TGF-β signaling and epidermal carcinogenesis

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Abstract

TGF-β and related factors regulate a complex set of cellular processes including proliferation, differentiation and apoptosis, and play a crucial role in the development and homeostasis of most tissues including epithelia. During the last years the idea of a dual function of TGF-β in epithelial carcinogenesis has gained considerable support. TGF-β can act as a suppressor of carcinogenesis by inhibiting tumor growth, but it also stimulates malignant progression by inducing an epithelial-mesenchymal transition associated with the development of a highly aggressive undifferentiated type of carcinoma. We have analyzed the role of TGF-β in carcinogenesis using the mouse skin experimental model. We show that TGF-β blocks proliferation of pre-neoplastic epidermal keratinocytes

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while stimulates malignant progression of transformed keratinocytes containing a Ras oncogene. Since TGF-β activates both Smad and Ras signaling pathways in epidermal cells, we studied the involvement of these signaling cascades on the cellular responses of keratinocytes to TGF-β. Our results suggest that the Ras/MAPK signaling pathway plays a prominent role in TGF-β-stimulated malignant progression. In addition, we have uncovered a cross-talk between Smad and Ras signaling pathways by which Smad4 inhibits Ras activity in transformed keratinocytes.

Introduction

The transforming growth factor-β (TGF-β) family of cytokines regulates cell proliferation, differentiation, motility, adhesion, and death. About thirty members of the TGF-β family have been described, including bone morphogenetic proteins, activins and TGF-βs themselves, that play a prominent role in embryonic development, and in homeostasis and repair of most tissues in adult organisms (1, 2). Of these, TGF-β_1, the first specific TGF-β identified in mammalian tissues, is the focus of most recent studies on the involvement of TGF-β in epithelial cancer. TGF-β_1 has a dual and paradoxical role in carcinogenesis (see refs. 3-5 for recent reviews). It acts as a suppressor of tumorigenesis at early stages by virtue of its well established growth inhibitory and pro-apoptotic effects in epithelial cells (6, 7). As the tumor progresses, neoplastic cells attenuate or lose completely the growth inhibitory response exerted by the growth factor (8, 9), while production of TGF-β_1 is often increased in the tumors (10-12). Then, TGF-β_1 stimulates malignant progression. TGF-β_1 can act directly on the cancer cell enhancing cell migration and invasiveness, or can influence the tumor microenvironment, as for example promoting angiogenesis. In both cases, TGF-β_1 favours the metastatic spread of carcinomas. TGF-β_1 induces a reversible epithelial to mesenchymal transition (EMT) in a number of normal and transformed epithelial cell lines (13-17). EMTs have been accomplished in cultured cells by different experimental approaches, such as disturbance of the adhesive properties, the forced expression of several oncogenes, and treatment with growth factors that induce motility and dispersion of epithelial cells (18). EMTs are characterized by extensive changes in the expression of differentiation-specific proteins, such as downregulation of the cell-cell adhesion molecule E-cadherin, and a switch from a cytoskeleton composed of cytokeratins to one composed predominantly of vimentin (14). These phenotypic alterations have been well characterized in the mouse skin carcinogenesis model system in vitro and in vivo.

In the mouse skin model of carcinogenesis (see *Fig. 1*), tumor initiation is induced by a single topical application of the carcinogen dimethylbenzanthracene (DMBA), and involves a specific A-T oncogenic mutation in the codon 61 of the Harvey-Ras (H-Ras) gene (19, 20). Thereafter, repeated application of the tumor promoter TPA results in the outgrowth of highly differentiated benign papillomas. A small subset of papillomas eventually progress to malignant squamous cell carcinomas. The progression of benign to malignant tumors frequently involves mutational events leading to inactivation of the p53 gene (21). The latest stage of tumor progression is the development of spindle cell carcinomas, a highly malignant type of tumor formed by cells that have lost the epithelial phenotype and acquired fibroblastic characteristics (22, 23). Spindle carcinoma cells have altered expression of keratinocyte-specific markers, such as cytokeratins (24) and loss of E- and P-cadherin expression (25), and are characterized by expression of fibroblastic cell

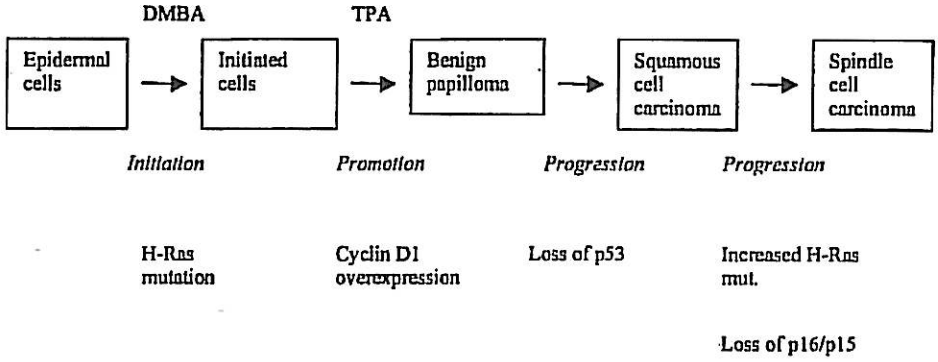


Figure 1. The mouse skin carcinogenesis model showing the main genetic alterations involved.

markers, such as vimentin (14, 17). The transition from a squamous to a spindle carcinoma correlates with amplification/overexpression of the mutant H-Ras gene or loss of the corresponding normal H-Ras allele (23, 26), and involves, at least in a proportion of cases, loss or missregulation of the INK4 locus which encodes p16INK4A, p15INK4B and p19ARF (27).

In this article, we summarize our studies on the cellular responses of normal and transformed mouse skin keratinocytes to TGF-β_1, and the signaling pathways involved.

TGF-β_1 modulates the phenotype of transformed keratinocytes

A summary of the responses triggered by TGF-β_1 in cell lines corresponding to different stages of mouse skin carcinogenesis is presented in *Table 1*.

While proliferation of immortalized and papilloma-derived keratinocytes is strongly inhibited at low concentrations of TGF-β_1 (0,5-1 ng/ml), carcinoma cell lines are only

Table 1. Differential responses of normal and transformed keratinocytes to TGF-β. The origin, presence of mutated H-Ras and p53 genes as well as the tumor phenotypes induced by the cell lines in nude mice are presented. Cell lines were characterized for their responses to TGF-β_1 using cell proliferation assays (growth inhibition), and measuring their abilities to repopulate a wound made in subconfluent cultures (migration) and to invade through the reconstituted basement membrane Matrigel (invasion). SCC I, well differentiated squamous cell carcinoma; SCC II, moderately differentiated SCC; SpCC, spindle cell carcinoma; ND, not determined.

Cell line	Origin	H-Ras mutation	P53 mutation	Tumor phenotype	TGF-β cell response	
					Growth inhibition	Migration/ Invasion
MCA3D	Immortalized	No	No	None	Strong	No
Pam212	Immorthlized	No	No	None	Strong	No
PB	Papilloma	No	No	None	Strong	No
MSCP1	Papilloma	Yes	ND	None	Strong	ND
PDV	Transformed in vitro	Yes	Yes	SCC I	Partial	Stimulated
HaCa4	Carcinoma	Yes	No	SCC II	Partial	Inhibited
Car C	Carcinoma	Yes	Yes	SpCC	Low	Inhibited

partially inhibited at higher concentrations (5-10 ng/ml) (9, 28). These results indicate that keratinocytes respond to TGF-β_1 depending on the particular differentiation state. Attenuation or loss of the antiproliferative response is a late event in carcinogenesis linked to malignant progression, and is not associated with the presence of an activating mutation in the H-Ras gene (9, 29). TGF-β_1, on the other hand, modulates the invasive behaviour of carcinoma cells depending on the cellular context. Thus, the well differentiated carcinoma cell line PDV responds to TGF-β_1 by enhancing cell migration and invasiveness (30), while TGF-β_1 decreases invasiveness of the two highly malignant fibroblastoid cell lines HaCa4 and Car C (31).

To unraveling the signaling pathways involved in the cellular responses of keratinocytes to TGF-β_1, we have focused on the MCA3D and PDV cell lines. MCA3D keratinocytes are non-tumorigenic and exhibit normal Ras and p53 genes, while PDV cells were derived from primary epidermal keratinocytes transformed in vitro by DMBA. PDV contains the same genetic alterations as found in squamous carcinomas induced in vivo by DMBA/TPA: a DMBA-specific codon 61 mutation in the H-Ras gene and inactivated p53 loci (21, 32), and produces low metastatic well differentiated SCCs upon injection in mice (32, 33). While chronic treatment of MCA3D keratinocytes with TGF-β_1 leads to growth arrest and cell death by apoptosis, PDV cells cultured under the presence of the growth factor elicit an EMT that involves changes in the expression of differentiation protein markers; i.e., loss of keratins and E-cadherin and induction of vimentin (14). TGF-β_1-treated PDV cells produces highly metastatic poorly differentiated carcinomas upon injection in nude mice (14, 33). We also evaluated whether TGF-β_1 was able to promote a squamous-spindle transition in vivo. To this aim, TGF-β_1 (50 ng) was injected four times (at two-days intervals) into well differentiated squamous cell carcinomas induced by PDV cells in nude mice. TGF-β_1-treated tumors showed significant changes in the pattern of differentiation compared with controls (tumors treated with PBS) with presence of sarcomatoid regions containing fusiform cells in the periphery of the tumors.

The differential response of MCA3D and PDV keratinocytes to TGF-β_1 allowed us to suggest for the first time a dual role for TGF-β_1 in mouse skin carcinogenesis (14). TGF-β_1 can act as a tumor suppressor at early stages (MCA3D) of carcinogenesis, but it also stimulates malignant progression at later stages (PDV) by promoting a transition from a squamous to a spindle tumor phenotype. This double function of TGF-β_1 in skin carcinogenesis was elegantly demonstrated by Akhurst and co-workers in transgenic mice overexpressing TGF-β_1 in the epidermis (34). The transgenic mice subjected to chemical carcinogenesis treatment with DMBA/TPA were more resistant to induction of benign papillomas than controls, but the conversion rate to malignant carcinomas was vastly increased, and there was also a higher incidence of spindle cell carcinomas. TGF-β_1 is also able to induce an epithelial-fibroblastic conversion in human mammary epithelial cells transformed by a Ras oncogene (15), and in several human carcinoma cell lines (16). These effects are direct and require signaling through TGF-β receptors (16, 17). In both mammary and skin models TGF-β_1 appears to cooperate with oncogenic Ras to stimulate malignant progression (15, 35).

The invasive response of transformed PDV keratinocytes to TGF-β_1 is associated with induction of extracellular matrix proteinases, such as urokinase (uPA) -and its inhibitor PAI-1- and the metalloproteinase MMP-9 (gelatinase B) (30, 36). uPA is a serine proteinase that converts the inactive plasminogen into the broad-spectrum proteinase

plasmin, which, in turn, degrades several components of the extracellular matrix and activates pro-metalloproteinases (37). Increased expression/secretion of uPA/PAI-1 and MMP-9 are linked to tumor invasion and metastasis in many types of human cancer. Interestingly, in human skin squamous cell carcinomas these proteinases are all expressed by cancer cells whereas in other types of cancer, such as colon adenocarcinomas and ductal mammary carcinomas, uPA and MMP-9 are expressed by non-neoplastic stromal cells (38). This observation acquires a special relevance taking into account that TGF-β_1 stimulates uPA and MMP-9 production in transformed but not in normal keratinocytes, and suggests a direct link between expression of these proteinases and the growth factor in vivo.

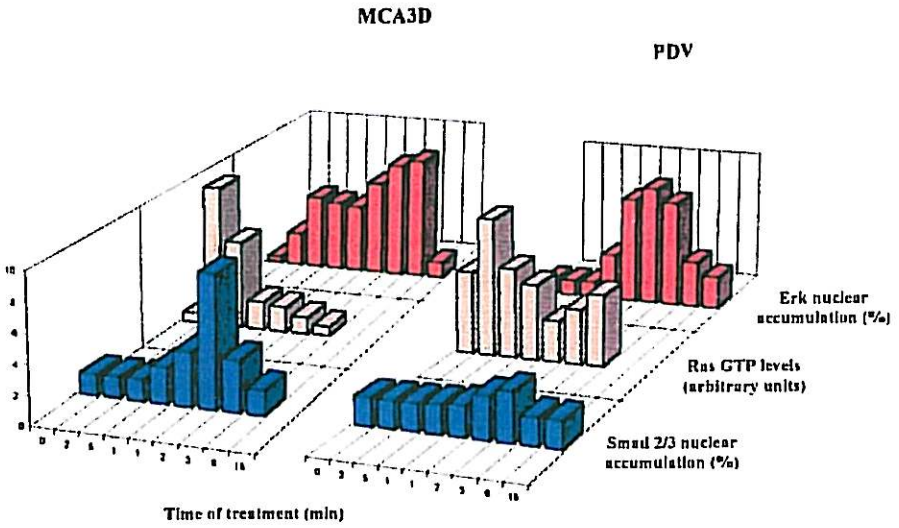


Figure 2. TGF-β_1 activates the Smad and Ras/MAPK signaling pathways in MCA3D and PDV keratinocytes. Endogenous Smad2, Smad3 and Erk 1, 2 proteins were visualized by immunofluorescence staining using anti-Smad2/Smad3 and anti-Erk1, 2 specific polyclonal antibodies in cells treated with TGF-β_1 (10 ng/ml) for the indicated times. The levels of active Ras-GTP were determined in the cell lysates by precipitating with the Ras-binding domain of Raf-1 (RBD) coupled to Sepharose followed by Western immunoblotting with a pan-Ras monoclonal antibody. Quantitation of the Ras-GTP levels was performed by densitometric analysis normalized for the levels of total Ras protein expression determined in the cell lysates.

TGF-β_1 activates simultaneously the Ras and Smad signaling pathways

TGF-β signals through formation of heteromeric complexes between cell-surface type I and type II serine/threonine kinase receptors. After activation by ligand binding, the signal transducing type I receptor phosphorylates Smad2 and Smad3 proteins that associate with Smad4 in the cytoplasm, and these complexes, then, translocate into the nucleus where they act as transcription factors (39, 40).

Nevertheless, TGF-β can activate Ras and several members of the mitogen-activated protein kinase (MAPK) family in different cell types, such as intestinal epithelial cells and fibroblasts (41). We have also demonstrated that TGF-β_1 activates Ras in epidermal keratinocytes (42). Ras activation by TGF-β_1 in mouse keratinocytes, as measured by the

levels of active Ras-GTP, is rapid and occurs before TGF-β_1-induced nuclear accumulation of Smad2/Smad3 (see *Fig. 2*). On TGF-β_1 addition, nuclear translocation of Smad2/3 and Erk1, 2, the end point of the MAPK cascade, coincides at about 20-30 min, suggesting that TGF-β_1 activates simultaneously the Smad and MAPK pathways.

PDV exhibits higher basal levels of activated Ras and Erk proteins than MCA3D cells. This is due to the presence of a mutated Ras oncogene. The expression of oncogenic Ras in PDV cells might be responsible for the constitutive inhibition of Smad signaling in these cells (only 40% of the cells accumulate Smad2/3 into the nucleus in response to TGF-β_1, see *Fig. 2*). A cross-talk mechanism between Smad and Ras signaling has been proposed (43) by which phosphorylation of Smad2/3 by Ras-activated MEK (MAPK kinase), at distinct sites from those phosphorylated by the TGF-β type I receptor, impairs their association with Smad4 and inhibits translocation of the complexes into the nucleus. However, controversial results have been obtained at this respect (44).

Studies with dominant-negative gene constructs. Cross-talk between Ras and Smad4

To analyze the involvement of Ras/MAPK signaling in the cellular responses of MCA3D and PDV keratinocytes to TGF-β_1, we interfered the Ras/MAPK pathway at different links of the signaling cascade. Thus, we used treatment with an inhibitor of MEK activity (PD098059), as well as treatment with antisense oligonucleotides directed against Erk1, 2, and transfection of a N17-Ras dominant-negative mutant gene construct to block Ras activation. A C-terminal truncated dominant-negative Smad4 (1-514) gene was also used to investigate the role of Smad signaling on the responses of keratinocytes to TGF-β_1. We examined both the antiproliferative and the migratory/invasive responses. In addition, we studied the induction of the cyclin-dependent kinase inhibitor p21Cip1, a mediator of the TGF-β_1 growth inhibitory response, as well as the expression/secretion of uPA and MMP-9 matrix proteinases which are associated with TGF-β_1-stimulated invasiveness.

The results of these studies are summarized in *Table 2*. They suggest a switch from the Smad to the Ras/MAPK signaling pathway, depending on the presence of a Ras oncogene, to mediate growth inhibition and p21Cip1 induction. Thus, both the growth inhibitory response and TGF-β_1-stimulated p21Cip1 expression are blocked in MCA3D keratinocytes transfected with the dominant-negative Smad4 construct, while in transformed PDV keratinocytes Smad4 is only partially involved in TGF-β_1-induced growth inhibition, and does not mediate enhancement of p21Cip1 levels by the growth factor (*Table 2*). TGF-β_1-mediated induction of P21Cip1 in PDV cells is inhibited by PD098059, indicating a requirement of MAPK activity (42). On the other hand, overexpression of oncogenic Ras in MCA3D cells by retroviral infection induced a high level of p21Cip1 expression regardless of blocking Smad 4 or not, indicating that a key factor for the different responses of MCA3D and PDV cells is the presence of an activated Ras gene in the latter cells.

Stimulation of PDV cell invasiveness and expression/secretion of uPA and MMP-9 by the growth factor are blocked by inhibiting Ras/MAPK activity (36, 45, see *Table 2*), suggesting that the Ras/MAPK pathway plays a crucial role on TGF-β_1-stimulated malignant progression. Furthermore, the blockade of Smad signaling in PDV cells by expression of a dominant-negative Smad4 construct constitutively hyperactivated

Table 2. Summary of studies about the effects of dominant-negative Smad4 and Ras mutant constructs on the response of MCA3D and PDV keratinocytes to TGF-β_1. Cell lines were stably transfected with either N17-Ras or Smad4 (1-514) dominant-negative gene constructs and the responses of the transfectant cell clones to TGF-β_1 studied respect to that of control clones transfected with the empty vector. The effect of the pharmacological inhibitor of MEK activity PD098059 was examined in the parental cell lines as well as in the transfectants. NA, not apply; ND, not determined.

	MCA3D		PDV	
TGF-β_1 cell response	**N17-Ras/ PD098059**	**Smad4 (1-514)**	**N17-Ras/ PD098059**	**Smad4 (1-514)**
Growth inhibition	Unaffected	Abrogated	ND	Partially abrogated
P21Cip1 induction	Unaffected	Blocked	Blocked	Unaffected
Enhanced migration/ invasiveness	NA	NA	Abrogated	Constitutively stimulated
UPA induction	NA	NA	Blocked	Constitutively stimulated
MMP-9 induction	NA	NA	Blocked	ND

Ras/MAPK signaling activity leading to a spontaneous EMT and increased migratory and invasive abilities. PDV cells with inactivated Smad4 function secreted high levels of uPA, and induced highly malignant spindle tumors in vivo. MCA3D cells expressing the Smad4 (1-514) construct, on the other hand, conserved their epithelial morphology and phenotypic characteristics (42). These results point again to an important role for the Ras/MAPK pathway in malignant progression. Furthermore, these data suggest a new type of crosstalk between Smad and Ras signaling by which Smad4, directly or indirectly, attenuates basal Ras activity in Ras-transformed epithelial cells.

This cross-talk might be physiologically relevant in certain type of carcinomas where oncogenic mutations in the Ras gene coincides with functional inactivation of Smad4. Smad4 (also named DPC4, deleted in pancreatic carcinoma locus 4) was originally identified as a tumor suppressor gene frequently inactivated in human pancreatic adenocarcinomas and colorectal cancer (46-48). Loss of Smad4 function appears to be a late event in carcinogenesis associated with progression to metastatic carcinomas (49, 50). Pancreatic and colon adenocarcinomas also exhibit a high rate of Ras (K-Ras) gene mutations with frequencies of about 90% and 50%, respectively, an event that occurs relatively early in carcinogenesis during development of late adenomas (51-53).

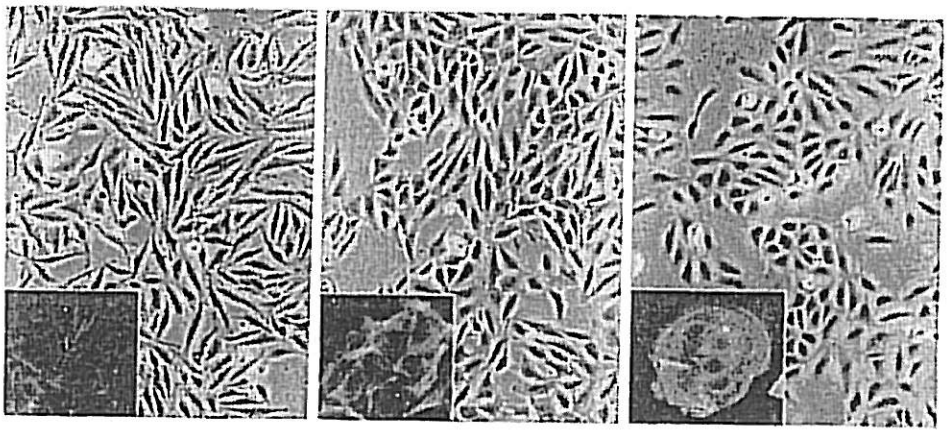
The presence of a Ras oncogene and loss of Smad4 function in carcinoma cells would cooperate for malignant progression by enhancing further the level of Ras/MAPK signaling activity. We have tested this hypothesis in the SW480 colon carcinoma cell line. SW480 colon carcinoma cells have accumulated a number of genetic alterations typical of colon carcinomas: an oncogenic mutation in the K-Ras gene, inactivation of the APC and p53 tumor suppressor genes, and are Smad4-deficient (54). Schwarte-Waldhoff and co-workers have shown that reconstitution of Smad4 in SW480 cells reduces uPA expression and suppress tumorigenicity in nude mice (55, 56). Furthermore, Smad4-reconstituted SW480 cells reverted from a fibroblastoid to an epithelial morphology and re-established functional adherens junctions mediated by cadherins (57). We have observed that re-

expression of Smad4 in SW480 cells diminishes the Ras-GTP levels and MAPK signaling activity leading to reduced expression/secretion of the cell-surface proteinase MMP-9 (DR, MI and MQ, manuscript submitted for publication). These results suggest again a cross-talk between Ras and Smad4, now in human transformed epithelial cells, by which Smad4 attenuates Ras signaling activity. However, Smad4-reconstituted SW480 cells did not recover the antiproliferative response to TGF-β_1 (54, 55, our own results) indicating that acquisition of TGF-β resistance and loss of Smad4 are independent events. Several additional experimental observations (reviewed in ref. 57) point to a function of Smad4 as a tumor suppressor independently of its role as a component of the TGF-β signaling pathway.

Rho GTPases come to scene

The Rho family of small GTPases: Rho, Rac and Cdc42, modulate an overlapping set of cellular responses, such as gene expression, cellular proliferation and actin-based cell motility (58, 59). As regulators of the actin cytoskeleton and cadherin junctions, the Rho GTPases should be implicated in EMTs, and are, therefore, candidates as downstream effectors in epithelial-mesenchymal transitions induced by TGF-β. Thus, RhoA appears to mediate TGF-β-induced EMT in nontransformed NmuMG mouse mammary cells (60). On the other hand, a cross-talk between Ras and Rho GTPases to modulate a wide range of essential biochemical pathways has been recognized for quite some time (58).

We have found an involvement of the small GTPase RhoA in mediating regulation of p21Cip1 expression by TGF-β_1 in PDV cells. TGF-β_1-mediated induction of p21Cip1 requires negative regulation of RhoA activity concomitantly to activation of Ras and MAPKs (MI, PF and MQ, unpublished results). In addition, preliminary experiments



N19-RhoA-PDV **PDV** **N63-RhoA-PDV**

Figure 3. Morphological changes induced in PDV keratinocytes by transfection of dominant-negative N19-RhoA and constitutively active N63-RhoA gene constructs. PDV cells were transfected with the above mentioned mutant genes cloned into the pcDNA3 vector. The figure shows phase-contrast micrographs of selected clones. F-actin fluorescence staining with phaloidin (insets) demonstrate significant changes in the organization of the actin cytoskeleton in the transfectants. Blockade of RhoA activity by the dominant-negative construct induces loss of cortical actin and stress fibers, suggesting increased cell motility. In contrast, expression of a constitutively active RhoA gene leads to a stronger cortical actin bundles and a more cohesive phenotype.

suggest an involvement of RhoA in the modulation of the epithelial phenotype in keratinocytes. Thus, transfection of PDV cells with a dominant-negative N19-RhoA gene construct induces a fibroblastoid morphology, while expression of a constitutively active N63-RhoA mutant gene results in a more cohesive and compact epithelial phenotype (see - *Fig. 3*). These findings point to a signaling cascade involving RhoA in epithelial-mesenchymal transitions induced by TGF-β_1 in transformed keratinocytes. Further experiments will be necessary to determine the relationships (cross-talk) between Ras and Rho GTPases on the signaling events triggered by TGF-β in epidermal cells.

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