Inhibition of endogenous SPARC enhances pancreatic cancer cell growth: modulation by FGFR1-III isoform expression

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Secreted protein acidic and rich in cysteine (SPARC) or osteonectin is a 32–33 kDa calcium-binding glycoprotein shown to associate with the cell membrane and membrane receptors (Yan and Sage, 1999). It belongs to a family of matricellular proteins and is divided into three modules that exert various functions (Bradshaw and Sage, 2001). It functions not only to modulate cell–cell and cell–matrix interactions, but also has de-adhesive and growth inhibitory properties in non-transformed cells (Tai and Tang, 2008). In cancer, SPARC may exert divergent actions reflecting the complexity of this protein (Clark and Sage, 2001). Thus, in certain types of cancers, such as melanomas and gliomas, SPARC is associated with a highly aggressive phenotype, whereas in others, mainly ovarian, neuroblastomas, and colorectal cancers, SPARC may function as a tumour suppressor (Tai and Tang, 2008). These functions are thought to be exerted in part by its ability to counteract effects of several growth factor families including fibroblast growth factors (FGFs) (Yan and Sage, 1999; Francki et al., 2003; Motamed et al., 2003).

Modulation of FGF actions by SPARC is mediated by FGF receptor (FGFR) 1, because FGFR1 was reported to be indispensable for SPARC-induced inhibition of FGF signalling (Motamed et al., 2003). The presence of several FGFR1 isoforms generated by alternative mRNA splicing makes the analysis of FGFR1 and SPARC interactions difficult. FGF actions strongly depend on the presence of specific FGFR isoforms and can be modulated by changes in isoform expression (Ornitz et al., 1996; Kornmann et al., 2001; Liu et al., 2007a). Alternative splicing of the second half of Ig-like domain III generates particular important isoforms, because this region determines ligand-binding specificity (Ornitz et al., 1996; Plotnikov et al., 2000). As a consequence, over-expression of FGFR1-IIIc in pancreatic cancer promoted tumourigenesis, whereas over-expression of FGFR1-IIIb inhibited the malignant phenotype (Kornmann et al., 2002; Liu et al., 2007b). In addition, several FGFs, signalling through FGFR1-IIIc, are highly over-expressed in human pancreatic ductal adenocarcinoma (PDAC) (Yamanaka et al., 1993). Several recent studies reported increased SPARC levels in human PDAC (Sato et al., 2003; Guweidhi et al., 2005) associated with poor prognosis (Infante et al., 2007). SPARC expression was absent in most of the cancer cells, but instead present at high levels in the peritumoural tissue harbouring fibroblasts and pancreatic stellate cells (PSCs) (Guweidhi et al., 2005; Infante et al., 2007).

Despite these recent efforts, the functions of SPARC and its associations with FGFR1-III isoforms in PDAC remain unclear. Therefore, the aim of this study was to elucidate SPARC functions and endogenous SPARC regulation depending on FGFR1-III isoforms expression in PDAC cells and its interaction with PSC cells.
MATERIALS AND METHODS

Cell culture

ASPC-1, BXPC-3, CAPAN-1, MIA PaCa-2, and PANC-1 human pancreatic cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), COLO357 and T3M4 human pancreatic cancer cells were a gift from M Korc (Dartmouth Medical School, Hanover, NH, USA). Human PSC were isolated and characterised as described earlier (Bachem et al, 2005). PANC-1, Mia PaCa-2, and COLO357 cells were grown in DME medium and ASPC, BXPC-3, CAPAN-1, and T3M4 cells in RPMI 1640 medium supplemented with 10% foetal bovine serum (FCS), penicillin G (100 units ml–1), and streptomycin (100 μg ml–1) termed as complete medium. PSCs were grown in complete DME/F12 (1 : 1) medium supplemented with Amphotericin B (250 μg ml–1). The respective media supplemented with 0.1% BSA, 5 mg ml–1 transferrin, 5 μg ml–1 selenium, penicillin G (100 units ml–1), and streptomycin (100 μg ml–1) were used as serum-free media. PANC-1 clones PF4 and PF40 (over-expressing FGFR1-IIIb), and PFC18 and PFC51 (over-expressing FGFR1-IIIc) were grown in complete DME medium with G418 (1.2 mg ml–1). SPARC shRNA-transfected cells were grown in complete RPMI and DME medium with G418 (0.8 and 1.2 mg ml–1, respectively) for ASPC-1 and PANC-1. Cells were maintained in monolayer culture at 37°C in humidified air with 5% CO2.

Establishment of cell clones over-expressing FGFR1-III variants

The establishment of the FGFR1-IIIb and -IIIc PANC-1 clones over-expressing the full-length cDNA of human FGFR1-IIIb or -IIIc expressed in a modified pSVK3 vector under the control of the simian virus 40 early promotor in a stable manner was described earlier (Liu et al, 2007b; Chen et al, 2008).

Establishment of cell clones expressing SPARC shRNA

Validated SureSilencing human SPARC shRNA and control plasmids were from SuperArray Bioscience Corp. (Frederick, MD, USA). ASPC-1 and PANC-1 cells were transfected in a stable manner using lipofectamine (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Transfected cells were selected with G418 (0.8 and 1.2 mg ml–1 for ASPC-1 and PANC-1, respectively) for 14 days before isolation of individual clones.

Immunoblot analysis

Total cell lysates were prepared and followed by immunoblot analysis as described (Liu et al, 2007b). A rabbit polyclonal antibody (SPARC, sc-25574, from Santa Cruz) was used (1 : 200) to detect SPARC protein. Bound antibodies were visualised using...
enhanced chemiluminescence. To confirm equal loading, membranes were stripped for 30 min at 50 °C
in complete medium in the absence or presence of SPARC. To assess the effect of SPARC on FGF-induced proliferation, cells (10,000 per well) were propagated for 48 h in serum-free medium including the indicated factors. FGF1 (recombinant human FGF acid, R&D Systems) and FGF2 (recombinant human FGF basic, R&D Systems) were added in the presence of heparin (1 μg ml⁻¹).

To assess N-linked protein, glycosylation cells were incubated in the absence or presence of tunicamycin (5 μg ml⁻¹, T7765 Sigma) for 24 h as described (Liu et al., 2007a). To assess the effect of kinase inhibitors, cells were incubated in the absence or presence of the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (Calbiochem, Darmstadt, Germany) and the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (Calbiochem) for 24 h (Liu et al., 2007a).

Single cell movement

Cells (50,000 per well) were seeded onto fibronectin-coated (5 μg ml⁻¹ in PBS) six-well plates and grown for 20 h. Cells were then incubated in the presence or absence of SPARC (5 μg ml⁻¹) for 24 h. Cell movement during that period was monitored by an Olympus IX81 motorised inverted microscope taking pictures every 10 min (Liu et al., 2007b). The total distance of individual cells covered within 24 h was determined using the ImageJ 1.32 program (NIH, Bethesda, MD, USA).

Cell migration assay

The ability of cells to migrate through filters was measured using a BioCoat Matrigel invasion chamber (BD Biosciences, San Jose, CA, USA). Cell culture inserts with an 8 μm pore size PET membrane were used according to the protocol of the manufacturer. The bottom chamber included medium (0.75 ml) containing 10% FCS, whereas cells (1.0 × 10⁶ suspended in 0.5 ml of medium containing 1% FCS) were seeded into the upper chamber and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then incubated in the presence or absence of exogenous SPARC (5 μg ml⁻¹) for another 24 h. Remaining cells on the upper surface were mechanically removed. Membranes were then washed, fixed, and stained by Diff-Quik (Medion Diagnostics, Dübening, Switzerland). The number of cells that migrated to the lower surface of the filters was determined by counting stained cells under a light microscope.

Anchorage-independent growth assay

Basal anchorage-independent cell growth was assessed by a double-layer soft-agar assay as described (Kornmann et al., 2002). Briefly, cells were suspended in complete medium containing 0.3% agar and seeded in triplicate in six-well plates onto a base layer of complete medium containing 0.5% agar. One milliliter of complete medium containing 0.3% agar was added every 5 days. After 14 days, 300 μg MTT per well was added to stain vital colonies for 24 h before counting by microscopy.

In vivo tumourigenicity assay

To assess the effect of expression of SPARC on xenograft formation, 10⁵ cells per site were injected s.c. into two sites of 4- to 6-week-old female athymic (nude) mice. Animals were monitored for tumour formation every 4 days. Tumour size was measured in three dimensions. Tumour volume was determined by the equation vol = l × w × d × 0.5, where l is the length, w is the width, and d is the diameter. Animals had to be killed 12 weeks after injection according to our animal protocol (#718) if neither tumour volume (≥ 2 cm³) nor skin ulcerations prompted earlier termination.

Immunohistochemistry of xenograft tumours

SPARC expression in control-transfected (n = 10) and FGFR1-IIIb over-expressing (n = 10) xenograft tumours (Liu et al., 2007b)
was determined by immunohistochemical analysis. SPARC immunohistochemistry was performed using formalin-fixed and paraffin-embedded sections using a rabbit polyclonal antibody detecting human SPARC (1:2000, sc-25574 from Santa Cruz) in combination with a secondary biotynylated goat anti-rabbit antibody and a Vectastain Elite ABC kit (Vector Lab, Burlingame, CA, USA) according to the protocol of the manufacturer.

Detection of SPARC in conditioned medium

Indicated cells were grown in complete medium to 70% confluency in 10 cm dishes. After washing twice with PBS, cells were incubated for 48 h in 10 ml of serum-free medium containing proteinase inhibitors as described (Kornmann et al., 1997). For immunoblot analysis, conditioned medium of five dishes was collected and incubated at 4°C overnight after adjusting the pH to 7.4 and adding 50 μl slurry heparin sepharose (CL-6B, Pharmacia Biotech, Piscataway, NY, USA) to pull-down calcium-binding proteins (Kornmann et al., 1997). The beads were collected by centrifugation, washed three times with 0.45 M NaCl/20 mM Tris–HCl (pH 7.4), and resuspended in 2× Laemmli buffer. Samples were boiled for 5 min and subjected to immunoblot analysis.

Statistics

The results were expressed as mean expression levels (±s.d. or s.e.m.). Student’s t-test or rank sum test was used for statistical analysis. A P-value < 0.05 was taken as level of significance (two sided).

RESULTS

Expression of SPARC and its effects on proliferation and migration in cultured PDAC cells

A clear signal of SPARC protein, migrating under reducing conditions at 43 kDa, and mRNA was found in ASPC-1 and PANC-1 cells (Figure 1A), whereas very weak signals of SPARC...
mRNA were observed in the remaining five cell lines (Figure 1A, lower panel). In contrast, higher levels of SPARC were found in human PSCs (Figure 1A). Independent of endogenous SPARC expression, exogenous SPARC inhibited proliferation of PDAC cells. This effect was most pronounced in COLO-357 and PANC-1 cells (Figure 1B). Exogenous SPARC (5 \( \mu \text{g m}^{-1} \)) also reduced proliferation of CAPAN-1 (\(-21 \pm 0.1\%\) s.e.m.) and MIA PaCa-2 cells (\(-20 \pm 1.8\%\) s.e.m.), but was without marked effect in ASPC-1 (\(-12 \pm 0.4\%\) s.e.m.), BxPC-3 (\(-7.5 \pm 1.1\%\) s.e.m.), and T3M4 cells (\(0.7 \pm 2.1\%\) s.e.m.).

The effects of exogenous SPARC (5 \( \mu \text{g m}^{-1} \)) on single cell movement and migration were investigated in PANC-1 and MIA PaCa-2 cells. SPARC reduced the distance covered within 24 h by 38% and 28% in PANC-1 and MIA PaCa-2 cells, respectively (Figure 1C). Cell migration was inhibited in the presence of SPARC by 58% and 26%, respectively (Figure 1D).

**Inhibition of endogenous SPARC expression**

To determine the importance of endogenous SPARC expression for the malignant phenotype of cultured PDAC cells, ASPC-1 and PANC-1 cell clones were established expressing SPARC shRNA in a stable manner. Screening of the clones was performed by histochemical analysis of SPARC protein expression of tumours expressing either FGF1-IIIb (PF4, PF40) or FGF1-IIIc (PF18c, PF51c) was characterised. Expression of SPARC was enhanced in FGF1-IIIb over-expressing clones, whereas SPARC expression was decreased in FGF1-IIIc over-expressing clones (Figure 5A).

This was confirmed in a xenograft model in vivo. Immunohistochemical analysis of SPARC protein expression of tumours over-expressing FGF1-IIIb (Liu et al, 2007b) revealed that SPARC protein was up-regulated in FGF1-IIIb over-expressing tumours (Figure 3B).

Incubation with tunicamycin, an inhibitor of glycosylation, showed that up-regulated SPARC protein in FGF1-IIIb over-expressing PANC-1 cells was a glycosylated protein similar to wild-type cells (Figure 5C).

**Effects of FGFR1 expression on SPARC modulation of FGF1 and FGF2**

Modulation of FGF actions by SPARC was reported to depend on FGFR1 expression (Motamed et al, 2003). It is not known, however, whether differences among the existing FGFR1 variants in mediating SPARC-modulated FGF actions. Therefore, we next investigated SPARC modulation of FGF-depended proliferation in regard to FGFR1-III isoform expression. Wild-type PANC-1, control-transfected (PN5), FGF1-IIIb over-expressing (PF4), and FGF1-IIIc over-expressing (PFc51) cells were incubated with FGF1 and FGF2 in the presence and absence of SPARC. As reported earlier, over-expression of FGF1-IIIc (PFc51) resulted in enhanced FGF1- and FGF2-induced proliferation in comparison with wild-type, control-transfected, and FGF1-IIIb (PF4) over-expressing cells (Figure 4). FGF2 effects were not markedly altered by exogenous SPARC. Irrespective of the FGF1-III isoform and the degree of FGF1-induced proliferation, the inhibitory effects of SPARC on FGF1-mediated proliferation seemed to be slightly more pronounced in FGF1-IIIb over-expressing clones compared with cells expressing lower levels of FGF1 (Figure 4). This observation did not reach any significance.

**Effect of FGFR1-III domain expression on SPARC expression**

Over-expression of FGF1-IIIb and FGFR1-IIIc enhanced the malignant phenotype of PDAC cells (Liu et al, 2007b). In the next set of experiments, endogenous SPARC expression in PANC-1 cells over-expressing either FGF1-IIIb (PF4, PF40) or FGF1-IIIc (PF18c, PF51c) was characterised. Expression of SPARC was enhanced in FGF1-IIIb over-expressing clones, whereas SPARC expression was decreased in FGF1-IIIc over-expressing clones (Figure 5A).
The mechanism of SPARC up-regulation in FGFR1-IIIb-expressing clones is unclear. We showed recently that FGFR1-IIIb up-expression was accompanied by up-regulation of p38-MAPK and JNK activities (Liu et al., 2007a, b). Incubating FGFR1-IIIb over-expressing PF4 and PF40 cells with increasing concentrations of the p38-MAPK inhibitor SB203580 (10, 20, and 40 μM) and the JNK inhibitor SP600125 (1, 2, and 4 μM) revealed that SB203580 reduced expression of SPARC, whereas SP600125 was without effect. In Figure 5D, the effects of 20 μM SB203580 and of 2 μM SP600125 are shown for FGFR1-IIIb over-expressing PF4 and PF40 and wild-type PANC-1 cells. Indicated cells were incubated in the absence (−) and presence (+) of SB203580 (20 μM) or SP600125 (2 μM) for 24 h before analysis.

**DISCUSSION**

SPARC is a matricellular protein with antiproliferative and counteradhesive functions (Yan and Sage, 1999). Its function in cancer is discussed in a very controversial manner (Clark and Sage, 2008; Podhajcer et al., 2008; Tai and Tang, 2008). In certain types of cancers, such as melanomas and gliomas, SPARC is associated with a highly aggressive tumour phenotype. In others, mainly ovarian, neuroblastomas, and colorectal cancers, SPARC may function as a tumour suppressor (Tai and Tang, 2008). We showed in this study that exogenous SPARC can inhibit cell proliferation, single cell movement, and migration of cultured PDAC cells. These exogenous SPARC functions were independent of endogenous SPARC expression. As reported (Sato et al., 2003; Guwedhi et al., 2005), the majority of the cell lines did not express endogenous SPARC, probably as a result of aberrant hypermethylation (Sato et al., 2003; Cheetham et al., 2008).
Inhibition of endogenous SPARC in cultured human PDAC cells by small hairpin RNA enhanced cell proliferation, migration, colony formation, and xenograft formation. These results indicate that endogenous SPARC may act as a tumour suppressor in PDAC cells, a function SPARC also has in ovarian, neuroblastomas, and colorectal cancers (Tai and Tang, 2008). This function of a tumour suppressor is also supported by our finding that inhibition of endogenous SPARC resulted in a doubling of the mitogenic activity that endogenous SPARC may act as a tumour suppressor in human PDAC cells. Future studies in human pancreatic cancer could aim at the design of treatment strategies specifically targeting SPARC–FGFR1 interactions.

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Down-regulation of SPARC in pancreatic cancer cells is believed to depend on DNA methylation (Sato et al, 2003; Guweidhi et al, 2005; Infante et al, 2007; Cheetham et al, 2008). Another reason contributing to the down-regulation of SPARC in human pancreatic cancer cells may be the over-expression of FGFR1-IIIc. FGFR1-IIIc is the predominant FGFR1 isoform expressed in PDAC (Kornmann et al, 2002). FGFR1-IIIb usually expressed in cells of epithelial origin is almost absent in PDAC (Kornmann et al, 2002). Re- or over-expression of FGFR1-IIIb in PDAC cells resulted in a marked up-regulation of endogenous SPARC in vitro and in vivo. In contrast, an additional over-expression of FGFR1-IIIc further lowered endogenous SPARC expression. We recently showed that over-expression of FGFR1-IIIb in PDAC cells reverted the malignant phenotype inhibiting proliferation, single cell movement, and migration in vitro, as well as xenograft formation and growth in vivo (Liu et al, 2007b). On the other hand, it is well known that over-expression of FGFR1-IIIc enhances the malignant phenotype of PDAC (Wagner et al, 1998; Kornmann et al, 2002). FGFR1-IIIb over-expression in PDAC cells resulted in strong p38-MAPK and JNK activation (Liu et al, 2007a, b). In this study, we also investigated the effects of specific inhibitors of these kinases on endogenous SPARC levels. Our results showed that endogenous SPARC levels could be down-regulated by inhibition of p38-MAPK, but not of JNK. Therefore, our observations suggest that modulation of endogenous SPARC expression may be one of the mechanisms resulting in the different phenotypes seen for the FGFR1-III domain variants and that the observed FGFR1-IIIb-induced induction of endogenous SPARC is mediated through p38-MAPK.

Recent studies investigating SPARC expression in human pancreatic tissues reported high levels of SPARC in the surrounding stromal tissue harbouring fibroblasts and PSCs, whereas SPARC was often absent in the cancer cells (Guweidhi et al, 2005; Infante et al, 2007). High SPARC expression in the stroma portended a poor patient prognosis (Infante et al, 2007). We showed in our study that PSCs express higher levels of endogenous SPARC than cultured PDAC cells and that SPARC is detectable in the conditioned medium of PSCs. Our study also revealed that conditioned medium of pancreatic cancer cells down-regulated endogenous SPARC expression of PSCs. In contrast, co-culture of fibroblasts with the presence of PDAC cells augmented SPARC expression in fibroblasts (Sato et al, 2003), suggesting that high SPARC expression in the tumour stroma may be mainly a result of augmented SPARC expression in stromal fibroblasts.

In summary, we showed that inhibition of endogenous SPARC enhances the malignant phenotype of PDAC cells and showed that endogenous SPARC expression is regulated by FGFR1 domain III isoform expression. On the basis of these observations, we conclude that endogenous SPARC levels can contribute to the reversion of the malignant phenotype and may, therefore, act as a tumour suppressor in human PDAC cells. Future studies in human pancreatic cancer could aim at the design of treatment strategies specifically targeting SPARC–FGFR1 interactions.

Figure 6 Secretion and regulation of SPARC protein expression in human pancreatic stellate cells (PSCs). (A) Detection of SPARC protein in conditioned medium. Conditioned medium of PSCs, PANC-1, and COLO-357 was collected for 48 h and subjected to immunoblot analysis after pull down of heparin-binding proteins by heparin sepharose. Cell lysates of PSCs were used as positive control (control), medium alone as negative control (medium). (B) Regulation of endogenous SPARC expression in PSCs. PSCs were incubated (treatment) with conditioned medium of PSCs, COLO-357, or PANC-1 cells for 48 h. SPARC immunoblot analysis of total PSC cell lysates after incubation is shown in the upper panel, relative SPARC protein expression after densitometric analysis in the lower panel. The results in the lower panel are mean SPARC levels (± s.d.) in relation to PSC (100%) of three separate experiments. *P<0.05 compared with control (PSC).
