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Down-regulation of osteopontin mediates a novel mechanism underlying the cytostatic activity of TGF-β

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Abstract

Purpose Loss of a cytostatic response to TGF-β has been implicated in multiple hyper-proliferative disorders, including cancer. Although several key genes involved in the cytostatic activity of TGF-β have in the past been identified, its exact mode of action is yet to be elucidated. A comprehensive understanding of the mechanisms underlying the cytostatic activity of TGF-β may open up new avenues for the development of therapeutic strategies.

Methods Quantitative real-time RT-PCR was used to assess osteopontin (*OPN*) gene expression in human hepatoma-derived Huh-7 and lung adenocarcinoma-derived A549 cells. Reporter assays using an *OPN* promoter-luciferase construct and its mutated counterparts were performed to assess its transcriptional activity. Binding of Smad4 to the *OPN* gene promoter was investigated using chromatin immunoprecipitation (CHIP). The putative role of Smad4 in *OPN* gene expression down-regulation was also assessed using a shRNA-mediated knockdown strategy. The anti-proliferative effect of TGF-β on different cancer-derived cell lines was determined using the cell proliferation reagent WST-1.

Results We found that the *OPN* expression levels dose-dependently decreased in TGF-β-treated Huh-7 and A549 cells. Our reporter assays indicated that this TGF-β-induced repression occurred at the transcriptional level, and could largely be abrogated by disruption of an element (TIE2) similar to the TGF-β inhibitory element found in other TGF-β-repressed genes. Our CHIP assay revealed that the Smad protein complex specifically binds to the *OPN* gene promoter, and that the TGF-β-mediated inhibition of *OPN* was lost upon shRNA-mediated knockdown of Smad4. Moreover, we found that the deregulation of *OPN* gene expression by TGF-β occurred concomitantly with loss of the TGF-β anti-proliferative response, whereas a neutralizing anti-OPN antibody partially restored this response.

Conclusions Our results indicate that the *OPN* gene is a direct target of Smad-mediated TGF-β signaling, implying that *OPN* expression inhibition serves as a novel mechanism underlying the cytostatic activity of TGF-β.

Keywords TGF-β · Smad · Cytostatic effect · Osteopontin

1 Introduction

Transforming growth factor-β (TGF-β) is a plurifunctional cytokine that intricately controls multiple fundamental cellular processes, including proliferation, migration, differentiation and survival [1–4]. TGF-β signaling is triggered by its binding to TGF-β receptors, which initiates Smad2/3/4 complex formation followed by translocation to the nucleus where it regulates the transcription of target genes [5, 6]. Under normal and premalignant conditions, TGF-β plays a role in maintaining tissue homeostasis by inducing tumor-suppressive effects, including cytostasis, differentiation and apoptosis, whereas also a tumor-promoting role of TGF-β has been observed in the context of advanced cancer [7–9]. The cytostatic effect of TGF-β involves activation of the cyclin-dependent kinase (CDK) inhibitors p15^{Ink4B}, p21$^{WAF1/Cip1}$ and p57^{Kip2}, and repression of the proto-oncogene *c-Myc*, the inhibitors of

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differentiation Id1, Id2 and Id3 and the CDK activator CDC25A [10–12]. Loss of cytostatic responses to TGF-β have been implicated in multiple hyper-proliferative disorders such as cancer [13–15]. Estrogen, a new member on the list of putative cancer-causing agents, disrupts TGF-β signaling, renders cells insensitive to growth inhibition by TGF-β and potentially accounts for the development of hormone-related malignancies such as breast, endometrial and uterine cancers [16]. Activation of TGF-β has, on the other hand, been found to be at least partially involved in anti-estrogen-mediated growth inhibition. A selective estrogen receptor modulator, raloxifene, which exerts estrogen-antagonistic effects on normal uterine endometrium and breast tissues, has e.g. been found to inhibit estrogen-related cell proliferation and endometrial carcinogenesis [17–19].

Although the molecular mechanism employed by TGF-β to switch from a tumor suppressor to a tumor enhancer is yet to be clarified, the escape from TGF-β-mediated growth constraints in cancer cells can largely be attributed to mutation and/or functional inactivation of TGF-β receptors or alterations of downstream mediators in the Smad signaling pathway [20]. This escape provides the cancer cell with the ability to use TGF-β as an oncogenic factor promoting motility, invasion, metastasis and epithelial-to-mesenchymal transition [21, 22].

Osteopontin (OPN) is a secreted multifunctional glycoprotein that has been implicated in disparate processes such as inflammation, wound healing, bone formation and remodeling, as also tumor growth and metastasis [23–25]. By acting through two cell adhesion molecules, i.e., integrin and CD44, OPN can initiate a complex signaling cascade promoting proliferation, migration and invasion of tumor cells, inhibiting apoptosis, and facilitating extracellular remodeling and angiogenic processes [26, 27]. Consistent with these observations, clinical statistical analyses have shown that the expression level of OPN correlates well with tumor grade and progression in multiple cancers such as breast, stomach, lung, prostate, liver and colon cancer [28–32], implying the utility of OPN as a molecular target for anti-cancer therapy.

Here, we identify OPN as a putative player in the cytostatic response to TGF-β. Our data emphasize the possibility to develop novel drugs targeting OPN for the treatment of human malignancies.

2 Materials and methods

2.1 Plasmid constructs

The reporter vector pOPN1-luc, and its 5′-truncated derivatives, have been described before [33]. pOPN-lucmTIE1, pOPN-lucmTIE2 and pOPN-lucmTIE1 + 2 are identical to pOPN1-luc, except for mutations introduced to disrupt the putative TIE1 and TIE2 sites individually or in combination. These mutations were generated by PCR-directed mutagenesis. Briefly, using pOPN1-luc as template, two overlapping PCR fragments were amplified using the following primers: for pOPN-lucmTIE1, 5′-ctcggtaccTAGGTAATAGTA TTGCA-3′ and 5′-CATCCTCCAATAACACAGGGAGGC-3′, or 5′-CCCTGTGTTATTGGAGGATGTCTG-3′; and 5′-atagagctcTGCCTCCTCCTGCT-3′; for pOPN-lucmTIE2, 5′-ctcggtaccTAGGTAATAGTATTGCA-3′ and 5′-TGCTGACAAATAAGCCCTCCCAGA-3′, or 5′-GGAGGGCTTATTTGTCAGCAGCAG-3′ and 5′-atagagctcTGCCTCCTCCTGCT-3′. Each two PCR fragments were re-amplified by overlap extension, after which the resulting products were digested with Kpn I and Sac I and inserted into a pGL3-basic vector. The sequences of the PCR-mutated regions and the presence of the expected mutations were confirmed by Sanger sequencing.

For construction of plasmids expressing shRNA against Smad4, sense and antisense oligonucleotides of self-complementary sequences containing Smad4 target sequences (sh-Smad4A: 5′-GGTGTGCAGTTGGAATGTA-3′; sh-Smad4B: 5′-GGTGGAGAGAGTGAAACAT-3′), containing cohesive ends for BamH I and EcoR I sites at their 5′- and 3′-ends, were synthesized and annealed. After gel electrophoresis purification, the annealed oligonucleotides were ligated into a pshuttleU6 vector that was opened up with the same enzymes [34]. The cloned sequences were confirmed by Sanger sequencing.

2.2 Cell lines and culture conditions

All cancer-derived cell lines used (Huh-7, QGP-1, A549, OUR-10 and PA-1) were purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 % fetal bovine serum (FBS) and 50 u/ml penicillin and streptomycin at 37 °C in a 5 % CO_2 humidified atmosphere.

2.3 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analyses were performed using an EpiXplore ChIP assay kit according to the manufacturer's instructions (Clontech) with minor modifications [35]. Briefly, 5×10^6 cells were fixed with formaldehyde and cell lysates were sonicated to shear DNA to lengths between 200 and 1000 bp. This sheared DNA was immunoprecipitated using a goat polyclonal IgG directed against Smad4 (sc-1909) or a control goat IgG (sc-2028). After de-crosslinking and purification with proteinase K and RNaseA, the DNAs were extracted and amplified using sets of primers for the putative TIE sites in the OPN gene promoter (5′-ACTGTAGATTGTGTGTGTGC-3′ and 5′-

CTGCCTCCTCCTGCTGCTGC-3′) and the *c-Myc* gene promoter (5′- CCCGAGCTGTGCTGCTCGCG-3′ and 5′-GCTGGAATTACTACAGCGAG-3′). Input controls were included in the PCR reactions. The PCR products were resolved in 2 % agarose gels and visualized by ethidium bromide staining.

2.4 Transient transfection and luciferase assays

Cells were seeded at a density of 1×10^5 per 1 ml medium per well in 12-well plates 24 h before transfection. Next, plasmid DNAs were transfected into cells using Effectene (Qiagen). In each transfection a pRL-TK (Promega) vector was co-transfected as an internal control to normalize the transfection efficiency. The cells were harvested 48 h post transfection after which cell lysates were prepared for luciferase assays using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a GloMax® 20/20 Luminometer (Promega).

2.5 Cell proliferation assay

Cells were seeded in 96-well plates at a concentration of 1×10^4 cells/well in 100 μl medium. After 24 h, TGF-β1 (Pepro Tech) was added to the wells at various concentrations. After an additional 72-h culture period, cell growth was measured using cell proliferation reagent WST-1 (Roche) according to the manufacturer's instructions.

2.6 ELISA

For OPN detection, supernatants were collected from cell cultures after 48 h in the absence or presence of TGF-β1. The OPN levels were determined using an ELISA kit (R&D Systems).

2.7 Real-time RT-PCR

Cells were lysed with TRIzol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. After treatment with RNase-free DNase (Promega) the RNA (250 ng) was used for first-strand cDNA synthesis at 42 °C for 60 min using MMLV Reverse transcriptase (Invitrogen). Real-time quantitative PCR using a Power SYBR Green PCR Master Mix (Applied Biosystms) was performed for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min in a 96-well format on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). The primer sequences used were as follows: OPN forward, 5′- ACTCGTCTCAGGCCAGTTG-3′, OPN reverse, 5′-CGTTGGACTTGGAAGG-3′; GAPDH forward, 5′-TGATGACATCAAGAAGGTGG-3′, GAPDH reverse, 5′-TCCTTGGAGGCCATGTGGGC-3′.

3 Results

3.1 OPN is down-regulated by TGF-β in A549 and Huh-7 cells

Our previous microarray-based gene profiling analyses revealed that decreased *OPN* expression in compound-treated A549 human lung adenocarcinoma-derived cells was associated with the activation of multiple TGF-β regulated target genes (unpublished results). This finding promoted us to hypothesize that *OPN* may act as a downstream target in the TGF-β signaling cascade. To address this issue, *OPN* mRNA expression levels were determined by real-time RT-PCR in human Huh-7 hepatoma-derived cells that were cultured in the absence or presence of recombinant TGF-β1. It was found that TGF-β, but not BMP-2, treatment led to a reduction in *OPN* mRNA levels in a dose-dependent manner (Fig. 1a). Similar results were obtained using a pOPN1-luc reporter assay, in which the luciferase gene is directed under the control of a human *OPN* gene promoter [29]. We found that OPN-directed luciferase expression in transfected A549 cells was dose-dependently suppressed by TGF-β1 treatment (Fig. 1b), while no effect was observed using an empty vector (not shown). Concordantly, a dose-dependent decrease in endogenous *OPN* mRNA accumulation was observed in TGF-β-treated A549 cells (Fig. 1c). These results confirm transcriptional regulation of the *OPN* gene by TGF-β. The TGF-β concentration required to inhibit *OPN* expression in A549 cells was higher than that in Huh-7 cells, which may be attributed to the poorer responsiveness of A549 cells to TGF-β (see below).

3.2 Identification of a putative TIE motif in the *OPN* promoter

In order to dissect the transcriptional suppression of *OPN* by TGF-β, we generated several 5′ deletion constructs of its promoter (Fig. 2a) and assessed the activity of each construct in Huh-7 cells treated with either the vehicle or TGF-β1 (3 ng/ml). By using these truncated constructs, we observed varying basal promoter activities, with the shortest construct (−180 to 23) resulting in the lowest activity. However, we found that TGF-β inhibited the activity of each construct with a similar magnitude (Fig. 2a), suggesting that the TGF-β responsive element(s) must reside downstream of −180. A web-based computer analysis of the region ranging from −180 to 23 indicated the presence of two TIE-like sites at −57 to −48 (designated TIE1) and −16 to −7 (designated TIE2) from the transcription start site, which are similar to the TIE sequence reported in the TGF-β responsive *c-Myc* and *Stromelysin-1* gene promoters. To assess whether these putative TIE sites are implicated in the observed TGF-β-induced *OPN* suppression, we mutated these elements individually (pOPN-
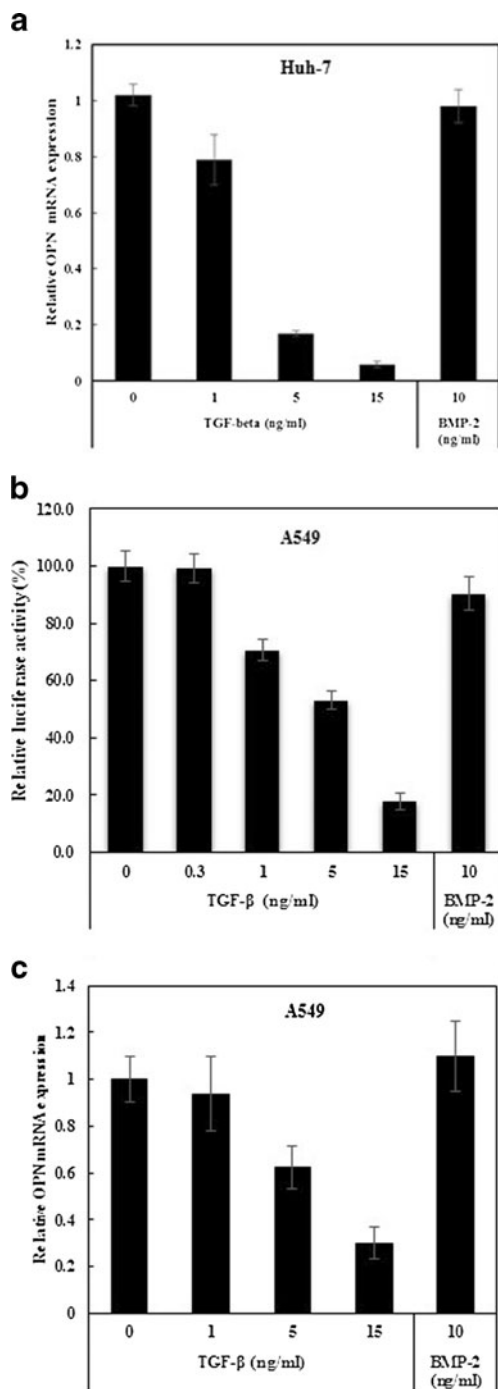
Fig. 1 Transcriptional down-regulation of the *OPN* gene by TGF-β. Huh-7 cells (**a**) or A549 cells (**c**) were cultured in the absence or presence of various concentrations of TGF-β1 or BMP-2 for 24 h, after which RNA was isolated and subjected to quantitative real-time PCR. All samples were normalized to the *GAPDH* expression level. Each experiment was carried out in triplicate. (**b**) A549 cells were transfected with pOPN1-luc and cultured in the absence or presence of various concentrations of TGF-β1 or BMP-2. Luciferase activities in the lysates were measured 48 h post transfection. Renilla luciferase activities of co-transfected pRL-TK vectors were used to normalize the transfection efficiencies. Normalized luciferase activities from mock-treated cells were set at 100 %, and those from the others were expressed as relative percentages. The results are presented as means ± standard deviations of three independent triplicate transfections

lucmTIE1 and pOPN-lucmTIE2) or in combination (pOPN-lucmTIE1 + 2) using site-directed mutagenesis. Compared to the wild-type construct (pOPN1-luc), we found that the pOPN-lucmTIE2 construct exhibited a higher basal activity and, importantly, we found that TGF-β-mediated *OPN* repression was largely abolished by the mutation introduced in pOPN-lucmTIE2, while the mutation introduced in pOPN-lucmTIE1 did not significantly affect the responsiveness to TGF-β1 treatment (Fig. 2b). Simultaneous ablation of both TIE1 and TIE2 in pOPN-lucmTIE1 + 2 did not further attenuate *OPN* suppression by TGF-β. These data suggest that TIE2 is the major element responsible for TGF-β-induced repression of *OPN* gene transcription.

### 3.3 Smad binds to the putative TIE motif in the *OPN* promoter

The data presented above suggest that a putative TIE motif in the proximal region of the *OPN* promoter is responsible for its suppression by TGF-β. Next, we set out to investigate whether *OPN* acts as a downstream target within the TGF-β signaling pathway involving specific recognition by the Smad complex. For this purpose, we performed a ChIP assay in TGF-β-treated Huh-7 cells. To this end, cross-linked chromatin was incubated with an anti-Smad4 antibody, or normal goat IgG as a negative control, and the immunoprecipitated DNAs were detected using PCR primers flanking the TIE-like sequences in the *OPN* gene promoter. PCR products were readily detected in the immunoprecipitates recovered with the anti-Smad4 antibody, while no amplified products were observed in the immunoprecipitates recovered with the control goat IgG (Fig. 3a). As a positive control, we analyzed the same immunoprecipitates for the presence of *c-Myc* promoter sequences, a well-documented TGF-β target gene. As expected, we found efficient amplification of promoter fragments using the *c-Myc* primer pair, while no amplified fragments were detected using a *β-actin* primer pair, which served as a TGF-β unresponsive control. This result indicates a specific binding of the Smad complex to the *OPN* gene promoter.

3.4 Smad4 knockdown abolishes TGF-β-mediated *OPN* gene repression

To further confirm that *OPN* acts as a downstream target of TGF-β/Smad signaling, we employed shRNA-mediated Smad4 silencing. pOPN1-luc was transfected with a construct expressing a negative control (sh-scramble) or two independent Smad4 short hairpin sequences (sh-Smad4A and sh-Smad4B), which resulted in a moderate knock-down of Smad4 as detected by quantitative real-time RT-PCR (Fig. 3b, upper panel). In the absence of TGF-β, the *OPN* gene promoter activities in cells transfected with the sh-Smad4 constructs were higher than in those transfected with
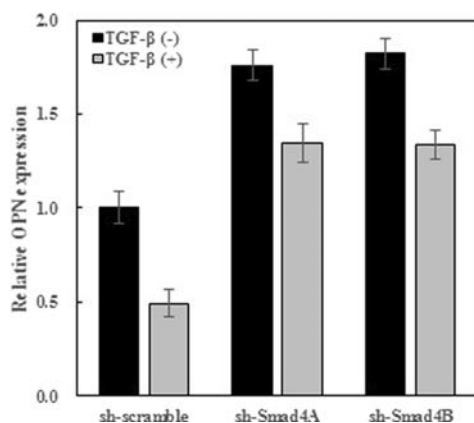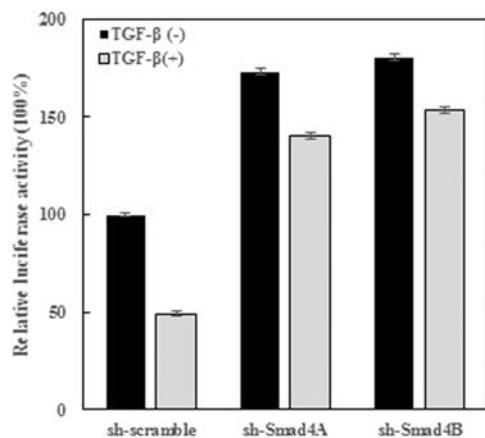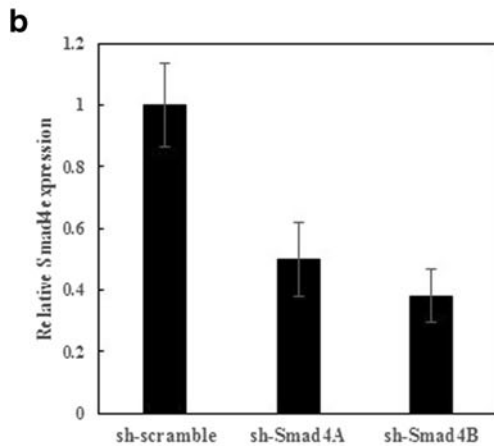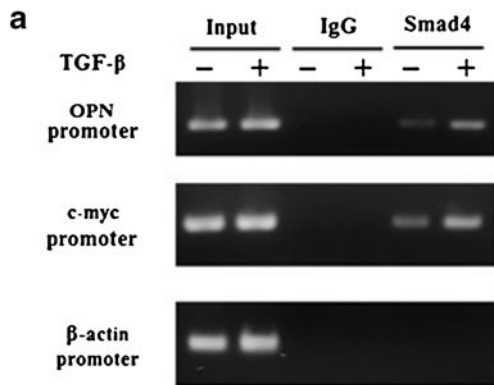
Fig. 2 Delineation of an *OPN* gene promoter sequence motif responsible for TGF-β-induced inhibition. Huh-7 cells were transiently transfected with reporter vectors under the control of various *OPN* gene promoter truncation mutants (**a**), or its mutants pOPN-lucmTIE1, pOPN-lucmTIE2 or pOPN-lucmTIE1 + 2, in which the putative TIE sites 1 and/or 2 were mutated as indicated (**b**), followed by culture in the absence or presence of 3 ng/ml TGF-β1 for 48 h. Relative luciferase activities were determined and calculated as described in the legend of Fig. 1. The results are derived from three independent triplicate transfections
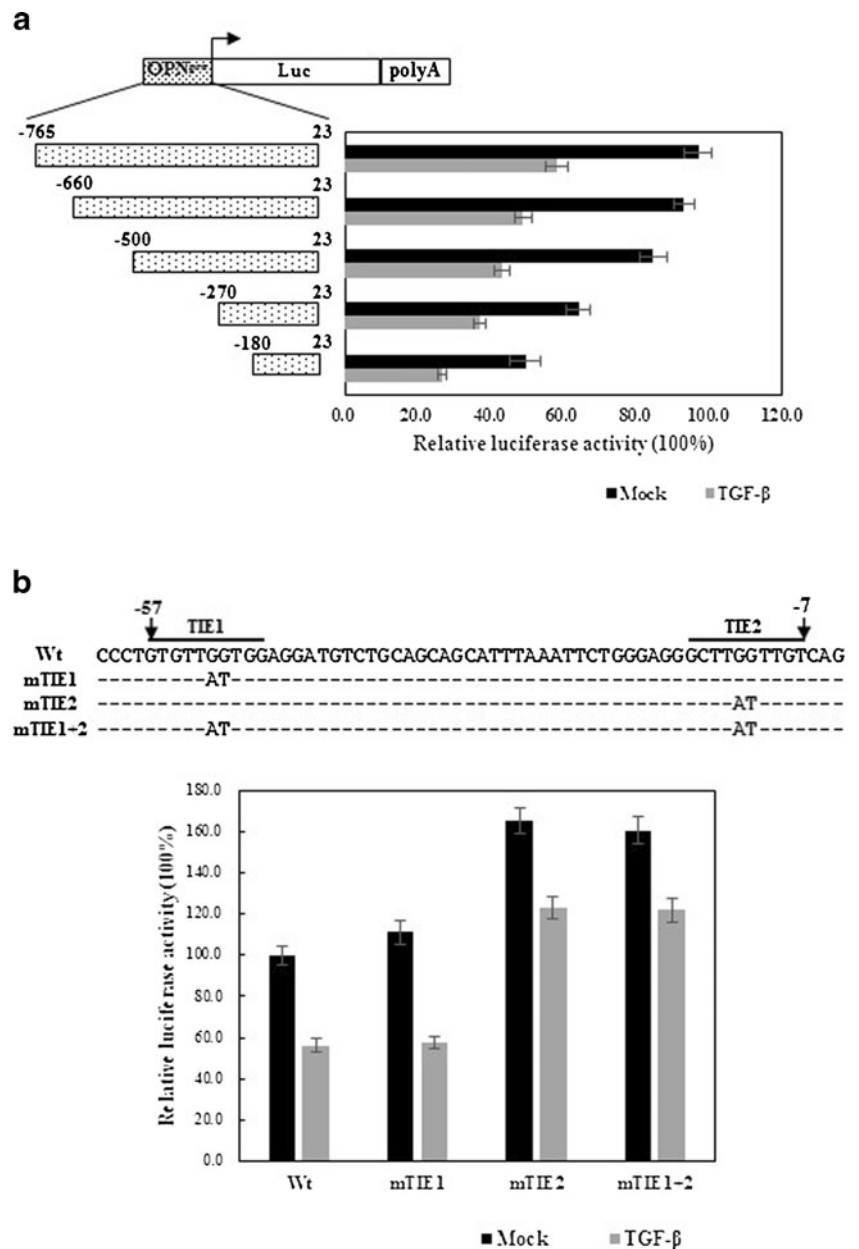
the negative control, which may be attributed to a de-repression of *OPN* gene transcription by endogenous TGF-β in the cells in which Smad4 was knocked down (Fig. 3b, middle panel). After a 48-h exposure to TGF-β1, the suppression of the *OPN* gene promoter activity in TGF-β-exposed cells was largely attenuated when Smad4 was knocked down by either sh-Smad4A or sh-Smad4B. Likewise, *OPN* gene expression analysis by quantitative real-time RT-PCR revealed that the inhibition of *OPN* mRNA expression by TGF-β was partially abrogated in cells transfected with sh-Smad4A or sh-Smad4B (Fig. 3b, lower panel). Together, these data provide additional evidence indicating that the repression of *OPN* by TGF-β is Smad-dependent.

3.5 Physiological relevance of *OPN* suppression in the TGF-β-mediated cytostatic response

Considering previous finding that the intensity of the TGF-β-induced anti-proliferative response varies greatly from cell to cell, and accumulating evidence correlating the *OPN* expression level with the proliferative and metastatic potential of multiple cancer cells [24, 36], we hypothesize that *OPN* down-regulation may be an important denominator in the cytostatic activity of TGF-β. To address this issue, multiple cancer-derived cell lines with varying *OPN* expression levels were tested. After a 72-h culture in the presence of increasing concentrations of TGF-β (0.1, 0.3, 1, and 3 ng/ml), cell growth was determined using the proliferation reagent WST-

1. By doing so, a high and modest growth inhibitory effect was observed in Huh-7 and QGP-1 cells, which express low (2.4 μg/ml supernatant) and moderate (18.4 μg/ml supernatant) levels of OPN, respectively (Fig. 4a). On the other hand, A549, OUR-10, and PA-1 cells that express high levels of OPN (226.3, 254.8 and 217.3 μg/ml supernatant, respectively) were fully resistant to TGF-β-induced grow inhibition. These results indicate a negative correlation between *OPN* expression and the anti-proliferative response to TGF-β, suggesting that de-repression of *OPN* by TGF-β may be associated with resistance to a TGF-β-mediated cytostatic response. In addition we found that depletion of OPN in the supernatant using a neutralizing antibody partially restored the anti-proliferative effect of TGF-β in A549 cells (high OPN-producing cells) (Fig. 4b), suggesting that down-regulation of OPN by TGF-β is required for an efficient inhibition of cell proliferation. Together, these data indicate that deregulated *OPN* expression coincides with a poor response to a TGF-β-induced anti-proliferative effect, implying that OPN acts as an important mediator of the cytostatic activity elicited by TGF-β.

4 Discussion

The TGF-β/Smad cytostatic activity is mediated by transcriptional regulation of multiple genes involved in cellular growth control and differentiation. In addition to cell cycle inhibition, TGF-β can down-regulate the expression of growth-promoting genes including *c-Myc* and *Id1-3* [7]. Here, we identified *OPN*, a gene implicated in tumor progression and metastasis, as a new player in TGF-β mediated cytostasis. Specifically, we found (1) that TGF-β can suppress *OPN* gene expression, (2) that Smad4 can bind to the *OPN* gene promoter upon TGF-β treatment and that elimination of a TIE-like element largely abolishes *OPN* gene suppression by TGF-β, (3) that shRNA-mediated knockdown of Smad4 can abrogate
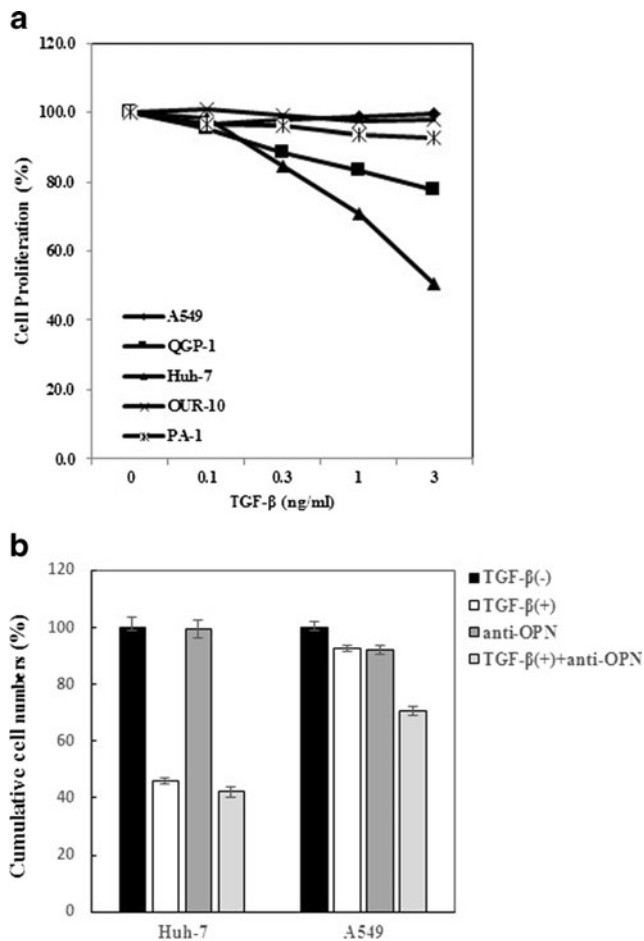
Fig. 4 **a** Effect of TGF-β on the growth of different cancer-derived cell lines. The indicated cell lines were cultured in various concentration of TGF-β1, and viable cells were quantified after 4 days. Cell numbers in TGF-β1 treated cultures were normalized to those of untreated cultures (set at 100 %). Data are derived from representative experiments performed in triplicate. **b** Partial restoration of TGF-β responsiveness after incubation with an anti-OPN antibody. Huh-7 or A549 cells were cultured in the absence or presence of TGF-β1 (3 ng/ml) and an anti-OPN antibody either alone or in combination. Viable cell counts were determined and calculated as described under (**a**)

the *c-Myc* promoter, where they coordinately mediate transcription inhibition. Another example in which gene repression by TGF-β has been well-documented is *Id1*, which is referred to as a 'self-enabling' gene response. Repression of *Id1* expression by TGF-β has been found to be modulated by association of an inhibitory complex consisting of TGF-β-activated Smads and ATF3, a co-repressor which itself is a target of TGF-β [38]. Here, we show that Smad4 binds to the *OPN* proximal promoter region upon TGF-β treatment, and that the TIE-2 motif within this region is critical for *OPN* gene repression by TGF-β. Although we failed to obtain evidence for the presence of (co)factors binding to the TIE-2 motif in a preliminary electrophoretic mobility shift assay (EMSA; data not shown), we can currently not rule out a role of such a (co)factor in the transcriptional down-regulation of the *OPN* gene by TGF-β. Additionally, it has been reported that genes containing clustered copies of TGF-β responsive elements in their promoter regions may be regulated by Smad-only complexes [39]. Since activated Smad complexes consist of Smad oligomers, the presence of multiple TGF-β responsive elements may enable multiple MAD homology (MH1) domain-DNA interactions by the same Smad complex. It is thus conceivable that the *OPN* gene promoter may contain other Smad-binding elements in the vicinity of the TIE motif identified here, and that *OPN* gene repression is brought about by cooperative interactions between these elements with the Smad complex only, without engagement of any co-factor as DNA-binding partner. Further experiments are underway to assess whether a sufficient binding affinity and selectivity for target genes such as *OPN* can be achieved by Smad oligomers interacting with multiple TGF-β responsive elements located in an optimal orientation and at an optimal distance on target gene promoters. Previously, Smads have been shown to associate with histone deacetylase (HDAC) activities through the MH1 domain, but whether these Smads directly interact with HDACs is as yet unclear [40]. Alternatively, it has been found that Smads can interact with co-repressors that recruit HDACs [41, 42]. Irrespective of whether recruited directly by Smads or indirectly by co-repressors, HDACs have been suggested to be mechanistically involved in TGF-β-induced transcription repression. This may also be the case for the *OPN* gene repression observed here, as it was recently shown that trichostatin A (TSA) treatment, a potent HDAC inhibitor, can increase *OPN* mRNA and protein expression levels [43].

It has been reported that the proximal region (−94 to −24) of the human *OPN* gene promoter can bind several transcription factors including c-Myc, which leads to *OPN* transcription up-regulation in malignant astrocytic cells [44]. Thus, teleologically, *OPN* repression may be an indirect effect of TGF-β via attenuating *OPN* activation by c-Myc. In view of the fact that *OPN* inhibition was largely abrogated in pOPN-lucmTIE2, in which the putative c-Myc-binding site is intact, we conclude that the possibility that the *OPN* repression

OPN gene suppression by TGF-β and (4) that this abrogated *OPN* gene suppression was concomitant with resistance to TGF-β growth inhibition, which could partially be restored by OPN depletion using a neutralizing antibody. Therefore, we propose that *OPN* inhibition by TGF-β represents an important denominator in the cytostatic activity of TGF-β, and that defects in the TGF-β/OPN regulatory axis may, at least partially, account for TGF-β unresponsiveness.

Recruitment of co-repressors to target gene promoters has been found to be essential in the transcriptional regulation of TGF-β-repressed genes. Transcriptional repression of e.g. the *c-Myc* gene by TGF-β has been well-documented [37]. In this example, it has been found that Smad3 and Smad4 interact with E2F4/5 and the Rb family co-repressor p107 at the TIE of

observed here occurred as a secondary consequence of c-Myc inhibition by TGF-β may be excluded.

The down-regulation of *OPN* by TGF-β observed here is inconsistent with earlier reports indicating that *OPN* may be activated through the BMP-2 and TGF-β signaling pathways. Shi et al. showed that Smad4 interacts with the transcriptional repressor Hoxa-9 in response to TGF-β stimulation, thereby inhibiting its binding to Hox-responsive elements and, consequently, de-repressing the *OPN* gene promoter [45]. Through another study by Somerman's group it was found that BMP-2 can activate *OPN* transcription via stimulating the binding of Smad proteins to the targeting sequence within the promoter [46]. The basis for the discordance between the results presented here and those by others is currently unknown. While the *OPN* promoter and the cells used here are of human origin, their conclusions were based on *OPN* gene promoter sequences amplified from mouse genomic DNA, in combination with mink lung epithelial cells (Mv1Lu) and mouse osteoblastic cells (MC3T3 E1 cells), respectively. Thus, the discrepancy noted may at least partially be attributable to a cell type-specificity of TGF-β signaling. Ample studies have shown that the cellular context is a crucial determinant in TGF-β signaling and that the effects of TGF-β on transcription can be positive or negative depending on both the target gene and the cellular context involved [47–49]. A good example is the *Id1* gene, which is inhibited by TGF-β in mammary epithelial cells [38], but is induced in metastatic breast cancer cells [50]. Further studies are underway to determine whether *OPN* can be added to the growing list of genes that are differently regulated by TGF-β in different cells.

The role of OPN in cancer progression has received considerable attention in recent years. OPN has e.g. been shown to determine the growth capacity of various cancers and to correlate with enhanced tumor progression and metastasis. It has been found that the *OPN* gene is responsive to various signal transduction pathways such as receptor tyrosine kinase, G-protein coupled, Wnt/β-catenin, NF-κB and estrogen signaling pathways. The tumor-promoting activities of these pathways can partially be attributed to aberrant *OPN* gene expression. The utility of OPN as a diagnostic and prognostic biomarker has been demonstrated in malignancies of different origin, including gynecological malignancies. Additionally, enhanced *OPN* expression has been reported to be associated with endometriosis, a benign condition that resembles invasive carcinomas in some respects. Statins, a class of cholesterol lowering drugs, have recently attracted attention as a therapeutic option for both cancerous and noncancerous diseases, such as endometriosis [51–53]. *OPN* expression reduction by statins may contribute to their anti-proliferative/pro-apoptotic and anti-angiogenic effects [54]. Nonetheless, our results suggest that *OPN* gene deregulation may be one of the factors contributing to tumor progression in cells with defective TGF-β signaling due to mutational inactivation or deregulated expression of components within its pathway.

In conclusion, we found that *OPN* acts as a downstream target negatively regulated by TGF-β, and we provide evidence suggesting that defects in *OPN* expression regulation are linked to the refractory phenotype of cells to TGF-β-mediated growth inhibition. Our findings emphasize the significance to develop therapeutic modalities targeting OPN for the control of malignant diseases.

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