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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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Table of Contents

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).

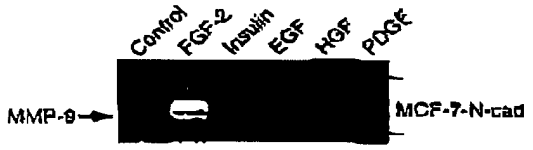

Figure 2. N-cadherin stimulated MMP-9 secretion is FGF-2 specific. MCF-7-N-cad cells were treated for 18 hr with the indicated growth factors at 50 ng/ml and MMP-9 activity was assessed by zymmography. FGF-2 stimulated robust MMP-9 secretion only in MCF-7-N-cad cells and not in control MCF-7-neo cells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

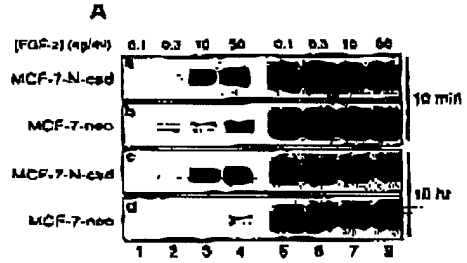

Figure 3 FGF-2 activation of MAPK is enhanced and sustained in the presence of N-cadherin. (A) MCF-7-N-cad (a and c) or MCF-7-neo (b and d) were treated with increasing concentrations of FGF-2 for 10 min (a and b) or 18 hr (c and d) in the presence of 5 μg/ml Heparin. Cell lysates were by immunoblotting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

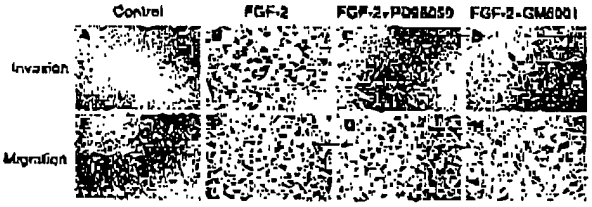

Figure 4. FGF-2 stimulated invasion of MCF-7-N-cad cells is mediated by MAPK-ERK-MMP-9 activation. MCF-7-N-cad cells were untreated (A and E) or treated for 18 hr with 50 ng/ml FGF-2 and 5 μg/ml Heparin (B and F) in the presence of either 40 μM of PD98059 (C and G), 2μM GM6001 (D and H). Cells were assayed 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 (Surokin 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.

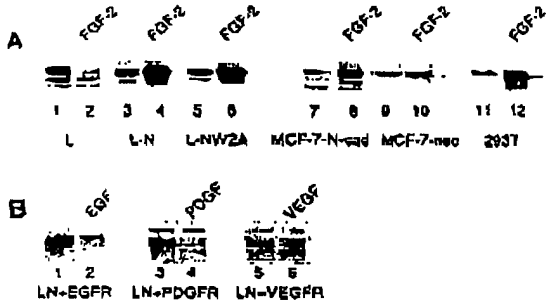

Figure 5. N-cadherin prevents FGF-2 induced FGFR downregulation but not that of other receptors (A) Mouse L-cell fibroblasts (L, lanes 1-2) or L-cells stably transfected with N-cadherin (LN, lanes 3-4) or a non-adhesive N-cadherin mutant (LNW2A, lanes 5-6) were transiently transfected with FGFR-Flag 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°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.

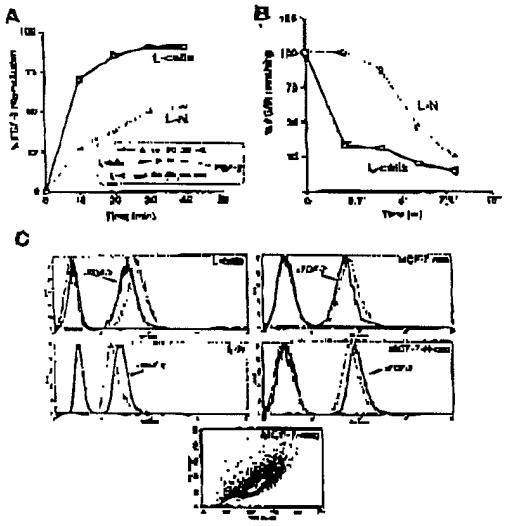

Figure 6. N-cadherin attenuates FGF-2-induced FGFR-1 internalization and degradation. A. Rate of internalization of FGF-2 in L-cells (squares) versus LN cells (diamonds) was determined by cell surface stripping of bound FGF-2 at various times points after saturation with FGF-2. (Inset) Immunoblots showing 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, β-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).

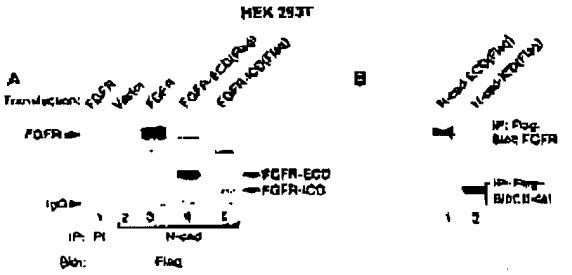

Figure 7 . N-cadherin and the FGFR interact exclusively via the extracellular domain. (A) Transfected Flag-tagged FGFR (lane 3) or FGFR-ECD (lane 4) but not FGFR-ICD (lane 5) co-precipitate with endogenous N-cadherin in HEK 293T. No co-immunoprecipiation was seen with preimmune sera (lane1) or with vector 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 β-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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