Metastasis is driven by sequential elevation of H-ras and Smad2 levels

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Metastasis is a multistep process that involves local tumour invasion followed by dissemination to, and re-establishment at, distant sites. Here we show that during multistage tumorigenesis, discrete expression thresholds of activated Smad2 and H-ras are sequentially surpassed, driving tumour progression through distinct phases from a differentiated squamous carcinoma to a motile invasive stage, followed by an overt change from epithelial to mesenchymal cell type, finally culminating in metastatic tumour spread. Smad2 activation alone induces migration of tumour cells. Elevated H-ras levels, however, are required for nuclear accumulation of Smad2, both of which are essential for the epithelial–mesenchymal transition (EMT). Having undergone EMT, fibroblastoid carcinoma cells with elevated levels of activated Smad2, gain the capability to spread to a wide variety of tissues by a further increase in Smad2 expression. These findings have far-reaching implications for the prevention of tumour growth, invasion and metastasis.

Cancer development and metastasis is a multistep process that involves local tumour growth and invasion followed by dissemination to, and re-establishment at, distant sites. The ability of a tumour to metastasize is the major determinant of cancer-patient mortality. Elucidating the molecular pathways essential for tumour metastasis is, therefore, a high priority in cancer biology, as well as for small-molecule drug design.

Transforming growth factor β (TGFβ) has been implicated in both tumour suppression and progression[1]. Although TGFβ inhibits growth of early carcinomas[2,3], later in tumorigenesis, and in co-operation with Ras, it can induce an epithelial–mesenchymal transition (EMT) towards an invasive, metastatic tumour phenotype[3–8]. The mechanisms by which TGFβ can act both to promote and inhibit tumour progression have been extensively studied but remain unclear. Many studies have suggested that TGFβ promotes EMT[5,6,8–11]. But most of these studies have focused on one particular aspect of tumour invasion rather than complete transformation to a mesenchymal cell type. These include increased cell motility, characterized by changes in the actin cytoskeleton and adoption of an invasive phenotype. Ultimately, these cells undergo a fate change from an epithelial-like cell state that expresses multiple markers of epithelial origin, towards a fibroblastic cell type that shows drastic alterations in the cytoskeleton and cell-membrane components. In particular cell types, features resembling an EMT can be induced by TGFβ alone[4,5], by Ras–Raf signalling[12], or cooperatively by both pathways[6]. Several mechanisms of cooperation between Ras and TGFβ have been proposed, including prevention of TGFβ-induced apoptosis by Raf[13] or phosphatidylinositol-3-OH kinase (PI(3)K) activation[14], repression of TGFβ–Smad signalling by oncogenic Ras[15], or a requirement for Raf mitogen-activated protein kinase (MAPK) signalling leading to the upregulation of TGFβ required for an EMT[12]. Interestingly, the same signalling pathways are involved in inducing a cell fate change to the mesoderm lineage during *Xenopus* development[16–20].

The relevance of these biological changes to *in vivo* models of multistage tumour progression has not been adequately investigated. We have exploited a series of well-characterized tumour cell lines derived from sequential stages of mouse skin carcinogenesis[21,22] to study the significance of changes in threshold levels of H-ras and TGFβ activation during progression to the metastatic phenotype. Early-stage mouse skin papillomas induced by successive treatment with initiators and promoters of carcinogenesis can progress to form squamous carcinomas and subsequently to undifferentiated spindle-cell tumours that resemble fibrosarcomas. These tumours are initiated by activating mutations in the *Hras1* gene, which undergoes a series of increases in gene copy number and/or expression during tumour progression[21]. We have used clonally related squamous and spindle cells derived from the same primary tumour to demonstrate that increases in the threshold levels of activated Smad2 and H-ras drive tumour progression from a differentiated squamous carcinoma to a motile invasive stage, through overt EMT, culminating in excess Smad2-dependent metastatic tumour spread. Activation and nuclear accumulation of Smad2 observed in spindle cell lines is also seen during tumour progression of chemically induced mouse skin tumours to the spindle phenotype *in vivo*, demonstrating the relevance of these observations to the process of tumour invasion and metastasis *in vivo*.

Results

Endogenous TGFβ signalling is activated in spindle tumour cells. We investigated the functions of TGFβ signalling in EMT during tumour invasion and metastasis using a well-characterized series of cell lines derived from murine keratinocytes and primary skin tumours that are representative of various stages of tumour progression (Fig. 1a). In particular, the squamous carcinoma cell clone B9 was isolated from the same primary tumour as the two spindle clones, A5 and D3. The common clonal origin of the three cell lines is demonstrated by identical mutations in *Hras1* and *Trp53* genes[22]. These matched cell lines thus enabled us to investigate the mechanism of a conversion to a spindle phenotype: increased motility, the switch to the invasive phenotype and the onset of metastasis.

In cells derived from benign tumours and squamous carcinoma cells, both the basal and TGFβ-induced transcriptional activity of a TGFβ-responsive plasminogen activator inhibitor type 1 (PAI1)–luciferase gene reporter construct[23] was relatively low. In

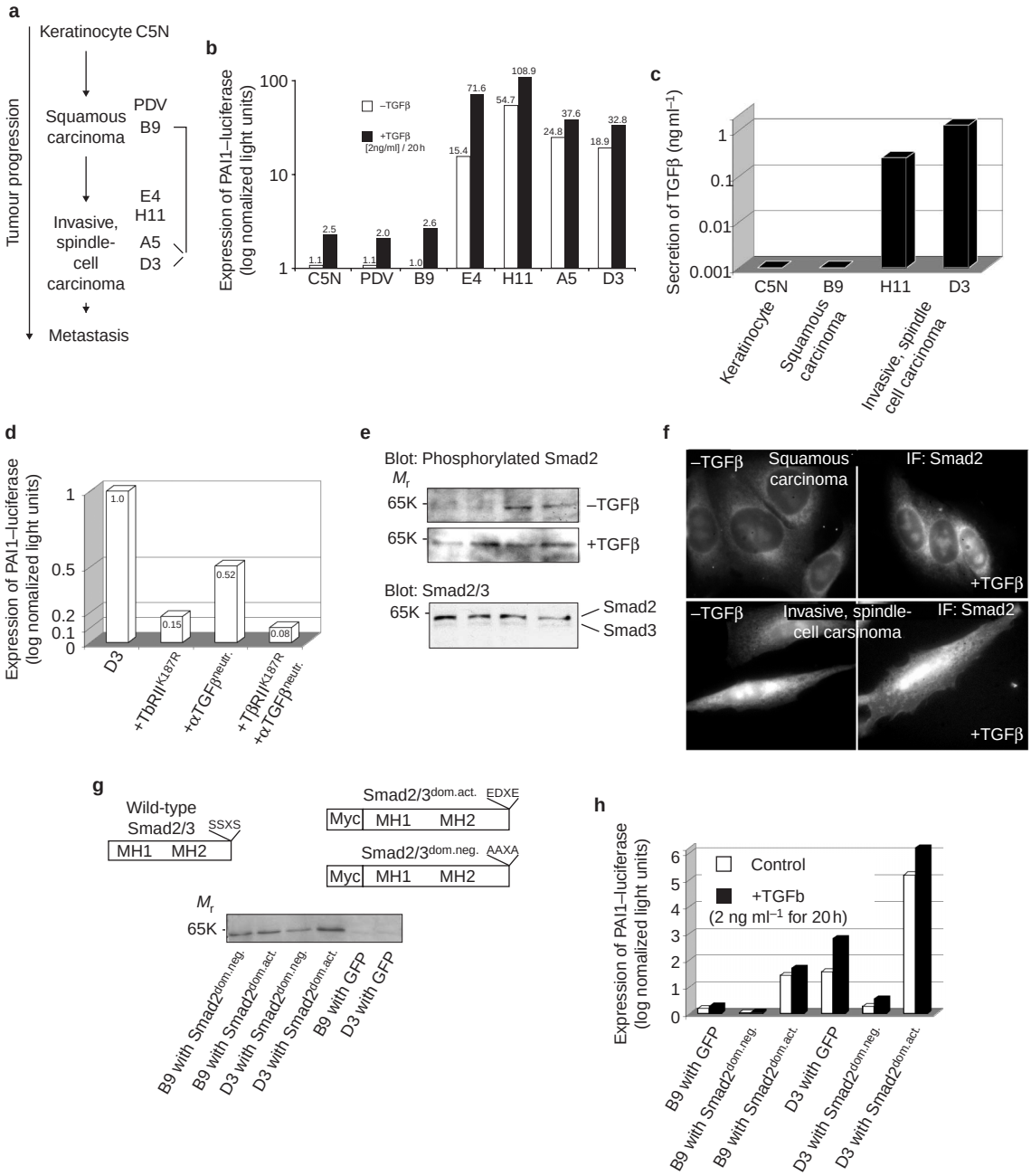

Figure 1 **Smad2 activation is elevated in invasive tumour cells.** **a,** Keratinocyte and tumour cell lines used in this study; tumour-progression stages are indicated. **b,** TGFβ-responsive PAI1–luciferase reporter activity in the various cell lines (note Log scale of x-axis). **c,** ELISA quantification of total TGFβ1 levels secreted by various cell lines. **d,** Activity of the PAI-1–luciferase reporter in spindle-cell carcinoma cells in response to expression of a dominant-negative receptor, TβRII^{K187R} and/or after exposure to TGFβ neutralizing antibodies. **e,** Western analysis for endogenous phosphorylated Smad2 (upper panels) and endogenous total Smad2 and Smad3 (lower panel); tumour cells were stimulated with exogenous TGFβ1 (middle panel). Relative molecular masses (M_r) are indicated. **f,** Immunofluorescent (IF) subcellular localization of total Smad2/Smad3 in non-invasive and highly invasive tumour cells, either untreated, or after a 20 min exposure to TGFβ1. **g,** Structure of recombinant Smad2 variants that harbour mutations in the TGFβ-inducible phosphorylation sites. Myc, c-myc epitope. Expression of Smad2 mutants transfected into non-invasive (B9) and highly invasive (D3) tumour cells. Smad2 immunoprecipitates were western blotted with an α-human c-myc antibody. Control lanes contain lysates from cells infected with GFP. **h**, Modulation of the TGFβ responsive PAI-1 promoter by the expression of Smad2 mutants. The magnification in **f** is 40x.

contrast, transcriptional activity of the reporter in the spindle cell clones was dramatically increased, both in the presence and absence of exogenous TGFβ (Fig. 1b). These results suggested that endogenous TGFβ signalling is activated in spindle cell lines.

In the panel of murine carcinomas, spindle tumours secreted significant amounts of TGFβ, whereas squamous carcinoma cells did not secrete detectable levels of TGFβ (Fig. 1c). Transfection of a dominant-negative TGFβ receptor, TβRII^{K187R}, or applying TGFβ neutralizing antibodies, reduced the activity of the PAI1–luciferase reporter (Fig. 1d), suggesting that activation of the endogenous

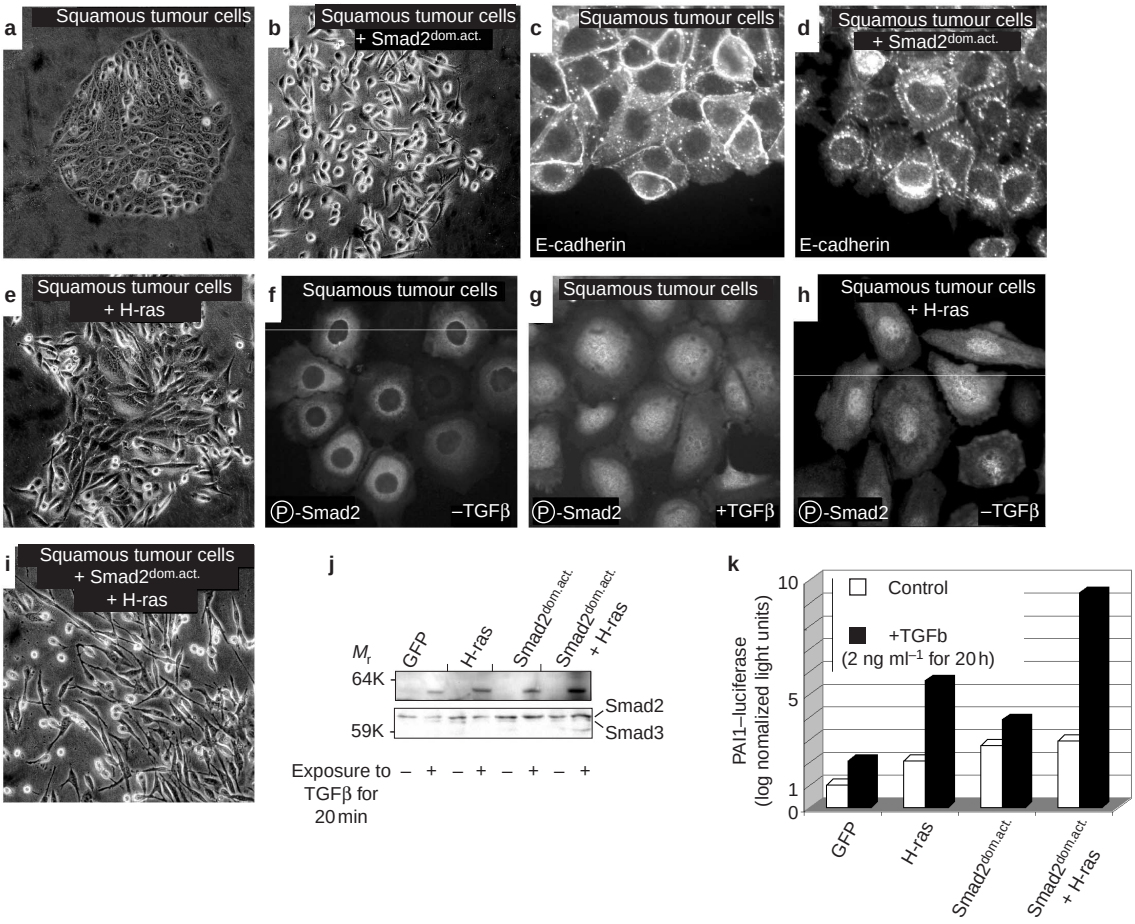

Figure 2 **Co-expression of H-ras and Smad2 is sufficient to induce EMT of squamous carcinoma cells. a,** Phase-contrast photomicrograph of control squamous carcinoma cells B9 or the same cells expressing Smad2$^{dom.act.}$ (**b**), H-ras (**e**), or co-expressing Smad2$^{dom.act.}$ and H-ras (**i**). **c**, **d**, Subcellular localization of E-cadherin in control squamous carcinoma cells B9 (**c**), or B9 cells expressing Smad2$^{dom.act.}$ (**d**). **f–h,** Subcellular localization of Smad2 in B9 cells in the absence (**f**), or in the presence of TGFβ (**g**), or overexpressing a H-ras mutation (RasV12) (**h**). **j,** Western blot for phosphorylated Smad2 (upper panel) and total Smad2 and Smad3 (lower panel). **k**, Activity of the PAI1–luciferase reporter in the cells (**a**, **b**, **e**, **i**) in the presence or absence of exogenous TGFβ. Magnifications: **a**, **b**, **e**, **i**, 25x; **c**, **d**, **f–h**, 40x;)

TGFβ signalling pathway in spindle tumours (Fig. 1b) was due, in part at least, to autocrine stimulation by TGFβ.

TGFβ stimulates phosphorylation and nuclear accumulation of the transcription factors Smad2 and Smad3 (ref. 24). Smad2 is expressed throughout skin tumour progression (Fig. 1e), in contrast to Smad3, which is expressed at comparatively low levels (Fig. 1e and data not shown). To further characterize the TGFβ–Smad signalling pathway we examined Smad2 phosphorylation and nuclear-localization status before and after stimulation with TGFβ. By both criteria, there was evidence that Smad2 is constitutively activated in invasive spindle tumour cells (Fig. 1e,f).

Activation of Smad2 alone stimulates cell migration, but not EMT. Retroviral transduction of Smad2$^{dom.act.}$ (Fig. 1g,h) into B9 squamous carcinoma cells resulted in cell migration (Fig. 2a,b), and inhibited cell-cycle progression (data not shown). In contrast, expression of Smad2$^{dom.neg.}$ (Fig. 1g,h) inhibited cell migration even in the presence of TGFβ (data not shown). Although Smad2$^{dom.act}$ induced a dramatic relocalization of E-cadherin from the adherens junctions to the cytoplasmic pool (Fig. 2c,d), similar to squamous carcinoma cells treated with TGFβ (ref. 8 and data not shown), no changes in the absolute levels of cytokeratins, vimentin or E-cadherin were detected in either Smad2$^{dom.neg.}$- (not shown) or Smad2$^{dom.act.}$-transduced cells (Fig. 3a). TGFβ and Smad2 were, therefore, not sufficient to induce a complete EMT of squamous carcinoma cells.

Mutant H-ras must co-operate with activated Smad2 to induce an EMT. The mutant *Hras1* allele present in the squamous B9 cells is amplified and over-expressed in the A5 and D3 spindle cell lines — the same is true of almost all spindle tumours[21] (S. Frame, R. Crombie and A. B., unpublished observations). We asked if overexpression of mutant H-ras could cooperate with upregulation of TGFβ signalling to induce EMT in B9 squamous carcinoma cells. Overexpression of mutant H-ras in squamous tumour cells stimulated TGFβ-induced transcription (Fig. 2k). In squamous carcinoma cells, stimulation with TGFβ induces carboxy-terminal phosphorylation of Smad2 (Fig. 2j) and its accumulation in the nucleus (Fig. 2f,g). Overexpression of mutant H-ras also induced nuclear accumulation of phosphorylated Smad2 without significantly increasing the C-terminal phosphorylation of Smad2 itself (Fig. 2h,j). Overexpression of mutant H-ras alone did not induce changes in cell shape or in the expression of intermediate filaments that indicate an EMT (Figs 2e and 3a). Strikingly, co-expression of mutant H-ras and Smad2$^{dom.act.}$ induced the cells to adopt a spindle-cell morphology (Fig. 2i). At the same time, epithelial intermediate filaments were replaced by mesenchymal filaments, such as vimentin (Fig. 3a). There was also *de novo* expression of α-smooth

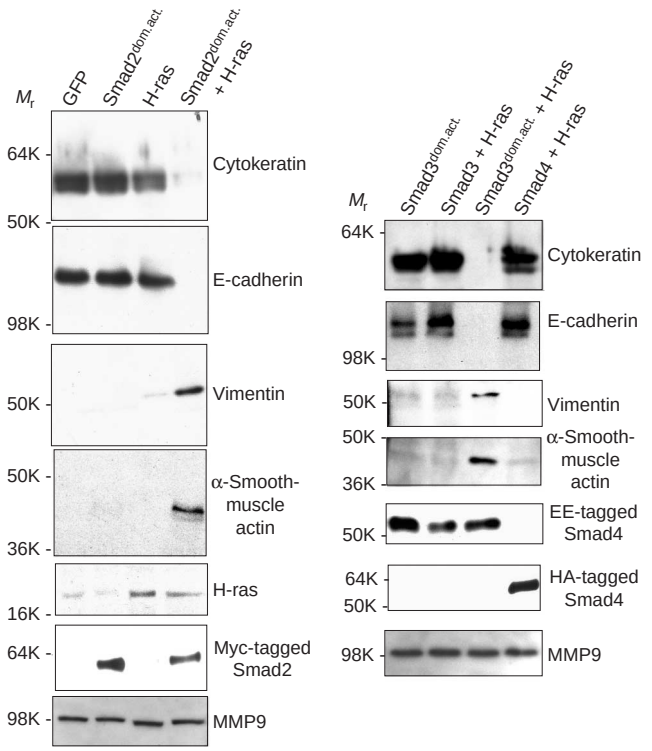

Figure 3 **Smad2 and/or Smad3 cooperate with H-ras to induce EMT of squamous carcinoma cells. a, b,** Western-blot analysis of epithelial (cytokeratin and E-cadherin) and mesenchymal (vimentin and smooth muscle actin) marker proteins, and detection of the Smad mutants using epitope tags, in squamous carcinoma cell lysates expressing combinations of wild-type or mutant Smads 2, 3 and 4, and H-ras.

muscle actin, a specialized mesenchymal marker. Interestingly, Smad3$^{dom.act.}$ also cooperated with H-ras to induce EMT (Fig. 3b). But there EMT could not be induced by either Smad3$^{dom.act.}$ alone, or by co-expression of H-ras with wild-type Smad2, Smad3 or Smad4 (Fig. 3b). An increase in the dosage of the mutant *Hras1* allele, together with the upregulation of TGFβ and Smad2 or Smad3 signalling, is therefore sufficient to induce overt EMT in squamous tumour cells that can't be induced to undergo EMT by TGFβ alone[8].

Activated Smad2 is essential to maintain the spindle phenotype. We next investigated whether Smad signalling is essential for maintenance of the invasive spindle-cell phenotype. Expression of Smad2$^{dom.neg.}$ in invasive spindle cells caused a dramatic reversion to a more cuboidal, epithelial phenotype (compare Fig. 4a,b) and inhibition of the activity of the TGFβ-responsive promoter (Fig. 1h). TGFβ and Smad2 or Smad3 are known to induce cell-cycle arrest in epithelial cells[25,26]. Accordingly, inhibiting aberrant TGFβ signalling by Smad2$^{dom.neg.}$ expression relieved this checkpoint and restored cell-cycle progression (Fig. 4d). Importantly, Smad2$^{dom.neg.}$ expression restored many of the features of a basic epithelial gene-expression profile in the spindle cells, such as the re-expression of E-cadherin, α2 and α5 integrins and cytokeratin 18, and simultaneously reduced the expression of the mesenchymal filament proteins, vimentin and α-smooth muscle actin (Fig. 4f). Spindle cells transfected with the Smad2$^{dom.act.}$ had elevated TGFβ reporter gene activity (Fig.1h) and continued to display a mesenchymal spindle-cell morphology (Fig. 4c) and gene-expression profile (Fig. 4f). We thus conclude that both the induction and reversal of spindle carcinoma formation can be accomplished by manipulating Smad2 signalling.

Smad2 activation promotes tumour invasion *in vivo*. Squamous cells, derived by infecting spindle tumour cells with Smad2$^{dom.neg.}$, lost the invasive capacity characteristic of the parental cell line when tested for invasion in collagen matrices (Fig. 4g,h). Furthermore, surface expression of αvβ3 integrin, which has been associated with the invasive migratory phenotype of tumour cells[27], was completely lost in these Smad2$^{dom.neg.}$-reverted populations (Fig. 4e,f). In contrast, expression of Smad2$^{dom.act.}$ enhanced the invasiveness of the parental spindle cell (Fig. 4g,i).

The parental spindle-cell population or its Smad2-transfected derivatives were injected intraperitoneally or subcutaneously into athymic mice. Squamous cells expressing Smad2$^{dom.neg.}$ lost the ability to form tumours compared with the parental or Smad2$^{dom.act.}$ spindle lines, and formed only small cellular nodules at the injection site or on the mesenteric surfaces (data not shown). In contrast, both the parental spindle cells and the population expressing Smad2$^{dom.act.}$ formed tumours at both locations. The primary subcutaneous tumours produced from Smad2$^{dom.act.}$-transduced cells, although significantly smaller than those from parental control cells, were far more invasive, readily penetrating the overlying skin and underlying muscle fascia (Fig. 5a,b, and data not shown).

High levels of activated Smad2 drive tumour extravasation and metastasis. The skin-derived mouse spindle tumour cells used in this experiment are capable of forming lung metastases after intravenous injection, but at exceedingly low frequency. The control spindle cell population caused no macroscopic metastases six weeks after intravenous injection of 1×10^6 cells into athymic mice (Fig. 5c,e). In contrast, cells expressing Smad2$^{dom.act.}$ formed a high number of perivascular metastatic deposits in the lungs of recipient animals (Fig. 5d,f). The observed gain of metastatic capacity of Smad2$^{dom.act.}$-expressing tumour cells could result from increased extravasation into the lung tissue, or from an increased capacity to establish progressively growing nodules after tissue dissemination. We therefore analysed the mechanism of this metastatic switch by investigating the kinetics of dissemination of tumour cells to the lung. Parental and Smad2$^{dom.act.}$-expressing tumour cells could both still be detected at the subcutaneous injection site several days after injection (Fig. 5g). But after intravenous injection, only cells expressing Smad2$^{dom.act.}$ were found in the lung (Fig. 5h). Moreover, these metastasizing cells were seen in the lung as early as 20 minutes after injection. We conclude that additional expression of Smad2 in already invasive cells accomplishes a second critical step in metastasis, namely, extravasation into the target tissue.

High levels of Smad2 and phosphorylated Smad2 located in the cytoplasm of tumour cells have been found in human breast-tumour samples[28]. To analyse whether endogenous Smad2 hyperactivation and nuclear accumulation occurs during normal tumour progression, we analysed primary tumour material from chemically induced mouse skin tumours, for the status of Smad2 activation (Fig. 6). In all differentiated tumours and squamous carcinomas, phosphorylated Smad2 and Smad2 total protein was predominantly localized in the cytoplasm (Fig. 6a,b). Target genes such as Smad7 were not induced (Fig. 6c). In the spindle tumour cells, however, phosphorylated Smad2 was predominantly nuclear (Fig. 6d,e) and Smad7 was strongly induced (Fig. 6f).

Discussion

Our data support a multistep model for tumour progression and metastasis that involves a progressive increase in the threshold levels of H-ras and Smad2 activity. Duplication, or in some cases amplification, of mutant *Hras1* has been observed in mouse and human tumours (reviewed in refs 29,30). TGFβ1 is frequently overexpressed in human tumours[31] and its expression is associated with poor prognosis[32,33]. H-ras has been reported to antagonize growth inhibition and apoptosis induction by TGFβ, as well as certain aspects of Smad2-mediated transcription[6,12,13,15]. In a cell-culture model for EMT, Ras-induced PI(3)K activity rescues cells from

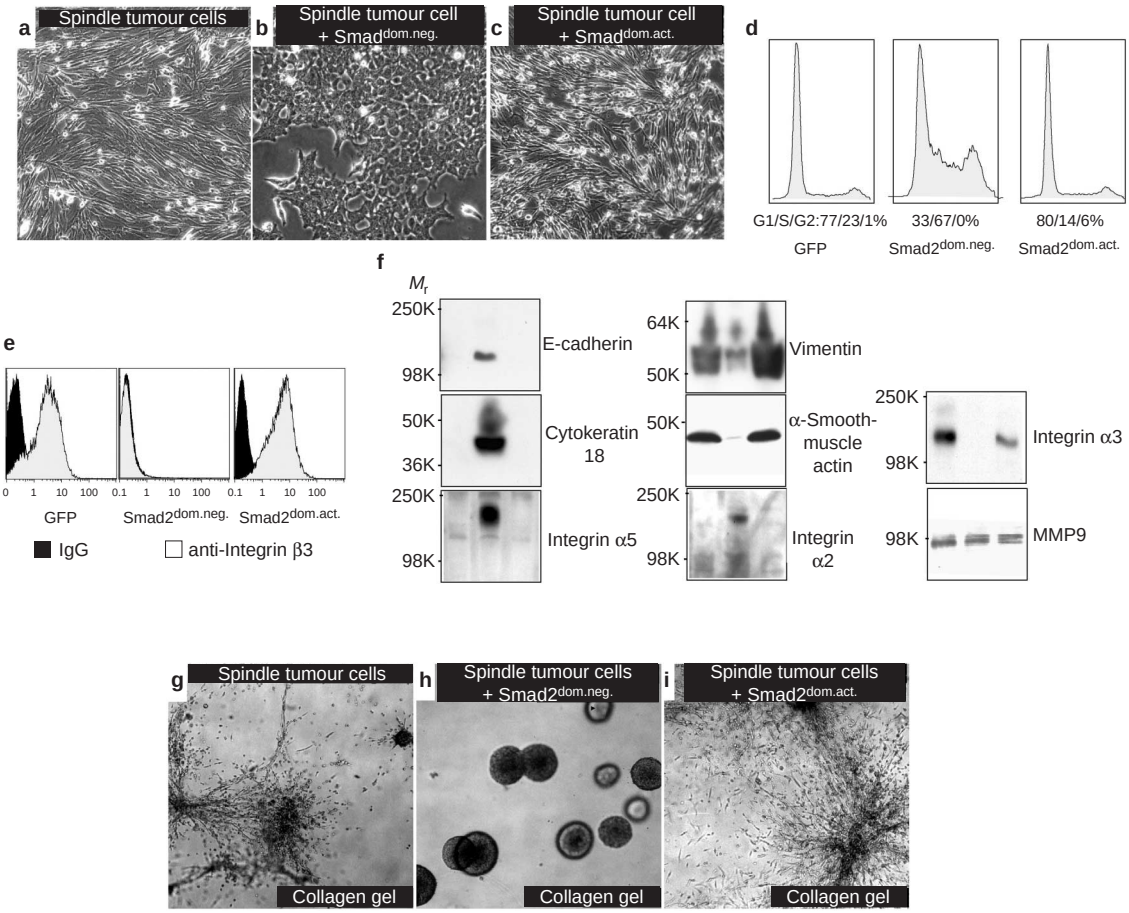

Figure 4 **Smad2 inhibits proliferation but is necessary for the EMT and for tumour cells to become invasive.** Phase-contrast photomicrographs of invasive D3 spindle tumour cells (**a**), and their derivatives expressing Smad2$^{dom.neg.}$ (**b**) or Smad2$^{dom.act.}$ (**c**). **d**, FACS cell-cycle profiles and cell-cycle distribution of the invasive spindle tumour cells and their derivative tumour cells (as shown in **a–c**). GFP indicates control, wild-type cells. **e**, Surface expression of integrin αvβ3, as assessed by FACS analysis, is repressed in Smad2$^{dom.neg.}$ transfectants (grey profile integrinβ3, black profile isotype control). **f**, Western-blot analysis for epithelial (cytokeratin and E-cadherin,) and mesenchymal (vimentin and smooth muscle actin) marker proteins from parental spindle carcinoma cells (GFP, control) and derivatives expressing the Smad mutants. **g–h**, *In vitro* invasion of tumour cells into three-dimensional collagen gels. Parental spindle cells D3 (**g**) show invasion into the gel, seen as individual elongated cells migrating away from the main colony. Smad2$^{dom.act.}$ stimulates even greater migration and invasion (**i**) than the parental cell line (**g**). Smad2$^{dom.neg.}$ (**h**) inhibits invasion of tumour cells, leading to compact epithelial colonies that remain localized on the collagen. Magnifications: **a–c**, 20×; **b**, **g–i**, 10×.

TGFβ-induced apoptosis and cell-cycle arrest, whereas Raf kinase activation is necessary for EMT induction[14]. Our results provide a new view of Ras and Smad cooperating to promote tumour development by demonstrating that H-ras induces nuclear accumulation of phosphorylated Smad2 and consequent upregulation of Smad2-mediated transcription. Preliminary analysis of gene-expression patterns in the cell lines described here, using complementary DNA microarrays, have shown, for example, that elevated TGFβ signalling in spindle cells continues to be reflected in increased expression of pro-apoptotic genes such as *Bax* and *Bad,* and that expression of these genes is downregulated by introducing the dominant-negative Smad 2 construct (data not shown). The lack of overt apoptosis in spindle cells may, therefore, result from concomitant upregulation of survival signals induced by the elevated H-ras levels in the same cells, which is consistent with one model of cooperation[14]. We have previously proposed that sequential increases in H-ras levels during tumour progression may be required to activate different effector pathways to provide the necessary survival signals at later stages of tumourigenesis[29]. Interestingly, spindle tumour cells are more sensitive than corresponding squamous cells to apoptosis being induced by PI(3)K inhibitors (S. Frame and A. B., unpublished observations), consistent with the possibility that PI(3)K activation occurs at the higher threshold level of H-ras signalling and is required for survival of spindle cells that overproduce TGFβ. The coordinate upregulation of both Ras and TGFβ can be seen as a mechanism that allows the tumour cell to adopt the cell-fate change and invasive properties required for progression without dying as a consequence of increased levels of pro-apoptotic signals.

The majority of genes whose expression increases dramatically during EMT are regulated cooperatively by Smad2 and H-ras, whereas there is only a small number of H-ras-induced genes that are repressed by TGFβ (M.O., unpublished observations). Taken together, the changes in gene-expression patterns indicate that both mechanisms — co-operativity of H-ras and Smad2 on certain promoters and antagonism on others — are functional during H-ras- and TGFβ-driven tumour progression.

Interestingly, spindle tumour cells that express higher levels of TGFβ1 target genes proliferate more slowly *in vitro* than their derivatives in which TGFβ signalling is blocked. Nevertheless, the former show enhanced tumour formation *in vivo*. These findings suggest that selective pressure for tumour outgrowth favours acquisition of invasive properties over increased cell-cycle progression.

These results provide a fascinating parallel with developmental systems, where both threshold levels of Ras[16] or MAPK[17] activity

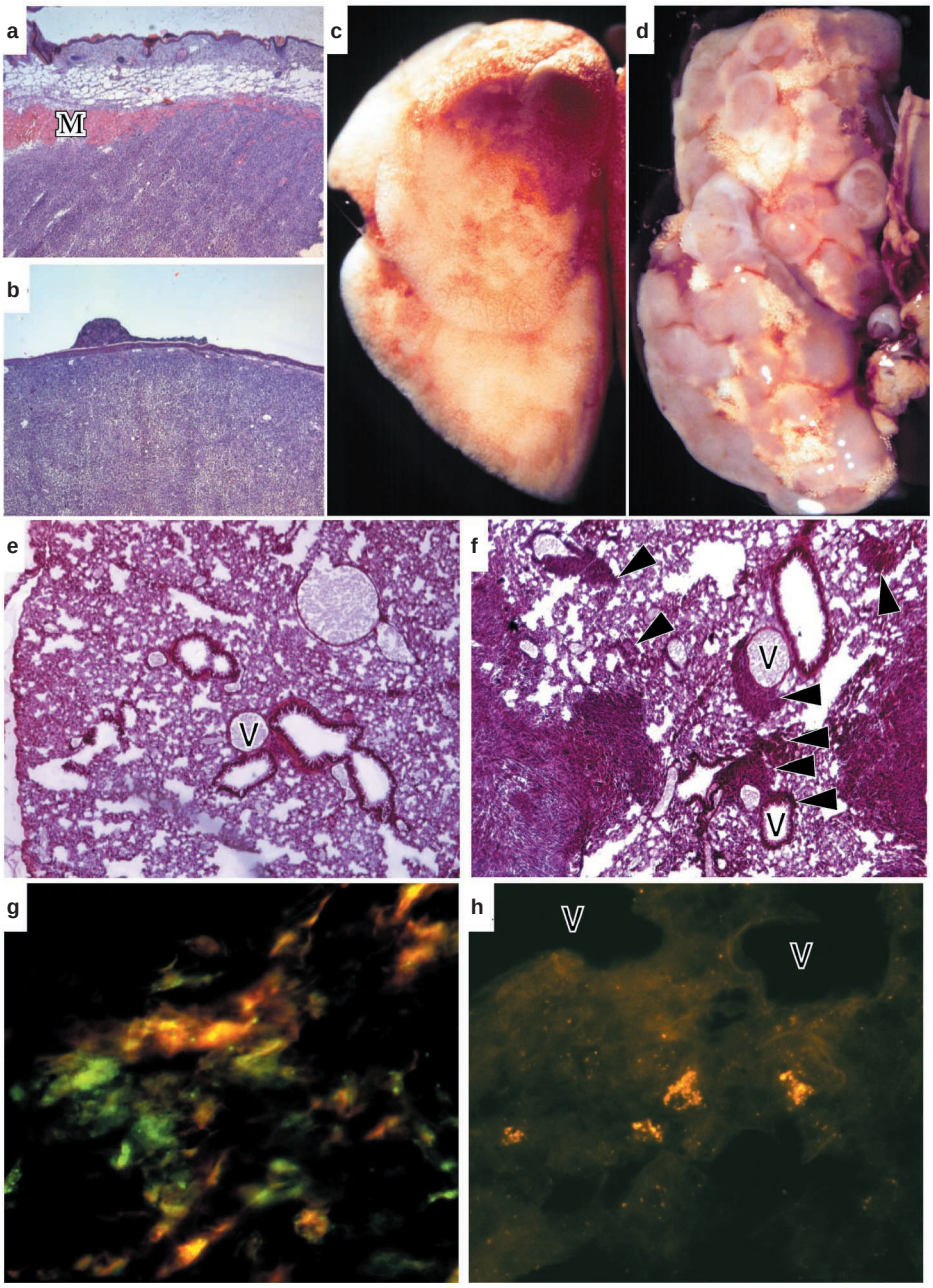

Figure 5 **Expression of Smad2$^{dom.act}$ augments metastasizing capability of spindle tumour cells. a, b,** *In vivo* invasion of tumour cells upwards through the dermis after subcutaneous injection. Smad2$^{dom.act.}$ (**b**) increases invasion compared to the parental line (**a**), evidenced by complete penetration through the muscular (M) and fatty layers up towards and through the epidermal surface. Smad2$^{dom.neg.}$ completely inhibited tumour formation (data not shown). Lungs from mice injected intravenously with 1×10^6 parental spindle carcinoma cells D3 (**c, e**), or their derivatives expressing Smad2$^{dom.act.}$ (**d, f**), and examined six weeks after injection. Note multiple metastatic nodules in (**d**) compared to (**c**), and perivascular metastases (arrowheads) in (**f**). **g, h,** Cryosections through the skin (**g**) and lung (**h**) of mice after injection of a 1:1 mixture of $2x10^6$ GFP-labelled parental spindle carcinoma cells D3 and orange-labelled Smad2$^{dom.act.}$-expressing cells. (**g**), 1 day after subcutaneous injection and (**h**) 20 min after intravenous injection. Both cell types are found in the dermis after subcutaneous injection, but strikingly, only Smad2$^{dom.act.}$-expressing cells are established in the lung 20 min after intravenous injection. Magnifications: **a**, **b**, 10×; **c**, **d**, 1.25×; **e**, **f**, 20×; **g**, **h**, 40×)

together with threshold levels of the TGFβ family members, activin and nodal[18,34], control cell-fate decisions during *Xenopus* mesoderm induction. Both activin or nodal receptors and TGFβ receptors activate Smad2 (ref. 35), and Smad2 is required for mesoderm induction in mice[20] and is sufficient to induce mesoderm in ectodermal explants in *Xenopus*[19]. Moreover, whereas low levels of Smad2 can induce lateral mesoderm in *Xenopus*, higher doses are required to induce dorsal mesoderm[19]. Similar to the changing threshold levels of Smad2 activity that regulate developmental processes, during tumour progression intermediate levels of Smad2 cooperate with mutant H-ras to induce spindle-cell transformation (EMT) and invasiveness of tumour cells. But even higher levels of Smad2 activity need to be surpassed to facilitate a high frequency of metastasis, caused by increased penetration through blood-vessel walls and dissemination into the target tissue.

We propose that differing threshold levels of Smad2 and Ras

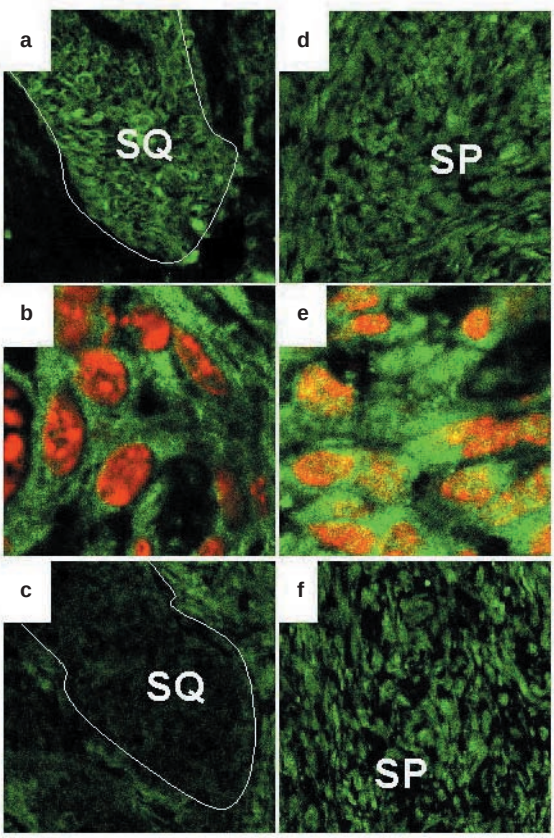

Figure 6 **Spindle, but not squamous, carcinoma cells show nuclear localization of phosphorylated Smad2 *in vivo*.** Cryosections of chemically induced primary squamous skin tumours (**a–c**) and spindle cell tumours of the skin (**d–f**). Phosphorylated Smad2 (**a,b,** and **d,e**) is predominantly localized in the cytoplasm of squamous tumour cells but in both cytoplasm and nucleus of spindle cell tumours (nucleus counterstained with DAPI). Smad2 target genes, such as Smad7 are specifically induced in spindle tumour cells (**f**), but expressed at low levels in squamous carcinomas and keratoacanthomas (**c**). Magnifications: a, d, 20×; b, e, 10×; c, f, 20×.

activity are important for accomplishing the sequential steps of metastasis, and suggest that this pathway will provide important targets for drug discovery, which should lead to new possibilities for the control of tumour spread. □

Methods

Cell culture and retroviral infections

The previously well-characterized cell lines C5N, PDV, B9, E4, H11, A5 and D3, which represent different stages of mouse skin tumorigenesis, were used in this study. Note that B9, A5 and D3 were isolated from the same tumour and are clonally related[22], as indicated by connecting lines in Fig. 1a. Tumour cell lines were grown in DMEM, containing 10% foetal calf serum (FCS).

Activating or inactivating mutants of Smad2 and Smad3 were generated by site-directed mutagenesis. The serine phosphorylation sites (SSMS, using single-letter amino acid code) at the C-terminus of Smad2 were mutated to acidic residues (EDME) to generate a constitutively activate molecule, Smad2$^{dom.act.}$ or to alanine residues that prevent phosphorylation and generate a dominant-negative molecule, Smad2$^{dom.neg.}$ (ref. 24; Fig. 1e,f and data not shown).

Parental cells were transduced with supernatants from BOSC23 high-titre retrovirus-producing cells expressing the respective Smad2 mutants or green fluorescent protein (GFP) as a control, as previously described by us[7]. Non-clonal populations were selected by culture in G418 media or by cell sorting using GFP. For co-expression experiments all recombinant plasmids were simultaneously transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA), followed by high G418 drug selection. Expression levels from exogenous constructs were verified by western-blot analyses. Cell culture, collagen-invasion assays, western blotting and immunofluorescence were performed as described previously[7]. All transduction experiments with the Smad mutant constructs were performed with either B9 (representative of a squamous cell line) or D3, H11 or A5 (representative of spindle cell lines). PAI-1 reporter assays were performed essentially as described after transient transfection of the cell lines[6,23]. Results were normalized for transfection efficiency by co-transfection and assay of a cytomegalovirus promoter (CMV)-β-galactosidase reporter. All experiments were performed in triplicate and repeated at least twice.

Antibodies

Antibodies used for western blot, fluorescence-activated cell sorting (FACS) and immunofluorescence analysis were: keratins (CRP, Denver, CO; Sigma, St Louis, MO), vimentin (Sigma), α-smooth muscle actin (Sigma), integrins (Transduction, Lexington, KY), Smad2 and Smad3 (Transduction; Santa Cruz, Santa Cruz, CA), Smad7 (Santa Cruz) and epitope tags (CRP). TGFβ neutralizing antibody (R & D, Minneapolis, MN) and human TβRII-Fc chimera (R & D), were used up to 50 ng ml^{-1} in culture medium.

Enzyme-linked immunosorbent assay (ELISA)

Cells were washed thoroughly in PBS, and cultured in DMEM without serum for 16 h. The medium was harvested and assayed for total TGFβ activity after acid-activation, using the Quantikine ™ ELISA kit (R & D).

Animal experiments

All cell injections were performed using female Balb/c *nu/nu* athymic mice. In general 1×10^6 cells were injected. Animals were killed when the tumours reached 1 cm in diameter, or if the mice showed signs of physical stress, or at the end of the observation period. All animal experiments were performed in triplicate and repeated at least twice.

Chemical carcinogenesis

Mice (NIH/Swiss, 8 week-old females) received a single topical dose of 7,12-dimethyl benzanthracene (DMBA; 25 μg per mouse in 200 μl acetone) and, starting one week after initiation, animals were promoted by topical application with 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA; 200 μl of 10^{-4} M solution in acetone) twice weekly for 20 weeks. Tumours were allowed to progress to the carcinoma stage, diagnosed initially according to clinical criteria and thereafter the diagnosis was confirmed by histological analysis and the tumour grade determined. All tumours were snap frozen and frozen-fixed sections were subjected to immunofluorescence analysis.

Metastasis assays

Cells were fluorescently labelled by incubating them with the lipophilic dyes Sp-DiOC18(3) (green) or Sp-DiIC18(3) (orange) (Molecular Probes, Eugene, OR) at 2 pg ml^{-1} for 24 h. Residual dyes were washed away with PBS. Within 4 h of labelling, 1×10^6 cells were injected either intravenously or subcutaneously into mice. The lungs and skins of ten mice at each of two time points (20 min and 24 h) were harvested, and unstained cryo-sections were simultaneously analysed for green and orange fluorescent cells.

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