TGFβ signaling is necessary for carcinoma cell invasiveness and metastasis

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Background: Invasive growth of epithelial tumor cells, a major cause of death from cancer in humans, involves loss of epithelial polarity and dedifferentiation. Transforming growth factor β (TGFβ) is regarded as a major tumor suppressor during early tumor development because it inhibits cell-cycle progression and tumor growth. Many dedifferentiated, late-stage tumors are resistant to growth inhibition by TGFβ, however, and even secrete TGFβ. In line with this, TGFβ is involved in angiogenesis, wound healing and epithelial–mesenchymal transition (EMT) during development. Ha-Ras-transformed mammary epithelial cells (EpRas) undergo TGFβ-induced EMT maintained via a TGFβ autocrine loop. Thus, we have analyzed whether signal transduction by the TGFβ receptor (TGFβR) is required for local tumor cell invasion and metastasis.

Results: A dominant-negative type II TGFβR (TGFβRII-dn) was expressed using retroviral vectors in EpRas cells and highly metastatic mesenchymal mouse colon carcinoma cells (CT26). In both cell types, TGFβRII-dn blocked TGFβR signaling and heavily delayed tumor formation. In EpRas cells, TGFβRII-dn prevented EMT. In the dedifferentiated mesenchymal CT26 cells, TGFβRII-dn caused mesenchymal-to-epithelial transition and inhibited their *in vitro* invasiveness in several assays. In addition, TGFβRII-dn completely abolished metastasis formation by CT26 cells. Furthermore, several human carcinoma lines lost *in vitro* invasiveness when treated with neutralizing TGFβ antibodies or soluble receptor variants. Finally, human colon carcinoma cells (hnPCC) expressing a mutated, non-functional TGFβRII were non-invasive *in vitro*, a defect restored by re-expressing wild-type TGFβRII.

Conclusions: Cell-autonomous TGFβ signaling is required for both induction and maintenance of *in vitro* invasiveness and metastasis during late-stage tumorigenesis. TGFβRII therefore represents a potential target for therapeutical intervention in human tumorigenesis.

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Background

Epithelial tumors (carcinomas) comprise more than 80% of the tumors occurring in man and arise through a multistep process, as best exemplified in human colon cancer [1] and mouse skin tumor progression [2]. During this process, epithelial polarity is gradually lost, organ structure is disrupted and epithelial cells dedifferentiate. Progression to metastatic carcinomas requires additional changes, such as proteolytic degradation of the basement membrane, conversion from a sessile to a migratory phenotype, survival in the blood stream and formation of metastases at distant sites [3].

Members of the transforming growth factor β (TGFβ) superfamily are important regulators of embryonic development, controlling determination of cell fate during embryogenesis and inducing mesoderm formation and epithelial-to-mesenchymal transition (EMT) [4,5]. TGFβs are also important as key regulators in carcinogenesis [6,7]. Widely diverse functions in tumor development are ascribed to TGFβ, its receptor (TGFβR) and to signal transduction intermediates (SMADs) activated by TGFβ signalling [8]. TGFβ negatively regulates cell-cycle progression in epithelial cells, involving Cdk inhibitors such as p27 and $p15^{INK4B}$ [9,10]. Tumor cell growth at early stages is inhibited by TGFβ [6], suggesting that TGFβ and its downstream effectors act as tumor suppressors. TGFβR and components of the TGFβ signal transduction pathway (Smad4/DPC4) are mutated in certain human tumors [11–13] and most human tumor cells are resistant to TGFβ-induced growth inhibition [14].

Positive effects of TGFβ-induced signals are of similar importance for TGFβ function in carcinogenesis. Many late-stage or even metastatic human tumors overexpress TGFβ [15–18]. Resistance to TGFβ-mediated growth inhibition may arise from mutations of proteins within the TGFβ signalling pathway, but can also involve

downstream cell-cycle regulators such as $p15^{INK4b}$, $p16^{INK4a}$ or Cdc25A [10,19,20]. Mutations in the genes encoding TGFβR type II (TGFβRII) or DPC4 are restricted to specific tumor types [11–13]. Furthermore, chemically induced tumors in heterozygous $TGF\beta1^{+/-}$ mice do not lose the second copy of the TGFβ1 gene [21]. Finally, TGFβ actively promotes angiogenesis and endothelial cell spreading, wound healing and immunosuppression [22–24], in accord with a positive role for TGFβ family members in carcinogenesis. In line with this, TGFβ promoted squamous-to-spindle cell transitions in a transgenic mouse model for multistage skin carcinogenesis [6,7].

Recently, we described a mouse mammary carcinoma model — Ha-Ras-expressing mammary epithelial cells (EpRas) — in which TGFβ actively promotes tumor progression [25]. TGFβ induced fully polarized, non-invasive epithelial cells to acquire a mesenchymal, spindle cell phenotype (that is, EMT). The mesenchymal cells obtained after EMT *in vitro* or cultivated from tumors were invasive in several *in vitro* assays. They displayed autocrine production of TGFβ, which is required to maintain the mesenchymal, spindle-like, invasive cell phenotype, as neutralizing antibodies to TGFβ caused reversion to a polarized, epithelial phenotype. TGFβ production by the tumor cells and acceleration of EMT by exogenous TGFβ could also be demonstrated *in vivo* [25].

These results raised the issue of whether TGFβ secreted by TGFβ-resistant human tumors may be functionally important for promoting late-stage tumor progression. According to the tumor suppressor model, exposure to TGFβ might select for cells that are incapable of transducing TGFβ-mediated signals and are thus resistant to TGFβ-induced growth arrest. This would facilitate the accumulation of further mutations favoring EMT or squamous-to-spindle cell transitions. Alternatively, continuous signaling through TGFβ-activated signaling pathways (for example those caused by an autocrine TGFβ loop) might be necessary to induce and maintain an invasive tumor cell phenotype. Furthermore, the TGFβ produced by human tumors *in vivo* might just act in a paracrine fashion, for example on tumor stroma, endothelial cells and cells of the immune system.

In this paper, we have investigated the possible, cell-autonomous function of TGFβ during invasion and metastasis of late-stage epithelial tumor cells. A dominant-negative version of TGFβRII (TGFβRII-dn) was employed to inhibit TGFβR signaling in EpRas cells and highly metastatic mouse colon carcinoma cells, which display a spindle-like, mesenchymal phenotype and are resistant to TGFβ-mediated growth inhibition (CT26 and C26 cells). TGFβRII-dn retarded tumor formation by the epithelial EpRas cells and prevented EMT *in vivo*. In the mesenchymal colon carcinoma cells, TGFβRII-dn induced mesenchymal-to-epithelial transition (MET) and abolished invasiveness *in vitro*. In mice, tumor outgrowth of TGFβRII-dn-expressing CT26 and C26 cells was retarded or even inhibited and metastatic spreading of residual tumor cells was completely abolished. These effects were cell autonomous, as GFP-labeled cells expressing TGFβRII-dn failed to contribute to primary tumors or metastases caused by unmodified tumor cells.

Interference with potential autocrine stimulation by TGFβ in human tumor cells inhibited local invasiveness of several unrelated human tumor cell lines *in vitro*. Colon tumors of the hereditary nonpolyposis colorectal cancer (hnPCC) group expressing a dominant-negative mutation of TGFβRII [12] displayed an intrinsically low invasive potential. The invasiveness of these cells could be restored, however, by re-expressing a wild-type TGFβRII. Thus, a major function of TGFβ is to act as a cell-autonomous promoter of late-stage human tumor development.

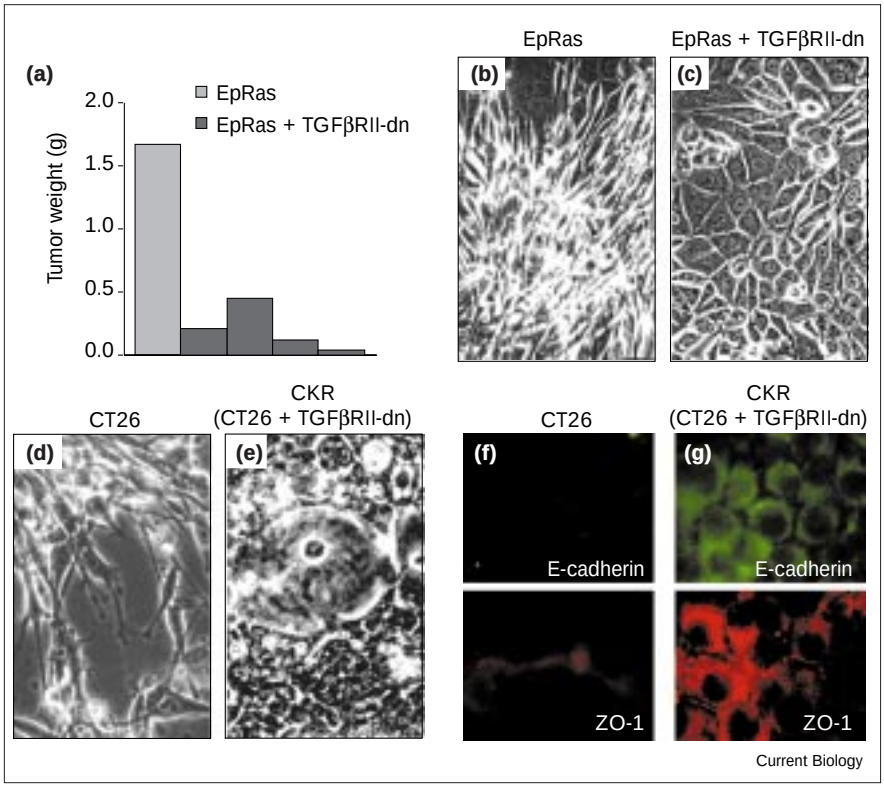
Results

Inhibition of TGFβ signaling prevents EMT in epithelial cells and causes MET in mesenchymal spindle tumor cells

EpRas cells are induced to undergo EMT by exogenously added or stroma-derived TGFβ *in vitro* and *in vivo* [25]. We investigated whether inhibition of signal transduction by TGFβR would interfere with tumor growth and inhibit EMT *in vivo*. TGFβ acts by binding to TGFβRII, a receptor serine/threonine kinase [26]. The ligand–receptor complex heterodimerizes with TGFβR type I (TGFβRI), thereby initiating signal transduction [27]. A dominant-negative, kinase-dead form of TGFβRII (TGFβRII-dn) [28] was stably expressed in EpRas cells using retroviral vectors. Cells infected with an empty vector formed tumors when injected subcutaneously into mice. Upon re-cultivation, these control tumor cells showed the expected, mesenchymal-like cell type (Figure 1b) [25]. All cell lines expressing TGFβRII-dn showed a severely reduced tumor outgrowth compared to EpRas cells (Figure 1a) and cells re-cultivated from the small tumor nodules displayed a polarized, epithelial phenotype (Figure 1c). Thus, TGFβRII-dn-expressing EpRas tumor cells are both strongly retarded in tumor outgrowth and unable to undergo EMT *in vivo*.

Next, we asked whether TGFβ signaling might also be required to maintain the already established, invasive spindle cell phenotype of late-stage tumor cells. The murine cell line CT26, derived from a chemically induced colon carcinoma [29], is highly tumorigenic in syngeneic or nude mice. It also induces blood-borne lung metastases from the primary tumor site with high incidence. In culture, CT26 cells display a spindle cell phenotype (Figure 1d) and fail to express basolateral epithelial markers such as the junctional proteins E-cadherin and

Figure 1



Inhibition of TGFβ signaling interferes with EMT and tumor growth of EpRas cells and causes MET in mesenchymal colon carcinoma cells (CT26). **(a–c)** TGFβRII-dn prevents EMT and retards tumor growth in v-Ha-Ras-expressing mammary epithelial cells. (a) Control EpRas cells or EpRas cells stably expressing a dominant-negative TGFβRII (TGFβRII-dn) were injected into nude mice (three mice per clone). Mean tumor weights obtained four weeks after injection are shown. Cells re-cultivated from the control tumors had undergone EMT (b), whereas cells grown from the tumor nodules induced by TGFβRII-dn EpRas cells retained an epithelial phenotype (c). **(d–g)** Expression of TGFβRII-dn in spindle-shaped CT26 tumor cells reverted them towards an epithelial phenotype. CT26 cells showed a mesenchymal-like phenotype in phase micrographs (d) and failed to express the epithelial markers E-cadherin and ZO-1 (f). The TGFβRII-dn-expressing CT26 clone CKR2 regained epitheloid morphology, formed hemicysts (e) and expressed E-cadherin and ZO-1 (g).

ZO-1 (Figure 1f). Expression of TGFβRII-dn in CT26 cells (CKR cells) caused reversion of the mesenchymal, spindle cell phenotype of CT26 cells towards a cobblestone, epitheloid morphology (Figure 1e) and upregulation of junctional cell adhesion molecules such as E-cadherin and ZO-1 (Figure 1g). These cells formed cell junctions sufficiently functional to support the formation of fluid-filled domes (hemicysts), suggesting partial epithelial polarization (Figure 1e). These results indicate that interference with TGFβ signaling induces MET in late-stage, metastatic carcinoma cells.

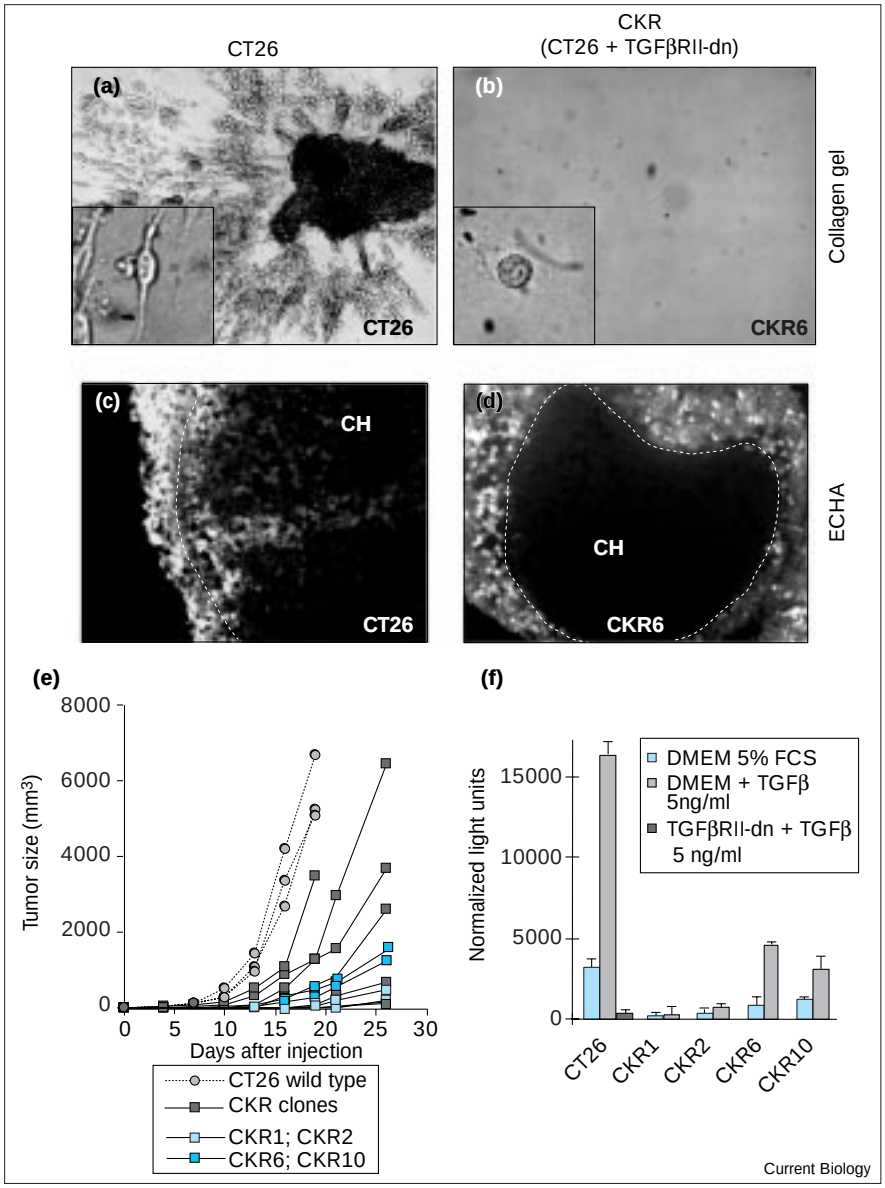
TGFβ induces *in vitro* invasiveness independent of cell-cycle control

The CT26 colon carcinoma cells and their TGFβRII-dn-expressing derivatives (CKR cells) are resistant to the cell-cycle arrest induced by TGFβ in normal epithelial cells (data not shown). We therefore analyzed whether TGFβ signaling was still required for *in vitro* invasiveness of CT26 cells. Strikingly, the TGFβRII-dn-expressing CKR cells completely lost their ability to undergo invasive growth *in vitro*. This result was obtained both in three-dimensional collagen gels (Figure 2b) and in the embryonic chicken heart invasiveness assay (ECHA; Figure 2d). As expected, the parental CT26 cells showed massive invasive growth in both assays (Figure 2a,c). These experiments show that CT26 colon cancer cells retain a functional TGFβ signaling pathway and require TGFβ-dependent signaling for invasiveness, despite the fact that they have become completely resistant to TGFβ-dependent inhibition of cell-cycle progression.

Inhibition of TGFβ signaling retards or abolishes tumor formation and prevents metastasis *in vivo*

CT26 cells cause rapidly growing tumors upon subcutaneous injection into syngeneic Balb/C mice (Figure 2e) or nude mice (data not shown). In all 15 CT26 cell clones stably expressing TGFβRII-dn (CKR cell clones), tumor growth was delayed or inhibited. Ten CKR clones gave rise to subcutaneous tumors of varied, but always greatly reduced, size and growth rate (Figure 2e). Furthermore, five of the fifteen CKR clones tested did not show any tumor formation in Balb/C mice, even up to 6 months after injection. To analyse whether TGFβR signaling was still intact in CT26 cells, but abolished by TGFβRII-dn expression, the effect of TGFβ on a TGFβ-responsive promoter element was determined in these cells. In the CT26 control cells, TGFβ strongly stimulated transcription from a transiently transfected PAI-1 reporter gene construct (Figure 2f), suggesting intact TGFβR signal transduction via the SMAD pathway. In contrast, both basal and TGFβ-induced PAI-1 reporter gene transcription were strongly

Figure 2



TGFβ signaling in CT26 cells is required for invasiveness *in vitro* and tumor outgrowth *in vivo*. (**a–d**) *In vitro* invasiveness assays. CT26 cells (a,c) showed invasive growth in three-dimensional collagen type I gels (a) or in the chicken heart invasiveness assay (ECHA; cryosections of heart fragments) (c). The TGFβRII-dn-expressing CT26 clone CKR6 (b,d) failed to show *in vitro* invasiveness in both assays. (Insets show higher magnifications.) CH, chicken heart fragment (the dotted line marks the border of the fragment). (**e**) Tumor outgrowth after subcutaneous injection into nude mice. Tumor outgrowth from control CT26 cells (circles) is shown as is the delayed or absent tumor outgrowth from various CT26 clones (CKR) expressing TGFβRII-dn (squares). Note that clones with medium-level TGFβRII-dn expression (CKR clones 6/10, verified by inhibition of PAI-1 reporter gene transcription – see panel f) showed slow tumor growth, whereas clones with high-level TGFβRII-dn expression (clones CKR 1/2 – see panel f)) failed to form tumors. (**f**) PAI-1 reporter gene expression was measured in CT26 control cells and in several TGFβRII-dn-expressing CT26 clones also analyzed for tumor formation (CKR clones 1–10), both before and after TGFβ induction. As a positive control, CT26 cells were co-transfected with a TGFβRII-dn expression construct. Note that suppression of PAI-1 reporter gene expression and inhibition of tumor formation correlates clearly in the clones analyzed – see (e). Mean values and standard deviations (error bars) are shown from at least three independent determinations.

inhibited in all TGFβRII-dn-expressing CKR clones, often to an extent obtained in CT26 cells transiently transfected with a TGFβRII-dn expression construct (Figure 2f). The complete (CKR clones 1/2) or partial (CKR clones 6/10) inhibition of TGFβ-dependent PAI-1 promoter activation correlated closely with the complete or partial inhibition of *in vivo* tumor growth in the same clones (compare Figure 2 parts e,f).

CT26 cells show a high ability to form blood-borne lung metastases. We therefore analyzed whether the development of such metastases in CT26 tumor-bearing animals would also require signaling by the TGFβ–TGFβR complex. In a first set of experiments, primary tumors grown from TGFβRII-dn-expressing CKR cells and CT26 control cells were surgically removed at a given size (2 cm^3). Thus, we avoided any influence of tumor size on metastasis probability, creating an experimental setting closely resembling the situation in tumor patients. Tumors derived from CT26 cells caused lethal lung metastases three weeks after tumor excision (6 weeks after subcutaneous injection of mice with 1×10^6 cells). All CKR cell clones — even those that did form primary tumors — completely failed to form lung metastases after tumor excision, even after observation times up to 9 months (Table 1).

Finally, we sought to determine whether TGFβ signal transduction was also required for evasion of the metastatic

cells from blood vessels at presumptive metastasis sites (extravasation). Different numbers of TGFβRII-dn-expressing CKR cells and control CT26 cells were injected intravenously into nude mice and syngeneic Balb/C mice. In control CT26 cells, this caused lethal lung metastasis in all animals within 2–4 weeks (Table 1), whereas most (95%) of the CKR clones failed to form any metastases. The remaining 5% of the CKR clones, which did form metastases with a significant delay, had all regained responsiveness to TGFβ with respect to transient PAI-1 gene transcription upon recultivation from the metastases. Thus, these cells had probably been selected for loss of TGFβRII-dn expression *in vivo*.

These experiments show that invasiveness *in vitro*, rapid tumor formation in mice (requiring local tumor cell spreading) and formation of blood-borne lung metastases require a functional TGFβ–TGFβR complex and active TGFβ-mediated signal transduction.

Cell-autonomous TGFβ signaling is required for contribution of tumor cells to tumors and metastases

In the previous experiments, cell lines stably expressing TGFβRII-dn were used that might have undergone alterations caused by prolonged cultivation, cloning and drug selection for TGFβRII-dn expression. More importantly, our experiments did not rule out the possibility that inhibition of TGFβR signaling mainly affects stroma–tumor cell interactions and interferes with the recruitment of the required tumor stroma by the injected cells. To address these issues, we co-expressed TGFβRII-dn and the green fluorescent protein (GFP) in a second mouse colon cancer cell line (C26), which metastasizes even more aggressively than CT26 cells [30]. The C26 cells were infected with a retrovirus expressing TGFβRII-dn and GFP. Control cells were infected with a retrovirus expressing GFP only. Two days after viral infection, the unselected C26 cell populations contained about 5% GFP-positive cells.

Table 1

Formation of lung metastases is dependent on TGFβRII signal transduction.

(a) Metastasis formation from subcutaneous tumors after tumor excision

	CT26	CT26 + TGFβRII-dn
Lethal lung metastasis	100% (6)	0% (18)
Survival time*/observation time† (days)	24*	266†

(b) Lung metastasis from intravenously injected tumor cells

	Number of injected cells	CT26	CT26 + TGFβRII-dn
Lethal lung metastasis		100% (12)	8.3% (24)
Delay (days)	5×10^4	14 (12)	n.a. (6)
	5×10^3	22 (18)	65, $n = 2$ (18)

(a) Metastasis formation of CT26 and CKR (CT26 cells expressing TGFβRII-dn) cells after surgical removal of primary tumor. Numbers of injected animals are given in brackets. *The maximum survival time or †observation time after excision of the primary tumor is given.
(b) Metastasis formation of intravenously injected CT26 and CKR cells. Numbers of injected animals are given in brackets; n.a., not analyzed (no animals died from metastasis); $n = 2$, 2 out of 18 animals died from metastases.

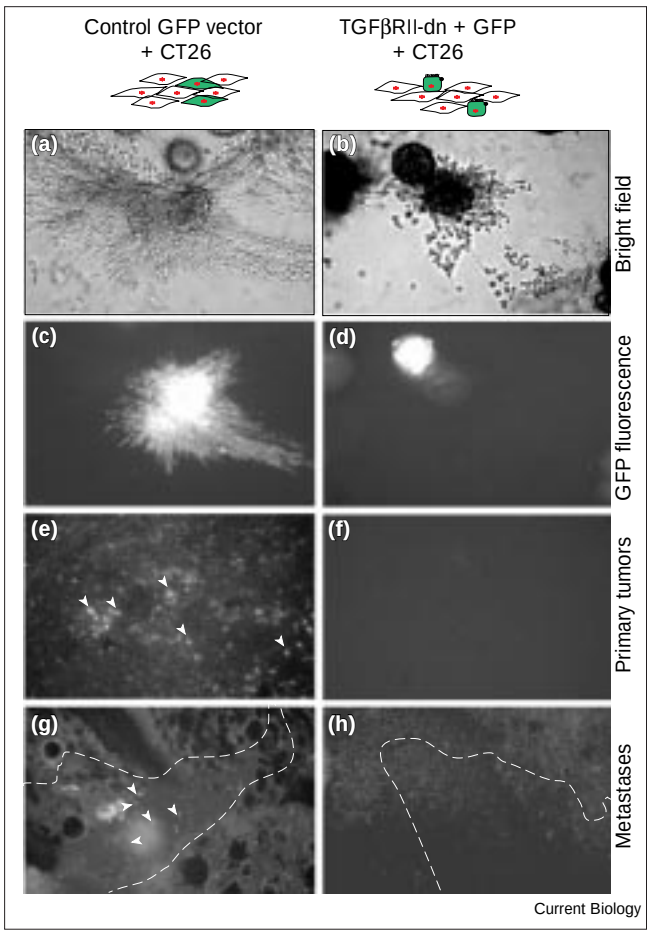
The usefulness and efficiency of GFP as a marker for TGFβRII-dn expression and prevention of TGFβ signaling was first tested *in vitro*. C26 cells infected with the GFP control vector exhibited a highly invasive, spindle-like cell phenotype in collagen gels, regardless of whether they did or did not express GFP (Figure 3a,c). In contrast, C26 cells expressing both GFP and TGFβRII-dn formed tight nodules unable to invade the collagen gel even when surrounded by invasive, uninfected C26 cells (Figure 3b,d).

This technique also allowed us to determine the fate of GFP-positive, TGFβRII-dn-expressing or control cells in an environment of unmodified tumor cells, both in subcutaneous tumors and lung metastases induced by these cell preparations. Upon subcutaneous injection into syngeneic mice, both cell preparations invariably gave rise to primary tumors and large lung metastases within 3 weeks in all animals, but only the control cells expressing GFP alone contributed significantly to primary tumors or lung metastases (Figure 3e,g, arrowheads). In contrast, C26 cells expressing both TGFβRII-dn and GFP were undetectable in both primary tumors and metastases (Figure 3f,h). Thus, interference with TGFβ-induced signal transduction confers a strong, cell-autonomous growth/migration disadvantage for C26 colon carcinoma cells *in vivo*.

Finally, we asked whether freshly infected, TGFβRII-dn-expressing or control cells were able to form blood-borne metastases after intravenous injection in the absence of any primary tumor. C26 cells infected with GFP alone or both TGFβRII-dn and GFP were sorted by fluorescence-activated cell sorting (FACS) for GFP fluorescence, and 1×10^6 cells were subsequently injected into nude mice (Figure 4). The C26 cells infected with GFP alone gave rise to heavily enlarged lungs due to innumerable metastasis nodules within 4 days after injection (Figure 4a). In contrast, no lung metastases were detected even microscopically after injection of 1×10^6 FACS-sorted C26 cells with both TGFβRII-dn and GFP, even after prolonged observation (Figure 4b).

In conclusion, maintenance of EMT, local tumor cell spreading and blood-borne transfer of metastatic cells to distant organs required sustained, cell-autonomous TGFβ signaling in two murine tumor/metastasis models. TGFβRII-dn expression strongly interfered with these

Figure 3



Cell-autonomous TGFβ signaling is required for *in vitro* invasiveness, tumor growth and metastasis formation. C26 cells were infected with retroviruses encoding **(a,c,e,g)** GFP alone or **(b,d,f,h)** GFP plus TGFβRII-dn. Cell populations containing 5–10% GFP-positive cells were then used in the various assays (in the diagrams at the top of the figure, GFP-positive cells are colored green). (a–d) Invasion properties in three-dimensional collagen type I gels, analyzed 7 days after seeding. In GFP-only C26 cell populations, both GFP-positive and GFP-negative cells show invasive growth (a,c). In the TGFβRII-dn plus GFP C26 cells, GFP-positive cells form tight clumps, whereas GFP-negative cells invade the gel (b,d). (e–h) Epifluorescence of unstained cryosections revealed the contribution of GFP-positive cells to tumors (e,f) and metastases (g,h; the border of metastases is marked by the dashed line) after subcutaneous injection of both cell populations into mice. Note the significant contribution of GFP-only C26 cells (some of the GFP-positive cells are marked by arrowheads) to tumors (e) and metastases (g). In contrast, no detectable contribution was seen of TGFβRII-dn plus GFP C26 cells to tumors (f) and metastases (h).

processes, establishing it as a potential drug target for therapeutical intervention with carcinogenesis.

TGFβ-induced signal transduction is important for late stages of human carcinogenesis

To address the issue of whether EMT is a significant event in human carcinogenesis and whether human tumors require TGFβ-dependent signal transduction for EMT and invasive cell behavior, two approaches were followed. Firstly, primary human tumor samples were histochemically stained for EMT-specific markers as well as TGFβ production. Secondly, invasive human tumor cell lines were tested for their dependence on autocrine TGFβ secretion for matrix invasion *in vitro*.

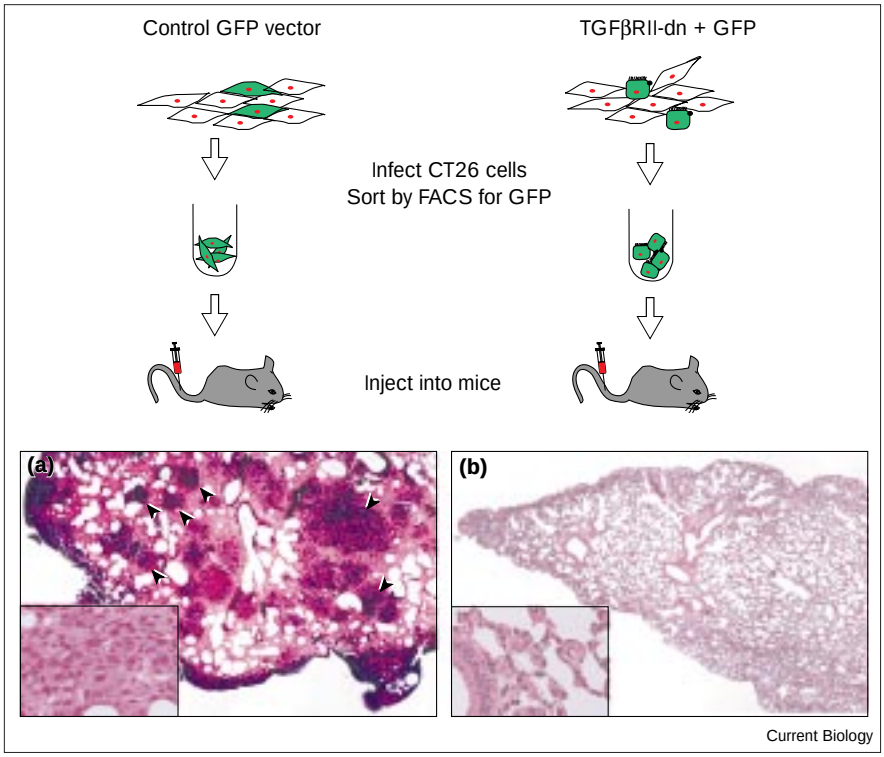
Occurrences of EMT and spindle cell carcinomas are considered to be rare events in human carcinogenesis. Studies using markers for EMT applicable to fixed tumor material (such as co-expression of the mesenchymal marker vimentin with epithelial markers such as basal cytokeratins [25]) are largely missing, however. Consecutive serial sections from 31 renal carcinomas and 65 mammary carcinomas of different histopathological types were histochemically stained for vimentin and basal cytokeratins. Co-staining of vimentin and cytokeratins in a significant fraction of the tumor cells was seen in 74% of the renal carcinomas and 25–30% of the mammary carcinomas (Table 2). A clear increase in the frequency of EMT (65%) was seen in the few (five) completely dedifferentiated mammary tumors analyzed. All mammary tumors tested (65) were positive for expression of TGFβ, both by histochemical analysis using antibodies detecting TGFβ 1, 2 and 3 and by *in situ* hybridization and reverse transcription–PCR (Table 2). In most tumors, TGFβ expression was restricted to the actual tumor cells and was low or absent in the tumor stroma (Table 2 and data not shown). These results indicate that EMT might be a more frequent event in human tumors than previously thought.

We then tried to interfere with the potential autocrine TGFβ stimulation of three unrelated human carcinoma lines that showed *in vitro* invasiveness in collagen gels. Exposure of the nasopharyngeal carcinoma KB (Figure 5a,b), the kidney carcinoma MZ-1795 (Figure 5c,d) and the mammary carcinoma T47D (data not shown) cell lines to neutralizing anti-TGFβ antibody or the soluble, extracellular domain of TGFβRII (data not shown) inhibited invasive growth in the collagen gels (Figure 5b,d). Untreated control cells, however, showed massive, invasive growth under the same conditions (Figure 5a,c).

Invasiveness is impaired in human colon tumors (hnPCC) bearing TGFβRII mutations

In certain gastric cancers and hnPCC, both alleles of TGFβRII are frequently inactivated and even rendered dominant-negative by mutation ([12], see Discussion). In addition, overexpression of wild-type TGFβR in respective tumor cell lines rendered them unable to form tumors [31]. These data, establishing the TGFβRII gene as a tumor suppressor gene, seemed at first sight incompatible with our idea that signal transduction through TGFβR was required for tumor progression. To test whether the

Figure 4



TGFβ signaling is required for metastasis in the absence of a primary tumor. C26 cell populations expressing (a) GFP alone or (b) GFP plus TGFβRII-dn were sorted by FACS for GFP expression (GFP-positive cells are colored green). Sorted cells (1×10^6) from each preparation were intravenously injected into mice. Histological sections from lungs processed 4 days after cell injection are shown. Note numerous large metastases in (a) (some nodules are indicated by arrowheads) and a histologically normal lung in (b). Insets show higher magnifications.

constitutive inactivation of TGFβRII in these cell lines resulted in a less invasive tumor phenotype, we analyzed the hnPCC-derived colon cancer cell lines DLD1 (Figure 5e) and HCT116 (data not shown) in *in vitro* matrix invasion assays. The invasive potential of both hnPCC-derived cell lines was very low and comparable to invasive human tumor cell lines after TGFβ neutralization. These low invasive properties *in vitro* were mirrored by a slow tumor growth and very inefficient metastasis formation *in vivo* (data not shown). We then restored TGFβ signaling in DLD1 cells by expressing a wild-type copy of TGFβRII driven by a Zn^{2+}-inducible metallothionine promoter. Expression of low levels of TGFβRII in the absence of Zn^{2+} was sufficient to restore TGFβ-inducible stimulation of a PAI-1 reporter construct (data not shown), proving that TGFβ-dependent signal transduction was intact under these conditions. When analyzed for their invasive properties, these DLD1 cells expressing low levels of wild-type TGFβRII showed a drastically enhanced *in vitro* invasiveness as compared to the parental cells (Figure 5f). In contrast, Zn^{2+} treatment of the same cells resulted in high-level TGFβRII expression and rendered the cells strongly susceptible to TGFβ-induced growth arrest and apoptosis (data not shown).

In conclusion, our data suggest that TGFβ produced by human tumor cells induces and maintains tumor cell invasiveness in an autocrine, cell-autonomous fashion. Human tumor cells constitutively mutated in their TGFβRs show impaired invasiveness, which is restored by re-expression of TGFβRII.

Discussion

Late-stage events in carcinogenesis require cell-autonomous TGFβ signaling

The major finding in this paper is that inhibition of TGFβ signaling by various means can revert mesenchymal, invasive tumor cells to non-invasive, sometimes even epithelial, cells. Expression of TGFβRII-dn abolishes tumor cell growth and invasiveness *in vitro* and *in vivo* even when the modified cells are surrounded by unmodified, actively growing and metastasizing tumor cells. This suggests that the contribution of TGFβ signaling to invasion is a cell-autonomous process, the simplest mechanism being an autocrine loop involving TGFβ and TGFβR.

Results from ourselves and others suggest a role for TGFβ signaling in the progression of tumors to late stages. Particularly good examples are keratinocyte tumors and prostrate cancers. In Ras-induced murine skin cancer, expression of TGFβ in transgenic mice under the control of a keratin promoter inhibits formation of benign papillomas, but increases progression of the residual benign tumors to spindle cell carcinomas, which also upregulate TGFβ-3 expression [6]. Also, expression of TGFβRII-dn in a squamous carcinoma cell

Table 2

Expression of EMT markers and TGFβ in human tumors.

(a) Co-expression of vimentin and basal cytokeratins

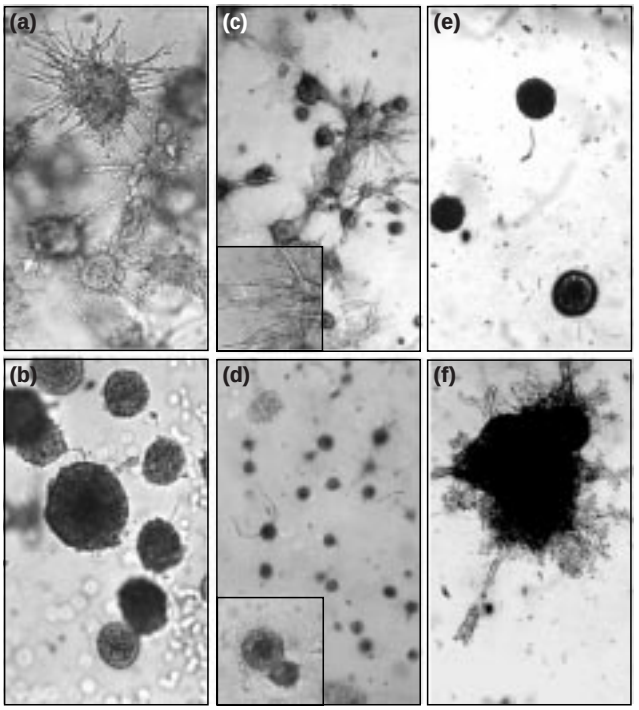
Tumor type	Number of tumors analyzed	Number of tumors co-expressing vimentin/cytokeratin*
Renal cell carcinomas	31	23/31 (74%)
Mammary tumors	65	19/65 (29%)
Fibroadenoma	3	2/3 (66%)
Invasive ductal carcinoma	34	8/34 (24%)
Invasive lobular carcinoma	26	7/26 (27%)
Invasive ductal–lobular carcinoma	1	1/1
Unknown	1	1/1

(b) Expression of TGFβ 1/2

Tumor type	Antibody staining†	*In situ* hybridization‡
Mammary tumors		
Fibroadenoma	4/4 (100%)	
Invasive ductal carcinoma	33/33 (100%)	13/13 (100%)
Invasive lobular carcinoma	23/23 (100%)	9/9 (100%)
Invasive ductal–lobular carcinoma	1/1 (100%)	

*Histochemical staining of tumor sections with vimentin and cytokeratin antibodies [25] using consecutive sections. Colocalization of cytokeratin and vimentin was verified by double immunofluorescence staining of the same sections with vimentin and cytokeratin antibodies. †Histochemical staining with anti-TGFβ1 and anti-TGFβ2 antibodies (Santa Cruz); confirmed with double immunofluorescence using cytokeratin and TGFβ antibodies. ‡*In situ* hybridization was performed as described in [25], using a probe (pmTGFβ) detecting TGFβ 1, 2 and 3. Adjacent serial sections stained by immunohistochemistry confirmed the result in all cases. Two additional analyses were performed. First, reverse transcription–PCR on invasive ductal and invasive lobular carcinomas using primers specific for human TGFβ1: 11 of the cases that were positive with antibody also yielded the expected reaction product. Second, expression of TGFβ in tumor stroma: the stroma was weakly stained by antibody in 10/22 cases where tumor cells stained clearly positive. The stroma was negative in the remaining 12 cases. From a similar analysis by *in situ* hybridization: 3/22 cases showed weak staining in the stroma.

Figure 5



TGFβ-dependent invasive properties *in vitro* of human epithelial tumor cell lines containing or lacking an intact TGFβRII. **(a–d)** Invasive human carcinoma cells rendered non-invasive by interference with TGFβ signaling. Cells from the nasopharyngeal carcinoma line KB (a,b) and the kidney carcinoma line MZ-1795 (c,d) were cultivated in serum-containing collagen gels in the presence of high levels of control antisera (a,c) or of TGFβ-neutralizing antibody [25] (b,d). Note inhibition of invasive growth by neutralizing anti-TGFβ antibody. Similar results were obtained with the T47D mammary carcinoma cell line and employing a soluble extracellular fragment of TGFβRII (data not shown). **(e,f)** Human colon carcinoma cells mutated in the TGFβRII gene are non-invasive *in vitro*, a defect restored by exogenous wild-type TGFβRII. When seeded into collagen gels under identical conditions to those in (a,c), cells from the hnPCC colon cancer cell lines DLD1 (e) or HCT116 (data not shown) failed to invade the collagen gel. DLD1 cells expressing an exogenous wild-type TGFβRII at low levels regained the ability to invade the collagen gel (f).

line prevented the spontaneous progression of these cells to a spindle cell phenotype [7]. In a murine prostate cancer model and in human prostate tumors, early benign tumors expressed TGFβ mainly in the tumor stroma and extracellular space around the epithelial tumor cells. In contrast, late-stage carcinoma cells produced TGFβ intracellularly [32,33]. In addition, cell lines from primary tumors retained TGFβ-dependent growth inhibition, whereas respective lines from progressed, metastatic tumors had lost this property [34].

Intracellular TGFβ expression was also seen in our own studies using renal cancers and late-stage, invasive breast cancers (Table 2). Many of these tumors also co-expressed vimentin and cytokeratin, suggesting that they had undergone EMT (Table 2). Thus, EMT, as diagnosed by vimentin/cytokeratin co-expression, seems to be a rather frequent event in human carcinomas. A possible reason that this was not described earlier is that histological criteria do not discriminate between mesenchymal tumor stroma and epithelial cells after EMT. Thus, carcinoma cells after EMT may often have been misdiagnosed as tumor stroma.

Tumor suppressor mutations in components of the TGFβ signaling pathway are rare events in human tumors

Currently, TGFβ itself, its receptors and the TGFβR-activated signal transducers Smad2 and Smad4/DPC4 are mainly thought to function as tumor suppressors [12,13,21].

Heterozygous deletion of TGFβ1 in mice leads to reduction of *in vivo* TGFβ levels and enhanced susceptibility to chemical carcinogenesis [21]. The second copy of TGFβ1 is not lost during tumor progression [21], however, suggesting that TGFβ may also have positive functions (such as stimulation of invasiveness) in these tumors.

TGFβRII is inactivated in certain gastric cancers and in hnPCC occurring in families with genetic defects in DNA repair (replication error, RER) [12]. In sporadic colon carcinomas [35] or even in lung cancers observed in patients with RER [36], no mutations in TGFβRII are observed. Thus, the function of TGFβR as a tumor suppressor is restricted to very specific tumor types [12,36]. As the RER defect pre-exists in the hnPCC patients and is responsible for the TGFβRII mutations, loss of TGFβRII function may be an early event in hnPCC. This would prevent selection for mutations specifically inactivating cell-cycle effectors downstream of TGFβ signaling which would allow TGFβ signaling to remain intact in the majority of invasive carcinoma cells.

Likewise, loss or mutation of Smad4/DPC4 mainly occurs in pancreatic cancer, but is infrequent or not found at all in other tumor types [11,13,37–42]. This implies that loss-of-function mutations in the TGFβR signaling pathway, rendering the tumor cell insensitive to TGFβ-induced growth arrest, may sometimes occur as an early event in specific tumor types, but does not argue against the concept proposed here, that late events in carcinogenesis are dependent on a functional TGFβ signal transduction pathway.

Conclusions

In most human tumors, cells first become resistant to TGFβ-mediated cell-cycle inhibition/apoptosis and later undergo progression towards invasiveness and metastasis [1]. In this paper, we have shown that signal transduction by TGFβ receptors is required for induction and maintenance of EMT and *in vitro* invasiveness in several unrelated human and murine epithelial tumor cell lines. Cell-autonomous TGFβ signaling is similarly required for induction and maintenance of rapid tumor growth and metastasis in mice. Interference with TGFβ signal transduction reverts highly metastatic, mesenchymal tumor cells to non-invasive ones and reverts EMT, inducing formation of epithelial cells from tumor cells with mesenchymal properties.

Our data raise the concept that cell-autonomous signaling via TGFβ, typically through an autocrine loop, is required for tumor cells to both invade locally and metastasize to distant organs. TGFβ-induced EMT, a process that reflects the phenotypic and developmental plasticity of epithelial cells, occurs during normal palate development [15–18,25,43] and constitutes an essential mechanism required for tumor cell invasion and metastasis *in vivo*. This suggests that TGFβRII could be a potentially interesting drug target for therapeutic intervention in late-stage carcinogenesis.

Materials and methods

Cell culture and retroviral constructs

Cells were cultured as previously described [25]. Briefly, all cell types were propagated on plastic dishes in DMEM containing 10–20% fetal bovine serum (FCS). Collagen gel cultures were performed [25] using a serum-free medium adapted to mammary cell culture (MEGM, Promocell) supplemented with FCS up to 10%, with TGFβ-neutralizing antibodies (mix of Genzyme and R&D antibodies [25]) or with TGFβRII soluble extracellular fragments (Sigma). To express TGFβRII-dn in cells, a TGFβRII-K277R cDNA [28] was cloned into the retroviral vector pBabe-Puro [44] or a corresponding vector containing green fluorescent protein (EGFP, Clontech) as a replacement for the puromycin selection cassette. Cells of the Bosc23 packaging cell line [45] were transiently transfected with the respective constructs and the target cells infected with cell-free supernatants from these cells.

Analysis of TGFβ responses

For PAI-1 promoter assays, cells were subjected to lipofectamine-mediated (Gibco) transfection with a construct expressing luciferase under the control of a triplicated TGFβ-responsive element derived from the PAI-1 promoter (3TPlux [28]). The experimental data were normalized for transfection efficiency to protein content and the activity of a co-transfected CMV–β-galactosidase control reporter construct. The chicken heart fragment invasion assays were performed as described previously [25].

Tumor inductions, tumor removal and metastasis scoring

1×10^6 of the various tumor cells were subcutaneously injected into nude mice or immune-competent syngeneic Balb/C mice. Tumors were surgically removed at a size of 2 cm^3 and only animals that were free of any local recurrence were further analyzed for the induction of metastasis. For quantification and characterization of tumors and metastases, see [25] and figure legends. All *in vivo* experiments were performed using at least three animals per cell type analyzed. Mean values of the data obtained are depicted.

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