Silencing of FLRG, an Antagonist of Activin, Inhibits Human Breast Tumor Cell Growth

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Abstract
Activin, a member of the transforming growth factor β (TGFβ) superfamily, regulates diverse processes, such as cellular growth and differentiation. There is increasing evidence that TGFβ and its signaling effectors are key determinants of tumor cell behavior. Loss of sensitivity to TGFβ-induced growth arrest is an important step toward malignancy. We previously characterized FLRG as an extracellular antagonist of activin. Here, we show that activin-induced growth inhibition is altered in FLRG-expressing breast cancer lines. Silencing FLRG induced growth inhibition, which is reversible upon addition of exogenous FLRG. We showed that FLRG silencing effects resulted from restoration of endogenous activin functions as shown by increased levels of phosphorylated smad2 and up-regulation of activin target gene transcripts. Furthermore, the growth inhibition induced by FLRG silencing was reversible by treatment with a soluble form of type II activin receptor. Finally, a strong expression of FLRG was observed in invasive breast carcinomas in contrast with the normal luminal epithelial cells in which FLRG was not detected. Our data provide strong evidence that endogenous FLRG contributes to tumor cell proliferation through antagonizing endogenous activin effects. [Cancer Res 2007;67(15):7223–9]

Introduction
Activins, members of the transforming growth factor β (TGFβ) superfamily, control many physiologic processes, including cell proliferation and differentiation, immune response, motility, and adhesion. Ligation of activin to its specific serine/threonine kinase receptors induces phosphorylation of the intracellular mediators, smad2 and smad3. These phosphorylated (phospho-) smads interact with smad4, migrate to the nucleus, and regulate gene transcription in association with cofactors in a cell-type specific manner (1). TGFβ and activin are potent growth inhibitors in most cell types and contribute to normal cell homeostasis. Alterations of TGFβ signaling result in progression of various tumors. TGFβ superfamily members and their associated signaling components have been implicated in both cancer onset and progression. Indeed, loss of responsiveness to TGFβ (mutations, loss of receptor expression, or inactivation of smad proteins) has been described in many tumor types (2, 3). Whereas several studies have focused on the role of TGFβ, little is known about the role of activin in carcinogenesis. Loss of expression of activin and activin receptors and alterations of smad signaling have been associated with poor outcome in human breast cancer (4, 5). Thus, impairment of the activin signal transduction system may also contribute to oncogenic progression.

Inhibins and activins, expressed as dimeric proteins in numerous human tissues, are members of the TGFβ family. Inhibins are dimers of an α subunit and either a βA or βB subunit, whereas activins are homodimers or heterodimers of the βA or βB subunits. Activin bioavailability is regulated by various extracellular binding proteins (6). Whereas activin-inhibin antagonism is dependent on their competitive binding to type II activin receptor, follistatin and FLRG (also named FSTL3) bind activin with high affinity, thereby preventing its binding to membrane receptors (7–9). Several reports have underlined the importance of the activin-follistatin system in normal and tumor tissues (10, 11). However, the role of this balance in tumor cells has been poorly investigated (12).

Activin displays antiproliferative properties in several cell types (13, 14). Both follistatin and FLRG are extracellular inhibitors of activin signaling. It thus seems important to consider these factors when studying the alteration of responsiveness to activin in cancer cells. We previously reported that FLRG expression is widely variable in human tumor cell lines from different tissue origins (15). Here, we show that FLRG overexpression could contribute to tumor progression through inhibition of endogenous activin functions. Using small interfering RNA (siRNA), we observed that FLRG silencing induced significant growth inhibition, which is reversible upon addition of exogenous FLRG. We showed that this effect resulted from the restoration of activin functions. Finally, FLRG overexpression was observed in primary breast epithelial tumor cells but not in their normal counterpart. Our data provide for the first time strong evidences that endogenous FLRG contributes to tumor cell proliferation through antagonizing activin effects.

Materials and Methods
Cells and cytokines. All cell lines used in this study were purchased from American Type Culture Collection. HeLa (cervical carcinoma), HepG2 (hepatocarcinoma), MDA-MB231, MDA-MB453, and MDA-MB459 (breast cancer) cell lines were grown in DMEM (Cambrex) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 units/mL). MCF7 cells (breast cancer) medium was supplemented with 0.01 mg/mL insulin and 16 μg/mL d-glutathione. MDA-MB361 cells (breast cancer) were grown in DMEM with 20% FBS, streptomycin (100 μg/mL), and penicillin (100 units/mL). MCF7 cells (breast cancer) medium was supplemented with nonessential amino acids (Cambrex). Reconstituent human FLRG, activin A, and soluble type II activin receptor (sActRII) were purchased from R&D Systems.

Activin A ELISA assay. Supernatants were collected from confluent cultures and tested in duplicate for Activin A concentrations using a specific two-site enzyme immunoassay purchased from Serotech.

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Transfection assays. Two siRNA sequences targeting human FLRG mRNA were designed by Eurogentec. Oligonucleotide sequences and nucleotide positions were siRNA-1: 5'-ACAACAAGCUACCUCAAU dTdT 3' (nt 645–663); siRNA-2: 5'-AGAUCAACCCUCCUGGCUU dTdT 3' (nt 246–264), located on human FLRG sequence (accession no. U76702). The day before transfection, cells were plated at 150,000 cells per 1.5 mL per well in six-well plates. Transfection was done using LipofectAMINE 2000 (Invitrogen) in UltraMEM medium (Cambrex) containing 50 nmol/L of scramble or siRNA-FLRG according to the manufacturer's instructions. After 5 h, the transfection medium was replaced by complete DMEM supplemented or not with FLRG (300 ng/mL) or sActRII (500 ng/mL).

Proliferation assays. SiRNA-treated cells were harvested from six-well plates 48 h posttransfection and seeded (1500 cells per 0.2 mL) in triplicate in 96-well plates. Uptilblue (20 µL; Interchim) was added to each well for the last 5 h of culture. The fluorescence intensity was monitored at 530-nm excitation wavelength and 590-nm emission wavelength in a multiwell plate reader (CytoFluor, PerSeptive Biosystem).

Reverse transcription-PCR analysis. Total RNA was isolated using Tri Reagent (Sigma). All reagents for reverse transcription-PCR (RT-PCR) were purchased from Invitrogen. RNA samples (1 µg) were treated with DNaseI, randomly primed and reverse transcribed with SuperScript II reverse transcriptase, according to the manufacturer's instructions. Reverse transcription reactions were carried out in a total volume of 20 µL, and 1.5 µL of cDNA was amplified by PCR using the Platinum Taq Polymerase kit for 25 cycles. Primers and PCR conditions used in this study are described elsewhere (16), except for p21: 5'-GTCCGTCAGAACC- CATCGGCG-3' (forward) and 5'-TAGAAAATCTGT CATC TTGTCGTCG-3' (reverse). PCR products were resolved on ethidium bromide–stained agarose gels and visualized under UV light.

Western blot analysis. Supernatants were collected from confluent cultures for basal expression or 48 h after transfection in siRNA assays. Cell pellets were lysed on ice in 200 to 500 µL of lysis buffer consisting of 50 mmol/L Tris-HCl [pH 7.5, 150 mmol/L NaCl, 1% NP40, 1 mmol/L NaF, 1 mmol/L Na3VO4 supplemented with complete mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail I (Sigma)]. Protein concentrations were determined through a bicinchoninic acid assay (Pierce). A 30-µg to 50-µg fraction of the total protein or 45 µL of culture supernatants was size-separated on a 12% SDS-PAGE, electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) for 1 h at 100 V, 250 mA. Membranes were blocked for 30 min in TBS–0.1% Tween containing 5% nonfat dry milk (w/v) before incubation with specific antibodies. The rabbit anti-FLRG serum (1:2,000) was obtained by immunization with a glutathione 5-transferase–FLRG fusion protein. Antifollistatin (AF669) polyclonal goat antibody (0.5 µg/mL) was purchased from R&D Systems. Phospho-smad2 (3101S; 1:1,000) and smad2 (3102; 1:1,000) antibodies were purchased from Cell Signaling Technology. p27 (SX53G8; 1:250), p21 (1:1,000; SX118), rabbit anti-mouse horseradish peroxidase (HRP) (PO260; 1:10,000) and goat anti-rabbit HRP (PO448; 1:10,000) antibodies were purchased from DAKO-Cytomation. Immunoblotted proteins were visualized using the chemiluminescence Western blotting detection system (Santa Cruz Biotechnology).

Immunostaining. Immunocytochemistry experiments on cell lines were done using an antiactivin (βA, MCA95OST, Serotec) and the Ultratech HRP kit (Coulter, Immunotech) according to the manufacturer's instructions. Briefly, cells were grown on coverslips, fixed in cold methanol for 2 min, washed in PBS, saturated in protein blocking agent, and incubated 1 h in PBS containing the primary antibody (1.25 µg/mL). After washing, coverslips were sequentially incubated with the biotinylated secondary antibody, the avidin-streptavidin complex solution, and revealed by addition of the substrate. After washing, coverslips were counterstained with hematoxylin and mounted over microscope slides (Starfrost).

Paraffin-embedded breast tumor tissues fixed in Bouin Hollande solution were used for analysis. The pathologist selected representative areas from the tumor and, whenever possible, from the normal tissue surrounding the
invasive carcinoma. Three cores from invasive carcinoma and two cores from normal tissue were collected to build the Tissue Microarray block containing 20 different tumors and three normal breasts. The block was sectioned at 4-µm thickness. After deparaffinization and rehydration, slides were boiled in 10 mmol/L citrate buffer (pH 6.0) using a microwave oven for 15 min at 750 W, then incubated in 5% hydrogen peroxide to block endogenous peroxidases.Slides were incubated for 1 h at room temperature in the presence of anti-FLRG (R&D Systems), anti-follistatin (R&D Systems) diluted at 1.25 µg/mL, or anti-activin antibodies diluted at 1:50 (hMCA950ST, Serotec). After washing in PBS, a biotinylated secondary antibody bound to a streptavidin-peroxidase conjugate (LSAB+ kit, Dako) was added. Adding the substrate 3,3-diaminobenzidine revealed bound antibody. After washing, slides were counterstained with hematoxylin and independently analyzed by two investigators.

Results

FLRG, follistatin, and activin basal expression. We previously reported that FLRG transcript levels were highly variable, ranging from barely undetectable to very strongly expressed in human tumor cell lines from different tissue origins (15). To assess the role of the activin-FLRG/follistatin system in breast cancer cells, we first analyzed the expression of FLRG, follistatin, and activin in seven breast cancer cell lines and in HeLa and HepG2 cell lines. Western blot analysis of culture supernatants revealed a significant level of FLRG expression in HeLa, MCF7, MDA-MB436, MDA-MB459, and HS578T cells, whereas significant expression levels of follistatin isoforms were detectable only in MDA-MB459 and HS578T cells (Fig. 1A). Activin expression analyzed by Western blot using culture supernatants failed to detect any signal, whereas immunostaining analysis showed a strong cytoplasmic staining in all the cell lines analyzed (Fig. 1B). Altogether, these results indicated that in the cell lines analyzed, the activin-FLRG/follistatin system mainly involved FLRG as extracellular antagonist of activin.

Altered response to activin in FLRG-positive cells. The antiproliferative effects of activin have been described in normal and tumor epithelial cells (13, 17). However, a loss of responsiveness to activin, resulting from alterations of the signaling components, has been frequently observed in breast cancer cells (4, 18). The activin-FLRG antagonism led us to analyze the proliferative response of FLRG-expressing cell lines under activin treatment (Fig. 2A). Whereas a time-dependent and dose-dependent growth inhibition was observed in HepG2 cells referred to as activin responders (7), the other cells exhibited only weak (MCF7, MDA-MB459) or undetectable (HeLa, MDA-MB436, HS578T) responses. To examine whether this absence of response resulted from alterations of the activin signaling pathway, FLRG-expressing lines were tested for their ability to phosphorylate smad2 under activin treatment, using HepG2 cells as a positive control (Fig. 2B). Cells were washed to remove endogenously secreted FLRG and were cultured with activin and/or FLRG. A strong activin-induced smad2 phosphorylation was observed at each time point and in all cell lines. Accordingly, no smad2 phosphorylation was detectable in HS578T cells, reported elsewhere to be unresponsive to activin due to a low level of expression of activin receptors (18). As expected, the activin-induced phosphorylation of smad2 was inhibited by recombinant FLRG (Fig. 2B). These results showed that despite the absence of detectable activin-induced growth inhibition, the activin signaling pathway was functional in all the tested cell lines, except for HS578T. However, one cannot exclude that an alteration could have occurred downstream of smad2 phosphorylation, which would explain the absence of detectable activin-induced growth

Figure 2. Altered antiproliferative response to activin in FLRG-expressing cells. A, cell lines were cultured with indicated doses of activin, and proliferation was assessed at indicated days. Points, mean values representative of three independent experiments done in triplicate; bars, ± SD. B, activin-induced phosphorylation of smad2. After extensive washing to remove endogenous FLRG, cells were cultured in X-VIVO serum-free medium (Cambrex) with or without activin (25 ng/mL) and/or FLRG (100 ng/mL). Levels of phospho-smad2 were determined by Western blot analysis using an anti-phospho-smad2 antibody. Membranes were subsequently incubated with anti-smad2 antibody.
inhibition. To rule out this hypothesis, RT-PCR analysis were done and showed that smad4 was expressed in all the tested cell lines. Furthermore, proliferation assays carried out with high concentration (200 ng/mL) of activin revealed a significant growth inhibition in HeLa cells (data not shown). Altogether, these data indicated that FLRG could be the endogenously secreted factor that prevents the antiproliferative effect of exogenous activin despite a functional activin signaling pathway.

**Silencing of FLRG inhibits cell proliferation.** To determine the involvement of FLRG in cell proliferation, we silenced FLRG expression in activin-expressing cell lines (HeLa, MCF7, MDA-MB436, and MDA-MB459). Two siRNAs targeting FLRG mRNA sequence were designed, and a standard scramble siRNA was used as control. Transfection with siRNA-1 or siRNA-2 induced a significant down-regulation of FLRG expression at both transcript (Fig. 3A, left) and protein levels (Fig. 3A, right), with a higher efficiency of siRNA-2 compared with siRNA-1. To examine the effect of FLRG silencing, cells were assayed for proliferation (Fig. 3B). A significant growth inhibition was observed in all siRNA-treated cells and compared with scramble-treated cells. Consistent with the efficiency of the two siRNAs, a strongest effect was observed when using siRNA-2. To ascertain that the growth inhibition specifically resulted from FLRG silencing, siRNA-2-transfected HeLa and MCF7 cells were complemented with exogenous FLRG and assayed for proliferation (Fig. 3C). In the presence of FLRG, the proliferation of FLRG-silenced cells was restored to the level of scramble-transfected cells for both cell lines, demonstrating that the antiproliferative effect of siRNA treatment specifically resulted from the down-regulation of FLRG expression. Interestingly, no growth inhibition was observed in siRNA-treated HS578T cells (data not shown) despite a significant FLRG silencing (Fig. 3A), excluding any siRNA-related toxicity. Altogether, these results indicated that endogenous FLRG contributes to spontaneous growth of HeLa, MCF7, MDA-MB436, and MDA-MB459 cells, probably through antagonizing endogenous activin and that HS578T cell proliferation was independent of FLRG.

**FLRG silencing restores activin function.** To test our hypothesis, despite the strong activin staining observed by immunocytochemistry (Fig. 1B), we first validate the presence of secreted activin in cell supernatants. Using a specific two-site enzyme immunoassay, we detected secreted activin A in HeLa, MCF7, MDA-MB436, and MDA-MB459 at concentrations ranging from 0.2 to 2 ng/mL (±0.005). We then analyzed the expression of activin target genes and the phosphorylation status of smad2 in FLRG-silenced cells. The level of phospho-smad2 was increased in siRNA-1–treated or siRNA-2–treated cells compared with scramble-treated cells (Fig. 4B). These results provide the first evidence that the level of FLRG expression can influence the phosphorylation status of smad2 in activin-expressing cells. We further analyzed the expression of activin target genes (Fig. 4A). An up-regulation of p21 transcripts was observed in siRNA-treated cells compared with scramble-treated cells. This result was confirmed at the protein level by Western blot analysis (Fig. 4B). Furthermore, an increased level of p27 protein was also observed in siRNA-treated cells (Fig. 4B). The up-regulation of these negative cell cycle regulatory proteins was in accordance with the growth inhibition observed in FLRG-silenced cells. Two additional activin transcriptional targets, ActRI (19) and follistatin (7), were up-regulated in FLRG-silenced cells (Fig. 4A). Interestingly, this observation indicated that both positive (ActRI) and negative (follistatin) activin regulatory elements are concomitantly up-regulated upon FLRG silencing. As expected, FLRG silencing did affect neither the transcript levels of activin target genes nor the proliferation of siRNA-treated HS578T cells (Fig. 4A). In accordance with our hypothesis, treatment of siRNA-treated HeLa cells with a sActRII
markedly attenuated the siRNA-induced growth inhibition (Fig. 4C). Altogether, our results show that the growth inhibition induced by FLRG silencing resulted from activation of endogenous activin signaling.

**FLRG overexpression in primary breast tumor cells.** Our *in vitro* observations prompted us to study the pattern of FLRG expression in a series of primary breast tumors using immunohistochemistry. We analyzed a series of 34 invasive breast carcinomas and compared them to normal tissue. In normal mammary tissue, FLRG exhibited a strong cytoplasmic staining, which was restricted to the basal myoepithelial cells in contrast to the surrounding luminal epithelial cells, in which no FLRG was detected (Fig. 5).

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**Figure 4.** Restoration of activin functions in FLRG-silenced cells. RT-PCR (A) and Western blot (B) analysis were done 48 h after transfection of siRNA or scramble-treated cells. Phosphorylation of smad2 and expression of activin target genes were analyzed. C, sActRII (500 ng/mL) was added on the day of transfection and maintained when seeding cells for proliferation tests. Proliferation was measured at days 3 and 4 (*D3 and D4*). Points, mean values of representative of three independent experiments done in triplicate; bars, ± SD.

**Figure 5.** Immunostaining of primary breast normal and tumor samples. Immunostaining of FLRG, follistatin, and βA subunit are shown (magnification, ×40) in normal breast tissue and invasive breast carcinoma expressing FLRG or not. Arrow, endothelial cells staining by FLRG.
A strong staining was also detected in endothelial cells but not in stromal cells. Twenty-four of thirty-four invasive carcinomas expressed high levels of FLRG in infiltrative carcinoma cells. The luminal phenotype of tumor cells was confirmed by the absence of cytokeratin 5-6 staining (data not shown) indicating that FLRG-expressing tumor cells were not derived from basal cells (20, 21). We further analyzed FLRG, follistatin and activin patterns of expression in 22 invasive breast carcinomas and compared them with normal tissue. As previously described (4, 22), in normal tissue, activin was significantly expressed in luminal cells but was not detected in basal cells. A similar pattern of expression was observed for follistatin (Fig. 5). In contrast to FLRG, no marked variation of activin and follistatin expression was observed in tumor cells when compared with normal tissue, except for two samples in which follistatin staining was undetectable (Table 1). Altogether, these observations revealed that FLRG was overexpressed in tumor cells compared with their normal counterpart, strengthening the idea that FLRG may play a major role in antagonizing activin in breast tumor cells.

**Discussion**

Activin signaling has been associated both with mammary cell growth inhibition and regulation of mammary glandular development. Activin effects are regulated at the extracellular level by FLRG and follistatin. Alterations of activin signaling in cancer cells are usually linked to mutations or aberrant regulation of signaling components, such as receptors and smad proteins (4, 5, 22). In the present study, we evidence a direct correlation between FLRG expression and the absence of activin-induced growth inhibition in mammary tumor cells. We show that FLRG-expressing cells display an altered response to activin and that FLRG silencing restores endogenous activin function. These data are the first evidence that endogenously produced FLRG contributes to tumor cell spontaneous growth and that the level of FLRG expression can markedly influence the proliferative potential of cancer cells. Follistatin overexpression has been reported by others in melanomas and hepatocarcinomas, but the correlation between follistatin level of expression and activin function has not been analyzed (12, 23, 24). Here, we show that both normal and tumor luminal cells express activin and follistatin in contrast to FLRG, which expression is seen in carcinomatous cells but not in normal cells. These results, together with data obtained with cell lines, indicate that the activin-FLRG/follistatin system mainly involves FLRG as extracellular antagonist of activin and that FLRG overexpression results in the alteration of activin signaling in breast tumor cells. Overexpression of Cripto, a protein belonging to the epidermal growth factor–colony-forming cell family, is found in several human tumors, including breast cancers, and is associated with the development of mammary tumors in mice (25). Cripto acts as a coreceptor for Nodal, a member of the TGF-β superfamily, and has also been shown to regulate signaling of other TGF-β ligands, such as activin. Despite some discrepancies regarding the molecular mechanisms, two groups (26, 27) have clearly identified Cripto as a novel extracellular negative regulator of activin signaling. Therefore, disequilibrium of the activin-Cripto balance might represent an additional mechanism in promoting transformation.

Our previous finding of high FLRG expression in different tumor types (15) suggests that this phenomenon is probably not tissue-restricted. The broad tissue distribution of activin and FLRG and their production as secreted proteins indicate that both normal and tumor cells can be targeted by a disruption of the activin-FLRG

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balance. The role of the activin in angiogenesis and carcinogenesis has been reported. Because activin plays a role in angiogenesis and carcinogenesis through inhibiting endothelial cell proliferation (28, 29), FLRG expression may also promote tumor progression through the modulation of activin effects on endothelial cell proliferation and angiogenesis.

In summary, our study indicates that FLRG expression represents a novel mechanism that promotes tumor cell proliferation through counteracting activin effects. The analysis of a large panel of clinically characterized breast cancer specimens should allow the evaluation of the prognosis and/or diagnosis value of FLRG expression status and should provide new insights into the frequent unresponsiveness of tumor cells to activin. The sensitivity of mammary tumor cells to activin suggests the activin-FLRG system as a promising target for therapeutic intervention.

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