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Smad2 suppresses the growth of Mv1Lu cells subcutaneously inoculated in mice

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Abstract

Smad2 and Smad3 are intracellular signal transduction proteins of importance in transforming growth factor-β (TGFβ)-mediated inhibition of epithelial cell proliferation. Inactivating mutations in the *Smad2* and *Smad3* genes have been found in various human malignancies. Here, we show that expression of Smad2 leads to the inhibition of growth of Mv1Lu cells inoculated with Matrigel subcutaneously (s.c.) in severe combined immunodeficient (SCID) mice. In histological appearance, the Matrigel plugs with Smad2-transfected cells showed strongly reduced cell density, proliferation and angiogenesis compared with the small tumour nodules of similar size formed by the vector- or Smad3-transfected cells. The histological appearance of vector- and Smad3-transfected cells inoculated in mice was identical. Overexpression of Smad2 and Smad3 in Mv1Lu cells led to the inhibition of cell growth in three-dimensional cultures when compared with vector-transfected cells. Overexpression of Smad2 and Smad3 also decreased the hyperphosphorylation of pRb in Smad-transfected cells. Thus, increased expression of Smad2 leads to inhibition of Mv1Lu cell proliferation and a reduction in the growth of the Smad2-expressing cells inoculated in mice.

Keywords: Smad2; Smad3; Growth; Proliferation; pRb

1. Introduction

Transforming growth factor-β (TGFβ) is a potent inhibitor of epithelial cell proliferation that binds to and activates specific serine/threonine kinase receptors, leading to subsequent phosphorylation of Smad2 and Smad3 proteins. Smad proteins are key intracellular signal transducers downstream of TGFβ receptors [1–4]. Disruption of TGFβ signalling contributes to uncontrolled cell proliferation, and inactivating mutations in TGFβ receptors and Smad proteins have been found in many human cancers [5–7]. Therefore, components of the TGFβ signalling pathway are putative tumour suppressor genes.

Mutations in *Smad2* and *Smad3* have been found in a subset of colorectal and breast carcinomas [5,8,9]. Infrequently, *Smad2* and *Smad3* mutations have been found in head, neck, oesophageal squamous cell carcinomas [10], hepatocellular carcinomas [11], sporadic gastric carcinomas [12], human lung cancers [13], and in tumours of the colon, breast, lung and pancreas [14]. However, in a series of other studies, no mutations affecting *Smad2* or *Smad3* have been observed [15–19]. So far, the mutations observed in human cancer led to loss of function of Smad proteins [20–24]. Moreover, inactivation of Smad signalling can occur through accelerated degradation of Smad proteins [21,25] or by interaction with transcriptional repressors [26,27].

Smad proteins are important mediators in TGFβ-controlled expression of cell cycle regulators, such as the CDK inhibitors p21 [28], p15 [29] and p57 [30]. Loss of Smad function relieves the cell cycle inhibitory effect of TGFβ, and gain of Smad function inhibits cell cycle progression, as has been shown in cultured cells [31, 32]. However, the effect of gain-of-function of Smad activity *in vivo* remains to be explored.

Homozygous deletion of *Smad2* in mice leads to embryonic lethality [33]. Heterozygous deletion of *Smad2* and *APC* resulted in acceleration of intestinal

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tumour formation [34]. Increased incidence of metastatic colorectal cancer in *Smad3*-deficient mice has been reported [35]. However, other studies have failed to demonstrate increased tumour incidence in *Smad3* null mice [36,37]. Overexpression of wild-type Smad4 in tumour cells led to decreased tumour growth through inhibition of angiogenesis [38]. Keratinocyte invasiveness was increased upon overexpression of Smad2 with mutated C-terminal serine residues, the EDXE mutant [39]. The mechanism behind this effect has to be examined, as in culture Smad2-transfected keratinocytes showed a decreased rate of proliferation [39].

2. Materials and methods

2.1. Cell culture

Mv1Lu mink lung epithelial cells, stably transfected with the empty pMEP4 vector, pMEP4-Flag-Smad2, or pMEP4-myc-Smad3, respectively, have been described previously in Ref. [40].

2.2. Tumour formation assay

The animal work in this study was approved by the local board of animal experimentation and carried out in accordance with the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines [41]. All manipulations were performed in isoflurane gas anaesthesia (Forene, Abbott). Upon arrival from the supplier (M&B, Denmark), the animals were acclimated, caged in groups of five and had their backs shaved. Five 6–8 weeks old female Fox-Chase severe combined immunodeficient (SCID) mice were injected subcutaneously (s.c.) with 3×10^6 Mv1Lu cells transfected with empty vector, Flag-Smad2, or myc-Smad3, respectively, in 100 μl Dulbecco's Modified Eagle's Medium (DMEM) in the absence (left flank) or presence (right flank) of 50% Matrigel (v/v) (Becton Dickinson). The experiment was repeated twice; in total, we analysed 11 animals for vector-transfected cells, 9 animals for Flag-Smad2-transfected cells, and 8 animals for myc-Smad3-transfected cells. Fourteen hours prior to sacrifice, three animals in each group received 60 mg kg^{-1} bromodeoxyuridine (BrdU) in 0.9% NaCl (w/v) by intraperitoneal (i.p.) injection. After sacrifice by carbon dioxide asphyxia, the tumours were excised, photographed, and measured with calipers. Tumour volume was calculated according to the formula $V=\pi/6\times a^2\times b$ where a represents the shorter and b the longer dimension of the tumour. Results were presented as means and standard error of the mean (SEM). One tumour from each group was minced, digested with collagenase and retrieved to tissue culture in DMEM supplemented with 10% foetal calf serum (FCS) (v/v) and 300 units/ml hygromycin. To accumulate sufficient numbers of cells, retrieved cells were cultured for 2 to 4 weeks. The other tumours were fixed overnight in 4% paraformaldehyde (PFA) (v/v) and embedded in paraffin. Sections were cut at 4 μm on Superfrost Plus slides (Histolab, Sweden).

2.3. Immunohistochemistry

For the detection of capillary blood vessels, sections were deparaffinised and pretreated by boiling in 10 mM citrate buffer pH 6.0 for 2×7 min at 750 W in a microwave oven. Tissue peroxidase activity was quenched by incubation in 3% H_2O_2 in phosphate-buffered saline (PBS) (v/v) for 10 min, followed by blocking in 1% bovine serum albumin (BSA) (w/v). Immunohistochemistry was performed with a goat anti-mouse CD31/platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody (sc-1506, 1:100, Santa Cruz). Positive reactions were developed using 3,3′-diaminobenzidine (DAB) (Vector) as a peroxidase substrate. Immunohistochemical detection of BrdU was performed on citrate-pretreated sections preincubated in 55% formamide/SSC/0.1% Tween-20 (v/w/v) for 30 min at 72 °C. Staining with a monoclonal mouse anti-BrdU antibody (1:50; Becton Dickinson) was done in a NexES immunostainer equipped with a DAB substrate kit (Ventana Medical Systems, Tucson, AZ, USA). Omission of the primary antibody, in the case of CD31/PECAM-1 staining, or replacement of the primary antibody with an irrelevant mouse IgG, in the case of BrdU staining, were used as negative controls. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) staining for the detection of apoptotic cells in tumour sections was performed as described in Ref. [42], with the omission of Proteinase K digestion. Nick-end labelling was carried out using TdT Enzyme and digoxigenin-11-dUTP (Roche) as recommended by the manufacturer. Positive nuclei were stained with peroxidase-coupled F_{ab} fragments raised against dUTP-digoxigenin (1:500; Roche) and DAB peroxidase substrate. Sections were counterstained in Mayer's haematoxylin, dehydrated and coverslipped in Mountex resin (Histolab, Sweden). Images were captured under a microscope (VANOX-T, Olympus) equipped with a FUJIX HC-300Z digital camera (Fuji), and processed in Adobe Photoshop 4.0.

2.4. Immunoprecipitation

Smad expression was analysed by pretreatment of Mv1Lu transfectants for 3–4 h with 50 μM $ZnCl_2$, followed by metabolic labelling with [^{35}S]methionine, lysis, immunoprecipitation using anti-Flag (M5, 1 μg/ml, Kodak) and anti-myc (9E10, 1 μg/ml, Santa Cruz) antibodies, separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and

analysis by FUJIX BAS-2000 phosphorimager (Fuji, Japan), as previously described in Ref. [40]. Quantification of ^{35}S-labelled and immunoprecipitated Flag-Smad2 and myc-Smad3 was performed using the AIDA software (IMG, GmbH, Germany).

2.5. Proliferation assay

The anchorage-independent growth assay was performed as described in Ref. [43], except that the cells were seeded at a higher density (40 000 cells/0.5 ml in 24-well plate wells). Matrigel (50% in culture medium, (v/v) or methyl cellulose (3% in culture medium, (w/v)) was used instead of agar. Cells were cultured for 14 days, with regular inspection of colony formation and counting colonies under a microscope.

2.6. Western blotting

Mv1Lu cells stably transfected with Flag-Smad2, or myc-Smad3, or with empty vector were starved for 24 h in serum-free DMEM. After stimulation with 10% serum (v/v) for 2 h, cells were lysed and subjected to SDS–PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and probed with anti-pRb antibodies (1 μg/ml, G3-245, PharMingen). The bands were visualised by enhanced chemiluminiscence detection. Equal loading of samples was evaluated by re-probing the same membrane with an anti-α-tubulin antibody (TU-02, Santa Cruz Biotechnology).

3. Results

3.1. Expression of Smad2 inhibits growth of cells inoculated subcutaneously in mice

To investigate the effect of Smad2 and Smad3 expression on growth of Mv1Lu cells in Matrigel plugs, Mv1Lu cells transfected with empty pMEP4 vector (Mv1Lu-vector cells), Flag-Smad2 (Mv1Lu-Smad2 cells), or myc-Smad3 (Mv1Lu-Smad3 cells), were injected s.c. in SCID mice in the absence or presence of 50% Matrigel. Upon sacrifice, no tumours had been formed in absence of Matrigel by any Mv1Lu transfectant, whereas small tumour-like nodules were retrieved from vector-transfected Mv1Lu cells (17 ± 2 mm^3), Mv1Lu-Smad2 cells (10 ± 4 mm^3), and Mv1Lu-Smad3 cells (17 ± 3 mm^3). Similar results were obtained in two independent experiments; we analysed 11 animals for vector-transfected cells, 9 animals for Mv1Lu-Smad2 cells, and 8 animals for Mv1Lu-Smad3 cells. The expression of Flag-Smad2 and myc-Smad3 was verified by immunoprecipitation of the respective Smads in lysates from metabolically labelled cells which were prepared for inoculation in mice (Fig. 1a, upper panel), and from cells retrieved from digested Matrigel plugs (Fig. 1a, lower panel) confirming the presence of Flag-Smad2 and myc-Smad3 expressing cells in the Matrigel plugs. Quantification of expressed Flag-Smad2 and myc-Smad3 showed similar levels of expression (Fig. 1b). Upon induction with $ZnCl_2$, the cells strongly expressed Flag-Smad2 or myc-Smad3, respectively, but low levels of Smad protein expression were also observed also in the absence of the exogenous inducer. An effect of $ZnCl_2$ on the appearance of a non-specific band co-migrating with myc-Smad3 was observed. Smad protein expression in the absence of $ZnCl_2$ was higher when the cells were kept in a dense culture, compared with a sub-confluent culture (data not shown). Thus, the level of Smad protein expression was moderately increased over the level of endogenous Smads in the absence of induction with $ZnCl_2$.

To characterise the effects of Smad2 and Smad3 on growth in Matrigel plugs, we sectioned and immunostained retrieved plugs derived from vector-transfected as well as Flag-Smad2- and myc-Smad3-transfected Mv1Lu cells using markers for capillary blood vessels (CD31), proliferation (BrdU), and apoptosis (TUNEL). We found that Flag-Smad2-expressing cells gave rise to small islands of cells in a scant, fibroblast-populated Matrigel stroma (Fig. 2a–d). No CD31-positive vessels with a lumen were found in the Smad2 tumours, although involuted CD31-positive structures were dispersed in the surrounding stroma, indicating an anti-angiogenic effect of Smad2 expression (Fig. 2b). Proliferating, BrdU-positive cells were present in some, but not all, of the Mv1Lu-Smad2 cell islands (Fig. 2c), and TUNEL-positive cell debris abundantly surrounded these islands (Fig. 2d). Vector-transfected and Smad3-expressing Mv1Lu cells gave rise to mixed nodules, having a cell-dense surface layer and a Matrigel-containing core with duct-like formations and cell islands (Fig. 2e–h, data not shown). Blood vessels with normal morphology, as well as involuted CD31-positive structures, were found side-by-side in the central part of the Mv1Lu-Smad3 Matrigel plugs (Fig. 2f). The rate of apoptosis of Mv1Lu-Smad3 cells, evaluated by TUNEL staining, was similar to that of the vector-transfected cells (Fig. 2h, data not shown). Taken together, these findings indicate that the expression of Smad2, but not Smad3, results in decreased proliferation and angiogenesis, while increasing the apoptosis of Mv1Lu cells inoculated in mice.

3.2. Expression of Flag-Smad2 or myc-Smad3 inhibits anchorage-independent growth of Mv1Lu cells

In an attempt to mimic the *in vivo* environment of inoculated cells, where the cells would be deprived of a solid support characteristic of a two-dimensional culture, we performed a three-dimensional culture in

Matrigel or in methylcellulose of Mv1Lu-Smad2, Mv1Lu-Smad3 or Mv1Lu-vector cells. Cells transfected with empty vector formed colonies in Matrigel after 3–4 days in culture (Fig. 3a, data not shown). At that time, no colonies were observed for the Smad-transfected cells (Fig. 3b, c, data not shown). We did not observe significant cell death in any of the cell lines, suggesting that Smad expression decreased the proliferation rate of cells. After 14–17 days, small colonies were observed for cells transfected with myc-Smad3, but no colonies were observed for the Flag-Smad2-transfected cells (Fig. 3). Colonies of vector-transfected cells were growing to a colony size of more than 120 cells per colony, and almost no single or non-dividing cells were observed (Fig. 3a). Some of the big colonies lost their compact structure, and single cells surrounding the colonies were observed. Similar results were obtained when the cells were cultured in methylcellulose (Fig. 3d–f). It should be noted, that methylcellulose does not contain biologically active substances, compared with Matrigel. In correspondence to the cell growth data in mice, the rate of colony formation by the Mv1Lu-vector cells in methylcellulose was lower than in Matrigel. The first colonies were observed after 7–10 days in culture. The difference in colony formation in Matrigel compared with methylcellulose suggests that Matrigel supports the proliferation of Mv1Lu cells, but the inhibition of colony formation by Mv1Lu-Smad2 and Mv1Lu-Smad3 cells is an intrinsic property of those cells due to the transfection of Smad proteins.

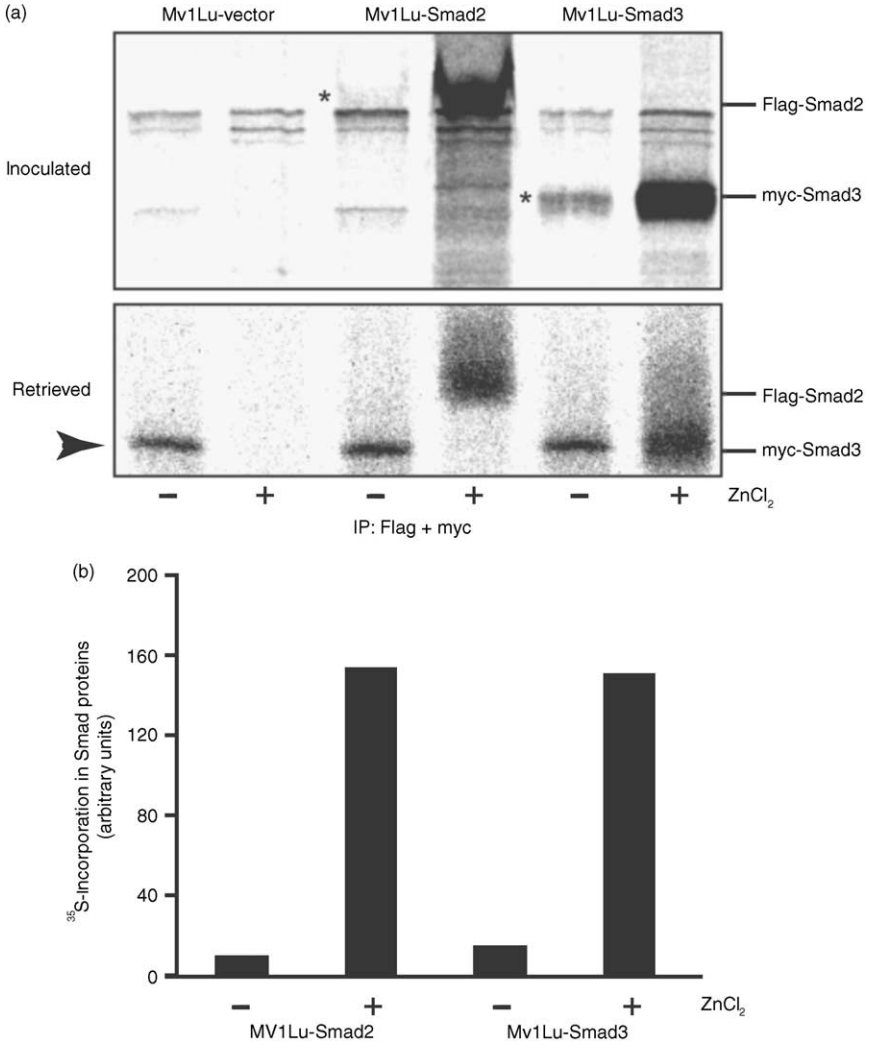

Fig. 1. Expression of Flag-Smad2 and myc-Smad3 in stably transfected Mv1Lu cells. (a) Mv1Lu cells, stably transfected with Flag-Smad2, myc-Smad3 or empty pMEP4 vector, were kept in culture at high density before inoculation (inoculated) or were retrieved from tumours (retrieved), then labelled with [^{35}S]methionine and treated with 50 μM $ZnCl_2$ for 4 h to induce the expression of Smad proteins as indicated. Cells were lysed and Smad proteins were immunoprecipitated with their respective anti-tag antibodies. Migratory positions of Flag-Smad2 and myc-Smad3 are indicated. Arrowhead indicates the migratory position of a non-specific band in the lower panel. (b) Quantification of immunoprecipitated Flag-Smad2 and myc-Smad3 from ^{35}S-labelled cells retrieved from tumours (a; lower panel) showed similar levels of expression of Smad proteins.

3.3. Hyperphosphorylation of pRb is decreased in cells transfected with Flag-Smad2 or myc-Smad3

In order to explore the mechanism whereby proliferation of Smad-transfected Mv1Lu cells is inhibited, we analysed the pRb phosphorylation status in proliferating cells transfected with empty vector or Smad proteins (Fig. 4). pRb is a key check-point in regulation of the cell cycle, and the phosphorylation state of pRb defines its functional activity [44]. We found that pRb was hypophosphorylated in the Mv1Lu-Smad2 and Mv1Lu-Smad3 cells, while in vector-transfected cells a significant fraction of pRb was hyperphosphorylated. Moreover, when cells were treated with serum, phosphorylation of pRb increased in Mv1Lu-vector cells, but no significant changes were observed for the Smad2- or Smad3-transfected cells (Fig. 4). Thus, the level of pRb phosphorylation correlated with rates of cell proliferation in three-dimensional culture and was dependent on Smad2 or Smad3 expression. The difference in histological appearance of Matrigel plugs formed by Mv1Lu-Smad2 and Mv1Lu-Smad3 cells (Fig. 2) suggests that other mechanisms, in addition to the hypophosphorylation of pRb, may be involved when cells are inoculated into mice.

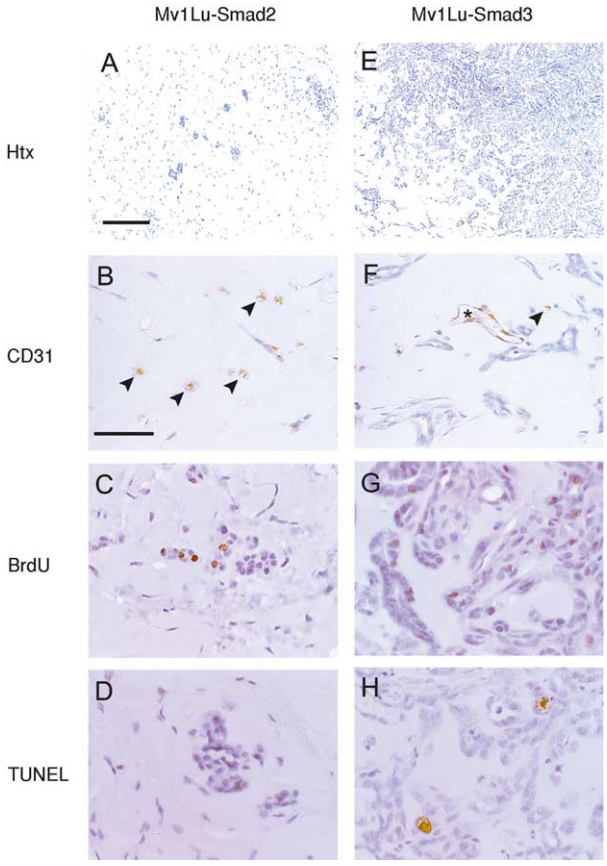

Fig. 2. Expression of Flag-Smad2 inhibits the growth of Mv1Lu cells injected subcutaneously (s.c.) in mice. Matrigel plugs retrieved 12 weeks after inoculation with Mv1Lu-Smad2 (a–d) or Mv1Lu-Smad3 (e–h) were sectioned and stained in haematoxylin (Htx) (a, e), stained for capillary blood vessels using anti-CD31 antiserum (b, f), for proliferating cells using anti-bromodeoxyuridine (BrdU) antibody (c, g) and for apoptotic cells by TUNEL (d, h). * ; lumen of vessels; arrowheads show involuted, CD31-positive cells. Matrigel plugs formed by vector transfected Mv1Lu cells were identical in appearance to Mv1Lu-Smad3 plugs (data not shown). Representative images from one of two independent inoculations are shown.

4. Discussion

Loss of Smad-dependent signal transduction results in the abrogation of most of the functions of TGFβ, such as the control of cell growth. TGFβ/Smad-dependent growth inhibition is claimed to be of importance in the prevention of tumorigenesis [6,44,45]. Here, we explored the roles of Smad2 and Smad3 in the inhibition of tumour formation *in vivo* by injecting Mv1Lu cells stably transfected with these Smad proteins into immunodeficient mice. We observed the formation of relatively small tumour nodules, with no differences between the nodules formed by Smad2-, Smad3- or vector-transfected cells. However, differences between Mv1Lu-Smad2 and Mv1Lu-Smad3 tumour-like nodules were found upon histological examination (Fig. 2a–h). Matrigel plugs with Mv1Lu-Smad2 cells contained fewer cells, compared with Mv1Lu-Smad3 cells. Differences in the degree of vascularisation and the appearance of apoptosis were also detected (Fig. 2a–h). These differences are not likely to be a result of gene dosage, as the levels of Flag-Smad2 and myc-Smad3 expression were approximately the same, as determined by immunoblotting with two different antibodies raised against each protein and by quantification of immunoprecipitated ^{35}S-labelled Smad proteins (Fig. 1b): with anti-tag (Fig. 1a), and with antibodies to endogenous proteins (data not shown). It is also unlikely that the differences are due to the different tags on Smad2 and Smad3, since the different tags did not affect the functions of Smad2 and Smad3 in numerous assays, including TGFβ1-dependent induction of Smad phosphorylation [1–6]. Differences between Smad2 and Smad3 can be anticipated from biochemical and gene targeting studies; Smad3 binds DNA directly, while Smad2 does not. The two proteins are also differently regulated, and their respective knock-out phenotypes are different [1–6,33]. Essential roles for Smad3 in TGFβ-induced apoptosis [40] and Smad2 in cell migration [23] have been shown. Direct comparison of primary fibroblasts from mice with targeted deletions of Smad2 or Smad3 [46], or phenotypes of the fruit fly upon transfection of Smad2 or Smad3 [47], have demonstrated the specific functions of each of the Smads. Our results provide further evidence that Smad2 and Smad3 possess a different tumour suppressor potential, Smad2 having stronger effect than Smad3.

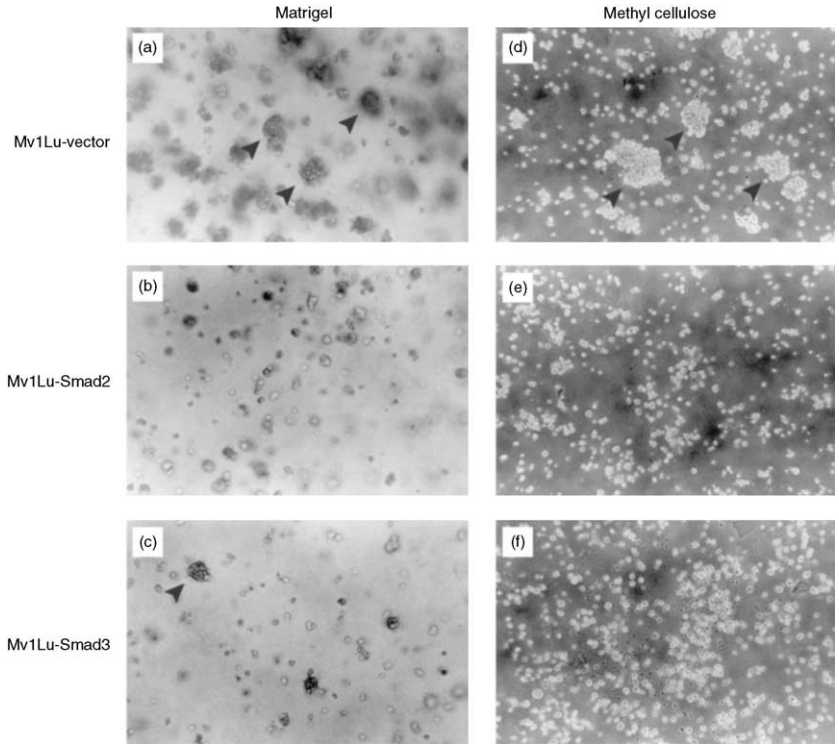

Fig. 3. Transfection of Flag-Smad2 or myc-Smad3 inhibits anchorage-independent proliferation of Mv1Lu cells. Anchorage-independent growth of cells was evaluated in Matrigel (a–c) and in methylcellulose (d–f) cultures. Cells stably transfected with empty vector formed colonies in Matrigel (a) or in methylcellulose (d), while proliferation of cells transfected with Flag-Smad2 (b, e) or myc-Smad3 (c, f) was inhibited. Colonies in vector-transfected cells (a, d) and small and rare colonies in myc-Smad3-transfected cells in Matrigel (c) are shown by the arrowheads. Cells were cultured in 50% Matrigel (a, b, c) or in 3% methylcellulose (d, e, f). Microphotographs of colonies of a representative experiment out of three assays are shown.

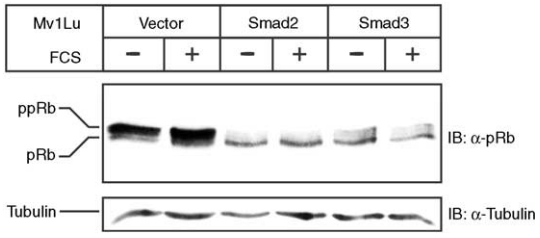

Fig. 4. Hyperphosphorylation of pRB is inhibited in cells transfected with Flag-Smad2 or myc-Smad3. Vector-Mv1Lu, Smad2-Mv1Lu and Smad3-Mv1Lu cells were treated with foetal bovine serum (FBS) (5%) as indicated, and pRb was detected in whole cell lysates by Western blotting (IB) with anti-pRb antibodies. To control for equal sample loading, the same membrane was re-probed with anti-α-tubulin antibodies. Migration positions for hyper- (ppRb) and hypo-phosphorylated pRb, and for α-tubulin are shown. A representative experiment out of three assays is shown.

In order to identify a molecular mechanism of suppression of cell growth in Matrigel plugs by Smad2, we investigated how apoptosis, proliferation and angiogenesis were affected by the expression of the Smads. Immunostaining of sections could not identify which of these activities was the most important for the suppression of cell growth. Comparison of the proliferation rate of Flag-Smad2-, myc-Smad3- or vector-transfected cells in two-dimensional culture by measurement of the [^{3}H]thymidine incorporation rate did not show any significant differences (data not shown). Thus, we investigated how transfection of Smad2 or Smad3 affects Mv1Lu cells in conditions which mimic the *in vivo* situation. Three-dimensional culturing of cells may provide an environment that mimicks *in vivo* conditions. The anchorage-independent growth assay allows the evaluation not only of the cell proliferation rate, but also of the cell death rate, as dead cells are easily detectable in the three-dimensional matrix. We did not observe any significant cell death of Smad-transfected cells, compared with the Mv1Lu-vector cells, while the difference in the rate of colony formation indicated an effect on cell proliferation (Fig. 3).

In a study of similar design, aiming to evaluate the role of Smad4 in tumorigenesis, no significant effects of Smad4 on cell proliferation or extracellular matrix formation were found [38]. However, expression of thrombospondin-1 and vascular endothelial growth factor (VEGF) were affected, leading to decreased angiogenesis, which was considered as the main tumour suppressor mechanism of Smad4. While the effects of Smad2 or Smad3 expression on vascularisation and apoptosis in tumour nodules can not be excluded, our attempts to determine whether conditioned medium of Smad-transfected Mv1Lu cells increased endothelial cell

proliferation did not provide consistent results (data not shown). Our results therefore suggest that the most profound effect of the Smads is on cell proliferation.

Overexpression of Smad2 with mutated C-terminal serine residues indicated a role for Smad2 in the invasiveness of transformed keratinocytes [39]. On the other hand, overexpression of the dominant-negative Smad2 AAXA mutant led to the restoration of cell cycle progression in invasive spindle tumour cells [39]. The latter finding is in agreement with our results obtained with Mv1Lu epithelial cells.

TGFβ inhibits the cell cycle through various mechanisms, including induction of the expression of CDK inhibitors and cdc25, inhibition of expression of cyclins E and A, and downregulation of c-myc [1–6,31]. Most of these mechanisms converge on the regulation of phosphorylation of pRb [44]. A pRb-independent mechanism of cell cycle regulation was reported, although its importance remains to be evaluated [48]. On the contrary, the role of pRb-dependent regulation of the cell cycle by TGFβ is well established.

We report here, a potent inhibition of the steady-state or serum-stimulated pRb phosphorylation upon increased Smad expression, suggesting a possible mechanism of the growth suppressive action of Smad proteins (Fig. 4). However, histological differences between the tumours formed by Mv1Lu-Smad2 and Mv1Lu-Smad3 cells (Fig. 2) suggest that other mechanisms, that have yet to be identified, modulate the behaviour of cells inoculated in mice, and that these mechanisms can be differentially regulated by Smad2 and Smad3. These mechanisms may be dependent on the requirement of a substrate for cell attachment, as we observed differences in the proliferation rate between vector-transfected cells and cells transfected with Smads in a three-dimensional culture, but not in a two-dimensional culture. Such mechanisms may be identified through comparison of global changes in mRNA and protein profiles specific for Smad2- or for Smad3-dependent signalling.

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