPK by pheromones in yeast discriminates between mating and invasive growth as a result of transient versus persistent activation of the ERK kinase homolog (Sabbagh et al., 2001). Thus, while different stimuli may lead to MAPK phosphorylation, differences in the duration of the signal can result in diversity of cellular programs (Marshall, 1995; Schaeffer and Weber, 1999). Others have also shown that growth factor stimulation of invasive growth was promoted by increased and sustained MAPK in breast tumor cells (Krueger et al., 2001). While we have focused on the MAPK-ERK pathway, we do not exclude the involvement of other MAP kinases such as the p38 and JNK stress-induced cascades in cancer invasion, of which the latter was shown to influence cell motility in response to cytokines (Xia et al., 2000).

Exposure of cells or tissues to growth factors leads to the internalization of receptors by a clathrin-dependent mechanism and to receptor degradation by the endocytic pathway (Ceresa and Schmid, 2000). Our study suggests that N-cadherin enhances FGFR-1 signaling by attenuating ligand-induced internalization. In support of our findings, others have suggested that MAPK activity could be sustained as a result of reduced internalization or overexpression of cell surface receptors (Marshall, 1995). The mechanism whereby N-cadherin inhibits FGFR-1 internalization remains to be determined. One possibility is that N-cadherin perturbs the assembly of clathrin-adaptor complexes with FGFR-1, thus preventing the endocytosis of ligand-occupied FGFR into the clathrin-coated vesicles.

We found that the FGF receptor interacts extracellularly with N-cadherin via its first two Ig-like domains, D1 and D2. In contrast, D3 does not appear to be involved in the association of N-cadherin with FGFR-1. It is thought that D1 of FGFR-1 interacts intramolecularly with the ligand binding sites in D2 and D3 and interferes with FGF binding (Plotnikov et al., 1999). Thus, binding of N-cadherin to D1 and D2 may prevent intramolecular association of these domains, relieving FGFR-1 autoinhibition, and resulting in enhanced sensitivity to ligand. In support of this model, MAPK-ERK activation occurred at substantially lower levels of FGF-2 in cells expressing N-cadherin than in control cells. The importance of D1 in modulating FGFR-1 function is underscored by the fact that D1 deletions have been observed in pancreatic and squamous tumors as a result of alternative FGFR-1 mRNA splicing, giving a possible selective advantage to tumors (Kobrin et al., 1993; Yamaguchi et al., 1994). We thus hypothesize that tumor cells may acquire N-cadherin as an alternative or additional mechanism to reverse the negative effect of D1 on FGFR-1 and enhance receptor outputs.

The interaction between N-cadherin and FGFR-1 has been suggested to be mediated by the HAV motif within D2 of FGFR-1 and the extracellular domain 4 (EC4) of N-cadherin (Kim et al., 2000; Williams et al., 2001). We found that mutagenesis of HAV to HEV did not disrupt the physical association of FGFR-1 with N-cadherin, suggesting that the HAV in FGFR-1 may not be required for the functional cooperation of these molecules. In support of our finding, the crystal structure of FGFR-1 indicates that the HAV tri-peptide may not engage in protein interaction, since it is buried in a hydrophobic pocket (Plotnikov et al., 1999). Since neither D1 or D2 alone were capable of recognizing N-cadherin, we propose that a larger site on the FGFR-1, contributed by D1 and D2, interacts with the EC4 domain of N-cadherin.

While we emphasize the critical role played by the MAPK-ERK pathway in breast cancer invasion in response to N-cadherin and FGF-2, we do not rule out other signaling pathways that may contribute to or prevent metastasis. Blockade of MAPK-ERK signaling in N-cadherin expressing cells resulted in suppressing the FGF-2-induced invasion and MMP-9 expression, but not migration, suggesting that a pathway other than ERK might be transducing the motility of cells in response to FGF-2. Consistent with this hypothesis, the FGFR has been shown to stimulate the PI3-kinase pathway, known to play a role in cell motility (Sotsios and Ward, 2000; Wu et al., 2000). Another potential candidate could be PLCγ, which is also thought to regulate cancer cell motility and invasion (Kassis et al., 1999; Wu et al., 2000) as well as neurite outgrowth dependent on N-cadherin and the FGFR (Doherty and Walsh, 1996). We hence speculate that combined signaling by FGFR-1 and N-cadherin may involve mobilization of several downstream molecules, including MAPK-ERK, PI3-kinase, and PLCγ, to produce an invasive outcome. On the other hand, the fact that no correlation between sustained ERK activation and invasion was found in the MDA-MB-134 cancer cells suggests perhaps that some tumor cells are deficient in downstream targets of ERK

that transactivate MMP-9 gene transcription or have developed additional pathways that counteract metastasis.

Finally, we postulate that the FGFR may either suppress or activate invasive growth depending on the adhesion molecule that it binds to. Namely, while N-CAM was shown to restrict tumor invasion by inducing FGFR-4 signaling (Cavallaro et al., 2001), N-cadherin is shown to promote invasion by enhancing FGFR-1 signaling. The opposite effects of N-CAM and N-cadherin on tumor metastasis may be due to differential mobilization of downstream signaling events (Cavallaro et al., 2001).

Altogether, our findings provide valuable insights into the molecular aspects of tumor metastasis. It appears that the association of adhesion molecules with growth factor receptors provides tumor cells with an important platform to create diversity in signaling events that differentially lead to adhesion, migration, invasion, and metastasis.

Experimental procedures

Cell lines

Cell lines were obtained from the American Type Culture Collection (ATCC) except as noted. The TSU.Pr-1 bladder carcinoma cell line (van Bokhoven et al., 2001) was from Dr. John Isaacs at Johns Hopkins University. The melanoma cell line 888 was from Dr. Paul Robins at the National Institutes of Health. The melanoma cell line MeWo was from Dr. Soldano Ferrone from the Roswell Park Cancer Institute. L cell lines were from Dr. David Colman at Mount Sinai School of Medicine.

Reagents

Growth factors were from Pepro Tech. Biocoat inserts were from Becton-Dickinson. PD 98059 was from NEB. GM 6001 was from Calbiochem. Monoclonal anti-N-cadherin antibodies were from Zymed laboratories. Polyclonal anti-N-cadherin antibodies were from Dr. David Colman. Anti-phospho-MAPK (ERK-1/2) was from NEB and anti-total-MAPK from Santa-Cruz. Monoclonal anti-Flag-tag antibody and anti-α-tubulin were from Sigma. Polyclonal anti-FGFR-1 and 2 and EGFR and VEGFR antibodies were obtained from Santa Cruz. Polyclonal antibodies to the FGFR-1 extracellular domain, monoclonal anti-FGF-2, anti-phosphotyrosine, and anti-PDGFR antibodies were from Upstate Biotechnology.

Constructs

Expression plasmids for the human EGFR or PDGFR α or β and a retroviral construct expressing constitutively active MEK1 (MEKEL) were from Dr. Stuart Aaronson (Mount Sinai School of Medicine). A dominant negative MEK1 was from Dr. Silvio Gutkind (National Institutes of Health). The human VEGFR construct was from Dr. Bruce Terman (Albert Einstein College of Medicine). The full-length human FGFR-1 cDNA was from Dr. Joseph Schlessinger (NYU).

The FGFR-1 full length and extracellular (FGFR-ECD) cDNA were subcloned by PCR into Not 1/Bam H1 restriction sites downstream of the CMV promoter and upstream of the c-terminal Flag-tag sequence in the expression vector pFLAG-CMV-5a. The intracellular domain of FGFR-1 (FGFR-ICD) was subcloned by PCR into a pFLAG-CMV-1 vector (Sigma). This construct included an N-terminal Flag-tag sequence fused to the signal sequence of preprotrypsin. Expression plasmids of FGFR-1-ECD deletions of Ig-like domains 1–3 (ΔD1, ΔD2, and ΔD3) and site-directed mutagenesis (HAV to HEV) were constructed using PCR and three-fragment ligation. Mutation of HAV to HEV in FGFR-1-ECD was performed by PCR site-directed mutagenesis with single nucleotide mutation and ligated to a Hind III/BamH1 cleaved pFLAG-CMV-5a vector. Full-length Flag-tagged human N-cadherin (hN-cad) was produced by PCR of the human N-cadherin cDNA (Dr. John Hemperly, Becton-Dickinson). Flag-tagged human N-cadherin extracellular domain (N-cad-ECD) was created by a similar strategy. N-cadherin intracellular domain (N-cad-ICD) was ligated in pFLAG-CMV-1, which contains an N-terminal Flag-tag sequence fused with the signal sequence of preprotrypsin.

Immunoblotting and immunopreciptation

Cells were serum starved for 18 hr before adding growth factors. Cells were extracted in solubilization buffer (50 mM Tris-HCL [pH 7.5], 150 mM NaCl, 0.5 mM $MgCl_2$, 0.2 mM EGTA, 1% Triton X-100) including protease inhibitors. Twenty μg of protein was loaded on 7.5% SDS-polyacrylamide gels and transferred to nylon membranes. Blots were probed with indicated antibodies and developed with chemiluminescence. Immunoprecipitations were done by incubation of 1–2 mg lysate from each indicated cell line, with primary antibody overnight at 4°C and addition of 5 mg Protein-A-sepharose beads for 1 hr at 4°C, followed by three washes in solublization buffer and extraction of beads in 30 μl sample buffer at 100°C for 5 min.

Invasion and migration

The ability of cells to migrate through Matrigel-coated filters (invasion) or through uncoated fiters (migration) was determined as described (Hazan et al., 2000).

Substrate gel electrophoresis

Secreted metalloproteinases were detected by zymmography (Nakajima et al., 1995) as described (Hazan et al., 2000).

Receptor internalization

L and LN cells were transiently transfected with FGFR-1 and seeded in 24 well plates at 5×10^4 per well until confluence and were then serum-starved and incubated with 0.5 ml DMEM/1% BSA including 100 ng/ml FGF-2 and 5 μg/ml heparin for 2 hr on ice. Cells were then washed with cold PBS to remove unbound FGF-2 and placed at 37°C for various time points. At each time point, the remaining surface-bound FGF-2 was stripped from the cell surface using ice cold 20 mM acetic acid/2 M NaCl for 2 min (Wong et al., 2002), TCA-precipitated, electrophoresed, and immunoblotted with an antibody to FGF-2. To confirm that all samples contained the same number of cells, the levels of α-tubulin were determined in each monolayer after stripping of FGF-2. Blots were scanned and FGF-2 levels were quantified by densitometry. Data were expressed as the percent of the levels of FGF-2 before transfer of cells to 37°C.

Receptor degradation

L and LN cells were transfected as above and incubated with DMEM with 20 μg/ml cycloheximide and 100 ng/ml FGF-2 for 1 to 9 hr. At each indicated time, cell lysates harvested, the protein was determined, and 30 μg was electrophoresed and immunoblotted with anti-FGFR-1 antibodies. Immunoblots were scanned and the intensity of the protein bands was determined by densitometry. Data were normalized to the values of endogenous α-tubulin and expressed as the percent of the levels of FGFR-1 before addition of FGF-2.

FACS analysis

Adherent monolayers of each indicated cell line that were incubated in serum-free media for 18 hr with or without 100 ng/ml FGF-2 and 5 μg/ml heparin were made into single cell suspensions using 5 mM EDTA/PBS. Cells were washed in PBS and 1×10^6 cells incubated for 30 min at 4°C in 0.5 ml PBS/0.5% BSA containing either a 1:100 dilution of an anti-FGFR-1 extracellular peptide antibody (UBI), or of a preimmune anti-sera (UBI). Cells were washed in cold PBS and incubated with secondary anti-rabbit-FITC antibodies at 1:100 in 0.5 ml PBS/0.5% BSA for 30 min on ice, washed in cold PBS, and analyzed by flow cytometry. The histogram plots for the FGF-2 treated and untreated samples labeled with the anti-FGFR-1 antibody were superimposed on to those from identical samples stained with the preimmune antibody using the Cell Quest software program. Experiments were performed at least in triplicate. Increase or decrease in FGFR surface expression was expressed as the ratio of the mean channel number of cells stained with anti-FGFR-1 after culture in the presence or absence of FGF-2.

Luciferase assay

The MMP-9 promoter plasmid was obtained from Dr. Boyd at MD Anderson Cancer Center. Vector- and N-cadherin-expressing MCF-7 cells were transiently cotransfected with 0.5 μg of the MMP-9 reporter plasmid and 0.5 μg of MFG-β-galactosidase gene plasmid (Dr. Toru Ouchi, Mount Sinai School of Medicine) using Lipofectamine-plus (Gibco). Cells were serum-starved 24 hr posttransfection for 4 hr and treated with or without FGF-2

(100 ng/ml) and heparin (5 μg/ml) in serum-free media for an additional 18 hr. Cell lysates were processed for luciferase activity.

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