Biochemical and Biophysical Research Communications 443 (2014) 821–827

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A mitochondrial thioredoxin-sensitive mechanism regulates TGF-β-mediated gene expression associated with epithelial–mesenchymal transition

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ARTICLE INFO

Article history:
Received 4 December 2013
Available online 14 December 2013

Keywords:
TGF-β
ROS
TXN2
HMGA2
Fibronectin
Mitochondria

ABSTRACT

Transforming growth factor (TGF)-β is a pro-oncogenic cytokine that induces the epithelial–mesenchymal transition (EMT), a crucial event in tumor progression. During TGF-β-mediated EMT in NMuMG mouse mammary epithelial cells, we observed sustained increases in reactive oxygen species (ROS) levels in the cytoplasm and mitochondria with a concomitant decrease in mitochondrial membrane potential and intracellular glutathione levels. In pseudo ρ0 cells, whose respiratory chain function was impaired, the increase in intracellular ROS levels was abrogated, suggesting an important role of mitochondrial activity as a trigger for TGF-β-stimulated ROS generation. In line with this, TGF-β-mediated expression of the EMT marker fibronectin was inhibited not only by chemicals that interfere with ROS signaling but also by exogenously expressed mitochondrial thioredoxin (TXN2) independent of Smad signaling. Of note, TGF-β-mediated induction of HMGA2, a central mediator of EMT and metastatic progression, was similarly impaired by TXN2 expression, revealing a novel mechanism involving a thiol oxidation reaction in mitochondria, which regulates TGF-β-mediated gene expression associated with EMT.

1. Introduction

Transforming growth factor (TGF)-β superfamily members including TGF-β isoforms, activins, inhibins, and bone morphogenetic proteins regulate various cellular functions ranging from embryonic development to adult tissue homeostasis, while under pathological conditions, they contribute to aggravation of diverse disorders such as tissue fibrosis and autoimmune diseases [1]. In particular, a wealth of studies has implicated TGF-β in tumorigenesis and characterized its ostensibly ambivalent roles during the process, depending on the stage of tumor development. According to a widely accepted view, in an early stage of carcinogenesis, TGF-β can suppress the neoplastic transformation of epithelial cells through growth inhibitory and proapoptotic activities. In advanced cancers, however, where the growth inhibitory signal of TGF-β is interrupted and/or distorted through genetic mutations in the molecular machineries that receive and/or transmit TGF-β signals, TGF-β stimulates neoplastic transformation through its ability to induce epithelial plasticity, thus leading to epithelial–mesenchymal transition (EMT) and/or cell motility [2,3]. Accordingly, TGF-β-mediated EMT induction has been a focus of cancer biology to better understand cancer progression.

TGF-β signals primarily through two pathways, Smad and non-Smad pathways [4]. Recently, additional pathways evoked by reactive oxygen species (ROS) have been associated with TGF-β signaling. Indeed, several reports demonstrated that TGF-β stimulates intracellular ROS production in various types of cells after the seminal discovery in murine osteoblastic cells [5,6]. According to the reports, ROS-evoked signals play a role in TGF-β-mediated EMT in breast and renal tubular epithelial cells and cardiac and pulmonary fibrosis [7–10] dependently or independently of the Smad pathway, although molecular details remain to be elucidated in most cases.

In an effort to substantiate the biological significance of ROS signals in TGF-β biology, we studied an increase in ROS levels and the impact of a consequent change in cellular redox on TGF-β-mediated gene expression associated with EMT. A series of analyses with mammary epithelial cells undergoing EMT after TGF-β treatment revealed a sustained increase in ROS levels accompanying reduction of cellular redox in cells, highlighting mitochondria as important players. Of interest, a cysteine thiol-disulfide exchange reaction in mitochondria is suggested to be involved in TGF-β-mediated regulation of gene expression. As a prominent example,

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http://dx.doi.org/10.1016/j.bbrc.2013.12.050

induction of HMGA2, a high mobility group AT-hook 2, which mediates TGF-β-induced EMT, was suppressed by the exogenous expression of TXN2, a mitochondrial thioredoxin. In summary, mitochondria-directed cellular redox changes have emerged as important regulators in TGF-β-mediated gene expression associated with EMT.

2. Materials and methods

2.1. Cell culture

NMuMG mouse mammary epithelial cells were obtained from ATCC and cultured as described previously [11]. The cells were treated with TGF-β1 (R&D Systems, Minneapolis, MN, USA) in conditioned media. Chemicals [12] were pretreated for 30 min before TGF-β1 treatment. Establishment of pseudo ρ0 cells was described previously [13].

2.2. Expression plasmids

HyPer-C and -M retrovirus plasmids were constructed by inserting cDNA from pHyPer-cyto and -dMito (Evrogen, Moscow, Russia) [14] into pMXs-IN [15]. Lentiviral expression vectors, CSII-CMV-MCS-IRES2-Bsd and hTERT/pLXIN, were kindly gifted by Dr. H. Miyoshi (Riken BRC, Japan) and Dr. H. Tahara (Hiroshima University, Japan), respectively. The Tet-Off lentiviral expression vector, CSII-TREII, was constructed by ligating a tetracycline-inducible unit from pSIN-TREII and tTA advanced/pMXs-IP to CSII-CMV-MCS-IRES2-Bsd [16]. Human thioredoxin (TXN)2 cDNAs were amplified from the human mammary epithelial cell cDNA library and inserted into the CSII-TREII vector.

2.3. Infection

Retrovirus production and infection was described previously [16]. For the generation of lentivirus-expressing HEK293T cells (Riken BRC Cell Bank, Tsukuba, Japan), the CSII-TREII vector was cotransfected with packaging plasmids (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev) [17] by the calcium phosphate precipitation method. The conditioned medium containing viral particles was harvested 48 h after transfection and used to infect cells with 4 μg/ml polybrene and 10 ng/ml doxycycline (Dox). Successfully infected cells were selected and maintained with 5 μg/ml puromycin in the presence of 10 ng/ml Dox.

2.4. Immunoblotting

The immunoblotting procedure was described previously [11]. The primary antibodies used are listed in Supplementary data.

2.5. Luciferase reporter assay

Cells in 12-well plates were transiently transfected with 0.5 μg of luciferase constructs, together with 0.02 μg of phRL/CMV (Promega, Madison, WI, USA), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega), according to the manufacturer's instructions. SBE/Luc ($(CAGA)_{12}$ MLP Luc) was a generous gift from Dr. J.-M. Gauthier [18]. pAP1/Luc was purchased from Stratagene (La Jolla, CA, USA).

2.6. RNA extraction and real-time reverse transcription PCR

RNA extraction and real-time reverse transcription (RT) PCR were performed in the same manner as described previously [16]. Quantities of mRNA were normalized by mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used are described in Supplementary data.

2.7. Measurement of intracellular redox states and mitochondrial membrane potential

Measurement of the intracellular redox state with H_2DCF was described previously [19]. For the measurement of the subcellular redox state, cells were infected with the HyPer-C or -M retrovirus and after 24–48 h, emission at 490 nm was observed in a single cell at intervals of 4 h using a Nikon ECLIPSE TE2000-U microscope equipped with a high-speed cooled digital CCD camera (Nikon Corporation, Tokyo, Japan). Images were acquired using an AquaCosmos image acquisition and analysis system (Hamamatsu Photonics, KK Hamamatsu, Japan). Mitochondrial membrane potential ($\Delta\psi m$) and intracellular glutathione (GSH) levels were determined using the Mito-ID Membrane Potential Cytotoxicity Kit (Enzo Life Science, Farmingdale, NY, USA) and GSH-Glo Glutathione Assay Kit (Promega), according to the manufacturer's instructions, respectively.

3. Results

3.1. Increase in ROS levels in the cytoplasm and mitochondria of mammary epithelial cells treated with TGF-β

We examined the changes in intracellular ROS levels in TGF-β-treated NMuMG cells using two distinct types of fluorescent probes sensitive to cellular redox. One is H_2DCF, a chemical dye routinely used for ROS detection in cells, and the other is HyPer-C, a genetically encoded probe that is expressed as a compound protein consisting of a derivative of green fluorescent protein and a regulatory domain of a bacterial ROS sensor OxyR, directed to localize in the cytoplasm [14]. The results obtained from each probe are shown in Fig. 1A and B, respectively, indicating that the fluorescence intensities of both probes changed essentially in the same patterns. In normal cells, they underwent a sustained increase followed by a decline at 24 h, indicating that the cytoplasmic ROS levels were increased over 20 h after TGF-β treatment (Fig. 1A and B, Normal). The increase was biphasic with peaks observed at approximately 8 and over 16–20 h, implying that multiple ROS-generating systems were involved in the elevation under the conditions [20].

Among numerous potential sources, mitochondria are regarded as a major site of ROS production within cells. Thus, we tested their involvement in the above change using pseudo ρ0 cells in which the respiratory chain function is severely deteriorated [19]. In contrast to ROS levels in the normal cells, changes in ROS levels in the ρ0 cells appeared within a margin of error (Fig. 1A and B, ρ0). The absence of the response to TGF-β in the ρ0 cells was unlikely to be due to a fundamental loss of responsiveness to TGF-β because phosphorylation of Smad3 and the subsequent signal transduction were intact in the cells (Supplementary Fig. S1A and B). Taken together, mitochondrial activity was indispensable for the increase in the cytoplasmic ROS levels in the normal cells, and mitochondria were prospective sources and/or triggers of the increased cytoplasmic ROS levels.

Therefore, we next examined changes in mitochondrial ROS levels after TGF-β treatment using another genetically encoded fluorescent probe, HyPer-M [21], a mitochondria-targeted version of HyPer-C. TGF-β treatment caused an increase in fluorescence over several hours (Fig. 1C), suggesting that the mitochondrial ROS levels also increased under the conditions. However, different from the changes in HyPer-C (Fig. 1B), the intensity was elevated with

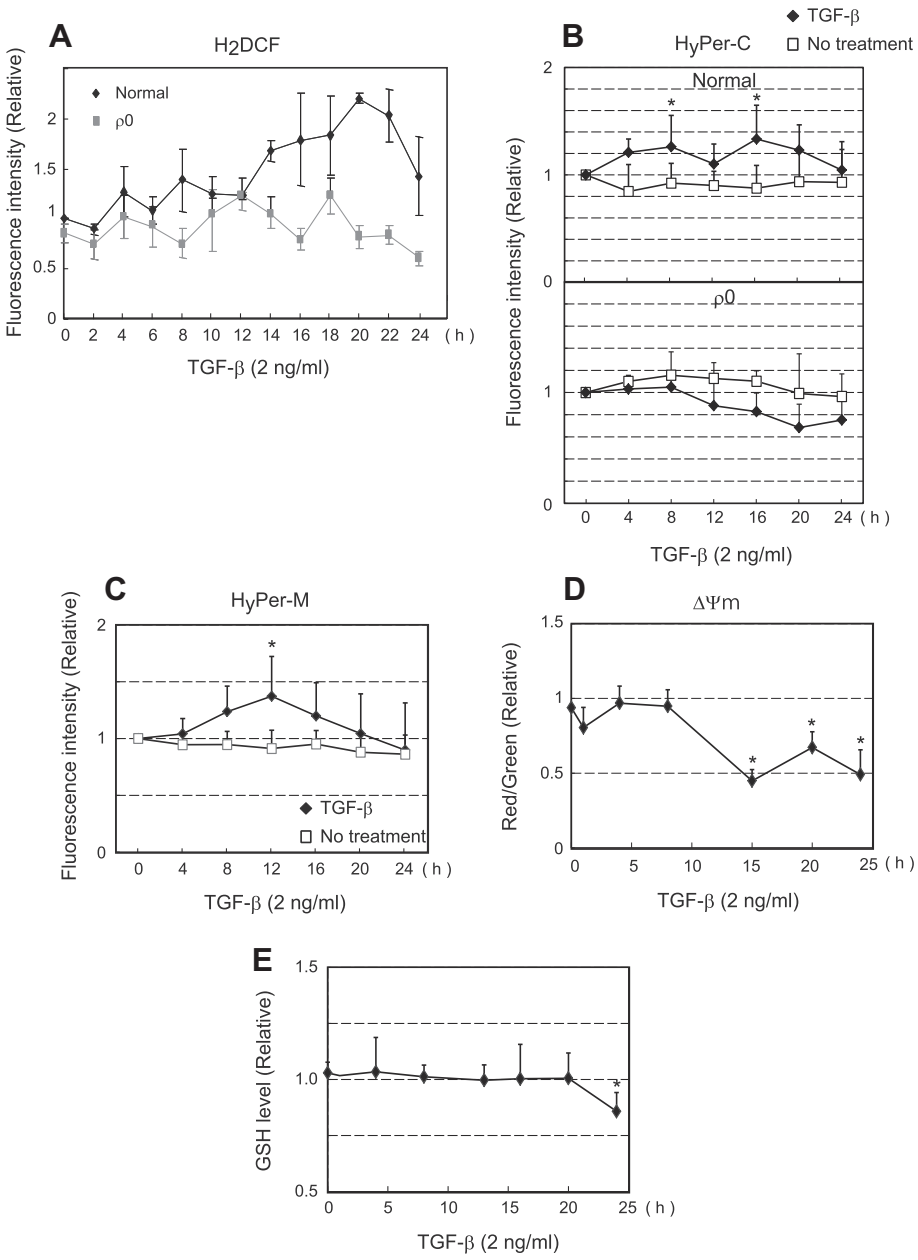

Fig. 1. Change in intracellular redox levels in NMuMG cells treated with TGF-β. (A) Normal and pseudo ρ0 cells were treated with TGF-β1 (2 ng/ml), and ROS levels were measured after the indicated periods with H_2DCF. At each time point, the images were captured in five fields, and the intensities of DCF from which the background was subtracted were averaged and normalized to those of an untreated sample. (B) HyPer-C-expressing normal and pseudo ρ0 cells were treated as in (A), and ROS levels in individual cells were determined by measuring the fluorescence intensity. After subtracting the background, the intensities were processed as in (A) at indicated periods. The experiments were repeated more than three times in which >5 cells were monitored. (C) HyPer-M-expressing normal cells were treated, and ROS levels were assessed as in (B). (D) Cells treated with TGF-β1 for the indicated periods as above were loaded with Mito-ID that fluoresces green as a monomer in the cytoplasm or orange as it aggregates in mitochondria in energized cells, respectively. Δψm was evaluated as a ratio of orange to green fluorescence. The measurement was performed in triplicate, and the ratios were averaged and normalized to those for the untreated sample. (E) In cells treated with TGF-β1 as above, the glutathione levels were determined using the GSH-Glo Glutathione Assay Kit, and the values were averaged and normalized to those for the untreated sample. Data are means ± SD values from at least three independent experiments. Error bars indicate SD. *means $p < 0.05$.

a single peak at approximately 12 h. The temporal discrepancy in the increase in ROS levels between the cytoplasm and mitochondria is seemingly ascribed to several causes, and this discrepancy is most probably caused by the involvement of multiple ROS-generating systems, including the electron transport chain (ETC), localized in either of the compartments with interdependent regulation, as described previously [20]. In mitochondria, concomitant with the increase in mitochondrial ROS levels, mitochondrial membrane potential (Δψm) sharply declined (Fig. 1D). In addition, the activity of complex I in ETC was decreased by TGF-β treatment for 4 h (Supplementary Fig. S1C). Thus, it is likely that under the conditions, ETC was initially affected to enhance ROS production in mitochondria, accompanying Δψm loss, which culminated in the eventual increase in ROS levels within cells.

Finally, a sustained increase in ROS levels was naturally assumed to shift cellular redox to an oxidative state. Indeed, the intracellular GSH levels, an index of the cellular redox state, decreased approximately 24 h after TGF-β treatment (Fig. 1E). Collec-

tively, we concluded that TGF-β-stimulated cells showed an increase in intracellular ROS levels over 20 h possibly through a mitochondrial-directed intricate interplay of ROS-generating machineries, consequently causing a shift in the cellular redox environment to the oxidative state.

3.2. Effects of changes in cellular redox on TGF-β-mediated gene expression in mammary epithelial cells undergoing EMT

NMuMG cells have been well characterized to respond to TGF-β and acquire a mesenchymal phenotype through EMT, which is accompanied by a distinctive set of gene expression [2,3]. In a further study, we investigated whether the above change in intracellular redox affected TGF-β-mediated gene expression in association with EMT. With chemicals modifying cellular redox and/or ROS signaling, we first found that the expression levels of fibronectin (FN) were sensitive to the chemicals. As shown in Fig. 2, FN expression but not that of plasminogen activator inhibitor (PAI)-1 and integrin α5 (Inta5) was blocked by an inhibitor of NFκB signaling, pyrrolidine dithiocarbamate (PDTC), and a superoxide anion scavenger, Tiron (Fig. 2). A sulfhydryl donor, *N*-acetyl cysteine (NAC), had no such effect. This may be explained by the recent finding that NAC treatment promoted oxidation in the mitochondria in spite of an increase in total intracellular pools for GSH [22]. Thus, it is possible that upregulation of a subset of genes by TGF-β is sensitive to the intracellular redox state during EMT.

Given the critical role of mitochondria in the intracellular redox change, we further studied the impact of the redox conditions in mitochondria on gene expression. Mitochondria-localizing superoxide dismutase MnSOD (Sod2) and mitochondrial thioredoxin TXN2 were expressed, and their effects on gene expression were evaluated. The effects of CuZnSOD (Sod1) and catalase expression were also observed for a comparison. The results indicated that while MnSOD overexpression, similar to CuZnSOD and catalase overexpression, had no overt effects (Supplementary Fig. S2A and B), TXN2 inhibited FN expression (Fig. 3A). PAI-1 expression was unaffected by TXN2, consistent with its insensitivity to the chemical inhibitors (Fig. 2). The effect of TXN2 was confirmed at a protein level (Fig. 3B), and moreover, recapitulated in human mammary epithelial cells (HMECs) (Supplementary Fig. S2C). It should be noted that the Smad pathway operated normally in the TXN2-expressing cells; phosphorylation of Smad3 (P-Smad3) occurred, and its transcriptional activities were upregulated by TGF-β treatment, as monitored using SBE/Luc [18] (Fig. 3C and D). The pathway upregulating AP1 transcriptional activity also appeared normal under the conditions (Fig. 3D).

In conclusion, these results reveal that the TGF-βsignaling pathway responsible for upregulating FN is sensitive to a redox shift or thiol oxidation in mitochondria.

3.3. TGF-β-mediated expression of a master regulator of EMT, HMGA2, is regulated by a TXN2-sensitive mechanism

HMGA2 is a non-histone nuclear protein with DNA binding ability that participates in a wide variety of gene transcription [23,24]. Of note, HMGA2 transactivates mesenchymal markers including FN as well as Snail-related zinc-finger factors (Snail and Slug), well-known transcriptional repressors of E-cadherin [25], and thus holds a key in EMT promotion. Given the sensitivity of FN expression to TXN2 and the role of HMGA2 in TGF-β-mediated FN induction [25], HMGA2 expression may be sensitive to TXN2. This was the case, as shown in Fig. 4A and B; TGF-β-mediated HMGA2 induction at both the mRNA and protein levels was significantly impinged by TXN2 expression. Consistently, the induction of downstream targets of HMGA2, Snail and Slug, was also repressed under these conditions (Fig. 4C), while that of Zeb1 and 2, which belong to the other zinc-finger transcription factors, remained unaffected. The other target of HMGA2, Twist, was not expressed at an appreciable level in this cell line. Taken together, a novel mechanism of TGF-β signaling has been uncovered in which mitochondria-based change in cellular redox regulates EMT-related gene expression typified by HMGA2 induction.

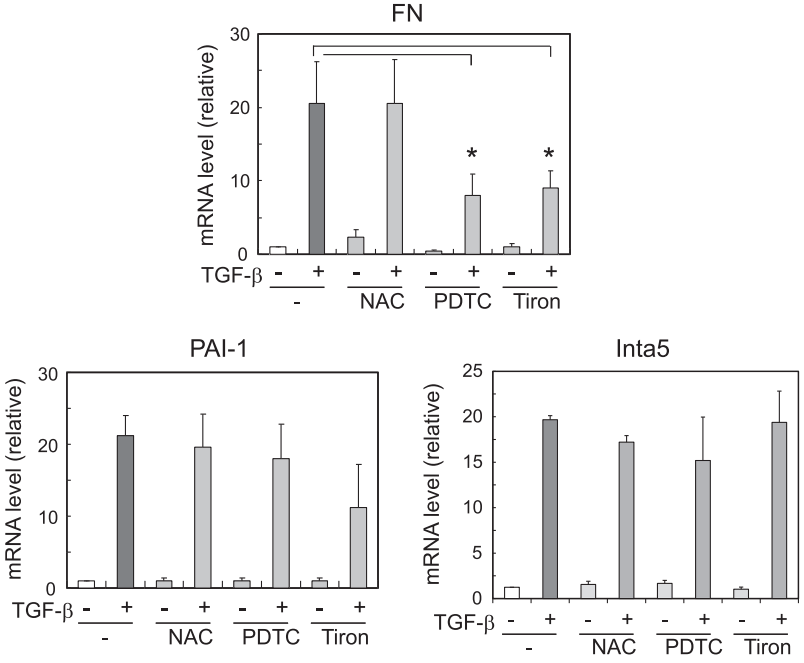

Fig. 2. Effect of pharmacological intervention with cellular redox signaling on TGF-β-mediated gene expression. NMuMG cells were treated with TGF-β1 (2 ng/ml) for 24 h with or without 10 mM NAC, 0.1 mM PDTC or 1 μM Tiron, and total RNA was extracted. mRNA levels were analyzed by real-time RT-PCR using primers as indicated. The ratio to the untreated sample is shown. Data are means ± SD values from at least three independent experiments. Error bars indicate SD. *means $p < 0.05$.

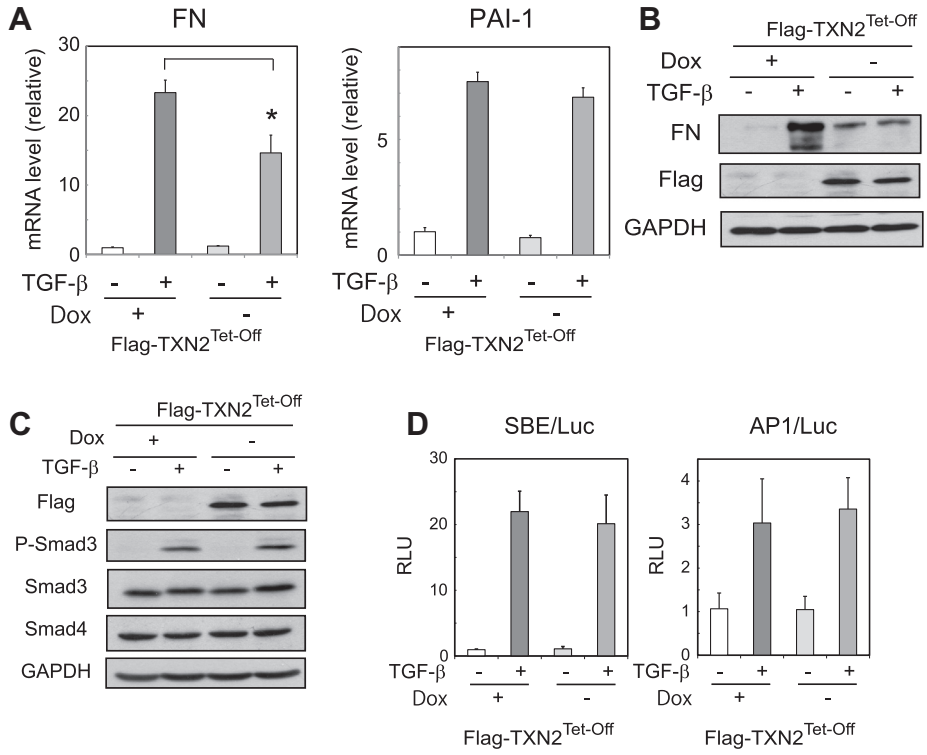

Fig. 3. Effect of TXN2 expression on EMT-related gene expression induced by TGF-β. (A) NMuMG cells infected with Flag-tagged TXN2 (Flag-TXN2)-encoding Tet-Off lentivirus were treated with TGF-β1 (2 ng/ml) for 24 h in the presence or absence of 10 ng/ml Dox, and specific mRNA levels were analyzed by real-time RT-PCR using primers indicated as in Fig. 2. (B and C) Cells as in (A) were treated with TGF-β1 for 48 h (B) or 2 h (C) in the presence or absence of 10 ng/ml Dox, and the lysates were subjected to immunoblotting with specific antibodies as indicated. GAPDH used as a loading control. (D) Cells as above were transiently transfected with SBE/Luc or AP-1/Luc together with phRL and treated with TGF-β1 in the presence or absence of 10 ng/ml Dox. After 24 h, the cells were lysed and assayed by the Dual luciferase assay. *Firefly* luciferase activities were normalized to *Renilla* luciferase activities, and the ratio to untreated cells in the presence of Dox is shown. Data are means ± SD values from at least three independent experiments. Error bars indicate SD. *means $p < 0.05$.

4. Discussion

EMT is initiated by loss of cell–cell adhesions in epithelial cells, which is caused by the suppression of junctional components, such as E-cadherin and ZO-1, followed by loss of polarity with concomitant rearrangement in the cytoskeletal network and acquisition of mesenchymal properties, such as production of matrix proteins including FN and collagen as well as metalloproteases [26]. TGF-β is involved in these processes with the predominant role of reprogramming nuclear transcriptional activities by regulating the expression and activity of several transcription factors, including Snail/Slug, ZEB1/2, and Twist proteins, the expression of which are all interconnected [2]. This group of regulators represses E-cadherin expression and induces mesenchymal genes, being central to the EMT processes [27]. HMGA2 is critically involved in these nuclear events during EMT induced by TGF-β. Concretely, HMGA2 is transactivated by Smads and in turn upregulates Snail and Twist in cooperation with Smads, leading to upregulation of other members of the regulators and the consequent nuclear reprogramming [2].

In the present study, we employed pharmacological and genetic strategies complementary to each other for evaluating the intracellular ROS levels and observed the long-lasting increase in the levels and eventual redox shift in mammary epithelial cells undergoing EMT by TGF-βtreatment (Fig. 1) Importantly, our results strongly suggest that the increased ROS levels and/or redox shift within cells are exploited by TGF-β to induce HMGA2 (Fig. 4). In other words, the cellular redox state has emerged as a modifier of the nuclear reprogramming in TGF-β-mediated EMT. A considerable number of reports have already suggested that TGF-β engages ROS signaling to regulate gene expression [6]. However, the focus of these previous studies was largely on the pro-fibrotic aspects of TGF-βand on the type of gene expression in fibroblastic cells in which ROS production is generally transient; the role of mitochondria in the production process is unclear in most cases except one [28]. It is likely that the spatial–temporal differences in ROS production have distinct effects on redox environments in cells, which modifies particular gene expression and ultimately molds cellular phenotypes in a distinctive manner.

In spite of a tremendous amount of literature implicating ROS signals in various biological circumstances, the evidence is not often fully convincing. For example, the involvement of ROS has often been deduced from the effects of low-molecular-weight chemicals, the prospective target of which is not absolutely specific. We approached the issue using redox-modifying enzymes including TXN2 that were exogenously expressed in cells. TXN2 and its cytoplasmic counterpart TXN1 are thioredoxins acting as antioxidants in a particular manner that facilitates the reduction of a disulfide bond on proteins by cysteine thiol–disulfide exchange [29]. The inhibitory effects of TXN2 on HMGA2 induction (Fig. 4) revealed that disulfide formation between cysteine residues on a particular protein localized in mitochondria is likely required for HMGA2 induction. The molecular details of the target will be addressed in a future study.

HMGA proteins are overexpressed in various cancers, which represents a constant feature of human malignancies [24]. Consistently, the experimental data so far strongly supports the critical role of HMGA in cancer cell proliferation and transformed phenotypes [30,31]. Especially, recent global gene profiling analyses revealed that HMGA2-mediated tumorigenesis is associated with

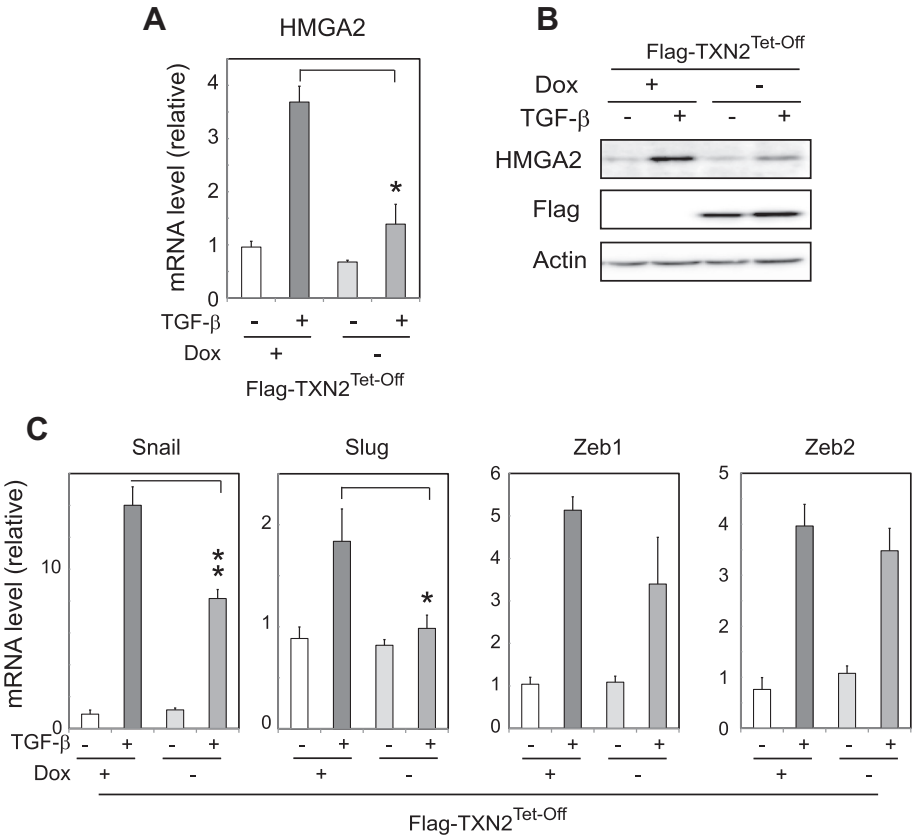

Fig. 4. Effect of TXN2 expression on HMGA2 and downstream gene expression induced by TGF-β. (A and B) Tet-Off TXN2-expressing NMuMG cells were treated as in Fig. 3, and mRNA and protein levels of HMGA2 were analyzed by real-time RT-PCR (A) and immunoblotting (B), respectively. (C) The cells were treated as above, and mRNA levels of EMT regulators were analyzed by real-time RT-PCR using specific primers as in Fig. 2. Data are means ± SD values from at least three independent experiments. Error bars indicate SD. *means $p < 0.05$.

expression changes in target genes and microRNAs that are involved in EMT [32]. Thus, HMGA expression is believed to be an excellent target for cancer treatment, in particular, for the prevention of progression and metastasis by interrupting the EMT processes. The present study highlights the importance of the redox-sensitive pathway and its unidentified target in this direction.

Acknowledgments

We thank Dr. H. Miyoshi for his continuous support with the viral expression systems. We also thank Dr. H. Tahara and Dr. J.-M. Gauthier for their generous gifts of the hTERT expression vector and SBE/Luc vector, respectively. We also thank Mr. Y. Ohmachi for contributing to this study as part of his bachelor degree.

This work was supported by a Grant-in-Aid for Young Scientist (B) (23790378), in part by a Grant-in-Aid for Scientific Research (C) (21570205), and also by a Private University High Technology Research Center Project matching fund subsidy from MEXT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.050.

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