GREMLIN MODULATES FIBROSIS IN RENAL CELLS VIA VEGFR2 PATHWAY AND INDEPENDENT OF ITS BMPS ANTAGONISTS EFFECTS.

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INTRODUCTION

Gremlin, also called down-regulated by mos (Drm) and initially known as induced in high glucose-2 (IHG-2), belongs to the cystine-knot superfamily that includes transforming growth factor-β (TGF-β) proteins. This protein has been highly conserved during evolution and has an important role in limb development and neural crest cell differentiation. However, in the adult kidney its role in normal and pathological conditions is still unclear. We have recently described that gremlin regulates profibrotic events in renal cells.

Gremlin belongs to a family of bone morphogenetic proteins (BMPs) antagonists and is highly conserved during evolution. Gremlin heterodimerizes with BMPs -2, -4 and -7, preventing their interactions with specific receptors and this capacity is thought to be responsible for the critical role of Gremlin during the process of nephrogenesis. However, BMP-independent mechanisms may mediate several Gremlin intracellular actions, such its ability to suppress tumorigenesis and modulation of angiogenesis. In this sense, it has recently been described that Gremlin-induced angiogenesis is mediated by binding to vascular endothelial growth factor receptor-2 (VEGFR2). Taken together these observations indicate that Gremlin may exert multiple functions in different physiopathology conditions via BMP-dependent and BMP-independent mechanisms.

METHODS

Cell culture studies

Murine renal cortical fibroblasts (TFB cell line) were grown in RPMI with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100U/ml penicillin, and 100 µg/ml streptomycin, in 5% CO_2 at 37°C. Human renal proximal tubular epithelial cells (HK2 cell line, ATCC CRL-2190) were grown in the above described conditions plus insulin transferrin selenite (ITS) and hydrocortisone. At 60-70% of confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments. Murine or human cells were treated with recombinant murine or human Gremlin at 5 ng/ml or 50 ng/ml, respectively (R&D). In some points, recombinant TGF-β1 (10 ng/ml; Peprotech) was used as positive control. In some experiments cells were preincubated with VEGFR2 kinase inhibitor SU-5416 (5 µM; Merck) and BMPs (10 µg/ml; Peprotech).

Chemical Cross-linking and coimmunoprecipitation assays

Before cross-linking and coimmunoprecipitation assays, HK2 cells were incubated with Gremlin for 5 min, and then used for further analysis. Briefly, the cells were washed three times with cold PBS (137 mM NaCl, 0.67 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and incubated for 30 min at 4°C with 1 mM DTSSP (3,3´-dithiobis[sulfosuccinimidylpropionate]) (Pierce Chemical Co) in PBS, followed by washing three times with Tris-buffered saline (TBS) (20 mM Tris-HCl, 100 mM NaCl, pH 7.5) before use in the following immunoprecipitation and immunoblot experiments. Cells were lysed in 500µl lysis buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM PMSF, 1% NP-40/IGEPAL, and a phosphatase-inhibitor cocktail (Set II, Calbiochem)], scraped off the dish and incubated 1 h to 4ºC with shaking. Cell lysates were precleared by incubating with 10 µl of protein A-agarose bead slurries (0.5 ml agarose/2 ml phosphate-buffered saline) for 30 min at 4°C, and then centrifuged three times for 5 minutes at 2500 rpm, to wash supernatants. Precleared lysates were incubated with a rabbit polyclonal anti-VEGFR2 agarose conjugated antibody (Santa Cruz, 20 µl) overnight at 4°C. The agarose beads were collected by centrifugation, washed three times with lysis buffer, resuspended in 2x Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Then, western blot was done using an anti- Gremlin antibody. For loading control, an anti-VEGFR2 antibody was employed.

Transfection, DNA Constructs and promoter studies

HK2 cells were transiently transfected for 24-48 hours with FuGENE (Roche), pCDNA3-Gremlin-myc-IRES2-eGFP plasmid (GREM-GFP) and/or pCDNA-FLAG-Smad7 expression vector or empty vector (pCDNA). The GREM-GFP was generated as follows: human gremlin cDNA purchased in the plasmid pCR4 TOPO from the Mammalian Gene Collection (NIH). PCR was performed with primers that included a c-myc tag in the 3´portion of gremlin and new NotI restriction site. The IRES-eGFP sequence was obtained by PCR with specific primers using a plasmid containing MecP2 IRES-eGFP as template. Gremlin-myc and IRES-eGFP were subcloned into a modified pCDNA3 vector. Smad-dependent promoter activation was evaluated by transfection of Smad/luc and TK-renilla as internal control.

AIM

To study the receptor and mechanisms involved in Gremlin-mediated fibrogenic events in renal cells

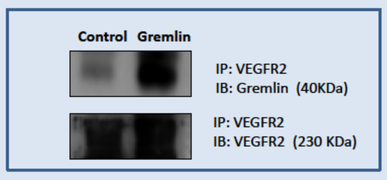
REFERENCES

Mitola S, Ravelli C, Moroni E, Salvi V, Leali D, et al. (2010) Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2. Blood. 4;116:3677-80.

Rodrigues-Diez R, Lavoz C, Carvajal G, Rayego-Mateos S, Rodrigues Diez RR, Ortiz A, Egido J, Mezzano S, Ruiz-Ortega M. Gremlin is a downstream profibrotic mediator of transforming growth factor-Beta in cultured renal cells. Nephron Exp Nephrol. 2012;122:62-74.

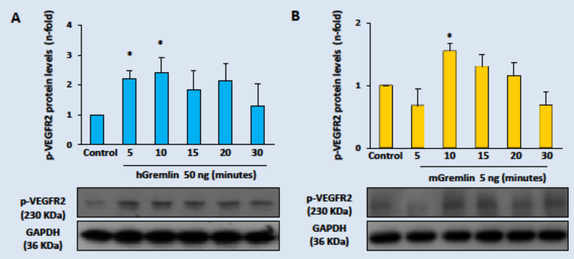
RESULTS

Gremlin binds to VEGFR2 and activates its signaling pathway in renal cells.



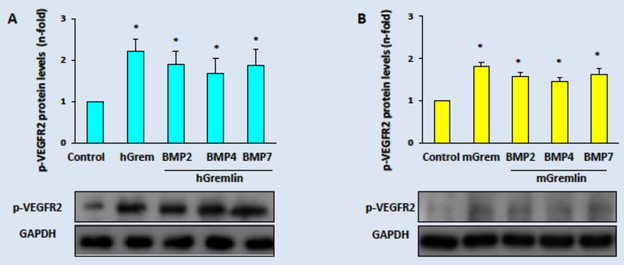
Gremlin-VEGFR2 complexes were found in human tubular epithelial cells. Serum-starved human tubular epithelial cells (HK2 cells) were stimulated with 50 ng/ml of human recombinant Gremlin for 5 min. Then, cells were treated with a cross-linker as described in Materials and Methods. Cell lysates were immunoprecipitated (IP) with an anti-VEGFR2 and then analyzed by Western Blot (IB) with anti-Gremlin antibody, to analyze the complexes formed.

Gremlin induces VEGFR2 phosphorylation in human tubular epithelial cells and in murine fibroblasts



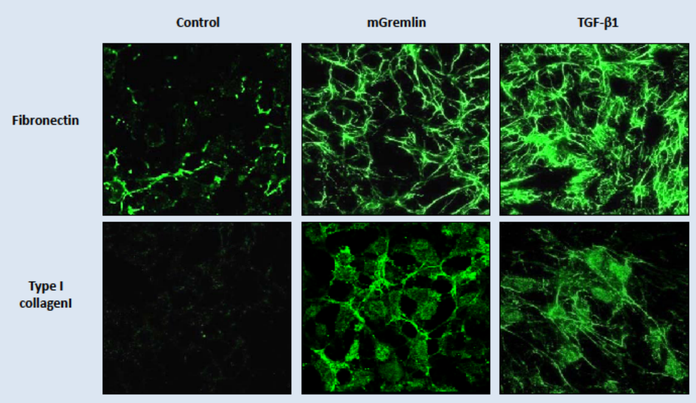
A. HK2 cells were treated with 50 ng/ml of human recombinant Gremlin for several times. VEGFR2 phosphorylation was assessed by western analysis, using antibodies against phosphorylated-VEGFR2. Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/GAPDH as n-fold over control. Figures show in upper panels representative autoradiograms and in lower panel data expressed as mean ± SEM of 3 independent experiments. *p<0.05 vs control. Similar results were observed in murine fibroblasts (**B**).

BMPs are not involved in Gremlin induced VEGFR2 activation

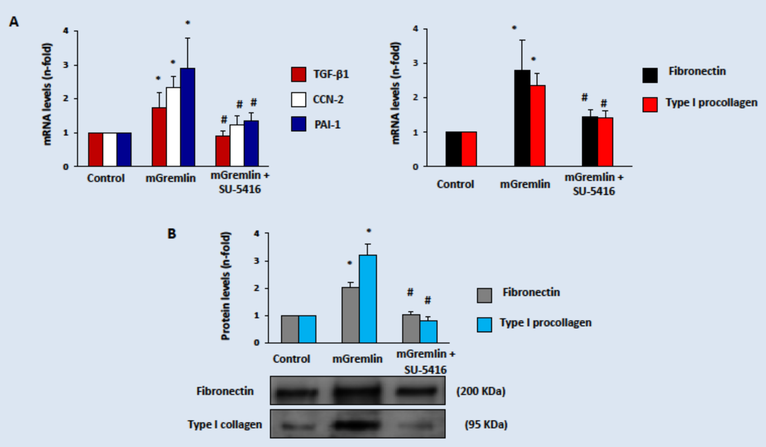


Gremlin induced VEGFR2 phosphorylation was independent of BMPs in human tubular epithelial cells. A. To determine the role of BMPs in Gremlin responses HK2 cells were preincubated with BMP-2, BMP-4 or BMP-7 at 10 µg/ml for 1 hour in serum-free medium, before the experiments. Then cells were stimulated with 50 ng/ml of human Gremlin for 5 min. Figure shows in the upper panel a representative western blot experiments and in lower panel data expressed as mean ± SEM of 3 experiments. *p<0.05 vs control. Similar results were observed in murine fibroblasts (**B**).

Gremlin increased the production of extracellular matrix proteins in murine renal fibroblasts.

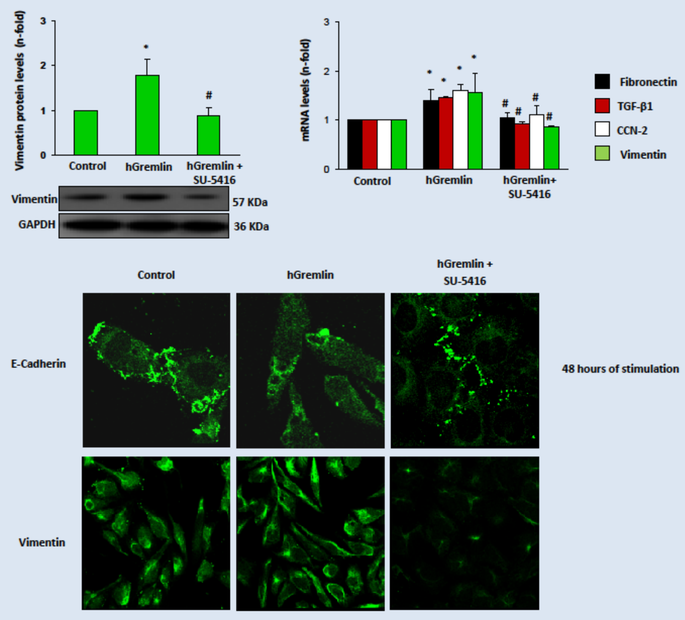


Gremlin via VEGFR2 increases the production of extracellular matrix proteins in TFBs

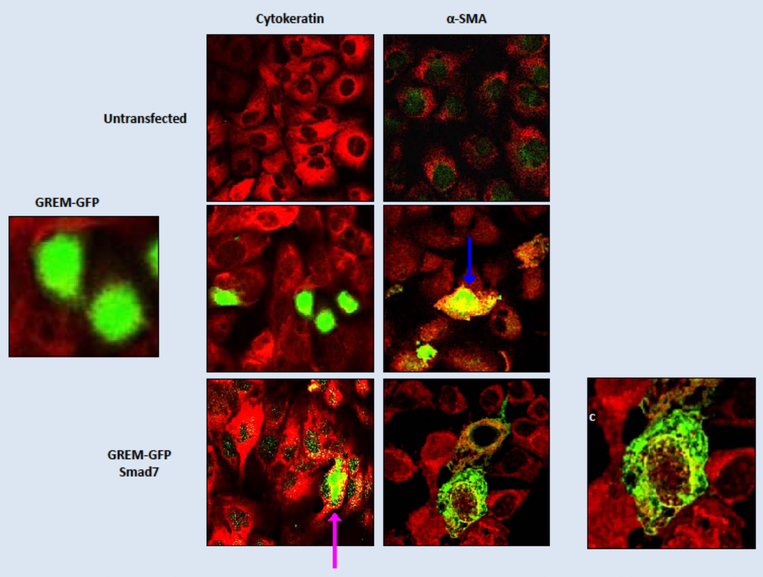


To determine the role of VEGFR2 in Gremlin responses cells were preincubated with VEGFR2 kinase inhibitor SU-5416 at 5 µM for 1 hour before the experiments. Then, cells were stimulated with or without Gremlin at 50 ng/ml for 24 (A, gene studies) or 48 (B, protein studies) hours in serum-free medium. Figures show data as mean ± SEM of 4 independent experiments. *p<0.05 vs. control; #p<0.05 vs Gremlin.

VEGFR2 activation participates in epithelial to mesenchymal transition and profibrotic events induced by Gremlin in human tubular epithelial cells.

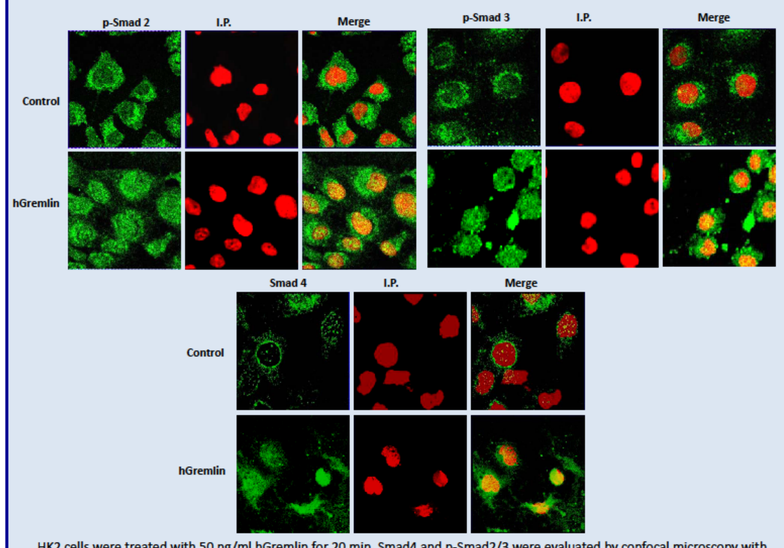


Gremlin via Smad regulates EMT in human tubular epithelial cells.

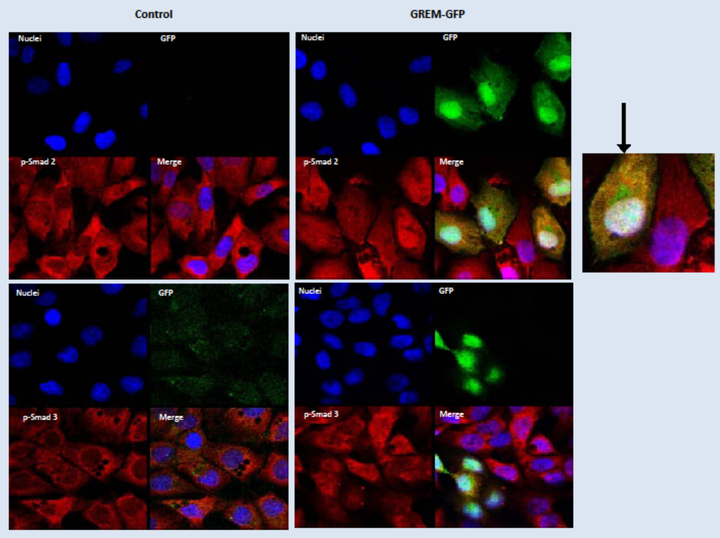


Cells were cotransfected with GREM-GFP and a Smad7 expression vector, that inhibits TGF-β/Smad-mediated transcriptional effects by interfering with receptor-mediated activation of R-Smad. EMT markers were evaluated after 48 hours. Gremlin transfected cells express GFP (green staining). Confocal microscopy analysis of cytokeratin and α-SMA immunofluorescence were performed using specific primary antibodies and an Alexa-633 secondary IgG (red staining). Figures show a representative experiment of 3 done.

Gremlin activates Smad pathway in human tubular epithelial cells.

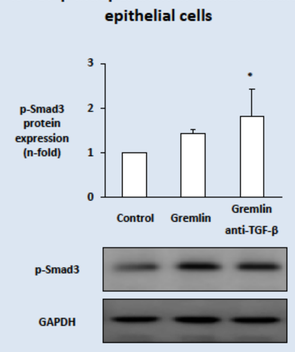


HK2 cells were treated with 50 ng/ml hGremlin for 20 min. Smad4 and p-Smad2/3 were evaluated by confocal microscopy with FITC-labeled secondary antibodies (green staining). Nuclei were stained with propidium iodide (in red). In the merge, the yellow staining indicates nuclear localization of Smad proteins. The results are representative of 3 independent experiments.

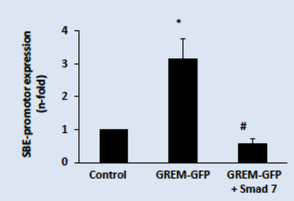


Cells were transfected with a Gremlin expression vector (GREM-GFP) for 24 hours and EMT changes were evaluated by confocal microscopy. Only in gremlin transfected cells (green staining by GFP) p-Smad2 was found in the nuclei. In the insert, the overlay image shows p-Smad2 nuclear localization in gremlin positive cells (arrow), that yielded a white tone in the nucleus, while in non-transfected cells blue nuclear staining was observed.

Early Smad activation induced by Gremlin is TGF-β independent in human tubular epithelial cells



Gremlin activates Smad-dependent gene trasnscription in human tubular epithelial cells.



HK2 cells were transfected with GREM-GFP, Smad/luc promoter and TK-renilla for 24 hours. In some points, cells were contransfected with Smad7. Then, luciferase/renilla activity was measured. Data of mean±SEM of 5 experiments done.
*p<0.05 vs. control. # p<0.05 vs. GREM-GFP.

CONCLUSION

In renal cells Gremlin regulates profibrotic factors, via VEGFR2 binding and independent on BMP antagonism.

Gremlin activates Smad pathway and increases profibrotic events through this molecular pathway.

We propose that gremlin, by VEGFR2 and activation of Smad signaling, increased profibrotic events and therefore could contribute to renal fibrosis.

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