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EXPERIMENTAL STUDY

Involvement of the gp130 cytokine transducer in MtT/S pituitary somatotroph tumour development in an autocrine-paracrine model

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Abstract

Objective: gp130 cytokines are placed as auto-paracrine regulators of pituitary function, since they, as well as their receptors, have been shown to be expressed in and to act in normal and tumoral anterior pituitary cells. The objective of this work was to study their involvement in a model that shows the interaction between different cellular types that participate in a tumorigenic process.
Design: The dependence of a pituitary somatotrophic cell line (MtT/S) on a gp130 cytokine-producing folliculostellate (FS) cell line (TtT/GF) for tumorigenesis *in vivo* has been described. In order to study the participation of gp130 cytokines in the auto-paracrine stimulation of MtT/S growth, we generated MtT/S gp130 sense (gp130-S) and gp130 antisense (gp130-AS) clones stably transfected with pcDNA3/gp130 sense and pcDNA3/gp130 antisense vectors respectively.
Methods and results: Functional characterization studies revealed that gp130-AS clones have an inhibited gp130 signalling, and proliferation studies showed that they have an impaired response to gp130 cytokines but respond normally to other independent stimuli. When injected into nude mice, MtT/S clones respond differently depending on cell number; at high concentrations MtT/S clones alone generated tumours equivalent in size to tumours derived from MtT/S plus TtT/GF cells. At low concentrations, MtT/S sense and control clones generated tumours of smaller size than tumours derived from these same clones plus TtT/GF cells, showing a dependence on FS cells. In both cases MtT/S gp130-AS clones had impaired tumour development. Furthermore, vessel density was significantly lower in tumours derived from gp130-AS plus TtT/GF cells.
Conclusions: This study underlines the importance of gp130 cytokines in proliferation and establishes its role in auto-paracrine pituitary growth regulation.

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Introduction

The gp130 cytokine family is composed of interleukin-6 (IL-6), leukaemia inhibitory factor (LIF), interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF), oncostatin M, cardiotrophin-1 (1), neuropoietin (2) and cardiotrophin-like cytokine, also known as stimulating neurotrophin-1/B cell-stimulating factor-3 (3). These cytokines bind to specific receptors and trigger the association of their alpha subunits with gp130 protein, which functions as an initial cellular signal transducer (4). The gp130 signal transduction pathway involves JAK (JAK1, JAK2 and TYK2) and STAT (STAT1 and STAT3) family proteins. STATs, once activated, dimerize and translocate into the nucleus and bind to promoter regions of specific response genes (5). The adaptor molecule, SHP-2, is recruited to the phosphotyrosine 759 residue in the stimulated gp130 and then undergoes tyrosine-phosphorylation by JAK1, JAK2 and TYK2 (1).

gp130 expression is ubiquitous, and its ability to respond differentially to each of the proteins that belong to the gp130 cytokine family is determined by the expression of the cytokines or their specific alpha receptor chains. This occurs also in the pituitary gland, where many studies have demonstrated not only the expression of gp130 mRNA (6) but also the expression of specific receptors and synthesis of gp130 cytokines in different types of pituitary cells (reviewed in 7–10), providing the cellular and molecular basis for an auto-paracrine mechanism of regulation of pituitary function. Particularly, several groups have demonstrated IL-6 production and the presence of IL-6 mRNA

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in anterior pituitary cells (11, 12). IL-6 production has been shown to be localized to folliculostellate (FS) cells in normal pituitary tissue (12), and to the tumour cells themselves in the case of pituitary adenomas (reviewed in 9, 13). The presence of LIF and LIF receptors has been demonstrated in developing human fetal pituitary and in normal and adenomatous adult human pituitary tissue (14). IL-11 and CNTF and their specific alpha chains are present in FS cells and lactosomatotrophic cells (15). Consistent with the cell line studies, IL-11 and CNTF expression were also detected in normal pituitary cells (15, 16) and the alpha chain specific for the CNTF receptor was detected in tumours secreting prolactin (PRL), growth hormone (GH) and in nonfunctioning tumours (17).

The influence of gp130 cytokines in pituitary cell growth has been shown by several studies. IL-6 regulates pituitary cell growth, stimulating proliferation of the GH3 cell line but, intriguingly, inhibiting normal anterior pituitary cell growth at the same concentrations (18). Furthermore, IL-6 has either inhibitory or stimulatory effects in different pituitary tumours (adrenocorticotrophin-, PRL-, GH-secreting and nonfunctioning adenomas), with no apparent association between the kind of response and tumour type or size. Using blocking antibodies it was observed that endogenous pituitary IL-6 regulates the early gene c-fos expression in adenoma explants, but not in monolayer cultures (19). This may indicate that cell–cell interactions are important for the control of c-fos expression and, hence, for the control of cell proliferation in the pituitary gland. IL-11 and CNTF stimulate the proliferation of FS and lactosomatotrophic cells (15), and IL-11 also induces the secretion of vascular endothelial growth factor (VEGF) by FS cells (15). IL-6 stimulates the angiogenic factor, VEGF, production in FS cells (20). FS cells also produce metalloproteinase inhibitors, which can contribute to angiogenic processes as well as to tumour invasion. LIF could also be considered an angiogenesis regulating factor, as this protein has an inhibitory effect on proliferation of aortic endothelial cells when produced by bovine pituitary cultures (21).

We have demonstrated that reduced levels of gp130 protein in GH3 cells (stable gp130 mRNA antisense clones) blocked *in vitro* cell growth stimulated by CNTF, and led to severely impaired *in vivo* tumour development in nude mice (22). These data provide evidence supporting a link between gp130 and pituitary abnormal growth. Participation of gp130 cytokines in pituitary gland tumorigenesis is further underlined by evidence indicating that the MtT/S somatotrophic cell line depends on the IL-6- and LIF-producing TtT/GF FS cell line to generate tumours in nude mice (23). Although the mechanism by which TtT/GF cells contribute to MtT/S cell tumorigenicity is not clear, one possible way might be through the liberation of cytokines and growth factors that stimulate MtT/S growth. In concordance with this hypothesis, it has been reported that MtT/S growth is enhanced *in vitro* by exogenous gp130 cytokines such as IL-6 (24).

According to this evidence, gp130 cytokines secreted by TtT/GF cells may have a central role in stimulating MtT/S *in vivo* tumorigenesis. Based on this hypothesis, we generated stable MtT/S clones that express different gp130 levels in order to study the involvement of gp130 cytokines in both the autocrine and paracrine regulation of somatotrophic cell growth.

Materials and methods

Materials

Materials and reagents, except where stated, were obtained from Sigma (St Louis, MO, USA), Invitrogen (Frederick, MD, USA) and Promega (Madison, WI, USA). Recombinant human IL-6 (rhIL-6) and recombinant human LIF (rhLIF) (R&D Systems, Minneapolis, MN, USA) were dissolved in PBS-BSA 0.05% and used in the indicated doses, in endotoxin-free conditions.

Cell culture

MtT/S cells, a rat somatotrophic pituitary cell line obtained from an oestrogen-induced somatotrophic tumour (25), MtT/S gp130 clones, generated as indicated below, and TtT/GF, a murine folliculostellate cell line obtained from a thyrotrophic pituitary tumour (26), were used. They were cultured in Dulbecco's modified Eagle's medium (DMEM) (pH 7.3) supplemented with 10% FCS, 2.2 g/l $NaHCO_3$, 10 mmol/l HEPES, 2 mmol/l glutamine and 105 U/l penicillin-streptomycin until they were confluent.

For *in vitro* experiments, cells were washed twice with phosphate-buffered saline (PBS) and then medium was replaced by an experimental medium consisting of the same supplemented DMEM media without FCS. Before and after the stimulation period, cell viability was routinely controlled to ensure that this parameter did not change during the experiment. Cell viability was determined microscopically after acridine orange-ethidium bromide staining.

Plasmids

The murine gp130 expression vector (pBMG gp130) was generously provided by Dr Matsumoto Hibi (27). The full-length cDNA (3 kb XhoI/XhoI fragment) was subcloned into pcDNA3 (Invitrogen) (pcDNA3/gp130 sense). The antisense expression vector was generated by subcloning of the 0.9 kb XhoI/BamHI fragment into pcDNA3 expression vector in reverse orientation (pcDNA3/gp130 antisense). cDNA insertions were verified by sequencing.

To measure STAT3 activity, the p4xm 67 TATA-tk-LUC, a luciferase (LUC) reporter construct with four copies of the STAT-3-binding sequence from the

interferon regulatory factor 1 (IRF-1), generously provided by Dr J E Darnell Jr (28), was utilized.

MtT/S gp130 stable clones

To obtain stable clones over- or downexpressing gp130 protein, pcDNA3/gp130 sense, pcDNA3/gp130 antisense or pcDNA3/control vector were used to transfect MtT/S cells by lipofectamine (Invitrogen) as previously described (22). After 48 h incubation, cells were transferred to selection medium containing 600 μg/ml G418. The stable clones that were obtained were cultured in medium with 300 μg/ml G418. pcDNA3/gp130 sense, pcDNA3/gp130 antisense and pcDNA3 stably transfected clones were denominated gp130-S, gp130-AS and gp130-C respectively. The levels of gp130 in these clones were tested by Western blot.

Transfection of MtT/S cells and reporter assays

Cell transfection was performed with lipofectamine using standard procedures, as previously described (29). After plating the cells in 6-well plates, they were transfected in DMEM without FCS for 6 h using 5 μl lipofectamine per well with 1.5 μg reporter plasmid, in conjunction with a control RSV-LacZ construction (0.5 μg). Cells were then washed and left for 20 h in DMEM supplemented with 10% FCS. Stimuli were added for 6 h in DMEM supplemented with 2% FCS. After treatment, LUC activity in cell lysates was measured as previously described (29) using the luciferase assay system (Promega) with a Junior luminometer (Berlthod, Bad Wildbad, Germany). β-Galactosidase activity used as a control for transfection efficiency (29) was measured as previously described and was used to standarize the results.

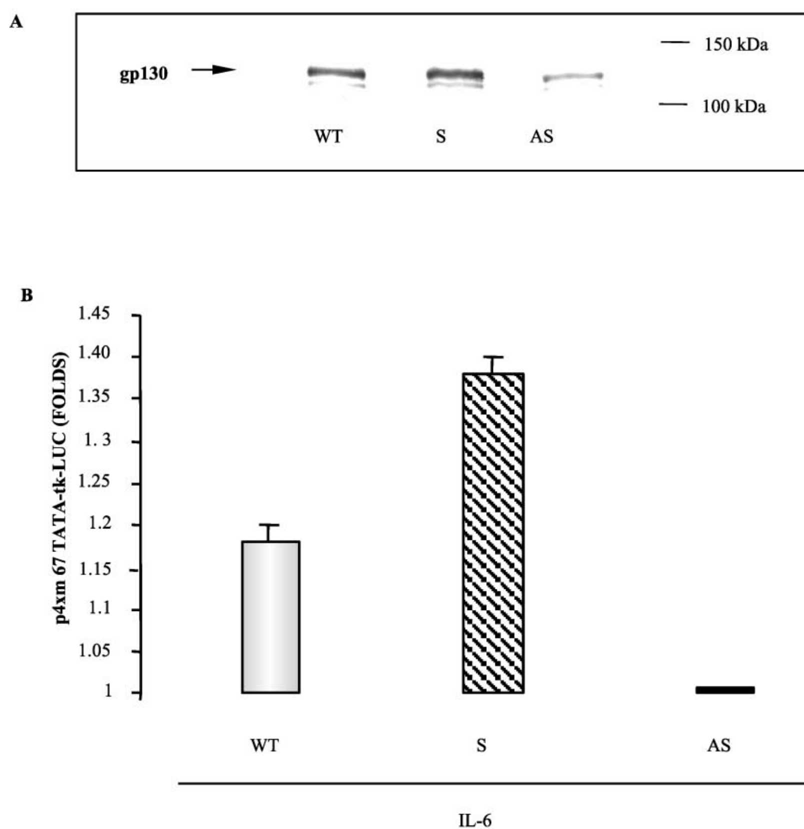

Figure 1 Characterization of gp130-S and gp130-AS stable clones. (A) Western blot analysis of MtT/S somatotrophic stable clones showing overexpressed or downexpressed gp130 protein levels. The bands corresponding to gp130 protein were detected by an anti-gp130 polyclonal antibody and are indicated by an arrow. Molecular weight markers are indicated on the right. (B) STAT-3-mediated transcriptional activation in response to IL-6 in gp130-S and gp130-AS stable clones. MtT/S stable lines were transiently transfected with plasmid DNA mixture containing 1.5 μg p4xm 67 TATA-tk-LUC and 0.5 μg pRSV-Lac Z. At 20 h after transfection, cells were treated with IL-6 (20 ng/ml) for 6 h. The values corresponding to luciferase were normalized with respect to β-galactosidase activity, and are expressed relative to values obtained from untreated cells. Results are expressed as means±S.E.M. ($n = 3$) of one representative mean of three independent experiments with similar results. WT, MtT/S wild-type cells; S, MtT/S cells transfected with pcDNA3/gp130 sense vector; AS, MtT/S cells transfected with pcDNA3/gp130 antisense vector. Results from one representative clone from different gp130-S ($n = 2$) and gp130-AS ($n = 2$) stable clones with similar results are shown. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector.

Western blot analysis

Cells were washed once with PBS (pH 7.0), and cell lysates were prepared in standard cracking buffer and boiled for 3 min. Equal levels of protein (30 μg) were analysed by 10% SDS-PAGE. Proteins were blotted onto nitrocellulose Western blotting membranes (Sigma) using standard procedures and anti-gp130 polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used. The antibody was detected with alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc.) followed by 5-bromo-4-chloro-3-indoly phosphate p-toluidine (BCIP) and nitroblue tetrazolium chloride (NBT) detection (Invitrogen).

Cell proliferation

Cell proliferation was measured with the WST-1 reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions and the reaction product was measured in an ELISA plate reader at 450 nm (29). Results were validated by total cell count. Acridine orange-ethidium bromide staining was used to rule out toxic effects.

In vivo *experiments in nude mice*

MtT/S cells, MtT/S gp130 clones and TtT/GF cells were collected by trypsinization, washed twice with PBS, resuspended in DMEM, and injected subcutaneously in variable numbers into the flanks of adult (20 g) female N:NIH nude mice. Animals were examined for tumour formation periodically, and tumour growth was determined as described (22, 29, 30). The experiments were stopped at 50 days after cell injection, when animals were killed and the tumours were extracted and immediately frozen for inmunohistochemical staining.

All experimental protocols were approved by the Ethical Committee on Animal Care and Use, University of Buenos Aires, Argentina.

Immunohistochemistry for S100, CD31 and determination of vascular density

For immunohistochemistry, 8-μm sections of shock-frozen MtT/S tumour tissue were thaw-mounted onto SuperFrost Plus slides (Menzel-Glaser, Hamburg, Germany), fixed in 4% paraformaldehyde in PBS, and stored in 96% ethanol at 4 °C until use.

An anti-mouse goat CD31 (PECAM-1) antibody (Santa Cruz Biotechnology Inc.), or an anti-mouse rabbit S100 antibody (Zymed Laboratories Inc., San Francisco, CA, USA), was used to detect intratumoral microvessels and the presence of TtT/GF cells in the tumours respectively. Slides were first incubated in horse serum diluted 1:10 for CD31 staining, or goat serum diluted 1:20 for S100 staining, in Tris–based buffer (TBS; pH 7.6). Subsequently, the slides were incubated overnight at 4 °C with CD31 primary antibody diluted 1:500, or with S100 primary antibody diluted 1:100. After three washes in TBS, the biotinylated horse anti-goat, or the biotinylated goat anti-rabbit secondary antibodies (dilution: 1:300) (Vector Laboratories Inc., Burlingame, CA, USA) were added for 30 min at room temperature. The slides were then washed again and were incubated for 30 min with the avidin-biotin-peroxidase complex (Vector Laboratories Inc.). Colour development was performed using 1 mg/ml diaminobenzidine (Sigma) with 0.01% hydrogen peroxide applied for 45 s or 3 min respectively. After washing in deionized water, the sections were counterstained with toluidine blue, fixed in Roti-Histol (Carl Roth, Karlsruhe, Germany) and coverslipped using Roti-Histokitt (Carl Roth). Negative controls were performed by omitting the primary antibody.

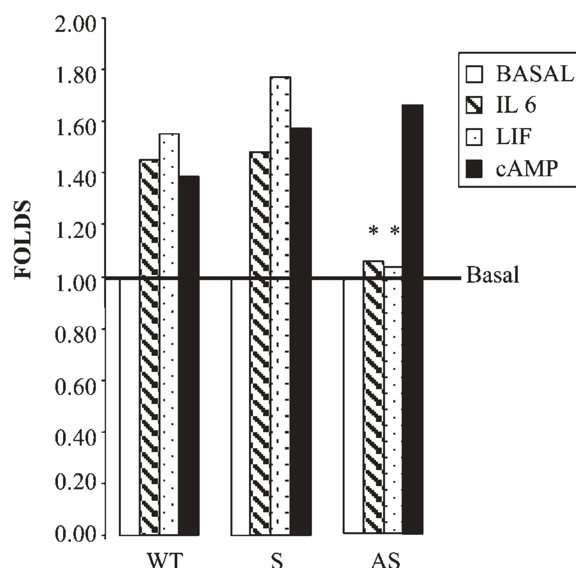

Figure 2 Proliferation of MtT/S wild-type cells (WT), gp130-S (S), and gp130-AS (AS) clones in response to IL-6 and LIF. MtT/S gp130 cell lines were seeded at 5000 cells/well in multiwell plates with 10% FCS. After attachment, cells were washed twice with PBS and incubated in FCS-free medium for 24 h. Cells were treated with IL-6 (100 ng/ml), LIF (10 ng/ml) or cAMP (500 μmol/l) for 72 h. Proliferation was measured by the WST-1 colorimetric method as detailed in Materials and methods. Values represent the average fold stimulation of five experiments with quadruplicate determinations. Results of averages with a variation from the mean of <10% are shown as folds. Absolute basal values (A_{450nm}) were MtT/S WT, 0.049±0.012; gp130-S, 0.047±0.014; gp130-AS, 0.057 ± 0.013. Similar results were obtained with IL-6 (20 ng/ml) and LIF (100 ng/ml). Results from one representative clone from different gp130-S ($n=2$) and gp130-AS ($n = 2$) stable clones with similar results are shown. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector. *$P > 0.05$ compared with the corresponding basal level; for all other stimulated bars $P < 0.01$ compared with the corresponding basal level (ANOVA with Tukey test).

For determination of vascular density, an investigator who was not informed about the type of tumour, counted vessels inside an area delimited by an eyepiece graticule 12.5 × 12.5 mm divided in 10 × 10 squares (Zeiss, Munich, Germany) at a × 200 magnification (× 20 objective and × 10 ocular). Vessel density was determined in 4 different tumours of each group (gp130-AS, gp130-S, MtT/S control-derived tumours) and in each tumour both single cells and cell clusters positive for CD31 were counted in 3 different areas. Results of vessel counting are expressed as means± S.E.M. of 12 areas counted.

Statistics

Statistics were performed by analysis of variance (ANOVA) in combination with the Tukey test. Data are shown as means±S.E.M.

Results

Generation and characterization of gp130 stable cell lines

Somatotrophic MtT/S stable clones that over- or down-express gp130 glycoprotein were generated by transfecting MtT/S cells with expression vectors containing gp130 cDNA in sense or antisense orientations. Control clones, generated by transfecting MtT/S cells with pcDNA3 empty vector, showed similar results to MtT/S wild-type cells in all experiments. For simplicity, results with wild-type cells are shown throughout the manuscript.

As shown in Fig. 1A, gp130 expression levels were elevated in gp130-S clones whereas they were reduced in gp130-AS clones compared with MtT/S wild-type cells. To test gp130 signalling pathway in MtT/S

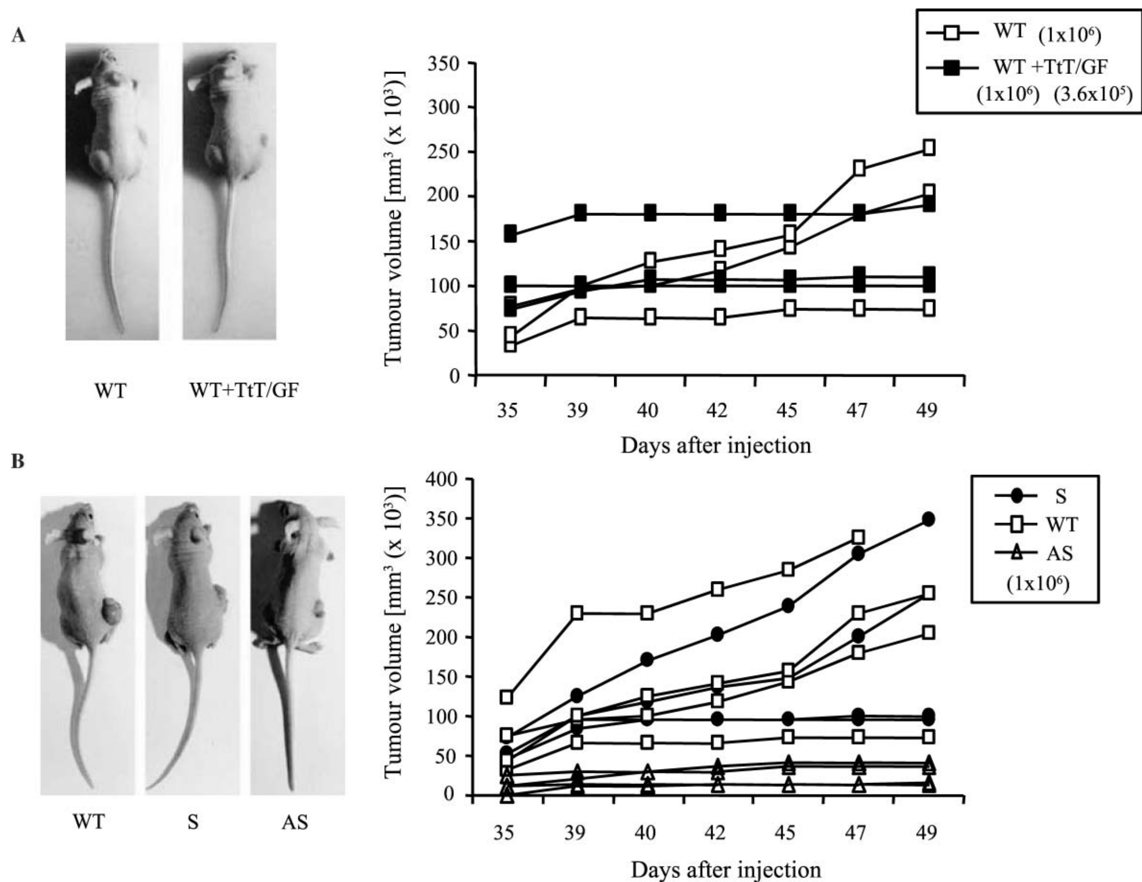

Figure 3 MtT/S cell tumorigenicity *in vivo* at high concentrations. (A) MtT/S wild-type cells were injected alone (1×10^6) or coinjected with TtT/GF cells (3.6×10^5) subcutaneously into athymic nude mice ($n = 3$, each group) as described in Materials and methods. The development of tumours in MtT/S wild-type cells alone or coinjected with TtT/GF cells started to be apparent at day 35 after injection. WT, MtT/S wild-type cells; WT + TtT/GF cells, MtT/S wild-type cells plus TtT/GF cells. (B) MtT/S wild-type cells, gp130-S or gp130-AS clones were injected (1×10^6) subcutaneously into athymic nude mice ($n = 4$, each group) as described in Materials and methods. The development of tumours in MtT/S wild-type cells and gp130-S cells started to be apparent at day 35 after injection, while gp130-AS cells showed delayed tumour formation. WT, MtT/S wild-type cells; S, gp130-S cells; AS, gp130-AS cells. Initial total cell number was controlled so as to be the same in all groups. For both (A) and (B), the clones preserved the expression of gp130-S or gp130-AS after *in vivo* injection. Results from one representative clone from different gp130-S ($n = 2$) and gp130-AS ($n = 2$) stable clones with similar results are shown. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector.

stable clones, a transient transfection with the p4xm 67 TATA-tk-LUC reporter construct was performed. STAT3-transcriptional activity was induced in response to IL-6 in MtT/S wild-type cells, this effect was slightly enhanced in gp130-S clones, and was efficiently reduced in gp130-AS clones (Fig. 1B).

Cell proliferation in MtT/S gp130 stable clones

No differences in basal proliferation were observed between different clones (Fig. 2). IL-6 and LIF significantly stimulated growth of MtT/S wild-type cells, and a similar response was found in gp130-S clones. In contrast, as shown in Fig. 2, the capacity of gp130-AS clones to respond to gp130 specific stimuli was abolished whereas they were able to respond to cAMP, a gp130-independent stimulus.

Auto-paracrine induction of in vivo *tumour formation depends on cell concentration and gp130 cytokines*

We first evaluated whether MtT/S cells would be capable of generating tumours *in vivo*, and if TtT/GF cells would induce MtT/S growth. Therefore, we injected MtT/S wild-type cells (1×10^6) into nude mice, alone or combined with TtT/GF cells (3.6×10^5). Visible tumours of similar size developed in mice injected either with MtT/S cells alone or combined with TtT/GF cells at these concentrations (Fig. 3A).

Next, we investigated whether MtT/S tumorigenic behaviour was mediated by gp130 cytokines, and if

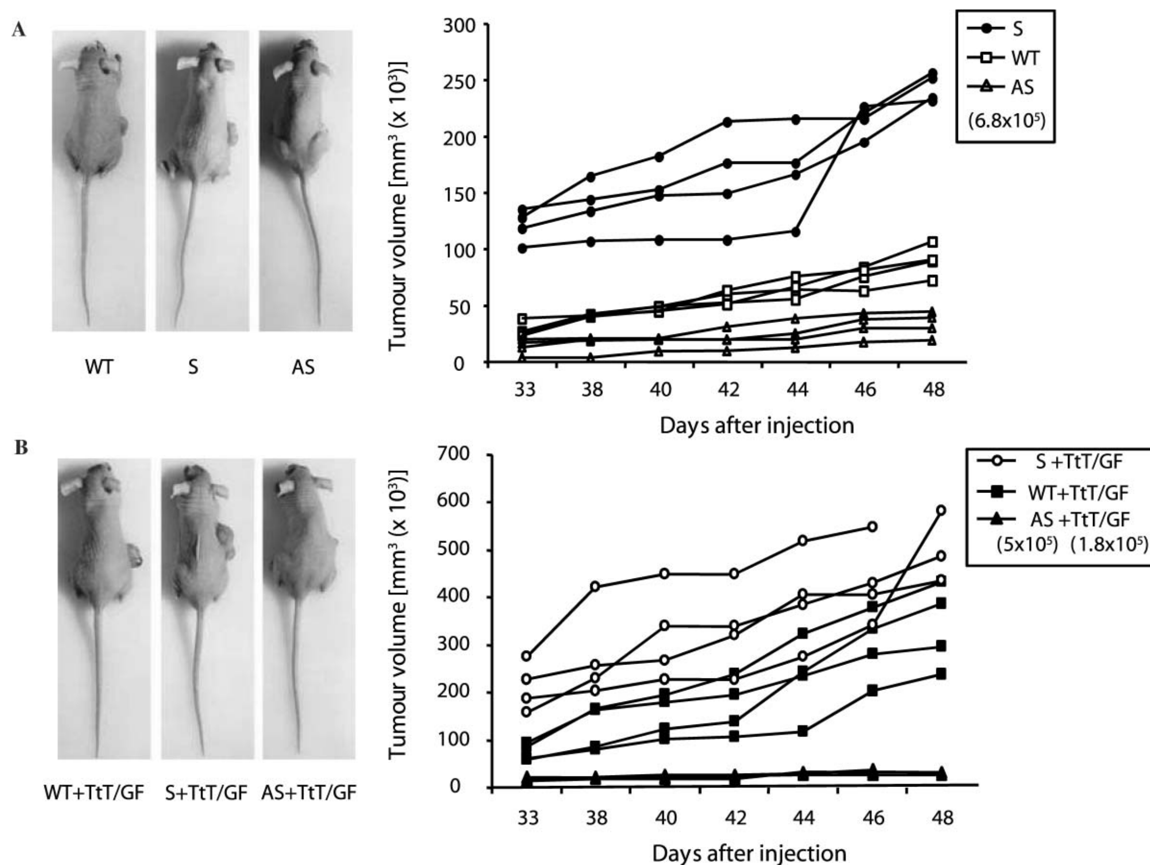

Figure 4 MtT/S cell tumorigenicity *in vivo* at low concentrations. (A) MtT/S wild-type cells, gp130-S or gp130-AS clones were injected (6.8×10^5) subcutaneously into athymic nude mice ($n = 4$, each group) as described in Materials and methods. The development of tumours in MtT/S wild-type cells and gp130-S cells started to be apparent at day 33 after injection. Tumours generated from MtT/S wild-type cells, gp130-S or gp130-AS clones alone at 5×10^5 cell number showed the same results as tumours generated from MtT/S clones at 6.8×10^5 cell number. (B) MtT/S wild-type cells, gp130-S or gp130-AS clones (5×10^5) were coinjected with TtT/GF cells (1.8×10^5) subcutaneously into athymic nude mice ($n = 4$, each group) as described in Materials and methods. The development of tumours in MtT/S wild-type cells and gp130-S clones coinjected with TtT/GF cells started to be apparent at day 33 after injection. For both (A) and (B): WT, MtT/S wild-type cells; S, gp130-S cells; AS, gp130-AS cells. Initial total cell number was controlled so as to be the same in all groups. The clones preserved the expression of gp130-S or gp130-AS after *in vivo* injection. Results from one representative clone from different gp130-S ($n = 2$) and gp130-AS ($n = 2$) stable clones with similar results are shown. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector.

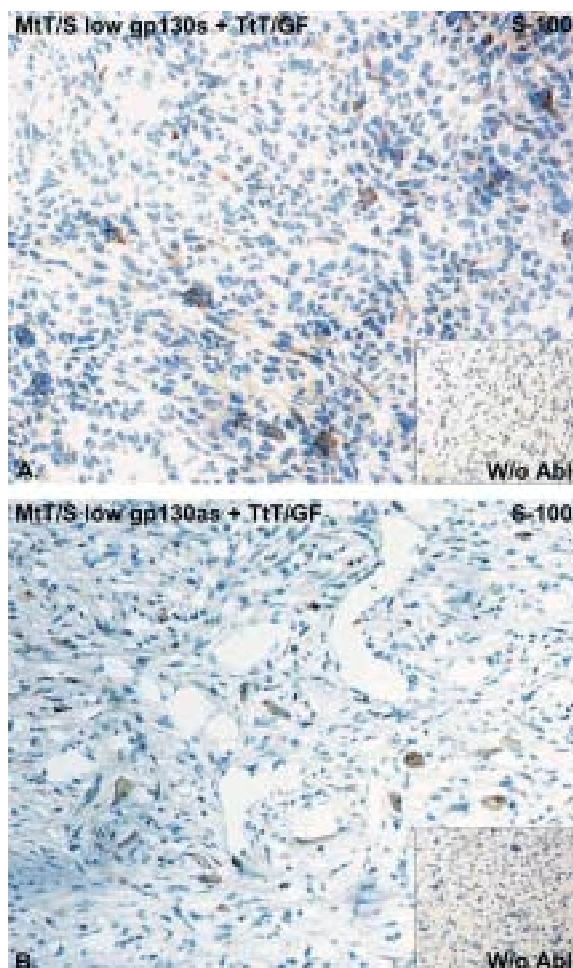

Figure 5 Distribution of folliculostellate TtT/GF cells in nude mice tumours composed of MtT/S cells injected at low concentration (5×10^5) and TtT/GF cells (1.8×10^5). (A) MtT/S gp130-S + TtT/GF-derived tumours; (B) MtT/S gp130-AS + TtT/GF-derived tumours. Immunostaining for the FS cell marker S100 (brown) is shown. No differences in the distribution of S100-immunopositive TtT/GF cells (brown) was observed. A similar scattered distribution of TtT/GF cells was also observed in MtT/S wild-type + TtT/GF tumours (not shown). Corresponding negative controls are inserted at the right corner of each picture. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector.

changing gp130 expression levels generated different tumorigenicity in MtT/S cells. We injected MtT/S wild-type cells, gp130-S or gp130-AS clones alone into nude mice and measured tumour growth. Tumours derived from MtT/S wild-type cells and gp130-S clones grew rapidly and reached similar sizes, considerably larger than gp130-AS-derived tumours (Fig. 3B).

We hypothesized that MtT/S tumorigenic behaviour might depend on whether the initial concentration injected was above or below a critical threshold that would determine their capacity to generate tumours independently of TtT/GF cells. Accordingly, we next tested if MtT/S cells were tumorigenic alone at low concentrations, and if in this case TtT/GF could stimulate MtT/S tumorigenic behavior. We then injected MtT/S wild-type cells, gp130-S or gp130-AS clones alone (6.8×10^5), or combined with TtT/GF cells (5×10^5 and 1.8×10^5 cells respectively) into nude mice. As shown in Fig. 4, at these concentrations TtT/GF cells were able to increase MtT/S tumorigenicity, as MtT/S wild-type cells and gp130-S clones when coinjected with TtT/GF cells generated tumours of a larger size than MtT/S wild-type cells and gp130-S clones injected alone. MtT/S tumorigenic behaviour was also mediated by gp130 cytokines at low concentrations, since growth was inhibited in gp130-AS clones (Fig. 4). Cells in the tumour were of MtT/S origin since S100 staining (a marker for TtT/GF cells) was the same in all groups (Fig. 5).

In order to study the influence of gp130 in vascularization, we next studied vessel density. In tumours derived from MtT/S wild-type cells and gp130-S cells injected at high concentrations, vessel density was increased when compared with tumours derived from MtT/S wild-type cells and gp130-S cells injected alone at low concentrations (Table 1). Also MtT/S wild-type cells and gp130-S cells coinjected at low concentrations with TtT/GF showed an increase in vascularization compared with MtT/S wild-type cells and gp130-S cells injected alone at low concentrations (Table 1). In the case of gp130-AS clones, they showed lower vessel density than MtT/S wild-type cells and gp130-S cells whether injected at high concentrations or coinjected at low concentrations with TtT/GF cells (Fig. 6, Table 1).

Table 1 Microvessel count in tumours derived from MtT/S clones. Vessel count was performed as described in the legend to Fig. 6 and detailed in Materials and methods. The number of vessels was significantly reduced in tumours derived from gp130-AS cells, both in the high cell concentration group with respect to tumours derived from gp130-S cells and MtT/S wild-type cells (*$P < 0.05$), and in the low cell concentration + TtT/GF group with respect to tumours derived from gp130-S cells (*$P < 0.05$) and MtT/S wild-type cells (**$P < 0.001$) (ANOVA with Tukey test).

	Wild type	gp130-S	gp130-AS
MtT/S low cell conc. (6.8×10^5)	26.667±1.202	22.499±0.167	24.499±0.834
MtT/S high cell conc. (1×10^6)	38.500±3.170	38.830±7.170	15.165±0.835*
MtT/S low cell conc. + TtT/GF (5×10^5) + (1.8×10^5)	41.830±6.500	34.000±7.000	17.000±4.000*and **

Discussion

Pituitary tumour generation is a complex process that may involve oncogene activation and tumour suppressor gene inactivation, as well as hormones, cytokines and growth factors as promoters of their development. Molecular mechanisms that contribute to tumour development are being studied.

gp130 cytokines play an auto-paracrine role in the regulation of pituitary function in physiological and pathophysiological conditions. Several studies provide the basis to this role reporting the expression and secretion of these cytokines and the expression of their receptors in normal pituitary gland and pituitary adenomas. In addition, regulatory involvement of these cytokines in processes such as hormone secretion, cell proliferation and tumorigenesis has been described (reviewed in 7–10, 31). In spite of the studies about the role of gp130 in adenoma development, models that would provide evidence of its role in the interaction and regulation between different cell types that participate in this process have not been studied.

Here we demonstrate the participation of gp130 in auto-paracrine stimulation of *in vivo* MtT/S cell growth using as model the dependence of the somatotrophic MtT/S cell line on the folliculostellate TtT/GF cell line to induce tumour formation in nude mice (23).

According to the characterization studies, MtT/S gp130-AS clones have lower gp130 expression and signalling activation in terms of STAT3 activity levels. We observed that the IL-6-stimulated increase in STAT3 activity levels was not very strong in control clones but was consistent and higher than in AS clones. It has also been reported that MtT/S cells respond to gp130 stimuli in proliferation studies (24). Our results show that gp130-AS clones have reduced proliferative capacity in response to gp130 stimuli compared with MtT/S wild-type cells or control (empty vector) and gp130-S clones. However, gp130-AS clones are still able to respond to gp130-independent stimuli, such as cAMP, which indicates that the effects of the lower amount of gp130 protein are indeed specific and critical for the actions of gp130 cytokines in the regulation of cell proliferation.

According to the threshold theory described in Results, MtT/S tumorigenic behaviour depends on the initial cell concentration; at high concentrations their *in vivo* growth would be mediated by either autocrine stimuli, circulating cytokines, or at this cell concentration they may become independent of gp130 cytokines. In these conditions, an additional induction of gp130 signalling pathway by TtT/GF cells does not affect MtT/S tumour development. MtT/S behaviour

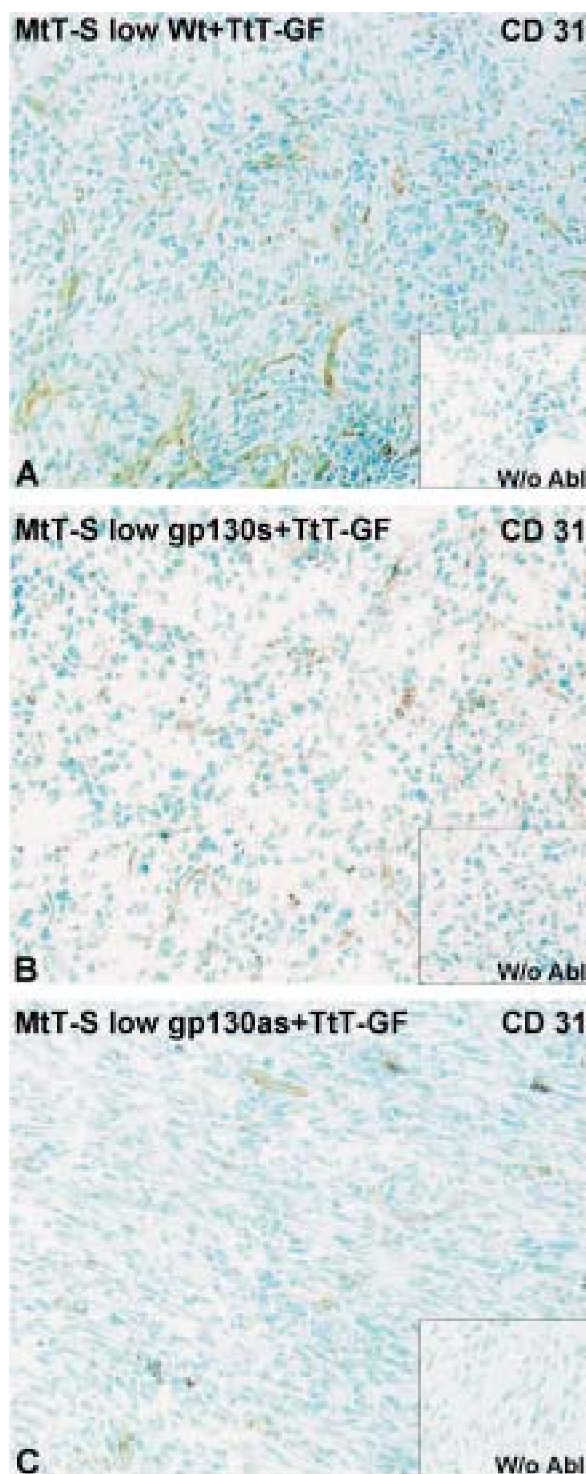

Figure 6 Density of microvessels in tumours derived from MtT/S cell at low concentration (5×10^5) + TtT/GF cells (1.8×10^5). (A) Wild-type cells-derived tumours; (B) gp130-S cells-derived tumours; (C) gp130-AS cells-derived tumours. Immunostaining for the endothelial cell marker CD31 (brown) is shown. The vessel density was significantly reduced in gp130-AS-derived tumours with respect to the gp130-S-derived tumours ($P < 0.05$) and MtT/S wild-type-derived tumours ($P < 0.001$) (ANOVA with Tukey test). Corresponding negative controls are inserted at the right corner of each picture. Similar results to wild-type cells were obtained with control cells ($n = 2$) transfected with empty vector.

at high concentrations is similar to that of GH3 cells (22). On the other hand, at low concentrations, TtT/GF cells are essential for MtT/S cells to develop their tumorigenic potential. At both concentrations, the role of gp130 cytokine in MtT/S growth is demonstrated, as gp130-AS clones generate smaller tumours compared with gp130-S, gp130-C, and MtT/S wild-type cells. Thus gp130 plays a critical role both in cellular models in which cells do not depend on FS cells (GH3 and MtT/S high concentrations) and in cells that depend on FS paracrine stimulus (MtT/S low concentrations), underlying the influence of gp130 in pituitary tumour lacto/somatotrophic development.

In immunohistochemistry studies of tumours derived from MtT/S clones, we found that vascularization in gp130-AS clones is not increased either in MtT/S at high concentrations or by the presence of TtT/GF cells. In contrast, MtT/S wild-type cells and gp130-S clones show an increase in vessel density when injected at high concentrations or coinjected with TtT/GF cells. This could be a direct effect due to suppression of gp130-mediated VEGF (15) and/or basic fibroblast growth factor production because of diminished gp130 levels, but an indirect effect due to lower tumour cell growth associated with lack of tumour expansion and hypoxia-induced neoangiogenesis cannot be ruled out.

In this study, we demonstrate a central role of gp130 cytokines in auto-paracrine stimulation of MtT/S growth, and postulate different models of cell behavior depending on cell concentration. The auto-paracrine somatotrophic cell-folliculostellate cell gp130 dependence described here constitutes an *in vivo* confirmation of the role of gp130 cytokine as a determinant of cellular behaviour and communication that leads to tumour development.

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