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RETRACTION

Retraction: ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC Cl3 thyroid cells

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Retraction of: *J. Cell Sci.* **121**, 477-486.

Journal of Cell Science was alerted by a reader to potential band duplication and manipulation in the above named paper. The journal contacted Dr Di Jeso, the corresponding author, to request the original data.

The authors were unable to provide all of the original data requested, or account for the potential blot duplication and manipulation. Although they provided new data that appear to support the conclusions, the journal has decided that, in the absence of the original data, the best course of action is to retract the paper, in agreement with Dr Di Jeso.

ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC Cl3 thyroid cells

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Summary

Conditions perturbing the homeostasis of the endoplasmic reticulum (ER) cause accumulation of unfolded proteins and trigger ER stress. In PC Cl3 thyroid cells, thapsigargin and tunicamycin interfered with the folding of thyroglobulin, causing accumulation of this very large secretory glycoprotein in the ER. Consequently, mRNAs encoding BiP and XBP-1 were induced and spliced, respectively. In the absence of apoptosis, differentiation of PC Cl3 cells was inhibited. mRNA and protein levels of the thyroid-specific genes encoding thyroglobulin, thyroperoxidase and the sodium/iodide symporter and of the genes encoding the thyroid transcription factors TTF-1, TTF-2 and Pax-8 were dramatically downregulated. These effects were, at least in part, transcriptional. Moreover, they were selective and temporally distinct from the general and transient PERK-dependent translational inhibition. Thyroid dedifferentiation was accompanied by changes in the organization of the polarized epithelial monolayer. Downregulation of the mRNA encoding E-cadherin, and up-regulation of the mRNAs encoding vimentin, α-smooth muscle actin, α(1)(I) collagen and SNAI1/SIP1, together with reformation of actin stress fibers and loss of trans-epithelial resistance were found, confirming an epithelial-mesenchymal transition (EMT). The thyroid-specific and epithelial dedifferentiation by thapsigargin or tunicamycin were completely prevented by the PP2 inhibitor of Src-family kinases and by stable expression of a dominant-negative Src. Together, these data indicate that ER stress induces dedifferentiation and an EMT-like phenotype in thyroid cells through Src-mediated signaling pathway.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/4/477/DC1

Key words: ER stress, Thyroid cells, Dedifferentiation, Epithelial-mesenchymal transition

Introduction

Newly synthesized secretory and transmembrane (cargo) proteins are co-translationally translocated into the lumen of the endoplasmic reticulum (ER), where oxidizing conditions and high calcium concentration provide a unique environment, crucial for formation of disulfide bonds and proper folding. In addition, the ER provides a machinery to assist protein folding and to assure that only correctly folded proteins can move on along the secretory pathway (reviewed in Ellgaard and Helenius, 2003). Many disturbances, including alterations of calcium homeostasis or redox status, increased cargo protein synthesis or/and altered glycosylation, result in accumulation of unfolded proteins in the lumen and trigger the unfolded protein response (UPR). This adaptive mechanism involves transcriptional induction of genes that enhance the ER protein folding capacity and promote ER-associated protein degradation (ERAD). Translation of mRNAs is also inhibited initially, thus reducing the load of newly synthesized proteins in the ER. Two other immediate responses to ER stress have been described recently: co-translational degradation of secretory proteins (Oyadomari et al., 2006) and IRE1-mediated degradation of ER-localized mRNAs (Hollien and Weissman, 2006). Activation of JNK, NF-κB and p38 also occurs. Finally, when ER stress is excessive or prolonged, cells activate the apoptotic program of cellular suicide (reviewed in Schroder and Kaufman, 2005).

Transmembrane ER proteins, such as IRE1α/β, PERK and ATF6, act as 'stress sensors' through their lumenal domain and transduce stress signals outside the ER through their cytosolic domain. In unstressed cells, BiP binds the lumenal domains of IRE, PERK and ATF6, preventing their dimerization and activation. When unfolded proteins accumulate in the ER, BiP releases from IRE1 and PERK, allowing their oligomerization and trans-autophosphorylation, and launching the UPR. IRE1 displays also endoribonuclease activity that, upon activation, splices mRNA encoding XBP-1 to produce a bZIP-family transcription factor that binds to promoters of ER chaperones and genes of the ERAD participants. In addition, the endoribonuclease activity of IRE1 is responsible for the degradation of ER-bound mRNAs. PERK is a Ser/Thr kinase that, upon activation, phosphorylates and inactivates the eukaryotic initiation factor 2α (eIF2α), thereby globally shutting off translation. However, certain mRNAs gain a selective advantage for translation, such as mRNA encoding ATF4, a bZIP-family transcription factor that regulates the promoters of UPR genes. Finally, release of BiP from the N-terminus of ATF6 frees the protein to translocate to the Golgi, where resident

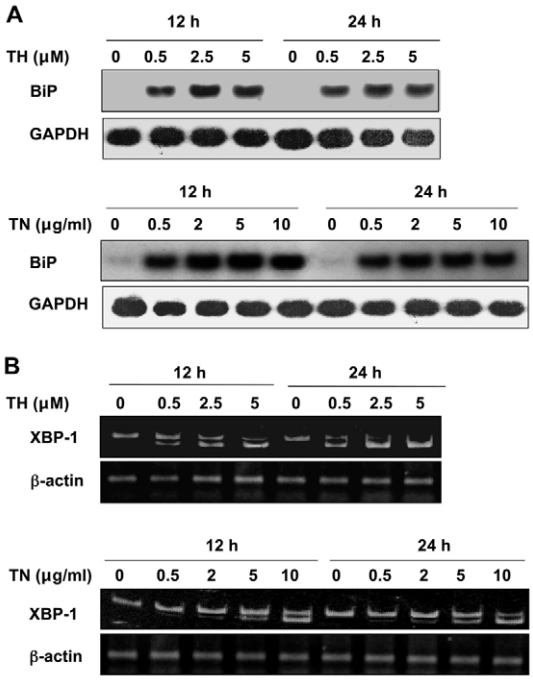
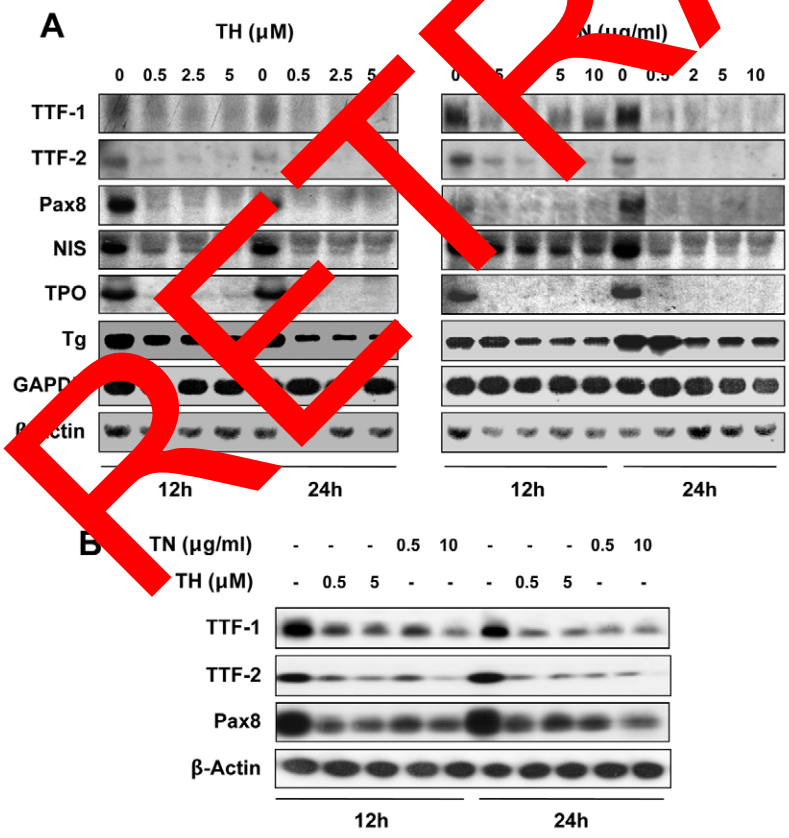


Fig. 1. Thapsigargin (TH) or tunicamycin (TN) induce ER stress in PC Cl3 cells. (A) TH and TN induce upregulation of mRNA encoding BiP. Northern blot analysis of total RNA extracted from PC Cl3 cells vehicle treated (0) or treated with increasing concentrations of TH or TN for 30 minutes, followed by 12 and 24 hours in medium without TH or TN. GAPDH was analyzed on the same filters. (B) RT-PCR analysis for XBP-1 and β-actin of the same total RNAs used in (A).



proteases cleave ATF6 at a juxtamembrane site, releasing this transcription factor, which induces XBP-1 transcription (Schroder and Kaufman, 2005).

Besides the above responses, systematic investigations of the variations of gene expression following ER stress have revealed an increased expression for ~14%, and a decreased expression for ~17%, of the total genes (6385) investigated (Kawai et al., 2004). Therefore, ER stress causes a dramatic re-programming of gene expression that goes well beyond the upregulation of genes involved in protein folding/ERAD. Moreover, a few recent papers report cellular dedifferentiation secondary to ER stress, in vivo and in vitro in chondrocytes, and in pancreatic beta cells (Yang et al., 2005; Tsang et al., 2007; Pi et al., 2007). Therefore, we wondered whether the negative effect on differentiation is a general phenomenon secondary to ER stress. To test this hypothesis, we decided to use the fully differentiated thyroid cell line PC Cl3 (Fusco et al., 1987). PC Cl3 cells would represent a good system to verify our hypothesis as the folding/misfolding of their major secretory product, thyroglobulin (TG) (which accounts for ~50% of the newly synthesized cargo proteins of the thyrocyte), and their differentiation have been extensively studied and characterized at the molecular level (Di Jeso and Arvan, 2004; Damante et al., 2006). Furthermore, we sought to extend our study to eventual changes in the organization of a polarized epithelial monolayer and to the signaling pathway(s) involved in these responses.

Results

TH and TN increase retention of TG in the ER and activate the UPR

ER stress is known to cause transcriptional reprogramming in eukaryotic cells. Therefore, we hypothesized that ER stress might cause changes in thyroid-specific gene expression. As a first step, we set up the experimental conditions able to cause protein misfolding and UPR activation in the ER of PC Cl3 cells. We tested the effect of the widely used ER stress-inducing agents thapsigargin (TH) and tunicamycin (TN) on the expression levels of BiP and the splicing of mRNA encoding XBP-1. As shown in Fig. 1A, treatments of 30 minutes with various concentrations of TH and TN, followed by 12 and 24 hours in medium without TH/TN, increased BiP mRNA, even at the lowest concentration investigated. We also examined XBP-1 activation by PCR amplification of XBP-1 cDNA. A dose-dependent increase in the spliced active form of XBP-1 mRNA (XBP-1s) was observed following TH and TN treatments (Fig. 1B).

ER stress was also evaluated by monitoring the intracellular fate of newly synthesized TG, which reflects its folding status (Di Jeso et al., 1998; Di Jeso et al., 2003; Di Jeso et al., 2005; Kim and

Fig. 2. TH and TN induce inhibition of thyroid differentiation markers in PC Cl3 cells. (A) Northern blot analysis of total RNA extracted from PC Cl3 cells vehicle treated (0) or treated with increasing concentrations of TH and TN for 30 minutes, followed by 12 and 24 hours in medium without TH or TN. (B) Western blots of total protein extracts from PC Cl3 cells vehicle treated or treated with 0.5 or 5 μM TH or 0.5 or 10 μg/ml TN for 30 minutes, followed by 12 and 24 hours in medium without TH or TN. Filters were probed with antibodies against TTF-1, TTF-2, Pax8 and β-actin.

Arvan, 1995). Pulse-chase experiments showed that the above-reported treatments with TH/TN inhibited TG secretion in a dose-dependent manner, with a residual secretion ranging from 40% to 3% (60 and 97% inhibition, respectively; data not shown).

ER stress results in decreased thyroid-specific gene expression in PC Cl3 cells

To study the effect of ER stress on thyroid-specific gene expression, we treated PC Cl3 cells with the same TH/TN concentrations used in Fig. 1 and performed northern blots. Our aim was to find the minimal effective concentrations and consequently to elicit a mild ER stress, trying to avoid the activation of apoptosis. TH/TN, even at the lowest doses, dramatically decreased mRNAs encoding the thyroid-specific markers thyroperoxidase (TPO), sodium/iodide symporter (NIS) and TG, whereas they had no effect on mRNAs encoding β-actin and GAPDH (Fig. 2A). Transcription of the genes encoding TG, TPO and NIS is directed by a combination of the thyroid-specific transcription factors TTF-1, TTF-2 and Pax-8 (Damante et al., 2001), and TH/TN decreased also mRNAs encoding these transcription factors (Fig. 2A). Consistent with these results, TTF-1, TTF-2 and Pax-8 protein levels, in total extracts from TH/TN-treated PC Cl3 cells, exhibited a dramatic decrease (Fig. 2B). Next, we sought to establish the temporal relationship between the observed downregulation of mRNA and protein levels and the translational inhibition operated by PERK. To this e[nd] cells were incubated in the absence or presence of 0.[5] μM TH for various times and the rate of protein synthesis was measured and compared with the rate of TG synthesis. As shown in Fig. 3A,B, TH treatment was associated with a profound and transient inhibition of protein synthesis, which was followed by a recovery. Strikingly, TG synthesis paralleled total protein synthesis at early times, but, at 12 and 24 hours, it dropped again (Fig. 3C,D). The 12/24 hours inhibition of TG synthesis was very likely secondary to the downregulation of its mRNA. The discrepancy between TG and total protein synthesis at late times also suggested that the mRNA downregulation, shown in Fig. 2A, was restricted to specific genes. These results suggested that decreased TTF-1, Pax-8 and TTF-2 caused transcriptional inhibition of the genes encoding TG, TPO and NIS. This appeared to be the case as both TH and TN reduced the activity of a NIS promoter-luciferase construct (Fig. 4A). Furthermore, TH/TN-induced downregulation of thyroid-specific transcription factors showed a transcriptional component. Nuclear run-on experiments showed decreased Pax-8 transcription initiation compared with that of GAPDH (Fig. 4B). These data indicate that ER stress induced by TH/TN inhibits thyroid-specific gene expression, at least in part, at the transcriptional level in PC Cl3 cells.

Importantly, thyroid dedifferentiation was not accompanied by apoptosis measured by FACS analysis of annexin V staining (2-3% of cells being apoptotic at 24 and 48 hours after TH/TN treatments and 4-5% of cells being apoptotic at 72 hours after treatments; supplementary material Fig. S1).

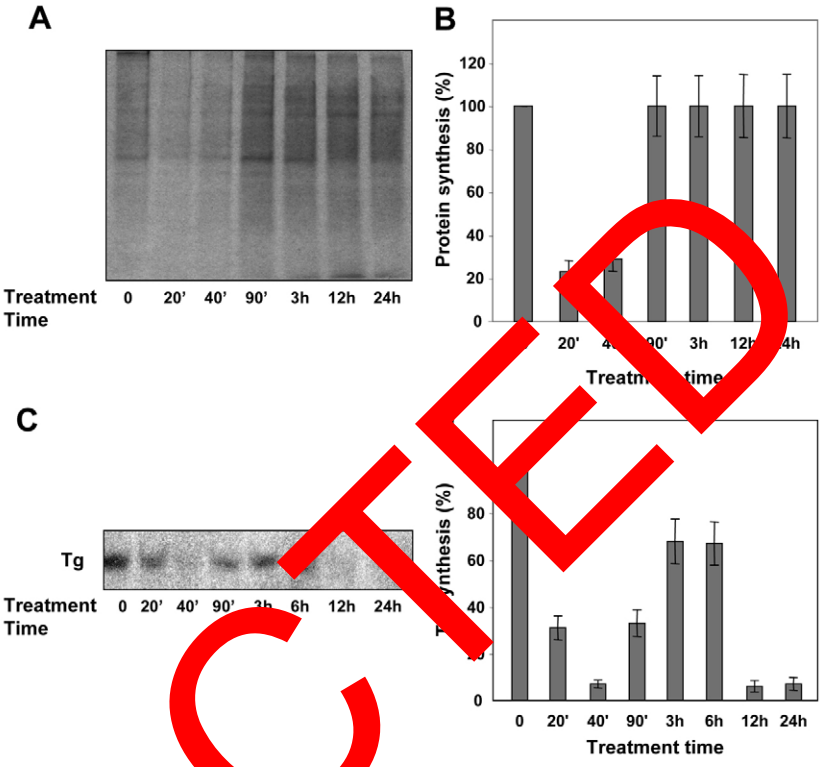


Fig. course of total [p]rotein and thyroglobulin (TG) synthesis following ER stress. (A) PC Cl3 cells treated with 0.5 μM TH for the times indicated and labeled for 5 minutes with [35S]-met/cys. Cell lysates were subjected to SDS-PAGE. (B) Values shown represent the mean (±s.d.) of three independent experiments. (C) PC Cl3 cells were treated and labeled as in (A), TG was immunoprecipitated and subjected to SDS-PAGE. (D) Values shown represent the mean (±s.d.) of three independent experiments.

ER stress induces an EMT-like phenotype in PC Cl3 and FRT thyroid cells

To investigate whether the dedifferentiation effect of ER stress involved alterations in the organization and function of the polarized epithelial monolayer, we analyzed E-cadherin expression and distribution in PC Cl3 cells. In normal conditions, E-cadherin was localized mainly at cell-cell borders (Fig. 5Ai). When cells were treated with TN (and TH; data not shown), the staining for E-cadherin decreased, suggesting a decreased level of expression (Fig. 5Aii). Furthermore, cells dramatically lost cell-cell contacts, with residual E-cadherin being localized at the remaining contacts (arrows in Fig. 5Aii). Next, we analyzed the organization of the actin cytoskeleton and compared it with expression of a differentiation marker (TG). In untreated cells, the TG signal showed a distribution characteristic of ER (Fig. 5Bi). Phalloidin staining showed that the distribution of F-actin was mainly cortical (Fig. 5Bii), with the result that the signals of TG and actin overlapped minimally (Fig. 5Biii). In cells treated with TN, as expected, TG was downregulated, with a few cells expressing various amounts of residual TG, very likely in the process of losing it (Fig. 5Biv, arrows). The distribution of F-actin changed dramatically, with loss of cortical actin and formation of stress fibers (Fig. 5Bv). Notably, in TN-treated cells, the residual TG expression correlated remarkably with partially formed, not fully formed, stress fibers and, albeit to a lesser extent, with residual cortical actin (Fig. 5Bv, arrows). As a result, the TG and actin signals remained distinct (Fig. 5Bvi). Furthermore, the morphology

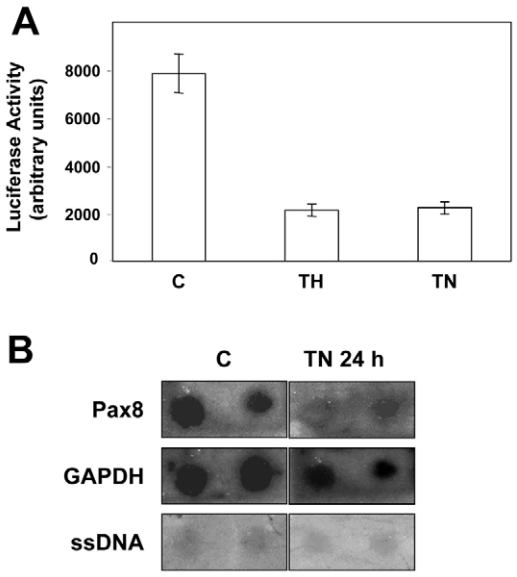


Fig. 4. ER stress induced by TH or TN inhibits thyroid-specific gene expression, at least in part, at the transcriptional level. (A) Relative luciferase activity of extracts of PC Cl3 cells transfected in triplicate with 2.5 μg NISLUC2 luciferase reporter plasmid. 24 hours after transfection, cells were vehicle treated ('C') or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 minutes and harvested after 24 hours in medium without TH or TN. Measurements were normalized for β-galactosidase activity driven by a co-transfected plasmid encoding RSV-βgal. Values shown (in arbitrary uni represent the mean (±s.d.) of at least three independent experiments. (B) n- on assay performed on nuclei prepared from PC Cl3 cells vehicle treated (') or treated with 0.5 μg/ml TN for 30 minutes, followed by 24 hours in medi without TN. Pax8 and GAPDH probes were immobilized on filters.

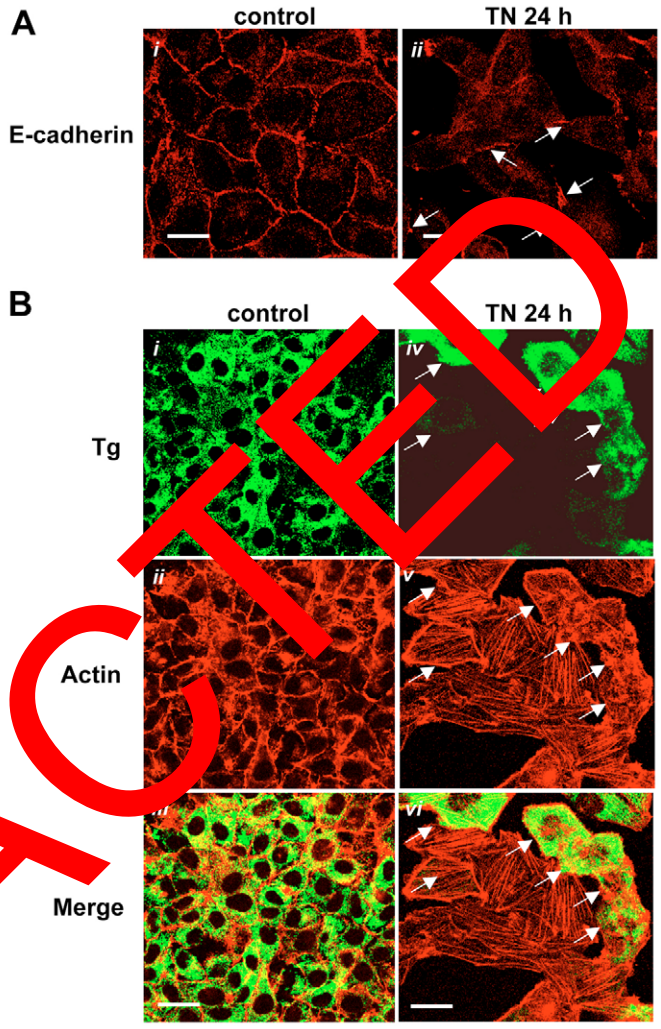


Fig. 5. ER stress induces downregulation of E-cadherin and formation of stress fibers in PC Cl3 cells. (A) PC Cl3 cells were grown on glass coverslips for 48 hours, then were vehicle treated (i) or treated with 0.5 μg/ml TN for 30 minutes, followed by 24 hours in medium without TN (ii). Cells were stained with antibodies against E-cadherin. Following TN treatment, the signal for E-cadherin decreased. Arrows in (ii) indicate residual E-cadherin localized at the remaining cell-cell contacts. Bars, 15 μm. (B) PC Cl3 cells were grown and treated as above. Cells were double stained with antibodies against TG and rhodamine-conjugated phalloidin. Bars, 30 μm (i, ii, iii), 15 μm (iv, v, vi). In control cells, rhodaminated phalloidin staining is mainly at the level of cortical actin. Following TN treatment, the signal for TG decreased and stress fibers were formed. Arrows indicate: the few cells expressing various amounts of residual TG (iv), the correlation between residual TG expression and partially formed, not fully formed, stress fibers (v) and, consequently, the lack of overlap between TG and actin signals (vi). A greater magnification for TN-treated cells was intentionally used to show better the coordinate variations of TG, cortical actin and stress fibers.

of treated cells changed from a round ar egular to olygonal and irregular shape. Next, we showed by vers ranscript (RT)-PCR and western blotting the ownregu of E-cadherin (Fig. 6A,B, respectively). PC 13 cells, in rmal growth conditions, expressed very l ba levels of vim in and N-cadherin (Fig. 6A). In fact, eak exp ion of vimentin has been found in differentiated pithelial cell Bindels et al., 2006; Kaimori et al., 200 Following TH/TN eatments, vimentin mRNA increased y 2-3 fold, whereas mRNA encoding N-cadherin did n hange (Fig. 6A). Moreover, by using the more sensitive real-tim T-PC we showed upregulation of α-smooth muscle actin (α-S and α(1) collagen (Fig. 6C), two additio markers of a pitheli mesenchymal transition (EMT) (Ka i and N son, 200 imori et al., 2007). BiP was used as itive co ol (Fig. 6C). Several transcription factors (S I1/sn , S AI2/slug and E12/E47) downregulate trans n of the gene encoding E-cadherin (Batlle et al., 2000; Comijn l., 2001; Hajra et al., 2002; Perez-Moreno et al., 2001). Thus, we m ured their mRNA levels in response to TH/TN. SIP1 and SNAI1 le els were increased 6 hours after TH/TN treatments and remained sustained after 24 (SNAI1) and 48 hours (SIP1) (Fig. 6D). There were no changes evident in SNAI2/slug and E12/E47 (data not shown).

As PC Cl3 cells express thyroid markers but display only a low level of cell polarity, we sought to extend our results to FRT cells that are well polarized both morphologically and functionally (Ambesi-Impiombato and Coon, 1979). They do not express, however, any thyroid marker, although they showed, at least in part, the thyrocyte phenotype when they were first established in culture (Ambesi-Impiombato and Coon, 1979). First of all, we tested whether TH/TN were able to induce the UPR in FRT cells. As shown in Fig. 7A, both agents increased the mRNA encoding BiP. Under normal growth conditions, FRT cells showed well-organized cell-cell junctions, as judged by the E-cadherin staining (Fig. 7Bi). FRT cells, like PC-Cl3 cells, showed cortical actin but not stress fibers (Fig. 7Bii), and thus F-actin staining overlapped quite well with E-cadherin staining (Fig. 7Biii). However, 24 hours after TN

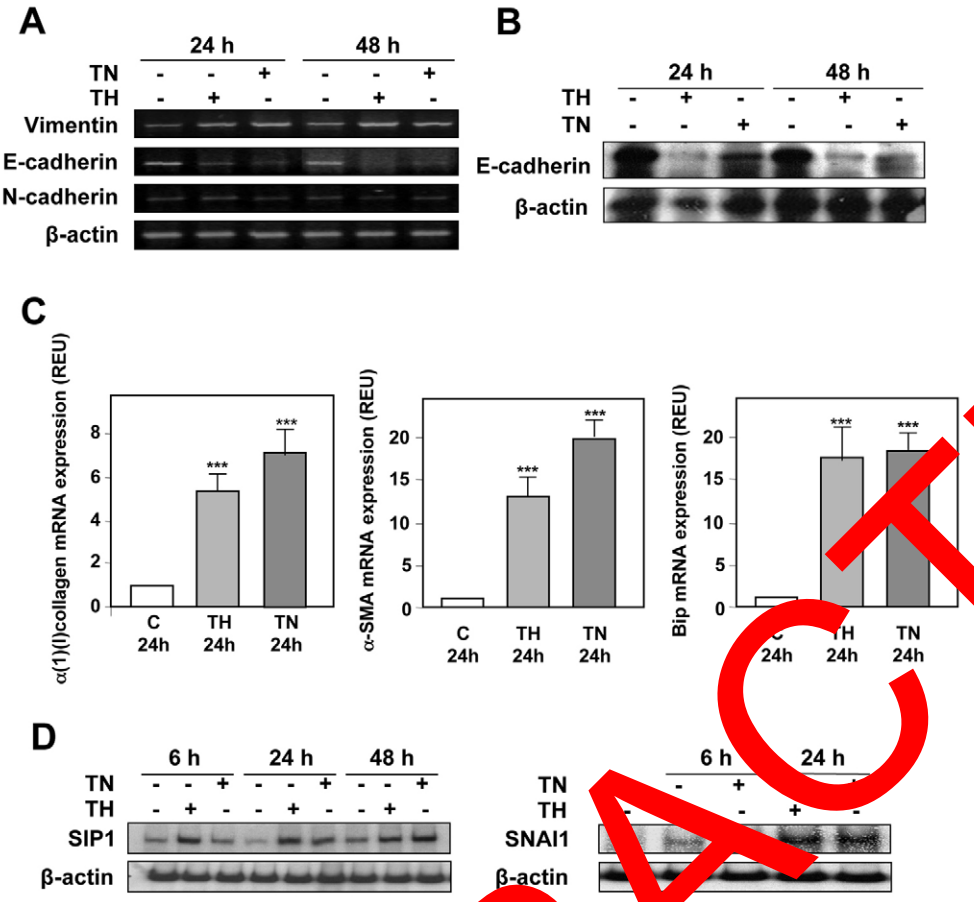


Fig. 6. ER stress upregulates EMT markers in PC CL3 cells. (A) RT-PCR analysis of total RNA extracted from PC Cl3 cells vehicle treated or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 minutes, followed by 24 and 48 hours in medium without TH or TN. (B) Western blot of total protein extracts from PC Cl3 cells vehicle treated or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 minutes followed by 24 and 48 hours in medium without TH or TN. Blots were probed with antibodies against E-cadherin and β-actin (control). (C) Real-time RT-PCR analysis of total RNA extracted from PC Cl3 cells vehicle treated or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 minutes, followed by 24 hours in medium without TH or TN. Each bar represents the mean±s.d. of four independent experiments, each performed in triplicate. β-actin was used as an internal standard (β-actin values were not affected by TH or TN treatments) (REU, relative expression units). Asterisks indicate statistically significant differences (***, $P<0.001$). (D) RT-PCR analysis of total RNA extracted from PC Cl3 cells vehicle treated or treated with 0.5 μM TH and 0.5 μg/ml TN for 30 minutes, followed by 6, 24 and 48 hours in medium without TH or TN.

treatment, E-cadherin staining decreased, becoming intermittent and jagged, indicating, as for PC Cl3 cells, downregulation of cadherin (Fig. 7Biv). Cortical actin decreased and stress fibers appeared (Fig. 7Bv). As a consequence of these changes, E-cadherin–actin signal overlay was strikingly lost (Fig. 7Bvi). Furthermore, mRNA encoding E-cadherin was markedly downregulated after TH/TN treatments, whereas mRNA encoding SNAI1 increased (Fig. 7C). Thus, ER stress induced by TH/TN caused, in both PC Cl3 and FRT cells, changes similar to those occurring during EMT.

Finally, we sought to test whether downregulation of E-cadherin influenced trans-epithelial resistance in FRT cells. FRT cells grown on bicameral systems are well polarized and consequently generate a high trans-epithelial resistance. This was established within 24-36 hours after confluency and reached a plateau in 2-3 days (not shown). At plateau, cells were treated with 0.5 μg/ml TN and trans-epithelial resistance was measured every 12 hours. As shown in Fig. 8A, control cells, once they had reached the plateau, did not show appreciable variations of trans-epithelial resistance, whereas cells treated with TN showed a marked decrease, more pronounced when TN was added simultaneously to the inferior and superior chambers. In such experimental conditions, cells were viable, did not show apoptotic death (data not shown) and the epithelial monolayer remained morphologically intact (Fig. 8B). Thus, we concluded that ER stress induced by TN/TH caused a disassembly of cell-cell junctions that was evident by morphological, biochemical and functional criteria.

ER stress induces dedifferentiation and an EMT-like phenotype in PC Cl3 cells through a Src-mediated signaling pathway

ER stress is known to activate a number of signaling pathways (Urano et al., 2000). To test the signal transduction pathway(s) mediating dedifferentiation signals, we performed pharmacological inhibition experiments. PC Cl3 cells were treated for 30 minutes with different concentration of inhibitors before the usual TH/TN treatments. After 24 hours in medium without TH/TN, but in the presence of the inhibitor, total RNA was extracted. Neither inhibitors of JNK (SP600125) and p38 MAPK (SB203580) nor an inhibitor of the phosphoinositide 3-kinase/AKT (Ly294002) pathways prevented downregulation of thyroid-specific genes (supplementary material Fig. S2). As TGF-β recapitulates most, if not all, of the effects we observed following ER stress (Thiery and Sleeman, 2006), we checked for the involvement of TGF-β–Smad. The TGF-β type I receptor inhibitor SB431542 did not prevent TH/TN-induced dedifferentiation (supplementary material Fig. S3A). In addition, TH/TN did not induce activity of a SBE4-Luc reporter construct (supplementary material Fig. S3B) and a Smad4 dominant-negative construct (Smad4-100T) did not prevent TH/TN-induced dedifferentiation (data not shown). These results suggested that TGF-β-Smad signaling was not involved. On the contrary, PP2 (an inhibitor of Src-family kinases) was very effective in preventing the downregulation of Pax-8 mRNA exerted by TH/TN (Fig. 9A). Interestingly, the same effect of PP2 was displayed by the EGF receptor inhibitor AG1478 (supplementary material Fig. S4).

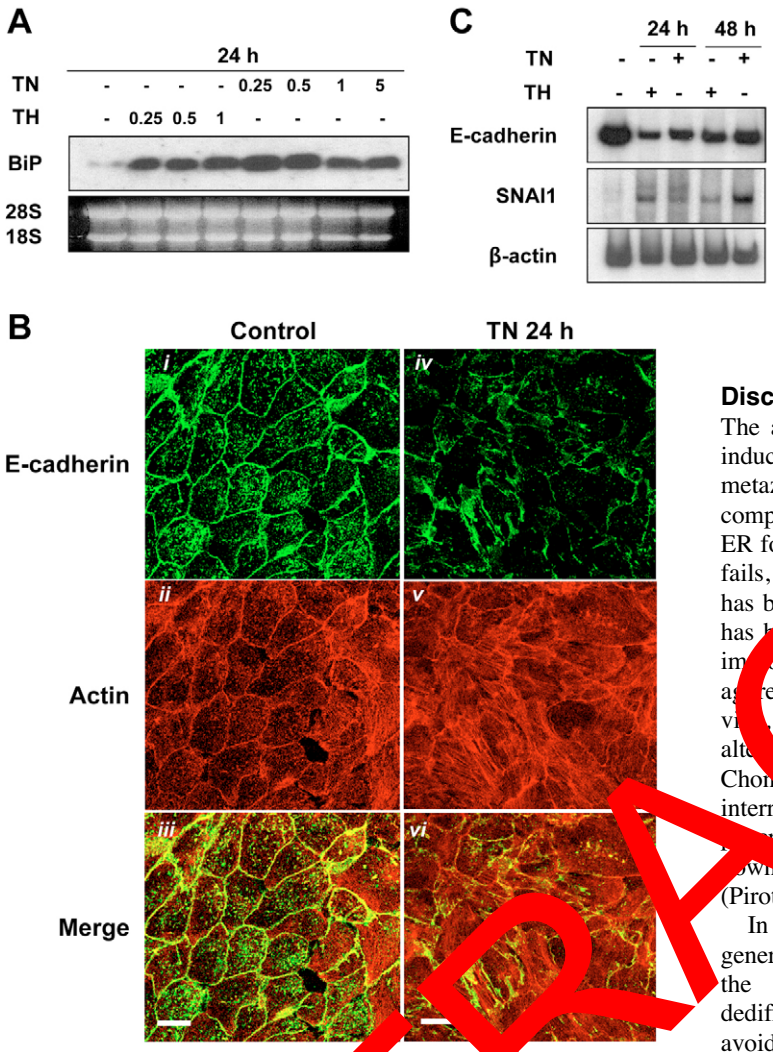


Fig. 7. ER stress induces an EMT-like phenotype in FRT cells. (A) Northern blot analysis of total RNA extracted from FRT cells vehicle treated or treated with increasing concentrations of TH and TN for 30 minutes, followed by 24 hours in medium without TH or TN. (B) FRT cells were grown on glass coverslips for 48 hours, then were vehicle treated or treated with 0.5 μg/ml TN for 30 minutes, followed by 24 hours in medium without TN. Cells were stained with antibodies against E-cadherin and rhodamine-conjugated phalloidin. Following TN treatment, the signal for E-cadherin increased and stress fibers were formed. Bars, 20 μm. (C) Northern blot analysis of total RNA extracted from FRT cells vehicle treated or treated with 0.5 μM TH or 0.5 μg/ml TN for 30 minutes, followed by 24 and 48 hours in medium without TH or TN.

Furthermore, PP2 prevented the changes in mRNAs encoding E-cadherin and SIP1 induced by TH/TN (Fig. 9B). Notably, induction of mRNA encoding BiP was not affected by PP2 pretreatment, indicating that PP2 did not prevent ER stress (Fig. 9A). Finally, as shown in Fig. 9C, TH/TN induced phosphorylation of c-Src at Tyr416 and this effect was completely abrogated when stimulation was carried out in the presence of PP2.

Next we generated PC Cl3 clones stably expressing a kinase-inactive Src protein (SrcDN) which effectively blocks the catalytic activity of endogenous Src (Migliaccio et al., 2005). Positive clones were selected on the basis of EGF-mediated c-Src phosphorylation at Tyr416. As shown in Fig. 10A, Tyr416-phosphorylation of Src was markedly increased by EGF stimulation in PC pSG5 and in clone 15. By contrast, EGF-dependent Tyr416-phosphorylation of Src was absent in clones 12 and 20, indicating the presence of a transdominant-negative effect. Finally, we tested TG and E-cadherin expression after TH/TN treatments. As shown in Fig. 10B, clones 12 and 20 exhibited a negligible decrease of both TG and E-cadherin, when compared with PC pSG5 and clone 15. Thus, we concluded that ER stress triggered by TH/TN induces both thyroid-specific dedifferentiation and an EMT-like phenotype in PC Cl3 cells through a Src-mediated signaling pathway.

Discussion

The accumulation of unfolded proteins in the lumen of the ER induces a coordinated adaptive program called the UPR. In metazoans, among other responses, the UPR includes a transcriptional component that upregulates expression of genes that enhance the ER folding capacity and promote ERAD. If the adaptive response fails, cells execute apoptosis. Recently, a new response to ER stress has been elucidated that entails an inhibition of differentiation. It has been shown that ER stress dedifferentiates both primary and immortalized chondrocytes, downregulating collagen II and aggrecan at the mRNA and protein levels (Yang et al., 2005). In vivo, in transgenic mice expressing mutant collagen X, ER stress alters chondrocyte differentiation and function (Tsang et al., 2007). Chondrocytes survive ER stress, but terminal differentiation is interrupted, producing a chondrodysplasia phenotype. Finally, pancreatic β-cells treated with cyclopiazonic acid show downregulation of genes related to differentiated β-cell functions (Pirot et al., 2007).

In this study, we tested the hypothesis that dedifferentiation is a general phenomenon linked to ER stress, perhaps instrumental to the survival function of the UPR. We reasoned that a dedifferentiating response would be protective to stressed cells, avoiding energy expenditure for the expression of genes that, in this condition, are unnecessary or even superfluous. Thus, ER stress might inhibit cell differentiation at the mRNA level in several cell types, eliciting a long-lasting response distinct from the general, transient, PERK-dependent inhibition of protein translation. Moreover, in the cited studies (Yang et al., 2005; Tsang et al., 2007; Pirot et al., 2007), the differentiation genes encode cargo proteins, resulting in a long-term reduction of ER-specific protein load. We used a thyroid cell line, PC Cl3, in which both protein folding/misfolding and differentiation have been well characterized at the molecular level and all thyroid markers are cargo proteins (Di Jeso and Arvan, 2004; Damante et al., 2001). TH/TN alter the folding pathway of TG (Di Jeso et al., 1998; Di Jeso et al., 2003; Di Jeso et al., 2005; Kim and Arvan, 1995) and, as a result, trigger the UPR (Leonardi et al., 2002) (and this study), as demonstrated by the upregulation of BiP and the splicing of mRNA encoding XBP-1. Without undergoing apoptosis, PC Cl3 cells dedifferentiate, downregulating the thyroid transcription factors and thyroid markers at the mRNA and protein levels. This represents a selective and long-term downregulation, clearly temporally distinct from the general and short-term shut-off of protein synthesis elicited by PERK (Fig. 3). The mechanism of this downregulation is, at least in part, transcriptional, not only for the thyroid markers, as expected, given the coordinate downregulation of the thyroid transcription factors, but also for the transcription factors themselves, as suggested by run-on experiments on Pax8.

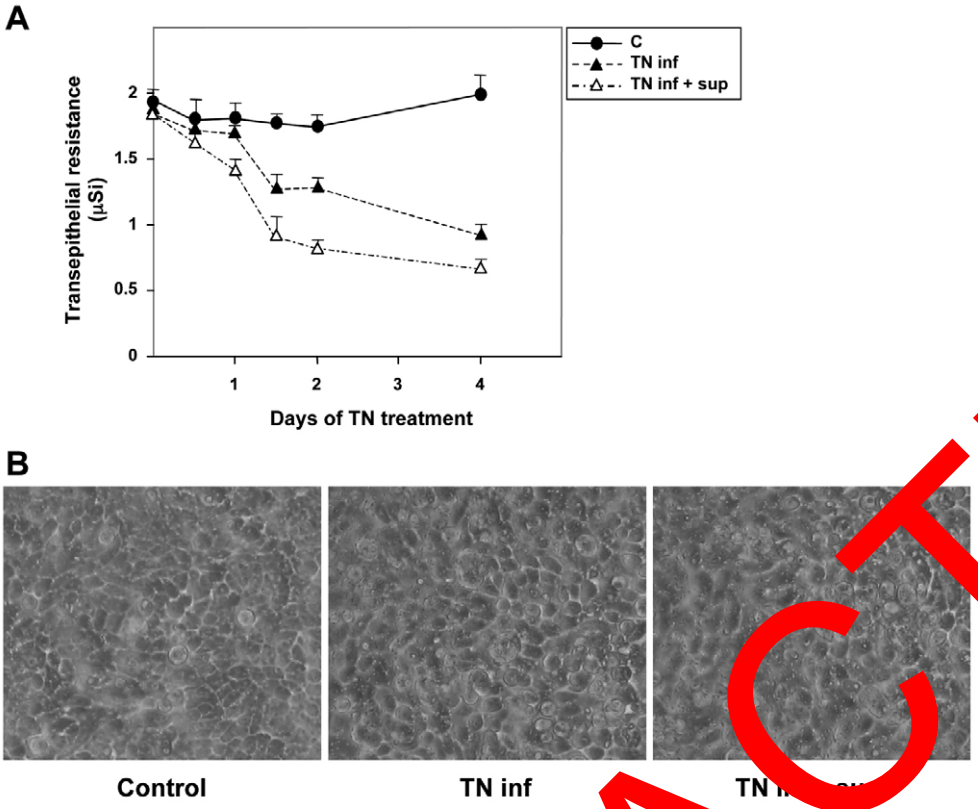


Fig. 8. TN decreases transepithelial resistance in FRT cells. (A) 2×10^6 cells were plated on 24.5 mm diameter Transwell filters. At confluency, transepithelial resistance was measured every 12 hours, until a plateau was reached (3-4 days). Then, cells were vehicle treated ('C') or treated with 0.5 μg/ml TN in the inferior chamber (TN inf) or in inferior plus superior chambers (TN inf + sup), and transepithelial resistance measured every 12 hours over a 4 day period. (B) FRT cells, vehicle treated and treated as in A, were observed daily by light microscopy to verify the integrity of the monolayer. At day four (last transepithelial resistance measurement), the cells were photographed.

Notably, Pax8 is the crucial factor for transcription of the genes encoding TG, TPO and NIS (Pasca di Magliano et al., 2000), although cooperativity has been reported between Pax8 and TTF1 (Miccadei et al., 2002). ER stress appears to induce dedifferentiation of those cell types whose phenotype is associated with expression of synthesis of numerous proteins either secreted or found on the cell surface, thus synthesized in the ER. It is likely that cells whose differentiation does not involve a lot of ER synthesis (e.g. smooth and skeletal muscle cells) will not dedifferentiate upon ER stress.

In this study, we report for the first time that, besides tissue-specific differentiation, ER stress negatively affects the organization of polarized epithelial cells. We performed these experiments not only in PC Cl3 cells but also in FRT cells that are morphologically and functionally better polarized (Ambesi-Impiombato and Coon, 1979). Indeed, we show that expression, localization and function of E-cadherin are dramatically impaired following ER stress in PC Cl3 and FRT cells. Interestingly, expression of vimentin, SMA and α(1)(I) collagen increases. We observe also changes in cell morphology and extensive reorganization of the actin cytoskeleton. These changes represent defining features of an EMT (Thiery and Sleeman, 2006). We also found induction of SNAI1 and SIP1 (PC Cl3 cells) and SNAI1 (FRT cells), transcription factors known to repress E-cadherin transcription (Batlle et al., 2000; Comijn et al., 2001), to induce vimentin expression (Bindels et al., 2006), to cause disappearance of cortical actin and formation of stress fibers (De Craene et al., 2005; see Fig. 5B, Fig. 7B) and, more generally, to induce an EMT (Barrallo-Gimeno and Nieto, 2005; Vandewalle et al., 2005). Therefore, ER stress-induced SNAI1/SIP1 might be responsible for the decreased level of E-cadherin, increased level of vimentin and disassembly of cortical actin/formation of stress fibers in PC Cl3 and FRT cells. In FRT cells, these changes cause a decrease of epithelial barrier function. We did not observe any variation in N-cadherin expression following ER stress. However, increased expression of N-cadherin is not the rule in cells undergoing an EMT. Indeed, the EMT comprises a wide spectrum of changes in epithelial plasticity, indicating that different 'subtypes' of EMT exist, differing in their progression towards a mesenchymal phenotype (Huber et al., 2005).

Strikingly, reorganization of the actin cytoskeleton and downregulation of thyroid markers (TG in Fig. 5B) coexist in the same cell. In addition, the gradual loss of TG expression correlates with a concomitant onset of actin reorganization (disappearance of cortical actin and formation of stress fibers), providing visual evidence of a possible link between these two processes (Fig. 5B, arrows). That a link between dedifferentiation and EMT might exist is suggested also by two recent reports (Yang et al., 2005; Seki et al., 2003). Thus, it has been reported that ER stress induces downregulation of mRNAs of the differentiation markers of prehypertrophic chondrocytes (collagen II, aggrecan) (Yang et al., 2005) and that, intriguingly, SNAI1 inhibits transcription of collagen II and aggrecan by binding to E-boxes in their respective gene promoters during chondrocyte passage from the prehypertrophic to the hypertrophic state (Seki et al., 2003). Thus, chondrocytes might experience ER stress in the passage from the prehypertrophic to the hypertrophic state (in a way similar to plasma cell differentiation) (Gass et al., 2004), and the resulting upregulation of SNAI1/snail links dedifferentiation to EMT.

That thyroid dedifferentiation might be mechanistically linked to an EMT-like phenotype is further strengthened by experiments

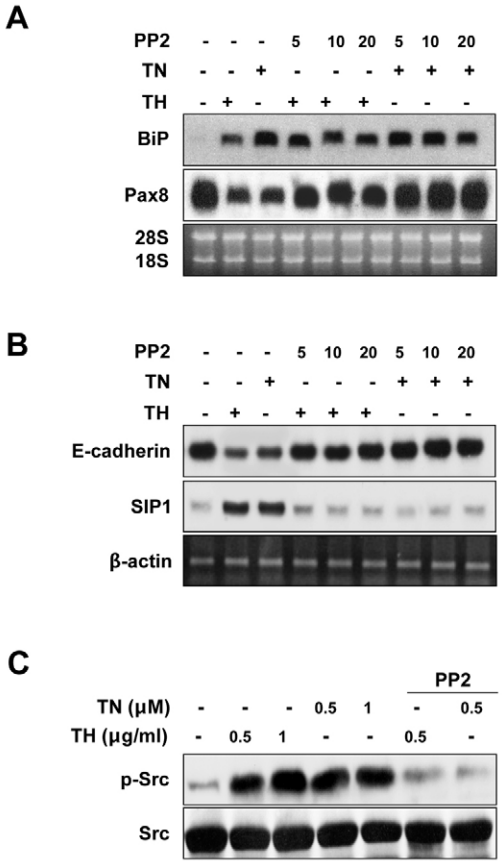


Fig. 9. c-Src is involved in ER stress-induced thyroid dedifferentiation. (A) Northern blot analysis of total RNA extracted from PC Cl3 cells pretreated or not for 30 minutes with 5, 10 or 20 μM PP2, then vehicle treated or treated for 30 minutes with 0.5 μM TH or 0.5 μg/ml TN in the presence or absence of PP2, followed by 24 hours in medium without TH or TN, but with the same filter was probed with Pax8 and BiP. (B) RT-PCR analysis of total RNA extracted from PC Cl3 cells treated as in A. (C) Western blot of total protein extracts from PC Cl3 cells starved for 48 hours in hormone and serum-free medium, pretreated with 5 μM PP2 or vehicle treated or treated for 30 minutes with 0.5 or 1.0 μM TH or 0.5 or 1 μg/ml TN in the absence or presence of 5 μM PP2. Filters were probed with antibodies against phosphorylated Src (p-Src; Tyr416) or total Src.

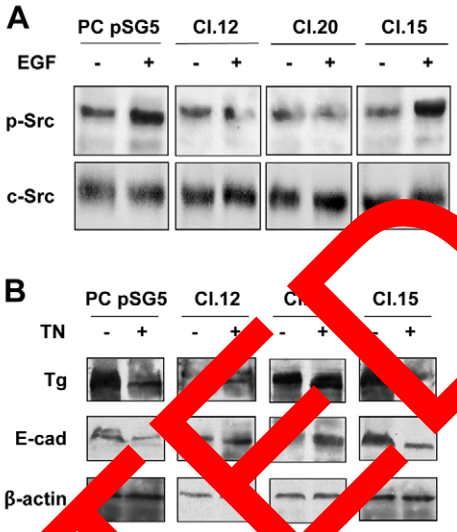


Fig. 10. Stable expression of SrcDN prevents TG or TN-induced downregulation of TG and E-cadherin. (A) Western blots of total protein extracts from PC pSG5 cells, clones (Cl.) 12, 20 and 15 starved for 48 hours in hormone- and serum-free medium, then vehicle treated or treated with 5 nM EGF for 5 minutes. Filters were probed with antibodies against phosphorylated Src (p-Src; Tyr416) or total Src (c-Src). (B) Western blots of total protein extracts from PC pSG5 cells and clones 12, 15, and 20 vehicle treated or treated with 0.5 μg/ml TG for 30 minutes, followed by 24 hours in medium with or without TN. Filters were probed with antibodies against TG, E-cadherin and β-actin.

exploring the signal transduction pathway(s) involved. We provide evidence that c-Src becomes activated following ER stress. Furthermore, activation of c-Src is required for downregulation of both thyroid markers and E-cadherin. Thus, when PC Cl3 cells are treated with PP2, or stably transfected with a SrcDN construct, ER stress trigger causes a decrease of Pax8, TG and E-cadherin mRNAs. Indeed, c-Src might be activated from the ER. Mutants of fibroblast growth factor receptor 3 (FGFR3) are retained in the ER and are capable of signaling to ERK1/ERK2 in a Src-dependent manner (Lievens et al., 2006). The ER-bound protein tyrosine phosphatase 1B (PTP1B) displays an activity that is instrumental in activation of c-Src, through dephosphorylation of the C-terminal tyrosine (Bjorge et al., 2000; Hernandez et al., 2006). ER stress might activate these pathways. Thus, ER stress, through tyrosine kinase receptors or PTP1B (or other mechanisms), might activate c-Src. The results shown in supplementary material Fig. S4 indicate that the EGF receptor is involved in thyroid dedifferentiation triggered by ER stress. As the EGF receptor activates Src (Bromann et al., 2004), very probably it functions, in the context of ER stress, upstream of c-Src and downstream of ER stress. Indeed, it is well known that thyroid cells express (and respond to) the EGF receptor (Miyamoto et al., 1988; Westermark et al., 1996).

Moreover, we suggest that activation of Src is upstream of SNAI1/SIP1 induction as expression of SNAI1/snail family members is downstream of stimulation of tyrosine kinase receptors (Savagner et al., 1997; Lu et al., 2003; Yang et al., 2006) and PP2 abrogates c-Src activation and SIP1 upregulation induced by TH/TN (Fig. 6). It is possible that abnormal activation of Src is responsible also for thyroid dedifferentiation as v-Src is able to dedifferentiate thyroid cells (Fusco et al., 1987). Another interesting possibility is that SNAI1/SIP1 themselves inhibit thyroid differentiation, acting as transcriptional repressors on promoter(s) of thyroid transcription factors, as has been shown in chondrocytes (Seki et al., 2003). By scrutinizing the Pax8 promoter (Okladnova et al., 1997), we have found a canonical AGGTG E-box located at position –6 from the main transcription start site and a CACCT E-box located in the first intron at +98 from the same main transcription start site. In fact, even a single E-box is sufficient for recruitment of SIP1 to the promoters of the genes encoding connexin 26 (Vandewalle et al., 2005) and E-cadherin (Comjin et al., 2001) and for significant repressive activity.

In conclusion, our results describe a new component of the cell response to ER stress. ER stress elicits survival as well as apoptosis. The final outcome depends on the combination between duration and intensity of the stress and the cellular background, with some cell types (neurons, for example) being more sensitive than others. Here, we show that, following ER stress, thyroid cells execute a dedifferentiation program,

involving tissue-specific proteins and epithelial tissue differentiation and organization, but they do not die. The tissue-specific dedifferentiation and loss of the epithelial organization appear to be linked. It is tempting to speculate that these changes might be part of an adaptive response that facilitates cell survival and recovery from ER stress.

Materials and Methods

Cell culture and TH/TN treatments

PC Cl3 cells were cultured as reported previously (Di Jeso et al., 1992). PC Cl3 stably transfected with dominant-negative Src (SrcDN) were cultured in the same medium and supplements plus 200 μg/ml hygromycin (Invitrogen). FRT cells were cultured in the same medium of PC Cl3 cells containing 5% FBS (Gibco). TH or TN (Calbiochem) were added to the medium for 30 minutes at a final concentration of 0.5 μM or 0.5 μg/ml, respectively, The medium was then replaced with medium without TH/TN until harvesting, as reported. To analyze polarity, cells were cultured on filters in Millicell HA bicameral systems (Millipore). Trans-epithelial resistance was measured using the Millicell-ERS apparatus (Millipore).

Plasmids and antibodies

The luciferase reporter plasmid NISLUC2 was provided by R. Di Lauro. The expression vector pSG5-SrcDN was provided by A. Migliaccio. SBE4-Luc and MBE6-Luc reporters (with three copies of the wild-type and mutant Smad binding site, respectively) were acquired from B. Vogelstein, and the Smad4 dominant-negative construct (Smad4-100T) was from L. Attisano. Antibodies used were directed towards the following proteins: TTF-1, TTF-2 and Pax8 (provided by R. Di Lauro), rat TG (Di Jeso et al., 1992), β-actin (Santa Cruz Biotechnology), E-cadherin (Cell Signaling Technology, Beverly, MA), v-Src (Calbiochem) and phosphorylated Src (Tyr416) (Cell Signaling, Danvers, MA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham.

Semiquantitative and real-time reverse transcription-PCR

RNA was reverse transcribed to cDNA by using random hexamers and the I… II reverse transcriptase system (Promega). 10% of the cDNA synthesis reacti… was submitted to semiquantitative PCR analysis by using Taq DNA Polym… se (Promega, Madison, WI). The following oligonucleotides were used: 5′-CC… GTTCAAGAACACCCGC and 5′-CAGCGGTGAGGTCAGGCTTG for vimen… 5′-CTCTGGACAGAGAAGCCATTG and 5′-CTGATGAT… TCATTGA… for E-cadherin; 5′-AGCCACAGCCGTCATCACAG and … AACTC… CACAGA… CACCGTGG for N-cadherin; 5′-GTCCATGCGAACT… CATCTGA… CGCTCT and 5′-GGCTTGCAGAATCTCGCCAC for SIP1… -ACCTTCC… CAGCCC-TACGACC and 5′-GTGTGGCTTCGGATGTGCAT… or SNAI… TGTGATTGAGAACCAGG and 5′-GAGGCT… GTGT… TA… G for XBP-1, 5′-ACCACCATGGAGAAGG and 5′-CTCAG… TAGCCCA… TGC for GAPDH. For real-time RT-PCR analysis, PCRs … performed usin… YBR Green mix (Invitrogen). Reactions were performe… Platinum SYBR G… qPCR Super-UDG using an iCycler IQ multicol… Real … PCR Detection S… m (Biorad, Hercules, CA). All reactions were performed in t… ate, and β-actin was used as an internal standard (β-actin valu… were not affected … H/TN treatments). Oligonucleotides used were: 5′-… GCTCCGGGCTCTG… G and 5′-GCCCATTC-CAACCATCACTCC f… -SMA; 5′-CGAGGGACC… AGGGAGAC and 5′-GGACCAGGAGGA… AGGAAG … α(1)(I) collagen; and 5′-GAGGACAA-GAAGGAGGATC… 5′-TTGG… TGAGTTGGTTC for BiP.

Immunofluorescenc…

1.5×10^5 … plated … 2 mm diam… glass coverslips. 48 hours later, cells were … le trea… or treated … th 0.5 …/ml TN or 0.5 μM TH for 30 minutes. The … dium was t… replaced w… ium without TN/TH and the cells incubated f… 4 hours. Cell… ere fixed for 20 minutes with 3% paraformaldehyde (Sigma) in P… containin… Cl, and 0.5 mM $MgCl_2$ (PBS-CM) at room temperature, was… twi… in 50 mM … Cl in PBS-CM and twice in PBS-CM. Cells were permea… d for 5 minutes in 0.5% Triton-X 100 (Bio-Rad) in PBS-CM and incubated … 30 minutes in 0.5% gelatin (Sigma) in PBS-CM. Cells were then incubated fo… hour with the primary antibodies diluted in 0.5% BSA (Sigma) in PBS. After thre… ashes with 0.2% gelatin in PBS-CM, cells were incubated for 20 minutes with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:50 in 0.5% BSA in PBS. To visualize actin filaments, permeabilized cells were incubated with a 1:100 dilution of rhodamine-conjugated phalloidin (Sigma) for 20 minutes. After final washes with PBS, the coverslips were mounted on a microscope slide and examined with a Zeiss 510 confocal laser scanning microscope. Samples were observed by three investigators, without knowledge of the experimental conditions.

Generation of stable clones and transient expression analysis

To generate PC Cl3 SrcDN stable clones, PC Cl3 cells were co-transfected using Lipofectamine 2000 (Invitrogen) with the plasmid pSG5-SrcDN (kinase-inactive form of Src, Lys259 changed to Met) and a plasmid with the gene encoding hygromycin resistance, or mock-transfected with pSG5 and the hygromycin resistance plasmid. Clones and control PC Cl3 (PC pSG5) were selected with 400 μg/ml hygromycin (Invitrogen). After 2 weeks, hygromycin-resistant clones were isolated and examined by western blot with monoclonal antibodies against v-Src (which revealed total cellular Src) and polyclonal antibodies against phosphorylated Src (Tyr416). For transient transfection analysis, cells were plated in six-well plates to ~80% confluence 24 hours before transfection. Cells were washed with serum-free medium before addition of 1 ml of plasmid-Lipofectamine mixture. The plasmid-Lipofectamine mixture was made … g 2.5 μg of luciferase reporter plasmids and 0.5 μg of pRSV-βga… th 5 μl … fectamine 2000 and 200 μl of serum-free medium for 30 minut… room temperat… before dilution with 800 μl of serum-free medium. Cel… ere incubated for 5 … rs at 37°C before addition of 4 ml complete medium. Aft… 4 hours, 0.5 μM T… or 0.5 μg/ml TN were added to the medium fo… 0 minut… The medium w… then replaced with medium without TH/TN … hours later, l… erase activi… were quantified by luciferase assay (Promes… and normalized fo… ctosid… activity (Promega).

Run-on assay

Twenty 100 mm diameter … ues of … cells were v… e treated or treated with 0.5 μg/ml TN for … minutes. The … ium was th… replaced with medium without TN and the … incubated for 24 … rs. N… were prepared with the Nuclei EZ Prep … ete … olation kit (Sig… lowing the manufacturer's instructions. Fo… e transc… on reaction, 200 … of nuclei were combined with 100 μl of 4× salt buffer (160 … M Tris pH 8.3, 600 mM NH_4Cl, 30 mM $MgCl_2$) and 100 μl of a ribonucleotide … (2.5 mM ATP, 1.25 mM GTP, 1.25 mM CTP and 25 …]UTP at 3000 Ci/… l) and the reaction was incubated at 27°C for … minutes. 8 μl of 1 mg/ml D… se I were added and the incubation was pr… nged for 10 minutes. 1/3 by volume of 1× extraction buffer (10 mM Tris pH 7… 15 mM EDTA, 3% … OS, 1 mg/ml proteinase K) was added, and the reaction w… incubated at 42°C … 3 hours. RNA was purified with an RNeasy Mini kit fro… QIAGEN, followi… the manufacturer's instructions. 500 ng of cDNA of a 0.3 k… agment downs… am of the paired box of mouse *Pax-8* (provided by M. Zannini, … 00 ng … GAPDH cDNA and of ssDNA were immobilized on nitrocellulo… eled nuclear mRNAs were incubated with filters in … idization buffer for 48 hours at 42°C. Finally, filters were washed in 0.2× … % SDS at 60°C and autoradiographed.

RNA extraction, northern and western blots, metabolic labeling and immunoprecipitation

Total RNA extraction, northern and western blots, metabolic labeling, and immunoprecipitation were carried out as reported previously (Ulianich et al., 2004; Di Jeso et al., 2005).

Statistical procedures

Data were analyzed with Statview software (Abacus Concepts) by one-factor ANOVA.

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