TGF-β1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells

Martin Oft,[1] Janos Peli,[2] Claude Rudaz,[2] Heinz Schwarz,[3] Hartmut Beug,[1] and Ernst Reichmann[2,4]

[1]Forschungsinstitut für Molekulare Pathologie, A-1030 Wien, Austria; [2]Institut Suisse de Recherches Experimentales sur le Cancer, CH-1066 Epalinges, Switzerland; [3]Max Planck Institut für Entwicklungsbiologie, 7400 Tübingen, Germany

Metastasis of epithelial tumor cells can be associated with the acquisition of fibroblastoid features and the ability to invade stroma and blood vessels. Using matched in vivo and in vitro culture systems employing fully polarized, mammary epithelial cells, we report here that TGF-β1 brings about these changes in Ras-transformed cells but not in normal cells. When grown in collagen gels in the absence of TGF-β, both normal and Ras-transformed mammary epithelial cells form organ-like structures in which the cells maintain their epithelial characteristics. Under these conditions, treatment of normal cells with TGF-β results in growth arrest. The same treatment renders Ras-transformed epithelial cells fibroblastoid, invasive, and resistant to growth inhibition by TGF-β. After this epithelial–fibroblastoid conversion, the Ras-transformed cells start to secrete TGF-β themselves, leading to autocrine maintenance of the invasive phenotype and recruitment of additional cells to become fibroblastoid and invasive. More important, this cooperation of activated Ha-Ras with TGF-β1 is operative during in vivo tumorigenesis and, as in wound healing processes, is dependent on epithelial–stromal interactions.

[*Key Words*: Mammary epithelial cells; Ras; TGF-β; autocrine loop; phenotypic plasticity; cell invasion]

Received June 13, 1996; revised version accepted July 30, 1996.

More than 80% of the tumors occurring in man are epithelial in origin. The generation of epithelial tumors (carcinomas) is a multistep process, as best exemplified in human colon cancer (Powell et al. 1993) and mouse skin tumor progression (Wright et al. 1994). Carcinomas are thought to originate from small foci of aberrant cells that develop into areas of benign epithelial hyper- or dysplasia. The progression of these hyperplastic areas to a carcinoma in situ, which may then acquire invasive and metastatic properties, requires a number of further changes in the tumor cells. Characteristically, these cells achieve the ability to proteolytically degrade their basement membrane, convert from a sessile to a migratory phenotype, survive in the blood stream, and form metastases at distant sites (Liotta et al. 1991; Liotta and Stetler-Stevenson 1991).

Although profound changes in gene expression are involved in the multiple alterations in cell architecture and behavior of malignant transformed cells, none of these newly acquired properties is unique to invasive tumor cells alone. Attachment to and proteolysis of basement membranes as well as the conversion from a sessile to a migratory phenotype also are important steps during normal processes such as trophoblast implantation, embryonic morphogenesis, mammary gland development, and tissue repair (Aznavoorian et al. 1993). The understanding of how these normal processes are deregulated in cell invasion and metastasis is crucial for a better understanding of cancer development and progression.

Recent work has unraveled molecular mechanisms involved in the modulation of the epithelial phenotype under normal and pathological situations (Reichmann et al. 1992; Birchmeier et al. 1993; Frisch and Francis 1994; Hartmann et al. 1994; Soriano et al. 1995). Only recently, transforming growth factor-β1 (TGF-β1) has been identified as another potent modulator of the phenotype of epithelial cells (Miettinen et al. 1994; Caulin et al. 1995; Zambruno et al. 1995). The TGF-β superfamily includes the various TGF-β genes as well as embryonic morphogens such as the activin family, Müllerian inhibitory substance, and the bone morphogenic protein (bmp) family that play crucial roles in regulating both embryonic development and tissue repair (Roberts and Sporn 1992). The TGF-β family itself consists of three highly homologous genes, TGF-β1, TGF-β2, and TGF-β3. TGF-βs inhibit the growth of many cell types, including epithelial cells, yet stimulate the proliferation of various mesenchymal cell types.

Interestingly, TGF-β has been found to be abundantly expressed in many epithelial tumors since its discovery, frequently acting in an autocrine fashion (Derynck et al.

[4]Corresponding author.

1985; Keski-Oja et al. 1987). TGF-βs inhibit the growth of normal epithelial cells and relatively differentiated carcinoma cells, whereas dedifferentiated tumor cells, lacking many epithelial characteristics, are usually resistant to growth inhibition by TGF-βs (Houssein et al. 1989; Murthy et al. 1989; Fynan and Reiss 1993). Furthermore, TGF-β1 can increase the invasive and metastatic potential of a mammary adenoma cell line (Welch et al. 1990), supporting a role for TGF-β1 in tumor progression. Finally, long-term treatment with TGF-β1 has allowed the selection of rare variants derived from epithelial tumor cell that exhibit a fibroblastoid phenotype (Caulin et al. 1995). Despite these observations, a satisfactory, mechanistic explanation for these effects of TGF-β in tumor cells is lacking.

Many human tumors harbor activated *ras* oncogenes as one of the multiple changes required for tumorigenesis (Bos 1989; Fearon and Vogelstein 1990). In human breast cancer, ras mutations are relatively rare (Kraus et al. 1984), but overexpression of nonmutated cellular Ras (Hand et al. 1984; Slamon et al. 1984; De Bortoli et al. 1985) and/or activation of upstream receptors hyperactivating signal transduction pathways, (for example, receptor tyrosine kinases of the EGF receptor family (De Bortoli et al. 1985; Kern et al. 1990; LeJeune et al. 1993), are frequent events (for review, see Clark and Der 1995). In addition, Ras is a key player in several cellular or animal models of carcinogenesis, for example, murine keratinocytes transformed by Ha-Ras (Buchmann et al. 1991; Caulin et al. 1995)

Although the high abundance of TGF-βs in tumor tissues, together with their known morphogenetic effects on epithelial cells, suggests a key role of these factors during tumor progression and metastasis, specific evidence for this notion is lacking. A potential clue is provided by the emerging concept that the events in a growing tumor closely resemble those in a wound that fails to heal (Dvorak 1986). TGF-βs play a pivotal role in wound healing and tissue repair (for review, see Border and Ruoslahti 1992), and it may be the deregulation of these TGF-β responses that significantly contributes to their progression toward more malignant stages.

In this paper we demonstrate that the Ha-Ras oncoprotein cooperates with endogenous TGF-β1 to cause an epithelial–fibroblastoid conversion (EFC) of mammary epithelial cells, both in vitro and upon tumor formation in mice. When cultured in type I collagen matrices in the complete absence of TGF-β, both nontumorigenic mammary epithelial cells (EpH4 cells) and their Ha-Ras-transformed counterparts (EpRas cells) develop into lumen-forming structures consisting of monolayers of polarized epithelial cells. In the presence of TGF-β1, however, EpRas cells form lumenless cords that consist of cells exhibiting fibroblastoid properties. In contrast, their normal counterparts are not affected in this fashion by TGF-β. The converted cells are highly invasive in collagen matrices, as well as in the chicken heart invasion assay. Once converted, the fibroblastoid cells themselves produce high levels of TGF-β1, but they revert to an epithelial phenotype upon exposure to a neutralizing antibody to TGF-β1. This indicates that the converted fibroblastoid phenotype is maintained by TGF-β1 that acts in an autocrine loop. Most important, we provide in vivo evidence that TGF-β1 both induces and maintains the invasive phenotype of Ha-Ras-transformed mammary epithelial cells in experimentally induced tumors.

Results

Ras-expressing polarized epithelial cells undergo EFC during tumor development

The work described here was prompted by our initial observation that Ras-transformed mouse mammary epithelial cells (EpRas cells) can display two widely different cell phenotypes. When grown on plastic substrates, these cells grow as ordered monolayers forming domes (hemicysts), indicative of a polarized epithelial phenotype (Fig. 1A,B). After injection into mice, however, these same polarized cells formed tumors consisting of depolarized, spindle shaped cells capable of invasive growth (Fig. 1A,C). To gain further insights into the mechanisms underlying this phenotypic plasticity, we studied the observed cell conversion in more detail, combining in vivo and in vitro experimental approaches. We used the cell clone EpH4, derived from a well characterized mouse mammary epithelial cell line (Reichmann et al. 1989, 1992; Strange et al. 1991). These cells exhibit a stable, polarized epithelial phenotype (Reichmann 1994; Fialka et al. 1996).

Tumorigenic subclones of EpH4 were generated by stable expression of the v-Ha-Ras oncogene using suitable retroviral vectors. After verification of v-Ha-Ras expression by Western blot analysis (data not shown), cells from seven clones (referred to as EpRas clones) were injected subcutaneously or directly into the mammary glands of BALB/c mice. Tumors formed regularly and were palpable 5–7 days after cell injection.

We compared the phenotype of these converted tumor cells with that of the original, differentiated clones. Before injection, all seven EpRas clones tested showed the expected polarized phenotype (Fig. 1B; Table 1). In contrast, when excised from tumors and recultured in the presence of G418, only converted, fibroblastoid cells were obtained (Fig. 1C; Table 1). Although fully retaining the expression of cytokeratins, these cells had lost many of their epithelial characteristics and gained the expression of various fibroblastoid markers (Fig. 1C; Table 1). To demonstrate the donor origin of the tumor cells and to show that no rearrangement or reintegration of the Ras-containing retrovirus had occurred during tumorigenesis and subsequent in vitro culture, we determined the integration pattern of the retroviral construct by Southern blot analysis. For this we analyzed pretumor cells, cells derived from a 15-day tumor and cells reisolated from a 30-day tumor. Identical integration patterns were obtained in all three cell types, using probes specific for the neomycin resistance gene or the ras gene (Fig. 1D). As expected, the pattern of viral integration sites was clone specific, whereas control cells showed no virus-specific signals (data not shown).

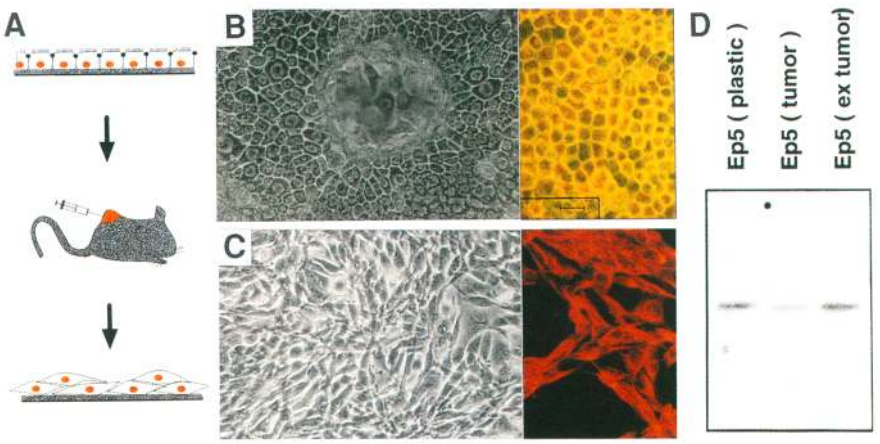

Figure 1. EpRas cells convert into fibroblastoid cells during tumor formation in mice. (*A*) Scheme illustrating the basic strategy used to study EFC of EpRas cells (7 different v-Ha-Ras-expressing cell clones) in vivo. (*B*) Before injection, clone Ep5 cells show dome formation on plastic and stain for both E-cadherin (FITC, green fluorescence) and cytokeratins (Texas Red). Note costaining of both proteins at the cell periphery (yellow staining). (*C*) Ep5 cells isolated from a tumor 28 days after cell injection. These cells show a fibroblastoid appearance and express cytokeratins but no E-cadherin (green fluorescence). (*D*) Southern blot analysis. One EpRas clone (Ep5) before injection (Ep5, plastic), recovered from the tumor (Ep5, tumor), and recovered from the tumor and recultivated in G418 for 5 days (Ep5, ex-tumor) shows the same retroviral integration pattern (detected with an NPT probe).

Sequence of events during EFC in vivo

We then examined if and at which stage of tumor development the subcutaneously injected epithelial EpRas cells underwent EFC. Three days after injection, EpRas cells formed smooth nodules in which the cells expressed characteristic cytokeratins but no vimentin (Fig. 2A). These epithelial cell nodules were already encapsulated by stromal cells (Fig. 2A). Cells grown out from these microtumors on plastic and in the presence of G418 still exhibited epithelial properties. Seven days after injection, cell scattering was observed at the tumor periphery (Fig. 2B). In this area the tumor cells intermixed with vimentin-positive stromal cells. However, the donor cells still expressed epithelial characteristics both in the tumor (Fig. 2B) and upon in vitro cultivation in G418 (data not shown).

Fifteen days after injection, three different cell types could be distinguished. About 20% of the tumor cells represented vimentin-positive stromal cells (green cells in Fig. 2C). Another 20% expressed cytokeratins only, indicating EpRas cells retaining an epithelial phenotype

Table 1. *Expression of epithelial and mesenchymal markers in polarized and fibroblastoid Ras-transformed mammary epithelial cells*

	EpRas (polarized)	EpRas (ex-tumor) (fibroblastoid)	EpRas (ex-tumor) (repolarized)	EpRas +TGFβ (fibroblastoid)
Epithelial markers				
Transepithelial Resistance[a]	+ +	–	+ + +	–
Lumen formation[b]	+ + +	–	+ + +	–
Cytokeratin[c]	+ + +	+	+ + +	+
E-cadherin[c]	+ + +	–	+ + +	–
ZO-1[c]	+ +	–	+ +	–
Dpp IV[c]	apical	random	apical	n.d.
Mesenchymal markers				
Fibronectin[c]	+/–	+ + +	+/–	+ + +
Vimentin[c]	+	+ + +	+	+ + +
Collagen I[c]	–	+	–	n.d.
TGF β production[d]	+	+ + +	n.d.	+ + +

For EpRas- and EpRas ex tumor cells, 3–7 different cell clones were investigated. The parameters analyzed did not vary significantly for these clones. Two or three independent determinations have been performed. EpRas (ex-tumor) cells were grown under Neo (Geneticin) selection for > 5 days and then cultivated for a further 9 days before analysis. Repolarized EpRas ex-tumor cells were analyzed either as a pool of clones after reversion to an epithelial morphology (Fig. 6D) or after derivation of mass cultures from such pools. Fibroblastoid EpRas cells were analyzed after retrieving these from collagen gels in which they were converted by TGF-β1.

[a] (+ + +) >2500 Ω × cm; (+ +) >1500 Ω × cm; (–) <200 Ω × cm.

[b] (+ + +) >95% of structures acquired lumina; (–) <5% of structures acquired lumina.

[c] (+ + +) >98% of cells strongly fluorescence positive; (+ +) >90% distinctly positive; (+) >90% weakly positive; (–) no specific staining; (n.d.) not determined.

[d] (+ + +) and (+) relative intensity of bands as judged on two different immunoblots (see also Fig. 7B).

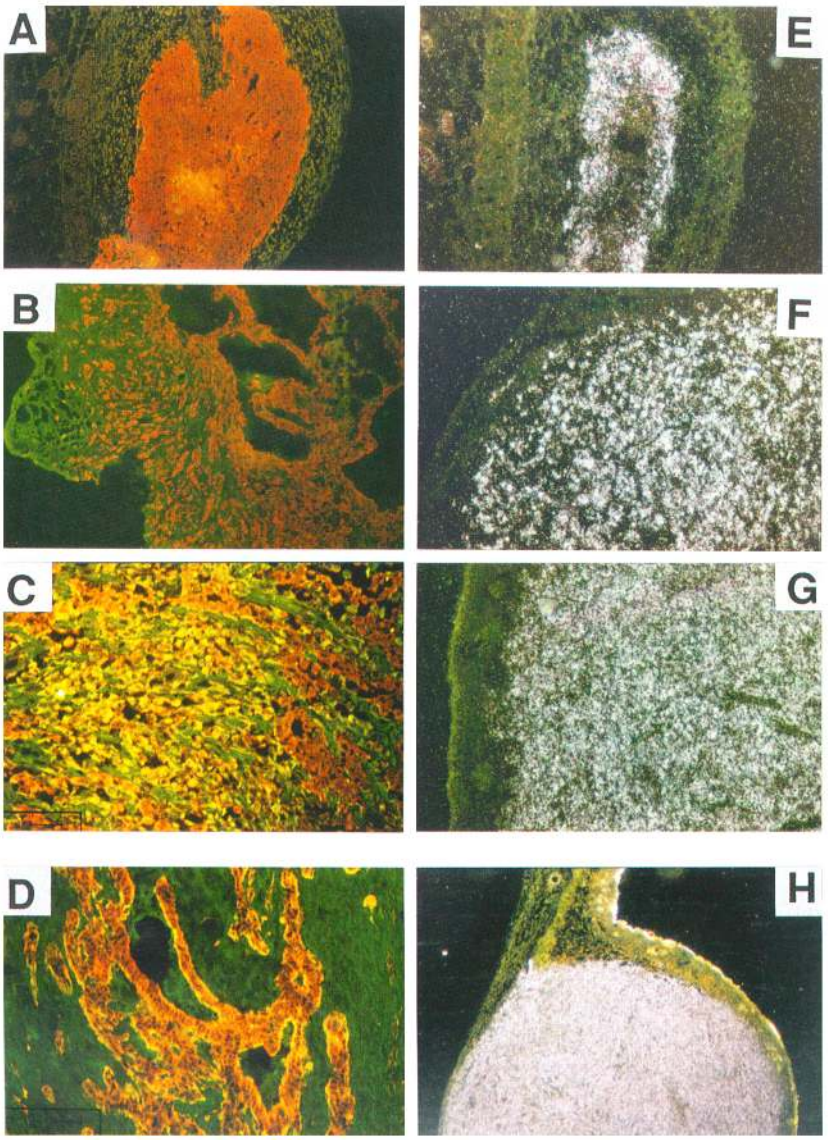

Figure 2. EFC during tumor development: time course and cell fate. Cryosections of EpRas tumors (clone Ep2) at day 3 (*A,E*), day 7 (*B,F*), day 15 (*C,G*), and day 28 (*H*) after injection. Cell structures formed by nontumorigenic EpH4 cells 15 days after injection (*D*). Sections were analyzed by immunofluorescence (*A–D*) and in situ hybridization (*E–H*). Sections were double-stained with antibodies to a 46-kD cytokeratin (Texas Red, red fluorescence) and vimentin (FITC, green fluorescence). Note that in 3-day tumors, injected epithelial cells (red) and host mesenchymal cells (green) are clearly separated. In 15-day tumors, high numbers of cytokeratin/vimentin double positive cells become visible. These cells have undergone EFC (yellow cells). RNA in situ hybridization employing an NPT probe confirms the donor origin of the tumor cells and shows the increasing density of tumor cells after EFC (*E–H*).

(red cells in Fig. 2C). The majority (50–60%) of the tumor mass, however, consisted of cells coexpressing cytokeratin and vimentin. These cells most likely represent converted or converting EpRas cells (yellow cells in Fig. 2C). Both the epithelial and the converted fibroblastoid cells were also obtained after G418 selection. Finally, in 5-week-old, fully developed tumors, the epithelial component could no longer be detected, neither in situ nor on plastic (data not shown). In contrast, parental EpH4 cells never formed tumors. When injected subcutaneously, EpH4 developed into sheets of epithelial cells that sometimes formed lumina and expressed cytokeratins 8 and 18 but no vimentin (Fig. 2D). These cells eventually disappear.

To identify donor cells in the three different tumor stages, we performed in situ hybridization using a probe specific to the neomycin phosphotransferase (NPT). These experiments revealed that all cytokeratin-expressing cells were of donor origin. Donor cell density increased with tumor size and was highest in large, fully progressed tumors (Fig. 2E—H).

Taken together, these data show that both Ras-expressing and control epithelial cells initially maintain an epithelial phenotype. During tumor progression, the Ras-transformed cells acquire fibroblastoid properties, whereas the nontumorigenic, parental cells stably maintain their epithelial characteristics.

TGF-β1 induces EFC in Ras-expressing but not in normal epithelial cells in vitro

To identify the mechanism underlying the EFC observed, we sought to use an experimental system to induce EFC in vitro under defined, and physiologically relevant conditions. For this, we grew normal EpH4 and their Ras-transformed counterparts in reconstituted collagen type I matrices in serum-free media. These condi-

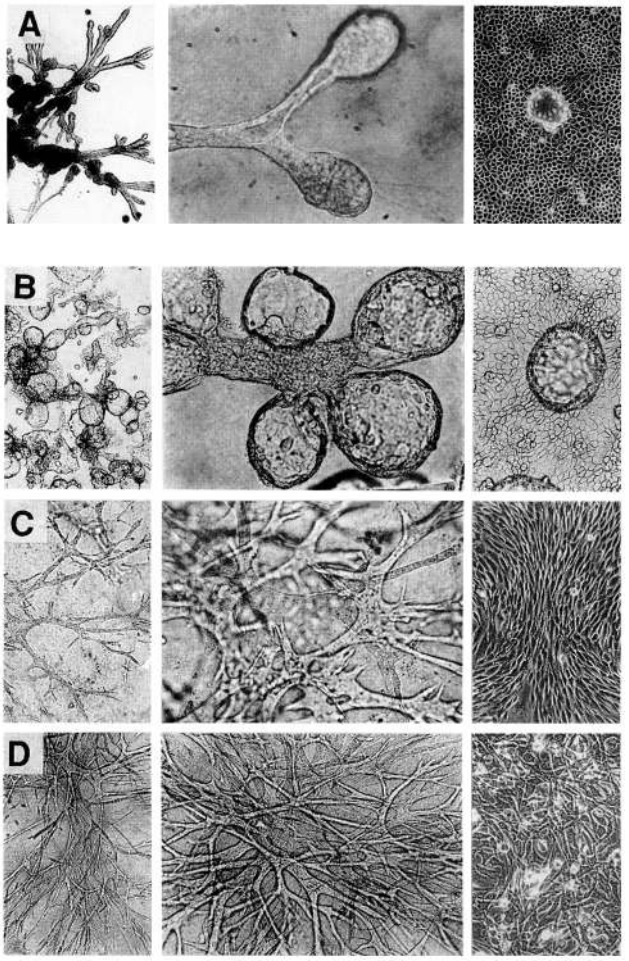

Figure 3. Lumen formation and epithelial polarity are disrupted by serum and TGF-β1. Nontumorigenic EpH4 cells (*A*) or tumorigenic EpRas cells (clone Ep5, *B–D*) were grown within collagen type I matrices. Macroscopic structures were photographed 8 days after plating at low and high magnifications (*left* and *middle*). Cells recovered from gels and cultured on tissue-culture plastic (*right*). (*A*) EpH4 cells form ducts and alveoli-like structures in serum-free collagen gels and a regular epithelial monolayer hemicysts on plastic. (*B*) Large ducts and cysts are formed by EpRas cells in serum-free collagen gels. (*C*) Addition of 10% FCS causes the cells to form invasively growing irregular cords lacking a lumen. On plastic these cells are spindle shaped. (*D*) TGF-β1 alone (5 ng/ml) causes EpRas cells to grow into invasive cords similar to those induced by FCS.

tions allowed us to use defined polypeptide growth factors and hormones known to be involved in the modulation of the epithelial phenotype. In such gels, normal EpH4 cells developed into organ-like ducts and terminal buds, similar to those formed by primary mammary epithelial cells (Fig. 3A). These structures could be efficiently induced to produce milk proteins upon addition of lactogenic hormones (data not shown). The same cells developed into the expected, regular dome-forming monolayers when isolated from the gel and grown on tissue culture plastic (Fig. 3A, right panel).

Surprisingly, the EpRas clones showed extensive lumen formation in these serum-free collagen gels. Lumina were already visible 2–3 days after seeding. Subsequently, >95% of these structures acquired relatively large cystic cavities (Fig. 3B, left and middle panel). On plastic these cells exhibited all the epithelial properties of their nontumorigenic counterparts (Fig. 3B, right panel).

However, EpRas cells behaved completely different when exposed to 10% fetal calf serum (FCS). Under these conditions, they formed elongated, invasively growing cords that never showed lumen formation. These cords were composed of nonpolarized cells that had lost many epithelial properties (Figs. 3C and 4) and behaved remarkably similar to the ex-vivo fibroblastoid tumor cells. These findings suggested that a factor (or factors) present in FCS synergized with the activated Ha-Ras oncoprotein.

To identify the factors involved, a number of growth factors (TGF-α, Heregulin, Scatter factor, acidic and basic FGF, PDGF, and TGF-β1) were added to Ras-transformed cells grown in collagen gels. Surprisingly, TGF-β1 was the only factor inducing striking and long lasting effects on EpRas cells. Upon TGF-β1 administration, these cells grew into elongated branching cords similar to those induced by FCS. On tissue culture plastic, these cells exhibited a clear fibroblastoid phenotype (Fig. 3D, right panel). In contrast, TGF-β1 did not induce EFC in EpH4 cells and other nontumorigenic mammary epithelial cell clones (data not shown). To demonstrate that the activity in serum promoting EFC was indeed TGF-β1, we added TGF-β1-neutralizing antibodies to cultures containing 5% FCS. Under these conditions, EpRas cells again formed cystic cavities closely resembling those shown in Figure 3B. These results clearly identify TGF-β1 as the cell-converting activity present in FCS.

We also determined the state of epithelial polarity in the structures formed by EpRas and EpH4 cells in collagen gels in response to TGF-β1. Ultrastructural and immunohistochemical analyses revealed that the tubular and cystic structures formed by EpH4 and EpRas cells in absence of TGF-β1 (Fig. 3A,B) consisted of a monolayer of polarized cells (Fig. 4A). The EpRas monolayers expressed abundant microvilli at their apical (lumenal) domain indicating a polarized organization (Fig. 4A). In addition, tight junctions, desmosomes (Fig. 4A), and the cell adhesion molecule E-cadherin (Fig. 4B) could be demonstrated at their respective locations. Similarly, the E-cadherin-associated protein β-catenin showed basolateral localization in most cells (Fig. 4C). Similar results were obtained with the EpH4 structures (data not shown).

In contrast, the cord-like structures induced in EpRas cells by TGF-β1 consisted of well-proliferating spindle shaped cells, which were only loosely attached to each other (Fig. 4D). None of the aforementioned epithelial characteristics were detected (Fig. 4D; Table 1), except a rudimentary and nonpolarized expression of E-cadherin (Fig. 4E). β-Catenin expression was greatly reduced and

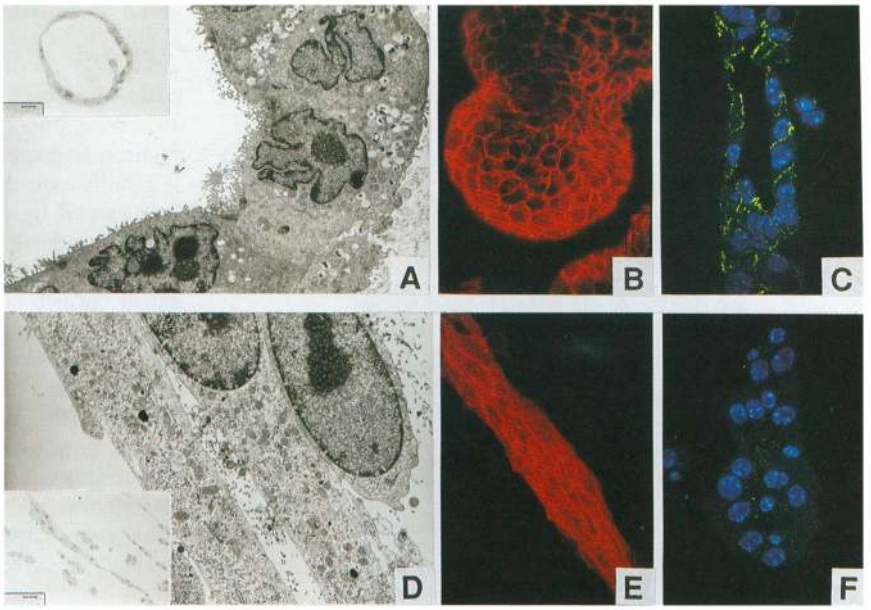

Figure 4. TGF-β1 disrupts cell polarity in Ras-transformed mammary epithelial cells. Cysts formed by EpRas cells (clone Ep6) in serum free collagen gels (*A–C*) and cords formed by the same cells under TGF-β1-treatment (*D–F*) were analyzed with respect to their epithelial organization. Sections through individual structures were photographed at high or low (*insets*) magnifications. (*A*) Transmission electron microscopy revealed that the cysts obtained in the absence of TGF-β1 consist of a monolayer of morphologically polarized cells, exhibiting apical microvilli exclusively toward the lumen. (*D*) In contrast, cords induced in the presence of TGF-β1 consist of loosely attached cells lacking microvilli, desmosomes, and tight junctions. (*B,E*) A cyst immunostained for the cell adhesion molecule E-cadherin shows lateral staining on individual cells (*B*). In cords, E-cadherin is reduced in expression and randomly distributed on the surface of fibroblastoid cells (*E*). (*C,F*) Lowicryl sections through structures similar to those shown in *B* and *E* stained by an anti-β-catenin antibody. Note the basolateral expression of β-catenin in most cells of the cyst (*C*) and the markedly reduced β-catenin expression that is now predominantly expressed in the cytoplasm (*F*).

mainly localized to the cytoplasm (Fig. 4F). In addition, these cells expressed the expected mesenchymal markers (Table 1). Interestingly, EpH4 cells retained their polar characteristics when treated with low concentrations of TGF-β1, whereas they underwent growth arrest and apoptosis at concentrations that efficiently caused EpRas cells to convert into a fibroblastoid phenotype (data not shown; M. Oft, and H. Beug, unpubl.).

These results indicate that Ras-transformed mouse mammary epithelial cells exhibit an extraordinary plasticity in phenotype ranging from polarized and sessile to fibroblastoid and migratory. TGF-β largely determines this phenotype. In nontumorigenic epithelial cells, such a dramatic TGF-β1-induced phenotypic plasticity is not observed.

Fibroblastoid EpRas cells are invasive

EpRas cells that had undergone EFC showed indications of invasive behavior. To gain conclusive evidence for this, we took advantage of the embryonic chicken heart invasion assay, the relevance of which for in vivo metastasis has been documented extensively (Mareel et al. 1979; Mareel 1983). In this assay, the penetration of cells into embryonic chicken heart fragments (Fig. 5A) was analyzed. To clearly identify the invading cells, these were labeled with a fluorescent dye (carboxy dichlorofluorescein diacetate). Parental EpH4 cells did not penetrate into the chicken heart tissue within the incubation period of 7 days (Fig. 5A,B). When three different, fully polarized EpRas clones were tested, only a few cells if any penetrated the heart tissue (Fig. 5C). The few invading cells stained strongly for vimentin but no longer for E-cadherin, confirming their conversion to a fibroblastoid phenotype (data not shown). Finally, the fibroblastoid cells recovered from tumors (ex-Tu cells), or cells induced to undergo EFC in vitro by application of TGF-β1, readily invaded the heart muscle tissue in large numbers (Fig. 5D). These results indicate that EpRas cells are highly invasive after induction of EFC in vivo or in vitro. In contrast, the nonconverted, epithelial EpRas cells show little invasiveness.

TGF-β1 maintains the fibroblastoid phenotype of converted EpRas cells via an autocrine loop

Having demonstrated that TGF-β1 induces EFC in Ras-transformed mouse mammary epithelial cells, the question arose as to whether TGF-β1 was also required for the maintenance of the converted, fibroblastoid phenotype. One possibility to explain the relative stability of this fibroblastoid phenotype was that the converted cells produced their own TGF-β1. To examine this, cells recovered from EpRas tumors were seeded at very low, clonal density in low serum media (1%) containing levels of TGF-β1 insufficient to maintain EFC. As shown in Figure 6A, the early, small clones consisted of cells with a fibroblastoid morphology. However, as the clones increased in size, the cells in essentially all clones gradually but synchronously reverted to an epithelial phenotype (Fig. 6B–D). Ten days after plating, the reversion to a polarized epithelial phenotype was essentially com-

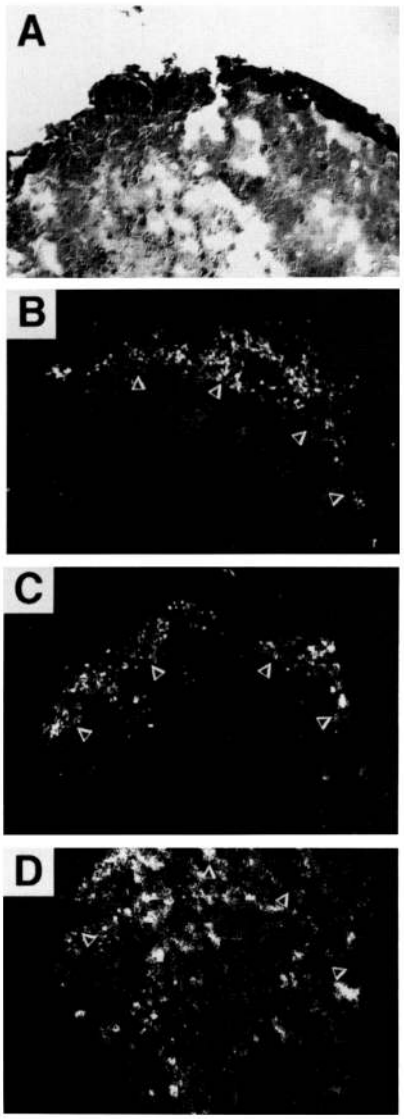

Figure 5. Fibroblastoid EpRas cells are highly invasive in the embryonic chicken heart assay. Fluorescently labeled cells to be tested for invasiveness were brought into contact with embryonic chicken heart fragments and sectioned 7 days thereafter. Nontumorigenic EpH4 cells did not invade the heart fragments (*A*,*B*) and nonconverted, epithelial EpRas cells exhibited only marginal invasiveness (*C*). In contrast, converted, fibroblastoid cells obtained after TGF-β1 treatment readily invaded the heart fragments (*D*). (Arrowheads) Edge of the chicken heart fragments.

plete (Fig. 6D; Table 1). This polarized phenotype was stable upon further subcultivation (Table 1).

To prove that the absence of TGF-β1 would indeed cause reversion to a polarized phenotype, freshly isolated ex-tumor cells were grown in serum-free collagen gels, in the presence or absence of TGF-β1-neutralizing antibodies. In the absence of antibodies, the fibroblastoid tumor cells formed the expected thin, invasively growing cords (Fig. 6E). In contrast, the same cells developed into hollow, cystic structures composed of an epithelial cell monolayers when treated with the antibodies for 8 days (Fig. 6F). These results indicate clearly that deprivation from TGF-β1 induces the converted, fibroblastoid EpRas cells to revert to an epithelial phenotype. Interestingly. this reversion proceeded in a synchronous and gradual fashion and most likely involved several cell divisions.

Finally, we determined whether EFC was indeed accompanied by the upregulation of TGF-β1 mRNA and protein and whether TGF-β1 was released into the culture medium. In one set of experiments, EpRas cells and EpH4 control cells were subjected to TGF-β1 treatment while growing sparsely on plastic. Alternatively, three different EpRas clones as well as the parental EpH4 clone were grown in serum-free collagen matrices. When treated with 5 ng/ml of TGF-β1 for 5–21 days, the EpRas clones underwent EFC under both conditions, whereas the nontransformed EpH4 cells maintained their epithelial phenotype.

In vitro converted fibroblastoid cells regularly expressed elevated levels of TGF-β1 mRNA, particularly

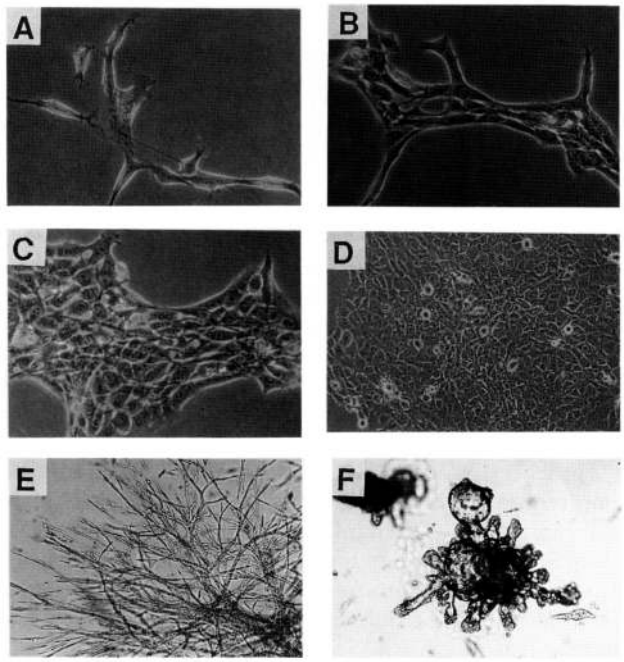

Figure 6. TGF-β1 maintains the fibroblastoid phenotype of converted EpRas cells via an autocrine loop. Fibroblastoid ex-tumor cells revert to an epithelial morphology at clonal density (*A–D*). EpRas cells after EFC were seeded at 500 cells per 100-mm dish in medium containing 1% FCS. Daily medium changes were performed to dilute potential autocrine factors. The same, representative clone was photographed at day 1 (*A*), day 3 (*B*), day 5 (*C*), and day 10 after plating (*D*). Note the gradual reversion of the fibroblastoid cells to an epithelial morphology. More than 95% of the clones obtained showed an identical behavior. (*E*,*F*) Fibroblastoid EpRas cells isolated from a tumor were selected in G418 for 5 days (to remove host-derived cells) and subsequently seeded into serum-free collagen gels. This was performed either in the absence (*E*) or presence (*F*) of TGF-β1-neutralizing antibodies. Note that the tumor cells develop into lumen-forming structures in the presence of a TGF-β-neutralizing antibody.

after prolonged TGF-β1 treatment (Fig. 7A). The same was true for EpRas cells that had undergone EFC in tumors (Fig. 7A). In contrast, epithelial EpRas cells produced only very low levels of TGF-β1 mRNA (Fig. 7A). As expected, parental EpH4 cells failed to produce detectable levels of TGF-β1 mRNA, even in response to prolonged TGF-β1 exposure. In analyzying three-dimensional structures retrieved from collagen gels (which consisted of relatively low cell numbers), similar results were obtained by semiquantitative PCR (data not shown).

Significant upregulation of TGF-β1 protein upon EFC was also demonstrated by immunoblotting of cell culture supernatants (Fig. 7B). In contrast, EpH4 cells failed to produce detectable levels of TGF-β1. Immunoblot analysis of TGF-β1 receptors revealed that the TGF-β1-receptor subunits I and II were expressed at similar levels in EpH4 cells and EpRas cells before or after EFC (Fig. 7C; data not shown).

Finally, we analyzed whether TGF-β1-induced EFC was accompanied by a release from the growth-inhibitory action of TGF-β1. For this, EpH4 control cells and both epithelial and converted, fibroblastoid EpRas cells were treated with TGF-β1 for 24 hr and then assayed for incorporation of tritiated thymidine. As expected, epithelial EpRas cells were inhibited to a similar extent by TGF-β1 as the parental EpH4 cell line (Fig. 7D). In contrast, EpRas cells after EFC were totally resistant to growth inhibition by TGF-β1 over a wide range of concentrations (Fig. 7D).

Taken together, these results suggest a dual role of TGF-β1 during tumor formation by EpRas cells. First, TGF-β1 is required for the induction of EFC. Second, an autocrine loop involving TGF-β1 maintains the converted, fibroblastoid, and TGFb-resistant phenotype and recruits further nonconverted EpRas cells to undergo this change.

TGF-β1 triggers EFC and cell invasiveness in experimentally induced tumors

In a last set of experiments, we investigated whether TGF-β1 was actually expressed in EpRas tumors and whether experimentally administered active TGF-β1 could also cause EFC and cell invasiveness in vivo. Tumors at early (4 days) and late (28 days) stages of development were analyzed for TGF-β1 expression, using RNA in situ hybridization and immunohistochemistry. In the early, 4-day tumors, elevated levels of TGF-β1 mRNA were detected in the outer periphery of the nodules formed by EpRas cells (Fig. 8A). Double immunofluorescence for TGF-β1 and neomycin phosphotransferase (NPT, which is exclusively expressed by the Ras-transformed donor cells) revealed that the great majority of donor cells (indicated by red staining for NPT) failed to produce TGF-β1 (green staining) at this stage of tumor development. However, host-derived nonepithelial cells located in the immediate tumor periphery were clearly

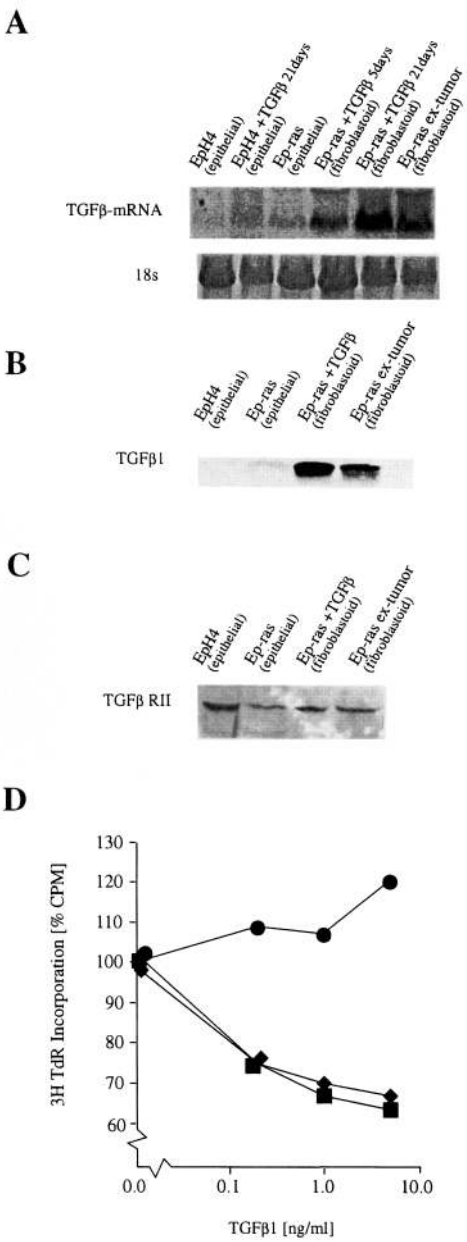

Figure 7. Expression of TGF-β1 and its receptor in epithelial and converted EpRas cells. (*A*) Nontumorigenic epithelial EpH4 cells, nonconverted epithelial EpRas cells (clone Ep5), and fibroblastoid EpRas cells (clone Ep5) (converted either in vivo or in vitro) were processed for Northern blot analysis. Note the significant increase in TGF-β1 mRNA in the fibroblastoid cells. (*B*) A dramatic up-regulation of TGF-β1 protein was demonstrated by Western blot in cell culture supernatants analyzing similar cell preparations as in *A*. The data shown in *B* were confirmed using two other EpRas clones (Ep2 and Ep6). (*C*) A Western blot using cell lysates from similar cell preparations as used in *B* was probed with an antibody to the TGF-β-receptor type II protein (TGFβRII). Note that receptor expression does not change during EFC. (*D*) EpH4 cells (■), epithelial EpRas cells (♦), and fibroblastoid EpRas cells (●) freshly isolated from a tumor were incubated with serial dilutions of TGF-β1 for 1 day and analyzed for [^{3}H]thymidine incorporation as described in Materials and Methods. Data are expressed as percent of the thymidine incorporation obtained in the absence of added TGF-β1.

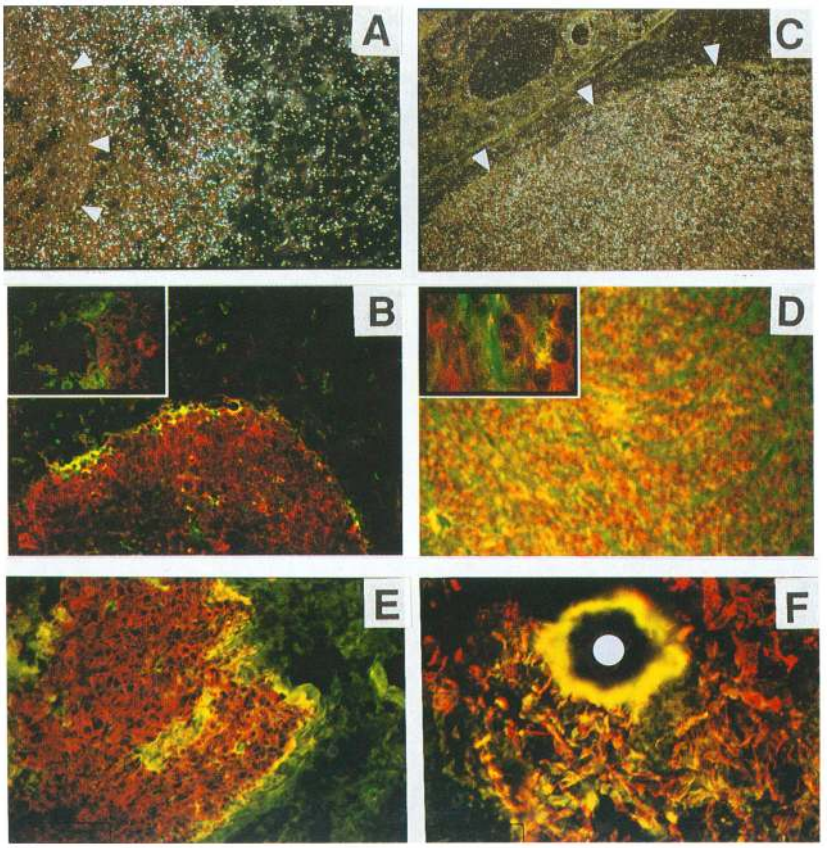

Figure 8. TGF-β1 triggers EFC conversion and cell invasiveness in experimentally induced tumors. (*A–D*) Cryosections of day 4 (*A,B*) and day 15 tumor stages (*C,D*). RNA in situ hybridization reveals that TGF-β1 expression at day 4 occurs at the outer tumor periphery (*A*) but in the entire tumor at day 15 (*C*). Arrowheads indicate the edge of the tumor. (*B,D*) Cryosections were stained by a TGF-β1 antibody (green fluorescence) and an anti-NPT antibody that recognizes the donor cells (red fluorescence). (*Insets*) Areas at higher magnification. Note that at early tumor stages TGF-β1 is exclusively produced by the tumor-surrounding stroma (*B*). In contrast, in 15 day tumors, TGF-β1 is also expressed in certain donor cells within the tumor tissue (*D*, yellow fluorescence). (*E,F*) Epithelial EpRas cells were injected subcutaneously into nude mice without (*E*) or together with three Elvax slow release pellets loaded with recombinant (active) TGF-β1 (*F*). Cryosections performed from 4-day tumors were double-stained by antibodies to cytokeratin (red) and vimentin (green). Note the dramatic cell scattering induced in the vicinity of the TGF-β1 releasing pellet (indicated by a white spot).

positive for TGF-β1 (Fig. 8B). In contrast, the 28-day, fully developed tumors showed a relatively high and uniform expression of TGF-β1 mRNA throughout the tumor area (Fig. 8C). In these tumors, the injected EpRas cells could be shown to produce TGF-β1 themselves, because they costained for NPT and TGF-β1 (Fig. 8D, yellow staining). Significantly, most of the cells producing TGF-β1 showed reduced cytokeratin expression, whereas the majority of cells highly expressing cytokeratins did not stain for TGF-β1 (data not shown). This provides additional evidence for the premise that the converted cells are indeed the ones producing TGF-β.

These findings suggest that host cells surrounding the tumor tissue initiate the cell conversion. In turn, the converted tumor cells produce TGF-β themselves, thus accelerating cell conversion and the subsequent invasion processes. To obtain direct proof for this notion, slow-release pellets loaded with recombinant human TGF-β1 were positioned in the vicinity of injected EpRas cells. Surprisingly, already 4 days after injection, EpRas cells situated in the close proximity of a TGF-β1 pellet had converted into irregularly shaped cells, showed extensive scattering and exhibited double positive staining for cytokeratin and vimentin (yellow cells; Fig. 8F). In contrast, the same cells injected in the absence of a TGF-β1 pellet (Fig. 8E) or together with a control pellet loaded with BSA (not shown) formed smooth homogeneous nodules of tightly associated, vimentin-negative cells. TGF-β1 pellets located near EpH4 cells failed to significantly affect the phenotype of these nontumorigenic cells (data not shown). These in vivo data are consistent with our in vitro findings and indicate a key role of TGF-β1 in regulating EFC during tumorigenesis by Ras-transformed mammary epithelial cells.

Discussion

Carcinogenesis involves multiple mutations in proto-oncogenes and tumor suppressor genes (Vogelstein and Kinzler 1993). Little is known, however, about how distinct oncogenic mutations correlate with defined changes in cell phenotype and how these changes contribute to tumor cell invasion and metastasis. In this paper we present evidence that the presence of TGF-β1 is absolutely required for oncogenic transformation of mammary epithelial cells by activated Ras. This is true both for in vitro cultures in collagen matrices and for developing tumors. In the absence of TGF-β1, Ras-expressing cells are similar to their nontransformed parental cells with respect to epithelial polarity. In the presence of TGF-β1, EpRas cells undergo EFC and become resistant to TGF-β-induced growth arrest, whereas parental EpH4 cells undergo growth inhibition and apoptosis. Most importantly, these converted fibroblastoid cells produce high levels of TGF-β1 themselves, thus maintaining

their mesenchymal and invasive characteristics via autocrine TGF-β1 stimulation.

EFC is a relevant mechanism in carcinogenesis

Our work originated from the observation that Ras-transformed mouse mammary epithelial cells express a polarized epithelial phenotype in culture but convert into invasive spindle cells during tumor formation in mice. Similar spindle cell tumors have been described that arise in the brain, skin, colon, and breast of both humans and animals (Guldberg 1923; Sandford et al., 1961; Sonnenberg et al. 1986; Buchmann et al. 1991; Stoler et al. 1993). Some investigators believe that spindle cell tumors are of fibroblastic origin, but a growing body of evidence suggests that these tumors originate from epithelial cells. In both animal and human tumors, cytokeratin-positive spindle-shaped cells often occur next to cytokeratin-positive cuboidal cells, and transitions between these two have been suggested by histological observations (Mareel 1980; Gabbert et al. 1985).

In our model system, the spindle cell tumors clearly originate from the donor epithelial cells. Tumor-derived spindle cells survive selection in G418 and still express cell and tissue-specific cytokeratins (46 kD and 50 kD), which confirm their epithelial origin. In addition, Southern blot analysis shows that the injected epithelial cells and the converted fibroblastoid tumor cells are of the same clonal origin and do not show reintegration of the retroviral vector into additional sites. Even more important, the EFC is not a rare progression event but rather involves the whole cell population. The fibroblastoid cells are stable in phenotype under standard culture conditions but can be efficiently reverted to a polarized, epithelial phenotype upon neutralizing the activity of TGF-β1. These data rule out a genetic change as being responsible for the cell conversion.

The question remains as to whether the observed cooperation of an activated Ras oncoprotein with endogenous TGF-β1 is relevant for human tumors. Overexpression of either activated or normal Ras has been observed in a broad range of human carcinomas, including those of the breast (Hand et al. 1984; Slamon et al. 1984; De Bortoli et al. 1985; Macauley and Pawson 1988). Moreover, autocrine ligand production, overexpression, and/or constitutive activation of receptor tyrosine kinases acting upstream of c-Ras (e.g., HER-1, HER-2) are frequent alterations in breast carcinomas (Kern et al. 1990; LeJeune et al. 1993; Clark and Der 1995). Because TGF-β1 is also often highly abundant in human tumors (Derynck et al. 1985; Keski-Oja et al. 1987; Thompson et al. 1991), it is likely that Ras and TGF-β1-induced signals may act synergistically not only in our mouse model, but also in human tumors.

Another important question is whether or not epithelial–mesenchymal transition in breast epithelial cells can be induced by other oncogenes and/or normal cellular mechanisms. First, long-term activation of a c-FosER fusion protein induced epithelial–mesenchymal transition similar to that seen in Ras-transformed epithelial cells (Reichmann et al. 1992). However, these cells were not tumorigenic in nude mice. In addition, TGF-β (Miettinen et al. 1994) and bFGF (Boyer et al. 1992) induced epithelial–mesenchymal transition in epithelial cell lines lacking an exogenous, activated Ras. In contrast to our system, however, these cell lines did not express a polarized epithelial phenotype, and the question of whether they contain an endogenous, activated Ras (or oncogenic mutations within the Ras pathway) has not been adressed.

TGF-β and Ras coregulate epithelial cell plasticity

In this paper we show that TGF-β1 induces EFC both in collagen matrices and during tumor development. Remarkably, this TGF-β1-induced cell conversion requires the cooperation of an activated Ras protein, because neither primary mammary epithelial cells nor the parental EpH4 cells undergo TGF-β1-induced EFC. Thus, we propose that EFC is caused by synergy between TGF-β1 and Ha-Ras-activated signaling pathways. This idea is supported by other reports showing that the effects exerted by activated Ras proteins are similar in action to members of the TGF-β family during myogenic differentiation (Payne et al. 1987) and mesoderm formation (Whitman and Melton 1992). In the latter process, signaling downstream of Ras (MAP kinase activation) was shown to be necessary (LaBonne and Whitman 1994; Umbhauer et al. 1995). In cardiac muscle, TGF-β1 up-regulates genes associated with the embryonic heart and with growth provoked by hemodynamic load. Both effects are mimicked at least in part by activated Ras (Parker et al. 1990; Thorburn et al. 1993), suggesting that, at least in certain biological systems, Ras and TGF-β may act synergistically.

When comparing our experimental mouse tumors to naturally occurring human tumors, one should keep in mind that such tumors do not necessarily contain all the genetic changes typical for immortalized established cell lines such as used herein (Pereira-Smith and Smith 1988). Whereas an activated Ras oncoprotein is sufficient to transform immortal fibroblast cell lines, nonimmortalized fibroblasts require additional oncogenic events, such as the cooperation between the Ras and Myc oncoproteins (Land et al. 1983). Even when such oncogene combinations are coexpressed, full transformation is still a rare event, implying that further mutations may be required (Macauley and Pawson 1988). These findings suggest that ras is a potent oncogene only in the presence of other critical changes, which may already have occurred in our Ras-transformed epithelial cells. However, these additional mutations are present in both control and Ras-transformed clones. On this defined, genetic background, we investigate late events in tumor progression such as dedifferentiation and invasiveness. Thus, our conclusions are probably not affected by the genetic differences between our model system and naturally occurring human tumor cells. In addition, our Ras-induced experimental tumors show many features of naturally

occurring tumors. These are VEGF expression in hypoxic areas and vascularization (G. Breier, S. Blum, J. Peli, W. Risau, and E. Reichmann, in prep.), TGF-β expression, recruitment of tumor stroma, changes in extracellular matrix composition and deposition, altered protease and protease inhibitor expression (C. Rudaz and E. Reichmann, unpubl.), and cell invasiveness. Interestingly, all these processes also occur during wound healing and are dependent on the expression and efficient activation of TGF-β (Roberts et al. 1986; Edwards et al. 1987; Laiho etal. 1987; Kim et al. 1989). Taken together, we postulate that tumor cell invasiveness is not just a result of specific mutations in individual cells, but rather a combination of these together with an aberrant response of the growing tumor cells to normal regulatory mechanisms such as those occurring in wound healing and tissue repair.

Ras alters the TGF-β reponsiveness of mammary epithelial cells: loss of epithelial polarity and gain of invasiveness and phenotypic plasticity

In serum-free or TGF-β-deprived collagen matrices, EpRas cells develop into dilated ducts and cystic cavities, whereas parental EpH4 cells or primary mammary epithelial cells form narrow, branching ducts and endbuds. In other systems, more dramatic effects of activated Ras on epithelial polarity have been described (for review, see Eaton and Simons 1995). Here, Ras transformation interferes with the establishment of apical polarity, while leaving the basolateral domain intact (Schoenenberger et al. 1994). However, because these experiments have been performed in the presence of serum (which itself contains TGF-β1), they are difficult to compare with our results in which we failed to observe obvious polarity defects. Because exogenous TGF-β1 completely disrupts cell polarity, it is possible that the partial loss of epithelial polarity in the Ras-transformed cell systems mentioned above may be attributable to the TGF-β1 levels present in serum.

In our system, TGF-β1 completely disrupted cell–cell adhesions and epithelial cell polarity, causing the cells to become spindle shaped and invasive. These changes are dependent on the continuous presence of TGF-β1, because the spindle cells revert into polarized epithelial cells upon addition of TGF-β1-neutralizing antibodies. The major, novel conclusion from these findings is that by addition or removal of TGF-β1, Ras-transformed cells can be switched back and forth between a noninvasive (polarized epithelial) and a highly invasive (fibroblastoid) phenotype. This phenotypic plasticity may be characteristic for invasively growing cells in general and may explain why invasive tumor cells often exhibit the migratory properties of fibroblasts. The reversibility of EFC shown here is also in accord with the possibility that the new environment of a distant organ may trigger the fibroblastoid, migratory cells to reaquire epithelial features after extravasation. This would explain why metastases of human carcinomas are often well differentiated.

Autocrine TGF-β1 production maintains the fibroblastoid phenotype

Another major, novel finding of this study is that EpRas cells had to undergo EFC to produce significant amounts of TGF-β1 themselves both in vitro and in vivo. Our work shows that tumor cells can maintain their fibroblastoid phenotype via the autocrine production of TGF-β1 and that this autocrine TGF-β loop had to be interrupted to allow the phenotypic reversion of the cells in vitro. The fact that once converted the fibroblastoid cells efficiently produce TGF-β1 themselves may also explain why the Ras-transformed cells in the tumors observed can convert so uniformly into spindle cells.

Polarized Ras-transformed epithelial cells did not express or release significant amounts of TGF-β1 shortly after their injection into mice. This raises the question as to how the conversion process may have been initiated. As seen by in situ hybridization and immunostaining, the stromal cells surrounding a 3- to 4-day-old microtumor produced the cytokine. These cells could be identified as fibrocytes and endothelial cells, but other cell types such as macrophages and lymphocytes are likely to have been present as well. All these cell types are known to produce and release TGF-β. Primary regulation of TGF-β action occurs through factors that control processing of the latent to the biologically active molecule. However, TGF-β activation in vivo is not well understood. In coculture systems, plasmin has been shown to activate latent TGF-β1 in a fashion that requires direct contact or close apposition of two different cell types (Antonelli-Orlidge et al. 1989; Sato et al. 1990). In our system, such cell–cell interactions causing TGF-β activation may include the encapsulation of the microtumor by stromal fibroblasts and, more important, the intermixing of donor tumor cells and host stromal cells during tumor development (see Fig. 2B). Latent TGF-β is apparently also activated by the extracellular matrix protein thrombospondin (TSP). In this case activation occurs in the soluble phase and does not require proteolytic activity (Schultz-Cherry et al. 1994). In fact, a supportive role for thrombospondin in cancer and increased thrombospondin levels in malignant breast cancers have been reported (Castle et al. 1991; Wong et al. 1992).

Our combined in vitro and in vivo results have led us to formulate the hypothetical model shown in Figure 9. TGF-β1 is initially provided by infiltrating nontumor cells, such as fibrocytes, endothelial cells, lymphocytes, and macrophages. The interaction of these different cell types with the tumor cells may trigger the efficient activation of TGF-β1. This in turn causes the epithelial tumor cells to lose polarized features and to dedifferentiate into migrating fibroblastoid cells. These cells gain invasive properties, become resistant to TGF-β-induced growth arrest, and begin to produce TGF-β1 themselves. The autocrine loop thus generated facilitates the maintenance of the fibroblastoid phenotype and the recruitment of additional cells into EFC (Fig. 9; see above). Our model agrees with findings of Thompson et al. (1992, 1993) who provide evidence that elevated TGF-β1 ex-

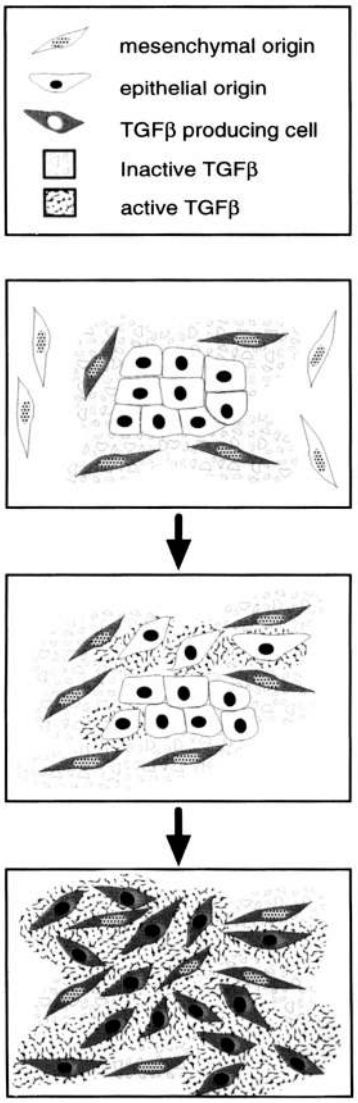

Figure 9. Model for TGF-β1 action in tumor development. In this hypothetical model, we postulate that TGF-β1 is initially provided by tumor-infiltrating cells, such as fibrocytes, endothelial cells, lymphocytes, and macrophages. The interaction of these different cell types with the (epithelial) tumor cells may trigger the efficient activation of TGF-β1. This in turn may cause certain epithelial tumor cells to convert to the fibroblastoid and invasive phenotype. These fibroblastoid cells then begin to produce TGF-β1 themselves, thus generating an autocrine loop that facilitates the maintenance of the fibroblastoid phenotype and the recruitment of additional cells into EFC and invasiveness.

pression is involved in the progression to malignancy in a mouse model for prostate cancer.

Since its discovery, TGF-β1 was found to be overexpressed and was postulated to act in an autocrine fashion in many different epithelial tumors (Derynck et al. 1985; Roberts et al. 1986). Our data reveal a clear functional role for such autocrine loops involving TGF-β. For instance, tumor cells of rather similar phenotype can produce high or low levels of TGF-β and can be either subject or resistant to growth inhibition by TGF-β. This otherwise puzzling observation makes sense if seen as a characteristic feature of tumor cell plasticity, allowing the cell to proliferate and migrate in widely different environments. Under certain conditions favoring EFC, autocrine TGF-β1 production would confer invasive properties to a transformed epithelial cell and maintain them until different environmental conditions favor reversion to a more differentiated phenotype. In such a scenario, tumor cells able to activate autocrine TGF-β1 production would even have a distinctly selective advantage in tumor progression.

Materials and methods

Cell culture

EpRas cells were generated by infecting the parental mammary epithelial cell line EpH4 (Fialka et al. 1996) with a helper-free retroviral vector expressing v-Ha-ras (Redmond et al. 1988). Selection and expansion of polarized epithelial clones were performed as described earlier (Reichmann et al. 1992). Briefly, cells were cultured on plastic dishes in growth medium [Dulbecco's modified Eagle medium (DMEM) containing 10% FCS (Boehringer Mannheim) and 20 mM HEPES] and subcultured at a 1:3 ratio twice a week. For induction of hemicyst (dome) formation, EpRas cells and the parental line EpH4 were cultured at high density for 1 week without subculturing.

Assay for TGF-β-dependent growth inhibition

Exponentially growing EpH4 cells, EpRas cells, and fibroblastoid EpRas cells after EFC were trypsinized, washed, and seeded in triplicate into the wells of 96-well tissue culture plates at 1×10^4 cells per well, using DMEM containing 5% FCS. Various concentrations (0.1 ng/ml–10 ng/ml) of TGF-β1 were added, and the cells were incubated for 24 hr. Cells were labeled for 3 hr with 0.8 mCi [^{3}H] thymidine per well ([^{3}H]-TdR, Amersham; ~30 Ci/mmole). Labeled cells were harvested onto glass fiber filters using an automated cell harvester (TOMTEC). The cell-bound radioactivity was determined in a 96-well scintillation counter (Wallac, Microbeta 1450).

Growth of organotypic cell structures in collagen gels

Semiconfluent cultures of the cells to be analyzed were trypsinized and adjusted to a final concentration of 4×10^4 cells/ml with ice-cold growth medium. Equal volumes of cell suspension and of a solution of acidified rat collagen type I (Sigma) were mixed at 4°C, dispensed on 35-mm tissue culture dishes, and allowed to solidify into a gel for 30 min at 37°C. To generate organotypic structures, the collagen gels were overlayed with a serum-free medium [MEGM (Promocell), containing bovine pituitary extract (BPE), recombinant epidermal growth factor (EGF), hydrocortisone, and insulin at concentrations suggested by the supplier]. When indicated, 5% or 10% fetal calf serum (FCS) or 5 ng/ml of recombinant TGF-β1 (a kind gift from Genentech, Inc., South San Francisco, CA) was added. The medium overlaying the collagen gels was changed every second day. To neutralize the TGF-β1 produced by the cells or

structures in the gel, a neutralizing, anti-TGF-β monoclonal antibody (Genzyme) or control antibodies were used at concentrations up to 50 ng/ml.

Tumor induction and reisolation of cells from tumors or collagen gels

Confluent EpRas or EpH4 cells were trypsinized and counted. Then, 1×10^5 cells suspended in 0.1 ml of phosphate-buffered saline (PBS) were injected subcutaneously or into the mammary gland of 5-week-old BALB/c or nude mice. The recipient mice were sacrificed after various time periods ranging from 3 to 28 days, and the tumors (or tissue areas containing the injected cells) were excised. For subsequent histological analysis, the tissue was immediately snap frozen in liquid nitrogen. To reisolate the tumor cells for further growth in tissue culture, the tissue was cut into small pieces under sterile conditions using opposing scalpel blades and digested with 2 mg/ml of collagenase type IA (Clostridiopeptidase A; Sigma) for 1 hr at 37°C. To remove remaining host cells lacking the retroviral neomycin or hygromycin resistance markers contained in the donor cells, the cells retrieved from the tumors were cultured for the first 5 days in the presence of G418 or hygromycin, respectively. Collagen gels were similarly digested with collagenase to reisolate cells for subsequent tissue culture.

Antibodies

Rabbit antisera to cytokeratin were described earlier (Reichmann et al. 1992). Rabbit antiserum and rat monoclonal antibodies against E-cadherin were a gift from Dr. Rolf Kemler (Max Planck Institut für Immunbiologie, Freiburg, Germany). Rabbit antiserum recognizing NPT was kindly provided by E.F. Wagner (Forschungsinstitut für Molekulare Pathologie, Vienna, Austria). The mouse monoclonal antibody against Vimentin V3B (Boehringer Mannheim), the rat monoclonal antibody against ZO-1 (Chemicon), the mouse monoclonal antibody against TGF-β 1–3 (Genzyme), the TGF-β 2,3 antibody (Genzyme), the polyclonal antiserum against activated TGF-β (Promega), the neutralizing rabbit polyclonal (R&D), and the mouse monoclonal antibodies (Genzyme) directed against TGF-β were purchased from the respective sources. Antibodies against the TGF-β1-receptor proteins TGF-β R1 (Alk-5) and TGF-β RII were obtained from Santa Cruz Biotech., Inc. and Upstate Biotech., Inc., respectively.

Sectioning and immunofluorescence

To retain optimal biological activity of RNA and proteins in the excised tissues and collagen gels, tumor material and collagen type 1 gels containing cell structures were snap-frozen in liquid nitrogen immediately after isolation. Prior to freezing, collagen gels were soaked for 2 min in medium containing 5% DMSO to prevent cell damage due to ice crystal formation. Cells grown on plastic or cryosections prepared from tumors or collagen gels were fixed and permeabilized for 15 min at −20°C using acetone/methanol mixed at a ratio of 1:1, air-dried, and stored at 4°C. First antibodies were applied for 1 hr at 37°C in PBS, usually containing gelatin, bovine serum albumin (BSA), and Tween 20 (0.2% each) to block unspecific antibody staining. The second antibodies, conjugated to FITC or Texas Red, were applied for 30 min at 37°C. The cells or sections were then mounted in Moviol 1-88 (Hoechst) and examined with a Zeiss Axiophot fluorescence microscope. Photographs were either taken conventionally or computer assisted, using a Kaf 1400 CCD camera (Photometrics) and the Adobe Photoshop 3.0 image processing program.

RNA in situ hybridization

For RNA in situ hybridization, cryosections were fixed in 4% paraformaldehyde in PBS, washed twice in PBS, prehybridized for 2 hr, and hybridized overnight to the respective ^{35}S-labeled riboprobe at 52°C in 50% formamide, 0.6 M NaCl. After washing under stringent conditions (T_m, −20°C) the sections were dipped in Kodak NTB liquid emulsion and exposed for 2 weeks. The slides were counterstained with hematoxylin/eosin and analyzed in bright- and dark-field illumination using a Zeiss Axiophot microscope.

To generate ^{35}S-labeled riboprobes, the hTGF-β1 cDNA (R&D) and the cDNA for NTP (exised from a suitable retroviral vector) were cloned into T_3–T_7 expression plasmids and transcribed in vitro in the presence of [^{35}S]UTP for antisense riboprobe and sense control probe.

Electron microscopy

Cells grown in collagen I gels were prefixed for 10 min in 3% paraformaldehyde in 0.2 M HEPES (pH 7.3) at room temperature. Cells were further fixed in 8% paraformaldehyde, 0.2 M HEPES (pH 7.3) for 30–60 min on ice. For immunocytochemistry, samples were dehydrated in ethanol at progressively lower temperatures, embedded in Lowicryl HM20 or K4M, and polymerized at −35°C by UV light (Schwarz et al. 1993) For ultrastructural studies, cells were postfixed with 1% osmium tetroxide in PBS (pH 7.2) for 1 hr on ice, block stained with 1% aqueous uranyl acetate for 1 hr, dehydrated in ethanol at room temperature, and finally, embedded in Epon. For immunocytochemistry, ultrathin sections were mounted on coverslips (Schwarz 1994) After blocking unspecific binding sites with 0.5% BSA and 0.2% gelatin in PBS, sections were incubated with rabbit anti-β-catenin antibodies and subsequently with Cy3-labeled goat anti-rabbit IgG. The labeled sections were stained with 4′,6-diamino-2-phenylindole (DAPI) to visualize nuclei in immunofluorescence microscopy.

Southern blot analysis

Total DNA of cells or tumor material was isolated and processed using standard procedures (Maniatis et al. 1982). DNA was extracted from cells before injection, from freshly excised tumor tissue (day 15 after injection), and from tumor tissue recultured in vitro in the presence of G418 for 5 days, digested with *Eco*RI (which cuts the retroviral vector only once), blotted onto a GeneScreen membrane, and hybridized with cDNAs encoding either the NPT or the v-Ha-ras gene.

Northern blot analysis

Northern blot analysis was performed as described elsewhere (Chomczynski and Sacchi 1987; Reichmann et al. 1992). Total RNA (10 μg per lane) was run on formaldehyde-containing denaturing gels, blotted to GeneScreen membranes, and hybridyzed with the entire coding region of hTGF-β1 cDNA (R&D) that displayed enough homology to mTGF-β1, mTGF-β2, and mTGF-β3 to recognize all three murine TGF-β isoforms.

Semiquantitative PCR

Total RNA from cells grown on plastic, in collagen gels, or from tumors was isolated and processed for semiquantitative PCR. A TGF-β1-specific fragment was amplified by RT–PCR under semiquantitative conditions, using β-actin primers as an internal control, as described by Leonard et al. (1993). Briefly, DNA was denatured at 94°C for 1 min, primers were annealed at 65°C

for 1 min, and the polymerase reactions were continued at 72°C for 1 min. The amplification was continued for 20 and 30 cycles. TGF-β-specific primers 5′-TGGACCGCAACAACGCCATC-TATGAGAAAACC-3′ (upstream) and 5′-TGGAGCTGAAG-CAATAGTTGGTATCCAGGGCT-3′ (downstream) (Clontech, Inc.) were used. The results of the PCR were quantitated on an ImageQuant PhosphorImager. The values were normalized to the respective control product (β-actin) and subsequently set in relation to the value from control 3T3 fibroblasts.

Immunoblotting

To analyze TGF-β1 in tissue culture supernatants, 2 ml of serum-free cell supernatants were concentrated to a final volume of 0.1 ml by ultrafiltration (Centricon 10, Amicon). The concentrated supernatants were mixed with fivefold concentrated SDS–polyacrylamide gel electrophoresis (PAGE) (sample buffer lacking mercaptoethanol and analyzed by SDS–PAGE (nonreducing conditions). Equal aliquots of protein (50 μg) were subjected to SDS–PAGE, and immunoblot analysis was performed as described previously (Hayman et al. 1993).

Embryonic chicken heart invasion assay

The embryonic chicken heart invasiveness assay was performed as described elsewhere (Behrens et al. 1993). To unambiguously discriminate invasive donor cells from chicken heart cells, the test cells were loaded with a vital, fluorescent dye before testing. For this, the cells were incubated for 1 hr in glucose-containing Hanks balanced salt solution containing 10 mM 5,6-carboxy-2′,7′-dichlorofluorescein diacetate-succinimidyl ester (Molecular Probes) and 0.2×10^6 M pluronic F127 (Hoechst). By this procedure, the fluorescent dye is covalently coupled to intracellular proteins without affecting cell viability or behavior, as judged by various differentiation and proliferation assays. After labeling, the cells were washed 3× in PBS for 5 min and twice in complete medium for 4 hr to remove uncoupled dye. The labeled cells were cultured for 24 hr at high density, scraped off the plastic dish, and brought into contact with precultured heart fragments from 9-day-old chicken embryos on top of a soft agar layer. The fragments with attached cells were collected after 7 days in culture, snap frozen in liquid nitrogen, cryosectioned, fixed in methanol/acetone, and fluorescent cells were determined by epifluorescence microscopy (Axiophot, Zeiss).

Implantation of TGF-β-loaded slow-release pellets into mice

To expose Ras-transformed mammary epithelial cells to activated TGF-β1 very early in tumor development, TGF-β1-loaded slow-release Elvax pellets and either EpRas epithelial cells or normal EpH4 cells were coinjected subcutaneously into mice. For control purposes, pellets loaded with BSA only were coinjected. The pellets were prepared and loaded according to the manufacturer's instructions.

Acknowledgments

We wish to thank Drs. Rolf Kemler and Erwin Wagner for their kind gift of antibodies. We thank Drs. Walter Birchmeier, Lucienne Juillerat, Anne-Catherine Andres, and Andrew Ziemieki for critical reading of the manuscript, Drs. Robert Friis and Rex Tyrrell for helpful comments and stimulating discussions, and Mrs. Christine Meyer and Jeannine Bamat for excellent technical assistance. This research was supported by the Swiss National Science Foundation (E.R.), the Deutsche Forschungsgemeinschaft (E.R.; M.O.), the Krebsforschung Schweiz (E.R.), and the Fondation Emma Muschamp (E.R.). M.O. and H.B. received support from the Forschungsförderungsfonds für die gewerbliche Wirtschaft, FFF 3/10628 and FFF 3/9743.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Antonelli-Orlidge, A., K.B. Saunders, S.R. Smith, and P.A. D'Amore. 1989. An activated form of transforming growth factor β is produced by cocultures of endothelial cells and pericytes. *Proc. Natl. Acad. Sci.* **86:** 4544–4548.

Aznavoorian, S., A.N. Murphy, S.W. Stetler, and L.A. Liotta. 1993. Molecular aspects of tumor cell invasion and metastasis. *Cancer* **71:** 1368–1383.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, R.F. Van, M.M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin ex in cells transformed with a temperature-sensitive v-SRC gene. *J. Cell Biol.* **120:** 757–766.

Birchmeier, W., K.M. Weidner, and J. Behrens. 1993. Molecular mechanisms leading to loss of differentiation and gain of invasiveness in epithelial cells. *J. Cell Sci.* (Suppl.) **17:** 159–164.

Border, A.I. and V. Ruoslahti. 1992. Transforming Growth Factor-β in disease: The dark side of tissue repair. *J. Clin. Invest.* **90:** 1–7.

Bos, J.L. 1989. Ras oncogenes in human cancer, a review. *Cancer Res.* **49:** 4682–4689.

Boyer, B., S. Dufour, and J.P. Thiery. 1992. E-cadherin expression during the acidic FGF-induced dispersion of a rat bladder carcinoma cell line. *Exp. Cell Res.* **201:** 347–357.

Buchmann, A., B. Ruggeri, S.A. Klein, and A. Balmain. 1991. Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-ras alleles on chromosome 7. *Cancer Res.* **51:** 4097–4101.

Castle, V., J. Varani, S. Fligiel, E.V. Prochownik, and V. Dixit. 1991. Antisense-mediated reduction in thrombospondin reverses the malignant phenotype of a human squamous carcinoma. *J. Clin. Invest.* **87:** 1883–1888.

Caulin, C., F.G. Scholl, P. Frontelo, C. Gamallo, and M. Quintanilla. 1995. Chronic exposure of cultured transformed epidermal cells to transforming growth factor β1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. *Cell Growth Differ.* **6:** 1027–1035.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid ganidinium thiocyanate - phenol - chloroform extraction. *Anal. Biochem.* **162:** 156–159.

Clark, G.J. and C.J. Der. 1995. Aberrant function of the Ras signal transduction pathway in human breast cancer. *Breast Cancer Res. Treat.* **35:** 133–144.

De Bortoli, M., H. Abou-Issa, B.E. Haley, and Y.S. Cho-Chung. 1985. Amplified expression of p21RAS protein in hormone-dependent mammary carcinomas of human and rodents. *Biochem. Biophys. Res. Commun.* **127:** 699–706.

Derynck, R., J.A. Jarret, E.Y. Chen, D.H. Eaton, J.R. Bell, R.K. Assoian, A.B. Roberts, M.B. Sporn, and D.V. Goeddel. 1985. Human transforming growth factor beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316:** 701–705.

Dvorak, H.F. 1986. Tumors: Wounds that do not heal. Similarities between tumor stroma generation and wound healing.

N. Engl. J. Med. **315:** 1650–1659.

Eaton, S. and K. Simons. 1995. Apical, basal, and lateral cues for epithelial polarization. *Cell* **82:** 5–8.

Edwards, D.R., G. Murphy, J.J. Reynolds, S.E. Whitham, A.J.P. Docherty, P. Angel, and J.K. Heath. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* **6:** 1899–1904.

Fearon, E.R. and B. Vogelstein. 1990. A genetic model for colorectal tumorigenesis. *Cell* **61:** 759–767.

Fialka, I., H. Schwarz, E. Reichmann, M. Busslinger, and H. Beug. 1996. The estrogen dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. *J. Cell Biol.* **132:** 1115–1132.

Frisch, S.M. and H. Francis. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **24:** 619–626.

Fynan, T.M. and M. Reiss. 1993. Resistance to inhibition of cell growth by transforming growth factor beta and its role in oncogenesis. *Crit. Rev. Oncog.* **4:** 493–540.

Gabbert, H., R. Wagner, R. Moll, and C.D. Gerharz. 1985. Tumor dedifferentiation, an important step in tumor invasion. *Clin. Expl. Metastasis* **3:** 257–279.

Guldberg, G. 1923. Changes in the skin and organs in white mice after painting with tar. *Acta Pathol. Microbiol. Scand.* **4:** 276–284.

Hand, P., A. Thor, D. Wunderlich, R. Maurano, A. Caruso, and J. Schlom. 1984. Monoclonal antibodies of pre-defined specificity detect activated RAS gene expression in human mammary and colon carcinogenesis. *Proc. Natl. Acad. Sci.* **81:** 5227–5231.

Hartmann, G., K.M. Weidner, H. Schwarz, and W. Birchmeier. 1994. The motility signal of scatter factor/hepatocyte growth factor mediated through the receptor tyrosine kinase met requires intracellular action of Ras. *J. Biol. Chem.* **269:** 21936–21939.

Hayman, M.J., S. Meyer, F. Martin, P. Steinlein, and H. Beug. 1993. Self renewal and differentiation of normal avian erythroid progenitor cells: Regulatory roles of the c-*erb*B/TGFα receptor and the c-*kit* /SCF receptor. *Cell* **74:** 157–169.

Hoosein, N.M., M.K. McKnight, A.E. Levine, K.M. Mulder, K.E. Childress, D.E. Brattain, and M.G. Brattain. 1989. Differential sensitivity of subclasses of human colon carcinoma cell lines to the growth inhibitory effects of transforming growth factor-beta 1. *Exp. Cell Res.* **181:** 442–453.

Kern, F.G., A.L. Cheville, and Y.L. Liu. 1990. Growth factor receptors and the progression of breast cancer. *Semin. Cancer Biol.* **1:** 317–328.

Keski-Oja, J., E.B. Loef, R.M. Lyons, R.J.J. Coffey, and H.L. Moses. 1987. Transforming growth factor and control of neoplastic cell growth. *J. Cell. Biochem.* **33:** 95–107.

Kim, S.J., K.T. Jeang, A.B. Glick, M.B. Sporn, and A.B. Roberts. 1989. Promoter sequences of the human transforming growth factor-β1 gene responsive to transforming growth factor β1 autoinduction. *J. Biol. Chem.* **264:** 7041–7045.

Kraus, M.H., Y. Yuasa, and S.A. Aaronson. 1984. A position 12-activated Ha-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. *Proc. Natl. Acad. Sci.* **81:** 5384–5388.

LaBonne, C. and M. Whitman. 1994. Mesoderm induction by activin requires FGF-mediated intercellular signals. *Development* **120:** 463–472.

Laiho, M., O. Saksela, and J. Keski-Oja. 1987. Transforming growth factor-β induction of type 1 plasminogen activator inhibitor. *J. Biol. Chem.* **262:** 17467–17474.

Land, H., L.F. Parada, and R.A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts require at least two cooperating oncogenes. *Nature* **304:** 596–602.

LeJeune, S., R. Leek, E. Horak, G. Plowman, M. Greenall, and A.L. Harris. 1993. Amphiregulin, epidermal growth factor receptor, and estrogen receptor expression in human primary breast cancer. *Cancer Res.* **53:** 3597–3602.

Leonard, M.W., K.C. Lim, and J.D. Engel. 1993. Expression of the chicken GATA factor family during early erythroid development and differentiation. *Development* **119:** 519–531.

Liotta, L.A. and W.G. Stetler-Stevenson. 1991. Tumor invasion and metastasis: An imbalance of positive and negative regulation. *Cancer Res.* **51:** 5054–5059.

Liotta, L.A., P.S. Steeg, and W.G. Stetler-Stevenson. 1991. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. *Cell* **64:** 327–336.

Macauley, A. and T. Pawson. 1988. Cooperative transforming activity of ras, myc and src viral oncogenes in nonestablished rat adrenocortical cells. *J. Virol.* **62:** 4712–4721.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mareel, M.M. 1980. Recent aspects of tumor invasiveness. *Int. Rev. Exp. Pathol.* **22:** 65–129.

———. 1983. Invasion in vitro. *Cancer Metastasis Rev.* **2:** 201–218.

Mareel, M., J. Kint, and C. Meyvisch. 1979. Methods of study of the invasion of malignant C3H mouse fibroblasts into embryonic chick heart in vitro. *Virchows Arch.* (B) **30:** 95–111.

Mareel, M.M., J. Behrens, W. Birchmeier, G.K. De Bruyne, K. Vleminckx, A. Hoodgewus, W.C. Fiers, and F.M. Van Roy. 1991. Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. *Int. J. Cancer* **47:** 922–928.

Miettinen, P.J., R. Ebner, A.R. Lopez, and R. Derynck. 1994. TGF-beta induced transdifferentiation of mammary epithelial cells to mesenchymal cells: Involvement of type I receptors. *J. Cell Biol.* **127:** 2021–2036.

Murthy, U., M.A. Anzano, and R.G. Creig. 1989. Expression of TGF-alpha/EGF and TGF-beta receptors in human colon carcinoma cell lines. *Int. J. Cancer* **44:** 110–115.

Parker, T.G., S.E. Packer, and M.D. Schneider. 1990. Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. *J. Clin. Invest.* **85:** 507–514.

Payne, A., E.N. Olson, P. Hsiau, R. Roberts, M.B. Perryman, and M.D. Schneider. 1987. An activated c-Ha-ras allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawal. *Proc. Natl. Acad. Sci.* **84:** 8956–8960.

Pereira-Smith, O.M. and J. Smith. 1988. Genetic analysis of indefinite division in human cells: Identification of four complementation groups. *Proc. Natl. Acad. Sci.* **85:** 6042–6046.

Powell, S.M., G.M. Petersen, A.J. Krush, S. Booker, J. Jen, F.M. Giardiello, S.R. Hamilton, B. Vogelstein, and K.W. Kinzler. 1993. Molecular diagnosis of familial adenomatous polyposis. *N. Engl. J. Med.* **329:** 1982–1987.

Redmond, S.M., E. Reichmann, R.G. Mueller, R.R. Friis, B. Groner, and N.E. Hynes. 1988. The transformation of primary and established mouse mammary epithelial cells by p21-Ras is concentration dependent. *Oncogene* **2:** 259–265.

Reichmann, E. 1994. Oncogenes and epithelial cell transformation. *Sem. Cancer Biol.* **5:** 157–165.

Reichmann, E., R. Ball, B. Groner, and R.R. Friis. 1989. New mammary epithelial and fibroblastic clones in coculture form structures competent to differentiate functionally. *J. Cell Biol.* **108:** 1127–1138.

Reichmann, E., H. Schwarz, E.M. Deiner, I. Leitner, M. Eilers, J. Berger, M. Busslinger, and H. Beug. 1992. Activation of an inducible c-FosER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. *Cell* **71:** 1103–1116.

Roberts, A.B. and M.B. Sporn. 1992. Mechanistic interrelationships between two superfamilies: The steroid/retinoid receptors and transforming growth factor-beta. *Cancer Surv.* **14:** 205–220.

Roberts, A.B., M.B. Sporn, R.K. Assoian, J.M. Smith, N.S. Roche, L.M. Wakefield, U.I. Heine, L.A. Liotta, V. Falanga, J.H. Kehrl, and A.S. Fauci. 1986. Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci.* **83:** 4167–4171.

Sandford, K.K., T.B. Dunn, B.B. Westfall, B.B. Covalesky, A.B. Covalesky, L.T. Dupree, and W.R. Earle. 1961. Sarcomatous change and maintenance of differentiation in long-term cultures of mouse mammary carcinomas. *J. Natl. Cancer Inst.* **26:** 1139–1193.

Sato, Y., R. Tsuboi, R. Lyons, H. Moses, and D.B. Rifkin. 1990. Characterization of the activation of latent TGF-beta by cocultures of endothelial cells and pericytes or smooth muscle cells: A self-regulating system. *J. Cell Biol.* **111:** 757–763.

Schoenenberger, C.A., A. Zuk, G.M. Zinkl, D. Kendall, and K.S. Matlin. 1994. Integrin expression and localization in normal MDCK cells and transformed MDCK cells lacking apical polarity. *J. Cell Sci.* **107:** 527–541.

Schultz-Cherry, S., S. Ribeiro, L. Gentry, and J.E. Murphy-Ullrich. 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. *J. Biol. Chem.* **269:** 26775–26782.

Schwarz, H. 1994. Immunolabelling of ultrathin resin sections for immunofluorescence and electron microscopy. In *Electron microscopy 1994 ICEM* (ed. B. Jouffrey and C. Colliex), Vol. 63, pp. 255–256. Les editions de physique, Les Ulis, Paris, France.

Schwarz, H., A. Mueller-Schmidt, and W. Hoffmann. 1993. Ultrastructural localization of ependymins in the endomeninx of the brain of the rainbow trout: Possible association with collagen fibrils of the extracellular matrix. *Cell Tissue Res.* **273:** 417–425.

Slamon, D.J., J.B. de Kernion, I.M. Verma, and M.J. Cline. 1984. Expression of cellular oncogenes in human malignancies. *Science* **224:** 256–262.

Sonnenberg, A., H. Daams, J. Calafat, and J. Hilgers. 1986. In vitro differentiation and progression of mouse mammary tumor cells. *Cancer Res.* **46:** 5913–5922.

Soriano, J.V., M.S. Pepper, T. Nakamura, L. Orci, and R. Montesano. 1995. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. *J. Cell Sci.* **108:** 413–430.

Stoler, A.B., F. Stenback, and A. Balmain. 1993. The conversion of mouse skin squamous cell carcinomas to spindle cell carcinomas is a recessive event. *J. Cell Biol.* **122:** 1103–1117.

Strange, R., F. Li, R.R. Friis, E. Reichmann, B. Haenni, and P.H. Burri. 1991. Mammary epithelial differentiation in vitro: Minimum requirements for a functional response to hormonal stimulation. *Cell. Growth Differ.* **2:** 549–559.

Thompson, A.M., D.J. Kerr, and C.M. Steel. 1991. Transforming growth factor beta 1 is implicated in the failure of tamoxifen therapy in human breast cancer. *Br. J. Cancer* **63:** 609–614.

Thompson, T.C., L.D. Truong, T.L. Timme, D. Kadmon, B.K. McCune, K.C. Flanders, P.T. Scardino, and S.H. Park. 1992. Transforming growth factor beta 1 as a biomarker for prostate cancer. *J. Cell. Biochem.* (*Suppl.*) **16H:** 54–61.

———. 1993. Transgenic models for the study of prostate cancer. *Cancer* (Suppl.) **71:** 1165–1171.

Thorburn, A., J. Thorburn, S.Y. Chen, S. Powers, H.E. Shubeita, J.R. Feramisco, and K.R. Chien. 1993. HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. *J. Biol. Chem.* **268:** 2244–2249.

Umbhauer, M., C.J. Marshall, C.D. Mason, R.K. Old, and J.C. Smith. 1995. Mesoderm induction in Xenopus caused by activation of MAP kinase. *Nature* **376:** 58–62.

Vogelstein, B. and K.W. Kinzler. 1993. The multistep nature of cancer. *Trends Genet.* **9:** 138–141.

Welch, D.R., A. Fabra, and M. Nakajima. 1990. Transforming growth factor beta stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc. Natl. Acad. Sci.* **87:** 7678–7682.

Whitman, M. and D.A. Melton. 1992. Involvement of p21ras in Xenopus mesoderm induction. *Nature* **357:** 252–254.

Wong, S.Y., A.T. Purdie, and P. Han. 1992. Thrombospondin and other possible related matrix proteins in malignant and benign breast disease. An immunohistochemical study. *Am. J. Pathol.* **140:** 1473–1482.

Wright, J.H., S. McDonnell, G. Portella, G.T. Bowden, A. Balmain, and L.M. Matrisian. 1994. A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progression. *Mol. Carcinog.* **10:** 207–215.

Zambruno, G., P.C. Marchisio, A. Marconi, C. Vaschieri, A. Melchiori, A. Giannetti, and M. De Luca. 1995. Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: Implications for wound healing. *J. Cell Biol.* **129:** 853–865.

TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells.

M Oft, J Peli, C Rudaz, et al.

Genes Dev. 1996, **10:**
Access the most recent version at doi:10.1101/gad.10.19.2462