Notch Activation Suppresses Fibroblast Growth Factor-dependent Cellular Transformation*

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Aberrant activations of the Notch and fibroblast growth factor receptor (FGFR) signaling pathways have been correlated with neoplastic growth in humans and other mammals. Here we report that the suppression of Notch signaling in NIH 3T3 cells by the expression of either the extracellular domain of the Notch ligand Jagged1 or dominant-negative forms of Notch1 and Notch2 results in the appearance of an exaggerated fibroblast growth factor (FGF)-dependent transformed phenotype characterized by anchorage-independent growth in soft agar. Anchorage-independent growth exhibited by Notch-repressed NIH 3T3 cells may result from prolonged FGFR stimulation caused by both an increase in the expression of prototypic and oncogenic FGF gene family members and the nonclassical export of FGF1 into the extracellular compartment. Interestingly, FGF exerts a negative effect on Notch by suppressing CSL [CBF-1/RBP-Jk/KBF2 in mammals, Su(H) in Drosophila and Xenopus, and Lag-2 in Caenorhabditis elegans]-dependent transcription, and the ectopic expression of constitutively active forms of Notch1 or Notch2 abrogates FGF1 release and the phenotypic effects of FGFR stimulation. These data suggest that communication between the Notch and FGFR pathways may represent an important reciprocal autoregulatory mechanism for the regulation of normal cell growth.

Notch receptors and their ligands are components of an evolutionarily conserved signaling pathway that regulate cell proliferation, differentiation, and survival in a cell- and tissue-specific manner (for reviews, see Refs. 1 and 2). Notch receptors and their ligands are structurally conserved transmembrane polypeptides, and four Notch receptors (Notch1–4) and six ligands (Delta1–4, Jagged1, and Jagged2) have been identified in vertebrates to date (3–8). Upon ligand binding, the intracellular domain of the Notch receptor is released by proteolytic cleavage and becomes a nuclear transcriptional regulator by interacting with members of the CSL (CBF-1/RBP-Jk/KBF2 in mammals, Su(H) in Drosophila and Xenopus, and Lag-2 in Caenorhabditis elegans) family of transcription factors (9). Notch receptors have also been reported to regulate cellular processes through CSL-independent pathways that may involve interactions with other signaling molecules such as nuclear factorκB and Src (10–12). Phenotypic analysis of mice null for Notch receptors or their ligands emphasizes the requirement for proper Notch signaling not only during development but also in the adult (13–17). Indeed, aberrant Notch signaling has been implicated in several human pathological conditions including the development of the CADASIL (18) and Alagille syndromes (19, 20) and the formation of neoplasias in mice and humans (21–23).

We have reported previously that suppression of endogenous Notch signaling mediated by the ectopic expression of either an extracellular and soluble form of Jagged1 (sJ1)1 or dominant-negative mutants of Notch1 (dnN1) or Notch2 (dnN2) induces dramatic changes in the NIH 3T3 cellular phenotype in comparison with NIH 3T3 cells stably transfected with the empty vector (vector control). These changes in cellular phenotype include chord formation on collagen matrices; increased Src activation and enhanced phosphorylation of the Src substrate, cortactin; a decrease in the formation of actin filaments and focal adhesion sites; impaired migratory ability; and increased survival at high cell densities (11, 24, 25). In contrast, NIH 3T3 cells stably expressing either constitutively active mutants of Notch1 (caN1) or Notch2 (caN2) display a phenotype similar to that exhibited by the vector control cells (11).

Our initial interest in Notch originated from our observation that Jagged1 was an FGF response gene in human endothelial cells undergoing differentiation on fibrin clots (26). Because several of the phenotypic characteristics displayed by Notch-repressed cells were dependent on the activity of the FGFR effector molecule Src (11), we anticipated that communication between Notch and FGFR signaling pathways may also represent an important mechanism regulating cellular behavior in fibroblasts. Whereas several studies report that the expression of Notch and/or its ligands is correlated with activation of the FGFR signaling pathway (27–29) and vice versa (30), little is known about how interactions between these two important and ubiquitous pathways influence cellular phenotype, including growth. To address this question, we examined the effects of FGFR stimulation in combination with Notch repression or activation in NIH 3T3 cells. We decided to study these interactions in the NIH 3T3 cell because we had already identified

1 The abbreviated used are: sJ1, soluble Jagged1; BCS, bovine calf serum; caN, constitutively active Notch; dnN, dominant-negative Notch; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; RT-PCR, reverse transcription-PCR.
phenotypic characteristics associated with the down-regulation of Notch signaling (11), and the NIH 3T3 cell represents a relatively simple system because it primarily expresses transcripts encoding Notch1 and Notch2, but not Notch3 and Notch4. In addition, aberrant activation of both Notch (21–23) and FGFR (31–33) signaling pathways have been found to be associated with neoplastic growth, and the NIH 3T3 cell is uniquely sensitive to oncogene-mediated transformation (34).

We report that antagonistic interactions between the Notch and FGFR signaling pathways regulate anchorage-independent growth in murine fibroblasts. Stimulation of the FGFR pathway by exogenous FGF1 causes Notch-repressed cells to grow as detached spheroids in tissue culture and to aggressively form colonies in soft agar, phenotypic characteristics associated with cellular transformation (reviewed in Ref. 35). The transformed phenotype exhibited by Notch-repressed cells may be attributed to their maintenance of FGFR-generated signals generated by an increase in both the expression and release of FGF family members. Furthermore, FGF1 has an inhibitory effect on Notch/CSL-dependent transcription. In contrast, the expression of canN1 or canN2 protects the NIH 3T3 cell from FGF-induced anchorage-independent growth and suppresses the release of FGFR1 under normal growth conditions. These results suggest that cross-talk between the Notch and FGFR signaling pathways may represent an important autoregulatory mechanism that is involved in the regulation of cell growth.

EXPERIMENTAL PROCEDURES

Generation of Stable NIH 3T3 Transfectants—Stable NIH 3T3 transfectants for vector control, sJ1, dnN1, cnN1, and canN2 were obtained and screened for expression as described previously (11). The FGF1 mutant containing the 3′ signal peptide sequence of FGF4 (hst-βFGF4:FGF1) and FGF1 (pXZ38) stable transfectants were obtained and screened for expression as described in Ref. 31. Stably transfected constitutively active Ras (caRas) clonal populations were obtained by previously described methods (11) using the activated H-Ras p185E plasmid (Upstate Biotechnology).

 Colony Formation in Soft Agar—Clonal populations of NIH 3T3 cells stably transfected with vector control, sJ1, cnN1, canN2, dnN1, and hst-βFGF4:FGF1, or caRas were plated on 6-cm tissue culture dishes with 0.5% agar in an overlay containing Dulbecco’s modified Eagle’s medium (DMEM) plus 10% bovine calf serum (BCS; Hyclone), 0.3% agar at 1.5 × 10^5 cells/dish. As indicated, some dishes were also treated with 1 μM of the FGFR1-specific inhibitor, PD166866 (Ref. 36; a generous gift from R. L. Panek, Park-Davis), and/or 10 ng/ml recombinant human FGF1 and 10 units/ml heparin (Sigma). Cells were fed with 0.5 ml of media with or without FGF1 and/or the FGFR1-specific inhibitor every 3 days as indicated. Twenty days after plating, colonies were stained with p-iodonitrotetrazolium violet (Sigma) for visualization. Quantitation of colony formation was achieved by counting all p-iodonitrotetrazolium violet-stained colonies consisting of more than 4 cells under a Zeiss Stemi SV111 A0 dissecting microscope.

Analysis of FGF1-induced Spheroid Formation—Clonal populations of NIH 3T3 stable transfectants were resuspended and grown in media containing 10% BCS, 10% BCS plus 1 μM PD166866, 10% BCS plus 10 ng/ml recombinant human FGF1 and 10 units/ml heparin, or 10% BCS plus 10 ng/ml recombinant human FGF1, 10 units/ml heparin, and 1 μM PD166866 as indicated in the figure legend. Approximately 2 × 10^5 cells were plated per well (6-well dish), and 3 days after plating, phase-contrast micrographs of the cells were taken. Analysis of Spheroid Formation and Soft Agar Colony Growth in hst-βFGF4:FGF1 Transfectants after Adenoviral Transduction—hst-βFGF4:FGF1 stable transfectants were transduced with either adenovirus expressing lacZ, dominant negative FGFAR, cnN1, or canN2 as described below. After 24 h, the cells were plated on 6-well dishes at a concentration of 1 × 10^5 cells/well, and 3 days after plating, phase-contrast micrographs of the cells were taken. For colony formation in the soft agar assay, the hst-βFGF4:FGF1 transfectants were transduced with the indicated recombinant adenoviral vectors and plated into 0.33% agar 24 h after the transduction. Two weeks after plating, colonies were visualized by staining with p-iodonitrotetrazolium violet.

Analysis of the Expression of the cf/fgfr Gene Family Members—Total RNA from vector control, sJ1, canN1, and dnN1 stable transfectants was isolated using Tri Reagent™ (Sigma) according to the manufacturer’s protocol. CDNA was obtained from 5 μg of total RNA with SuperScript™ (Invitrogen) reverse transcriptase using an oligo(dT) primer. The following primers were designed from the RT and used for RT-PCR analysis (sense primers are indicated by (s); antisense primers are indicated by (as)): FGF1(s), 5′-ATGGCTGAAAGGGAGATCACACCAC-3′; FGF1(as), 5′-CGGCCGTTCAGCTCCGTTTC-3′; FGF2(s), 5′-ATGGGCCATCGACGCGCT-3′; FGF2(as), 5′-GAAAGAACGATGGTGCCTGAC-3′; FGF3(s), 5′-GAGGGCTGACCTCCTGCTGCTC-3′; FGF3(as), 5′-ACCAGGGGAGACCTGCTG-3′; FGF4(s), 5′-GATTACCCGCTGAGTGGCGAGG-3′; FGFR1(s), 5′-ACCAGGGGAGACCTGCTG-3′; FGFR1(as), 5′-GATTACCCGCTGAGTGGCGAGG-3′; FGFR2(s), 5′-GCAAGCTTCTCTAGTCTGAC-3′; FGFR2(as), 5′-GATAGTCATATATGCGACAGC-3′; FGFR3(s), 5′-GCAAGCTTCTCTAGTCTGAC-3′; FGFR3(as), 5′-GATAGTCATATATGCGACAGC-3′; FGFR4(s), 5′-GCAAAGCTTGAGTGGCGAGG-3′; FGFR4(as), 5′-GATAGTCATATATGCGACAGC-3′.

PCR amplification was performed for 45 cycles as follows: 40 s at 94 °C, 40 s at 50 °C (for FGF2, FGF3, FGF4, and FGF5) or at 55 °C (for FGF1, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, and FGF21) and 1 min at 72 °C. For FGF5, RT-PCR analysis was performed as described previously (37), and all amplified DNA was visualized with ethidium bromide on 1.5% agarose gels.

Analysis of FGF1 Release in Stable NIH 3T3 Cell Transfectants—Adenovirus vector expressing lacZ, FGF1, canN1, cnN1, canN2, or dnN2 was used as described previously (38) at a titer of ∼10^13 viral particles/ml. For adenoviral transduction, NIH 3T3 stable transfectants were incubated in serum-free medium with ∼10^3 viral particles/cell in the presence of poly-b-lysine hydrobromide (Sigma) (5 × 10^3 molecules/viral particle) for 2 h at 37 °C, after which the adenovirus-containing medium was removed and replaced with serum-containing medium (10% BCS) for an additional 24 h. The transduced cells were harvested by trypsin digestion and seeded for the heat shock experiments as described previously (39). After heat shock, the conditioned media from cells exposed to either 37 °C (normal conditions) or 42 °C (heat shock conditions) were treated with 0.1% dithiothreitol for 2 h at 37 °C, after which the conditioned media were harvested to heparin-Sepharose, and eluted from the column with 1.5 M NaCl. The eluants were recovered to 100 μl, and RT-PCR was evaluated by FGF1 immunoblot analysis as described previously (39).

Transfection Assays of CSL-regulated Transcription—NIH 3T3 cells were plated onto fibronectin-coated (10 μg/cm^2) 12-well tissue culture dishes and transiently transfected at ~80% confluency with 500 ng of a luciferase construct activated by four tandem copies of the CSL (CBF1) response element (40). 100 ng of the Tk Renilla (Promega) construct as an internal control for transfection efficiency, and 500 ng of either vector control, sJ1, or canN1 constructs using FuGENE 6 (Roche Molecular Biochemicals) per the manufacturer’s instructions. For analysis of canN1 activity in the background of NIH 3T3 stable lines, vector control, cnN1, sJ1, and hst-βFGF4:FGF1 stable transfectants were analyzed (40) at 10 ng/ml (fibronectin-coated) medium and transiently transfected with 500 ng of the CSL-luciferase construct, 100 ng of the Tk Renilla (Promega) construct as an internal control for transfection efficiency, and 200 ng of canN1. For all experiments, the medium was replaced 24 h after transfection with fresh Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% BCS or 10% BCS containing 10 μg/ml (fibroin-coated) medium and transiently transfected with 500 ng of the CSL-luciferase construct, 100 ng of the Tk Renilla (Promega) construct as an internal control for transfection efficiency, and 200 ng of canN1. For all experiments, the medium was replaced 24 h after transfection with fresh Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% BCS or 10% BCS containing 10 μg/ml (fibroin-coated) medium.

The efficiency of transcription was measured and normalized in relationship to the activity of pRL-TK Renilla, and the activity was reported as the ratio of luciferase/Renilla activity. The experiment was done in triplicate, and error bars represent the S.E.

Analysis of Jagged1 Expression—Vector control, sJ1, cnN1, Jagged1, hst-βFGF4:FGF1, and sJ1:cnN1 stable NIH 3T3 cell transfectants were plated on tissue culture dishes in either normal growth media.
FIG. 1. Spheroid formation is regulated by FGF1 and the Notch pathway. NIH 3T3 cell transfectants were grown in media containing either 10% BCS or 10% BCS with the addition of FGF1 (10 ng/ml) and heparin (10 units/ml), or 1 μM PD166866 as indicated. Phase-contrast photomicrographs (original magnification, ×40) of the cells 3 days after plating are shown.

RESULTS

Stimulation of the FGFR Pathway Potentiates a Transformed Phenotype in Notch-repressed Fibroblasts—In an effort to further our understanding of how interactions between the Notch and FGFR signaling pathways regulate cellular processes, we examined the response of Notch-activated and Notch-repressed NIH 3T3 cells to the addition of recombinant FGF1 to the growth media (Fig. 1). Surprisingly, NIH 3T3 cells in which endogenous Notch signaling was repressed (sJ1 and dnN1) formed multicellular, spheroid-like structures similar to those observed in NIH 3T3 cells stably expressing an oncogenic mutant of FGF1 engineered with the FGF4 signal peptide sequence (hst-β(FGF4):FGF1) to force constitutive secretion of FGF1 through the conventional ER-Golgi pathway (31). The cells contained within the spheroid structures were viable because they continued to proliferate over time and also grew as a monolayer when replated onto fresh tissue culture dishes in the absence of recombinant FGF1 in the growth media (data not shown). In contrast, vector control and caN1 NIH 3T3 cell stable transfectants did not form spheroids but instead continued to grow as a monolayer in the presence of recombinant FGF1. Spheroid formation was a specific response to FGF1 because treatment with the FGFR1-specific inhibitor PD166866 (36) completely abolished FGF1-induced spheroid formation in sJ1, dnN1, and hst-β(FGF4):FGF1 stable transfectants.

Cellular proliferation despite detachment from the extracellular matrix is indicative of anchorage-independent growth in most cell types and is an in vitro signature of the NIH 3T3 cell transformed phenotype (34). Therefore, we examined our Notch-activated and Notch-repressed cell lines for anchorage-independent growth in soft agar in both the presence and absence of exogenously added recombinant FGF1 (Fig. 2, A and B). Although we have previously reported (25) that sJ1 NIH 3T3 stable transfectants do not form colonies in soft agar when plated at low seed densities (100 cells/6-cm dish) in growth media containing 10% BCS, we have found during the course of these studies that sJ1 and dnN1 transfectants do form small, pinpoint-sized colonies in soft agar when plated at seed densities greater than 1,500 cells/6-cm dish. The addition of FGF1 to the growth media greatly exaggerated the size of the colonies formed by sJ1 and dnN1 transfectants so that they were clearly visible to the eye, although the number of colonies did not significantly increase. Indeed, the intensity of the transformed phenotype induced by FGF1 in the Notch-repressed NIH 3T3 cells resembled that exhibited by NIH 3T3 cells stably expressing either an oncogenic Ras construct (caRas) or an oncogenic mutant of FGF1 (hst-β(FGF4):FGF1). Interestingly, the size of the colonies also increased at seed densities of 5,000–10,000 cells/6-cm dish, even in the absence of exogenously added recombinant FGF1 (data not shown). In contrast, the addition of FGF1 had either no effect or only resulted in the formation of sparse and very small colonies in the caN1 and vector control transfectants. These transfectants also do not form colonies, regardless of plating concentration, in the absence of FGF1. NIH 3T3 cells stably expressing dnN2 but not caN2 also formed small colonies whose size was dramatically increased in the presence of FGF1 (data not shown). Similar to spheroid formation, FGF potentiation of soft agar growth in NIH 3T3 cells was a specific response to FGFR stimulation because the addition of the FGFR1 inhibitor PD166866 substantially reduced FGF1-mediated colony formation in sJ1, dnN1, and the hst-β(FGF4):FGF1 transfectants but had no effect on colony formation in the caRas cells. Treatment with PD166866 also inhibited small colony growth exhibited by sJ1 and dnN1 in the absence of FGF1. These data suggest that repression of endogenous Notch signaling sensitizes the NIH 3T3 cell to FGFR-mediated cellular transformation and that activation of the Notch signaling pathway may protect the NIH 3T3 cell from abnormal growth.

Activation of Notch Inhibits FGFR-mediated Transformation—To further explore the possibility that Notch signaling may protect the NIH 3T3 cell from FGFR-mediated anchorage-independent growth, we assayed the ability of sJ1 NIH 3T3 stable transfectants cotransfected with caN1 to form colonies in both the presence and absence of FGF1. Expression of caN1 in the sJ1 NIH 3T3 background dramatically inhibited, in terms of both number and size, colony formation that occurred in the presence or absence of FGF1. Indeed, the number of colonies formed by the sJ1:caN1 cotransfectants was similar to that observed in vector control and caN1 stable lines (Fig. 2, A and B). Unlike sJ1 single transfectants, sJ1:caN1 cotransfectants...
did not form spheroids in the presence of FGF1. However, these cells were less adherent to the tissue culture dish than the vector control or caN1 cells (Fig. 1). Although analysis of several different clonal cell lines of the various stable transfec-
tants used in these studies yielded similar results (data not shown), we wanted to confirm that constitutively active Notch could repress FGFR-mediated transformation by transducing hst-β(FGF4):FGF1 stable transfectants with adenovirus ex-
pressing either caN1 or caN2. Adenoviral expression of either caN1 or caN2, but not a lacZ control, reduced the number of colonies formed in soft agar to a level similar to that observed in the hst-β(FGF4):FGF1 NIH 3T3 cell transfectants transduced with either lacZ, dominant-negative FGFR1, caN1, or caN2. Positive colonies were visualized by staining with p-iodonitrotetrazo-
lium violet 2 weeks after plating. B, phase-contrast photomicrographs (original magnification, ×40) of hst-β(FGF4):FGF1 NIH 3T3 cell transfectants adenovirally transduced with either lacZ, dominant-negative FGFR1, caN1, or caN2.

**Fig. 2.** Anchorage-independent growth requires FGF1 signaling and Notch repression. As indicated in the figure, cells were grown in soft agar as described under "Experimental Procedures," with some dishes treated with 1 μM of the FGFR1-specific inhibitor, PD166866, and/or 10 ng/ml recombinant human FGF1 and 10 units/ml heparin as indicated. Cells were fed every 3 days, and colony number was quantified after 20 days. A, representative wells for each growth condition are shown (original magnification, ×4). B, the number of colonies was quantified by counting all stained colonies from two plates for each experimental condition. Graphed values are the means ± S.E. Error bars represent the S.E., and the data reflect a representation of one of several soft agar experiments conducted.

**Fig. 3.** The expression of activated Notch suppresses spheroid formation and colony growth in soft agar in hst-β(FGF4):FGF1 NIH 3T3 cell transfectants. A, colony formation in soft agar exhibited by hst-β(FGF4):FGF1 NIH 3T3 cell transfectants adenovirally transduced with either lacZ, dominant negative FGFR1, caN1, or caN2. Positive colonies were visualized by staining with p-iodonitrotetrazolium violet 2 weeks after plating. B, phase-contrast photomicrographs (original magnification, ×40) of hst-β(FGF4):FGF1 NIH 3T3 cell transfectants adenovirally transduced with either lacZ, dominant-negative FGFR1, caN1, or caN2.

**Notch Signaling Regulates the Expression of the** fgf**Gene Family**—Prior to this study, we reported (11) that Notch-repressed cells exhibit a pattern of tyrosine phosphorylation similar to that observed in NIH 3T3 cells stimulated by FGF1. In this study, we found that sJ1 and dnN1 cells form small, PD166866-sensitive colonies in soft agar even in the absence of exogenous FGF1. Taken together, these observations suggested that Notch-repressed transfectants were releasing FGF
Fig. 4. Regulation of FGF/FGFR gene expression by soluble Jagged1. A, the expression of fgf family members in vector control and sJ1 stable NIH 3T3 transfectants as determined by RT-PCR using primers and conditions described under “Experimental Procedures.” RT-PCR using primers specific for murine GAPDH was performed as a control for cDNA synthesis. B, the expression of fgfr family members in vector control and sJ1 NIH 3T3 stable transfectants as determined by RT-PCR using primers and conditions as described under “Experimental Procedures.” Each primer set was designed to detect both the 2- and 3-lg loop forms for fgfr1, fgfr2, and fgfr3 as well as the VT− and VT+ isoforms of fgfr1.

The fgfr1 primer set was designed to detect both the 2- and 3-lg loop forms for sJ1 NIH 3T3 stable transfectants as determined by RT-PCR using primers and conditions as described under Experimental Procedures. RT-PCR using primers specific for murine GAPDH was performed as a control for cDNA synthesis. The expression of fgfr family members in vector control and sJ1 NIH 3T3 stable transfectants as determined by RT-PCR using primers and conditions as described under “Experimental Procedures.” Each primer set was designed to detect both the 2- and 3-lg loop forms for fgfr1, fgfr2, and fgfr3 as well as the VT− and VT+ isoforms of fgfr1.

No differences were found in the expression pattern of the fgs family members in all cell lines examined, expression of mRNAs encoding fgs2, fgs7, and fgs10 are expressed in all of the cell lines examined, expression of mRNAs encoding fgs1, fgs3, fgs4, and fgs5 is limited to the sJ1 stable transfectants. In contrast, the pattern of fgs family gene expression in caN1 transfectants is the same as that seen in the vector control lines (data not shown). Transcripts encoding fgs6, fgs8, and fgs9 were not found in any of the cell lines examined by RT-PCR (data not shown). No differences were found in the expression pattern of the fgfrs because all cell lines examined expressed both the 2- and 3-lg loop isoforms of fgfr1, fgfr2, and fgfr3 as well as the VT− and VT+ isoforms (41) of fgfr1 (Fig. 4B). These data indicate that sJ1-mediated repression of endogenous Notch signaling regulates the FGFR signaling pathway by a mechanism that includes changes in fgs but not fgfr mRNA expression.

Repression of Notch Signaling Induces FGF1 Release—Although most of the fgs family members contain signal peptides that facilitate their secretion through the classical ER-Golgi exocytosis pathway, it is well established that the prototype members of the fgs gene family (FGF1 and FGF2) do not contain a signal peptide and are instead released by nonclassical mechanisms. Whereas the pathway utilized by FGF2 to gain access to the extracellular compartment is not known, FGF1 is released in response to environmental stress as a component of a copper-dependent, multiprotein aggregate that includes the p40 extravesicular domain of p65 synaptotagmin-1 and S100A13 (42–45). Given that FGF1 but not FGF2 is differentially expressed in the sJ1 stable transfectants (Fig. 4A), we examined these cells for FGF1 release. Because expression of the FGF1 steady-state translation product is undetectable in the NIH 3T3 cell (39) and very low in sJ1 transfectants (data not shown), we examined vector control, sJ1, caN1, dnN1, caN2, and dnN2 transfectants for the release of adenovirally transduced FGF1 under normal (37 °C) and heat shock (42 °C) conditions (Fig. 5). Whereas vector control, sJ1, and sJ1:caN1 transfectants were able to export FGF1 at similar levels into the extracellular compartment in response to temperature stress, a significant release of FGF1 at 37 °C was observed only in the sJ1 cells. Expression of caN1 into the sJ1 background (Fig. 5A, sJ1:caN1) significantly reduced FGF1 release at 37 °C, but not at 42 °C, indicating that activation of Notch attenuates FGF1 release under normal but not heat shock conditions. A role for Notch as a regulator of FGF export was further substantiated by the reverse experiment in which NIH 3T3 cells stably transfected with FGF1 were transduced with either the lacZ−, caN1−, dnN1−, caN2−, or dnN2-expressing adenovirus (Fig. 5B). Under these conditions, FGF1 was also released into the extracellular compartment in response to heat shock in all cell lines examined, but its release at 37 °C was limited to those cells expressing either dnN1 or dnN2.

Exogenous FGF1 Represses CSL-dependent Signaling—We have previously observed (11) that the ability of caN1 to up-regulate a CSL-luciferase reporter construct is significantly diminished in sJ1 stable transfectants in comparison with vector control cells (Fig. 6). Because FGF release appears to be up-regulated in Notch-repressed cells, we wanted to determine whether caN1/CSL-mediated transcription was negatively correlated with activation of the FGFR signaling pathway. The ability of caN1 to stimulate transcription of a CSL-luciferase reporter in hst−βFGF4:FGF1 transfectants was significantly reduced in comparison with its activity in vector control and caN1 stable transfectants (Fig. 6A). In addition, exogenous FGF1 also repressed caN1/CSL transcription in a dose-dependent fashion in caN1 transfectants (Fig. 6C). The low levels of endogenous CSL-dependent transcription in vector control and sJ1 NIH transfectants also displayed a dose response to exogenous FGF1, although the magnitude of the FGF1 effect was less than that achieved in the caN1 transfectants (Fig. 6, A and B). However, whereas caN1/CSL-dependent transcriptional activation is significantly repressed by FGF1, this repression is not complete. These results are interesting because the vector...
control cells (Fig. 2, A and B) still do not proliferate in response to exogenous FGF1 under conditions of soft agar growth. Therefore, it is possible that this relatively low level of conventional CSL-dependent activity is sufficient to repress the ability of the NIH 3T3 cell to respond to exogenous FGF1 as an agent of cell transformation.

FGF1 Up-regulates the Expression of Jagged1—Because we have previously reported that Jagged1 was an FGF response gene in human endothelial cells, we examined the NIH 3T3 stable transfectants used in this study for expression of Jagged1 by immunoblot analysis using an antibody directed against an epitope located within the intracellular C-terminal domain of full-length Jagged1 (Fig. 7). We found that Jagged1 expression was more pronounced in sJ1 and hst-β(FGF4):FGF1 transfectants than in vector control and caN1 transfectants grown in normal growth medium (Fig. 7, 10% Serum). The presence of FGF1 in the growth media increased Jagged1 expression in all stable lines examined, and caN1 still displayed the lowest level of Jagged1 expression. We have not found that NIH 3T3 cells express other Notch ligands including Jagged2, Delta1, Delta3, or Delta4 in either the absence or presence of FGF1 in the growth media (data not shown). In addition, we have also observed that steady-state levels of mRNA encoding Notch1 and Notch2 appear to be unchanged as determined by RT-PCR (data not shown), although slight differences in the expression of these transcripts detectable by more sensitive assays cannot be ruled out.

**DISCUSSION**

Whereas both the Notch and FGFR signaling pathways have long been recognized as important regulators of cell fate determination events in a variety of cell types, little is known about how interactions between these two major signaling pathways impact cellular processes. In this report, we present evidence that supports the existence of an important cellular mechanism that may be involved in balancing the signals generated by these signaling pathways. Interestingly, whereas the stimulation of the FGFR by FGF1 exerts a negative regulatory effect on Notch signaling by repressing Notch-mediated CSL transcription, Notch, in turn, tempers FGF-generated signals by regulating the extracellular appearance of fgf gene family members. In addition, whereas activated Notch also represses the effects of FGFR stimulation through an as yet unidentified mechanism other than FGF export, the perturbation of endogenous Jagged1/Notch signaling disrupts the equilibrium between the Notch and FGFR pathways and leads to the manifestation of a transformed phenotype.

Our results indicate that endogenous Jagged1/Notch signal-
The expression of this is probably not a contributing factor in our system because also been associated with cellular transformation (33, 46, 47), although aberrant expression of FGFRs and their isoforms has Notch2 interferes with FGFR-mediated cellular transformation already present in Notch-repressed cells. This is FGF export would then reinforce the inhibition of Notch/CSL activation of Notch/CSL-responsive genes. Although it is currently unknown which other signaling pathways are involved in facilitating cross-talk between Notch and FGFR, it is possible that suppression of FGF-mediated anchorage-independent growth by caN1 and caN2 stable transfectants continues to release FGF1 under heat shock conditions to the same extent as vector control transfectants argues against this possibility. Instead, it is more likely that repression of Notch in the sJ1, dnN1, and dnN2 stable transfectants induces a stress/survival response that enables FGF1 export.

Because expression of caN1 or caN2 strongly inhibited FGFR-mediated anchorage-independent growth in hst-β(FGFr4):FGF1 transfectants, it is likely that activated Notch1/Notch2 interferes with FGFR-mediated cellular transformation at a level other than its regulation of FGF release. Although aberrant expression of FGFRs and their isoforms has also been associated with cellular transformation (33, 46, 47), this is probably not a contributing factor in our system because the expression of ffgfr mRNA, including those transcripts that represent splice variants, is similar in all of the NIH 3T3 stable lines examined in this study. Although it is currently unknown which other signaling pathways are involved in facilitating cross-talk between Notch and FGFR, it is possible that suppression of FGF-mediated anchorage-independent growth by caN1 and caN2 may occur through its regulation of activator protein-1-dependent transcription. In fibroblasts, continuous exposure to FGF1 increases the transcription of fos (44), a polypeptide component of the activator protein-1 complex.

Notch activation has been reported to inhibit activator protein-1-mediated transcription in HeLa and human erythroblast cell line K562 (48), and caN1 suppression of activator protein-1 may be the underlying mechanism behind its inhibition of human papillomavirus-induced transformation in cervical carcinoma cells (49).

The unanticipated observation that FGF1 suppresses Notch1/CSL-mediated transcription suggests that Notch may also protect the NIH 3T3 cell from abnormal growth through the transcriptional regulation of Notch/CSL-responsive genes. FGF export would then reinforce the inhibition of Notch/CSL activation already present in Notch-repressed cells. This is consistent with the requirement for the Notch/CSL-dependent induction of p21waf1/cip for the stimulation of keratinocyte differentiation by the regulation of growth arrest (keratinocytes require exogenous FGF for cell division) (50). However, this regulatory mechanism may also contain a cell- and tissue-specific as well as an age-dependent component because in the developing tooth bud, FGF10 is able to induce the Notch/CSL-dependent transcription of hes1 (51), and this may be complicated by an additional level of specificity for some but not all of the 23 members of the fgg gene family.
tion with Notch receptors. Naturally occurring soluble forms of the Notch ligands arising from proteolytic cleavage (55) or perhaps differential miRNA processing (19, 26) have been identified, yet the functional activities of these modified ligands are not clear. The observation that soluble forms of Notch ligands have been demonstrated to be both agonists (19, 56, 57) and antagonists of Notch signaling (58–61) may be due to factors including oligomerization or immobilization of ligands (62–64).

Although it is possible that proteolytic cleavage of Notch ligands generates nonfunctional soluble fragments that reduce endogenous ligand availability (65), our data support a model where soluble ligands have significant activity in regulating Notch signaling. In addition, a preponderance of the human jagged1 Notch signaling. In addition, a preponderance of the human Notch ligands has been associated with neoplasms (61) may be due to factors perhaps differential mRNA processing (19, 26) have been identified, yet the functional activities of these modified ligands are not clear. The observation that soluble forms of Notch ligands have been demonstrated to be both agonists (19, 56, 57) and antagonists of Notch signaling (58–61) may be due to factors including oligomerization or immobilization of ligands (62–64).

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