Unfolded Protein Response Is Required for the Definitive Endodermal Specification of Mouse Embryonic Stem Cells via Smad2 and β-catenin Signaling

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Keyword: definitive endoderm specification, unfold protein response, embryonic stem cells

Background: Unfolded protein response (UPR) influences cellular differentiation and function.

Results: UPR-inducing agents enhance the differentiation of definitive endoderm cells from mouse ESCs. Inhibition of UPR prevents the specific differentiation.

Conclusion: UPR is required for the formation of definitive endoderm.

Significance: This study provides new molecular mechanism that interconnects UPR and the cell fate decisions in early embryonic development.

ABSTRACT

Tremendous efforts have been made to elucidate the molecular mechanisms that control the specification of definitive endoderm cell fate in gene-knockout mouse models and ESCs differentiation models, however the impact of unfolded protein response (UPR) due to the stress of the endoplasmic reticulum (ER) on the endodermal specification is not well addressed. We employed UPR-inducing agents, thapsigargin (TG) and tunicamycin (TM), in vitro to induce endodermal differentiation of mouse ESCs. Apart from the endodermal specification of ESCs, western blotting demonstrated the enhanced phosphorylation of Smad2 and nuclear
The inclusion of ER stress inhibitor tauroursodeoxycholic acid (TUDCA) to the induction cultures prevented the differentiation of ESCs to definitive endodermal cells even when Activin A was supplemented. Besides, the addition of TGF-β inhibitor SB431542 and Wnt/β-catenin antagonist IWP-2 negated the endodermal differentiation of ESCs mediated by TG and TM. These data suggest that the activation of UPR appears to orchestrate the induction of definitive endodermal cell fate of ESCs via both Smad2 and β-catenin signaling pathways. The prospective regulatory machinery may be helpful for directing ESCs to differentiate into definitive endodermal cells for cellular therapy in the future.

In the embryonic development the epiblasts migrate to the blastula and form the primitive streak, the mesoderm and endoderm are then generated(1). Several signaling pathways and cascades of transcription factors were reported to have roles in the induction and specification of definitive endoderm at different stages of development (2-6). TGF-β signaling was thought to be essential for the formation of definitive endoderm (7,8). Mutants of Nodal failed to form primitive streak and subsequently difficult to generate both mesoderm and definitive endoderm (9). Wnt/β-catenin signaling is also noted to be vital to the development of primitive streak and definitive endoderm (6,10). In homozygous Wnt3a-knockout embryos, epiblasts migrated to the primitive streak and diverted into neuroectodermal cells (11). In addition to Wnt/β-catenin and TGF-β pathways, FGF signaling has also been shown to regulate the formation of primitive streak and the specification of the definitive endoderm (3). Cross-talks among these signaling molecules appear to affect the process as well (3-6,12,13). However, the precise regulatory mechanism of the process of gastrulation is still unclear. Whether there are other factors involved in the specification of endodermal cell fate remains elusive.

ER provides a unique environment for protein folding, assembly and modification. Many insults, including disturbances of calcium homeostasis, changes in the redox potential, ionic strength and requirements of protein folding can pose stress to the ER. Upon ER stress, cells elicit a series of adaptive processes termed UPR to cope with ER stress to maintain the homeostasis of the ER. It was reported that three trans-membrane ER proteins can act as sensors of UPR, namely protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring protein-1 (IRES1) endonuclease/kinase and activating transcription factor-6 (ATF6) (14,15). Under a normal condition, binding immunoglobulin protein (Bip), which is a key ER chaperone, acts as a master regulator of UPR and binds to the luminal domain of ER sensors to prevent their activations. Upon ER stress, Bip is released from PERK, IRES1 and ATF6, leading to the activation of these UPR signaling pathways. Activated PERK kinase phosphorylates the eukaryotic translation-initiation factor 2α (eIF2α), thereby reducing translation-initiation and protein synthesis. Activated IRES1 endonuclease/kinase splices the mRNA-encoding X-box binding protein 1 (XBP-1) to allow the translation of mature XBP-1 protein, which mediates the up-regulation of UPR genes involved in protein folding and proteasome-dependent ER-associated degradation (ERAD). ATF6 transcription factor, which is released from Bip, translocates from ER to the Golgi apparatus where it is cleaved. The cleaved form of ATF6 then acts as an activated transcription factor to target genes augmenting the capacity of protein folding(15). In general, the UPR integrates pathways to efficiently process newly synthesized proteins in the ER.
and activates ERAD to remove mis-folded and excessive proteins to maintain the homeostasis. However, excessive and persistent ER stress can induce apoptosis (15).

A majority of studies reported in the literature focus on restoration of the homeostasis of ER by UPR and ER stress that may lead to life or death of cells (15). Recently, increasing data indicate that UPR play important roles in developmental and metabolic processes (16-21), and that ER stress influences chondrocyte differentiation and function (22). However, whether UPR due to ER stress could influence cell fate decisions of the ESCs to become endoderm at the stage of gastrulation is undetermined.

In the present study, we used mouse ESCs (mESCs) as a differentiation model for early cell lineage specification at the stage of gastrulation. We employed UPR stress-inducing agents, TG or TM, which activate UPR and found that these molecules enhanced the specification of definitive endoderm from ESCs by promoting the nuclear translocation of β-catenin and transiently activating Smad2 signaling.

EXPERIMENTAL PROCEDURES

Cell culture and induction of ER stress—Mouse ESCs E14Tg2a (CRL-1821, American Type Culture collection, Manassas, VA(23), CGR8 (gift of Austin Smith) were propagated as previously described (24,25). Embryoid bodies (EBs) were formed from ESCs by culturing in ESCs medium without leukemia inhibitory factor (LIF, Merck Millipore, Billerica, MA). For the induction of ESCs into a monolayer of endodermal cells, ESCs were allowed to grow overnight without feeder support in the LIF-supplemented ESCs medium. The medium was then replaced with freshly prepared serum-reduced differentiation medium of advanced RPMI 1640 enriched with 2% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). UPR-inducing agents, thapsigargin (TG) and tunicamycin (TM), at various concentrations were added to either EB cultures or endodermal induction cultures. For monolayer culture, cells were allowed to grow with 0.01 µM TG or 0.25 µg/ml TM for 24 hours, and then the cultures were further nurtured in the differentiation medium without TG/TM for 24 hours or grow in the presence of 100ng/ml Activin A for 48 hours. For the EB model, EBs were treated with 0.01 µM TG or 0.25 µg/ml TM for 48 hours.

In the study of TGF-β/Smad signaling, SB431542 at 10µM was added to the differentiation medium for an hour prior to TG or TM treatment. IWP-2 (Stemgent, San Diego, CA) which is an inactivator of Wnt production and secretion at 10 µM was added to the differentiation medium for an hour before TG or TM treatment to investigate the Wnt/β-catenin signaling. GSK-3α/β inhibitor CHIR99021 (Merck Millipore, Billerica, MA) at 1 µM was added to the differentiation medium for 48 hour. Chemicals were purchased from Sigma unless stated otherwise.

Definitive endodermal induction of ESCs into hepatocytic and pancreatic lineage cells—E14Tg2a ESCs were cultured in the differentiation medium supplemented with 0.01 µM TG or 0.25 µg/ml TM for 24 hours, and continued culture for 24 hours without TG/TM. For hepatocytic induction, differentiated cell were allowed to grow in the differentiation medium supplemented with 50 ng/ml bone morphogenetic protein 4 (BMP4, R&D Systems), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), 20 ng/ml Activin A and 10 ng/ml vascular endothelial growth factor (VEGF, R&D Systems) for three days. Thereafter, the medium was changed with DMEM/F12 medium supplemented with N2B27 (Life Technologies), 10 ng/ml hepatic growth factor (HGF, R&D Systems), 10 ng/ml bFGF and cultures continued for six days. Cells were further grown in
William’s E medium (Life Technologies) enriched with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 ng/ml epidermal growth factor (EGF, R&D Systems), 10 ng/ml HGF, 1 µM insulin, 0.5 mM Ascorbic Acid or Vitamin C (Vc, Sigma), 0.1 µM dexamethasone (Sigma) for six days. The cultures were maintained for 6-12 days in William’s E medium further supplemented with 10 ng/ml oncostatin m (OSM, PeproTech, Rocky Hill, NJ), 1 mM dexamethason and 1% insulin-transferrin-selenium (ITS, Life Technologies).

For pancreatic induction, differentiated cells were allowed to grow in DMEM supplemented with 2% FBS, 1% ITS, 2 µM retinoic acid (RA, Sigma) for one day. The medium was then replaced with freshly prepared DMEM enriched with 10% FBS, 1% ITS, 20 ng/ml EGF, 10 ng/ml bFGF and 50 ng/ml fibroblast growth factor 10 (FGF10, Sigma) and maintained for additional five days.

RT-PCR and real-time PCR analysis—RNA was extracted by using RNeasy Mini kit (Qiagen, Hilden, Germany) and transcribed to cDNA using PrimedScript™ RT Master Mix (Takara, Shiga, Japan). RNA integrity was confirmed by RT-PCR of a ubiquitous mRNA GAPDH. Results were confirmed in at least three separate analyses.

SYBR Green-based quantitative RT-PCR (Takara) was performed using an ABI Prism 7900 HT (Applied Biosystems) according to the manufacturer’s instructions. The relative expression level of each target gene was calculated by the comparative $C_T$ method and was normalized to GAPDH expression.

Western blotting — Western blot analyses were carried out as previously reported (26). The following antibodies raised from rabbit were obtained from Cell Signaling Technology to identify the specific proteins: rabbit anti-Bip, rabbit anti-p-eIF2α and eIF2α, rabbit p-GSK3β and GSK3β rabbit anti-p-Smad2 (Ser465/467) and Smad2, rabbit anti-GAPDH, and rabbit anti-β-catenin. Mouse anti-β-Actin was from Sigma, and Rabbit p21 was from Abcam.

Flow cytometry — For cell phenotype analysis, cultures were dissociated by trypsinization and washed with cold PBS. Having been fixed in 4% paraformaldehyde, cells were permeabilized with 0.2% cold Triton X-100. They were washed and separately stained with IgG or polyclonal goat anti-Sox17 (1:100, R&D Systems), rabbit IgG or polyclonal anti-β-catenin (1:100, Cell Signaling Technology), rabbit IgG or polyclonal anti-Pdx1 (1:100, Cell Signaling Technology), and mouse IgG or anti-CK8 (1: 50, Santa Cruz Biotechnology.), mouse IgG or anti-albumin (1:100, Novus Biologicals, Littleton, CO). After rinsed with PBS for three times, specific cell markers were detected by the corresponding secondary antibodies, Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated goat anti-mouse IgG, or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Life technologies), at 400-fold dilution in 3% BSA in PBS. Upon completion of washing, cell nuclei were counter-stained with 4, 6-diamidino-2-phenylindole hydrochloride (DAPI, Sigma). The immunostaining was visualized by using an inverted fluorescence microscope and the corresponding fluorescence filters.
rabbit anti-goat antibody diluted in 1% normal bovine serum. Upon completion of washing, labeled cells were re-suspended and at least $10^5$ events were acquired by using a FACS Calibur flow cytometer and analyzed using the software CellQuest Version 3.1 (BD Biosciences). Background of non-specific antibody uptake was evaluated by staining in parallel with FITC-conjugated isotype-matched control antibody.

For cell surface staining, cells were dissociated with 0.02% EDTA, and incubated with biotin-conjugated anti-E-cadherin mAb (ebioscience), after twice wash, cells were incubated with APC-conjugated streptavidin (ebioscience) and PE-conjugated anti-CXCR4 mAb (ebioscience).

**Luciferase reporter Assays** — E14Tg2a ESCs were transfected with 7TFP (7Xtcf promoter) from Addgene (27) and selected with puromycin to obtain wnt-responding ES cell line. TG/TM was added to the differentiation medium for 24 hours, and then the cells were harvested for luciferase activity analysis. The luciferase activities in the samples were measured using a dual-luciferase reporter assay system (Promega).

**Statistical analysis** — Data derived from at least three independent experiments were presented as mean ± standard deviation (SD), unless stated otherwise. The relative mRNA levels were quantified by using the $2^{-\Delta\Delta CT}$ method and averaged by normalization to GAPDH expression. Statistical significances were tested by Student’s t-test. The software SPSS 13.0 (SPSS Inc., Chicago, IL,) was used and p-values < 0.05 were considered statistically significant.

**RESULTS**

**Thapsigargin and Tunicamycin trigger ER stress and activate UPR** — ER stress influence expression of many gene and cellular differentiation and function (20,22,28,29). Therefore, we hypothesized that ER stress might simulate the specification of germ layer at the stage of gastrulation. First, we used ESCs as an in vitro differentiation model. Secondly, we set up an experimental condition to trigger ER stress which activates UPR. We tested the effect of widely used ER stress-inducing agents, TG and TM on two-day cultures of EBs derived from ESCs. RT-PCR demonstrated that the gene expressions of Bip and XBP-1(s), which is the active isoform of XBP-1 after splicing, were up-regulated in mouse ESCs-derived EBs treated with TG and TM in a dose-dependent manner (Fig. 1). Both compounds could activate UPR at low concentrations (TG: 0.01 µM, TM: 0.25 µg/ml) in two days.

**ER stress enhances endodermal differentiation of ESCs in EBs** — To study the effects of ER stress on the differentiation of ESCs, we differentiated ESCs in EB model. EBs derived from mouse ESCs were cultured in the differentiation medium with the supplement of either TG or TM for two days followed by four-day cultures in the medium with neither supplement. Real-time PCR for endodermal gene Sox17 and FoxA2 from cultures over the time course revealed that a dramatic increase of gene expression in treated cultures compared to those control cultures (Fig. 2). In addition, TG and TM also enhanced the expression of primitive endodermal genes, Sox7, Gata4 and Gata6 (Fig.2A). Conversely, down-regulation of gene expression of mesodermal T and Flk1, ectodermal Pax6 and Ck18, Epiblast marker Fgf5 and trophectodermal Cdx2 was observed in TG- and TM-treated cultures compared to those untreated counterparts, while the expression of pluripotent gene Oct4 became diminished in TG- and TM-treated cultures as compared to the control cultures (Fig. 2A).

To further observe the effect of TG/TM on the differentiation of EBs, 2d EBs were allowed to differentiate in the TG- or TM-supplemented differentiation medium for two days Real-time RT-PCR analysis demonstrated a significant increase of endodermal gene expression in the
cultures with TG and TM, compared to the control cultures, while T, Flk1, Fgf5 and Cdx2 expressions were remarkably down-regulated (Fig. 2B). In addition, immunofluorescence staining showed that the numbers of Sox17 and FoxA2 positive cells in the cultures treated with TG/TM were notably more than those in the control cultures (Fig. 2C). Taken together, these data suggest that ER stress-induced UPR enhances definitive endodermal differentiation and inhibits other germ layer commitments of ESCs in EBs of the mouse origin.

ER stress promotes definitive endodermal differentiation of ESCs in monolayer cultures – To further determine whether definitive endoderm differentiation was associated with UPR, we treated ESCs with Activin A Mouse ESCs were grown in the differentiation medium on monolayer in the presence of Activin A at a high concentration of 100 ng/ml for 72 hours. The gene expression of Bip and XBP-1(s) was up-regulated progressively in the first 36 hours, whereas the expressions of definitive endodermal genes, Sox17 and FoxA2, increased steadily up to 72 hours (Fig. 3A). These findings suggest that definitive endodermal lineage commitment of ESCs might be associated with UPR.

To confirm whether ER stress promote definitive endoderm differentiation, we adopted monolayer culture differentiation model. Quantitative real-time PCR for gene expression on monolayer cultures of ESCs, which grew in the presence of TG-/TM or Activin A, displayed a significant up-regulation of definitive endodermal genes, Sox17 and FoxA2, compared to those of control cultures (Fig. 3B) and the increases of the gene expression were more profound in monolayer cultures of ESCs induced with ER stress. In contrast, there was a down-regulation of the T, Flk1, Zic, Fgf5 and Cdx2 genes in the cells treated with TG/TM (Fig.3B).

Immunofluorescence staining of Sox17 yielded stronger green fluorescent signals in 24-hour TG- or TM-treated monolayer cultures of ESCs compared to those in the control cultures, confirming the up-regulated expression of Sox17 gene quantified by real-time PCR (Fig. 3C). The numbers of Sox17 positive cells in TG- or TM-treated monolayer cultures quantified by flow-cytometry were significantly more than those in control cultures (Fig. 3D).

On the other hand, Sox17 was also expressed in the visceral endoderm, which will form extra-embryonic yolk sac, however, definitive endoderm would eventually contribute to visceral organs, such as the liver and pancreas (30,31). To date, a few markers are available to distinguish visceral and definitive endoderm cells, among of theses markers, CXCR4 and E-cadherin double positive cells were reported to define definitive endodermal cells by FACS analysis (32). As shown in Fig. 3E, double positive cells in TG- or TM-treated monolayer cultures were remarkably more than those in control cultures. Moreover, TG-treated cultures yielded similar numbers of double-positive events, compared to cultures supplemented with Activin A.

To further validate the above results, CGR8 cells, another mESC line, were treated with TG/TM on monolayer culture. Quantitative real-time PCR results showed that expression of endodermal genes Sox17 and FoxA2 was significantly increased in the TG-/TM-treated cells compared to those in the control cultures (Fig. 4A). Inversely, mesodermal genes were downregulated in the TG/TM-treated cells compared to the control group (Fig.4A). Immunofluorescence staining showed that the numbers of Sox17 positive cells in the cultures treated with TG/TM were significantly more than those in the control cultures (Fig. 4B). In addition, flow cytometry assays with CXCR4 and E-cadherin antibodies also indicated that the numbers of double positive cells in TG- or TM-treated monolayer cultures were remarkably
more than those in the control group (Fig. 4C). These observations demonstrate that ER stress-induced UPR enhances definitive endodermal differentiation.

It is well-known that definitive endoderm cells can further differentiate into functional hepatocytes and insulin-secreting cells, which is of significance for regeneration medicine. Thus, it is important to evaluate the differentiation potency of the definitive endodermal cells from TG/TM-treated ESCs. To address the differentiating potential, we first treated mESCs with TG/TM to form definitive endoderm, and then kept the cells in the differentiation medium with the growth factors described in material and methods. In the control group, mESCs were first maintained in the differentiation medium without treatment, and then the growth factors were added. Immunofluorescence staining demonstrated the expression of cytokeratin 8 (CK8) and albumin in the cultures upon completion of the induction into hepatocytic lineage cells. The expression of pancreatic progenitor marker Pdx1 was also noted in the cultures upon completion of the differentiation to the pancreatic lineage (Fig. 4D).

**TGF-β/Smads signaling in definitive endodermal specification of ESCs under ER stress** – ER stress can activate a number of signaling pathways (22,33,34). Previous studies reported that TGF-β/Smad, Wnt/β-catenin, and FGF signaling pathways can regulate the formation of the definitive endoderm (2,4,7). To test which signal that activates ER stress is associated with definitive endoderm differentiation, we performed western blotting assays with the whole lysate from ESCs treated with TG/TM and found an active translation of Bip, which was not evident in the control cultures (Figure 5A). In addition, phosphorylated eIF2α was seen in treated cells, indicating that ER stress did activate UPR. It was also noted that phosphorylated Smad2 was up-regulated in cells treated with either TG or TM at 6 to 24 hours (Fig. 5A). To provide additional evidence for the activation of TGFβ/smad2 signaling pathway during ER stress, we analyzed the downstream target gene expression. TGF-β/smad signaling has been considered as a tumor suppressor pathway by inhibiting cell growth, which is mediated by upregulating of cyclin-dependent kinase inhibitors p15/INK4b and p21/WAF1 (35,36). We did find that expression level of p21 was upregulated, similar to that of p-smad2 (Fig. 5A).

To verify the role of Smad2 in definitive endodermal induction of ESCs under ER stress, 10 µM SB431542 was supplemented to ESCs cultures with TG or TM. Western blot showed that SB431542 did inhibit smad2 signaling activated by TG/TM (Figure 5B). Quantitative RT-PCR revealed a significant down-regulation of definitive endodermal genes, Sox17 and FoxA2 and up-regulation of Fgf5 in the cultures examined, compared to those in the control cultures (Figure 5C). These data suggest that ER stress enhances definitive endodermal specification of ESC via TGF-β/Smad2 signaling.

**Wnt/β-catenin signaling in definitive endoderm differentiation of ESCs under ER stress** – Apart from phosphorylated Smad2, which was up-regulated, we also found a down-regulation of phosphorylated GSK3α/β proteins in these cultures under ER stress (Fig. 5A), which phosphorylates cytoplasm β-catenin and subsequently degrades it by proteasome (37). As shown in Fig. 6A, immunostaining of β-catenin revealed that nuclear β-catenin in the cells treated with TG/TM for 24 hours was remarkably more than those in the control group. Quantification of the percentage of cells with β-catenin nuclear staining over the total β-catenin positive cells showed a significantly higher percentage in TG-treated (approximately 98.5%) or TM-treated (about 85.1%) than the control group (approximately 12.9%) (Fig 6B). The same results were observed by western blot.
analysis at the protein level (Fig. 6C).

To determine whether the wnt/β-catenin signaling also participated in the process of definitive endoderm induction from ESCs by UPR, we tested TCF luciferase wnt reporter activity in the treated cells lysates. The luciferase activity in the cell lysates of the TG/TM-treated cells for 24 hours was evidently enhanced compared to that of the control cultures (Fig. 6D). These findings indicate an involvement of β-catenin signaling in the definitive endoderm differentiation of ESCs under ER stress.

To provide more evidence that wnt/β-catenin mediated definitive endoderm differentiation induced by ER stress, we applied IWP-2 and CHIR99021 to the cell culture. Quantitative RT-PCR for gene expression from the differentiation cultures of ESCs exposed to 10 µM IWP-2 with and without TG or TM, displayed a significant down-regulation of, Sox17 and FoxA2, and up-regulations of Fgf5, compared to those in the control cultures (Fig. 6E). Inversely, a pattern of up-regulated expression of Sox17 and FoxA2 was observed in the differentiation cultures of ESCs treated with 1 µM GSK3α/β inhibitor CHIR99021 (Fig. 6E). The data suggest that the Wnt/β-catenin signaling may play a role in definitive endoderm differentiation of ESC under ER stress.

Inhibition of ER stress abolishes the differentiation of endoderm - To investigate whether UPR induced by ER stress is requisite for the definitive endodermal specification, we adopted ER stress inhibitor, tauroursodeoxycholic acid (TUDCA), to treat ESCs. Quantitative RT-PCR for gene expression on cell derivatives of ESC in differentiation cultures supplemented with 300 µM TUDCA for 48 hours. Down-regulation of ER stress-related genes, Bip and XBP-1(s), definitive endodermal genes Sox17 and FoxA2, and primitive endodermal genes and up-regulation of T and Fgf5 were observed (Fig. 7A). To further verify whether UPR is necessary for the specification of definitive endoderm, 300µM TUDCA together with 100 ng/ml Activin A were added to the ESC culture and maintained for 48 hours, Sox17 and FoxA2 were also found to be also significantly down-regulated (Fig. 7A). These data together indicate that inhibition of ER stress does nullify the definitive endodermal specification of ESCs.

DISCUSSION

To the best of our knowledge, this is the first report to show that ER stress-mediated UPR can stimulate the definitive endodermal specification of ESCs in in-vitro systems of induction cultures of EBs and monolayer of mouse ESCs. The definitive endodermal lineage commitment of mouse ESCs could be kindled by using low dose of ER stress agonists, 0.01 µM TG and 0.25 µg/ml TM, to induce ER stress without provoking remarkable cell apoptosis (data not shown). The supplement of ER stress inhibitor TUDCA to ESC cultures abolishes the definitive endodermal commitment even when Activin A was also added, which induces definitive endoderm (Fig. 7A) (8,38). These observations suggest that UPR is requisite for the definitive endodermal cell fate decision during the formation of gastrulation (Fig. 7B).

Previous studies reported that Nodal/Activin A (9), Wnt/β-catenin (2,12,39), TGF-β signaling pathways can regulate the formation of primitive streak and the specification of the definitive endoderm (3). To test the down-stream pathways controlling the specification of definitive endoderm which UPR participates, we performed western blotting experiments with the protein extracts of ESCs treated with TG/TM at time points from 0 hour to 24 hours and found that p-smad2 was upregulated and p-GSK3α/β was downregulated (Fig. 5A). These data indicated that TG/TM could specifically active TGF-β/Smad2 and Wnt/β-catenin pathways. To ascertain the signaling in the TGF-β/Smad2 and
Wnt/β-catenin pathways, we also assessed the TGF-β/Smad2 downstream gene p21 expression and β-catenin TCF luciferase reporter activity. The results showed that p21 was upregulated and that TCF luciferase activity was enhanced when the cells were treated with TG/TM. Additionally, we applied the TGF-β smad inhibitor SB431542, inactivator of Wnt production and secretion IWP-2 to ESC prior to the administration of pharmaceutical inducers of ER stress. These molecules were able to block the definitive endoderm specification of ESCs under ER stress. In agreement with our hypothesis, it has been reported that mutant mice which missed an allele of Smad2 and Smad3, display definitive endodermal defects (7). Activin induces definitive endoderm in ESCs via Smad2 pathway (8,38). In embryos lacking of β-catenin, the definitive endodermal derivative changed the fate to cardiac mesoderm (2). More importantly, Sox17, which regulates the transcription of endodermal genes, has been shown to be a downstream target of β-catenin (10,40). These studies together support that TGF-β/Smad2 and Wnt/β-catenin signaling pathways may be essential in the induction of definitive endoderm (8,41) and suggested that UPR acts on Smad2 and Wnt/β-catenin to induce the specification of definitive endoderm from the ESCs.

ER provides a unique environment for the production of secretory and membrane protein and processing of unfolded, mis-folded, degenerated and mutated proteins (15). Recent findings indicate that ER stress might alter gene expression beyond protein folding (34). ER stress inducers, TG and TM, are able to enhance the expression of differentiation genes in addition to genes encoding secretory polypeptides, membrane proteins and growth factors (18-20). A recent study shows that some extracellular matrix components are necessary for definitive endoderm differentiation from ESCs (42). Furthermore, there is several reports addressing the intracellular compartment of protein folding, which happens in the ER of stem and progenitor cells, in controlling cell fate decisions especially under the stress to restore equilibrium between ER load and protein folding efficiency (14,43,44). Therefore, we think that ER stress could change the expression of some genes controlling and determining the specification of endoderm. In summary, data of this study indicate ER stress-mediated UPR is requisite for the specification of endoderm cell fate in the early embryonic development via the synthesis and translocation of β-catenin from cytoplasms to nuclei and transient activation of Smad2. Definitive endoderm derived from ESCs which can then give rise to the internal organs including the liver and the pancreas. Further understandings of the controlling mechanisms of cell fate divisions of stem cells are helpful for the development of new treatment regimens for the patients who currently do not have much available therapeutic options.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**FIGURE 1.** TG or TM activates UPR in 2d EBs from mESCs. Representative images of RT-PCR products of *Bip* and *XBP-1* derived from two-day embryo bodies (EBs) developed from mouse ESCs (E14Tg2a) treated with and without 0.01 – 0.2 µM TG or 0.01 – 0.2 µg/ml TM for 24 hours and 48 hours, respectively. An up-regulation of gene expression of *Bip* and *XBP-1(s)* was noted in the treated cell samples. *XBP-1(u): uncut XBP-1. XBP-1(s): spliced XBP-1.*

**FIGURE 2.** TG and TM enhance the differentiation of endoderm derived from mESCs in EB models. A.Real-time PCR analysis for expression of marker genes in EBs differentiated in ES medium without
Lif in the presence of either 0.01 µM TG or 0.25 µg/mL TM for two days, and followed by four-day cultures in the same medium without the supplement. con, abbreviation for control cultures. The level of mRNA in ESCs was set as 1. B. Relative gene expression on 2d EBs grown in low serum differentiation medium with either TG or TM for two days. The level of mRNA in cells without the supplement was set as 1. Data were collected from at least three separate experiments and are shown as mean ± standard deviation (SD). *P<0.05, ** p<0.01 compared to controls. C. Immunofluorescence staining of Sox17 and FoxA2 positive cells in the 2d EBs cultures supplemented with TG/TM for two days. Scale bar is 50µm.

FIGURE 3. Characterization of definitive endodermal cell derivatives from differentiation cultures of ESCs in monolayer culture under ER stress. A. Relative gene expression of Bip, XBP-1(s), Sox17 and FoxA2 from mouse E14Tg2a ESCs cultured in differentiation medium supplemented with 100 ng/ml Activin A for 72 hours. B. Real time PCR analysis for marker gene expression in the cells treated with TG/TM (0.01 µM TG/TM for 24 hours and maintained in the medium without the supplement for 24 hours), or 100 ng/ml Activin A for 48 hours. C. Immunofluorescence staining of Sox17-positive cells in the cultures supplemented with either TG/TM or Activin A as panel B. D. Flow cytometry analysis for Sox17-positive cells in the cultures treated with TG/TM or Activin A as panel B. E. Flow cytometry assays for E-cadherin and CXCR4 double positive cells from the cultures treated with TG/TM or Activin A as panel B. Scale bar is 50µm.

FIGURE 4. Characterization of definitive endodermal cells derived from CGR8 mESC cells and developmental potential of TG/TM-induced definitive endoderm cells derived E14Tg2a ESCs. A. Induction of CGR8 mESC cells by TG/TM to differentiate into definitive endodermal cells in monolayer cultures. Real-time PCR analysis for marker gene expression in the cells treated with TG/TM for 24 hours and maintained in the medium without the supplement for 24 hours. B. Immunofluorescence staining of Sox17 in the cultures treated with TG/TM as panel A. C. Flow cytometry assays for E-cadherin and CXCR4 double positive cells in the cultures treated with TG/TM as panel A. D. Immunofluorescence staining of hepatocytic lineage markers, cytokeratin 8 (CK8), albumin and pancreatic progenitor marker Pdx1 in the cultures upon completion of the induction as described in the section “Experimental procedure”. Scale bar is 50µm.

FIGURE 5. Involvement of the TGF β/Smad2 signaling pathway during the definitive endodermal specification of ESCs under ER stress. A. Western blotting of proteins extracted from ESCs treated with TG or TM at 6 to 24 hours. GAPDH was loaded as the loading control. B. Western blot assay for the cell lysate from mESCs treated with 10 µM Smad antagonist SB43152 and TG/TM. C. Real-time PCR assay for marker gene expression of the cells treated with SB431542 as the above.

FIGURE 6. Involvement of the Wnt/β-catenin signaling during the definitive endodermal commitment of ESCs under ER stress. A. Immunofluorescence staining of β-catenin in ESC in differentiation cultures supplemented with TG/TM for 24 hours. B. Quantification of the percentages of cells with β-catenin nuclear staining over the total β-catenin positive cells. Five to six representative visual fields for each of the groups were counted. C. TCF luciferase wnt reporter assays of cell lysates from TG/TM treated cells. D. Western blotting of β-catenin extracted from cell derivatives of the nucleus and cytoplasm from ESCs under the same differentiation conditions for β-catenin staining.
TATA-binding protein (TBP) and β-actin were used as loading controls for nuclear protein and cytoplasmic protein, respectively. E. Real-time PCR for marker gene expression in the cultures treated with 10 µM IWP-2 with TG/TM or 1 µM GSK-3α/β inhibitor CHIR 99021. Scale bar is 20µm.

FIGURE 7. Inhibition of ER stress hinders the formation of definitive endoderm. A. Real-time PCR for marker gene expression in the ESC differentiation cultures supplemented with 300 µM TUDCA, in the presence or absence of Activin A for 48 hours. B. Schematic representation of enhanced induction of definitive endodermal cells from mESCs via UPR induced by TG/TM.

Figure 1
Figure 3

A

B

C

D

E
Figure 5

A

B

C

Figure 6

A

B

C

D

E
Unfolded Protein Response Is Required for the Definitive Endodermal Specification of Mouse Embryonic Stem Cells via Smad2 and beta-catenin Signaling

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