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Abbreviations: ECM: extracellular matrix; MMP: matrix metalloproteinase; TGF-β: transforming growth factor-β

Abstract

We previously identified constitutive Smad signaling in human melanoma cells despite resistance to TGF-β control of cell proliferation. This led us to investigate the effect of inhibitory Smad7 overexpression on melanoma cell behavior. Using the highly metastatic cell line, 1205-Lu, we thus generated melanoma cell clones constitutively expressing Smad7, and their mock-transfected counterparts. Stable expression of Smad7 resulted in an inhibition of constitutive Smad2/3 phosphorylation, and in a reduced TGF-β response of Smad3/Smad4-driven gene transactivation, as measured using transfected Smad3/4-specific reporter gene constructs. Smad7 overexpression, however, did not alter their proliferative capacity and resistance to TGF-β-driven growth inhibition. On the other hand, expression of Smad7 efficiently reduced the capacity of human melanoma cells to invade Matrigel in Boyden migration chambers, while not affecting their motility and adhesion to collagen and laminin. Gelatin zymography identified reduced MMP-2 and MMP-9 secretion by Smad7-expressing melanoma cells as compared with their control counterparts. Smad7-expressing melanoma cells exhibited a dramatically reduced capacity to form colonies under anchorage-independent culture conditions, and, when injected subcutaneously into nude mice, were largely delayed in their ability to form tumors. These results suggest that TGF-β production by melanoma cells not only affects the tumor environment but also directly contributes to tumor cell aggressiveness through autocrine activation of Smad signaling.

Introduction

Melanocytes are derived from the neuroectoderm but, like epithelial cells, are highly sensitive to growth inhibition by transforming growth factor-β (TGF-β), a mechanism thought to be an essential tumor suppressor mechanism (Gold, 1999; Rodeck et al., 1994). In contrast and similar to carcinomas, many malignant melanomas exhibit various degrees of resistance to the growth inhibitory effects of TGF-β (MacDougall et al., 1993; Rodeck et al., 1994) and secrete high amounts of TGF-β which paracrine effects may contribute indirectly to tumor progression by attenuating anti-tumor immunity and favoring peri-tumoral angiogenesis (Akhurst & Derynck, 2001; Siegel & Massague, 2003). Also it has been shown that TGF-β overexpression in melanoma cells results in the activation of their surrounding stromal cells, whose remodeled extracellular matrix (ECM) creates a favorable environment for melanoma cell survival and increases metastasis formation (Berking et al., 2001). TGF-β signal transduction occurs via heterotetrameric serine/threonine kinase receptors (TβRI and TβRII) on the cell surface, which specifically phosphorylate the cytoplasmic proteins Smad2 and Smad3. These receptor-regulated Smads are ligand-specific and, upon activation, associate with Smad4, a common partner to all receptor-regulated Smads activated by the various ligands of the TGF-β family, to form heterocomplexes that translocate into the cell nucleus to regulate target gene transcription, either directly or in association with other transcription factors (Javelaud & Mauviel, 2004; Shi & Massague, 2003). The inhibitory Smad, Smad7, binds activated TβRI to prevent phosphorylation of Smad2/3 and recruits E3 ubiquitin-ligases such as Smurf1, Smurf2 and WWP1, to the activated TGF-β receptor complexes, leading to their proteasomal degradation. Smad7 may also interact with GADD34, the regulatory subunit of the protein phosphatase PP1, recruiting the

catalytic subunit of the phosphatase to TβRI, leading to its inactivation (Komuro et al., 2004; Nakao et al., 2002; Shi et al., 2004). High expression of the proto-oncogene *c-ski*, whose protein product is a transcriptional co-activator capable of exerting an inhibitory activity on Smad2- and Smad3-dependent transcription, has been described in numerous melanoma cells, and could be involved in their resistance to the antiproliferative activity of TGF-β by inhibiting TGF-β-induced expression of $p21^{WAF1}$ (Reed et al., 2001). However, we previously identified that melanoma cell-derived TGF-β contributes to high constitutive Smad3-dependent transcriptional activity, underscoring the notion that intact, or exacerbated, Smad signaling occurs throughout tumor progression in the melanocytic cell system (Rodeck et al., 1999). Therefore, the question arises whether certain autocrine transcriptional effects by tumor-derived TGF-β are beneficial to melanoma development. In this study, we have attempted to determine how altering Smad3/4 signaling in melanoma cells may affect their tumoral behavior *in vitro* and *in vivo*. Our strategy consisted in stably overexpressing the inhibitory Smad7 in melanoma cells and evaluating various biological parameters important for tumorigenicity, including proliferation, adhesion, migration, and invasive capacity. We demonstrate that Smad7 strongly affects MMP-2 and MMP-9 production by melanoma cells, accompanied by dramatically reduced invasive capacity *in vitro*, reduced anchorage-independent growth, and abolished subcutaneous tumor growth in nude mice.

Results and Discussion

Melanoma cells are known to secrete large amounts of TGF-β whose autocrine activity leads to constitutive Smad signaling (Rodeck et al., 1999) and correlates with their aggressiveness (Wakefield & Roberts, 2002). We thus stably overexpressed the inhibitory Smad7 in the aggressive metastatic human melanoma cell line 1205-Lu, as an approach to characterize the role of endogenous Smad signaling on melanoma cell behavior. 1205-lu cells are highly resistant to the TGF-β-antiproliferative activity and display a strong basal Smad3/Smad4 transcriptional activity (Rodeck et al., 1999).

1205-Lu cells were transfected with either empty pcDNA or pcDNA-Smad7 encoding Smad7 (Nakao et al., 1997), both carrying a neomycin-resistance gene. Following selection with G418, a mock population and several Smad7-expressing stable clones were generated. Among them, clones S7.a, S7.b and S7.c were used for subsequent experiments. Smad7 expression, as compared to parental and mock-transfected 1205-Lu cells, was verified by western blotting (Figure 1A, upper panel). Note that endogenous Smad7 was not detectable in either parental or mock-transfected 1205-Lu whereas all three S7 clones expressed high levels of the protein. Smad7 expression translated in dramatically reduced phosphorylation levels of Smad2/3 compared to the high amounts of constitutive P-Smad2/3 observed in either parental or mock-transfected cells (central panel). The latter result is consistent with our previous demonstration of high constitutive Smad3-dependent transcription in 1205-Lu cells (and other human metastatic melanoma cell lines) (Rodeck et al., 1999). At the mean time, total Smad3 levels remained unchanged (bottom panel). These results indicate efficient reduction of constitutive TGF-β signaling by Smad7 at the receptor level. All Smad7-overexpressing clones exhibited dramatically reduced transcriptional responses to exogenous TGF-β, as measured in transient

cell transfection experiments with the Smad3/4-specific reporter construct $(CAGA)_9$-luc (Dennler et al., 1998) (Figure 1B). Of note, Smad7 gene expression, measured by semi-quantitative RT-PCR, was efficiently activated by TGF-ß in mock-transfected cells, while the high levels of expression achieved in Smad7-transfected cells could no further be elevated by TGF-ß treatment (not shown). Despite the dramatic changes in their basal levels of Smad3 phosphorylation, Smad7-overexpressing clones did not exhibit any modification in their proliferative rate (Figure 1C), consistent with the initial observation that exacerbated Smad-driven transcription in human melanoma cell lines does not correlate with their proliferative potential and with their degree of resistance to TGF-β-induced growth inhibition (Rodeck et al., 1999).

The influence of Smad7 overexpression in melanoma cells was then examined on several aspects of tumor cell behavior *in vitro*. As shown in Figure 2A, Smad7-overexpressing clones exhibited a strongly reduced capacity to penetrate Matrigel™ in a Boyden chamber assay, as compared to both untransfected and mock-transfected parental 1205-Lu cells. On the other hand, adhesion of melanoma cells to either laminin or collagen was not modified by Smad7 (Figure 2B). Likewise, cell migration, as estimated in a scratch wound assay, was not significantly altered by Smad7 overexpression (Figure 2C). Similar negative results were obtained when migration was assayed using uncoated Boyden chambers (not shown), suggesting that the reduction of the invasive capacity of melanoma cells by Smad7 may be due to an altered capacity to remodel the ECM rather than from modifications of their adhesive and migratory functions. Indeed, both basal and exogenous TGF-β-induced secretion of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 was strongly diminished in all three Smad7-overexpressing clones (Figure 3A). The expression and activity of these MMPs is known to be

significantly elevated in aggressive melanomas and to contribute to their invasive capacity and metastatic potential (Hofmann et al., 2000; Ludwig et al., 2002). RT-PCR analysis indicated a reduction of both basal and TGF-β-induced MMP-9 mRNA levels in Smad7-overexpressing clones (Figure 3B), while MMP-2 mRNA levels remained unchanged (Figure 3C). Densitometric quantitation of each PCR product (Figure 3D), indicated a 50-60% reduction in basal MMP-9 mRNA steady-state levels while basal MMP-2 mRNA levels remained unchanged. Likewise, induction of MMP-9 expression by TGF-β dropped from 4-fold in mock-transfected cells to about 2.5-fold in Smad7-expressing cells. Several conclusions can be drawn from these experiments. Firstly, these data indicate that Smad7 affects MMP-2 secretion, without alteration of MMP-2 mRNA steady-state levels. Secondly, it appears that basal MMP-2 expression in 1205Lu melanoma cells may not depend on autocrine Smad3/4-dependent transcription, as it was not affected by Smad7 overexpression. However, MMP-2 secretion was reduced by Smad7 overexpression, while enhancement by TGF-β was still possible, suggesting that mechanisms other than Smad signaling may be involved for the latter phenomenon.

Marimastat, one of the several pharmacological inhibitors of MMPs that have been developed as potential antitumor drugs and predominantly targets MMP-2 and MMP-9 (Stamenkovic, 2000), potently inhibited the invasion of Matrigel™ by mock-transfected cells, indicating that MMPs play a key role in their invasive capacity (Figure 3C). On the other hand, the reduced invasive capacity of Smad7-transfected clones was no further diminished by Marimastat, suggesting that reduced MMP secretion as a result of Smad7 overexpression is responsible for the altered invasive potential of the S7 clones *in vitro*.

We next tested the effects of Smad7 on melanoma cell tumorigenicity in an anchorage-independent growth assay. As shown in Figure 4A, despite some variability between clones,

Smad7 severely reduced colony formation, from 30% for clone S7.a to about 90% for clones S7.b and S7.c. Smad7-overexpressing clones were then tested against mock-transfected cells for their capacity to form tumors *in vivo*, in a xenograft transplantation model using nude mice. Representative results obtained with clone S7.a are shown in Figure 4B. Specifically, after subcutaneous injection of $4x10^6$ (4 mice per group), S7.a cells exhibited a considerably reduced capacity to form a primary tumor. No significant growth of the injected S7.a cells was observed over a 40-day period, at the end of which mice injected with mock–transfected cells exhibited tumors with a volume of up to 600 mm^3 and were sacrificed. Similar differences between the S7.a clone and mock-transfected cells were obtained when injecting $1x10^6$ cells instead of $4x10^6$ (not shown). Likewise, clone S7.c did not generate any tumor within the same time frame (not shown).

Taken together, our current results suggest that various cellular functions implicated in melanoma development may be under the control of autocrine TGF-β and may be inhibited by Smad7 expression. We observed that the reduction in the tumorigenicity of Smad7-expressing melanoma cells was not associated with a reduction of their proliferative potential and escape from TGF-β growth control. However, inhibition of Smad3/4 signaling by Smad7 in melanoma cells was accompanied by a strong reduction in MMP expression and capacity to invade a basement membrane-like matrix *in vitro*, a reduced capacity to grow under anchorage-independent conditions, and an absence of tumor growth in nude mice. These results are in accordance with those by Berking et al. (Berking et al., 2001) who very elegantly demonstrated that peritumoral stroma ECM remodeling in response to TGF-β secreted by melanoma cells provides a significant survival advantage to the malignant melanocytes resulting in increased metastatic activity. It will be interesting to determine whether autocrine TGF-β signaling may

also control the expression of survival and/or pro-angiogenic factors by melanoma cells. In this context, it should be noted that Smad7 overexpression did not modify the expression of the three TGF-β isoforms in 1205Lu melanoma cells, as estimated by RT-PCR (not shown).

Of note, our current identification of MMP-9 as a specific gene target inhibited by Smad7 is interesting as MMP-9 may non only contribute to ECM invasion but also to peritumoral angiogenesis via its capacity to increase the bio-availability of the angiogenic factor VEGF (Bergers et al., 2000), as reduced angiogenesis around Smad7-expressing melanoma cells, in addition to their observed diminished capacity to grow under anchorage-independent conditions, would be a logical hypothesis to explain the lack of tumor growth in nude mice.

In conclusion, our results raise the interesting possibility that pharmacologic activation of Smad7 expression in melanoma cells could represent an efficient mean to attenuate the aggressiveness of these tumor cells. In this context, we recently identified halofuginone (dl-trans-7-bromo-6-chloro-3-[3-(3-hy-droxy-2piperidyl)acetonyl]-4(3H)-quinazolinone hydrobromide), as a new inhibitor of TGF-β signaling, acting via reduction of TβRII expression and induction of Smad7, thereby opposing the Smad-dependent pro-fibrotic activities of TGF-β in a murine model of radiation-induced fibrosis (Xavier et al., 2004). Future directions to our work will include the mapping of gene expression patterns affected by Smad7 in melanoma cells, and the testing of Smad7-inducing compounds against melanoma development.

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Legends to Figures

Figure 1: Smad7 expression in melanoma cells alters endogenous and exogenous TGF-β signaling without affecting cell proliferation

A. The 1205-Lu human metastatic melanoma cell line derived from a selection of cells metastatic to the lung by serial passage through athymic mice was cultured in W489 medium supplemented with 4% FCS (MacDougall et al., 1993). Generation of Smad7 expressing clones (S7.a, S7.b and S7.c) and Mock-transfected (M) cells was performed by transfection of the parental (P) cells using Fugene™ (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol, with the pcDNA-Smad7 and pcDNA vectors, respectively. Stable transfectants were selected with, and maintained in the presence of, 0,7 mg/ml G418 (Gibco BRL, Gaithersburg, MD). Protein extraction and Western blotting were performed as previously described (Javelaud et al., 2003). Polyclonal anti-Smad7 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Smad3 and anti-β-actin were from Zymed (San Fransisco, CA) and Sigma-Aldrich, respectively. The rabbit anti-phospho-Smad2/3 antibody (Daniels et al., 2004) was a gift from E. Leof (Mayo Clinic College of Medicine, Rochester, MN). B. Subconfluent melanoma cell cultures (mock and Smad7 clones) were transfected using Fugene with 0.2 μg of $(CAGA)_9$-luc vector together with 0.1 μg of pRL-TK Renilla luciferase expression vector (Promega, Madison, WI). Four hours after transfection, cultures were left untreated or were stimulated with TGF-β (10 ng/ml, R&D System Inc., Minneapolis, MN). Luciferase activities were measured in cell extracts 20h after transfection. Luciferases' activities were determined with the Dual Luciferase Reporter Assay System (Promega) and

transfection efficiency was normalized on the basis of Renilla luciferase expression. Results are the means ± s.e.m. of three independent experiments and are expressed as % of maximal activity. C. Mock (solid squares), S7.a (open circles) and S7.c (open triangles) cells ($1x10^3$ cells/well) were cultured in 96-well plates in 1% serum-W489 medium for 5 days. Each day, the number of viable cells was determined in triplicate using the colorimetric MTS/formazan assay (Promega) according to the Manufacturer's instructions. Experiments were repeated at least three times with similar results.

Figure 2: Expression of Smad7 alters the invasive capacity of melanoma cells without modifying their adhesion to ECM and motility

A. Tissue culture transwell inserts (8-µm pore size, Falcon, Franklin Lakes, NJ) were coated for 3 h with 10 µg of growth factor-reduced Matrigel™ (Biocoat, BD Biosciences, San Jose, CA) in 100µl of PBS at 37°C. The chambers were air-dried for 16 h, the Matrigel™ barrier was then reconstituted with 100 µl DMEM for 24 h at 37°C, and chambers were placed into 24-well dishes containing 750 µl of W489 medium supplemented with 0,1% FCS. Cells ($5x10^4$) were added to the upper well of each chamber in 500 µl of serum-free W489. After a 24h-incubation period, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells on the underside of the membrane were fixed, stained with Diff-Quik™ (Dade Behring, Düdingen, Switzerland) and counted by bright-field microscopy at x200 in six random fields. Results are the means ± s.e.m of three independent experiments. B. Mock-transfected cells (open bars) and S7.a cells (solid bars) were seeded onto uncoated (-), laminin- (lam), or collagen-coated (coll) plates (10 µg/ml, Sigma-Aldrich, St. Louis, MO). Adherent cells were counted 4h later. Results of counting are the mean ± s.e.m. of three independent experiments.

C. Confluent cells culture were wounded with a pipette tip, following which cell culture medium was replaced with fresh medium, and wound closure was monitored by microscopy. Representative micrographs for mock-transfected (M) and two Smad7-expressing clones, taken immediately (0), 24, or 48h after wounding, are shown. Experiments were repeated at least three times with similar results.

Figure 3: Smad7 expression reduces MMP secretion: correlation with invasion inhibition

A. Production of MMP-2 and MMP-9 by parental (P), mock-transfected (M), and Smad7-overexpressing 1205-Lu cells. Cells were cultured for 48h without serum and the conditioned media were analyzed by gelatin zymography in 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma-Aldrich). B. Total RNA was isolated from mock-transfected and S7.a cells non-treated or left treated with TGF-β for 48h using an Rneasy™ kit (Qiagen GmbH, Hilden Germany). Following reverse transcription of 0.5 μg of RNA, cDNAs were amplified using a Multiplex PCR kit (35 cycles for MMP-9 and GAPDH, 30 cycles for MMP-2, Tm 60°C) according to the manufacturer's protocol (Qiagen). Primers used for PCR were as follow: MMP-9 sense (5'-CATTCAGGGAGACGCCCA-3'), MMP-9 antisense (5'-AACCACGACGCCCTTGC-3'), MMP-2 sense (5'-CTGGCTTTTCACTGCTGGCT-3'), MMP-2 antisense (5'-TGCTAAGTAGAGTGAACAGGG-3'), GAPDH sense (5'-CGGATTTG GTCGTATTGGGC-3'), and GAPDH antisense (5'-GTCATACCAGGAAATGAGCTTG-3'). PCR products were separated on 2% agarose gels, stained with ethidium bromide. C. Densitometric scanning of PCR products using a Gel-Doc™ apparatus and its associated software (Bio-Rad). Fold-induction of MMPs is calculated after correction against GAPDH expression levels in the same samples. D. Cells ($5x10^4$/well) were plated onto Matrigel™-coated

cell culture inserts with or without Marimastat (20μM, British Biotechnology, Oxford, UK). After 24h, cells that had migrated through the filters were fixed, stained with Diff-Quik™ and counted by bright-field microscopy at x200 in six random fields.

Figure 4: Smad7 expression inhibits the tumorigenicity of 1205-Lu cells

A. Mock- and Smad7-transfected 1205-Lu melanoma cells were seeded into 24-well plates in W489 medium, 0.5% agar (Sigma-Aldrich), supplemented with 4% FCS on top of a 1% agar bed in similar medium. The cultures were incubated for 28 days and the total number of colonies in each well was determined using a phase contrast microscope (Nikon France, Rollay, France). Results are expressed as the mean $\pm$ s.e.m. of three independent experiements. B. 8-week old Swiss *nu/nu* (nude) female mice were housed at the animal facilities of the Curie Institute, Orsay, France, in specific pathogen-free conditions. Their care was in accordance with the institutional guidelines of the French Ethical Committee (Ministère de l'Agriculture et de la Forêt, Direction de la Santé et de la Protection Animale, Paris, France) and under supervision of authorized investigators. $4x10^6$ cells (mock- or Smad7-transfected, clone S7.a) in logarithmic growth phase were injected subcutaneously into the flank of nude mice. The viability of cells, estimated by Trypan blue exclusion before injection into mice, was over 95%. Twice weekly, tumors were measured with calipers, and their volume was calculated according to the formula $V= a \times b^2 \times 0.5$ where a is the largest diameter and b the smallest diameter of the tumor. Solid symbols: volume of tumors generated from mock cells; open symbols: volume of tumors generated from S7.a cells.

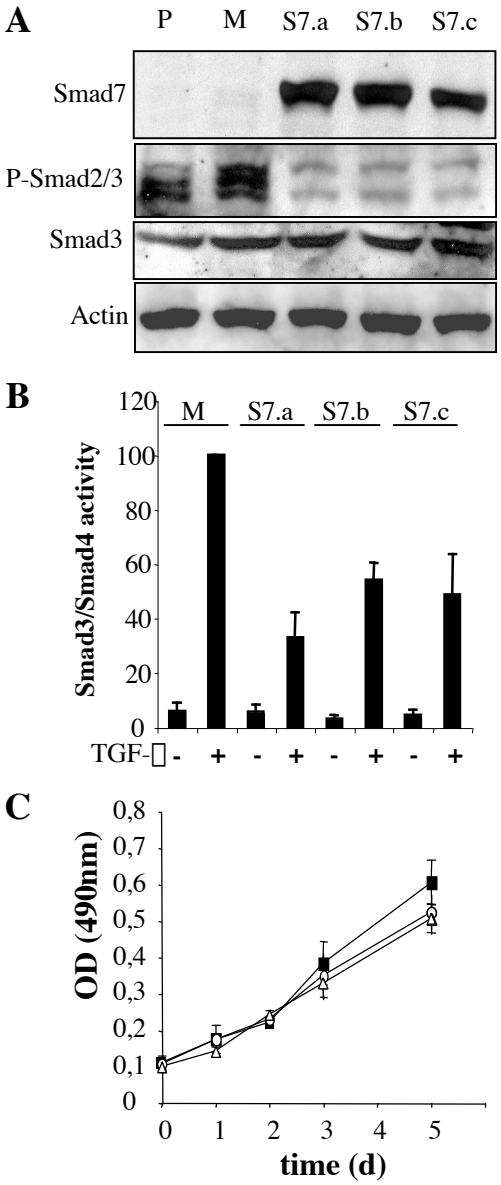

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Figure 1

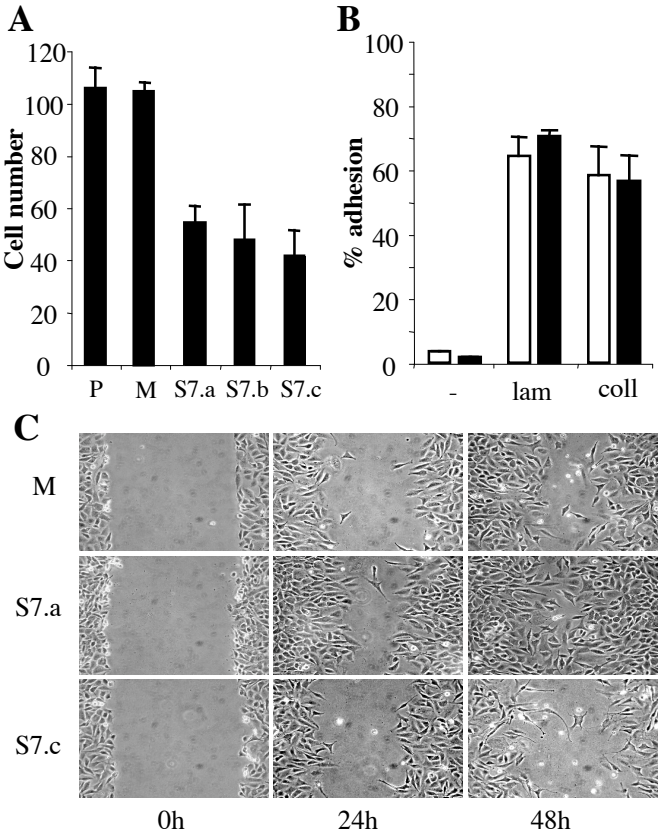

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Figure 2

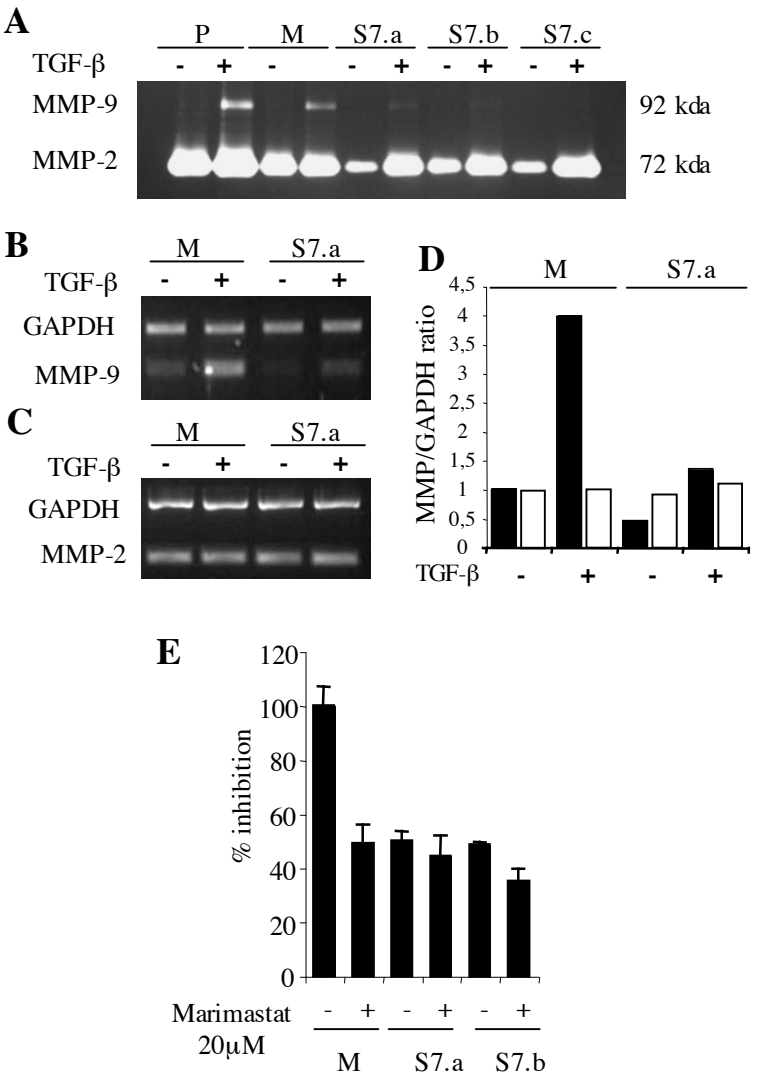

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Figure 3

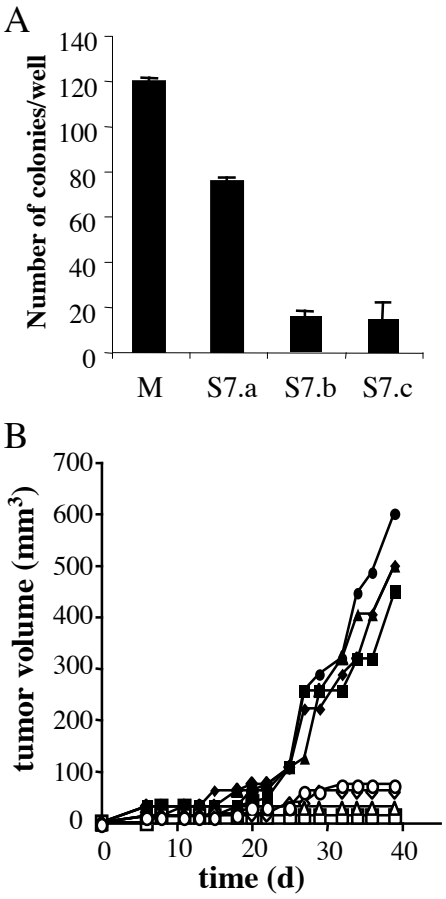

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Figure 4