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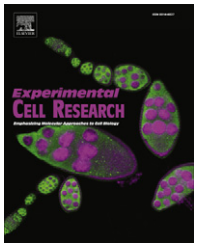

Research Article

Reactive oxygen species stimulates epithelial mesenchymal transition in normal human epidermal keratinocytes via TGF-beta secretion

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ABSTRACT

Epithelial to mesenchymal transition (EMT) plays an important role in tumor progression, and is an early step in carcinogenesis. Although reactive oxygen species (ROS) are known to be implicated in EMT in many tumor cell types, its exact role in EMT initiation in normal human cells, especially epidermal keratinocytes (NHEKs), remains unknown. To clarify whether ROS induce EMT in NHEKs, and to establish how ROS regulate EMT, we examined the effect of hydrogen peroxide (H_2O_2) on the expression of molecules involved in EMT and cell morphology in NHEKs. H_2O_2 altered the expression of EMT biomarkers, including downregulation of epithelial cadherin and upregulation of α-smooth muscle actin, through a transcriptional modulator, Snail1. H_2O_2 also induced epithelial to fibroblast-like morphological changes, together with upregulation of EMT biomarkers, and promoted phosphorylation of ERK1/2 and JNK in a time-dependent manner. Interestingly, H_2O_2 stimulated the expression and secretion of TGF-β1 in NHEKs. Exogenous TGF-β1 also induced the expression of EMT biomarkers. In contrast, neutralizing antibody anti-TGF-β1 antibody or inhibitor of TGF-β receptor type I suppressed the expression of EMT biomarkers. Our results suggest that ROS stimulated TGF-β1 secretion and MAPK activation, resulting in EMT initiation in NHEKs.

Introduction

Reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2) can cause severe damage to DNA, protein, and lipids. Increased levels of ROS produced during normal cellular metabolism such as mitochondrial electron transport, and from environmental stimuli such as cytokines, UV radiation, perturb the normal ROS balance and shift cells into a state of oxidative stress [1]. Oxidative stress is believed to contribute to the etiology of various degenerative diseases such as diabetes, atherosclerosis, arthritis, cancer, and the process of aging. There have been several reports indicating that elevated ROS has been found in many types of tumor cells, and contributes to carcinogenesis and metastasis [2–5]. Metastasis is a complicated pathological process consisting of several stages. Initially, the primary cancer cells lose cell-to-cell contact, exhibit epithelial to mesenchymal transition (EMT), and promote their capability to migrate into the surrounding tissue. Subsequently, extracellular matrixes around the primary tumor areas are remodeled by proteinases such as matrix metalloproteinases, facilitating the tumor cell to intravasate into the circulation.

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EMT, originally identified as a crucial differentiation and/or morphogenetic process during embryogenesis, is now recognized as a process that contributes to wound healing, tissue remodeling, fibrosis, and metastatic malignancies. In particular, several reports have suggested that the process of EMT may be crucial for tumor progression, invasion and metastasis [6–8]. EMT is a transdifferentiation process by which epithelial cells lose their epithelial characteristics and acquire a mesenchymal phenotype, and is characterized by changes in cell morphology, disruption of tight junctions and adherent junctions, and decreased expression of epithelial biomarkers, such as epithelial cadherin (E-cadherin), zonula occludens-1 and other molecules. Furthermore, EMT is associated with increased expression of mesenchymal biomarkers, such as fibronectin, alpha-smooth muscle actin (α-SMA) and vimentin. This mesenchymal phenotype correlates with the capacity of cells to migrate to distant organs and, therefore, with the initiation of metastasis [9]. A growing number of transcriptional molecules have been found to be involved in the EMT process [10]. Among them, ectopic expression of Snail has been reported to suppress E-cadherin expression, leading to a full EMT phenotype, whereas silencing of Snail expression reverses this process [11]. Snail expression has been detected in a number of different human carcinoma and melanoma cell lines. More importantly, it is expressed at the invasive front of epidermoid carcinomas and has been associated with carcinoma metastasis.

Although ROS have been recognized as an inducer of EMT via Snail expression in various tumor cells, it remains unknown whether ROS can induce EMT and how it exactly regulates EMT in normal human cells, especially epidermal keratinocytes (NHEKs).

EMT is triggered by the interplay of extracellular signals and many kinds of secreted soluble growth factors, such as transforming growth factor-β (TGF-β), epithelial growth factor, fibroblast growth factor, hepatocyte growth factor and platelet-derived growth factor. TGF-β regulates cell proliferation, differentiation, migration, extracellular matrix production, apoptosis and tumorigenesis [12]. It is also a potent inducer of EMT, and it has long been recognized that through EMT induction, TGF-β can promote tumor metastasis and invasion [7,13]. In contrast, blockage of TGF-β signaling can decrease tumor cell motility and metastasis [14]. On the other hand, TGF-β is not sufficient for EMT in human mammary epithelial cells and instead requires collaboration and/or enhancement of the other signaling molecules for complete EMT [15]. Therefore, the exact role of ROS in TGF-β-associated EMT is unclear in NHEKs. The aim of present study was to clarify whether ROS contributes to TGF-β-associated EMT in NHEKs. We have investigated the effect of H_2O_2 on the expression of EMT biomarkers and cell morphology in NHEKs.

Materials and methods

Reagents

Recombinant human TGF-β1 was purchased from Peprotec Co., Ltd. (Minneapolis, MN, USA). Anti-phospho- ERK1/2, -JNK and −p38 antibodies were purchased from Cell Signaling Tec. (Delaware, CA, USA). Anti-E-cadherin and α-SMA antibodies were purchased from Dako Co., Ltd. (Tokyo, Japan) and BD Bioscience Co., Ltd. (Tokyo, Japan), respectively. All other antibodies were purchased from R&D Co. (Minneapolis, MN, USA). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

NHEKss were purchased from Lonza Co., Ltd. (cat no.00192627, Tokyo, Japan) and cultured in KGM2 medium (Lonza Co., Ltd.). The experiments were only used the 2–5 passage cells, which keep the characteristics of normal human keratinocytes, but not fibroblastic and/or mesenchymal cells. The cells were incubated in culture medium with or without 100–800 μM H_2O_2 for 0–72 h. In some experiments, cells were incubated in culture medium supplemented with 1–100 ng/ml TGF-β1.

RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse-transcriptase, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). To detect mRNA expression, we selected specific primers based on the nucleotide sequence of cDNA. The cDNA was amplified by PCR under the following conditions: 1 min denaturation at 95 °C, 1 min annealing at 58 °C, and 1 min extension at 72 °C, for 32 cycles. PCR products were subjected to electrophoresis on 2% agarose gels and visualized after staining with ethidium bromide. The signal intensity of each PCR product was quantitatively measured with Scion image software (version 4.02, NIH, Bethesda, MD) and normalized to that of GAPDH mRNA. The sequences of the primers used were as follows: human E-cadherin, 5′-CGACAAAGGACAGCCTATTT-3′ (forward), 5-TCTCCATTGGATCCTAAACT-3′ (reverse); human α-SMA, 5′-GCCATGTATGTGGCTATTCA-3′ (forward), 5′-TGACAGGACGTTGTTAGCAT-3′ (reverse); human Snail1, 5′-GCCTGGGTGCCCTCAAGAT-3′ (forward), 5′-TTGTGGAGCAGGGACATTCG-3′ (reverse); human GAPDH, 5′-CCTCTCCAGAACATCATTCC-3′ (forward), 5′-CCTCTCCAGAACATCATCC-3′ (reverse).

Western blot analysis

Cells were lysed in TNT buffer containing 20 mM tris–HCl, pH 7.5, 200 mM NaCl, 1% Triton-X, 1 mM dithiothreitol, and protease inhibitors (Roche, Basel, Switzerland). Protein content was measured with a protein assay kit (Pierce, Hercules, CA, USA), according to the manufacturer's protocol. Twenty micrograms of each protein was subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins were then electrophoretically transferred to PVDF membrane at 100 V for 1 h at 4 °C. The membrane was then incubated with primary targeted antibodies diluted in 5% skimmed milk solution plus 0.01% azide overnight at 4 °C. Blots were washed in TBST (10 mM tris–HCl, 50 mM NaCl, 0.25% Tween-20) for 30 min at room temperature, then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or mouse IgG secondary antibodies, which were diluted 1:2500 in 5% of non-fat dry milk-TBST, and developed using an enhanced chemiluminescence system (GE Healthcare, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The amount of human TGF-β1 secreted into culture medium during H_2O_2 treatment in NHEKs for 0–72 h was determined using ELISA kits (Bender MedSystems, Vienna, NJ, Austria).

Immunocytochemistry

Cells were cultured on Lab-Teck chamber slides (Falcon Co., Ltd.) and fixed with 4% paraformaldehyde-PBS 0 and 72 h after treatment with 500 μM of H_2O_2. After blocking of non-specific binding with rabbit serum, the slides were incubated with primary antibodies against E-cadherin (BD Bioscience Co., Ltd., Tokyo, Japan) and α-SMA (Dako Co., Ltd., Tokyo, Japan) overnight at 4 °C. After the slides were rinsed three times with PBS, the targeted antigens in the cells were detected with Alexa Fluor 488 or 568-conjugated anti-mouse IgG antibodies for 40 min at room temperature. Nuclear staining was performed with DAPI dye, and cells were imaged with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). The excitation beam was produced by 380/488/596-nm lasers and delivered to the specimens via a Zeiss Apochromat objective. Emitted fluorescence was captured using ZEN 2008 software (Carl Zeiss).

Statistical analysis

Data were expressed as mean ± standard error. Differences were analyzed with paired one-way ANOVA, where appropriate P values of <0.05 were considered to be significant.

Results

ROS upregulated EMT biomarkers

It is known that ROS induces EMT in many carcinoma cells, but the effect of ROS on normal epithelial cells, such as keratinocytes, is unclear. Recently, we have reported that treatment of H_2O_2 (800 μM) had effect on oxidation to DNA and produced 8-oxo-7, 8-dihydroguanine in NHEKs [16]. To clarify the effect of ROS on EMT in normal epithelia, we first examined the effect of H_2O_2 (800 μM) on the expression of EMT biomarkers in NHEKs using semi-quantitative RT-PCR methods and western blot analysis. H_2O_2 downregulated the expression of epithelial cell marker E-cadherin mRNA in NHEKs in a time-dependent manner (Fig. 1A and B). In contrast, H_2O_2 upregulated the expression of mesenchymal cell marker α-SMA mRNA together with Snail1 mRNA expression for EMT promotion. The changes in EMT biomarkers were observed 12 h after treatment with H_2O_2 and at concentrations higher than 300 μM (Supplemental Fig. 1A). Similar to the PCR results, H_2O_2 downregulated the expression of E-cadherin protein and upregulated α-SMA and Snail1 protein expression in NHEKs (Fig. 1C). Furthermore, H_2O_2 also altered the expression of EMT biomarkers, in the dose-dependent manner in squamous carcinoma cells (Supplemental Fig. 1B)

ROS slowly induced MAPK activation in a time-dependent manner

ROS elevation has been reported to enhance MAPK activity for malignant progression of mouse keratinocyte cell lines [17]. To

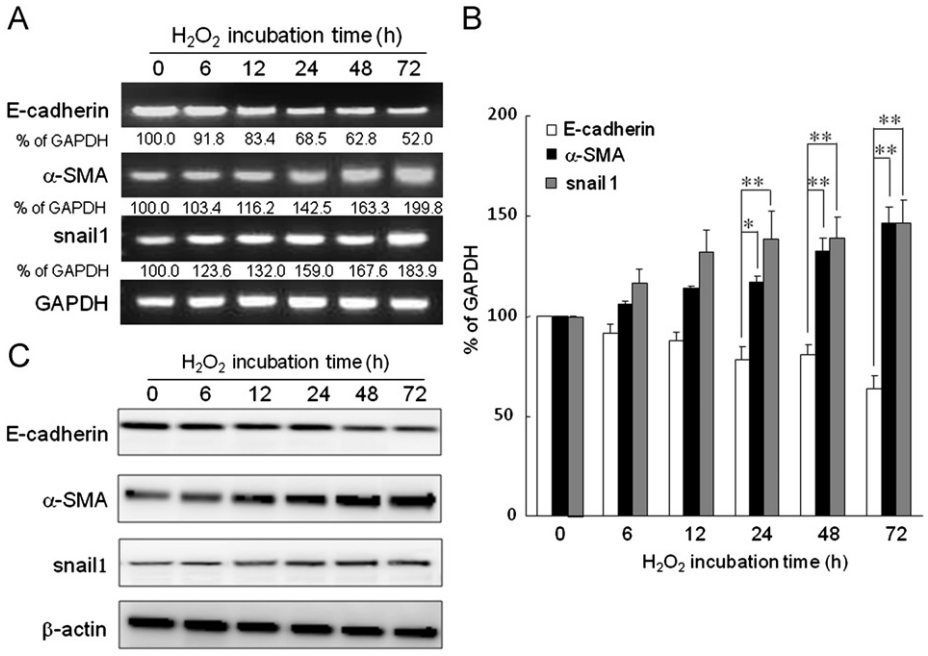

Fig. 1 – *ROS promoted changes in EMT biomarkers, such as loss of E-cadherin and gain of α-SMA expression in NHEKs*. (A) NHEKs were incubated with H_2O_2 (800 μM) for the indicated times. Expression of E-cadherin, α-SMA, Snail1 and GAPDH mRNA was analyzed by RT-PCR. Changes in EMT markers were observed 12 h after treatment with H_2O_2 at concentrations greater than 100 μM. Numbers below the gels represent the intensity of each targeted mRNA relative to GAPDH mRNA. (B) Expression levels of E-cadherin, α-SMA and Snail1 mRNA were analyzed semi-quantitatively by RT-PCR. Data shown are the mean from six culture wells (mean ± SEM). ∗ and ∗∗ indicate $P<0.05$ and $P<0.01$, respectively. (C) Total cell lysates were prepared and western blot analyses carried out using targeted and β-actin antibodies. NHEKs were incubated with H_2O_2 (800 μM) for the indicated culture times. Similar results were obtained in five independent experiments.

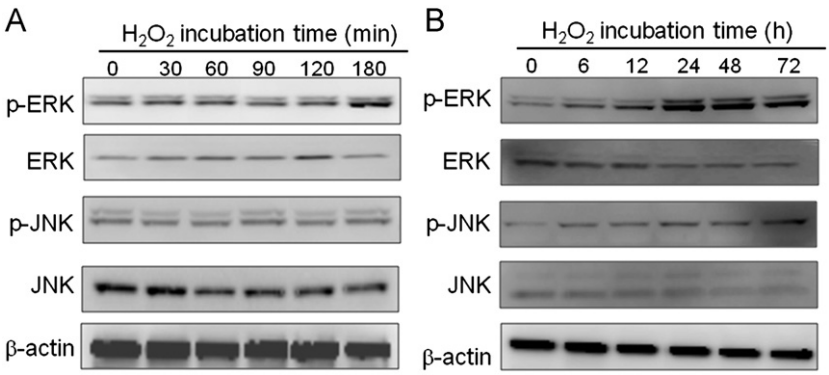

Fig. 2 – *ROS slowly induced MAPK activation in a time-dependent manner.* NHEKs were incubated with H_2O_2 (800 μM) over short-term (A) or long-term (B) incubation times. Total cell lysates were prepared and western blotting carried out using targeted and β-actin antibodies for the indicated culture times. Similar results were obtained in four independent experiments.

assess which downstream molecules of ROS were activated, we examined the effect of H_2O_2 on the expression and phosphorylation of MAPKs in NHEKs using western blot analysis; H_2O_2 had no effect until 180 min after treatment (Fig. 2A). However, long-term treatment with H_2O_2 significantly increased the phosphorylation of ERK1/2 and JNK together with a decrease in the expression of total ERK1/2 and JNK in a time-dependent manner 12 h after treatment (Fig. 2B). H_2O_2 had little effect on the phosphorylation and expression of p38 MAPK in NHEKs (data not shown). Furthermore, H_2O_2 had no effect on E-cadherin and α-SMA expression in the presence of PD98059 MAPK inhibitor (10 μM) (see supplemental Fig. 2A).

ROS induced the expression of TGF-β precursor and TGF-β1 secretion in NHEKs

There are many reports that TGF-β induced EMT in various tumor cells [18,19]. To investigate whether H_2O_2-induced EMT is associated with TGF-β in NHEKs, we examined that the effect of H_2O_2 on TGF-β1 expression and secretion using western blot analysis and ELISA. H_2O_2 increased the expression of TGF-β precursor in a time-dependent manner. Furthermore, H_2O_2 significantly stimulated the secretion of TGF-β1 until 72 h after stimulation (Fig. 3A). Since H_2O_2-stimulated TGF-β1 in an auto- and/or paracrine manner seemed to induce EMT in NHEKs, we examined the effect of blockage of secreted TGF-β1 on expression of EMT biomarkers. As a control, H_2O_2 also downregulated E-cadherin expression and upregulated α-SMA in the presence of non-immune mouse IgG (1 μg/ml). In contrast, the presence of neutralizing anti-TGF-β1 antibody (1 μg/ml) altered the expression of EMT biomarkers, such E-cadherin loss and α-SMA gain. Furthermore, treatment with SB43154 (10 μM), a TGF β receptor type I (TGFβRI) inhibitor, also suppressed EMT biomarker expression. These results suggest that H_2O_2 induced EMT in NHEKs through TGF-β1 auto- and/or paracrine stimulation.

TGF-β1 upregulated expression of EMT biomarkers in NHEKs

To confirm whether TGF-β1 induced EMT in an autocrine manner in H_2O_2-treated NHEKs, we examined the effect of TGF-β1 on EMT induction in NHEKs using semi-quantitative RT-PCR methods and western blot analysis. TGF-β1 downregulated the expression of E-cadherin mRNA in NHEKs in a time-dependent manner (Fig. 4A and B). In contrast, TGF-β1 upregulated the expression of α-SMA and Snail1 mRNA 12 h after treatment with H_2O_2. Similar to the PCR results, TGF-β1 downregulated E-cadherin protein expression and upregulated α-SMA and Snail1 protein expression in NHEKs (Fig. 4C).

ROS promoted mesenchymal- and/or fibroblast-like NHEKs morphology

Finally, we examined the effect of H_2O_2 on NHEKs morphology using an immunostaining method. After H_2O_2 treatment for 72 and 96 h, NHEKs changed from epithelial to mesenchymal- and/or fibroblast-like morphology (Fig. 5). These changes were accompanied with decreased E-cadherin staining and increased α-SMA staining following treatment with H_2O_2. In contrast, inhibition of TGFβRI signaling using a neutralizing antibody or a specific inhibitor blocked H_2O_2-induced morphological changes in NHEKs (data not shown).

Discussion

Although the involvement of ROS in EMT initiation has been reported in physiological conditions and pathological processes, its exact role in EMT processes remain unclear. In the present study, we demonstrated that: (1) ROS induced epithelial to mesenchymal- and/or fibroblast-like morphological changes in NHEKs together with upregulation in EMT biomarkers, such as decreased E-cadherin expression and increased α-SMA expression; (2) ROS induced MAPK activation in a time-dependent manner.; (3) ROS upregulated the expression of TGF-β precursors and TGF-β1 secretion in NHEKs; and (4) ROS induced the secretion of TGF-β1, which in turn activated MAPK, resulting in the initiation of EMT.

Recently, several reports have suggested that the process of EMT may be crucial for cancer progression [20–22]. In particular, during the progression to metastatic competence, carcinoma cells have been described to enter into the EMT program, allowing them to acquire features of mesenchymal-like cells that may be significantly related to cancer invasiveness, including changes in adhesive properties, activation of motility and the ability to degrade/remodel the extracellular matrix. Since EMT

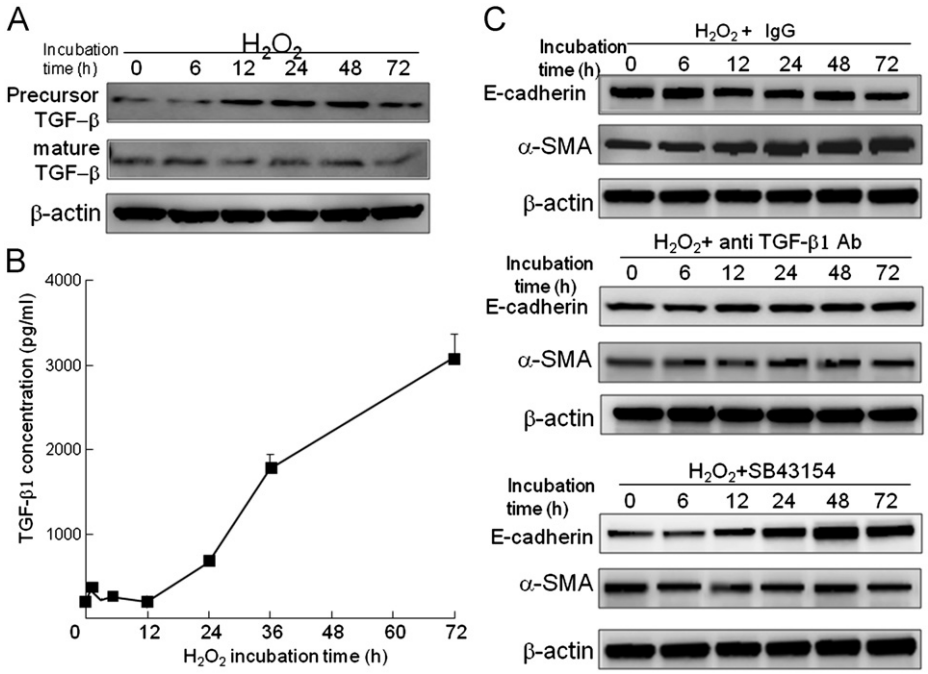

Fig. 3 – *ROS induced the expression of TGF-β1 precursor and TGF-β1 secretion in NHEKs.* **(A) NHEKs (2×10^4 cell/well) were incubated with H_2O_2 (800 μM) for 0, 3, 6, 12, 24, 36 and 72 h. Total cell lysates were prepared and western blotting carried out using antibodies against TGF-β1 and β-actin. TGF-β1 protein concentration in culture medium was determined by ELISA. Data represent the mean from six culture wells (mean ± SEM). (B) The effects of neutralizing TGF-β1 antibody and TGFβRI inhibitor on EMT biomarker expression. NHEKs (2×10^4 cell/well) were incubated with H_2O_2 (800 μM) in the presence of mouse IgG (1 μg/ml), neutralizing TGF-β1 antibody (1 μg/ml) or TGFβRI inhibitor, SB43154 (10 μM) for 0, 6, 12, 24, 48 and 72 h. Similar results were obtained in three independent experiments.**

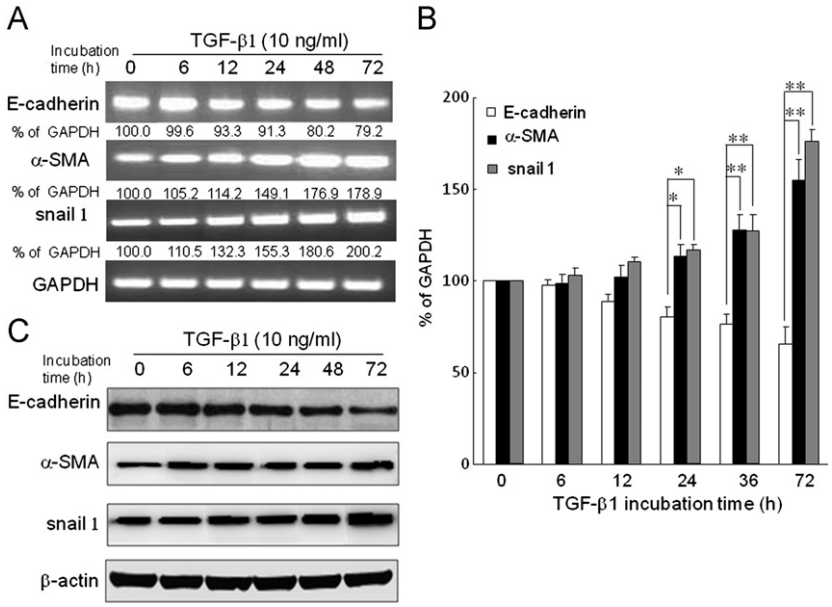

Fig. 4 – *TGF-β1 upregulated expression of EMT biomarkers in NHEKs.* **(A) NHEKs were incubated with TGF-β1 (10 ng/ml) for the indicated times in culture. Expression of E-cadherin, α-SMA, Snail1 and GAPDH mRNA were analyzed by RT-PCR. Changes in EMT markers were observed 12 h after treatment with TGF-β1 at concentrations higher than 30 nM. Numbers below the gels represent the intensity of each targeted mRNA relative to GAPDH mRNA. (B) Expression levels of E-cadherin, α-SMA and Snail1 mRNA were analyzed semi-quantitatively by RT-PCR. Data shown are the mean from six culture wells (mean ± SEM). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. (C) NHEKs were incubated with TGF-β1 (10 ng/ml) for the indicated culture times. Total cell lysates were prepared and western blot analysis carried out using targeted and β-actin antibodies. Similar results were obtained in five independent experiments.**

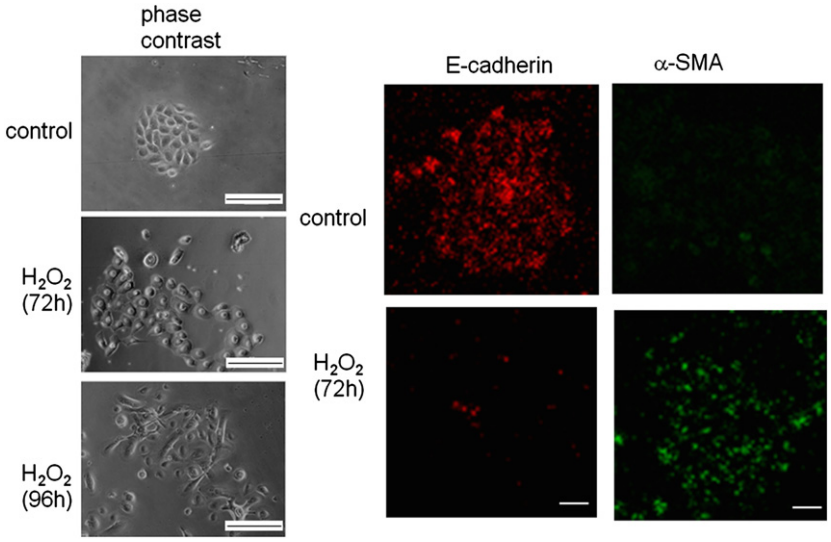

Fig. 5 – *ROS resulted in mesenchymal- and/or fibroblast-like NHEKs morphology.* NHEKs were cultured on glass slides and fixed after 72 and 96 h with or without H_2O_2 treatment. Slides were incubated with E-cadherin and α-SMA antibodies, and visualized with Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-mouse IgG. Left-hand panel indicates the phase contrast images with or without H_2O_2 treatment. Bars indicate 100 μm. Similar results were obtained in three independent experiments.

seems to represent part of the processes leading to tumor cell invasiveness and metastasis, the signaling pathways that regulate EMT during cancer progression have been well investigated [23–25]. Interestingly, it has recently been proposed that EMT may be a critical process of cancer stem cells in the context of tumor progression [26]. However, little is known as to how EMT is initiated and regulated during cancer progression.

Although there are many reports about tumor-associated EMT, it remains to be clarified what role EMT plays in normal cells. When squamous cell carcinoma (SCC), a malignant type of keratinocyte, was treated with H_2O_2, it showed an EMT phenotype the same as that observed in NHEKs (unpublished data). EMT of SCC is likely to be attributed to the features of normal keratinocytes. Repeated injury on skin or mucosa is known to be a risk factor for carcinogenesis; inflammatory reactions following injury could generate ROS, which may induce not only DNA mutations by oxidatively altered nucleotides but also EMT in keratinocytes. If mesenchymal-like keratinocytes after EMT are transformed by ROS-mediated DNA mutation, a poorly-differentiated type of SCC cell will occur. It is well known that poorly-differentiated SCC cells have more potential to invade and metastasize than well-differentiated SCC cells. Therefore, the present study of the molecular mechanisms of EMT in normal keratinocytes will also contribute to understanding invasion and metastasis of SCC.

ROS elevation has been reported to enhance MAPK activity in the malignant progression of keratinocyte cell lines [17]. MAPKs, including ERK, JNK and p38 [27,28], are known to be involved in tumor invasion and to be major signaling cascades for EMT and cell migration [29]. Recently, ROS-activated MAPK signaling was shown to be required for the migration of keratinocytes and smooth muscle cells [30–32]. Furthermore, TGF-β1-induced activation of MAPKs is reported to be sustained for a long period [33,34]. In the present experiments, ROS promoted continuously phosphorylated ERK1/2 and JNK for more than 72 h in NHEKs, while inhibition of MAPKs significantly suppressed ROS-induced expression of EMT biomarkers. Hence, MAPKs are thought to play an important role in EMT. However, it remains to be determined how MAPKs induce the expression of EMT biomarker molecules.

Recent studies have documented cross-talk between TGF-β and ROS signaling. Treatment with TGF-β1 promoted the production of ROS in renal tubular epithelial cells [35] and mouse hepatocytes [34], which in turn activated ERK1/2 and p38 MAPKs. The suppression of cellular ROS signaling with antioxidants can inhibit TGF-β1-induced EMT [36]. Inhibitors of NAPDH oxidase (diphenyleneiodonium and apocynin) also significantly inhibited TGF-β1-induced ROS. In the preliminary experiments, we found that TGF-β also production in NHEKs 72 h after stimulation (Supplemental Fig. 4). Furthermore, ROS have also been shown to be involved in matrix metalloproteinase 3-, and hypoxia-, and aldosterone-induced EMT [35,37,38]. Although these reports demonstrate that TGF-β1 induces ROS during the process of EMT, the present study shows for the first time that ROS induced TGF-β1 production and resulted in EMT in NHEKs. Inhibition of TGFβ1RI signaling suppressed ROS-induced expression of EMT biomarkers in NHEKs. These results suggest that ROS stimulates TGF-β1 expression, which activates MAPKs in an autocrine and/or paracrine manner leading to EMT. Therefore, once ROS has stimulated TGF-β1 expression, a vicious circle between ROS and TGF-β1 may promote EMT in NHEKs.

In conclusion, we demonstrate that ROS induces EMT in NHEKs through TGF-β1 secretion and MAPK activation such as ERK and JNK phosphorylation, and suggest that antioxidants and MAPK inhibitors may prevent EMT through both the TGF- β1and MAPK pathways and subsequent epidermal carcinogenesis.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2012.05.023.

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