Biochemical and Biophysical Research Communications 391 (2010) 1021–1027

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Decreased expression of glutaredoxin 1 is required for transforming growth factor-β1-mediated epithelial–mesenchymal transition of EpRas mammary epithelial cells

Eun Kyung Lee [a,1], Woo-Kwang Jeon [b,1], Min Young Chae [a,1], Hye-Young Hong [b], Youn Sook Lee [a], Jun Hwan Kim [a], Jae Young Kwon [a], Byung-Chul Kim [b,*], Seok Hee Park [a,*]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon, Republic of Korea

ARTICLE INFO

Article history:
Received 25 November 2009
Available online 5 December 2009

Keywords:
TGF-β1
Grx1
EMT
ROS

ABSTRACT

Transforming growth factor-β (TGF-β) is a cytokine important in inducing epithelial–mesenchymal transition (EMT), a crucial morphological event in a wide range of physiological and pathological cellular processes. In this study, we demonstrate that TGF-β1 induces the EMT phenotype through decreasing the expression of the glutaredoxin 1 (Grx1) gene, an anti-oxidant enzyme, in H-Ras transformed EpH4 mammary epithelial cells (EpRas), but not in the parental EpH4 cells. TGF-β1-induced reduction of Grx1 expression caused an increase of intracellular reactive oxygen species (ROS) in EpRas cells, and pre-treatment of the ROS scavenger *N*-acetylcysteine (NAC) inhibited TGF-β1-induced EMT. Grx1-overexpressing EpRas cells showed a reduction in intracellular ROS generation and suppressed the expression of mesenchymal markers upon treatment of TGF-β1. In addition, MEK/MAP kinase and phosphatidylinositol-3 kinase (PI3K) signaling were found to mediate the decrease in Grx1 expression upon TGF-β1 treatment, depending on the presence of Ras protein. Thus our findings strongly suggest that TGF-β1 promotes EMT by increasing intracellular ROS levels via down-regulation of the Grx1 gene in EpRas cells.

Introduction

TGF-β1 is a potent cytokine that regulates a wide range of cellular functions, including tissue morphogenesis, differentiation, and extracellular matrix remodeling [1,2]. Upon binding of TGF-β1 to its cognate receptors (type II and type I) with intrinsic serine–threonine kinase activities, TGF-β1 performs its effects mainly through the Smad-dependent canonical pathway [3,4]. Alternatively, TGF-β1 can activate a variety of other intracellular signaling pathways independent of the Smad proteins [3].

Among the biological functions exerted by TGF-β1, much attention has been paid to a distinct role of TGF-β1 as an inducer of epithelial–mesenchymal transition (EMT) in a number of tissues [5]. EMT, an important morphological event where epithelial cells convert to mesenchymal cells, has been recognized as a crucial process during embryonic development and tissue organization [6–8] and has been reported to be involved in cancer progression, giving rise to metastatic carcinomas [6,9,10].

Accumulating data indicate that TGF-β1-induced EMT in a variety of cells is likely to be dependent on Smad-dependent or Smad-independent signaling. For example, experiments using either Smad3 knock-out mice or overexpression of a TGF-β receptor type I mutant defective in Smad activation demonstrated a requirement for Smad signaling in mediating EMT [11–13]. Although the evidence implicating Smad-independent pathways is less substantial than Smad-dependent pathway, the Ras, RhoA, MAPK, PI3 kinase, Notch, and Wnt signaling pathways have been implicated in TGF-β1-induced EMT [5,14–19]. However, extensive cross-talks between Smad-dependent and -independent pathways make it difficult to define which pathway is predominant in TGF-β1-induced EMT.

Glutaredoxins (Grxs) are ubiquitous oxidoreductases belonging to the thioredoxin (Trx) superfamily [20]. Grx proteins constitute intracellular defense systems against oxidative stress, by mediating reversible electron transfer between reduced/oxidized glutathione (GSH/GSSG) and target proteins [20–23]. Thus Grx proteins are responsible for maintaining a constant cellular redox state by detoxifying reactive oxygen species (ROSs) and regulating redox-dependent signaling pathways [20,23,24]. In particular, the Grx1 gene, encoding the cytosolic dithiol glutaredoxin, is known to be induced by several cytokines including IL-4, IL-6, and IL-13 [25,26]. In contrast, Grx1 expression was reported to be decreased by TGF-β1, although the molecular mechanism and biological

* Corresponding authors. Fax: +82 33 242 0459 (B.-C. Kim), +82 31 290 7015 (S.H. Park).
E-mail addresses: bckim@kangwon.ac.kr (B.-C. Kim), parks@skku.edu (S.H. Park).
[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.009

meaning are yet unknown [25,27]. Based on various reports, Grx1 has been speculated to play a distinct role in inflammation, apoptosis, and tumor progression by inducing changes in the cellular redox system [20,28].

Although there are a considerable number of independent reports about TGF-β1, Grx1, and the EMT phenotype, little is known about the distinct role of Grx1 during TGF-β1-induced EMT in tumorigenesis. In this study, we demonstrate a novel mechanism underlying TGF-β1-induced EMT of H-Ras transformed EpH4 mammary epithelial cells (EpRas cells) via increasing intracellular ROS levels.

Materials and methods

Cell culture and establishment of stable cell lines expressing the Grx1 gene. EpH4 and EpRas mouse mammary epithelial cells were kindly provided by the Anita B. Roberts (National Cancer Institute, USA). Cells were maintained in DMEM with 10% FBS (Gibco–BRL). Production of the recombinant retrovirus encoding the Grx1 gene and generation of the EpRas-Grx1 stable cell line was performed as previously described [29]. EpRas cells stably expressing the Grx1 gene with an HA epitope were initially screened for resistance to puromycin, and verified through immunoblot analysis against the hemagglutin (HA) epitope.

Plasmids. To construct a recombinant retroviral plasmid expressing the Grx1 gene, the open reading frame of Grx1 tagged with HA epitope was amplified by specific primers (Forward primer, 5′-GAAA GATCTGCCGCCATGGATTATAAAGATGATGATGATAAATGAGCTCAAG AGTTTGTGAACTGC-3′; Reverse primer, 5′-CCGGAATTCTTACTGCAG AGCTCCAATCTG-3′), and cloned into the pMSCV-puro retroviral vector (Clontech), resulting in pMSCV-Grx1. Human Grx1 promoter was cloned into the pGL3-basic vector as previously described [30], generating pGL3-Grx1. PCR-generated sequences were verified by DNA sequencing.

Reagents, antibodies, and immunoblot analysis. Recombinant TGF-β1 was purchased from R&D systems. *N*-Acetylcysteine (NAC), reduced glutathione (GSH), and buthionine sulfoximine (BSO) were purchased from Sigma. The inhibitors LY294002, PD98059, and SB203580, were obtained from Calbiochem. Antibodies against the HA epitope, ZO-1, Vimentin, Grx1, Grx2, and α-smooth muscle actin (α-SMA) were purchased from Santa Cruz Biotechnology. Antibodies against E-cadherin and β-actin were obtained from BD Sciences and Sigma, respectively. Immunoblot analysis was done essentially as described [31].

Glutaredoxin assay. Cells were rinsed with ice-cold PBS and harvested in lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 5 mM NaF, 1 mM Na_3VO_4, plus 1:1000 protein inhibitor cocktail (Sigma)) and clarified by centrifugation. Protein concentration was measured by the Bradford assay (Bio-Rad). Grx enzymatic activity was determined with the standard 2-hydroxyethyl disulfide (HED) assay as previously described [32].

Statistical analysis. All data are shown as mean values ± SD and are representative of the results of three or more independent experiments. Statistical analysis was performed using the Student's *t*-test for paired data. Differences were considered significant at $P < 0.05$.

Details of materials and methods used in this study are provided in Supplementary materials and methods. Measurement of intracellular ROS, immunofluorescence, RT-PCR, and quantitative real-time PCR were performed using standard methods.

Results

TGF-β1-induced EMT in EpRas mammary epithelial cells is dependent on the high intracellular ROS levels

Polarized H-Ras-transformed mammary epithelial cells (EpRas) were previously established to analyze the role of Ras downstream signaling in epithelial cell plasticity and tumorigenesis [14,33]. In this system, it is known that TGF-β1 can induce the EMT phenotype in EpRas cells, but not in the parental EpH4 cells. However, little is known about the effect of ROS in EpRas cells during TGF-β1-induced EMT. To address the distinct role of ROS in this cellular event, we initially examined intracellular ROS levels in EpRas and the parental EpH4 cells. Flow cytometer analysis using the fluorophore 2,7-dichlorohihydrofluorescein diacetate (H_2DCFDA) showed that the intracellular ROS levels were higher in EpRas cells than in EpH4 cells (Fig. 1A). These results prompted us to hypothesize that TGF-β1-induced EMT in EpRas cells may be dependent on intracellular ROS levels. To confirm this possible role of ROS in EpRas cells, the expression of epithelial and mesenchymal markers were examined in the presence or absence of the ROS scavenger *N*-acetylcysteine (NAC). In the absence of NAC, TGF-β1 treatment caused decreased expression of the epithelial markers E-cadherin and ZO-1, and increased expression of the mesenchymal markers α-SMA and Vimentin (Fig. 1B). These results were consistent with typical EMT phenomena in EpRas cells. However, the changes in expression of epithelial or mesenchymal markers upon TGF-β1 treatment were inhibited upon treatment of NAC. The changes in expression of these markers were also confirmed by an immunofluorescence assay (Fig. 1C and Supplementary Fig. 1).

TGF-β1 treatment causes a decrease in Grx1 expression in EpRas cells

In order to explore the molecular reason(s) behind the difference of intracellular ROS levels in EpRas and EpH4 cells, we examined the activity or expression of proteins involved in defense mechanisms against ROS. The glutaredoxin 1 (Grx1) protein, an anti-oxidant enzyme, was found to have significantly decreased basal activity and expression in EpRas cells, compared to EpH4 cells, in contrast to glutaredoxin 2 (Grx2), which did not show a difference (Fig. 2A, B and Supplementary Fig. 2). The basal reduction of Grx1 protein levels and activity in EpRas cells compared to EpH4 cells may be due, to a certain degree, to overall lower transcription levels in EpRas cells (Fig. 2D). When EpRas cells were subjected to TGF-β1 treatment, a definite decrease in Grx1 mRNA and protein levels was observed (Fig. 2C–E). In contrast to Grx1, expression of the thioredoxin 1 (Trx1) gene, encoding another anti-oxidant enzyme, did not change upon TGF-β1treatment (Fig. 2C).

Stable expression of the Grx1 gene in EpRas cells blocks TGF-β1-induced EMT

From the results above, we hypothesized that the reduction of Grx1 expression induced by TGF-β1 in EpRas cells leads to significant augmentation of intracellular ROS levels, possibly to the threshold required for induction of the EMT phenotype. In order to verify our hypothesis, retroviruses expressing the Grx1 gene tagged with the HA epitope were infected into EpRas cells, and a stable cell line expressing the Grx1 gene, EpRas-Grx1, was isolated. Morphologically, these stable cells did not show the EMT phenomenon upon treatment of TGF-β1, compared with control cells expressing the empty vector (EpRas-pMSCV) (Fig. 3A). Moreover, following treatment of TGF-β1, immunoblot analysis of EpRas-Grx1 cells did not show the reduction of epithelial markers and augmentation of mesenchymal markers seen in control cells, indicating that EpRas-Grx1 cells are resistant to TGF-β1-induced EMT (Fig. 3B). These results were further confirmed by immunostaining for epithelial and mesenchymal markers in both EpRas-Grx1 and EpRas-pMSCV cells following TGF-β1 treatment (Fig. 3C and Supplementary Fig. 3A).

Next, we questioned whether overexpression of the Grx1 gene reduces the intracellular ROS level in EpRas-Grx1 cells. Flow cytometer analysis revealed that Grx1 expression significantly decreases the ROS level in EpRas-Grx1 cells, compared with EpRas-pMSCV cells

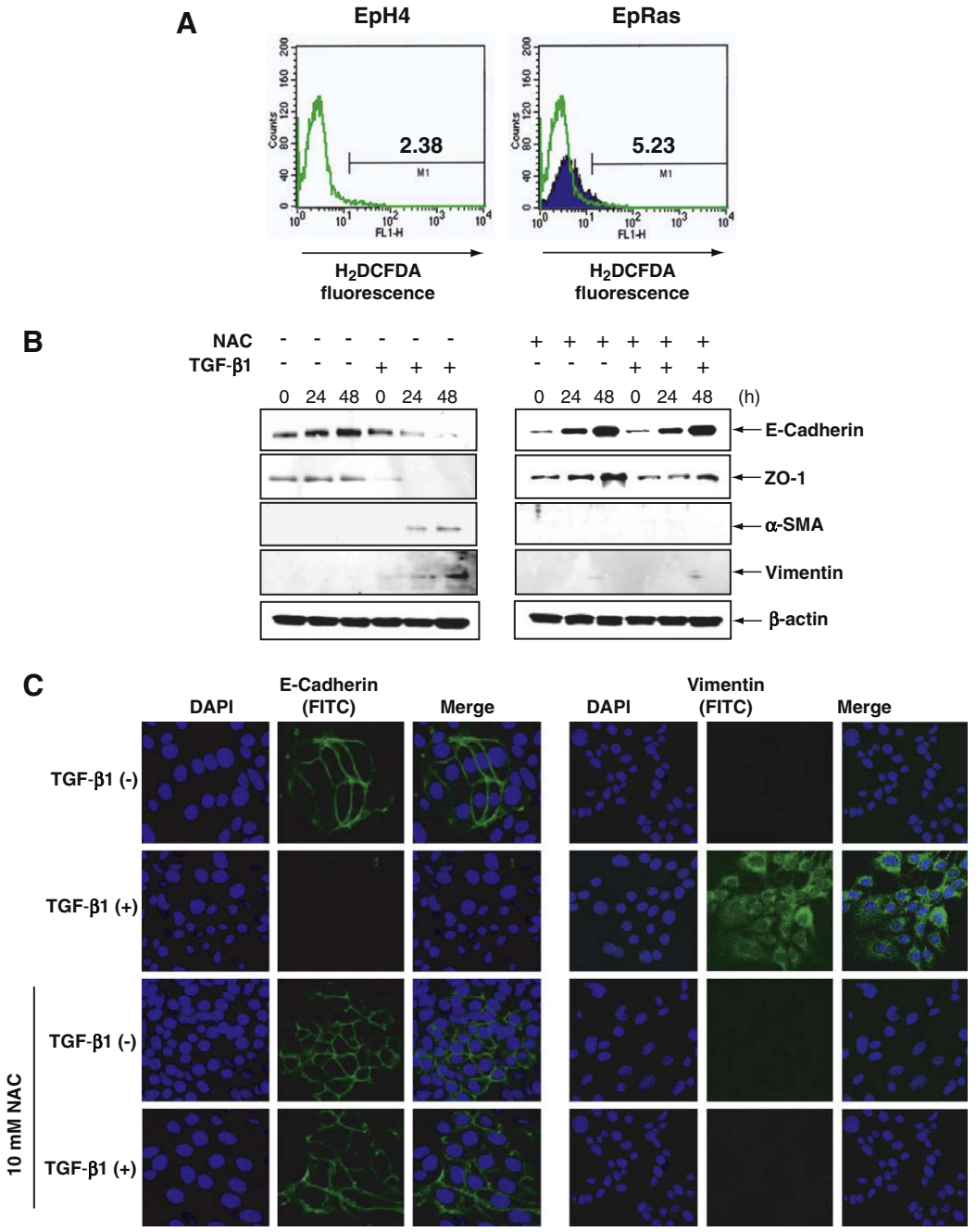

Fig. 1. TGF-β1-induced EMT of EpRas cells depends on the high intracellular ROS levels. (A) Intracellular ROS levels were higher in EpRas cells than in EpH4 cells. Both cell lines were pre-treated with the H_2O_2-sensitive fluorescence dye H_2DCFHDA for 20 min and the fluorescence was quantified by flow cytometry. (B) NAC treatment inhibits changes in expression of epithelial and mesenchymal markers during TGF-β1-induced EMT in EpRas cells. In the right panel, EpH4 and EpRas cells were pre-incubated with 10 mM NAC for 30 min and then treated with 5 ng/ml TGF-β1 for the indicated time periods. Expressions of epithelial and mesenchymal markers in both cells were monitored by immunoblot analysis. Expression of β-actin protein was used as a loading control. (C) The results seen in (B) were verified by immunofluorescence analysis. In the lower panels, EpH4 and EpRas cells were pre-incubated with 10 mM NAC for 30 min and then treated with TGF-β1 for 48 h. Expression of the E-cadherin and Vimentin proteins were observed by immunofluorescence. Nuclei are stained with DAPI. Original magnification, 400×. All data are representative of at least three independent experiments.

(Fig. 3D). In addition, the anti-oxidant activity of the Grx1 protein was greatly increased to a level similar to the EpH4 cells (Fig. 3E). These results indicate that TGF-β1-induced EMT of EpRas cells is mediated by the augmentation of intracellular ROS levels caused by decrease in Grx1 gene expression. These findings were also supported by experiments adding either L-buthionine-(*S*,*R*)-sulfoxamine (BSO) or reduced glutathione (GSH), as an ROS producer or ROS reducer, respectively (Supplementary Fig. 3B). BSO treatment of EpRas cells further increased expression of the mesenchymal marker, α-SMA, and GSH addition dramatically decreased α-SMA expression. Thus our present results demonstrate that the decreased expression of the Grx1 gene by TGF-β1 treatment affects the intracellular ROS level and induces EMT in EpRas mammary epithelial cells.

TGF-β1-induced decrease in Grx1 expression requires the activation of MEK/MAPK and PI3K signaling

The activation of MEK/MAPK signaling and PI3K signaling are required for TGF-β-induced EMT and the protection of TGF-β-induced apoptosis, respectively, in EpRas cells [14]. Thus it is possible

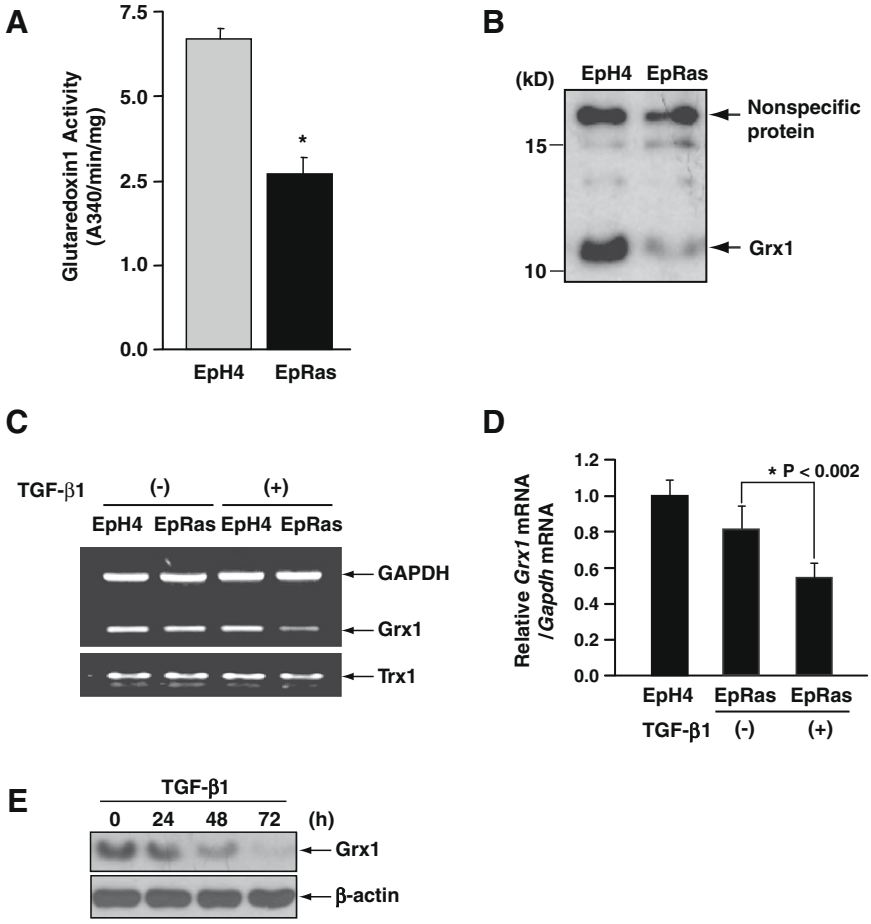

Fig. 2. TGF-β1 decreases expression of the Grx1 gene in EpRas cells, but not in EpH4 cells. (A) Endogenous glutaredoxin 1 activity is higher in EpRas cells than in EpH4 cells. (B) Immunoblot analysis of endogenous Grx1 protein in EpH4 and EpRas cells. Expression of non-specific protein was used as a loading control. (C) Grx1 mRNA levels were reduced in EpRas cells following TGF-β1 treatment. Grx1 and Trx1 mRNA levels were monitored by RT-PCR analysis after EpH4 and EpRas cells were treated with 5 ng/ml TGF-β1 for 48 h. Expression of the GAPDH gene was used as a loading control. (D) Relative levels of Grx1 mRNA in EpRas cells further decreased upon TGF-β1 treatment. Grx1 mRNA levels in EpRas cells were analyzed by quantitative real-time RT-PCR in the absence or presence of TGF-β1. TGF-β1 treatment was performed at 5 ng/ml for 48 h. As a control, Grx1 mRNA levels in EpH4 were also measured by real-time RT-PCR. GAPDH expression was used for normalization. The results are shown as mean value ± SD of three independent experiments (*$P < 0.002$). (E) Decrease in endogenous Grx1 protein levels upon increase of TGF-β1 treatment time in EpRas cells. EpRas cells were treated with 5 ng/ml TGF-β1 for the indicated time periods, and the expression of endogenous Grx1 protein was observed by immunoblot analysis. All data in this figure are representative results of at least three independent experiments.

that the TGF-β1-induced decrease of Grx1 expression leading to EMT in EpRas cells is mediated by MEK/MAPK signaling. To verify this possibility, we investigated expression of the Grx1 gene upon treatment with specific inhibitors to selectively suppress MEK/MAPK, PI3K, or p38 MAPK signaling pathways in EpRas cells. As shown in Fig. 4A, treatment of the MEK/MAPK inhibitor PD98059, or the PI3K/Akt inhibitor LY294002, blocked the reduction of Grx1 mRNA levels upon TGF-β1 treatment in EpRas cells. These results indicate that the decrease in expression of the Grx1 gene induced by TGF-β1 is mediated by the MEK/MAPK and PI3K signaling pathways. To further confirm the involvement of MEK/MAPK and PI3K signaling, a full-length Grx1 promoter–luciferase reporter and an oncogenic H-Ras expression plasmid were co-transfected into EpH4 cells in the presence of either PD98059 or LY294002. The activity of the Grx1 promoter was repressed by TGF-β1 only together with oncogenic H-Ras protein (Fig. 4B). However, this decreased activity of the Grx1 promoter was inhibited by either PD98059 or LY294002 (Fig. 4B).

Discussion

Epithelial–mesenchymal transition (EMT) is a crucial morphological event contributing to cancer progression in pathophysiological conditions [6,9,10]. It is generally recognized that TGF-β plays an important role in the progression of EMT. Here we describe a distinct mechanism mediating TGF-β1-mediated EMT in H-Ras transformed mouse mammary epithelial cells (EpRas). EpRas and their parental EpH4 cells are a useful model system to study the molecular mechanism causing EMT. Using EpRas cells, it was initially reported that the cooperative actions of TGF-β1 and oncogenic H-Ras promote the EMT phenotype as well as the protection of TGF-β1-induced apoptosis [14].

In this study, we provide evidence that an increased level of ROS contributes to TGF-β1-induced EMT in EpRas cells. Furthermore, we show that the increased ROS levels upon treatment of TGF-β1 are due to the decreased levels of Grx1 protein, which has anti-oxidant activity. This is the first report showing that down-regulation of the Grx1 gene can induce the EMT phenotype in EpRas cells. It is notable that the reduction of basal Grx1 protein in EpRas cells compared to EpH4 cells was to an extent that could not be fully explained by differences in Grx1 mRNA levels. These results imply the possibility that the decreased basal activity of Grx1 protein in EpRas cells may, to some degree, be due to the degradation of Grx1 protein at the post-transcriptional level. More interestingly, the further reduction of Grx1 mRNA levels by TGF-β1 was dependent on the presence of oncogenic Ras. That is, decreased expres-

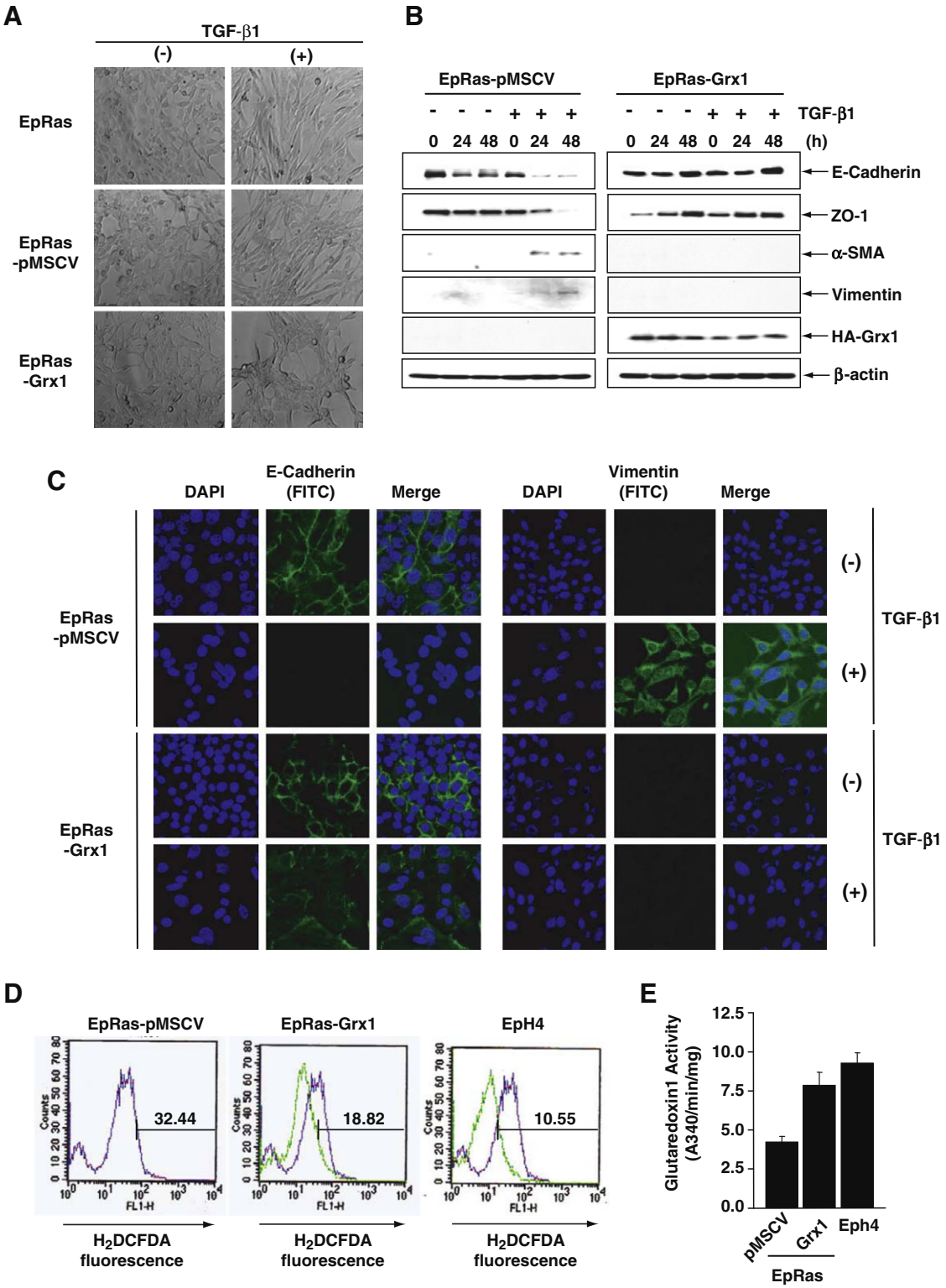

Fig. 3. Overexpression of the Grx1 gene inhibits TGF-β1-induced EMT of EpRas cells via decreasing intracellular ROS. (A) EpRas cells stably expressing the Grx1 gene (EpRas-Grx1) did not exhibit the morphological changes seen in EpRas cells stably expressing the empty vector (EpRas-pMSCV) or in parental EpRas cells following TGF-β1 treatment. TGF-β1 treatment was performed at 5 ng/ml for 48 h. (B) Immunoblot analysis of EpRas-Grx1 cells did not show the reduction of epithelial markers and augmentation of mesenchymal markers seen in control EpRas-pMSCV cells after TGF-β1 treatment. Cells were treated with 5 ng/ml TGF-β1 for the indicated time period. Expression of the β-actin protein was used as a loading control. (C) The results seen in (B) were verified by immunofluorescence analysis. EpRas-pMSCV and EpRas-Grx1 cells were treated with 5 ng/ml TGF-β1 for 48 h. Expressions of E-cadherin and Vimentin proteins were observed by immunofluorescence. Nuclei are stained with DAPI. Original magnification, 400×. (D) Intracellular ROS levels in EpRas-pMSCV, EpRas-Grx1 and EpH4 cells. All cells were pre-treated with the H_2O_2-sensitive fluorescence dye H_2DCFHDA for 20 min and the fluorescence was quantified by flow cytometry. (E) Glutaredoxin 1 activities of EpRas-pMSCV, EpRas-Grx1and EpH4 cells. All data in this figure are representative results of at least three independent experiments.

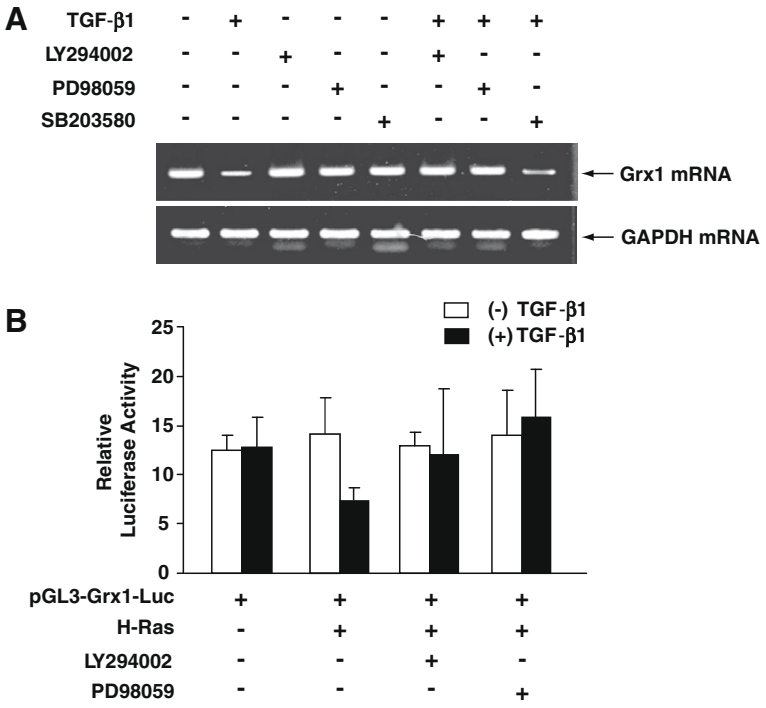

Fig. 4. The decrease in Grx1 expression upon TGF-β1 treatment is mediated through MEK/MAPK and PI3K signaling in EpRas cells. (A) Expression of Grx1 mRNA was monitored by RT-PCR analysis. EpRas cells were pre-treated with 10 μM LY294002, 10 μM PD98059, and 10 μM SB203580 for 2 h, respectively, and then treated with 5 ng/ml TGF-β1 for 48 h. Expression of GAPDH mRNA was used as a loading control. The data are representative of the results of three independent experiments. (B) pGL3-Grx1-Luc reporter plasmid, which contains the full-length Grx1 promoter, was transiently transfected into EpH4 cells together with an oncogenic H-Ras expression plasmid. After transfection, cells were pre-treated with 10 μM LY294002 and 10 μM PD98059 and then treated with 5 ng/ml TGF-β1 for 24 h. Luciferase activities were normalized on the basis of TK-*Renilla* luciferase expression to adjust for variation in transfection efficiency. The results shown are the mean value ± SD of three independent experiments.

sion of Grx1 mRNA levels by TGF-β1 was not detected in EpH4 cells, indicating that TGF-β1-induced expression of the Grx1 gene is regulated by a Ras-dependent signaling pathway. Our findings that decreased Grx1 expression is mediated by MEK/MAPK and PI3K signaling support this speculation.

Although numerous data indicate that intracellular ROS levels are involved in tumorigenesis, the direct link between intracellular ROS and EMT has not been well characterized. Several recent studies indicated a relationship between ROS and EMT. In particular, Felton et al. showed that inhibition of intracellular ROS generation during TGF-β1 treatment blocked aveolor EMT [34]. In addition, Zhang et al. showed that the increased labile iron pool by TGF-β1 treatment promotes the production of ROS and subsequently induced EMT [35]. These findings are similar with our present results. The novel finding in our study is the characterization of the mechanism by which intracellular ROS levels are increased in TGF-β1-induced EMT of EpRas cells. Increased ROS generation upon TGF-β1 treatment was specifically linked to Grx1 protein expression levels, but not to thioredoxin (Trx) protein expression. Therefore, our results extend our knowledge about the molecular mechanism of EMT mediated by increased ROS levels. However, we do not exclude the possibility that other factors could also contribute to the augmentation of intracellular ROS levels.

In addition, our findings that the reduced expression of Grx1 mRNA is mediated by MEK/MAPK and PI3K signaling in EpRas cells suggests that the increased ROS levels are involved in the protection of TGF-β1-induced apoptosis as well as EMT. Previously, it was reported that MAPK is required for EMT, whereas PI3K signaling protects from TGF-β-induced apoptosis and causes cell scattering in EpRas cells [14]. Based on these data, the decreased expression of Grx1 might be upstream of TGF-β1-induced EMT or protection of cell death in EpRas cells.

In conclusion, we propose that an increased level of ROS, due to the decrease of Grx1 expression by TGF-β1, is a crucial event causing EMT in EpRas mammary epithelial cells. Furthermore, we demonstrate that the reduced expression of Grx1 is dependent on the presence of oncogenic H-Ras protein and mediated through MEK/MAPK and PI3K signaling. Therefore, modulations of the intracellular ROS level or Grx1 expression may be important therapeutic strategies for the treatment of EMT-related human diseases such as cancer.

Acknowledgments

We thank Dr. Min Sung Choi for critical reading of the manuscript. This work was supported by a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (00000490 to S.H.P.) and by a Korea Science Engineering Foundation (KOSEF) Grant (ROA-2007-000-20047-0 to S.H.P.) funded by the Korean Government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.009.

References

[1] G.C. Blobe, W.P. Schiemann, H.F. Lodish, Role of transforming growth factor beta in human disease, N. Engl. J. Med. 342 (2000) 1350–1358.
[2] S. Dennler, M.J. Goumans, P. ten Dijke, Transforming growth factor beta signal transduction, J. Leukoc. Biol. 71 (2002) 731–740.
[3] R. Derynck, Y.E. Zhang, Smad-dependent and Smad-independent pathways in TGF-beta family signalling, Nature 425 (2003) 577–584.
[4] C.H. Heldin, K. Miyazono, P. ten Dijke, TGF-beta signalling from cell membrane to nucleus through SMAD proteins, Nature 390 (1997) 465–471.
[5] A. Nawshad, D. Lagamba, A. Polad, E.D. Hay, Transforming growth factor-beta signaling during epithelial–mesenchymal transformation: implications for embryogenesis and tumor metastasis, Cells Tissues Organs 179 (2005) 11–23.

[6] A. Moustakas, C.H. Heldin, Signaling networks guiding epithelial–mesenchymal transitions during embryogenesis and cancer progression, Cancer Sci. 98 (2007) 1512–1520.
[7] D. Shook, R. Keller, Mechanisms, mechanics and function of epithelial–mesenchymal transitions in early development, Mech. Dev. 120 (2003) 1351–1383.
[8] M. Iwano, D. Plieth, T.M. Danoff, C. Xue, H. Okada, E.G. Neilson, Evidence that fibroblasts derive from epithelium during tissue fibrosis, J. Clin. Invest. 110 (2002) 341–350.
[9] S. Grunert, M. Jechlinger, H. Beug, Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis, Nat. Rev. Mol. Cell. Biol. 4 (2003) 657–665.
[10] J.P. Thiery, Epithelial–mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (2002) 442–454.
[11] S. Saika, S. Kono-Saika, Y. Ohnishi, M. Sato, Y. Muragaki, A. Ooshima, K.C. Flanders, J. Yoo, M. Anzano, C.Y. Liu, W.W. Kao, A.B. Roberts, Smad3 signaling is required for epithelial–mesenchymal transition of lens epithelium after injury, Am. J. Pathol. 164 (2004) 651–663.
[12] S. Itoh, M. Thorikay, M. Kowanetz, A. Moustakas, F. Itoh, C.H. Heldin, P. ten Dijke, Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses, J. Biol. Chem. 278 (2003) 3751–3761.
[13] E. Piek, A. Moustakas, A. Kurisaki, C.H. Heldin, P. ten Dijke, TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells, J. Cell Sci. 112 (1999) 4557–4568.
[14] E. Janda, K. Lehmann, I. Killisch, M. Jechlinger, M. Herzig, J. Downward, H. Beug, S. Grunert, Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways, J. Cell Biol. 156 (2002) 299–313.
[15] M. Davies, M. Robinson, E. Smith, S. Huntley, S. Prime, I. Paterson, Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways, J. Cell. Biochem. 95 (2005) 918–931.
[16] N.A. Bhowmick, M. Ghiassi, A. Bakin, M. Aakre, C.A. Lundquist, M.E. Engel, C.L. Arteaga, H.L. Moses, Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism, Mol. Biol. Cell 12 (2001) 27–36.
[17] A.V. Bakin, A.K. Tomlinson, N.A. Bhowmick, H.L. Moses, C.L. Arteaga, Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration, J. Biol. Chem. 275 (2000) 36803–36810.
[18] J. Zavadil, L. Cermak, N. Soto-Nieves, E.P. Bottinger, Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition, EMBO J. 23 (2004) 1155–1165.
[19] K. Kim, Z. Lu, E.D. Hay, Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT, Cell Biol. Int. 26 (2002) 463–476.
[20] C.H. Lillig, C. Berndt, A. Holmgren, Glutaredoxin systems, Biochim. Biophys. Acta 1780 (2008) 1304–1317.
[21] M.D. Shelton, P.B. Chock, J.J. Mieyal, Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation, Antioxid. Redox Signal. 7 (2005) 348–366.
[22] P. Ghezzi, Regulation of protein function by glutathionylation, Free Radic. Res. 39 (2005) 573–580.
[23] M.M. Gallogly, J.J. Mieyal, Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress, Curr. Opin. Pharmacol. 7 (2007) 381–391.
[24] J.J. Song, J.G. Rhee, M. Suntharalingam, S.A. Walsh, D.R. Spitz, Y.J. Lee, Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H_2O_2, J. Biol. Chem. 277 (2002) 46566–46575.
[25] N.L. Reynaert, E.F. Wouters, Y.M. Janssen-Heininger, Modulation of glutaredoxin-1 expression in a mouse model of allergic airway disease, Am. J. Respir. Cell. Mol. Biol. 36 (2007) 147–151.
[26] Y. Takashima, K. Hirota, H. Nakamura, T. Nakamura, K. Akiyama, F.S. Cheng, M. Maeda, J. Yodoi, Differential expression of glutaredoxin and thioredoxin during monocytic differentiation, Immunol. Lett. 68 (1999) 397–401.
[27] M. Peltoniemi, R. Kaarteenaho-Wiik, M. Saily, R. Sormunen, P. Paakko, A. Holmgren, Y. Soini, V.L. Kinnula, Expression of glutaredoxin is highly cell specific in human lung and is decreased by transforming growth factor-beta in vitro and in interstitial lung diseases in vivo, Hum. Pathol. 35 (2004) 1000–1007.
[28] M.D. Shelton, J.J. Mieyal, Regulation by reversible S-glutathionylation: molecular targets implicated in inflammatory diseases, Mol. Cells 25 (2008) 332–346.
[29] B.C. Kim, M. Mamura, K.S. Choi, B. Calabretta, S.J. Kim, Transforming growth factor beta 1 induces apoptosis through cleavage of BAD in a Smad3-dependent mechanism in FaO hepatoma cells, Mol. Cell Biol. 22 (2002) 1369–1378.
[30] Y. Urata, Y. Ihara, H. Murata, S. Goto, T. Koji, J. Yodoi, S. Inoue, T. Kondo, 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells, J. Biol. Chem. 281 (2006) 13092–13102.
[31] K.C. Choi, Y.S. Lee, S. Lim, H.K. Choi, C.H. Lee, E.K. Lee, S. Hong, I.H. Kim, S.J. Kim, S.H. Park, Smad6 negatively regulates interleukin 1-receptor-Toll-like receptor signaling through direct interaction with the adaptor Pellino-1, Nat. Immunol. 7 (2006) 1057–1065.
[32] H.J. Kang, S.M. Hong, B.C. Kim, E.H. Park, K. Ahn, C.J. Lim, Effects of heterologous expression of thioredoxin reductase on the level of reactive oxygen species in COS-7 cells, Mol. Cells 22 (2006) 113–118.
[33] M. Oft, J. Peli, C. Rudaz, H. Schwarz, H. Beug, E. Reichmann, TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells, Genes Dev. 10 (1996) 2462–2477.
[34] V.M. Felton, Z. Borok, B.C. Willis, *N*-Acetylcysteine inhibits alveolar epithelial–mesenchymal transition, Am. J. Physiol. Lung Cell. Mol. Physiol. 297 (2009) L805–L812.
[35] K.H. Zhang, H.Y. Tian, X. Gao, W.W. Lei, Y. Hu, D.M. Wang, X.C. Pan, M.L. Yu, G.J. Xu, F.K. Zhao, J.G. Song, Ferritin heavy chain-mediated iron homeostasis and subsequent increased reactive oxygen species production are essential for epithelial–mesenchymal transition, Cancer Res. 69 (2009) 5340–5348.