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A role for Id in the regulation of TGF-β-induced epithelial–mesenchymal transdifferentiation

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Abstract

Epithelial–mesenchymal transdifferentiation (EMT) is a critical morphogenic event that occurs during embryonic development and during the progression of various epithelial tumors. EMT can be induced by transforming growth factor (TGF)-β in mouse NMuMG mammary epithelial cells. Here, we demonstrate a central role of helix–loop–helix factors, E2A and inhibitor of differentiation (Id) proteins, in TGF-β-induced EMT. Epithelial cells ectopically expressing E2A adopt a fibroblastic phenotype and acquire migratory/invasive properties, concomitant with the suppression of E-cadherin expression. Id proteins interacted with E2A proteins and antagonized E2A-dependent suppression of the E-cadherin promoter. Levels of Id proteins were dramatically decreased by TGF-β. Moreover, NMuMG cells overexpressed Id2 showed partial resistance to TGF-β-induced EMT. Id proteins thus inhibit the action of E2A proteins on the expression of E-cadherin, but after TGF-β stimulation, E2A proteins are present in molar excess of the Id proteins, thus over-riding their inhibitory function and leading to EMT.

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Abbreviations: TGF-β, transforming growth factor-β; TβR-I, TGF-β type I receptor; BMPs, bone morphogenetic proteins; HLH, helix–loop–helix; Id, inhibitor of differentiation; EMT, epithelial–mesenchymal transdifferentiation; Tet, tetracycline; TRITC, tetramethylrhodamine B isocyanate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Introduction

Transforming growth factor (TGF)-β, a prototypic member of the TGF-β superfamily, exhibits a broad range of biological activities. It has an antiproliferative effect on divergent cell types, including epithelial cells, endothelial cells and hematopoietic cells. Thus, perturbations of TGF-β signaling result in the progression of certain tumors. In contrast to its antiproliferative effects on most cell types, TGF-β also promotes growth of mesenchymal cell types, including fibroblasts, chondrocytes and osteoblasts. Moreover, if cells acquire resistance to the growth inhibitory effects of TGF-β, TGF-β contributes to the progression of tumors through the induction of angiogenesis, extracellular matrix accumulation and immunosuppression.[1–4]

Members of the TGF-β superfamily, including TGF-βs, activins, bone morphogenetic proteins (BMPs) and growth/differentiation factors, transmit their pleiotropic effects through transmembrane serine/threonine kinase type I and type II receptors.[1,5] Upon ligand-induced heteromeric complex formation between type I and type II receptors, the type I receptor becomes phosphorylated and activated by the type II receptor kinase, and mediates specific intracellular signals. Smads are the central signal mediators of the TGF-β superfamily activated by type I receptor kinases. Smads are subdivided into three subtypes: receptor-regulated Smads (R-Smads), common-mediator Smad (Smad4) and inhibitory Smads (Smad6 and Smad7). R-Smads are further subdivided into two subtypes: Smad2 and Smad3 for TGF-β and activin signaling pathways and Smad1, Smad5 and Smad8 for BMP signaling pathways. Among the three different classes of Smads, only R-Smads interact directly with activated type I receptors and become activated through phosphorylation of a conserved SSXS motif in their C-termini. R-Smads then form heteromeric complexes with Smad4, followed by nuclear translocation and regulation of gene transcription in collaboration with other transcription factors and transcriptional regulators.[5]

During the processes of embryonic development and wound healing and reorganization in adult tissues, epithelial cells may lose their epithelial polarity and acquire mesenchymal phenotypes. In addition, the invasion process of tumor cells involves the loss of cell–cell interaction together with acquisition of migratory properties, and is often associated with epithelial–mesenchymal transdifferentiation (EMT) of

cells.[6,7] Formation of tight cell–cell adhesion is mainly dependent on the E-cadherin system both in embryonic and adult epithelial cells. Therefore, loss of E-cadherin-mediated interaction is an essential process for EMT that takes place during early normal embryonic development as well as during invasion of tumor cells into adjacent connective tissues. TGF-β has been identified as an important molecule inducing EMT both *in vivo* and *in vitro*.[8] TGF-β2 is a candidate inducer of EMT in the atrioventricular tissues of the embryonic heart,[9] whereas TGF-β3 is responsible for EMT at palate fusion.[10] The mouse mammary epithelial cell line, NMuMG, has been shown to undergo EMT following TGF-β treatment. However, in other epithelial cells, including immortalized hepatocytes and MDCK cells, a synergistic effect between the H-Ras signal and the TGF-β signal appears to be required for the induction of a full EMT that occurs, at least in part, via the expression of a zinc-finger factor, Snail.[11–15]

The E2A proteins belong to the class I basic helix–loop-helix (bHLH) family of transcriptional regulatory proteins, which function as dimers with the class II bHLH through an HLH domain. The E2A gene encodes two alternatively spliced products, E12 and E47, which differ in bHLH domains and hence their DNA-binding properties.[16] E2A proteins have been shown to be involved in the regulation of the expression of p21 in HeLa cells and MG63 cells, leading to growth arrest.[17] Recently, it has been shown that E2A proteins participate in the repression of E-cadherin expression by direct binding to the E-cadherin promoter region during the process of EMT in epithelial cells.[18] The transcriptional activity of E2A proteins can be negatively regulated by dimerization with another subclass of HLH factors, inhibitor of differentiation (Id) proteins, which contain functional HLH dimerization motifs but lack the basic DNA-binding region.[19,20] More recently, Id expression has been demonstrated to be upregulated by BMP and downregulated by TGF-β through a transcriptional repressor, ATF-3, in various cell types.[21]

In the present study, we investigated the regulatory mechanisms by which TGF-β was able to induce EMT in NMuMG cells. We show that the *E2A* gene products associate constitutively with, and are inactivated by Id proteins in NMuMG cells, leading to the expression of E-cadherin and maintenance of the epithelial phenotype. In contrast, down-regulation of Id expression by TGF-β generates E2A that is free from Id proteins, thus allowing E2A to become active and induce EMT through repression of E-cadherin expression.

Results

EMT of NMuMG by TGF-β

TGF-β has been reported to induce EMT of NMuMG cells.[22] In untreated NMuMG cells, the characteristic epithelial phenotype with a strong cortical and diffuse cytoplasmic actin staining was observed by staining with tetramethylrhodamine B isocyanate (TRITC)–phalloidin, as shown previously (Figure 1a and Piek *et al*.[22]). Treatment with TGF-β dramatically induced actin fiber formation typical of transdifferentiation from the epithelial to mesenchymal phenotype, whereas BMP-4 failed to do so. In addition, staining of E-cadherin, a representative marker for differentiated epithe-

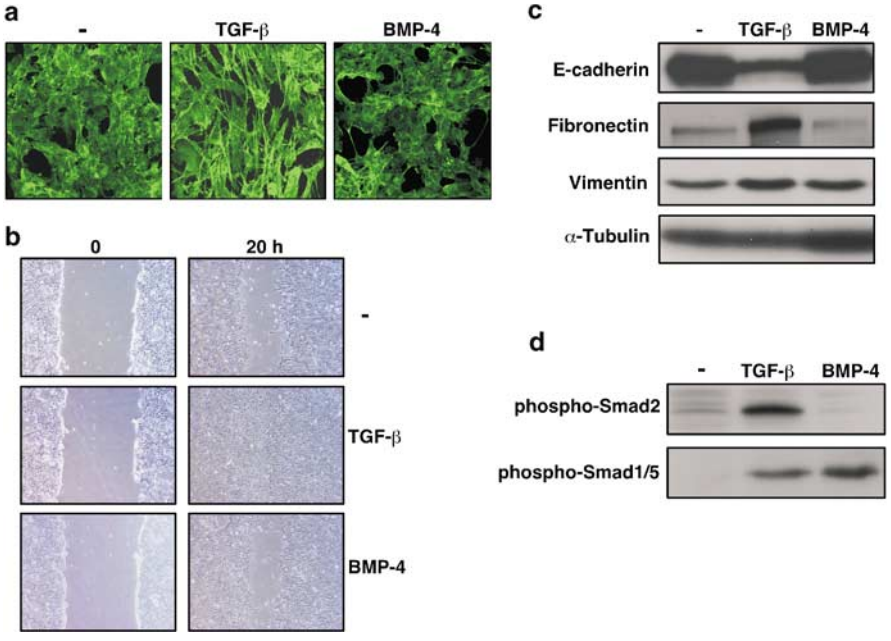

Figure 1 Induction of EMT by TGF-β. (**a**) Organization of the actin cytoskeleton in NMuMG cells. Cells were grown in the absence or presence of 100 pM TGF-β or 1.4 nM BMP-4 for 24 h, followed by direct staining of the actin cytoskeleton using TRITC–phalloidin as described in Materials and methods. (**b**) Migratory behavior of NMuMG cells in the absence or presence of TGF-β and BMP-4. NMuMG cells were stimulated with or without 100 pM TGF-β or 1.4 nM BMP-4. The migratory behavior was analyzed in an *in vitro* wound model. Cells grown at 80% confluency were scratched by a pipette tip, and photographs were taken immediately after the incision (0) and after 20 h. (**c**) Immunoblotting of the indicated proteins from cells treated with or without TGF-β or BMP-4. α-Tubulin was used as a loading control for whole-cell extracts. (**d**) Phosphorylation of Smad2 and Smad1/5 by TGF-β and BMP-4. Cells were stimulated for 1 h with 100 pM TGF-β or 1.4 nM BMP-4. Immunoblotting was performed by anti-phospho-Smad2 antibody and anti-phospho-Smad1/5 antibody as described in Materials and methods

lial cells, was decreased and diffusely localized in the cytosolic area after treatment with TGF-β, whereas it was localized at the plasma membrane in the absence of TGF-β (data not shown).

We also analyzed the migratory property of NMuMG cells by a wound culture assay (Figure 1b). Cells were scratched to produce wounds, and ligands were added to the culture medium. NMuMG cells treated with TGF-β showed highly migratory behavior at 20 h postincision, whereas BMP-4 was unable to significantly induce cell migration. These findings suggest that only TGF-β is able to elicit EMT in NMuMG cells.

Various epithelial and mesenchymal markers were quantitatively examined by immunoblotting whole-cell extracts from NMuMG cells (Figure 1c). Treatment with TGF-β for 48 h resulted in the downregulation of E-cadherin expression with concomitant upregulation of representative markers for fibroblastic cells, that is, fibronectin and vimentin. In contrast, BMP-4 had no effect on the regulation of the expression of these proteins. We confirmed that in NMuMG cells, TGF-β induced phosphorylation of Smad2 and Smad1/5, whereas BMP-4 induced that of Smad1/5 (Figure 1d). These results suggest that TGF-β induces EMT of NMuMG cells possibly through the Smad2/3 pathway.

Regulation of expression of Id proteins by TGF-β

Since TGF-β is a potent cytokine that induces phenotypic transformation of epithelial cells, we investigated TGF-β-regulated genes in HaCaT human keratinocyte cells by DNA microarray analysis (Akiyoshi *et al*.[23] and data not shown). Several representative genes were induced or suppressed by TGF-β in HaCaT cells; among those, we found that Id genes were slightly downregulated at 4 h after stimulation with TGF-β, but were dramatically upregulated by BMP-4. In contrast, our microarray data from MG63 human osteosarcoma cells[24] revealed that both TGF-β and BMP induced expression of Id genes at the same time point (our unpublished observations), suggesting the possibility that the difference in the expression of Id genes might influence differences in responses to TGF-β, for example, in phenotypic morphology, between epithelial cells and mesenchymal cells.

To examine whether the expression of Id proteins is regulated by treatment with TGF-β, we performed immunoblotting and reverse transcription-polymerase chain reaction (RT-PCR) analyses of NMuMG cells incubated with or without TGF-β or BMP-4. Interestingly, Id1 expression was increased at early time points (1 and 4 h in the protein and 1 h in the mRNA) of TGF-β stimulation, and decreased thereafter, whereas the levels of Id2 protein and mRNA were repressed at 4 h and subsequently repressed more strongly (Figure 2a and b). Basal levels of the Id3 protein seem also to be downregulated by TGF-β, particularly after 24 h, and suppression of its mRNA was observed more clearly (Figure 2a and b). In contrast, BMP-4 treatment showed upregulation of Id1, Id2 and Id3 until 4 h of BMP stimulation, and declined at 24 h, indicating that BMPs have effects distinct from TGF-β on Id1, Id2 and Id3 expression. In addition, we examined the expression profile of E2A protein in NMuMG cells, because E2A proteins were isolated as a transcriptional repressor interacting with the E-cadherin promoter by the yeast one-hybrid system,[18] and are inactivated by interaction with Id proteins. The expression level of E2A proteins was determined by reimmunoblotting using the same membrane that had been used for the detection of Id proteins, but it was not significantly affected by TGF-β or BMP-4 in NMuMG cells (Figure 2a).

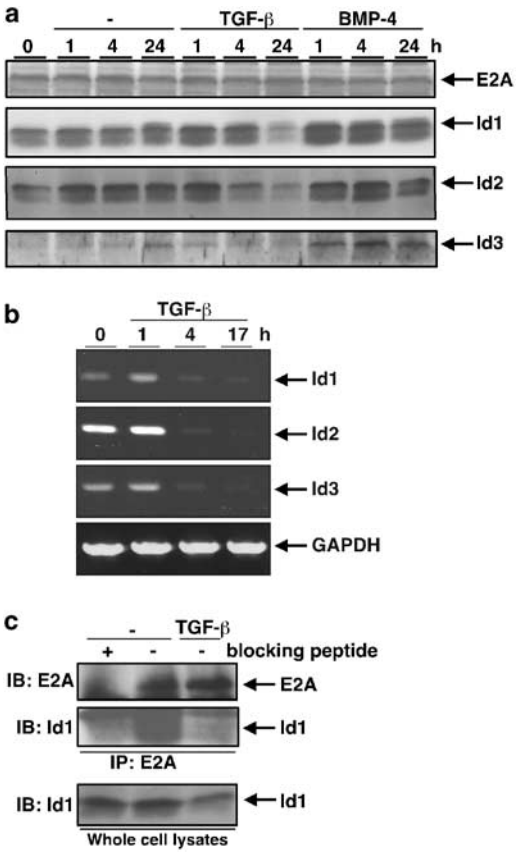

Figure 2 Expression profiles of Id and E2A proteins upon TGF-β or BMP treatments. (**a**) Expression profiles of Id1-3 and E2A proteins. Extracts from NMuMG cells treated with 100 pM TGF-β or 1.4 nM BMP-4 for the indicated time periods were prepared and then subjected to immunoblotting for E2A, Id1, Id2, and Id3. (**b**) Expression profiles of Id1-3 mRNA. RNA samples from NMuMG cells treated with TGF-β were analyzed by RT-PCR for expression of Id1-3 and housekeeping gene GAPDH. (**c**) Interaction of endogenous E2A with endogenous Id1. NMuMG cells were seeded at a density of 5×10^5 in a 10 cm dish, and cultured overnight before stimulation with 100 pM TGF-β. After 24 h, cell extracts were prepared and processed to immunoprecipitation or were directly separated by SDS-PAGE. Endogenous E2A was immunoprecipitated with anti-E2A antibody pretreated with or without the E2A-blocking peptide, followed by immunoblotting with the same antibody (top panel). Endogenous Id1 co-immunoprecipitated with E2A was immunoblotted with the same anti-Id1 antibody (middle panel). Id1 from whole-cell lysates was also detected by immunoblotting with anti-Id1 antibody (bottom panel)

We further examined the possibility that, after 24 h of TGF-β stimulation, Id proteins complexed with E2A proteins would be decreased compared with those of TGF-β-untreated cells. To address this, endogenous E2A proteins were immunoprecipitated with anti-E2A antibody and co-immunoprecipitated Id proteins were evaluated by immunoblotting. Despite no clear difference in the levels of immunoprecipitated E2A protein (Figure 2c, top), a great reduction in the association of Id1 with E2A was observed in NMuMG cells treated with TGF-β for 24 h, consistent with the repressed expression of Id1 as

determined by immunoblotting using whole-cell lysates (Figure 2c, bottom). Taken together, these findings demonstrate that prolonged TGF-β treatment results in the down-regulation of Id proteins and that E2A proteins are dissociated from Id proteins by TGF-β treatment in NMuMG cells.

Repression of E-cadherin promoter activity by TGF-β

To determine whether TGF-β regulates the promoter activity of E-cadherin, NMuMG cells were transfected with an E-cadherin promoter fused to a luciferase reporter gene, and luciferase activities were determined after 36 h stimulation of TGF-β. Similar to the data obtained from immunoblotting (Figure 1c), the E-cadherin promoter was suppressed by TGF-β, but not by BMP-4 (Figure 3a and data not shown). When constitutively active TGF-β type I receptor (TβR-I) was transfected, a reduction of E-cadherin promoter activity was also observed, although it was slightly less potent than the TGF-β ligand (data not shown). These findings indicate that TGF-β represses E-cadherin promoter activity in NMuMG cells.

Effects of E2A proteins on E-cadherin promoter activities

Next, we focused on the molecular function of E2A proteins in suppressing E-cadherin promoter activity. Transient transfection of E12 or E47 expression vectors into NMuMG cells showed that both E12 and E47 repressed E-cadherin promoter activity (Figure 3b). Since both of these proteins interact with Id1, Id2 and Id3 (data not shown), we next cotransfected E2A constructs together with Id expression vectors to determine whether Id proteins can inactivate E2A function. As shown in Figure 3b, the reduction in luciferase activity induced by E12 or E47 was restored by Id1 or Id2. The expression levels of transfected Id1 and Id2 proteins were almost the same as the levels determined by immunoblotting (data not shown). Similar studies in MDCK cells showed that Id3 is able to fully overcome the E47-induced repression of E-cadherin promoter (Figure 3c). These results indicate antagonistic effects of Id proteins on E2A-regulated transcriptional activities.

Effect of stable expression of Id2 on EMT in NMuMG cells

To further investigate the roles of Id2 in the regulation of EMT, we established NMuMG cells retrovirally infected with pBabe vector (NMuMG-TRE cells) and pBabe-Flag-Id2 (NMuMG-Id2 cells), in which the expression of Id2 is under the control of a Tet-repressible promoter. Among the three Id proteins, Id2 was used in the following experiments, because Id2 was significantly repressed by TGF-β (Figure 2a and b), and abolished the transcriptional repression induced by E2A (Figure 3b). After complete removal of Tet from the culture medium, expression of Id2 was induced and could be detected by immunoblotting (Figure 4a). No leaky expression was seen in the presence of 1 μg/ml Tet. Id2 was seen dominantly in the nuclei, detected by immunostaining with anti-Flag antibody (data not shown). TGF-β still repressed endogenous Id1 and Id2 expression and maintained a steady

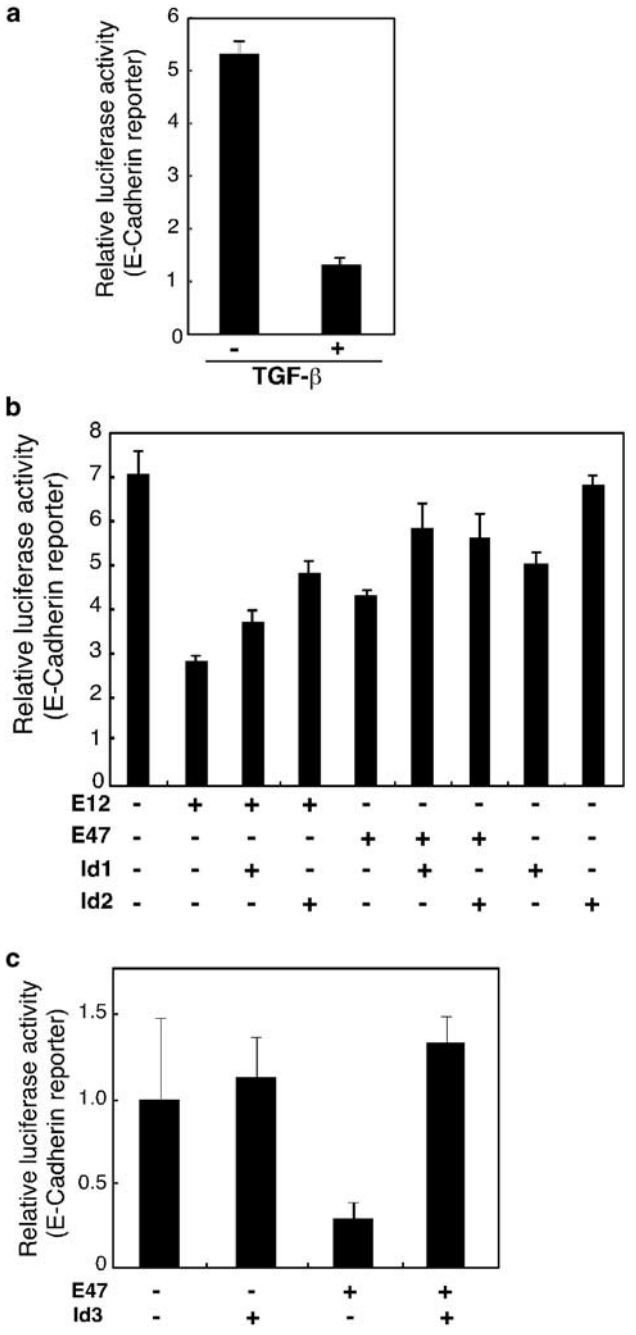

Figure 3 Effects of TGF-β and E2A on E-cadherin promoter activity. (**a**) Effect of TGF-β on E-cadherin promoter activity. NMuMG cells transfected with E-cadherin-luciferase construct were stimulated or not with 100 pM TGF-β for 24 h, and then assayed for luciferase activities. (**b** and **c**) Inhibition of E12- and E47-dependent suppression of E-cadherin promoter activity by Id proteins. NMuMG (**b**) and MDCK (**c**) cells were cotransfected with 0.3 μg of E-cadherin-luc in combination with pcDNA3-E12, pcDNA3-E47, pcDNA3-Id1, pcDNA3-Id2 and pcDNA3-Id3, as indicated. The cells were harvested 36 h (**b**) and 24 h (**c**) after transfection and then assayed for reporter gene expression. Empty vector was included to adjust the amounts of DNA used

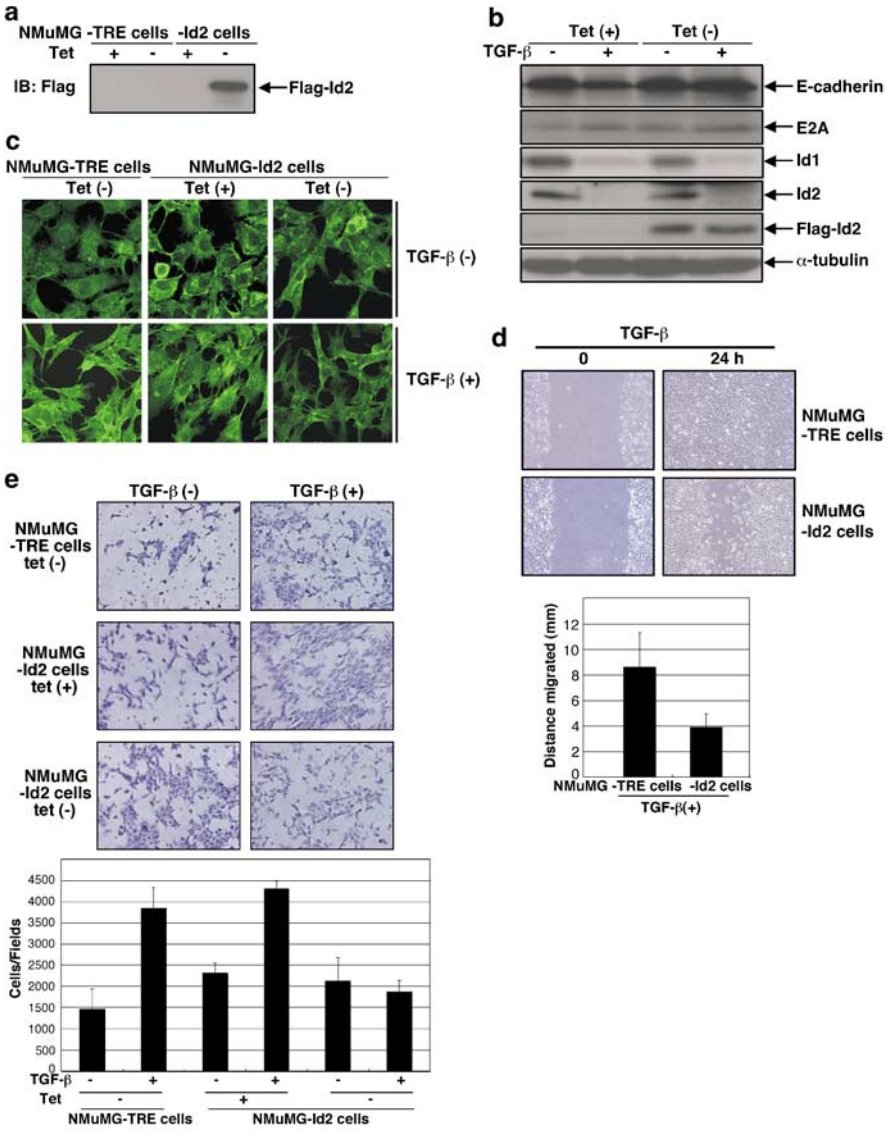

Figure 4 Effects of Id2 on EMT. (**a**) NMuMG cells preinfected with pBabe-tTA were reinfected with empty retrovirus (NMuMG-TRE cells) or with retrovirus encoding Flag-Id2 (NMuMG-Id2 cells) under the control of Tet. After 24 h of culture with or without 1 μg/ml Tet, cell extracts were prepared and analyzed by immunoblotting using anti-Flag antibody. (**b**) Immunoblotting of the indicated proteins from NMuMG-Id2 cells treated with or without TGF-β. Cells cultured in the absence or presence of 1 μg/ml Tet for 6 h were stimulated or not with TGF-β for 24 h, and extracted for immunoblotting using the antibodies indicated. α-Tubulin was used as a loading control for whole-cell extracts. (**c**) Effect of stably expressed Id2 on EMT. Cells cultured overnight with or without 1 μg/ml Tet were stimulated with 100 pM TGF-β for 24 h, and TRITC–phalloidin staining was carried out as shown in Figure 1a. (**d**) The migratory behavior of NMuMG-TRE cells and NMuMG-Id2 cells. Cells cultured for 12 h without 1 μg/ml Tet were scratched and simultaneously stimulated with 100 pM TGF-β. Photographs were taken immediately after the incision (0) and after 24 h (top). The migration of wound edges was measured at three random points on the photograph and shown in the bottom. Each experiment was performed in triplicate. (**e**) NMuMG cells precultured for 12 h with or without 1 μg/ml of Tet were treated with or without 40 pM TGF-β. After 6 h, cells were seeded into the chamber and cultured for 12 h in the presence or absence of TGF-β. The cells that had invaded to the lower surface of the filter were stained with Giemsa (shown in the top), photographed and quantitated by visual counting (bottom)

or very slightly higher state of E2A expression under these conditions (Figure 4b).

Figure 4c shows the representative morphology of the NMuMG cells ectopically expressing Id2 with or without TGF-β treatment. Upon TGF-β stimulation, typical mesenchymal phenotype was observed in the morphology of the NMuMG-TRE cells and NMuMG-Id2 cells not expressing Id2 (Tet (+)), whereas a moderate suppression of TGF-β-induced actin fiber formation was detected by phalloidin staining in NMuMG-Id2 cells expressing Id2 (Tet (−)). Moreover, repression of E-cadherin induced by TGF-β was suppressed in the Id2-expressing cells (Figure 4b), suggesting that ectopic expression of Id2 inhibits EMT induced by TGF-β.

We have also examined the migratory and invasive properties of control and Id2-expressing cells upon TGF-β stimulation. At 12 h after withdrawal of Tet, NMuMG-Id2 and NMuMG-TRE cells were scratched to produce wounds, and TGF-β was added to the culture media. After 24 h, NMuMG-Id2 cells expressing Id2 showed a low migratory behavior compared with NMuMG-Id2 cells cultured in the presence of

Tet and NMuMG-TRE cells (Figure 4d and data not shown). Similar to the migratory behavior, TGF-β-induced invasive properties, detected in control cells and NMuMG-Id2 cells in the presence of Tet, were clearly suppressed by ectopic expression of Id2 (Figure 4e). Total cell numbers of NMuMG-Id2 cells treated with TGF-β in the absence of Tet were almost the same as that in the presence of Tet (data not shown), meaning that low migratory and invasive properties in NMuMG-Id2 (without Tet) are not due to higher sensitivity to growth inhibition of the Id2-expressing cells induced by TGF-β. Therefore, these findings suggest that Id2 could block TGF-β-induced migration and invasion of NMuMG cells.

Overexpression of E12 facilitates EMT in NMuMG cells

To further examine the role of Id proteins, the function of the Id proteins was suppressed by overexpression of E12 in NMuMG cells. After infection with pBabe vector or pBabe-Flag-E12, cell extracts were prepared and expression of Flag-E12 was analyzed by immunoblotting. As shown in Figure 5a, NMuMG cells expressing E12 maintained low levels of E-cadherin expression in the presence and absence of TGF-β. Moreover, a high migratory behavior was observed in the E12-expressing cells compared to the pBabe vector-infected cells (Figure 5b). These results support the role of Id proteins as targets of TGF-β and as negative regulators of TGF-β-induced EMT in NMuMG cells.

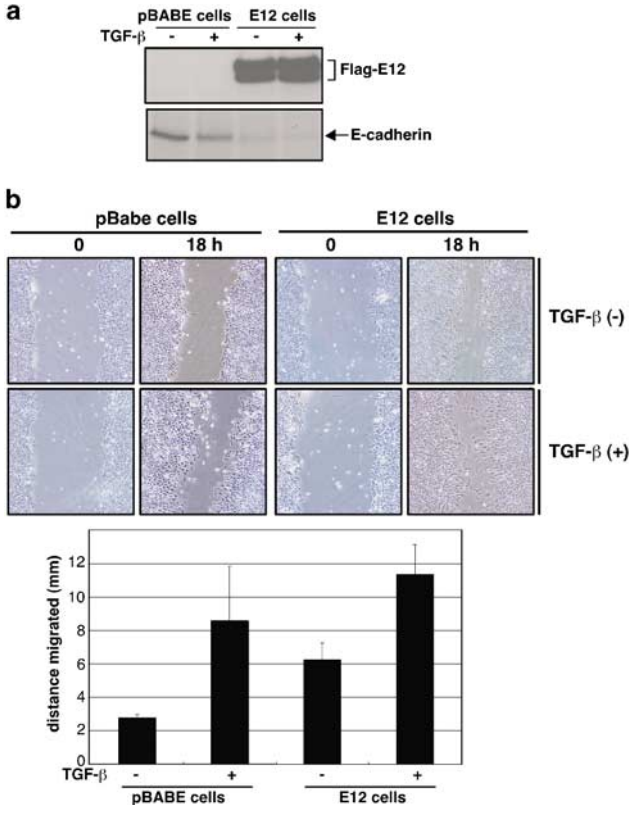

Figure 5 Effect of E12 overexpression on EMT. (**a**) Repression of E-cadherin expression in E12-infected cells. NMuMG cells were infected with pBabe vector (pBabe cells) or pBabe-Flag E12 (E12 cells). Expression of Flag-E12 and the endogenous E-cadherin levels were analyzed by immunoblotting. (**b**) The migratory behavior of pBabe cells and E12 cells, in the absence and presence of TGF-β, were analyzed by a wound assay (top) and quantitated (bottom) as described in Figure 4d

SB431542, a TβR-I inhibitor, blocked TGF-β-induced EMT and restored the expression of E-cadherin and Id2 proteins

Finally, we examined the effect of SB431542, a specific inhibitor of TβR-I, on TGF-β-induced EMT and expression of E-cadherin and Id proteins. As shown in Figure 6a and b, SB431542 almost completely blocked EMT induction and migration, and restored E-cadherin and Id2 expression regulated by TGF-β. These results suggest that TGF-β inhibitors might be used for the treatment of certain cancers through regulating EMT and cell migration.

Discussion

In the present study, we investigated the role of HLH transcriptional factors, Id and E2A, in the regulation of TGF-β-induced EMT in NMuMG cells. We found that down-regulation of Id proteins is, at least in part, required for TGF-β-induced EMT.

Among the Id protein family, TGF-β differentially regulates their expression, although their responses to BMP-4 are almost the same (Figure 2a). TGF-β downregulated only the Id2 protein after 4 h of treatment; thereafter, it downregulated Id1 and Id2. In addition, the basal level of Id3 in NMuMG cells was only weakly detected by immunoblotting when compared with the other Id proteins, and TGF-β treatment was able to repress Id3 basal levels (Figure 2a). These findings suggest

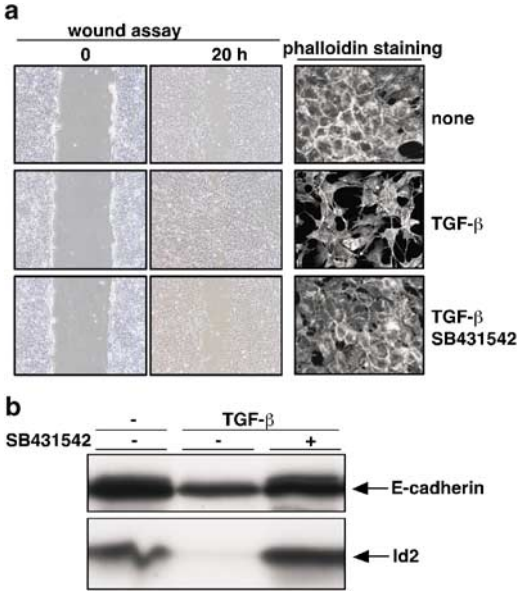

Figure 6 Effect of SB431542 on EMT. (**a**) Migratory behavior and organization of the actin cytoskeleton of NMuMG cells. NMuMG cells were stimulated with or without 100 pM TGF-β in the presence or absence of 1 μM of SB4331542 as described in Materials and methods. Cells were photographed immediately after the incision (left) and after 20 h (center), and stained with TRITC–phalloidin (right). (**b**) Immunoblotting of the indicated proteins from cells treated with or without TGF-β and SB431542

that TGF-β regulates the expression of Id genes by different mechanisms. It should be noted, however, that TGF-β repress the expression of all three Id proteins at later periods (after 24 h) in NMuMG cells. Recently, Kang *et al*.[21] reported that expression of Id1 by TGF-β is regulated by a transcriptional factor ATF-3 in epithelial cells. In contrast, Id2 is upregulated by c-myc oncoprotein in cancer cells and in cells treated with serum.[25,26] More recently, it has been shown that Id2 expression is dependent on myc–max transcriptional complexes in HaCaT and NMuMG cells.[27] In contrast to Id1 and Id2, the molecular mechanism by which TGF-β regulates the expression of Id3 is still unclear. In B-lymphocyte cells, stimulation by TGF-β for 1 h increased the expression of Id3 mRNA, and Id2 was also upregulated by TGF-β.[28] Taken together, the mechanisms by which TGF-β regulates the expression of Id proteins may be different between each Id protein, and may be dependent on cell type.

The E2A proteins strongly repressed the promoter activity of the E-cadherin gene and its protein level in NMuMG cells (Figures 3b and 5a), as well as in MDCK cells (Figure 3c, see also Perez-Moreno *et al*.[18]). Overexpression of either Id1 or Id2 in NMuMG cells partially restored the expression of E-cadherin, while Id3 overexpression fully derepress E-cadherin promoter activity in MDCK cells. These findings suggest that Id proteins suppress the activity of E12/E47 proteins to inhibit E-cadherin promoter activity, and that molecules other than Id proteins may be required for this effect in a cell type-specific context. One of the candidate molecules may be Twist.[29] Twist was identified as an inhibitory molecule for E2A, and was shown to bind directly to E12 to inhibit the expression of the muscle creatine kinase gene.[30] Previous studies showed that Twist may be involved in the inhibition of differentiation of multiple cell lineages, especially mesenchymal cells, including muscle, cartilage and bone cells.[29] It would be interesting to examine whether Twist acts as a modulator of E2A action in NMuMG cells.

For repression of E-cadherin in the process of EMT, the zinc-finger factors, Snail[14,31] and Slug,[32–34] and the Smad-interacting protein 1 (SIP1)[35] have been characterized previously. All of these proteins have been shown to bind directly to the promoter of the E-cadherin gene through specific E-boxes and to suppress its expression in various cell types. In fact, overexpression of these factors in NMuMG cells reduced E-cadherin promoter activity to levels similar to those induced by E2A proteins (data not shown). However, upregulation of these factors by TGF-β was not detected by RT-PCR (data not shown), and functional collaboration of these factors with Id proteins was not observed in NMuMG cells by monitoring for luciferase activity. Interestingly, we found that SIP1, but not Slug or Snail, interacted weakly with E2A in transfected COS-7 cells. Thus, an intriguing possibility is that E2A may cooperate with SIP1 in the regulation of E-cadherin expression, at least in cell-specific contexts.

Involvement of Smad pathways in the regulation of EMT is still controversial. RhoA has been shown to be a key player in exhibiting EMT by TGF-β, and its activity is independent of the inhibitory effect of Smad7.[36] However, we have observed that TGF-β-induced EMT in NMuMG cells was completely blocked by infection with Smad7 or Ski adenoviruses (data not shown) in agreement with other reports.[22,37] In addition, downregulation of Id proteins by TGF-β was completely inhibited by these treatments. Taken together, these results support that TGF-β-induced EMT in NMuMG cells is highly dependent on the Smad pathways.

EMT is thought to be a phenomenon that is important for development in early embryonic stages and for the invasion and metastasis of cancer cells. Our results using a TGF-β receptor kinase inhibitor indicate that inhibition of TGF-β signaling may block the EMT and migration/invasion induced by TGF-β, suggesting that these inhibitors may be used for the treatment of certain cancers. In conclusion, we propose that E2A proteins regulate the steady-state level of E-cadherin in NMuMG cells through binding to Id proteins, thus maintaining the original epithelial cell morphology. After downregulation of Id proteins induced by TGF-β or after constitutive expression of E2A, the E2A proteins would be in molar excess of the inhibitory Id proteins, leading to transdifferentiation from epithelial to mesenchymal phenotypes in NMuMG cells.

Materials and Methods

Reagents, constructs and antibodies

TGF-β3 and BMP-4 were purchased from R&D Systems (Minneapolis, MN, USA). The E-cadherin promoter (pGL2-pEDwt) containing the mouse promoter from -178 to $+92$ bp was fused to a luciferase reporter gene.[32] The cDNA constructs encoding E12 and E47 were from Dr. C Murre (University of California, CA, USA). The Id1 gene was obtained from Dr. H Weintraub (Fred Hutchinson Cancer Research Center, WA, USA), and the Id2 gene was as described previously.[38] Anti-phospho-Smad2 antibody and anti-phospho-Smad1/5 antibody were purchased from Cell-Signaling (Beverly, MA, USA). Anti-Id1, -Id2, -Id3 and -E2A antibodies and the blocking peptide against E2A were from Santa Cruz (Santa Cruz, CA, USA). Anti-E-cadherin antibody and anti-fibronectin antibody were purchased from BD Pharmingen (Transduction Laboratories, Lexington, KY, USA) and Calbiochem (San Diego, CA, USA), respectively. Anti-vimentin antibody was from Dako (Kyoto, Japan) and anti-α-tubulin from Sigma-Aldrich (St. Louis, MO, USA). SB-431542 was synthesized as described[39] and stored as a solution in DMSO. This solution was used after diluting with the medium for each assay.

Cell culture

Mouse NMuMG mammary epithelial cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l. glucose, 10% fetal bovine serum (FBS), 10 μg/ml insulin, 100 U/ml penicillin and 100 mg/ml streptomycin. MDCK cells were grown in DMEM in the presence of 10% FBS, 10 mM glutamine and antibiotics. Cells were grown in a 5% CO_2 atmosphere at 37°C.

RT-PCR analysis

Total RNA was isolated from NMuMG cells with Isogen (Nippon Gene, Tokyo, Japan) and first-strand cDNA was synthesized using the Superscript First-strand synthesis System (Invitrogen, Carlsbad, CA, USA) with random hexamer primers as described by the manufacturer's instructions. The expression of Id1, Id2, Id3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was compared by semiquantitative RT-PCR analysis. A mouse GAPDH was used to normalize the amount of total cDNA in each sample. PCR conditions were 95°C (1 min), 25 cycles

of 95°C (15 s), 60°C (30 s), 72°C (1 min) for GAPDH, Id1 and Id2, and 30 cycles for Id3, followed by 10 min at 72°C. PCR primers used in this experiment were: f-Id1, 5′-AGGTGAACGTCCTGCTCTACGA-3′; r-Id1, 5′-CAGGATCTCCACCTTBCTCACT-3′; f-Id2, 5′-CCGGATTCAGCATGAA-AGCCTTCAGTCCG-3′; r-Id2, 5′-CCTTCGAATTAGCCACAGAGTACT-TT-3′; f-Id3, 5′-CCTCTTGGACGACATGAACCA-3′; r-Id3, 5′-TTCAGGC-CACCCAAGTTCAGT-3′; f-mGAPDH, 5′-TGCAGTGGCAAAGTGGAG-ATT-3′; and r-mGAPDH, 5′-TTGAAGTCGCAGGAGACAACCT-3′. PCR products were separated by electrophoresis in agarose gel (2%) and visualized with ethidium bromide.

Transfection, immunoprecipitation and immunoblotting

NMuMG cells and MDCK cells were transiently transfected using Fugene 6 (Roche Molecular Biochemicals) and Lipofectamine Plus (Gibco BRL), respectively. Immunoprecipitation and immunoblotting were performed using anti-Flag M2 antibody (Sigma-Aldrich) or other antibodies as described previously.[40] NMuMG cells or retrovirus-infected cells were grown to 90% confluency. After stimulation with 1.4 nM (50 ng/ml) BMP-4 or 100 pM TGF-β, cells were put on ice, rinsed with PBS (−) and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodiumdeoxycholate and 0.1% SDS) for immunoblotting or in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1.5% aprotinin and 1 mM phenylmethylsulfonylfluoride) for immunoprecipitation. Cell lysates were quantitated by Bradford analysis for protein content and extracts containing equal amounts of total proteins were processed to SDS gel electrophoresis, followed by semidry transfer of the proteins to Pall Fluorotrans-W membrane (Pall corp.). Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) containing 5% skim milk or 5% polyvinylpyrrolidone (Calbiochem).[41] The detection of immunoblotted proteins was performed by enhanced chemiluminescence.

Luciferase assay

NMuMG and MDCK cells were transiently transfected with appropriate combinations of reporter constructs (pGL2-pEDwt), expression plasmids and pcDNA3.0. The total amount of transfected DNAs was the same in each experiment. Luciferase activities were assayed by the luciferase assay system (Promega) and normalized to sea-pansy luciferase activity under the control of the thymidine kinase promoter.

Retrovirus vectors and generation of stable cell lines

pBabe-TRE was constructed by inserting a hygromycin resistance gene, followed by TRE and the minimal CMV promoter from pTRE (Clontech), between the modified gag gene and 3′-UTR of pBabe-puro. pBabe-tTA was constructed by inserting a puromycin resistance gene, followed by the CMV promoter and the tTA gene from pTET-OFF (Clontech), between the modified gag gene and 3′-UTR of pBabe-puro.[42] The cDNAs encoding Flag-tagged Id2 and E12 were subcloned into pBabe-TRE. To obtain stable cell lines, 293T cells were first cotransfected with pBabe-tTA and ecotropic-helper plasmids by Fugene 6 to produce culture supernatants containing virus. NMuMG cells were then infected with retrovirus by culturing for 2 days in the conditioned media with 8 μg/ml of polybrene before selection with 3 μg/ml of puromycin. Several puromycin-resistant clones were established and analyzed for inducible efficiencies after infection with pBabe-TRE-EGFP. The clones most efficiently expressing tTA, clones 9 and 15, were further infected with retrovirus in the conditioned media derived from 293T cells cotransfected with pBabe-TRE, pBabe-Flag-Id2 or pBabe-Flag-E12 and ecotropic-helper plasmids. After reselection with 1 mg/ml of hygromycin, the double resistant clones were maintained in DMEM supplemented with 4.5 g/l glucose, 10% FBS, 1 μg/ml tetracycline (Tet), 1 mg/ml hygromycin and 3 μg/ml puromycin, before use in further experiments.

Transdifferentiation of NMuMG cells and analysis by fluorescence microscopy

NMuMG cells were seeded at a density of 0.75×10^5 cells/well on glass coverslips in six-well plates. Approximately 6 h later, the medium was changed and cells were further cultured for 18 h. Cells were then stimulated with BMP-4 or TGF-β in the normal culture medium containing 10% FBS. The hygromycin and puromycin double resistant clones of NMuMG cells infected with pBabe-tTA and either pBabe-TRE or pBabe-Flag-Id2 were maintained in DMEM supplemented with 4.5 g/l glucose, 10% FBS, 10 μg/ml insulin and antibiotics (1 mg/ml hygromycin and 3 μg/ml puromycin) in the presence of 1 μg/ml Tet. Similar to normal NMuMG cells, the infected NMuMG cells were stimulated with TGF-β after they had been seeded in the presence or absence of Tet. After 24–36 h of stimulation, cells were treated to allow direct fluorescence of the actin cytoskeleton with 0.25 mM TRITC-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO, USA) as described previously.[22] All specimens were mounted onto glass slides and observed by confocal laser scanning microscopy.

Migration assay

NMuMG cells and NMuMG cells infected with pBabe-TRE, pBabe-Flag-Id2 or pBabe-Flag-E12 were seeded at 1.5×10^5 cells/well in six-well tissue culture plates. After cells reached 80% confluency, a wound was incised with a pipette tip in the central area of culture and 100 pM TGF-β or 1.4 nM BMP-4 was added. Photographs were taken under phase-contrast microscopy immediately after the incision, and after 18–24 h of ligand stimulation. When a wound was incised, an edge of the wound was marked with a thin line on the bottom of each well. After 18–24 h, the migration of wound edges (healing the wound area) from the line was measured at three random points.[43]

Invasion assays

Cell invasion assays were performed using a Cell Culture Insert (8 μm pore size; FALCON). Type IV collagen (BD Biosciences) was added to the Cell Culture Insert (5 μg/well) and dried. Cells pretreated with or without 40 pM TGF-β were split and reseeded in each well at a concentration of 4×10^4 cells/well in the presence or absence of TGF-β. After 12 h, the cells that had not invaded were removed from the upper face of the filters using cotton swabs, and the cells that had invaded to lower surface of the filter were fixed in methanol and stained with Giemsa. Invasion was quantitated by visual counting after photographed. The mean of three random fields was obtained for each well. Each experiment was performed in triplicate.[44]

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