# Transforming growth factor-β and epidermal growth factor synergistically stimulate epithelial to mesenchymal transition (EMT) through a MEK-dependent mechanism in primary cultured pig thyrocytes

**Mats Grände[1,*], Åsa Franzen[2], Jan-Olof Karlsson[1], Lars E. Ericson[1], Nils-Erik Heldin[2] and Mikael Nilsson[1]**

[1]Institute of Anatomy and Cell Biology, Göteborg University, Göteborg, and [2]Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University Hospital, Uppsala, Sweden

*Author for correspondence (e-mail: mats.grande@anatcell.gu.se)

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## Summary

**Enhancement of tumor cell growth and invasiveness by transforming growth factor-β (TGF-β) requires constitutive activation of the ras/MAPK pathway. Here we have investigated how MEK activation by epidermal growth factor (EGF) influences the response of fully differentiated and growth-arrested pig thyroid epithelial cells in primary culture to TGF-β1. The epithelial tightness was maintained after single stimulation with EGF or TGF-β1 (both 10 ng/ml) for 48 hours. In contrast, co-stimulation abolished the transepithelial resistance and increased the paracellular flux of [$^3$H]inulin within 24 hours. Reduced levels of the tight junction proteins claudin-1 and occludin accompanied the loss of barrier function. N-cadherin, expressed only in few cells of untreated or single-stimulated cultures, was at the same time increased 30-fold and co-localised with E-cadherin at adherens junctions in all cells. After 48 hours of co-stimulation, both E- and N-cadherin were downregulated and the cells attained a fibroblast-like morphology and formed multilayers. TGF-β1 only partially inhibited EGF-induced Erk phosphorylation. The MEK inhibitor U0126 prevented residual Erk phosphorylation and abrogated the synergistic responses to TGF-β1 and EGF. The observations indicate that concomitant growth factor-induced MEK activation is necessary for TGF-β1 to convert normal thyroid epithelial cells to a mesenchymal phenotype.**

Key words: Claudin, E-cadherin, EGF, EMT, Erk, N-cadherin, Occludin, TGF-β, Thyroid

## Introduction

The differentiated epithelial phenotype is characterised by structural and functional polarisation of the cell surface into apical and basolateral domains, and formation of a junctional complex that mediates strong intercellular adhesion and paracellular tightness (Cereijido et al., 2000). The tight junction consists of a multiprotein complex comprising the transmembrane proteins occludin and claudins that exert fence and gate functions, and a set of cytoplasmic proteins (ZO-1, ZO-2, ZO-3, and others) that link the junction to microfilaments and regulate its assembly (Tsukita et al., 2001). The epithelial adherens junction has a similar structural organisation in which E-cadherin forms homophilic contacts between neighbouring cells, and the catenins mediate the connection of the E-cadherin cytoplasmic tail to the submembranous cytoskeleton (Nagafuchi, 2001). E-cadherin is a member of the classical cadherin family and has a critical role in the development of cell polarity and to establish firm adhesion (Vleminckx and Kemler, 1999). E-cadherin is also involved in growth control by contact inhibition and maintenance of epithelial tightness, thus influencing the overall differentiation state of the cells.

Epithelial to mesenchymal transition (EMT) designates cell changes characterized by loss of the epithelial phenotype and is encountered in many biological processes, such as embryonic development and cancer progression (Hay, 1995; Birchmeier et al., 1996; Thiery and Chopin, 1999; Savagner, 2001). The mechanisms that govern EMT are not fully understood, although a primary event is downregulation of E-cadherin. Transforming growth factor-β (TGF-β) is one of the main EMT-inducing factors in both normal and pathological conditions. Expression profiling analysis has identified a great number of genes that are turned on or off by TGF-β (Zavadil et al., 2001), but for most of the corresponding proteins their contributions to EMT are yet to be established. Although E-cadherin expression is lost during TGF-β-induced EMT (Miettinen et al., 1994), Rho-dependent cytoskeletal rearrangements (Bhowmick et al., 2001a) and altered integrin binding to extracellular matrix (Bhowmick et al., 2001b) also seem to be required. TGF-β-induced EMT has been linked to activation of Smad (Bhowmick et al., 2001b; Piek et al., 1999) and phosphatidylinositol 3-kinase (PI3K) (Bakin et al., 2000) signalling pathways. Interestingly, the ability of TGF-β to promote tumor cell invasiveness depends on constitutive

activation of MAPK by mutated Ras (Fujimoto et al., 2001; Ellenrieder et al., 2001). This raises the possibility that ligands to the receptor tyrosine kinase (RTK) family of growth factor receptors might interact with TGF-β, by autocrine or paracrine mechanisms, to co-operatively stimulate EMT.

TGF-β has profound effects on the growth and function of thyroid epithelial cells, and EMT-like responses have been reported (Toda et al., 1997). However, using reconstituted pig thyroid follicles primary cultured in collagen gel, we did not observe any morphological signs of EMT after TGF-β1 stimulation unless epidermal growth factor (EGF) was present (Nilsson et al., 1995). Thus, when EGF was added along with TGF-β1 the thyrocytes became spindle-shaped and migrated out of the follicles into the surrounding matrix. Moreover, ultrastructural analysis suggested that cell-cell contacts of the follicular epithelium were disrupted at sites of active cell movements only in co-stimulated cultures. The present study, conducted on confluent pig thyrocytes grown on filter, was undertaken to characterise the co-operative effects of TGF-β1 and EGF on the thyroid epithelial phenotype and to investigate the possible role of MEK activation. We find that TGF-β1 and EGF synergistically promotes EMT, comprising loss of expression of E-cadherin and the tight junction proteins occludin and claudin-1, whereas separately the growth factors are ineffective. The joint action also involves a transient expression of N-cadherin that is localised to cell-cell contacts before E-cadherin is being downregulated. Moreover, specific inhibition of EGF receptor tyrosine kinase or MEK activities completely abrogates the mesenchymal conversion of the thyroid epithelial cells in response to TGF-β1 and EGF co-stimulation.

## Materials and Methods

### Cell culture and experimental conditions

Pig thyrocytes were isolated from thyroid glands as previously described (Nilsson and Ericson, 1994). In brief, the glands were minced and incubated in collagenase followed by repeated mechanical disintegration. Single cells and tissue remnants were removed by serial centrifugation and filtration adjusted to enrich follicle segments, which were seeded in Transwell bicameral chambers (Corning Costar, Cambridge, MA) pre-coated with collagen I (Roche, Basel, Switzerland). The cells were cultured in minimum essential medium (MEM) with the addition of fetal bovine serum (FBS; 5%), penicillin (200 U/ml), streptomycin (200 U/ml) and fungizone (2.5 μg/ml), in humidified atmosphere (5% $CO_2$) at 37°C. All culture reagents were from Gibco (Paisley, UK). Confluent cells were stimulated with EGF (10 ng/ml; Roche), TGF-β1 (10 ng/ml; Sigma, St Louis, MO) or co-stimulated with both EGF and TGF-β1 in low serum (0.5% FBS) conditions. In some experiments, the cultures were in addition incubated with 1.5 μM of the EGF receptor kinase inhibitor AG1478 (Biomol Research Laboratories, Plymouth Meeting, PA), 25 μM of the MEK inhibitor U0126 (Biomol Research Laboratories), 5 μM LY294002 (Sigma), 100 mM wortmannin (Sigma), or 50 μM of the caspase inhibitor zVAD-fmk (Calbiochem, Nottingham, UK). Experiments were conducted at least three times for the different assays employed.

### Transepithelial electrical resistance and flux of radiotracer

The electrical resistance established across confluent cultures grown on filter was measured with an ohmmeter (Millcell ERS; Millipore, Bedford, MA) at different times after growth factor addition. Paracellular permeability was estimated by measuring the transepithelial flux of [$^3$H]inulin (0.1 μCi/ml) after 24 hours stimulation with one or both of the growth factors. The radiotracer was added to the apical chamber and the amount of radioactivity recovered in the basal chamber after incubation for 20 minutes at 37°C was measured in a LKB Wallac scintillator.

### Antibodies

Antibodies used for western blotting and immunofluorescence were directed towards the following proteins: E-cadherin (Transduction Laboratories, Lexington, KY), occludin (Transduction), claudin-1 (Zymed, San Francisco, CA), N-cadherin (3B9; Zymed), β-actin (AC-15; Sigma), Erk1 (sc-94; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-p44/42 MAPK (#9101S; Cell Signalling Technology, Beverly, MA), Smad2 (DQQ) and phosphorylated Smad2 (PS2; both Smad antibodies kindly provided by C-H Heldin, Uppsala, Sweden). In addition, a pan-cadherin antibody raised against the conserved cytoplasmic domain of N-cadherin (CH-19; Sigma) was used. Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Dako (Glostrup, Denmark). The biotin-conjugated anti-mouse antibody was obtained from Amersham Biosciences (Uppsala, Sweden).

### Western blot analysis

Cells were solubilized in lysis buffer consisting of 62.5 mM Tris, 2% sodium dodecyl sulphate (SDS) and a mixture of protease inhibitors: 5 μg/ml each of leupeptin and aprotinin (Sigma) and 0.4 mM 4-(2-aminoethyl)-benzolsulfonylfluoride (Pefabloc; Roche). The protein concentration was determined with the Micro BCA™ protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). The samples were diluted with equal volumes of supplementing lysis buffer (62.5 mM Tris, 20% glycerol, 10% 2-mercaptoethanol, 0.075% bromphenol blue and 2% SDS) and further diluted to equal concentrations. After boiling for 8 minutes, the samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-Protean II, 4-15% polyacrylamide; Bio-Rad, Hercules, CA) and transferred to nitro-cellulose filters (0.45 μm) in a mini trans-blot cell (Bio-Rad). Filters were blocked in a buffer consisting of 5% fat-free dried milk in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6), and then incubated with primary and secondary antibodies for 1 hour each. Antibody incubations were followed by washings 6×5 minutes in TBS-Tween. The immunolabelled proteins were detected by enhanced chemiluminescense (ECL; Amersham Biosciences). Molecular weights were estimated by comparison with pre-stained SDS-PAGE molecular weight standards (Bio-Rad). Densitometric evaluation of immunoblotted proteins were performed on a GS-700 Imaging Densitometer (Bio-Rad) using the Molecular Analyst software. Western blot analysis of Erk and phospho-Erk was done as previously described (Franzen and Heldin, 2001).

### Immunofluorescence

Filter-grown cells were fixed in ice-cold ethanol for 20 minutes and washed twice in PBS, pH 7.4. Unspecific binding was blocked by preincubation with avidin-biotin blocking reagents (Vector, Burlingame, CA) for 2×10 minutes and with a buffer consisting of 5% fat-free dried milk, 0.1% gelatin and 7.5% sucrose in PBS, for 10 minutes. The cells were further incubated in sequence with primary antibodies for 1 hour, secondary biotin-conjugated antibodies for 30 minutes and fluorescein-isothiocyanate (FITC)-conjugated streptavidin for 30 minutes. Immunolabelled cells were counterstained with DAPI (Sigma) to show nuclear morphology. The filters were cut out of the inserts, mounted with Vectashield™ (Vector), and examined and photographed in a Nikon Microphot FXA epifluorescence microscope equipped with a QLC100 confocal laser scanning module (VisiTech International, Sunderland, UK). Images

were processed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

### [$^3$H]Thymidine incorporation

Confluent cells were stimulated with EGF and TGF-β1 under low serum conditions (0.5% FBS) and [$^3$H]thymidine (1 μCi/ml) was added to the culture medium of the basal chamber for 0-24 and 24-48 hour periods. Incorporation was determined after fixation of the cells in 10% trichloroacetic acid, and extensive washout of free radioactivity. The filter was cut from its holder, solubilised in 1 ml Soluene-100 (Packard Instrument Company, Downers Grove, IL) and analysed by liquid scintillation in a Packard gamma-counter.

### Caspase-3 activity

Cultures treated with EGF and TGF-β1 for 24 or 48 hours were gently washed twice in MEM and frozen at –20°C. Cells were thawed and extracted in 200 μl of 50 mM Tris-HCl (pH 7.3) containing 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.2% CHAPS, 3 mM $NaN_3$, 1 mM PMSF, pepstatin (1 μg/ml), leupeptin (2.5 μg/ml), and aprotinin (10 μg/ml), and incubated at 37°C for 45 minutes with occasional stirring. After centrifugation for 5 minutes in a Microfuge, 50 μl supernatant aliquots were mixed with 50 μl of 50 μM DEVD-AMC (Calbiochem, La Jolla, CA) a synthetic caspase-3 substrate, and 4 mM DTT dissolved in extraction buffer without CHAPS, and incubated in a non-transparent (white) microtiter plate (Microfluor; Dynatech, VA) placed in a Perkin-Elmer LS 50B luminescence spectrometer. Cleavage of the substrate was measured at room temperature at an excitation wavelength of 380 nm (slit 10 nm) and an emission wavelength of 460 nm (slit 15 nm). The degradation was followed at intervals of 2-3 minutes at a rate, which was linear for at least 2 hours. Data from samples were obtained after approximately 60 minutes and zero time values were subtracted from each well. Standard curves with AMC in the appropriate buffer were used to express the data in pmoles AMC formed per minute and mg protein or filter. Addition of a known amount of AMC to the complete incubation mixture demonstrated a recovery better than 95%. Protein concentration was determined as described above.

### Electron microscopy

Filter-grown cells were fixed for 1 hour in 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4, followed by post-fixation for 1 hour in 1% $OsO_4$, dehydration in ethanol series, and embedding in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Philips 400 T electron microscope.

## Results

### TGF-β1 and EGF reciprocally inhibits thyroid cell growth and apoptosis

The growth response of primary cultured pig thyrocytes to EGF and TGF-β stimulation was investigated. Before addition of growth factors, the filter-cultured cells were largely growth-arrested due to high cell density. As shown in Fig. 1A, EGF (10 ng/ml) increased the incorporation of [$^3$H]thymidine after 24 hours and more pronouncedly after 48 hours, thus enhancing the proliferation rate within the confluent cell monolayer. TGF-β1 present for 48 hours abolished both baseline and EGF-induced DNA synthesis (Fig. 1A). In order to evaluate if apoptosis was simultaneously induced, the degradation of a caspase-3 specific synthetic substrate was measured by fluorometry after 24 and 48 hours of growth factor stimulation. TGF-β1-treated cultures showed an increased caspase-3 activity at both time intervals (Fig. 1B). The caspase-3 activation by TGF-β1 was low compared to that induced by pro-apoptotic agents like staurosporine, and only a minority of the cells displayed apoptotic nuclei profiles (data not shown; manuscript in preparation). Caspase-dependent proteolysis was noted also after EGF stimulation for 48 hours (Fig. 1B). Interestingly, the low caspase-3 activity recorded in co-stimulated cultures was not different from that of control cultures (Fig. 1B). These findings show that single stimulation with EGF or TGF-β1 induces growth and survival responses typical of each factor and, moreover, that reciprocal inhibitory effects are encountered when the factors are added together.

### TGF-β1 and EGF synergistically induce loss of the thyroid epithelial phenotype

Confluent thyrocytes grown on filter formed a polarised monolayer (Fig. 2A) that displayed a high transepithelial resistance (Fig. 3A) and a diffusion barrier to radiolabeled [$^3$H]inulin (Fig. 3B). Single stimulation with TGF-β1 or EGF

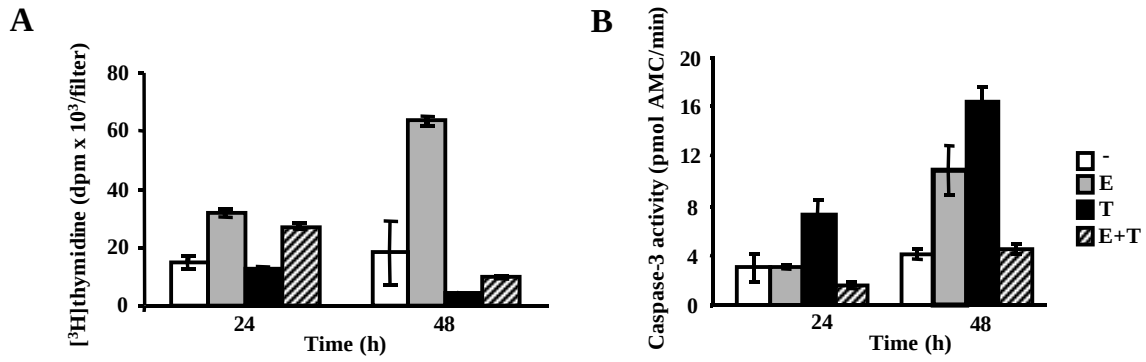


**Fig. 1.** Effect of TGF-β1 and EGF on cell proliferation and apoptosis in filter-cultured pig thyroid epithelial cells. (A) [$^3$H]Thymidine incorporation. Confluent cells stimulated with EGF (10 ng/ml) or TGF-β1 (10 ng/ml) alone or in combination were simultaneously exposed to 1 μCi/ml [$^3$H]thymidine, added to the basal medium of the bicameral chamber, for 0-24 or 24-48 hours, after which incorporated radioactivity was counted. Mean±s.d. ($n$=3). (B) Caspase-3 activity. Filter cultures were lysed after growth factor stimulation at the indicated times and the degradation rate of a caspase-3 specific fluorogenic substrate (DEVD-AMC) was measured. Mean±s.d. ($n$=3).

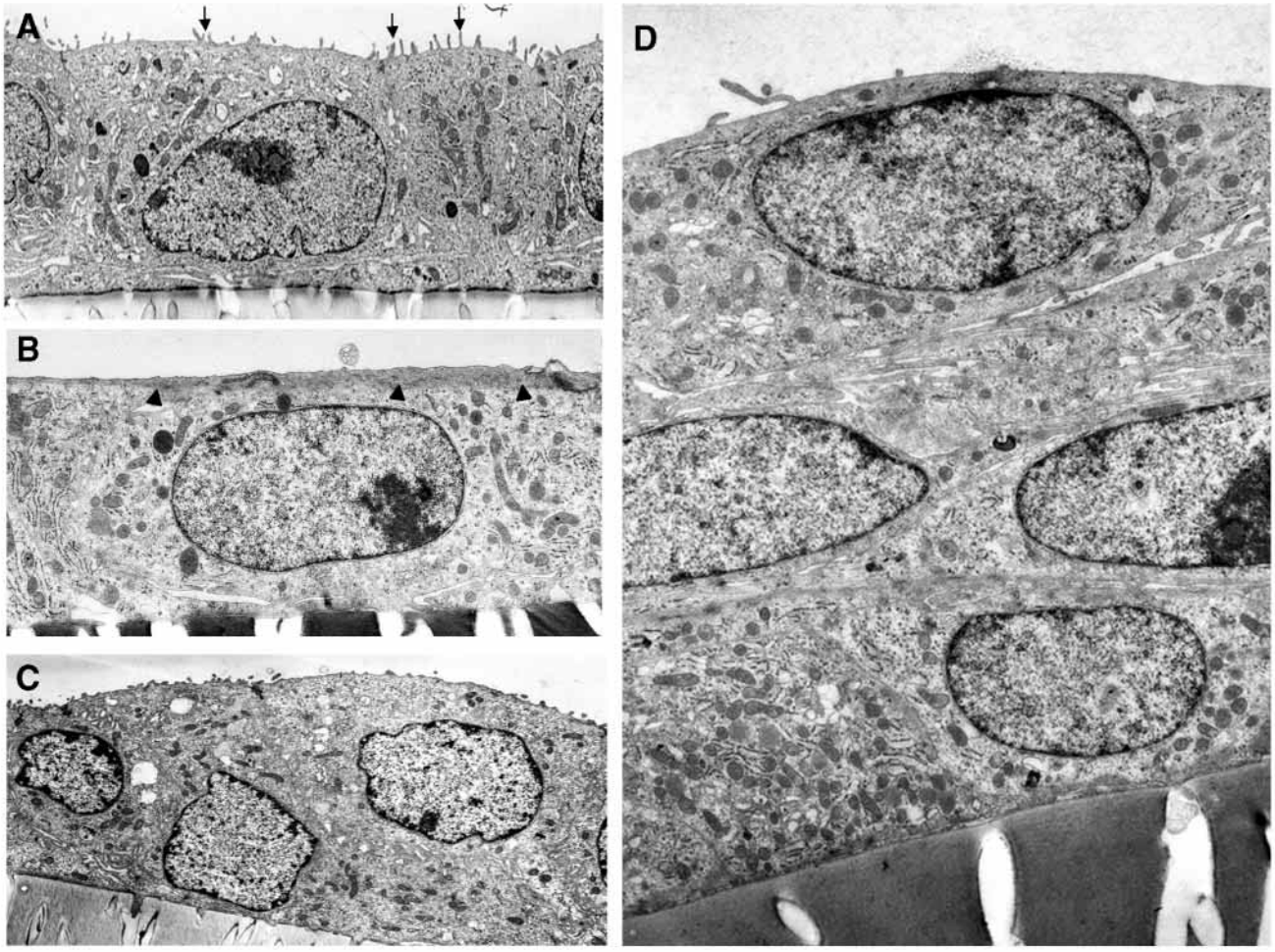


**Fig. 2.** Electron micrographs of filter-cultured pig thyrocytes after TGF-β1 and EGF stimulations. (A) Portion of an untreated cell monolayer. The cells are polarised with numerous microvilli (arrows) at the apical plasma membrane. (B) Cells exposed to TGF-β1 (10 ng/ml) for 48 hours. The monolayer organisation is similar to that of control cultures. Apical microvilli are lost and the appearance of submembranous microfilament condensations is evident (arrowheads). (C) Cells treated with EGF (10 ng/ml) for 48 hours. The cells are tall and crowded due to increased proliferation, but the monolayered epithelium is largely maintained. The apical plasma membrane displays microvilli. (D) Cells co-stimulated with TGF-β1 and EGF (both 10 ng/ml) for 48 hours. The cells are flat and elongated and extend on top of each other in multiple layers. Distinct junctional complexes are not present.

for 48 hours caused distinct subcellular changes but the general epithelial phenotype of the cells was largely maintained (Fig. 2B,C). Either factor alone did not change the epithelial barrier function during the same time interval (Fig. 3A,B). In contrast, co-stimulated cells gradually attained a flattened and elongated shape, and after 48 hours extensive multilayering was observed (Fig. 2D). The epithelial disorganisation was preceded by a reduced transepithelial resistance that was evident already after 8 hours and fully developed after 24 hours (Fig. 3A). The failure of co-stimulated cultures to keep a barrier function was confirmed by [$^3$H]inulin flux measurements (Fig. 3B). Cells exposed to both TGF-β1 and EGF had less developed junctional complexes as judged by electron microscopy (data not shown). Western blot analysis of transmembrane tight junction proteins showed that both occludin and claudin-1 were downregulated in the co-stimulated cells (Fig. 4). TGF-β1 alone had no effect on the occludin and claudin-1 levels, whereas EGF selectively increased the expression of claudin-1 (Fig. 4; see also Fig. 7A).

### TGF-β1 and EGF co-stimulate transient N-cadherin expression preceding E-cadherin downregulation

As the morphological changes of co-stimulated thyrocytes resembled that of EMT we examined whether the expression or localisation of E-cadherin, a major determinant of the epithelial phenotype, were simultaneously altered. As shown in Fig. 5A (upper panel, left), untreated confluent cells on filter uniformly expressed E-cadherin along the entire cell periphery, corresponding to the expected location of epithelial adherens junctions. Single stimulation with EGF or TGF-β1 for 8-48 hours did not change the total level of E-cadherin (Fig. 5B, left panel) nor its junctional localisation (shown for 24 hours stimulations in Fig. 5A). Similarly, after co-stimulation with TGF-β1 and EGF for 24 hours both the expression (Fig. 5B) and localisation (Fig. 5A, upper panel, right) of E-cadherin were maintained. However, loss of E-cadherin expression was evident after co-stimulation for 48 hours (Fig. 5B). Together, these findings suggested that the impaired barrier function resulting from the combined TGF-β1 and EGF stimulation was

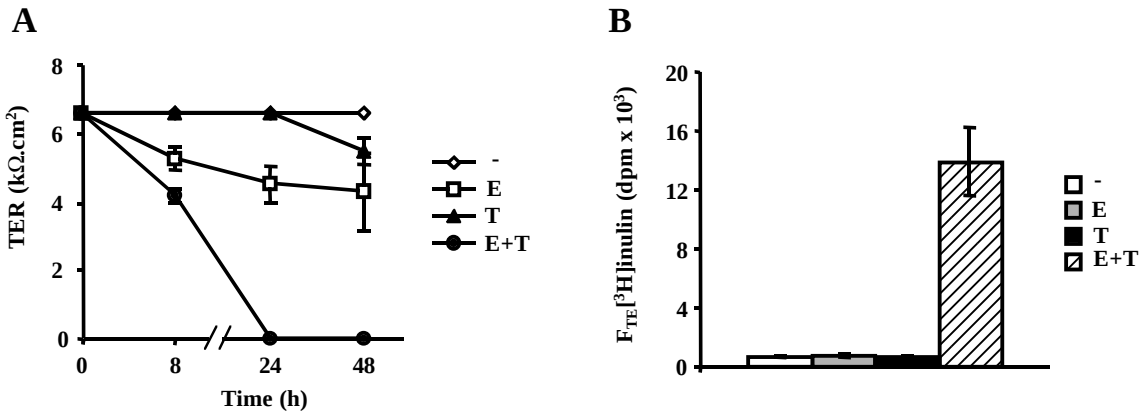


**Fig. 3.** Effects of TGF-β1 and EGF on epithelial barrier function in filter-cultured thyrocytes. (A) Transepithelial resistance (TER) measured consecutively in the same cultures at the indicated times. Mean±s.d. (*n*=3). (B) Apical-to-basal transepithelial flux of [$^3$H]inulin (0.1 μCi/ml) recorded after growth factor stimulation for 24 hours. Mean±s.d. (*n*=3). E, EGF (10 ng/ml); T, TGF-β1 (10 ng/ml).

not secondary to major changes in E-cadherin-mediated adhesion.

N-cadherin, normally present mainly in neuronal and muscle tissues, is aberrantly expressed in epithelial tumours and currently discussed as a mechanism by which carcinoma cells acquire a migrating and invasive phenotype. It was therefore of interest to investigate if N-cadherin might be up-regulated also in normal epithelial cells undergoing EMT. We found only minute amounts of N-cadherin in untreated cultures (Fig. 5B), and the expression was restricted to a limited number of cells within the monolayer (Fig. 5A, lower panel). Single stimulation with TGF-β1 for 8-48 hours had no effect, whereas EGF caused a small increase in total N-cadherin expression (Fig. 5B). In marked contrast, EGF and TGF-β1 added together induced a 30-fold increase in N-cadherin already after 24 hours (Fig. 5B). Immunofluorescence revealed that all co-stimulated cells were recruited to express N-cadherin that largely co-localised with E-cadherin at the adherens junctions (Fig. 5A). However, the expression of N-cadherin was transient, and after 48 hours treatment with TGF-β1 and EGF N-cadherin was hardly detectable (Fig. 5B). The loss of N-cadherin coincided with that of E-cadherin (Fig. 5B). Interestingly, low molecular immunoreactive bands were regularly detected for both E- and N-cadherin (Fig. 5C), suggesting that proteolytic degradation likely contributed to the decreased expression. The cadherin cleavage was unaffected by long-term incubation with the caspase inhibitor zVAD-fmk (data not shown).

### Involvement of MEK-mediated Erk phosphorylation in synergistic response to TGF-β1 and EGF

Investigation of the Ras/MAPK signalling pathway showed that EGF rapidly induced Erk phosphorylation (Fig. 6A) that sustained at high levels for several hours (Fig. 6B). TGF-β1 alone also phosphorylated Erk but only to a small degree that lasted for less then 30 minutes (Fig. 6A). Moreover, TGF-β1 counteracted the EGF-induced Erk phosphorylation (Fig. 6B). Nevertheless, maintained EGF receptor signalling was required to obtain a synergistic response, as both loss of barrier function and increased expression of N-cadherin was completely blocked by the EGF receptor inhibitor AG1478 (data not shown). The fact that TGF-β1 only partly inhibited the Erk activation by EGF (Fig. 6B) suggested that residual MEK activity might be critical to transduce the EMT-promoting signal(s). To investigate this possibility cells were co-stimulated with TGF-β1 and EGF in the presence of the MEK inhibitor U0126. As shown in Fig. 7A, U0126 blocked both the up-regulation of N-cadherin and the downregulation of occludin and claudin-1. In addition, the transepithelial resistance was maintained at a high level when the MEK blocker was added to co-stimulated cultures (data not shown). As expected, EGF-induced phosphorylation of Erk was completely inhibited by U0126 (Fig. 7B). The importance of EGF receptor signalling through the Ras/MAPK/Erk pathway was further emphasised by the finding that blocking of PI3K signalling by co-incubation with LY294002 did not at all influence the synergistic effects of TGF-β1 and EGF, for

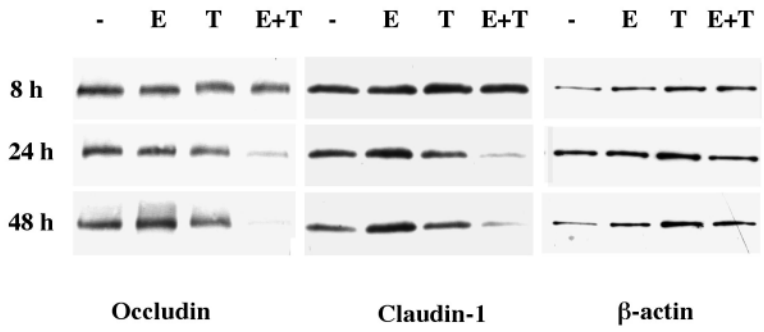


**Fig. 4.** Expression of occludin and claudin-1 proteins in growth factor-stimulated thyrocytes. Cultures were exposed to TGF-β1 (10 ng/ml) and/or EGF (10 ng/ml) for the indicated times, after which cell lysates at equivalent protein concentrations were analysed with SDS-PAGE and western blotting. β-actin was used as an internal control for equal protein loading. E, EGF; T, TGF-β1.

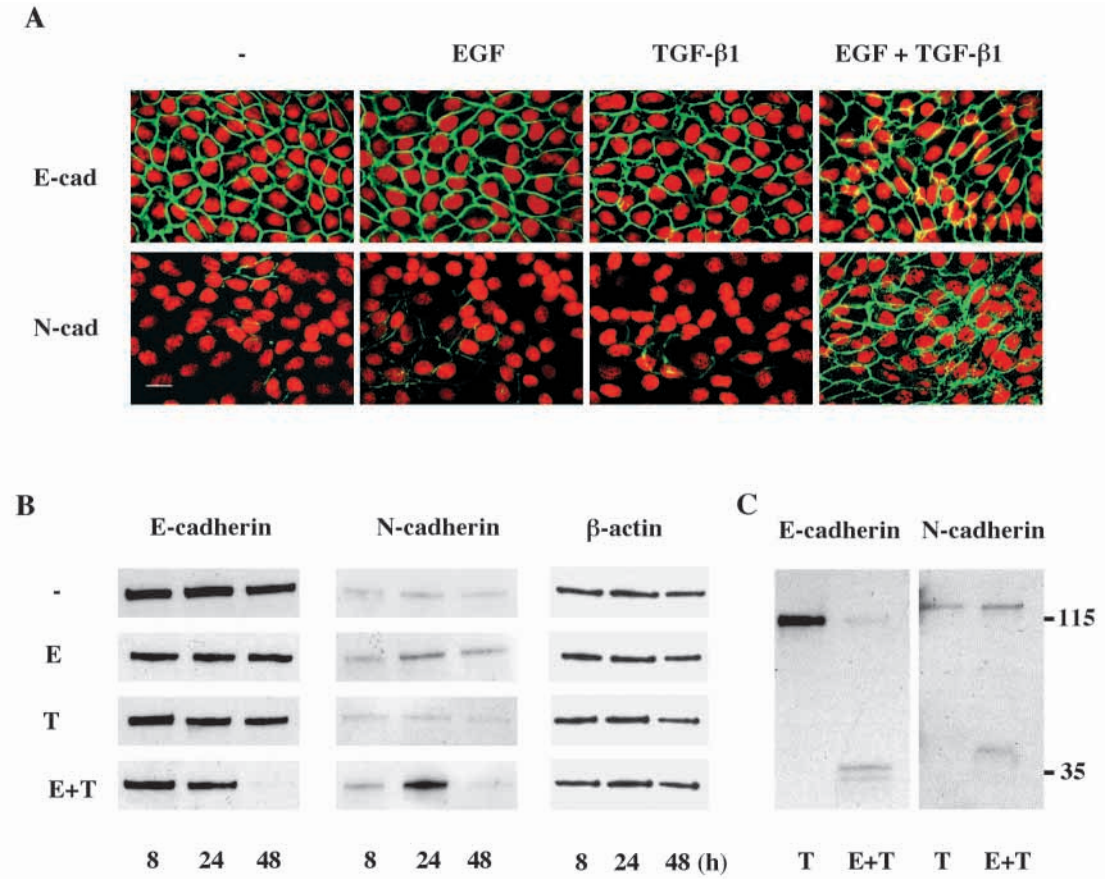


**Fig. 5.** Expression of E-cadherin and N-cadherin in growth factor-stimulated thyrocytes. (A) Immunofluorescent staining of filter-cultured cells treated with TGF-β1 (10 ng/ml) and EGF (10 ng/ml) for 24 hours. (Upper panel) E-cadherin (E-cad); (lower panel) N-cadherin (N-cad). Specimens were counterstained with DAPI (red) to visualise the cells' nuclei. N-cad was immunolocalised with clone CH-19, which is a pan-cadherin antibody raised against a sequence of the highly conserved cytoplasmic domain of N-cadherin. CH-19 gives results identical to that of the 3B9 monoclonal N-cadherin antibody in western blot analysis, but was used for immunofluorescence due to superior staining intensity. Bar, 15 μm. (B) Western blot analysis of E-cadherin and N-cadherin (using clone 3B9) after stimulation with TGF-β1 (T; 10 ng/ml) and/or EGF (E; 10 ng/ml) for the indicated times. β-actin indicates equivalent protein loading. (C) Appearance of distinct small size bands with E- or N-cadherin immunoreactivity after co-stimulation for 48 hours.

example, on N-cadherin expression (Fig. 7C). Wortmannin, another PI3K inhibitor, was also without effect although at the same time a typical inhibition of PI3K-dependent endosomal transport was evident (data not shown). Lastly, TGF-β1-induced Smad2 phosphorylation was found to be unaffected by EGF co-stimulation (data not shown), suggesting a potential role of Smad-dependent TGF-β1 receptor signalling in the synergistic induction of EMT in thyroid cells.

## Discussion

TGF-β and EGF are known to exert mutually antagonistic effects on cell cycle control and apoptosis in normal epithelial cells. In the present study, conducted on highly differentiated pig thyroid follicular cells in primary culture, we show that TGF-β1 and EGF in addition synergistically promote the loss of tight junctions and epithelial-type adherens junctions and the development of a spindle-shaped, fibroblastoid morphology. The phenotypic change of co-stimulated cells is accompanied by a pronounced but transient increase in the expression of N-cadherin. In contrast, thyrocytes exposed to either TGF-β1 or EGF maintain the epithelial properties. From this we conclude that phenotypic changes suggestive of EMT in normal thyroid cells requires growth factor co-operation.

Several in vitro studies have previously shown that TGF-β is capable of inducing EMT (Potts et al., 1991; Miettinen et al., 1994; Toda et al., 1997; Piek et al., 1999; Bhowmick et al., 2001a). It is therefore somewhat surprising that the epithelial phenotype was maintained in thyrocytes stimulated with TGF-β1 alone; a gradual loss of epithelial-specific markers was only observed when TGF-β1 was added together with EGF. At the same time TGF-β1 induced other effects, such as generalised cytoskeletal reorganisation, inhibition of growth and a low rate of apoptosis, indicating that the cells were highly sensitive to TGF-β1 also in the absence of EGF. It is possible that the inability of TGF-β1 to induce EMT without simultaneous EGF co-stimulation is related to use of primary cultured cells that were growth-arrested and fully differentiated and therefore might be more prone to resist EMT signals than, for instance, cell lines. This further suggests that TGF-β and EGF receptor signalling pathways converge on a critical step without which EMT cannot be fully developed in normal cells. Interestingly,

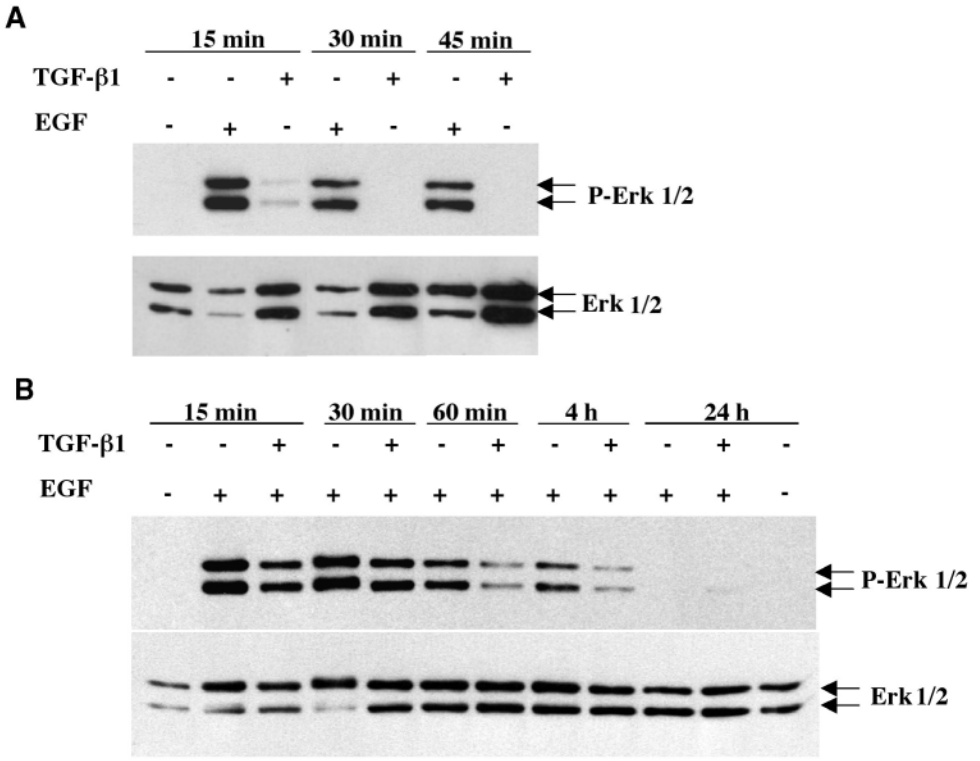


**Fig. 6.** MAPK signalling in pig thyrocytes treated with EGF and TGF-β1. (A) Phosphorylation of Erk by TGF-β1 or EGF. Cells were stimulated with TGF-β1 (10 ng/ml) or EGF (10 ng/ml) for 15-45 minutes and then solubilised. Proteins were detected by antibodies against phosphorylated Erk (P-Erk 1/2) and total Erk (Erk 1/2). Note diminutive amounts of Erk phosphorylation in TGF-β1-treated cells only after 15 minutes. (B) Effect of TGF-β1 on EGF-induced Erk phosphorylation. Cells were exposed to EGF in the presence or absence of TGF-β1 for the indicated times and then analysed for the presence of phosphorylated Erk and total Erk as outlined in A.

a similar interplay of post-receptor signals that enhance the EMT response to TGF-β has recently been shown for Ha-Ras transformed cells (Fujimoto et al., 2001). Moreover, MEK inhibition prevents the ability of TGF-β to stimulate invasiveness in cancer cell lines that harbour Ras mutations (Ellenrieder et al., 2001). Long-term stimulation of the Ras/MAPK pathway, either by ligand-dependent RTK stimulation (mimicked in this study) or constitutively activating mutations of downstream signalling molecules, might therefore constitute a necessary component of the EMT response to TGF-β. In tumor cells, this mechanism may explain the paradoxical stimulation of tumor growth by TGF-β in advanced tumour stages (Cui et al., 1996; Park et al., 2000; Pasche, 2001).

It has recently been evident that TGF-β may also activate the Ras/MAPK pathway and phosphorylate Erk (Mulder, 2000) that otherwise primarily is targeted by mitogenic RTK signals. The mode of action on MEK seems to differ considerably between experimental systems: TGF-β-induced Erk phosphorylation may be rapid and hence probably direct (Mulder, 2000) or delayed in comparison with that observed after, for example, EGF stimulation (Zavadil et al., 2001). A delayed response may indicate an indirect effect working through an enhanced autocrine activity of another growth factor, as has been shown for basic fibroblast growth factor produced by TGF-β-treated fibroblasts (Finlay et al., 2000). In the present study, TGF-β1 was found to rapidly phosphorylate Erk but only to a limited amount and lasting for not more than 15 minutes. On the contrary, TGF-β1 inhibited the pronounced activation of Erk by EGF. This inhibition was however not total, and a basal level of Erk phosphorylation sustained for several hours in the co-stimulated cells. Importantly, the synergistic changes elicited by TGF-β1 and EGF was completely blocked by the addition of U0126, indicating that MEK activity was required to elicit EMT. The fact that single stimulation with EGF had no drastic effect on the epithelial properties indicates that activation of the Ras/MAPK pathway is necessary but not sufficient to dedifferentiate primary cultured thyroid cells towards a mesenchymal phenotype. Clearly, other signalling pathways known to transduce TGF-β1 and EGF effects may also be involved in EMT. For example, disassembly of adherens junctions preceding cell scattering in response to hepatocyte growth factor has been shown to require activation of both MAPK and PI3K to be fully developed (Potempa and Ridley, 1998), A pivotal role for PI3K in TGF-β-induced EMT has also been reported (Bakin et al., 2000). However, we could not demonstrate any effects of wortmannin and LY294002 on the synergistic response to EGF and TGF-β1. Yet, these PI3K inhibitors typically blocked early to late endosomal transition in pig thyrocytes in the same culture system (data not shown). Preliminary investigation of Smad proteins downstream of TGF-β1 receptor activation indicated that Smad2 phosphorylation was not counteracted by simultaneous EGF stimulation. Smad-activated protein(s) are therefore putative EMT co-effectors of interest to explore in future experiments.

The superior role of E-cadherin in regulating the development and maintenance of the epithelial phenotype is well known (Vleminckx and Kemler, 1999). Conversely, downregulation of E-cadherin, recently shown to be mediated by the Snail/Slug family of transcription factors (Batlle et al., 2000; Cano et al., 2000; Carver et al., 2001), is a key event in EMT (Savagner, 2001). In accordance with previous findings (Brabant et al., 1995), we did not observe any effect of EGF on the protein expression level of E-cadherin, and single stimulation with TGF-β1 was also ineffective. This finding

further indicates that each factor alone were unable to induce EMT in thyrocytes. In marked contrast, co-stimulation with EGF and TGF-β1 caused a nearly complete loss of E-cadherin after 48 hours. However, this did not correlate with the loss of the epithelial barrier function, which was initiated much earlier, suggesting that other mechanisms may be operating early during EMT induction. That the function of E-cadherin, and hence the differentiated epithelial phenotype in general, may be impaired by post-translational mechanisms in response to peptide growth factors like EGF have been reported in many studies. The present finding of increased N-cadherin expression taking place concomitantly with the major drop in transepithelial resistance provides another possible mechanism. Before stimulation only a few cells scattered within the monolayer were N-cadherin positive. After co-stimulation for 24 hours all cells displayed N-cadherin that was recruited to the cell borders and co-localised with E-cadherin. TGF-β1 has previously been reported to induce N-cadherin expression in the NMuMG mammary epithelial cell line (Bhowmick et al., 2001a). In our primary culture system EGF rather than TGF-β1 seemed to upregulate N-cadherin but only to a minor extent and, importantly, both factors were required to substantially increase the expression by as much as 30 times or more. Studies on cancer cell lines indicate that N-cadherin-mediated adhesion is linked to a more malignant and invasive behaviour (Islam et al., 1996; Hazan et al., 1997; Tran et al., 1999; Hazan et al., 2000). The ability of N-cadherin to increase the motility of tumour cells has been mapped to a distinct portion of its extracellular domain (Kim et al., 2000). Interestingly, the effect of N-cadherin on tumour cells seems to be independent of the presence of E-cadherin (Nieman et al., 1999). It is probable, therefore, that the transient expression of N-cadherin presently observed in normal thyrocytes in response to EGF and TGF-β1 actively takes part in the dedifferentiation process. The importance of cadherin switch, i.e. from E- to N-cadherin, in normal and pathological conditions designated by pronounced tissue reorganisation is increasingly recognised (Cavallaro et al., 2002). The present findings indicate the loss of E-cadherin in favour of N-cadherin expression is not necessarily synchronous. Rather, both cadherins were simultaneously downregulated at later times of growth factor-induced dedifferentiation. In view of the fact that very little information is available on the transcriptional control of N-cadherin expression, the present experimental system provides a model to elucidate this in more detail in the future.

Claudin-1 and occludin were gradually lost during TGF-β1 and EGF co-stimulation, whereas TGF-β1 alone had no effect and EGF in fact was weakly stimulatory on the expression level of claudin-1. To our knowledge, repression of these tight junction proteins during growth factor-induced EMT has not previously been reported. Being directly involved in the formation of epithelium-specific tissue compartment barriers, claudins and occludin are intimately connected to the epithelial phenotype. However, it is not known whether they also play a regulatory role in epithelial determination analogous to that of E-cadherin. That the levels of claudin-1 and occludin was substantially decreased already 24 hours after addition of TGF-β1 and EGF suggests that the mechanism is not secondary to the loss of E-cadherin. Interestingly, breakdown of tight junctions may be directly regulated by the

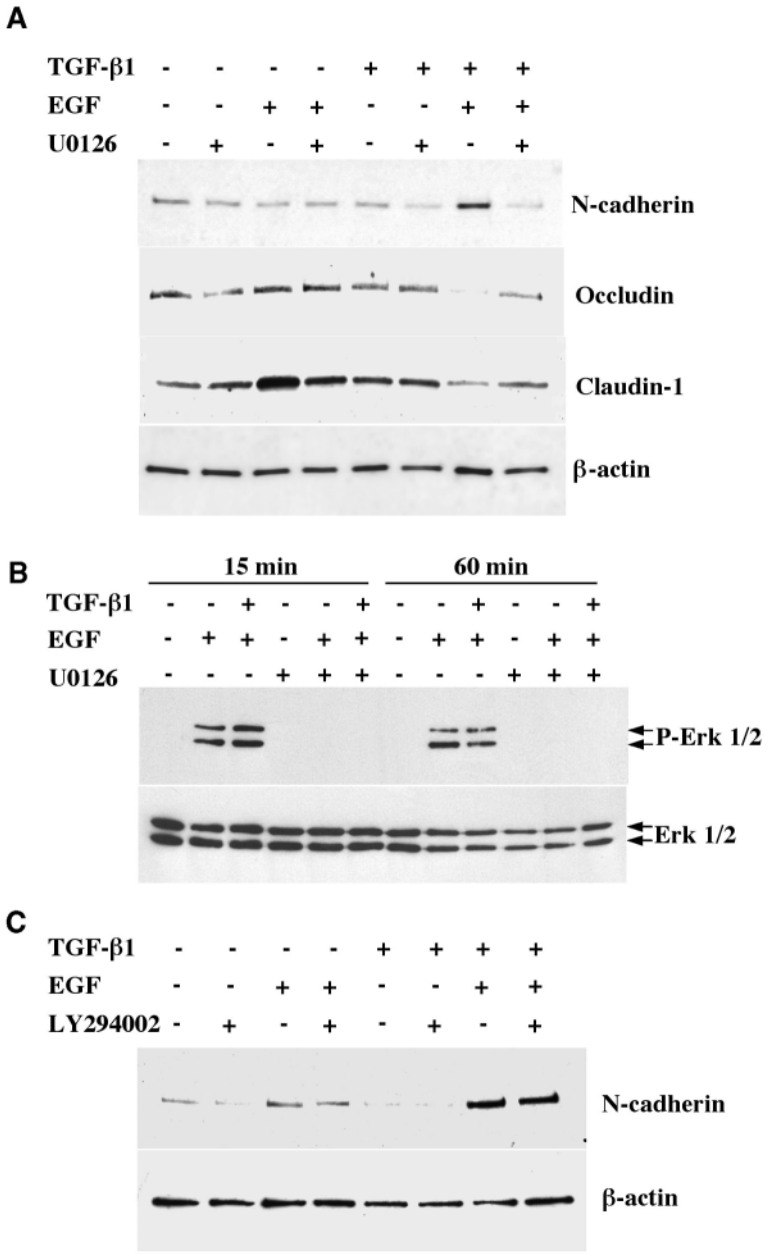


**Fig. 7.** Effects of MEK inhibitor U0126 and PI3K inhibitor LY294002. (A) Filter-grown cells were stimulated with TGF-β1 (10 ng/ml) or EGF (10 ng/ml) or both in the presence or absence of 25 μM U0126 for 24 hours, and then processed for western blot analysis of N-cadherin, occludin and claudin-1. β-actin (β-actin) was used as an internal control for equal protein loading. (B) Cells were exposed to EGF with or without TGF-β1 in the presence or absence of U0126 for the indicated times and then analysed for the presence of phosphorylated Erk (P-Erk 1/2). Total Erk (Erk 1/2) indicates equal protein loading. (C) Cells were exposed to EGF and TGF-β1 in the presence or absence of 5 μM LY294002. Cell lysates at equivalent protein concentrations were analysed for N-cadherin with SDS-PAGE and western blotting. β-actin was used as an internal control for equal protein loading.

Ras/MAPK pathway, as recently observed in Ras-transformed MDCK cells (Chen et al., 2000). Moreover, restoration of tight junctions involving relocalisation of both claudin-1 and occludin to cell-cell contacts by MEK inhibition preceded that of E-cadherin (Chen et al., 2000). Together, this supports the idea that tight junction disassembly during EMT occurs independently of impaired E-cadherin-mediated adhesion. Whether N-cadherin, in this study found to be targeted to the junctional complex at the time when the epithelial barrier function gradually is lost, influences the expression and assembly of tight junction proteins is presently unknown.

In the thyroid context, considering the outstanding capacity of the gland to increase in size and give rise to different histoarchitectual patterns, the results of the present study point on a possible mechanism that might operate in tissue remodelling and follicle neoformation in normal and pathological conditions. Indeed, EGF is inferred a role in both hyperplastic and neoplastic growth (Nilsson, 1995) and TGF-β is proven to be a key regulator in, for example, the development of thyroid fibrosis (Contempre et al., 1996). Thus, normal and highly differentiated epithelial cells in primary culture are here shown to be able to convert to a mesenchymal-like phenotype provided the appropriate stimulus. The importance of growth factor co-operation determining the state of cellular differentiation uncoupled from mitogenic and anti-mitogenic actions is emphasised.

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