Ras and TGFβ cooperate to regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways

Elzbieta Janda, Kerstin Lehmann, Iris Killisch, Martin Jechlinger, Michaela Herzig, Julian Downward, Hartmut Beug, and Stefan Grünert

Institute of Molecular Pathology, A-1030 Vienna, Austria
Signal Transduction Laboratories, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

Introduction
Carcinomas are the most frequent type of malignancies in humans. They arise by multistep mechanisms, involving five to eight genetic alterations in dominant oncogenes or tumor suppressor genes. These mutated genes cause alterations in at least six types of cellular mechanisms, which cooperate to generate a fully malignant carcinoma cell (for review see Hanahan and Weinberg, 2000). Of these events, local invasion and metastasis of tumor cells are the least well understood at the molecular level, although metastases cause ~90% of human cancer deaths. Particularly, it is unknown which and how oncogenes or tumor suppressors cause changes typical for invasive/metastatic cells such as loss of altered functionality of cadherins (Christofori and Semb, 1999), CAMs (Perl et al., 1999), integrins, and their extracellular matrix ligands (Lukashev andWEB, 1998), and extracellular proteases and their inhibitors (Bergers and COUSSENS, 2000). Except for E-cadherin (Christofori and Semb, 1999), mutations in these genes themselves have not been observed.

During normal development, tissue reorganization, wound healing, and carcinogenesis, epithelial cells may transiently or even stably lose epithelial polarity and acquire a mesenchymal phenotype. Depending on the cell model and type of exogenous signal applied, these phenotypical changes have been termed transformation, dedifferentiation, scattering, or epithelial mesenchymal transition (EMT). EMT is relatively ill defined, always involving a change to a fibroblastoid spindle-shaped cell morphology, whereas changes in epithelial/mesenchymal markers or gene expression may diver widely (for reviews see Hay, 1995; Thiery and Chopin, 1999). EMT is also still little understood in mechanistic...
Transforming growth factor β (TGFβ),* TGFβ receptor (TGFβ-R), and oncogenic Ras have been identified as important molecular players in EMT and metastasis (Oft et al., 1996, 1998) using well-polarized mammary epithelial cells (EpH4) (Reichmann et al., 1992) as a combined in vitro/in vivo model. EpH4 cells are nontumorigenic and require low amounts of TGFβ for organotypic tubular morphogenesis in type I collagen gels, whereas higher TGFβ concentrations induce cell cycle arrest and apoptosis (Oft et al., 1996). In contrast, EpH4 cells transformed by oncogenic Ha-Ras (EpRas) form rapidly growing tumors in mice and undergo EMT in response to TGFβ both in tumors and in collagen gels, giving rise to mesenchymal-like cells (EpXT) in both cases. These EpXT cells are characterized by a spindle-like morphology and loss of epithelial and gain of mesenchymal marker proteins, a phenotype stabilized by an autocrine TGFβ loop in vitro and in vivo (Oft et al., 1996).

Further work revealed that TGFβ-R signaling (Massague and Wotton, 2000) was required for EMT, invasion in vitro, and metastasis in vivo, using diverse murine and human cell systems (Oft et al., 1998). This tumor-promoting role of TGFβ did not agree with either the known growth-inhibitory tumor-suppressive function of TGFβ or inactivating mutations of Smad4 or the TGFβ-R in certain cancers, suggesting a function of these genes as tumor suppressors (Parsons et al., 1995; Schutte et al., 1996). However, more recent findings argue against TGFβ-R signaling module and TGFβ-R signaling.

Results

EMT and scattering: two distinct processes in epithelial plasticity induced by growth factors or oncogenes

In addition to oncogenic Ras (Oft et al., 1996), hepatocyte growth factor (HGF)/scatter factor (SF), fibroblast growth factor (FGF), and TGFβ alone induce mesenchymal features in diverse epithelial cell systems (Brinkmann et al., 1995; Pick et al., 1999; Thiery and Chopin, 1999). We investigated whether these different signals would induce similar or different types of mesenchymal phenotypes in EpRas cells. These represent Ha-Ras--transformed fully polarized EpH4 cells (Oft et al., 1996), spontaneously immortalized from mammary glands of midpregnant mice (Reichmann et al., 1992). EpRas cells were seeded in serum-free collagen gels and subjected to different factor treatments. HGF/SF induced EpRas cells to invade collagen gels and grow as disordered chords of nonpolarized cells (Fig. 1 A; true for >95% of >100 structures counted in two to three separate collagen gels; see Materials and methods). Upon HGF/SF removal, >90% of these factor-induced disordered structures reverted to hollow tubular structures after an additional 5 d (Fig. 1 B). The disordered collagen gel structures formed by the HGF/SF-treated cells continued to express β4-integrin, E-cadherin, and ZO-1 at the entire cell surface (indicating loss of polarity) and failed to express vimentin (Fig. 1 C, left; unpublished data). Upon factor withdrawal, β4-integrin regained polarized basal expression in the hollow tubules formed (Fig. 1 C, right). Likewise, E-cadherin and ZO-1 were again expressed basolaterally (Fig. 1 C, inset; unpublished data). Similar results were obtained using FGF (unpublished data).

Controls using EpRas cells undergoing EMT in response to TGFβ showed that the spindle-like phenotype induced by TGFβ treatment (Fig. 1 A, inset) persisted after factor removal (Fig. 1 B, inset) and involved loss of E-cadherin/β4-integrin, whereas vimentin was strongly induced (Fig. 1 D, left). Again, these marker changes persisted after TGFβ removal (Fig. 1 D, right). These results show that HGF and FGF reversibly induce a spindle-like migratory phenotype in EpRas cells, which is characterized by loss of polarity but involves no lasting changes in epithelial or mesenchymal marker expression. We refer to this phenotype as scattering, since FGF and HGF also induce “classical” scattering on plastic (unpublished data), and distinguish it from EMT, which is not re-

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*Abbreviations used in this paper: EMT, epithelial mesenchymal transition; Erk, extracellular signal–regulated kinase; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; P13K, phosphatidylinositol 3-kinase; SF, scatter factor; TGFβ, transforming growth factor β; TGFβ-R, TGFβ receptor.
versed upon factor removal and involves stable loss of epithelial markers and upregulation of mesenchymal markers.

To answer the question if and how TGFβ alone (Piek et al., 1999) would alter the phenotype of nontransformed EpH4 cells, we used EpH4 cells expressing a retrovirally transduced antiapoptotic Bcl-2 protein. This was necessary since doses of TGFβ (2–5 ng/ml) causing EMT in EpRas cells induced growth arrest and apoptosis in EpH4 cells. In the absence of TGFβ, the Bcl-2–expressing EpH4 cells formed tubular structures in collagen gels similar to EpH4 control cells (Fig. 1, E compared with F). When treated with TGFβ, the EpH4–Bcl-2 cells were apoptosis protected as expected (8% TUNEL-positive cells; Fig. 1 G, inset) and formed disordered structures consisting of migratory cells (Fig. 1 G), whereas control EpH4 cells underwent apoptosis (48% TUNEL-positive cells; Fig. 1 E, inset). However, in contrast to EpRas cells the TGFβ-treated Bcl-2–expressing EpH4 cells reverted to hollow tubular structures 4 d after TGFβ removal (Fig. 1 H).

Likewise, they maintained E-cadherin expression throughout the experiment, whereas vimentin expression was not detectable (unpublished data). In conclusion, TGFβ causes reversible scattering in EpH4 cells apoptosis protected by Bcl-2 but fails to induce EMT in the absence of oncogenic Ras, suggesting that Ras induces an EMT competent state in addition to prevent TGFβ-induced apoptosis.

Active oncogenic Ras is required for both induction and maintenance of EMT

Using a kinase-dead dominant negative mutant of the TGFβ-RII, we showed previously that maintenance of TGFβ-R signaling is required for EMT and metastasis (Oft et al., 1998). To address if EMT also required maintenance
Figure 2. Ras activity is required for induction and maintenance of EMT. (A–D) EpXT cells were seeded into collagen gels in the absence (A) or presence (B) of 10 μM Ras farnesylation inhibitor (L739749), and resulting structures were photographed after 6 d (as described in Materials and methods). Note the complete reversal of EpXT cells to tubular structures with lumina (white arrows), which persisted after removal of the inhibitor for 4 d (C). The inhibitor alone did not affect tubular structures formed by EpRas cells (B, inset) or after reversal (D, inset, control), whereas treatment of the reverted structures with TGFβ plus L739749 caused cell disintegration. (E and F) Immunostaining of frozen ultrathin sections of EpXT collagen gel structures before (E) or after treatment (F) with L739749 for 5 d. Sections were stained with
of oncogenic Ras activity, we used a specific nontoxic inhibitor of Ras-farnesylation (L739749 [Kohl et al., 1994]). L739749 reversed EMT in mesenchymal EpXT cells, causing reformation of hollow tubular structures (Fig. 2 B) from the unordered cords and strands of EpXT cells (Fig. 2 A) but did not affect EpH4 (Fig. 2 B, inset). After inhibitor withdrawal, the reverted cells maintained their polarized phenotype as indicated by persistence of tubular structures (Fig. 2 C). However, these structures could be reinduced to undergo EMT when again treated with TGFβ (unpublished data). This indicates that Ras inhibition reversed EMT completely, converting EpXT cells into cells with EpH4 cell properties. In line with this, reactivation of Ras in the reverted cells was insufficient for EMT, again requiring TGFβ as in the original EpRas cells (Oft et al., 1996). Furthermore, cotreatment of the reverted cells with L739749 and TGFβ resulted in cell death (Fig. 2 D), whereas their treatment with Ras inhibitor alone caused no phenotypical changes (Fig. 2 D, inset).

These results were confirmed by analyzing marker expression, using ultrathin cryosections stained with respective fluorescent antibodies (see Materials and methods) from similar collagen gel structures as shown in Fig. 2, A and B. As expected, the mesenchymal chords of untreated EpXT cells expressed vimentin and intracellular TGFβ (Fig. 2 E, top) and deposited fibronectin extracellularly (periphery of structures and intercellular spaces; Fig. 2 E, bottom). After treatment with L739749 for 5 d (Fig. 2 F), vimentin and TGFβ expression was no longer detectable in the hollow structures obtained (Fig. 2 F, top). Two additional mesenchymal markers, CD68 (macрослalin) and calgranulin A, were also downregulated (unpublished data). Furthermore, the L739749-treated cells reexpressed the tight junction marker ZO-1 (Fig. 2 F, bottom) and E-cadherin (unpublished data) in a polarized basolateral fashion. The presence of tight junctions in the epithelial tubules induced by L739749 were confirmed by EM, using immunogold staining for ZO-1, although the mesenchymal chords obtained in the absence of inhibitor failed to show any junction-like structures (unpublished data).

In conclusion, inhibition of Ras in EpXT cells completely restores a normal polarized epithelial phenotype, indicating that sustained oncogenic Ras and TGFβ-R signaling are required for induction and maintenance of EMT in EpRas cells.

**Ras downstream signaling required for EMT: analysis by low molecular weight inhibitors**

Oncogenic Ha-Ras activates multiple downstream signal transduction pathways, including the Raf/MAPK module and the PI3K-PKB/Akt pathway (for review see Rommel and Hafen, 1998). Both pathways were implied in cell transformation in vitro (Rodriguez-Viciana et al., 1997) and tumorigenesis (Webb et al., 1998). To dissect Ras-activated downstream pathways required for different aspects of Ha-Ras transformation in epithelial cells (EMT, scattering, and apoptosis protection), we employed two specific low molecular weight inhibitors selectively blocking either the MAPK pathway (PD98059) or PI3K signaling (LY294002).

The activity of the Mek1/MAPK- and PI3K-PKB/Akt pathways in EpRas and EpH4 cells and the ability of the two inhibitors to selectively suppress either pathway was analyzed by Western blots using phospho-specific antibodies to MAPK/extracellular signal–regulated kinase (Erk)1/2 and PKB/Akt. EpRas cells exhibited strongly elevated phospho-Erk/MAPK and phospho-PKB/Akt levels compared with EpH4 cells (Fig. 3 A, lanes E and R). These elevated levels were largely independent of cell density (unpublished data). Testing of the inhibitors in EpRas cells revealed that 10 μM PD98059 reduced MAPK phosphorylation to basal levels, whereas 40 μM completely abolished MAPK phosphorylation (unpublished data). Conversely, PKB/Akt phosphorylation levels were not affected by PD98059 (Fig. 3 A, left, lanes E and R; unpublished data). Conversely, 5 μM LY294002 added at 12-h intervals stably reduced elevated PKB/Akt phosphorylation in EpRas cells to basal levels (Fig. 3 A, middle; unpublished data), whereas 30 μM LY294002 essentially abolished PKB/Akt phosphorylation (Fig. 3 A, right). In contrast, even high levels of LY294002 had no effect on MAPK phosphorylation in EpRas cells (Fig. 3 A).

Next, we analyzed the effects of the two inhibitors on TGFβ-induced EMT in collagen gels (Fig. 3 B, top). 10 μM PD98059 prevented EMT in >95% of the structures, giving rise to hollow tubules (Fig. 3 B, top) typical for control EpRas cells (Fig. 3 A, inset). These inhibitor-treated structures failed to express vimentin while retaining E-cadherin (unpublished data). In contrast, 5 μM LY294002 did not affect TGFβ-induced EMT in EpRas cells (Fig. 3 B, bottom left; unpublished data), whereas 30 μM LY294002 (almost completely suppressing PI3K signaling; Fig. 3 A) caused cell disintegration (Fig. 3 B, bottom right). None of the inhibitors strongly altered formation of hollow structures in the absence of TGFβ, showing that they were nontoxic at the respective concentrations used (Fig. 3, A–D, insets).

The disintegration of EpRas cells observed after treatment with 30 μM LY294002 plus TGFβ was clearly due to strongly increased apoptosis as shown by TUNEL staining or dye exclusion (Fig. 3, C and D; see Materials and methods). In contrast, 10 μM PD98059 or 5 μM LY294002 plus TGFβ and LY294004, PD98059, or TGFβ added alone failed to significantly increase apoptosis (Fig. 3, C and D; unpublished data). In conclusion, high levels of Mek1/MAPK activity are necessary for induction and maintenance of EMT. Similar elevated levels of PI3K pathway activity are required for protection from TGFβ-induced apoptosis but not for EMT.

**Effectorspecific Ras mutants in vitro: MAPK hyperactivation is required for EMT, whereas PI3K signaling causes scattering**

To verify these results by an independent approach, two well-characterized Ras mutants were used, which selectively signal along the MAPK pathway, S35-V12–Ras (S35-Ras), or the
PI3K pathway, C40-V12–Ras (C40-Ras), due to specific amino acid changes in the effector loop of the Ras protein (Rodriguez-Viciana et al., 1997; Downward, 1998). These mutant proteins and the parental oncogenic Ras protein (V12-Ras) were overexpressed in EpH4 cells using respective retroviral constructs (see Materials and methods). Since V12-Ras did not reach similar expression levels in mass cultures as seen for v-H-Ras in EpRas cells (unpublished data), clones were selected expressing particularly high levels of V12-Ras, S35-Ras, and C40-Ras. In Western blots, S35-Ras–expressing clones showed elevated phosphorylation of Erk1 but only basal levels of PKB/Akt phosphorylation compared with empty vector controls. In contrast, the C40-Ras clones showed only basal level phosphorylation of Erks but elevated levels of PKB/Akt phosphorylation (Fig. 4 A). As expected, the V12-Ras control cells showed elevated levels of both phospho-Erk and phospho-PKB/Akt. Similar results were obtained with pools of >10 clones selected also for high level expression of V12-Ras, S35-Ras, and C40-Ras proteins (Fig. 4 B; unpublished data; see Materials and methods).

The Ras mutant-overexpressing clones (Fig. 4 A) were then characterized for their response to TGFβ. On porous supports (filters) allowing epithelial polarization, treatment of control V12-Ras cells and two S35-Ras–overexpressing clones with TGFβ for 7 d resulted in spindle-shaped, vimentin-positive, E-cadherin–negative cells (Fig. 4 C; unpublished data). In contrast, C40-Ras–overexpressing cells failed to lose E-cadherin or to upregulate vimentin but showed loss of polarity (redistribution of E-cadherin; Fig. 4 C). As expected, control cells not treated with TGFβ always expressed lateral E-cadherin and no vimentin (Fig. 4 C, insets).

In collagen gels, untreated S35-Ras cells resembled V12-Ras cells (e.g., forming distended tubular structures with large lumina in collagen gels), whereas C40 cells more closely resem-
bled EpH4 cells (thin tubules with tiny lumina; Fig. 5 A, top). Marker staining revealed basolateral E-cadherin staining and no expression of mesenchymal markers in all three cell types (Fig. 5 B, insets; unpublished data). Treatment of V12-Ras, S35-Ras, and C40-Ras cells with TGFβ resulted in unordered cell strands and cords with spindle-like cellular morphology (Fig. 5 A, middle). After withdrawal of TGFβ, these lumenless disordered structures persisted in the V12-Ras and S35-Ras cells, whereas C40-Ras cells reverted to thin hollow structures (Fig. 5 A, bottom). Likewise, TGFβ-treated S35-Ras cells showed loss of E-cadherin/β4-integrin staining and induction of the mesenchymal markers vimentin and CD68 (Fig. 5 B, top), a marker distribution persisting after removal of TGFβ (Fig. 5 B, bottom). In contrast, C40-Ras–expressing cells maintained nonpolar E-cadherin staining in the disordered structures induced by TGFβ, whereas mesenchymal markers remained undetectable (Fig. 5 B, top). Upon withdrawal of TGFβ, the C40-Ras structures regained epithelial polarity as indicated by lumen formation and basolateral E-cadherin staining (Fig. 5 B, bottom).

In conclusion, a hyperactive MAPK/Erk pathway plus TGFβ is sufficient to cause EMT, whereas elevated PI3K signaling plus TGFβ induces scattering, that is, spindle-shaped cells without loss of epithelial and gain of mesenchymal markers.

**Effectorspecific Ras mutants: protection from TGFβ-induced apoptosis requires PI3K signaling**

Oncogenic Ras and Raf abolish TGFβ-induced apoptosis in several epithelial cell systems (Oft et al., 1996; Lehmann et al., 2000). V12-Ras, S35-Ras, and C40-Ras cells (from both clones and clone pools; Fig. 4 A) were therefore tested for their apoptotic response upon TGFβ treatment. This was done using cells pregrown in collagen gels for 3–4 d (Fig. 6 A), since detection of apoptosis was much easier in these cultures than on plastic (Lehmann et al., 2000). Collagen structures were either collagenase digested and the cells subjected to TUNEL staining in suspension (Fig. 6 C, top) or TUNEL stained and counted in situ, that is, within the intact collagen gels (Fig. 6, B and C, bottom; see Materials and methods).

Both approaches yielded similar results. Already at low TGFβ levels (1 ng/ml), apoptosis of EpH4 control cells was enhanced significantly, whereas >95% of these cells were TUNEL positive and thus apoptotic at 5 ng/ml TGFβ (Fig. 6 C). EpH4 cells expressing V12-Ras and C40-Ras showed no significant elevation of apoptotic cells even at high TGFβ levels (Fig. 6 C).
concentrations. In contrast, S35-Ras cells were consistently sensitive to TGFβ-induced apoptosis but required higher TGFβ levels for complete apoptosis than EpH4 cells (Fig. 6, B and C). Thus, hyperactivation of the PI3K pathway is required for protection from TGFβ-induced apoptosis. In contrast, hyperactive MAPK signaling in S35-Ras cells failed to induce apoptosis protection at higher TGFβ levels.

**Effector-specific Ras mutants in vivo: elevated MAPK and PI3K signaling are each sufficient for tumor formation**

To analyze the importance of Ras downstream signaling pathways for tumor formation in vivo, clones strongly overexpressing V12-Ras, S35-Ras, and C40-Ras were injected into the mammary gland fat pads of nude mice. All cell types caused tumors with comparable efficiencies (16/16 injection sites; Fig. 7 A). Similar results were obtained with respective pools of clones overexpressing the different Ras signaling mutants except that tumor formation by cells from S35-Ras clone pools was significantly slower than from respective C40-Ras clone pools (unpublished data). Bcl-2–EpH4 control cells formed similarly small regressing nodules as EpH4 cells, which contained only well-polarized, E-cadherin–positive, vimentin-negative cells (unpublished data). Therefore, protection of EpH4 cells from TGFβ-dependent apoptosis is not sufficient for tumorigenesis.

To address which phenotypic changes of the Ras mutant-expressing cells accompanied tumor growth, we recultivated cells from 4–5-wk-old tumors, using G418 to select for ex-tumor cells of donor origin (see Materials and methods) and analyzed them for E-cadherin and vimentin expression by immunofluorescence. These ex-tumor cells closely resembled the respective Ras mutant-expressing cells subjected to TGFβ treatment in vitro (Fig. 4 C compared with Fig. 7 B). V12-Ras and S35-Ras ex-tumor cells showed a typical spindle-shaped morphology (unpublished data) and were vimentin-positive and E-cadherin–negative (Fig. 7 B, left and middle). In contrast, the C40-Ras ex-tumor cells showed...
epithelioid morphology (unpublished data) and E-cadherin staining, whereas vimentin was undetectable. In conclusion, the separate activation of either the PI3K pathway or the MAPK pathway is sufficient to confer tumorigenic potential to EpH4 cells, whereas EMT in vivo requires the MAPK pathway (see Discussion).

**Effector-specific Ras mutants in vivo: only hyperactive MAPK can induce metastasis**

Finally, we sought to determine whether or not V12-Ras–, S35-Ras–, or C40-Ras–expressing cells were able to form metastases. In line with metastasis formation in nude mice being a rare event (McClatchey, 1999), EpRas-derived primary tumors rarely progressed to metastasis before killing the animal. Resection of primary tumors to allow for late metastasis formation met with technical difficulties. Therefore, ex-tumor cells derived from two clones of S35-Ras cells and one clone each of C40-Ras and V12-Ras cells were injected into the tail veins of nude mice, an assay testing for survival of injected cells in the circulation and for evasion and colonization of distant organs. Most of the mice injected with V12-Ras or S35-Ras cells died between 2 and 5 wk, whereas all C40-Ras–injected mice survived until the end of the experiments (10 wk; Fig. 8 A). Upon histological analysis, the lungs of dead or moribund animals injected with V12-Ras or S35-Ras cells showed multiple metastases in all cases, often surrounded by blood vessels (Fig. 8 B, left). In contrast, lungs from C40-Ras–EpH4 injected animals were histologically free of metastases (Fig. 8 B, right), and no metastases were found in other organs (unpublished data). Similar results were obtained using pooled clones of V12-Ras–, S35-Ras–, or C40-Ras–EpH4 cells except that the S35-Ras cell-derived metastases appeared much later in line with the slower growth rate of the respective primary tumors (unpublished data).

These results indicate that metastasis formation seems to depend entirely on hyperactivity of the Raf/MAPK pathway, whereas a hyperactive PI3K pathway (in C40-Ras cells) is not sufficient for metastasis formation.
Discussion

In this paper, we utilize a well-characterized combined in vitro/in vivo model of mammary carcinogenesis (EpRas) to address the role of distinct Ras downstream signaling pathways in epithelial plasticity, tumorigenesis, and metastasis. We show that Ras-dependent hyperactive signaling of the Raf/MAPK pathway is required for EMT, whereas Ras activation of the PI3K pathway causes scattering and is required for protection from TGFβ/H9252-induced apoptosis. In vivo, a hyperactive PI3K pathway correlated with tumor formation, whereas a hyperactive MAPK pathway caused both tumors and metastasis. Thus, activation of two different Ras downstream signaling pathways during tumorigenesis and metastasis cause two clearly distinct changes toward a motile phenotype, only one of which (EMT) is associated with metastatic potential.

EMT and scattering, two distinct aspects of epithelial plasticity

Our work and work by others have established several operational criteria for EMT. These are (a) a requirement for cooperation of a hyperactive MAPK–Erk1/2 pathway with TGFβ–R signaling, (b) metastability, that is, persistence of the EMT phenotype after removal of TGFβ, and (c) drastic long-lasting molecular changes such as loss of E-cadherin and a switch to a mesenchymal gene expression pattern (Oft et al., 1996; Eger et al., 2000; Lehmann et al., 2000). These criteria apply only partially to epithelial plasticity changes (often also termed EMT) observed by others in different cell systems in response to Ras alone (Chen et al., 2000), Ras or Raf plus TGFβ (Oft et al., 1998; Lehmann et al., 2000), FGF, EGF, and SF/HGF (Thiery and Chopin, 1999; Boyer et al., 2000), and finally TGFβ alone (Piek et al., 1999; Bakin et al., 2000). These systems differ widely with respect to reversibility and switch from an epithelial to a mesenchymal gene expression program, their common denominator being morphological changes to a spindle-like phenotype.

These discrepancies may be explained by one major finding of this paper, showing that different signals can cause qualitatively different alterations of epithelial plasticity in the same cell type. In EpH4 cells, Ras or upstream receptor tyrosine kinases (e.g., activated HER2; unpublished data) plus TGFβ caused EMT. In contrast, HGF, FGF, or TGFβ alone caused scattering in apoptosis-protected EpH4 cells. Scattering involves a similar spindle-like migratory pheno-
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Type as EMT but involves only transient gene expression changes and is fully reversible. Thus, it is possible that some of the various types of epithelial plasticity termed EMT by others (see above) may correspond to scattering rather than EMT or represent hybrid phenotypes. Scattering may also occur during the initial stages of organotypic tubule formation in collagen gels (unpublished data), perhaps corresponding to tissue remodeling during outgrowth of glandular epithelial cells apparently dependent on PI3K plus Mek1/Erk signaling (Khwaja et al., 1998).

Multiple functions of Ras during EMT and tumorigenesis

Oncogenic Ras, the second player in EMT, displays diverse functions in EpH4 cells. First, Ras protects EpH4 cells from apoptosis, both by upregulation of PI3K and by downregulation of Fas receptor, rendering the cells refractory to Fas ligand (Peli et al., 1999). Second, oncogenic Ras or Raf alone cause more plastic cell–cell contacts, increased migration, and distended tubular or alveolar structures with large lumina in collagen gels (Oft et al., 1996; Lehmann et al., 2000). These changes may be due to Ras-dependent upregulation of N-cadherin expression in EpH4 cells, which functionally substituted for E-cadherin and contributed to the altered behavior of EpRas cells (unpublished data). In line with this, N-cadherin regulates cell motility and invasiveness during development and in various carcinoma cell lines (Radice et al., 1997; Nieman et al., 1999).

Importantly, even strongly overexpressed oncogenic Ras does not abolish epithelial polarity and organotypic structure formation on its own in several epithelial cell types (Lehmann et al., 2000; Gotzmann et al., 2002). The evidence from others with respect to this issue is controversial. In MDCK cells, Ras either caused loss of E-cadherin and ZO-1 expression and change to a fibroblastic morphology (Chen et al., 2000) or a partial loss of epithelial polarity, maintaining intact, tight, and adherent junctions (Schoenenberger et al., 1991). These discrepancies, probably due to heterogeneity of the MDCK cells studied or effects by TGFβ present in serum, underline the importance of using cell systems, which form organotypic epithelial structures under serum-free conditions where levels of TGFβ and other factors can be strictly controlled.

On the other hand, TGFβ-dependent EMT required strong overexpression of oncogenic Ras. This was shown in

Figure 8. **S35-Ras–but not C40-Ras–overexpressing EpH4 cells form lung metastases upon tail vein injection.** (A) Ex-tumor cells from the V12-Ras (black symbols), S35-Ras (red symbols), and C40-Ras clones (blue symbols) indicated were injected intravenously into four nude mice per cell type. Moribund mice were killed and analyzed for the presence of lung metastases. The two surviving mice injected with S35-Ras clones were killed at day 55 and also contained lung metastases. None of the four mice injected with C40-Ras cells (killed after 10 wk) showed any lung metastases. (B) Histological stainings of sections from lungs of uninjected animals (left) and S35-Ras (middle) and C40-Ras–injected mice (two animals each). Note large metastases (also around blood vessels) only in the S35-Ras–injected mice. Bar, 100 μm.
cells expressing low or intermediate Ras levels, which are protected from apoptosis and undergo scattering but not EMT in response to TGFβ (unpublished data). The same was true for saturating doses of TGFα (1 μg/ml) when added to EpH4 cells together with standard doses of TGFβ (unpublished data). These findings raise the question whether EMT requires sustained nuclear translocation of MAPK, occurring during neuronal differentiation of PC12 cells induced by overexpressed receptor tyrosine kinases (Traverse et al., 1994). It is also unclear which endogenous pathways cooperate with TGFβ family ligands when EMT is induced during development (Sun et al., 2000).

**Dissection of Ras downstream signaling**

A second important finding of our paper was that hyperactivation of the Raf/MAPK pathway caused EMT in vitro, and in vivo tumor formation and metastasis. Respective activation of the PI3K pathway was sufficient for tumorigenesis but not metastasis, causing both scattering and protection from TGFβ-induced apoptosis. These findings were obtained using both low molecular weight inhibitors of the two pathways and Ras effector mutants selectively activating these pathways. However, these mutants might signal through additional pathways, which we did not assay for. To study whether or not activation of the MAPK and PI3K pathways was sufficient for EMT and tumor formation, respectively, we are analyzing EpH4 cells expressing constitutively active PI3K variants and Mek1–Erk fusion proteins and an estrogen-inducible RafER fusion protein. Preliminary results suggest that RafER is not sufficient for EMT of EpH4 cells in serum-free collagen gels (unpublished data) in contrast to RafER-expressing MDCK cells, which underwent EMT in serum-containing cultures upon RafER activation (Lehmann et al., 2000). We currently analyze whether RafER-EpH4 cells require both TGFβ and basal PI3K signaling induced by endogenous RTK ligands, both obviously provided by serum.

So far, our studies did not address the role of the MAPK and PI3K pathways in controlling proliferation of EpRas cells either in collagen gels or in tumors. Our recent work revealed that a moderate upregulation of the PI3K pathway causes hyperproliferation of EpH4 cells in collagen gels and rapid tumor growth but not EMT. In contrast, cells showing a similarly upregulated MAPK pathway grow less rapid in collagen gels and form only slowly growing tumors, which nevertheless contained islands of mesenchymal tumor cells having undergone EMT (unpublished data). This indicates that tumor growth and EMT are controlled by independent pathways and makes it very unlikely that the lack of in vivo EMT in the C40-Ras–induced tumors is due to their slightly slower growth rate as compared with respective S35-Ras clones (Fig. 7 A).

**Prevention of TGFβ-dependent growth arrest/apoptosis without abolishing TGFβ-R signaling?**

TGFβ blocks cell cycle progression and causes apoptosis in most normal epithelial cells, suggesting that an important step in carcinogenesis is to overcome this antiproliferative effect of TGFβ (Fynan and Reiss, 1993). Multiple human tumors indeed carry inactivating mutations in Smad4 (Schutte et al., 1996) or even TGFβ-RII (Parsons et al., 1995). However, complete suppression of TGFβ-R signaling by such tumor suppressive mutations would also abolish the dominant oncogenic effects of TGFβ, for example, EMT and metastasis. This discrepancy is not yet fully explained by available data. However, the rare tumors with TGFβ-RII mutations have a low or absent ability to metastasize (Oft et al., 1998). Furthermore, TGFβ stimulation still induced a subset of TGFβ–specific promoters in MEF’s from Smad4−/− mice (Sirard et al., 1998), suggesting that mammalian cells might contain a second Smad4 family member already described in frogs (Smad4β/Smad10) (Howell et al., 1999). Finally, TGFβ-induced cell cycle arrest is also lost frequently by mutations in downstream effectors like p16ink4A/p15ink4B (Hall and Peters, 1996).

These results suggest that most carcinoma cells are selected for resistance to TGFβ cell cycle arrest while remaining sensitive for induction of migration/invasion by TGFβ. In line with this, murine and human cell lines completely resistant to cell cycle arrest by TGFβ retain apparently normal TGFβ-R signaling (Oft et al., 1998; McEarchern et al., 2001).

Obviously, oncogenic Ras completely substitutes for this type of tumor cell selection in the various epithelial cell models described above, including also strong protection from TGFβ-induced apoptosis. However, possible mechanisms for how Ras may modulate TGFβ-R signaling are still controversially discussed. Ras signaling was reported to attenuate Smad2/3 signaling via inhibitory phosphorylation, preventing nuclear translocation (Kretzschmar et al., 1999), but we and others failed to observe Ras-dependent inhibition of Smad2 or Smad3 nuclear translocation by elevated Ras or Raf activity in several systems (Lehmann et al., 2000; Liu et al., 2000). Therefore, the MAPK pathway might attenuate TGFβ responses via the induction and/or activation of new distinct transcription factors, corepressors, or coactivators recruited by Smads into a variety of transcriptional modules, which determine the diversity of responses to similar TGFβ signals in different cell types (Massague and Wotton, 2000).

**Metastasis: which EMT-related changes in gene expression are important?**

The results presented here strongly correlate metastasis to EMT, requiring cooperation of TGFβ-R–signaling with a hyperactive MAPK pathway and thus arguing for a dominant oncogenic role of the TGFβ-R pathway in addition to possible tumor suppressive functions. This positive role of TGFβ in EMT and metastasis correlates best with the defect of TGFβ-3−/− mice in palate development (Taya et al., 1999), which involves EMT, and by the defects in mesoderm induction in mice lacking Smad4 or Smad2 (Weinstein et al., 1998; Yang et al., 1998).

Which TGFβ–induced gene expression changes are important for EMT? One clear example is the loss of E-cadherin typical for EMT, which has been implicated in tumorigenesis and metastasis (Christofori and Semb, 1999). In line with this, the transcriptional repressor Snail blocks E-cadherin expression and causes a fibroblastoid phenotype and enhanced tumor progression in weakly tumorigenic car-
cinoma cell lines (Cano et al., 2000). However, mesenchymal FosER cells after EMT completely lack E-cadherin but form neither tumors nor metastases (Reichmann et al., 1992), indicating that loss of E-cadherin expression may be necessary but not sufficient for tumor progression.

Materials and methods

Cells and cell culture

The origin and culture conditions for EpH4 mouse mammary epithelial cells and their Has-Ras-transformed derivative EpRas have been described earlier (Reichmann et al., 1992; Oft et al., 1996). V12-Ras-, S35-Ras-, and C40-Ras-expressing cells were generated by retroviral gene transfer using retroviral vectors of V12-Ras and its two mutants S35-V12-Ras and C40-V12-Ras in pLXSN (Rodriguez-Viciana et al., 1997). Bcl-2-expressing cells were generated as above by transfer of pBabe-puro Bcl-2 (a gift from M. Bussinger, Institute of Molecular Pathology). Construct DNA was used to transfect cells from the Phoenix™ packaging cell line (grown in DME plus 10% FCS and 2 mM glutamine, after recent hygromycin and G418 toxicity selection for optimal virus production). After 2–3 d, virus supernatants from the transfected packaging cells were used to infect EpH4, which were then selected with 800 μg/ml G418 (5–6 d) or 2 μg/ml puromycin (3–4 d). Clones were isolated from retrovirally infected and drug-selected cell populations by seeding at low density, marking single adherent cells 6 h later, expansion for 7–9 d, and isolation of colonies using trypsin-soaked filter paper circles.

Cells from mouse tumours were recultivated from finely minced tumours by digestion in 2 mg/ml collagenase type VI (Sigma-Aldrich) plus 5% FCS for 30 min. Dispersed cells were seeded in Eagle’s medium plus 15% FCS, selected for 7 d in 800 μg/ml G418 (day 2–8), and subcultured at a ratio of 1:3 every 2 d. During the first 2 d, 2 mg/ml TGFβ were added to facilitate outgrowth of ex-tumor cells.

Reagents and antibodies

10 μg/ml stocks of recombinant TGFβ-1, acidic FGF-1, and HGF/SF (R & D Systems) were prepared according to manufacturer’s instructions, that is, FGF-1 solutions containing 1 μg/ml of heparin. The PHK3 and Mek-1 inhibitors LY294002 and PD98059 (Calbiochem) were kept as 20–50 μM stocks in DMSO. The Ras farnesylation inhibitor L 739.749 (Kohl et al., 1992), formaldehyde stocks stored at –20°C, and 0.05% Tween. Filters were then incubated with first antibody (diluted in blocking solution lacking nonimmune IgGs) for 1 h, washed five times with blocking solution (PBST, 0.1% gelatin plus 10% nonimmune bovine IgG). Gels were incubated with primary antibodies plus DAPI in blocking solution for 1 h at 37°C in a wet chamber, washed three times in PBST for 30 min, and postfixed with 4% PFA in PBST for 15 min at 23°C. After 30-min washes in TBST, PBST, and blocking solution, the gels were incubated with appropriate secondary antibody mixtures made up in blocking solution for at least 1 h or overnight at 4°C. Gels were washed three times in PBS, once in distilled water, and mounted in Mowiol (Hoechst). Confocal analysis was performed using a Leica TCS-NT confocal microscope (DAPI visualized by two-photon excitation microscopy using Coherent-Vitesse pulsed NIR laser). From representative gel structures, 5–10 horizontal scans using a 40× (1.3 NA) oil immersion objective were recorded for each channel and used to calculate an extended focus image with the respective software (exported as a TIFF file).

Cryosectioning and immunofluorescent staining of collagen gel structures

Cryosectioning was performed to visualize TGFβ and ZO-1 by antibody staining not possible using whole-mount gels. Collagen gels were fixed for 15 min at 4°C for 20% PFA in 250 mM Hapes followed by 8% PFA in 250 mM Hapes for 1 h and then washed in TBS. Individual structures were excised from the gel under a dissection microscope and placed into PVP/sucrose (Sigma-Aldrich) on ice. Blocks were incubated in PVP/sucrose overnight, mounted upright onto a cryo-nail, flash-frozen in liquid nitrogen, and stored in liquid nitrogen until further use. For cryosectioning, these nails were placed into a Leica Cryo-Microtome and semi-thin sections of 500–800 nm were placed on a gelatin-coated slide. Alternatively, ultrathin sections of 50–100 nm were placed on copper grids for EM analysis. Immunostaining of semi-thin sections was performed in small drops on the slides directly with the same solutions as mentioned above for whole-mount stainings. Samples were analyzed by conventional fluorescent microscopy, recorded with a monochrome digital camera, and the three channel color image was restored from the individual images.

Immunofluorescence of cells grown on porous supports

Cells were cultured on porous supports (cell culture inserts, pore size 0.4 μm, Becton Dickinson) for 2–7 d and fixed either at ~70% confluency (or as fully confluent well-polarized epithelial sheets). Filters were rinsed twice with Hank’s solution plus glucose or PBS, fixed in acetone/methanol (1:1) for 3 min at ~20°C, dried, washed with PBS, and blocked for 1 h in 0.2% BSA in PBS-containing 10% nonimmune goat or bovine IgGs (200 μg/ml) and 0.05% Tween. Filters were then incubated with first antibody (diluted in blocking solution lacking nonimmune IgGs) for 1 h, washed five

with minor modifications. Cells (in serum-containing medium) and rat tail collagen (3–4 mg/ml) (40236; Becton Dickinson) were mixed rapidly at 0°C (final collagen concentration 1.5 mg/ml), and 100-μl droplets containing between 2,000 and 3,000 cells were dispersed into 17-mm wells. When indicated, collagen solutions gels were supplemented with 10% vol/ vol Matrigel solution (GF reduced) (40 230; Becton Dickinson) directly before use. After solidification on a level surface at 23°C for 15–30 min, the gels were incubated at 37°C in a CO2 incubator for another 1–2 h and overlaid with 500 μl of serum-free medium (mammary epithelial basal medium) (C-21010; PromoCell GmbH) supplemented with growth factors according to manufacturer’s instructions. The batches of bovine pituitary extract had to be pretested for optimal performance. The medium overlaying the gel was changed 1 d after seeding and then every other day unless stated otherwise.

After allowing structure formation of the cells for 4–7 d, growth factors (20 ng/ml HGF, 20 ng/ml acidic FGF, and 5 ng/ml TGFβ unless stated otherwise) were added upon medium change every other day. Predetermined amounts of pharmacological inhibitors (20% higher final concentrations to correct for collagen gel volume) where added every 12 or 24 h for 5 d unless stated otherwise. Growth factors were withdrawn from collagen gels by switching them to medium without factors, changing medium after 24 h, and further cultivation for 2–5 d. In all experiments, at least 50–100 structures were inspected to quantify lumen-containing structures versus disordered strands or chords. If >90% of the structures were of one type, quantification is not further mentioned in the Results section.

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Collagen gel structures

Collagen gels (sometimes split in half by a scalpel) were fixed with 1% formaldehyde in 250 mM Hapes, pH 7.4, freshly diluted from 16% paraformaldehyde stocks stored at ~20°C. After 15 min at room temperature, the gels were washed once with each Tris- and phosphate-buffered saline plus 0.2% Tween 20 (TBST and PBST, respectively) and treated for 1 h at 4°C with blocking solution (PBS, 0.1% gelatin plus 10 μg/ml nonimmune bovine IgG). Gels were incubated with primary antibodies plus DAPI in blocking solution for 1 h at 37°C in a wet chamber, washed three times in PBST for 30 min, and postfixed with 4% PFA in PBST for 15 min at 23°C. After 30-min washes in TBST, PBST, and blocking solution, the gels were incubated with appropriate secondary antibody mixtures made up in blocking solution for at least 1 h or overnight at 4°C. Gels were washed three times in PBS, once in distilled water, and mounted in Mowiol (Hoechst). Confocal analysis was performed using a Leica TCS-NT confocal microscope (DAPI visualized by two-photon excitation microscopy using Coherent-Vitesse pulsed NIR laser). From representative gel structures, 5–10 horizontal scans using a 40× (1.3 NA) oil immersion objective were recorded for each channel and used to calculate an extended focus image with the respective software (exported as a TIFF file).

Western blot analysis

Cells were used for Western blot analysis of either serine-phosphorylated or total Erk and Ras proteins were 80% confluent and cultured in 4% FCS or starved for 48 h (for phospho-Akt and total Akt). Plates were washed in PBS and lysed on ice with kinase buffer (10 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 30 mM sodium pyrophosphate, 10 mM NaVO3, 2 mM o-acidic acid, 10 mM Pefablock® and cocktail of protease inhibitors Complete™ both from Boehringer), centrifuged at 12,000 g for 10 min at 4°C, and the pellet was discarded. Freshly prepared lysates were analyzed by 8–10% SDS-PAGE and immunoblotted as described by others (Yu and Sato, 1999). Total V12-Ras expression was analyzed by 12% SDS-PAGE followed by Western blot analysis as above.

Collagen gel cultures

Serum-free three-dimensional cultures of EpH4 cells, EpRas cells, and their derivatives were described as earlier (Oft et al., 1996, 1998)
times in PBS containing 0.05% Tween, treated with similarly prepared di-
lutions of secondary antibodies for 30 min, and washed again as above
(first wash containing DAPI; 1 mg/ml stock, final dilution 1:10,000). Alexa-
conjugated secondary antibodies against rabbit or mouse IgG (Molecular
Probes, Inc.) were diluted 1:1,000, whereas Cy3-conjugated goat anti–rab-
bbit or anti–mouse IgG (Jackson ImmunoResearch Laboratories) were di-
luted 1:300. Confocal microscopy was performed as above, recording only
single horizontal scans for each channel.

Apoptosis assays using cells and structures from collagen gels
2–3 d after seeding, cell structures within collagen gels were treated with
TGFβ plus or minus other factors/inhibitors for 2.5 or 4 d. After washing in
PBS, the structures were either processed to yield cell suspensions or
TUNEL stained in situ. To generate and stain cell suspensions, collagen
gels were digested with 2 mg/ml collagenase in 0.5 ml PBS for 10 min.
Pooled cells from 8 gels were spun down, washed in PBS, transferred into
v-shaped wells of a 96-well plate, fixed with 4% PFA for 1 h, treated with
0.1% Triton X-100 in 0.1% sodium citrate and subjected to the TUNEL as-
say (method for cells in suspension) (1684795; Roche) according to manu-
facturer’s instructions. After staining with DAPI in the last wash, TUNEL-
stained cell pellets were suspended in 30 μl Mowiol, spread on glass
slides, and apoptotic indices were determined by counting green versus
blue nuclei under the fluorescence microscope. For each point, at least
500 cells were scored. Mean values and SD were calculated from three in-
dependent determinations.

For TUNEL staining of collagen gels in situ, the collagen gels were fixed
with 4% PFA for 2 h, washed in PBS, and subjected to the TUNEL assay (as
above) according to the manufacturer’s instructions for tissue sections with
minor modifications. Briefly, 65 μl of reaction mix diluted 1:1 in PBS was
added to each collagen gel in 17-mm wells, and the wells were then cov-
ered with paraffin and incubated at 37°C for 2.5 h. Subsequently, labeled
collagen gels were washed three times in PBS and once with PBS contain-
ing DAPI. Collagen gels were spread on slides, mounted in Mowiol, and
TUNEL-positive cells (FITC-labeled) were scored against DAPI-stained nu-
clei under a fluorescence microscope (counting >1,000 cells from three to
six randomly chosen fields in two to three collagen gels). Mean apoptotic
indices ± SD are shown.

Trypan blue exclusion assay
4 d after the start of inhibitor treatment, collagen gels were rinsed in PBS,
incubated 5 min in 0.5 ml trypan blue solution 0.4% (T 8154; Sigma-
Aldrich), and rinsed twice in PBS. Thereafter, the percentage of stained
structures was determined under a bright field microscope, counting at
least 100 structures (“partially dead” and “dead” structures containing 25–
60% of trypan blue–positive cells).

Induction of tumors and metastases in mice
To test the various cell types for tumorigenicity, groups of three to six
Balb/C athymic nude mice (nu/nu); 5–9 wk old; obtained from Charles River
Wiga, GmbH, Sulzfeld, Germany) were used for each cell type per experi-
ment. Confluent cells from several V12-Ras, S35-Ras, and C40-Ras clones were
trypsinized and washed three times in PBS. Multiple doses of 104 cells
suspected in 25 μl PBS were injected into the 3rd and 4th mammary
and gland pairs of Metofane anaesthetized mice (shallow injection into the nip-
pale area). Initial tumor nodules were confined to the mammary fat pad in
almost all cases. Primary tumor growth was weekly measured with a cali-
per in mm. Mean tumor diameter was calculated as the sum of diameters
of all tumors detected divided by the number of injection sides. For the ex-
perimental metastasis assay, 105 ex-tumor cells derived from tumors in-
duced by the above clones (as described in Cells and cell culture) were
injected into mice (Charles River Wiga, GmbH). Lethality of injection due to lung me-
transfers was cut at 7.5-μm steps and stained with hematoxylin and eosin ac-
cording to standard protocols. The surviving mice were killed after 10 wk,
inspected visually for metastases in major organs, and the lungs were sub-
jected to histological analyses as above.

The authors thank Dr. R. Kemler for his gift of anti–E-cadherin antibodies
and useful discussions, Drs. G. Christofori, M. Baccarini, and R. Foisner
(all Vienna Biocenter, Vienna, Austria) for critically reading the article, and
Gabri Litos for excellent technical assistance.

E. Janda, S. Grunert, and H. Beug were supported by grants from the Eu-
orpean Union Training and Mobility of Researchers network (ERBFM-
RXCT-980197), the Austrian Research funding agency (FWF; SFB 006/
612), and the Austrian Industrial Research Promotion Fund (FFI project no.
803776).

Submitted: 13 September 2001
Revised: 4 December 2001
Accepted: 7 December 2001

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