

Hydrogen Peroxide Enhances TGF β -mediated Epithelial-to-Mesenchymal Transition in Human Mammary Epithelial MCF-10A Cells

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Abstract. *Aim: This study investigated the effect of reactive oxygen species (ROS) on transforming growth factor (TGF)- β -mediated epithelial-to-mesenchymal transition (EMT) in order to clarify the influence of ROS and TGF β on the induction of dysplasia and ultimately, tumorigenesis. Materials and Methods: Confluent MCF-10A human mammary epithelial cells were treated with H₂O₂ for 1 h, then reseeded at low density in the presence of TGF β and cultured until confluence. Results: Hydrogen peroxide (H₂O₂, 250 μ M) enhanced TGF β -mediated EMT, as evidenced by increased expression of EMT-associated transcription factors, which was accompanied by increased nuclear localization of phosphorylated SMAD family member 2 (SMAD2) and up-regulation of the TGF β signaling pathway components mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK). Pharmacological inhibition of MEK/ERK signaling partly reversed the effects of H₂O₂. Conclusion: H₂O₂ enhances TGF β -mediated EMT via SMAD and MEK/ERK signaling.*

Epithelial-to-mesenchymal transition (EMT) is involved in many biological processes, including embryogenesis, tissue fibrosis and cancer metastasis. EMT is characterized by loss of cell polarity and adhesion, resulting in changes in cell morphology and increased cell migration and size (1, 2).

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Activation of EMT-inducing transcription factors including SNAIL family transcriptional repressor 1 (SNAIL1), SNAIL2, and TWIST family bHLH transcription factor 1 (TWIST), among others, is thought to promote tumor initiation via negative regulation of the tumor suppressor p53 (3). Transgenic mice overexpressing resistance to audiogenic seizures (*Ras*) oncogene alone in mammary glands do not develop mammary carcinoma, while mice overexpressing *Twist1* in addition to *Ras* are susceptible to cancer (4). In addition, some EMT features, including loss of E-cadherin expression, are observed in some types of epithelial dysplasias (5). Expression of EMT-inducing transcription factors may also confer tumor cells with an advantage under conditions of stress by enabling oncogene-induced cell senescence and escape from apoptosis (6, 7).

Reactive oxygen species (ROS) are generated by normal cellular metabolism (mitochondrial electron transport chain) and in response to cytokines and environmental factors (ultraviolet or ionizing radiation); an increase in intracellular ROS production can lead to oxidative stress (8), which underlies degenerative diseases such as obesity, arteriosclerosis, rheumatoid arthritis, and aging. Increased ROS levels have been observed in many types of tumor cells, suggesting that they contribute to tumorigenesis and tumor metastasis (9, 10). Mesothelioma is caused by inhalation of asbestos fibers, which results in higher ROS levels and EMT induction (11, 12). These observations suggest that tumorigenesis is associated with the interaction of ROS and EMT.

Transforming growth factor (TGF)- β is a cytokine that plays an important role in cell growth inhibition, cell migration, tumor metastasis, extracellular matrix remodeling, and immunosuppression. The biological function of TGF β is context-dependent; for instance, it inhibits and promotes tumor cell growth at early and late stages of tumorigenesis, respectively (13). TGF β is activated by oxidative stress in various tissues, including mammary gland (14).

Table I. Primer pairs for quantitative reverse transcription-polymerase chain reaction.

Gene	Encoded protein		Primer ^a
<i>CDH1</i>	E-Cadherin	Forward	ATGGGCCCTTGGAGCCG
		Reverse	AAAATTCACCTGCCAGGACG
<i>SNAI1</i>	Zinc finger protein SNAI1	Forward	GGACCCACACTGGCGAGAAG
		Reverse	TGTGGAGCAGGGACATTCGG
<i>SNAI2</i>	Zinc finger protein SNAI2	Forward	TGTTGCAGTGAGGCAAGAA
		Reverse	GACCCTGGTTGCTTCAAGGA
<i>ZEB1</i>	Zinc finger E-box-binding homeobox 1	Forward	AACTGCTGGGAGGATGACA
		Reverse	TCCTGCTTCATCTGCCTGA
<i>ZEB2</i>	Zinc finger E-box-binding homeobox 2	Forward	TGGTCCAGAAGAAATGAAGGAAGAC
		Reverse	CTGCATGACCATCGCGTTCC
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	Forward	GAAGGTGAAGGTCGGAGTC
		Reverse	GAAGATGGTGATGGGATTC

^aBase sequences are written in the 5' to 3' direction.

We speculated that increased ROS and TGFβ cooperatively induce EMT to cause dysplasia and, ultimately, tumorigenesis. In order to test this hypothesis, the present study investigated the effect of hydrogen peroxide (H₂O₂), an ROS, on TGFβ-mediated EMT and molecular mechanisms underlying the event, as ionizing radiation has been shown to enhance TGFβ-mediated EMT in human mammary epithelial cells (15, 16).

Materials and Methods

Cell culture. Immortalized MCF-10A human mammary epithelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained as previously described (15). Confluent cells were treated with 50 or 250 μM H₂O₂ in culture medium for 1 h or with γ radiation (1 Gy; Gammacell, Best Theratronics, Ottawa, Canada) at a dose-rate of 0.9 Gy/min. The cells were then detached with a mixture of 0.05% trypsin and cell dissociation solution (Sigma-Aldrich, St. Louis, MO, USA) and counted, then sparsely reseeded with 400 pg/ml human recombinant TGFβ1 (R&D Systems, Minneapolis, MN, USA), except for immunofluorescence analysis (100 pg/ml). Cells were cultured until confluence (about 6 days); the medium containing TGFβ1 was changed every 48 h. To evaluate the effect of inhibitor on expression of E-cadherin, cells were treated with 10 μM of the MEK inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA).

Immunofluorescence analysis. Immunocytochemistry was carried out as previously described (15). Briefly, cells were grown on a 96-well plate (ibidi GmbH, Martinsried, Germany), and then fixed with ice-cold methanol. The cells were then labeled with antibodies against E-cadherin (610181; BD Biosciences, San Jose, CA, USA), N-cadherin (610921; BD Biosciences), phospho-(p-) SMAD family member 2 (SMAD2) (#3108; Cell Signaling Technology), or p-p44/42 MAPK (extracellular signal-regulated kinase, ERK1/2) (#4370; Cell signaling Technology) followed by Alexa 488-conjugated anti-mouse or -rabbit secondary antibody (Thermo Fisher Scientific, San Jose, CA, USA). Nuclei were counterstained with Hoechst33258 (Thermo Fisher Scientific). Images were

acquired using the IN Cell Analyzer 2000 automated fluorescence microscope (GE Healthcare, Indianapolis, IN, USA) or Opera Phoenix (Perkin Elmer, Waltham, MA, USA).

Western blotting. Western blotting was performed as previously described (17). Cells were lysed using Complete Lysis-M, EDTA-free (Roche diagnostics, Mannheim, Germany) according to manufacturer's instructions. Primary antibodies were listed as follows: mitogen-activated protein kinase kinase (MEK1/2) (#4694), p-MEK1/2 (#9154), p38 MAPK (#8690), p-p38 MAPK (#4511), ERK1/2 (#4695), p-ERK1/2, stress-activated protein kinase (SAPK)/JNK (#9252), and p-SAPK/JNK (#4668) (all from Cell Signaling Technology). β-Actin (A5316; Sigma-Aldrich) was used as a loading control.

RNA purification and quantitative reverse transcription (qRT)-polymerase chain reaction (PCR). RNA extraction and qRT-PCR were carried out as previously described (18) using the SYBR Green system (Thunderbird SYBR qPCR Mix; TOYOBO, Osaka, Japan) and specific primers (Table I) (4). Glyceraldehyde 3-phosphate dehydrogenase expression was used as an internal standard. Relative gene expression levels were calculated with the 2^{-ΔΔCT} method (19).

Statistical analysis. Results are presented as mean±SD of at least triplicate measurements of one representative from several independent experiments. Differences between groups were evaluated with Welch's *t*-test, and a value of *p*<0.05 was considered statistically significant.

Results

H₂O₂ enhances TGFβ-mediated EMT. We investigated the effect of H₂O₂ on TGFβ-mediated EMT. Confluent MCF-10A cells were treated with H₂O₂ for 1 h, and then trypsinized and replated at a low density in the presence or absence of TGFβ. The highest concentration of TGFβ was 400 pg/ml, which is not sufficient to induce EMT (15). Immunofluorescence analysis revealed that E- and N-

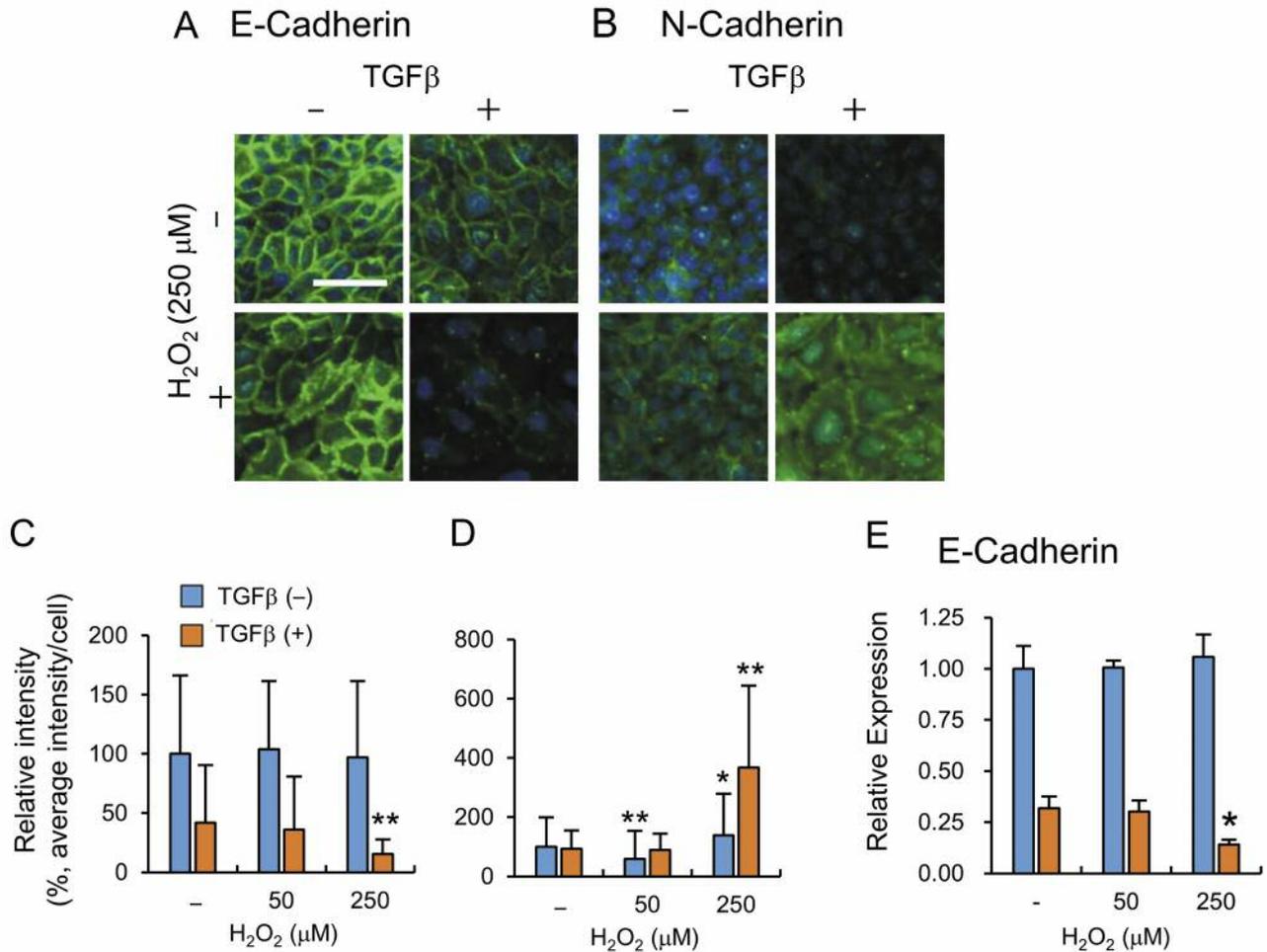


Figure 1. Immunofluorescence analysis of E-cadherin and N-cadherin expression at 144 h after H₂O₂ treatment. Representative images of E-cadherin (A) and N-cadherin (B) (green) are shown. Nuclei (blue) are stained with Hoechst33258. Bar, 50 μm. Quantification of E-cadherin (C) and N-cadherin (D) intensity. For each sample, 98 fields from the same area of each well were analyzed and average intensity of E- or N-cadherin per cell was determined using IN Cell Developer Toolbox software (GE Healthcare BioScience). **p*<0.05, ***p*<0.01 vs. corresponding negative control (-) of in the presence or absence of transforming growth factor β (TGF β). E: Quantitative reverse transcription polymerase chain reaction analysis of E-cadherin gene expression. Expression level was calculated relative to control cells at 144 h of triplicate measurements from one representative of three independent experiments. **p*<0.05 vs. corresponding negative control (-) of in the presence or absence of TGF β .

cadherin, markers for epithelial and mesenchymal phenotypes, respectively, were down- and up-regulated, respectively, after H₂O₂ treatment in the presence of TGF β (Figure 1A and B), although TGF β alone down-regulated E-cadherin expression to some extent. These changes were induced by H₂O₂ at concentrations greater than 50 μM, as determined using a high-throughput image analyzer (Figure 1C and D). E-Cadherin transcript levels were similar to the protein levels in each treatment group (Figure 1E).

Up-regulation of EMT-inducing transcription factors contributes to the enhancement of TGF β -mediated EMT by H₂O₂. We investigated the molecular basis for enhancement

of TGF β -mediated EMT by H₂O₂. Several transcription factors are known to inhibit E-cadherin expression and subsequently stimulate that of N-cadherin (20). Here we observed up-regulation of *SNAIL1*, *SNAIL2*, zinc finger E-box-binding homeobox 1 (*ZEB1*) and *ZEB2* transcripts 48 h after application of TGF β irrespective of H₂O₂ treatment or irradiation (data not shown). *SNAIL1*, *SNAIL2* and *ZEB1* levels were significantly increased in TGF β -treated cells 96 h after application of 250 μM H₂O₂ (Figure 2), while there was little change in the expression levels in cells that were not exposed to TGF β . Similar up-regulation was observed upon γ -radiation treatment, consistent with previous reports of increased *ZEB1* levels following irradiation (15).

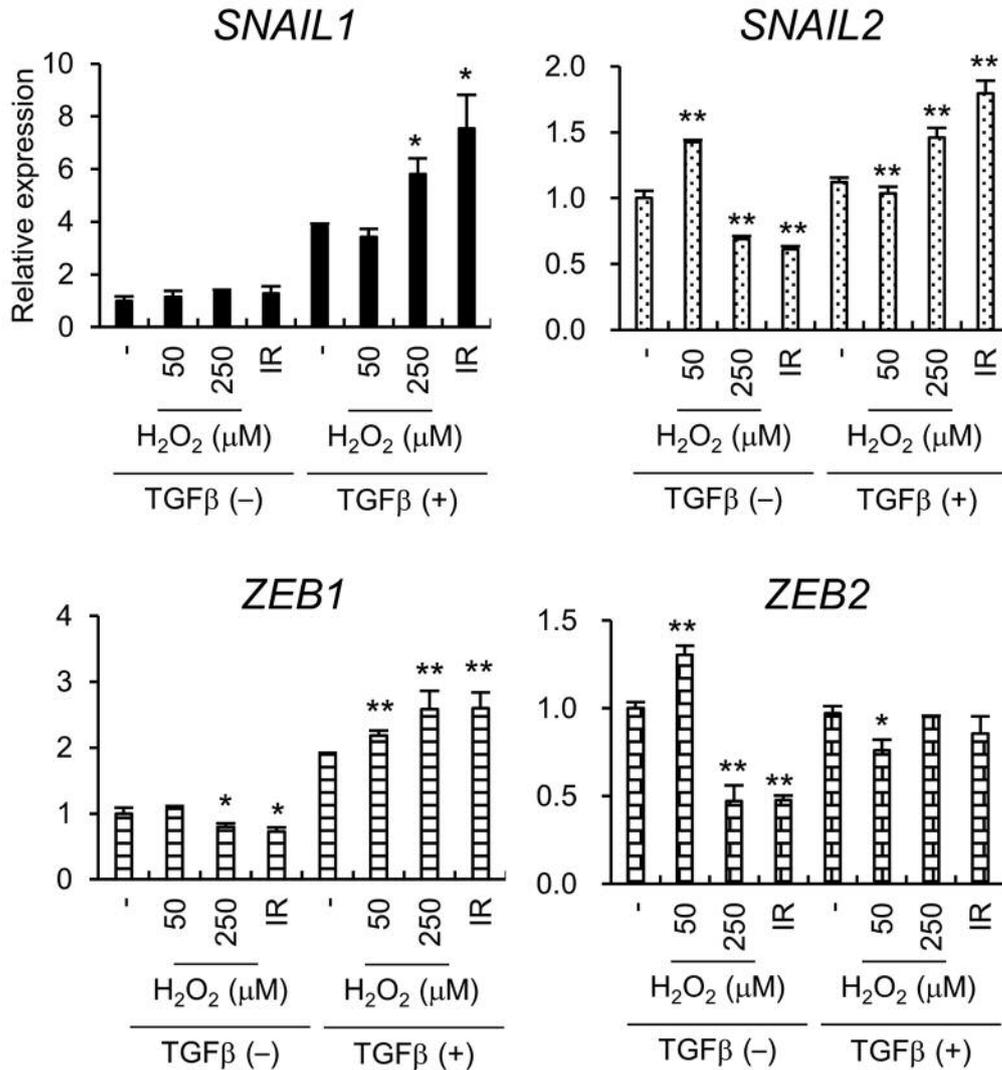


Figure 2. Quantitative reverse transcription polymerase chain reaction analysis of *SNAIL* family transcriptional repressor 1 (*SNAIL1*), *SNAIL2*, zinc finger E-box-binding homeobox 1 (*ZEB1*) and *ZEB2* expression at 96 h after H₂O₂ or radiation treatment (IR). Expression levels were calculated relative to control cells of triplicate measurements from one representative of three independent experiments. *p<0.05, **p<0.01 vs. corresponding negative control (-) of in the presence or absence of transforming growth factor β (TGFβ).

Nuclear localization of p-SMAD2 and MEK/ERK phosphorylation are involved in the enhancement of TGFβ-mediated EMT by H₂O₂. Finally, we investigated upstream signaling molecules of these transcription factors (*SNAIL1*, *SNAIL2* and *ZEB1*). Activation of SMADs and subsequent up-regulation of *SNAIL1*, *SNAIL2* and *ZEB1* expression is important for TGFβ-induced EMT signaling (13, 20). In this study, immunofluorescence analysis revealed an increase in nuclear localization of p-SMAD2 associated with the enhancement of TGFβ-mediated EMT at 72 h after H₂O₂ or radiation treatment (Figure 3).

MEK/ERK, JNK, and p38 are critical components of non-canonical, SMAD-independent TGFβ signaling (13). Of

these, ERK1/2 is also known to regulate *SNAIL1*, *SNAIL2* and *ZEB1* expression (21, 22). MEK/ERK phosphorylation was enhanced by both H₂O₂ and irradiation in TGFβ-treated cells (Figure 4), as previously reported (15). On the other hand, both JNK and p38 phosphorylation were unaltered (Figure 4). Moreover, immunofluorescence analysis revealed an increase in p-ERK1/2 expression associated with the enhancement of TGFβ-mediated EMT at 72 h after H₂O₂ or radiation treatment (Figure 5). The down-regulation of E-cadherin upon H₂O₂-induced enhancement of TGFβ-mediated EMT was partly but significantly abrogated by treatment with the MEK inhibitor U0126 (Figure 6A and B),

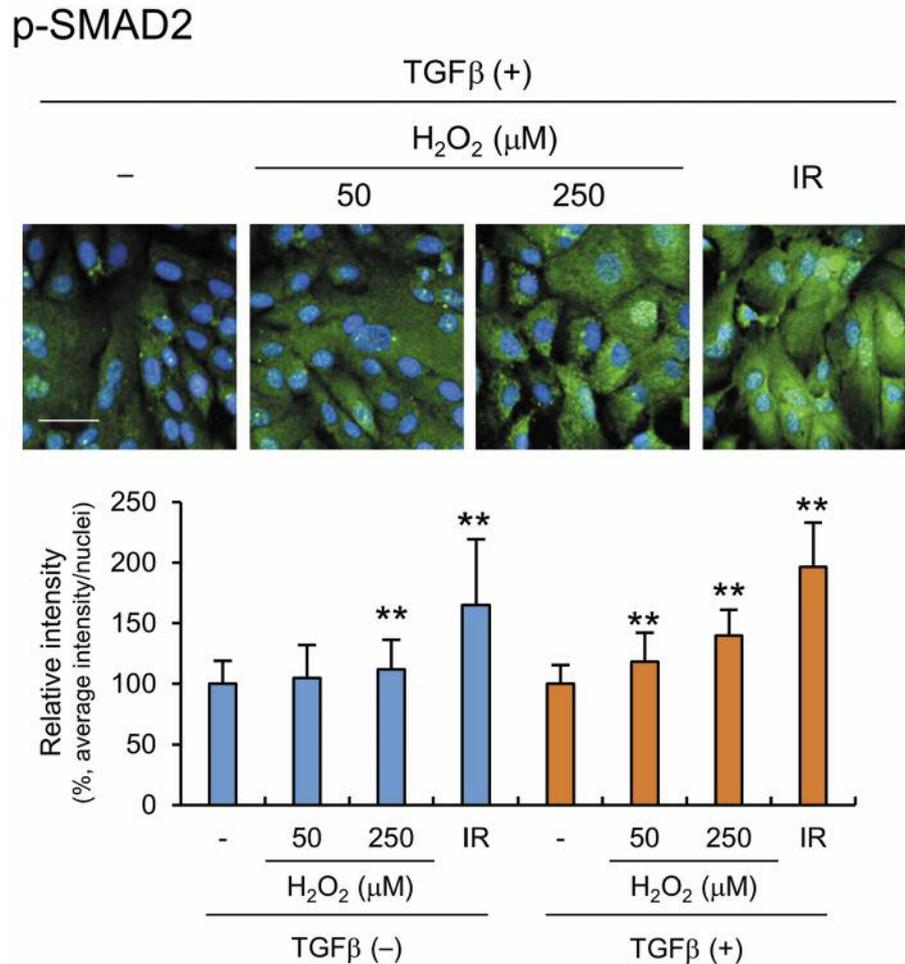


Figure 3. Immunofluorescence analysis of phospho-SMAD family member 2 (p-SMAD2) expression at 72 h after H₂O₂ treatment or irradiation (IR). Representative images of p-SMAD2 expression (green) are shown. Nuclei (blue) are stained with Hoechst33258. Bar, 50 μ m. Quantification of nuclear p-SMAD2 intensity. A total of 81 fields from the same area of each well was analyzed for each sample and average intensity of nuclear p-SMAD2 per cell were determined using the Harmony image-analysis software (Perkin Elmer). ** $p < 0.01$ vs. corresponding negative control (-) of in the presence or absence of transforming growth factor β (TGF β).

which also blocked ERK1/2 phosphorylation (Figure 6C). These results indicate that both SMAD and MEK/ERK pathways are critical for the enhancement of TGF β -mediated EMT by H₂O₂.

Discussion

The results presented here demonstrate that TGF β -mediated EMT in MCF-10A cells was enhanced not only by radiation exposure, which has been previously established, but also by H₂O₂ treatment at concentrations greater than 50 μ M. This was likely associated with the observed up-regulation of *SNAIL1*, *SNAIL2* and *ZEB1*, which encode EMT-inducing transcription factors. The induction of EMT was

accompanied by increased nuclear localization of phosphorylated SMAD2, as well as MEK/ERK activation, which is important for non-canonical, SMAD-independent TGF β signaling. These results indicate that the enhancement of TGF β -mediated EMT by H₂O₂ involves SMAD2 and MEK/ERK activation followed by upregulation of *SNAIL1*, *SNAIL2*, and *ZEB1*.

The binding of TGF β to its receptor and consequent activation of SMAD2/3, which is phosphorylated and accumulated in the nucleus, initiates EMT (13, 23, 24). It was reported that SMAD2/3 phosphorylation increased in cells exposed to radiation in the presence of TGF β (15). In the present study, we observed a significant increase in the nuclear localization of phosphorylated SMAD2 (Figure 3).

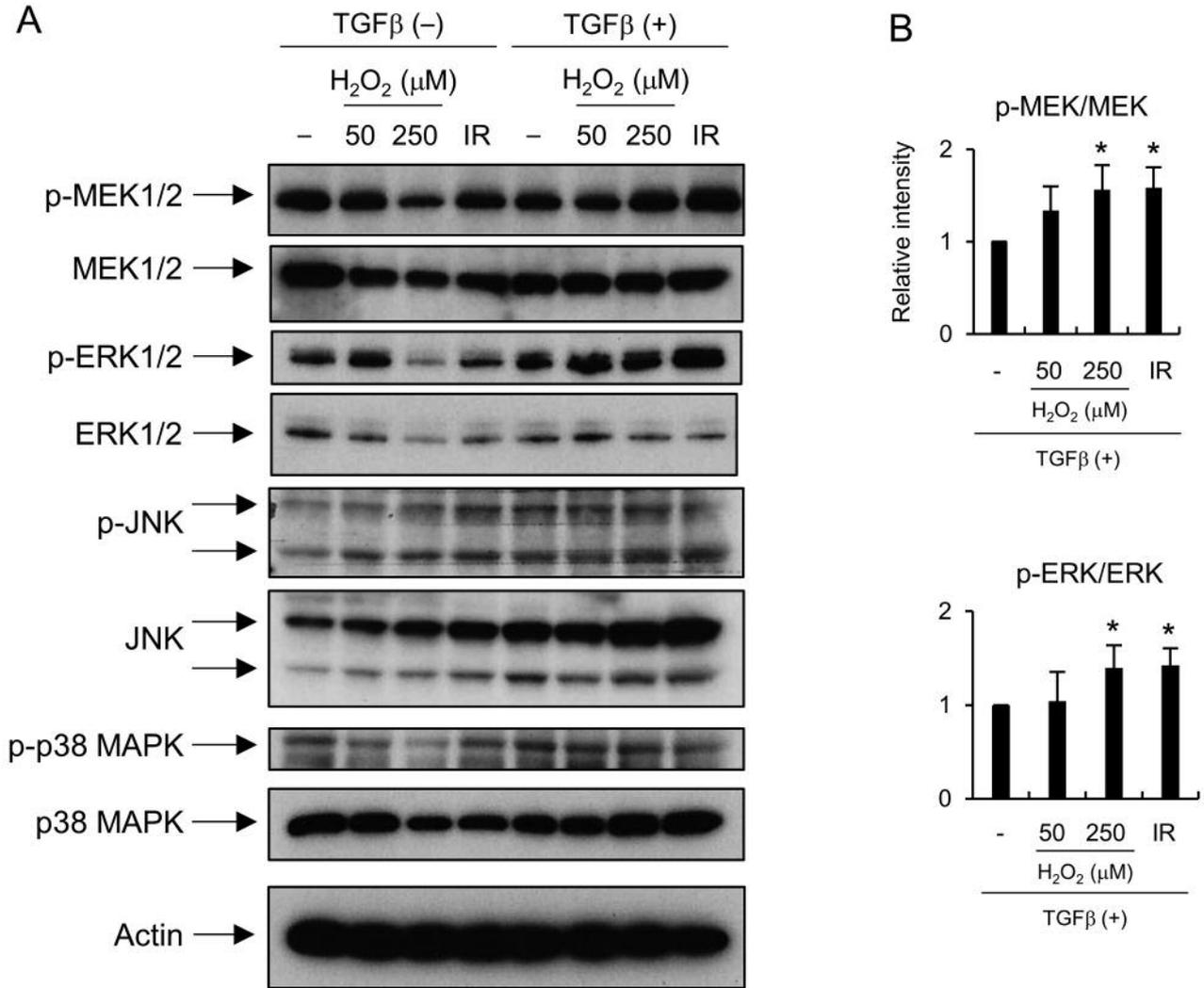


Figure 4. A: Western blot analysis of mitogen-activated protein kinase kinase (MEK), phospho-(p-) MEK, extracellular signal-regulated kinase (ERK1/2), p-ERK1/2, stress-activated protein kinase (SAPK)/JUN N-terminal kinase (JNK), p-JNK, p38 and p-p38 expression in MCF-10A cells 144 h after H₂O₂ treatment or irradiation (IR). The level of actin in each sample was used as an internal control. B: Densitometric analysis of the ratio of p-MEK to MEK and p-ERK1/2 to ERK1/2 of triplicate measurements from one representative of three independent experiments. *p<0.05 vs. negative control (-).

In addition, the TGFβ-SMAD pathway is correlated with other signaling molecules including MEK/ERK, JNK or p38 (25). In fact, MEK/ERK inhibition partly abrogated the enhancement of TGFβ-mediated EMT by H₂O₂, suggesting that both SMAD2 and MEK/ERK are important.

It is unclear how H₂O₂ activates SMAD and MEK/ERK signaling to enhance TGFβ-mediated EMT. However, SMAD activation might be caused via TGFβ, as TGFβ is induced by oxidative stress. Epidermal and platelet-derived growth factor receptors, which act upstream of MEK/ERK, are activated by H₂O₂ treatment in a ligand-independent manner (26, 27), while SRC and RAS are also required for

ERK1/2 activation induced by H₂O₂ (28). These are possible mechanisms of action that require further investigation.

Our results showed that MEK/ERK signaling is important for the enhancement of TGFβ-mediated EMT by H₂O₂, in agreement with previous observations that the MAPK pathway is activated by TGFβ via ROS in quiescent renal tubular epithelial cells (29). Most studies have evaluated EMT at relatively early time points and thus report the direct response of cells to oxidative stress. In contrast, our study evaluated the effects of oxidative stress in the daughter cells of those exposed to H₂O₂; the results suggest that the changes

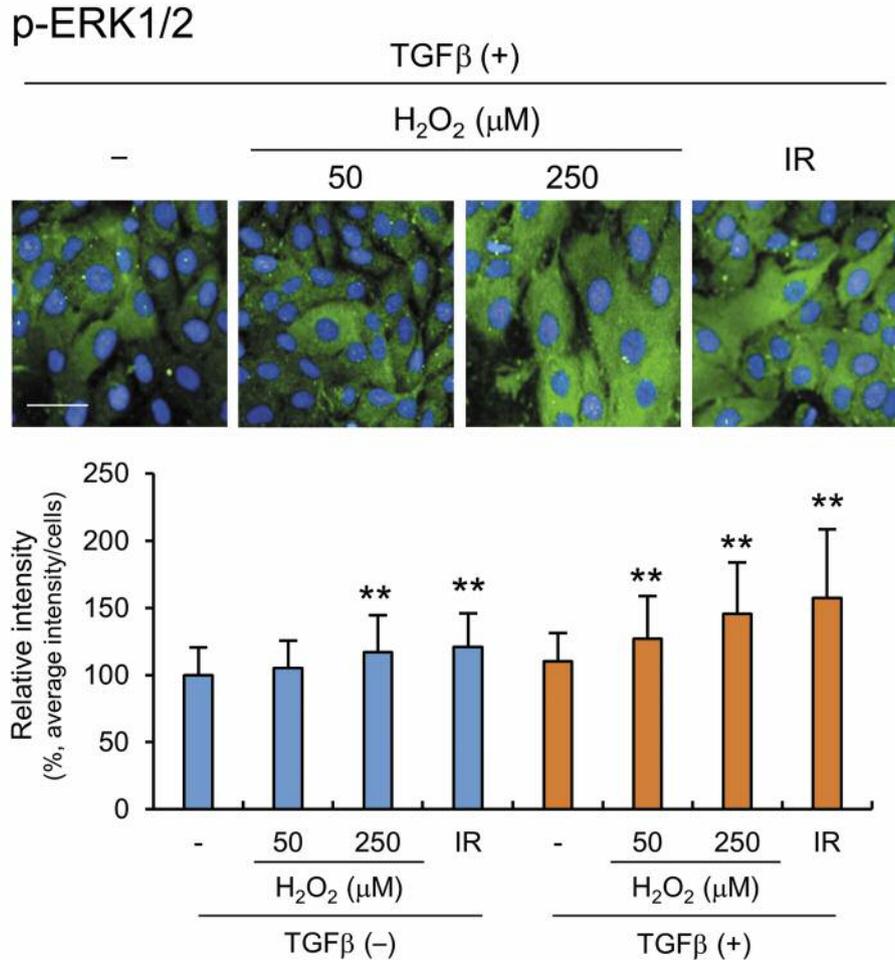


Figure 5. Immunofluorescence analysis of phospho-extracellular signal-regulated kinase (p-ERK1/2) expression 72 h after H₂O₂ treatment or irradiation (IR). Representative images of p-ERK1/2 expression (green) are shown. Nuclei (blue) are stained with Hoechst33258. Bar, 50 μ m. B: Quantification of p-ERK1/2 intensity. A total 81 fields from the same area of each well were analyzed for each sample and average intensity of p-ERK1/2 per cell were determined using Harmony image-analysis software. ** $p < 0.01$ vs. corresponding negative control (-) of in the presence or absence of transforming growth factor β (TGF β).

induced by H₂O₂ are transmitted to subsequent generations, which may ultimately contribute to tumorigenesis.

In summary, H₂O₂ was found to enhance TGF β -mediated EMT in MCF-10A cells, and this involved both SMAD and MEK/ERK signaling. Our finding that oxidative stress can enhance EMT in cooperation with TGF β suggests that these events (*i.e.* activation of EMT/EMT-inducing transcription factors) are involved in the induction of many types of cancer, and provides a basis for the development of therapeutic strategies for cancer that target ROS as well as TGF β .

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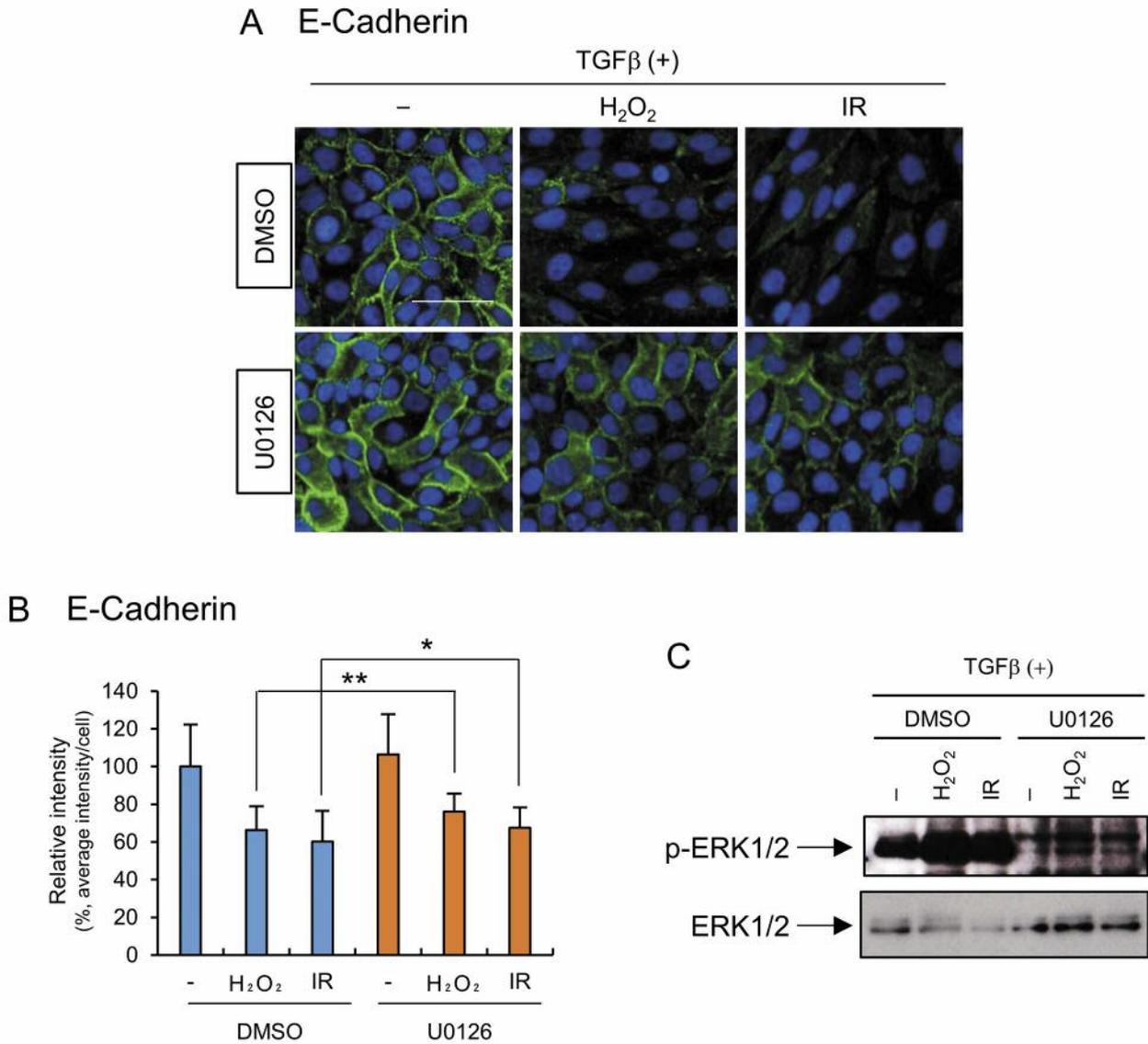


Figure 6. A: Representative images of E-cadherin expression (green) in cells treated with 10 μM U0126 for 24 h after 144 h of incubation with transforming growth factor β (TGFβ). Nuclei (blue) are stained with Hoechst33258. Bar, 50 μm. B: Quantification of E-cadherin intensity. Thirty-three sections from the same area of each well was analyzed for each sample and average E-cadherin intensity per cell was determined using Harmony image-analysis software. **p*<0.05, ***p*<0.01. C: Western blot analysis of extracellular signal-regulated kinase (ERK1/2) and phospho-(p-)ERK1/2 expression in MCF-10A cells treated with 10 μM U0126 for 8 h after 120 h of incubation with TGFβ. IR: Radiation treatment.

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