

## Local and Systemic Protumorigenic Effects of Cancer-Associated Fibroblast-Derived GDF15

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### Abstract

The tumor stroma is vital to tumor development, progression, and metastasis. Cancer-associated fibroblasts (CAF) are among the abundant cell types in the tumor stroma, but the range of their contributions to cancer pathogenicity has yet to be fully understood. Here, we report a critical role for upregulation of the TGF $\beta$ /BMP family member GDF15 (MIC-1) in tumor stroma. GDF15 was found upregulated *in situ* and in primary cultures of CAF from prostate cancer. Ectopic expression of GDF15 in fibroblasts produced prominent paracrine effects on prostate cancer cell migration, invasion, and tumor growth. Notably, GDF15-expressing fibroblasts exerted systemic *in vivo* effects on the outgrowth of distant and otherwise indolent prostate cancer cells. Our findings identify tumor stromal cells as a novel source of GDF15 in human prostate cancer and illustrate a systemic mechanism of cancer progression driven by the tumor microenvironment. Further, they provide a functional basis to understand GDF15 as a biomarker of poor prognosis and a candidate therapeutic target in prostate cancer. *Cancer Res*; 74(13); 3408–17. ©2014 AACR.

### Introduction

Cancer-associated fibroblasts (CAF) stimulate tumor growth and progression, angiogenesis, and metastasis, are involved in the attraction of different cell types into the tumor, and have been shown to modulate tumor drug uptake and drug sensitivity (1, 2). Characterization of CAF-derived factors can provide insight into tumor biology and identify novel potential cancer drug targets. Previous studies have demonstrated comparative gene-expression analyses of microdissected tumor stroma and normal stroma as a productive strategy for identification of functionally relevant CAF-derived proteins (3, 4).

GDF15 (growth/differentiation factor 15), also designated as macrophage inhibitory cytokine-1 (MIC-1), prostate-derived factor, placental bone morphogenic protein, placental transforming growth factor, and nonsteroidal antiinflammatory drug-activated gene 1 (NAG-1), is a divergent member of the BMP subfamily of the TGF $\beta$  superfamily. The protein is

expressed as a preprotein with an N-terminal propeptide and the mature GDF15 domain at the C-terminus (5).

Under physiologic conditions, GDF15 is expressed at low levels in most tissues and cell types, with the exception of the placenta and macrophages (6). However, its expression is markedly increased in cardiovascular disease, chronic inflammation, and injury (7–10). GDF15 is also overexpressed in prostate, colon, pancreas, thyroid, and breast carcinomas (10, 11). High expression levels within the primary tumor are associated with high GDF15 serum levels, suggesting continued release from the tumor into the blood stream. Importantly, enhanced GDF15 serum levels are associated with disease progression, shorter survival, and recurrence (9, 10, 12–14). Experimental and clinical studies have additionally implied a role for GDF15 in protection against chemotherapy (15, 16). GDF15 is thus becoming increasingly recognized as a potential clinically relevant predictive biomarker (10, 17). Support for this notion has recently been provided through the demonstration that an enhanced serum level of GDF15 is a predictor of all-cause mortality (18).

Pro- and antitumor activities have been described for GDF15 and a role in resistance to chemotherapy (11, 19–25). Overexpression of this factor reduces the tumorigenic potential of some cancer cells, but enhances the protumorigenic activity of other cancer cells (21, 26–30). Importantly, recent studies identified GDF15 as a prometastatic factor in different prostate cancer models (31, 32). Together, these findings suggest that GDF15 action is highly dependent on the cellular and microenvironmental context and on experimental conditions (6, 11).

The present study demonstrates higher *GDF15* mRNA levels in tumor stroma, as compared with normal stroma, in human prostate tissue. This finding was used as a starting point to

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investigate the functional roles of stroma-derived GDF15 in prostate tumorigenesis. For this purpose, fibroblasts were engineered to overexpress GDF15, and their functional properties were subsequently characterized *in vitro* and *in vivo*.

## Materials and Methods

### Parental and derived cell lines

Cells were kept under standard culture conditions. For details see Supplementary Material and Methods. The authenticity of LNCaP cells was confirmed by short tandem repeat (STR) profiling carried out at last in December 2013. The identity of LAPC-4 cells could not be analyzed because a reference for this cell line was lacking. NIH3T3 cells were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) that uses STR for the authentication of cell lines. Here, we used NIH3T3 derivatives established by viral transduction as described in Supplementary Material and Methods.

### Laser capture microdissection and qRT-PCR analysis

For details about laser capture microdissection (LCM), RNA isolation, and qRT-PCR expression analyses, we refer to the reference (3) and Supplementary Material and Methods where also primer sequences are listed.

### Prostate tissue microarray, *in situ* hybridization, and immunohistochemistry

For detailed information about the tissue microarray and mRNA *in situ* hybridization, we refer to Supplementary Material and Methods. Immunohistochemistry and immunofluorescence to detect GDF15, CD31, CD68, and PDGFR- $\beta$  were largely performed as described in the supplements of ref. 3. The staining was quantified by grading the fraction of stained area of each tumor section on a scale ranging from 0 (no signal) to 5 (signals distributed over the whole section) or by counting.

### ELISA for analysis of GDF15 protein expression

A total of  $1 \times 10^5$  cells, prostate cancer cells, or NIH-ctr and NIH-GDF15 fibroblasts were seeded per well of a 6-well plate. The next day growth medium was replaced by medium supplemented with 1% FBS. A total of 1.5-mL conditioned medium was collected after 24, 48, and 72 hours culture of these cells in low serum medium and sterile filtered. A total of 100- $\mu$ L aliquots of the conditioned medium were subjected to a GDF15-specific ELISA (R&D Systems) that was performed according to the manufacturer's protocol. GDF15 levels of blood serum samples collected from the xenograft studies at sacrifice were determined by subjecting 100  $\mu$ L aliquots of 1:4 diluted serum to the GDF15-specific ELISA (R&D Systems).

### Growth, migration, invasion, and clonogenicity assay

The growth of fibroblast and prostate cancer cells under different conditions was monitored using a crystal violet assay. Paracrine effects of fibroblasts on the growth of prostate cancer cells were evaluated by coculture of LNCaP-eGFP cells together with NIH-GDF15 or NIH-ctr cells. Cell number was derived by measuring GFP-fluorescence. The migration and invasion of LNCaP-eGFP cells cocultured with NIH-ctr or NIH-GDF15 fibroblasts was analyzed using Transwell chambers in a

6-well plate format containing 8- $\mu$ m pore-sized polycarbonate inserts (Corning Costar), with or without Matrigel. Analyses of effects of recombinant GDF15 (R&D Systems) on the invasion of prostate cancer cells were measured following labeling with Cell Tracker green fluorescence dye (Invitrogen). A more detailed description of the different assays is provided in Supplementary Material and Methods.

### Xenograft experiments

The animal experiments were conducted in accordance with national guidelines and approved by the Stockholm North Ethical Committee on Animal Experiments. The xenograft experiments followed procedures as described in ref. 3. "Instigator/responder" experiments followed a design introduced by McAllister and colleagues (33). In brief, a suspension of prostate cancer cells alone or admixed with fibroblasts ("instigator tumors") was injected in one flank of male SCID mice. Subsequently, GFP-expressing LNCaP cells mixed with Matrigel ("responder tumors") were injected in the contralateral flank of the same animal. Both instigator and responder tumors were resected when the size of the instigator tumor had reached a volume between 600 and 900 mm<sup>3</sup>. For further details, see Supplementary Material and Methods.

### Statistical analysis

The results of the different assays are expressed as mean and SEM. The statistical significance of differences was determined by a two-sided Student *t* test, one-way ANOVA with Newman-Keuls Multiple Comparisons post-tests, and two-way ANOVA with Bonferroni post-tests. The statistical evaluations were done using Sigma Stat software (Systat Software Inc.).

## Results

### GDF15 is overexpressed in human prostate cancer stroma

To identify novel factors expressed by the prostate tumor stroma, a microarray analysis was performed comparing normal and tumor prostate stroma isolated by LCM from tissue of four patients with prostate cancer (3). *GDF15* was one of the factors found to be highly upregulated (8-fold on the microarray) in prostate tumor stroma. Upregulation of *GDF15* in tumor stroma was confirmed, by qRT-PCR analysis, in six of eight cases of prostate cancer (Fig. 1A). Stromal *GDF15* expression levels increased with the histopathologic grade (Gleason 4 > Gleason 3 > normal) implying a role of stroma-derived *GDF15* in the course of prostate cancer progression (Fig. 1B).

To investigate the tissue distribution of GDF15 protein in prostate cancer, immunohistochemistry analyses were performed (Supplementary Fig. S1). In line with previous studies (5, 20), GDF15 protein was found abundantly in the prostate tumor epithelium with variation among the different samples. However, with a few exceptions, we did not detect GDF15 protein in stromal cells or the extracellular matrix (Supplementary Fig. S1).

In contrast, *in situ* hybridization of prostate cancer tissue microarrays revealed *GDF15* gene expression also in the stroma and not only in cancer cells (Fig. 1C). Stromal expression was predominantly detected in elongated, matrix-aligned cells

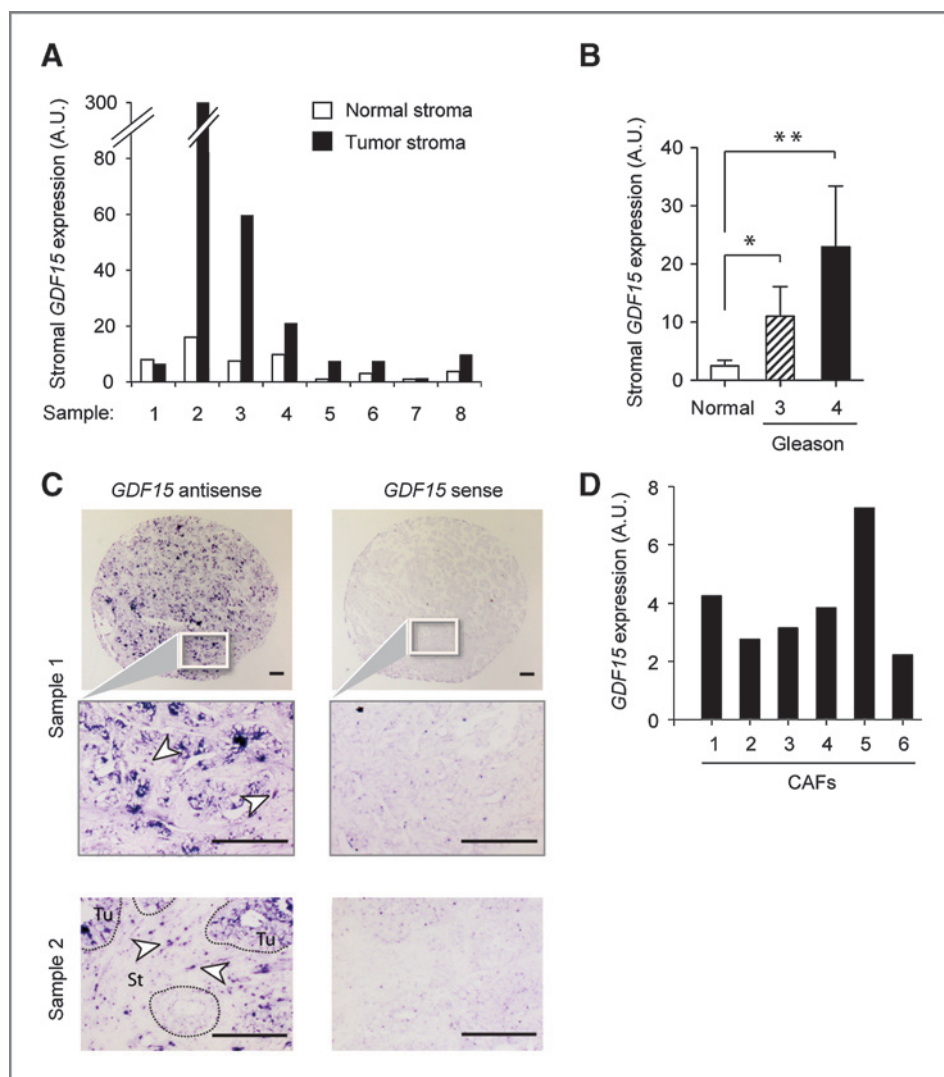


Figure 1. GDF15 is overexpressed in prostate cancer stroma. A, *GDF15* expression was analyzed with qRT-PCR in microdissected stroma of normal and cancerous tissue from 8 individual patients with prostate cancer. B, *GDF15* qRT-PCR analysis of microdissected prostate stroma comprising areas of normal and cancerous tissue (Gleason grades 3 and 4) derived from four different individuals. C, staining of prostate cancer tissue by *in situ* hybridization detecting different cell types expressing *GDF15*. Arrowheads, *GDF15* positivity in stromal, fibroblast-like cells. St, tumor stroma; Tu, tumor epithelium. Scale bar, 100  $\mu$ m. D, *GDF15* expression analysis of primary, human prostate CAFs by qRT-PCR. Statistics for B: \*,  $P = 0.0226$ ; \*\*,  $P = 0.0027$ . Error bars, SEM.

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resembling fibroblasts, vascular, and/or vasculature-associated cells (Fig. 1C). High *GDF15* expression in both stroma and epithelium was the most abundant staining pattern of the cases analyzed. Subsequent analysis of primary human prostate CAFs, established in our laboratory (34), confirmed that fibroblasts express *GDF15* as proposed by the *in situ* staining (Fig. 1D).

These findings implicate stromal cells and fibroblasts in particular as an important, previously unrecognized source of *GDF15* in prostate cancer.

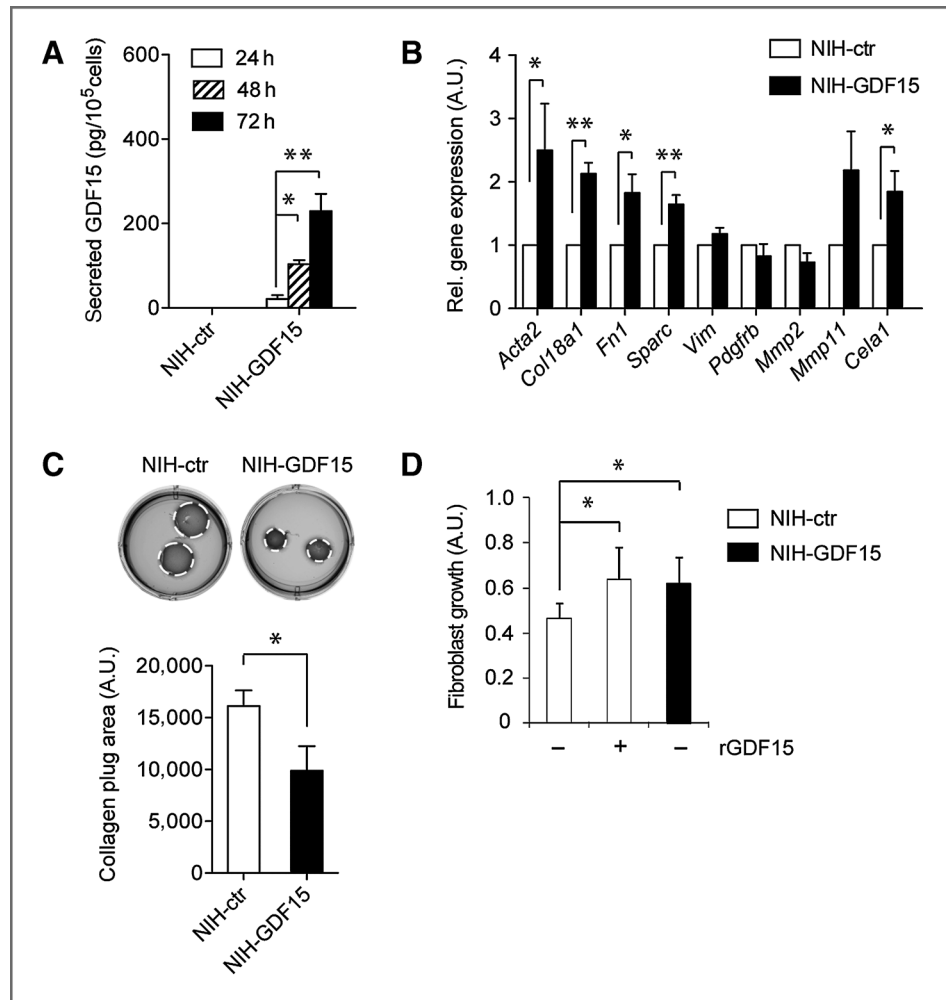
### Generation and characterization of fibroblasts overexpressing GDF15

The enhanced expression of *GDF15* by CAFs has previously not been recognized. Therefore, we decided to study the impact of fibroblast-derived *GDF15* on prostate cancer development.

NIH3T3 mouse fibroblasts stably expressing human *GDF15* (designated NIH-GDF15) were generated together

with a control cell line (named NIH-ctr). Expression analyses using species-specific primers (see Supplementary Materials and Methods for details) revealed that both NIH-ctr and NIH-GDF15 cells lack endogenous expression of *Gdf15*, but *GDF15*-fibroblasts express human *GDF15* abundantly (Supplementary Fig. S3B). *In situ* hybridization of fixed cells confirmed expression of *GDF15* by NIH-GDF15 but not NIH-ctr cells (Supplementary Fig. S2B). Consistent with the transcriptional profile, *GDF15* fibroblasts secreted increasing amounts of *GDF15* protein into the conditioned medium over the course of 3 days of culture, whereas the *GDF15* levels in medium from control fibroblasts were below detection level (Fig. 2A). The levels of secreted *GDF15* produced by the engineered fibroblasts were lower, but of similar magnitude, as those of the prostate cancer cells (Supplementary Fig. S3C) and primary prostate cancer cells (Supplementary Fig. S3D). Despite production of *GDF15*, no major protein accumulation was found in the engineered *GDF15* fibroblasts, in contrast to LNCaP prostate cancer cells (Supplementary Fig.

**Figure 2.** GDF15 stimulates proliferation of fibroblasts. **A**, measurement of GDF15 protein levels by ELISA in conditioned medium of engineered NIH3T3 derivatives expressing GDF15 (NIH-GDF15) or empty vector control (NIH-ctr) collected at the time points indicated. **B**, qRT-PCR analysis of CAF-marker expression by NIH-ctr and NIH-GDF15 fibroblasts. **C**, pictures of collagen discs (top) and quantification of the collagen disc surface area (bottom) from cultures of NIH-ctr and NIH-GDF15 cells embedded in collagen for 72 hours. **D**, the effect of 50 ng/mL recombinant GDF15 (rGDF15) on NIH-ctr cell growth was evaluated by measuring the cell number of stimulated and unstimulated cells following 4 days of culture in low serum. Under the same conditions, the effect of GDF15 overexpression on fibroblast growth was evaluated by comparing the cell number of NIH-ctr and NIH-GDF15 cells. The average of three (A–C) and four (D) independent experiments is shown. **A**, NIH-GDF15, 72 versus 24 hours; \*\*,  $P = 0.0077$  and 72 versus 48 hours; \*,  $P = 0.040$ . **B**, \*\*,  $P < 0.01$  and \*,  $P < 0.05$ . **C**, \*,  $P = 0.01$ . **D**, \*,  $P < 0.05$ . Error bars in each figure indicate SEM.



S2A). Notably, the staining pattern of NIH-GDF15 cells, with clear mRNA expression but no detectable intracellular protein accumulation (Supplementary Fig. S2), is similar to the pattern observed in prostate cancer tissue (Fig. 1C and Supplementary Fig. S1).

Further characterization of the engineered fibroblasts revealed significant induction of *Acta2* ( $\alpha$  smooth muscle actin) and upregulation of different transcripts encoding extracellular matrix proteins (*Col18a1*, *Fn1*, and *Sparc*) and matrix-remodeling enzymes (*Mmp11* and *Cela1*) in NIH-GDF15 compared with NIH-ctr cells (Fig. 2B). Moreover, GDF15 fibroblasts displayed an enhanced capacity to contract collagen (Fig. 2C). NIH-GDF15 cells also displayed an increased growth rate, and recombinant GDF15 enhanced the growth of NIH-ctr fibroblasts (Fig. 2D).

**Paracrine stimulation of prostate cancer cell growth, migration, and invasion by fibroblast-derived GDF15**

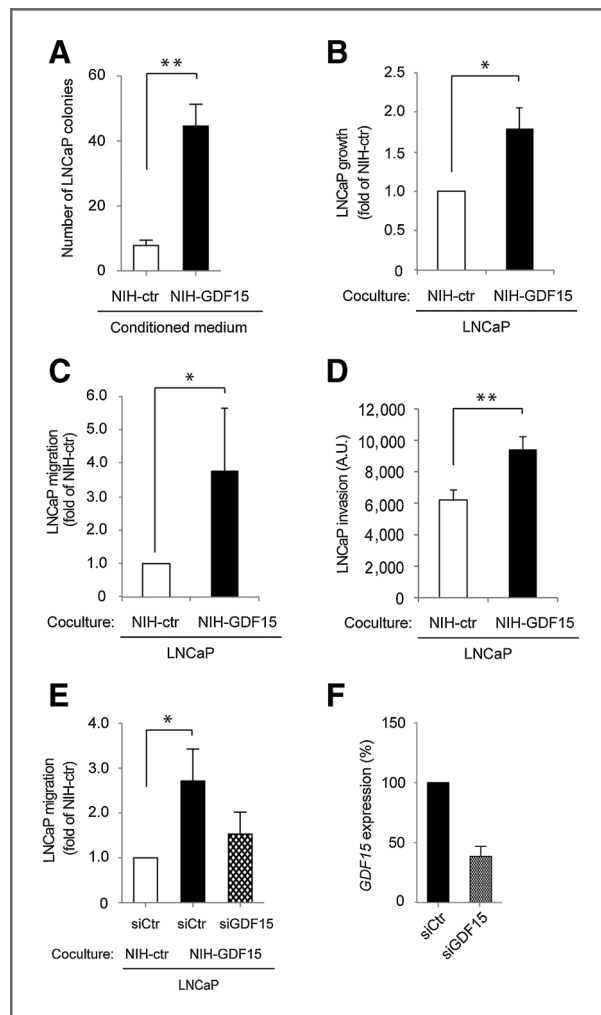
Since our tissue analysis of prostate cancer clinical samples revealed abundant expression of *GDF15* by cancer cells and fibroblasts, we set up a model system recapitulating this expression pattern. Therefore, the prostate cancer cell line

LNCaP was selected, which expresses and secretes GDF15 (5, 35; Supplementary Fig. S3A and S3C).

To analyze growth-stimulatory effects of the two types of fibroblasts, a clonogenic assay was performed. Conditioned medium from NIH-GDF15 was more potent than conditioned medium from control fibroblasts with regard to promote the growth of LNCaP cells (Fig. 3A). We also observed an increase in the number of LNCaP-eGFP cells when they were cultured together with GDF15-expressing fibroblasts as compared with control fibroblasts (Fig. 3B). A similar growth-stimulatory effect of GDF15 was found when these prostate cancer cells were stimulated with recombinant GDF15 protein (Supplementary Fig. S4A). Growth-promoting effects of recombinant GDF15 were also observed on LAPC-4 prostate cancer cells (Supplementary Fig. S4B), which lack expression of endogenous *GDF15* (Supplementary Fig. S3A).

GDF15 fibroblasts were more potently stimulating LNCaP cell migration and invasion, as compared with control fibroblasts (Fig. 3C and D). Knockdown of *GDF15* in NIH-GDF15 cells almost completely abrogated their potential to enhance LNCaP cell migration, confirming the GDF15 dependency of the increased paracrine potency of these cells (Fig. 3E and F).

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**Figure 3.** Overexpression of GDF15 in fibroblasts promotes proliferation, migration, and invasion of cocultured prostate cancer cells. **A**, to evaluate the effects of fibroblast-produced GDF15 on cancer cell growth, LNCaP cells were seeded in low density and cultured in conditioned medium derived from NIH-ctr or NIH-GDF15 fibroblasts grown in 10% FBS. The number of LNCaP colonies was counted after 13 days of culture. **B**, the number of LNCaP-eGFP cells was determined following coculture with NIH-ctr or NIH-GDF15 fibroblasts for 12 days. **C**, to analyze the effect of fibroblast-derived GDF15 on cancer cell migration, coculture experiments in Transwell chambers were performed using LNCaP-eGFP cells and unlabeled fibroblasts (NIH-ctr, NIH-GDF15). After 24 hours under coculture conditions, cancer cells from the lower compartment of the chamber were collected and the number of migrated cancer cells was quantified by measuring eGFP-fluorescence. **D**, the effect of NIH-ctr and NIH-GDF15 cells on LNCaP-eGFP invasion was analyzed by using the migration assay described above and coating the membrane separating the two chambers with a layer of Matrigel. **E**, the role of fibroblast-derived GDF15 as mediator of the promigratory effect of NIH-GDF15 was assessed in a coculture Transwell assay using LNCaP-eGFP cells and fibroblasts transfected with either nontargeting (siCtr) or GDF15-specific siRNA (siGDF15) as depicted in the figure. **F**, the efficacy of siRNA-mediated knockdown of GDF15 was determined by qRT-PCR analysis comparing cells transfected with nontargeting siRNA and GDF15-specific siRNA. The figure presents the average of three (**A**), four (**B**), six (**C**), and three (**D** and **E**) independent experiments, each performed in duplicates. **A**,  $P = 0.037$ . **B**,  $P = 0.011$ . **C**,  $P = 0.029$ . **D**,  $P = 0.003$ . **E**,  $P = 0.049$ . Error bars in each figure indicate SEM.

Furthermore, exogenous GDF15 exerted a proinvasive effect on LAPC-4 cells (Supplementary Fig. S4C).

To analyze the effects of fibroblasts on the secretome of LNCaP cells, a cytokine profiling experiment was performed. LNCaP cells exposed to conditioned medium from cultures of NIH-GDF15 cells secreted higher levels of different cytokines and chemokines (GM-CSF, CXCL1/GRO, IL12, CCL2/MCP-1, CXCL12/SDF-1, and CCL15/MIP-1 $\delta$ ) than those treated with conditioned medium from NIH-ctr cells (Supplementary Fig. S5A). Notably, these factors were also induced when LNCaP cells were exposed to conditioned medium from NIH-ctr cells supplemented with recombinant GDF15 (Supplementary Fig. S5A). An interactome pathway analysis demonstrated that the overexpressed cytokines are related to each other (Supplementary Fig. S5B).

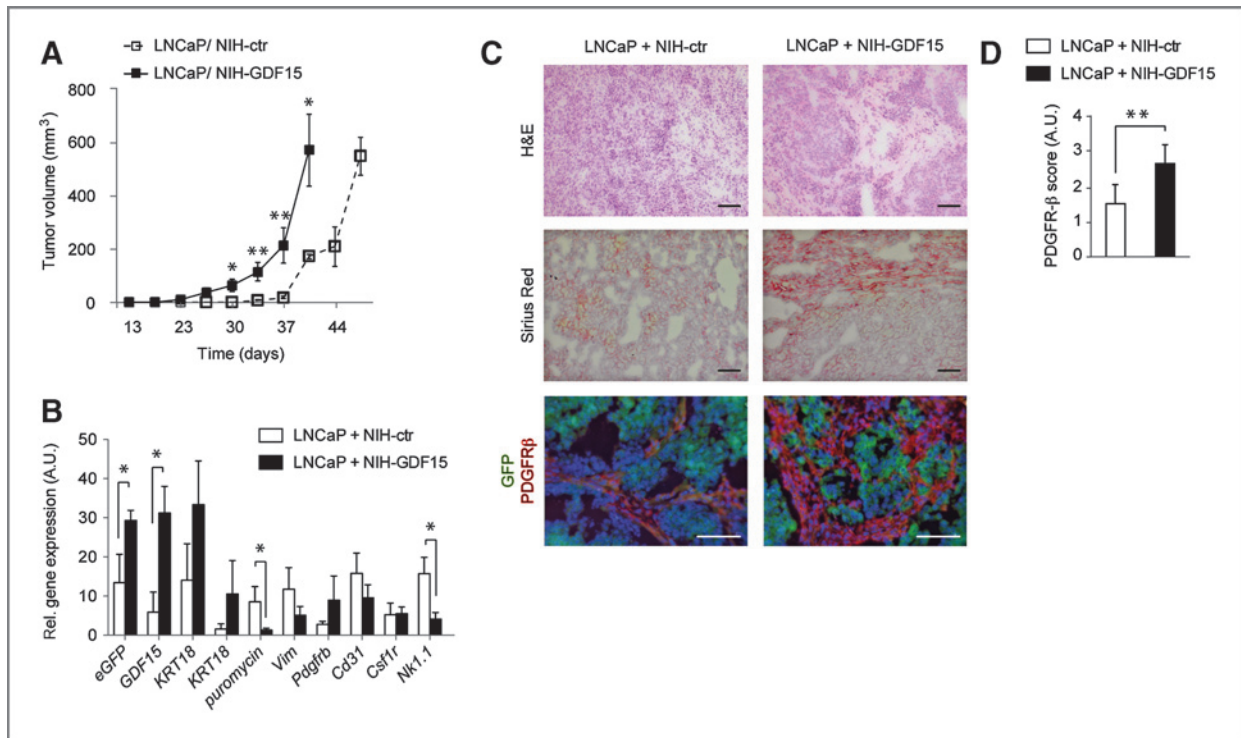
Altogether, these results demonstrated stimulatory effects of fibroblast-derived GDF15 on prostate cancer cell proliferation, migration, invasion, and cytokine release, thus implying an important instructive role of GDF15 in paracrine signaling.

#### GDF15-expressing fibroblasts promote the growth of tumor xenografts

The effect of fibroblast-produced GDF15 on tumor growth *in vivo* was analyzed by using an established fibroblast-dependent coinjection mouse model (36). LNCaP-eGFP prostate cancer cells were mixed with GDF15-expressing (LNCaP/NIH-GDF15) or control fibroblasts (LNCaP/NIH-ctr), and the cell mixtures were injected subcutaneously into SCID mice. As reported previously, LNCaP cells are not able to form tumors when injected alone (3). Importantly, tumors with GDF15-overexpressing fibroblasts demonstrated reduced tumor latency and an enhanced growth as compared with tumors with control fibroblasts (Fig. 4A). Moreover, increased serum levels of GDF15 were detected in mice injected with cancer cells and GDF15-expressing fibroblasts ( $5,500 \pm 1,100$  pg/mL in LNCaP/NIH-GDF15 vs.  $4,300 \pm 860$  pg/mL in LNCaP/NIH-ctr).

Analyses of tumors with a tumor volume in the range of 700 to 900 mm<sup>3</sup> did not reveal an enhanced ingrowth of vessels (*Cd31*) or macrophages (*Csflr*) in LNCaP/NIH-GDF15 tumors compared with LNCaP/NIH-ctr (Fig. 4B). The natural killer cell (*Nkl1*) and tumor stroma marker *vimentin* and *puromycin* (detecting the engineered mouse fibroblasts) were decreased in LNCaP/NIH-GDF15 tumors compared with the control. However, other stromal proteins such as collagens and PDGFR- $\beta$  were more abundantly expressed in LNCaP/NIH-GDF15 tumors (Fig. 4C and D), although *Pdgfrb* expression was not enhanced in NIH-GDF15 fibroblasts (Fig. 2B). This suggests that NIH-GDF15-derived signals enhance the recruitment of host cells contributing to a PDGFR- $\beta$ -positive stroma. Moreover, GDF15 levels and the fraction of epithelial markers, *eGFP*, *KRT8*, and *KRT18*, were also enhanced in LNCaP/NIH-GDF15 tumors (Fig. 4B), compatible with the strong stimulatory effect of GDF15 on prostate cancer cells *in vitro* (Fig. 3).

The xenograft experiment thereby demonstrated that overexpression of GDF15 increased the tumor-stimulatory effect of coinjected fibroblasts.



**Figure 4.** Fibroblast-derived GDF15 enhances tumor growth *in vivo*. A, a fibroblast-dependent tumor model was used to assess the effect of fibroblast-derived GDF15 on tumor growth *in vivo*. Tumor growth was followed after s.c. injection of LNCaP-eGFP cells combined with either GDF15-overexpressing fibroblasts (NIH-GDF15) or control fibroblasts (NIH-ctr) in male SCID mice. B, the expression of different epithelial, stromal, vascular, and immune cell marker in LNCaP/NIH-ctr and LNCaP/NIH-GDF15 tumors was analyzed by qRT-PCR. C, hematoxylin and eosin (H&E) and Sirius Red staining for collagen fibers (red), and immunofluorescence for PDGFR- $\beta$  (red) combined with LNCaP-derived eGFP (green) were used to study the histology of LNCaP/NIH-ctr and LNCaP/NIH-GDF15 tumors. D, semiquantitative analysis of PDGFR- $\beta$  expression in LNCaP/NIH-ctr and LNCaP/NIH-GDF15 tumors. In A, each group consisted of 12 mice. \*,  $P = 0.019$ ; \*\*,  $P = 0.006$ ; \*\*\*,  $P = 0.008$ ; \*,  $P = 0.033$  at days 30, 34, 37, and 41, respectively. D, \*\*,  $P = 0.004$ . Error bars indicate SEM.

**Fibroblast-derived GDF15 stimulates tumor growth at a distant site**

A clear association between serum levels of GDF15 and tumor progression has been reported for different tumor types (10). Another study showed that tumor-derived GDF15 can affect appetite regulation (37). This prompted us to investigate a potential systemic tumor-promoting activity of stromal GDF15. Therefore, the effect of fibroblast-derived GDF15 on tumor growth at a distant site was studied, adopting a previously described experimental approach (33). LNCaP cells were injected alone or together with GDF15-expressing (LNCaP/NIH-GDF15) or control fibroblasts (LNCaP/NIH-ctr) in one flank of a mouse to form instigator tumors. LNCaP cells, mixed with Matrigel, and injected in the contralateral flank of the same mouse, served as responder tumors (Fig. 5A).

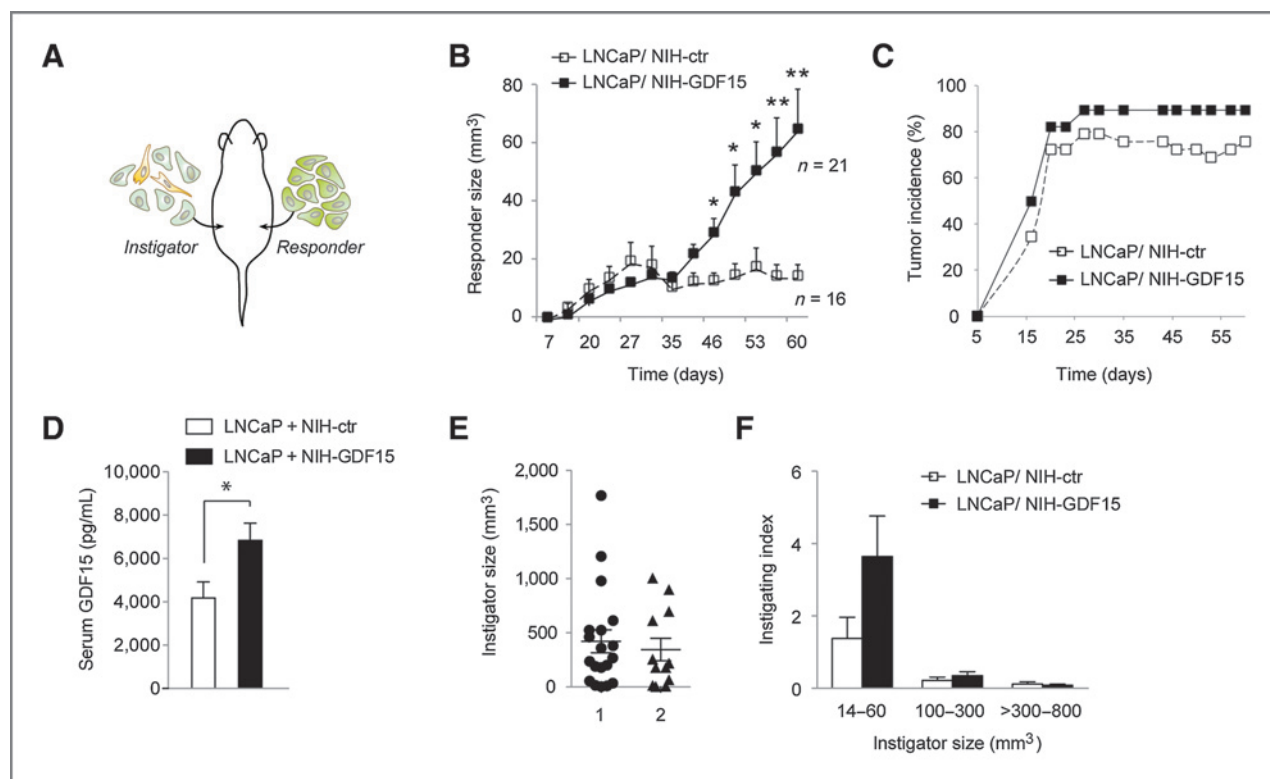
Interestingly, responder tumor growth was significantly enhanced in mice with LNCaP/NIH-GDF15-instigating tumors as compared with mice with LNCaP/NIH-ctr tumors (Fig. 5B). Moreover, LNCaP/NIH-GDF15 tumors stimulated the establishment of responder tumors more efficiently compared with LNCaP/NIH-ctr (Fig. 5C), and blood serum analyses revealed significantly higher circulating levels of GDF15 in mice with LNCaP/NIH-GDF15-instigating tumors

(Fig. 5D). Of note, GDF15 serum levels clearly correlated ( $r = 0.478$ ) with the size of responder tumors (Supplementary Fig. S6A).

Additional analyses were performed to investigate a potential relationship between the responder tumor growth rate and the size of instigator tumors. A grouping of animals according to instigator tumor size at day 46 did not result in a significant separation of responder tumors (Fig. 5E). Also, correlation analyses of sizes of instigator and responder tumors in individual mice did not indicate significant associations between sizes of responder and instigator tumors (Supplementary Fig. S6B). A comparison of the "instigator index" of three different size-matched groups of instigator tumors revealed that the enhanced instigating potency of LNCaP/NIH-GDF15 tumors was predominantly conferred by small (14–60 mm<sup>3</sup>) instigator tumors (Fig. 5F).

Finally, responder tumors in the group of LNCaP/NIH-GDF15-instigated tumors displayed a higher abundance of collagen fibers and vessels with a clear lumen, although the number of small vessels and macrophages was not different between the two groups (Supplementary Fig. S6C and S6D).

The instigator/responder experiment thus demonstrates an instigating effect of fibroblast-derived GDF15 and, in



**Figure 5.** GDF15 instigates tumor growth at a distant location. The systemic tumor-promoting capacity of GDF15 was evaluated by injecting male SCID mice with s.c. tumors at both flanks. **A**, schematic presentation of the experimental setup. The left flank was injected with LNCaP cells combined with either GDF15-expressing fibroblasts or control fibroblasts (designated as instigator tumors). The right flank was injected with LNCaP cells in Matrigel (designated as responder tumors). **B**, responder growth followed over time by palpation. Unfilled squares, responder growth with instigating tumors containing LNCaP combined with NIH-ctr cells; black squares, responder growth with instigating tumors containing LNCaP cells combined with NIH-GDF15 fibroblasts. **C**, the fraction of mice with palpable tumors is depicted as tumor incidence ( $n = 16$  for LNCaP/NIH-ctr;  $n = 21$  for LNCaP/NIH-GDF15). **D**, GDF15 blood serum levels of mice engrafted with LNCaP/NIH-ctr or LNCaP/NIH-GDF15-instigating tumors. **E**, size of the instigator tumors in animals with smaller than average responder tumors (group 1) and larger than average responder tumors (group 2). **F**, instigating index (ratio of the size of responder and instigator tumor) for three different pairs of size-matched instigating tumors. For **B**, the number of animals in each group is indicated in the figure. \*,  $P = 0.032$ ; \*,  $P = 0.026$ ; \*,  $P = 0.024$ ; \*\*,  $p = 0.0084$ ; \*\*,  $P = 0.0056$  at day 46, 50, 53, 57, and 60, respectively. **D**, \*,  $P = 0.01$ . Error bars in **B**, **D**, and **F** indicate SEM.

general terms, provides novel evidence for a role of cancer-associated fibroblasts in systemic protumoral signaling.

**Discussion**

This study identifies stromal cells as an important source of GDF15 in prostate tissue and describes fibroblast-derived GDF15 as a candidate regulator of prostate cancer progression. Fibroblasts expressing GDF15 were shown to stimulate the growth, migration, and invasion of prostate cancer cells under *in vitro* coculture conditions (Fig. 3). Moreover, coinjection of fibroblasts and cancer cells revealed a potent tumor growth-promoting effect of fibroblast-derived GDF15 in a mouse xenograft model, predominantly derived from paracrine effects (Fig. 4). Importantly, beside this local protumoral activity, GDF15-expressing fibroblasts also stimulate the outgrowth of distant, otherwise indolent cancer cells (Fig. 5). This study thereby demonstrates, for the first time, that tumor-instigation potency can be governed by stroma characteristics of the instigating tumor.

The expression of *Acta2* suggests that GDF15 fibroblasts share characteristics with myofibroblasts for which  $\alpha$  smooth muscle actin is the prototypical marker. We also noted increased expression of factors encoding extracellular matrix components and matrix-remodeling enzymes by GDF15 fibroblasts (Fig. 2B). Interestingly, the matrix composition of LNCaP/NIH-GDF15 instigator and responder tumors was also changed with a higher abundance of collagens (Figs. 4C and 5F). Future studies will hopefully address if GDF15-expressing fibroblasts also enhance matrix stiffness, which can stimulate cancer cell invasion (38, 39).

Analyses of *GDF15* transcripts in clinical samples demonstrated that this factor is expressed by stromal cells in addition to the previously recognized expression in epithelial cancer cells (Fig. 1C). The present study also demonstrated a more abundant *GDF15* expression in tumor stroma than in the stroma of nontumor tissue (Fig. 1A). In agreement with previous analyses of human prostate samples, we found abundant GDF15 protein localized to the cancer cell compartment (5, 20). However, low or no levels of GDF15 protein were found in the

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tumor stroma and fibroblasts overexpressing GDF15 by immunohistochemistry (Supplementary Figs. S1 and S2A). On the contrary, *in situ* analyses identified stromal beside epithelial GDF15 expression, indicating that the protein is efficiently secreted from stromal cells, including fibroblasts (Fig. 1C and Supplementary Fig. S2B).

Because the GDF15 gene is expressed in both stromal and epithelial cells in the majority of patient samples analyzed (Fig. 1C), this feature was incorporated in the *in vitro* and *in vivo* coculture models by using LNCaP prostate cancer cells known to produce and release active GDF15 (5, 35). The stimulatory effect of GDF15 on LNCaP cells (Fig. 3) is somewhat surprising in light of the GDF15 production of the LNCaP cells themselves. This could be a matter of dose-dependency, but could also reflect differences in bio-activity of the LNCaP-produced GDF15, as compared with the fibroblast-derived or recombinant GDF15, or differences in cellular responses to autocrine or paracrine GDF15 signaling. These issues warrant further analyses in future studies.

The analyses of the present study demonstrate associations between elevated stromal GDF15 and higher Gleason grade (Fig. 1B). Earlier epidemiologic data have also implied GDF15 as a factor contributing to more aggressive variants of prostate cancer (12, 40–43). It has been assumed that the underlying biology relates to the ability of GDF15 to enhance the malignancy of prostate cancer cells through local signaling in the primary tumors (Fig. 3 and ref. 19). Importantly, the data presented here (Fig. 5) suggest systemic effects of GDF15 as another component contributing to the associations between GDF15 serum levels and worse prognosis. Interestingly, recent data showed that enhanced circulating levels of tumor-derived GDF15 affect the regulation of appetite and induce rapid weight loss in a prostate cancer model, and patients with prostate cancer with cachexia display elevated GDF15 serum levels (37).

A number of nonexclusive principally distinct mechanisms can be envisioned for the increased instigating potency of LNCaP/NIH-GDF15 tumors (Fig. 5B). Either this reflects direct effects of circulating GDF15 on the malignant cells of the responder tumor, or alternatively, the increased instigating potency involves other systemically acting signaling factors induced by GDF15. For example, GDF15 signaling through fibroblasts stimulated the secretion of chemokines and cytokines from LNCaP cells (Supplementary Fig. S5). These factors act locally in an autocrine and paracrine fashion but also have the potential to act systemically (44, 45). The direct mechanism, on the other hand, is supported by blood serum analyses, showing a significant correlation between GDF15 serum levels and the size of responder tumors (Supplementary Fig. S6), and by the stimulatory effect of recombinant GDF15 on the prostate cancer cells *in vitro* and *in vivo* (Fig. 3 and 4). Some findings from the present study also support the notion that the effects of GDF15 on responder tumor growth involve mechanisms that are distinct from those involved in the stimulation of the instigating tumor. LNCaP/NIH-GDF15 primary tumors mostly display an earlier appearance, as compared with LNCaP/NIH-ctr control tumors (Fig. 4). In contrast, the two groups of responder

tumors differ pre-dominantly with regard to growth rate (Fig. 5B). Future studies on potential indirect instigating effects of fibroblast-derived GDF15 should consider the possibility of an involvement of mobilization of bone marrow-derived cells, as previously described in the case of the instigating effects of certain breast tumors (33, 46).

The analysis comparing the instigating capacity of size-matched LNCaP/NIH-ctr and LNCaP/NIH-GDF15 tumors strongly suggests that the increased instigating potency of LNCaP/NIH-GDF15 is not explained by their larger size (Fig. 5E). Furthermore, this analysis also demonstrated that the superiority of the LNCaP/NIH-GDF15 responder tumors is most apparent when small instigating tumors are compared (Fig. 5F), as also noted in the initial studies on tumor instigation (33, 46).

The findings of this study merit future validation in other cancer models. In spite of its limitation, the present model has been able to identify various distinct mechanisms for stroma-derived enhancement of tumor growth. For example, Yang and colleagues found a proangiogenic phenotype when connective-tissue growth factor was expressed by fibroblasts (47), and an earlier study from our laboratory demonstrated that the fibroblast-derived chemokine CXCL14 enhanced tumor growth in a manner involving increased vessel density and more prominent monocyte infiltration (3). None of these phenotypes were observed in the LNCaP/NIH-GDF15 tumors, where GDF15 seemed to predominantly act by directly affecting the tumor epithelial cells (Fig. 4B).

The findings of the present study, and earlier findings of a potential etiological role of GDF15 in disease, provide a strong rationale for identification of GDF15 receptors and their intracellular signaling. The recent description of an involvement of FAK and RhoA in GDF15-induced migration and invasion represents one example of progress in this area (31).

From a general perspective, the major finding of the present study is the demonstration of a stroma-derived tumor instigating effect. The study thereby adds new insight to an emerging and growing list of observations that imply the tumor stroma as an important regulator of metastatic potential (48, 49) and, at the same time, also provides support for the role of systemic signaling in metastasis. As such the findings should stimulate to continuous efforts to identify other novel stroma-derived regulators of metastasis that, like GDF15, could act as potential targets for novel antimetastatic therapies.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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