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Original Paper

SOCS1 Represses Fractionated Ionizing Radiation-Induced EMT Signaling Pathways through the Counter-Regulation of ROS-Scavenging and ROS-Generating Systems

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Key Words
Fractionated ionizing radiation (FIR) • Epithelial to mesenchymal transition (EMT) • Suppressors of cytokine signaling 1 (SOCS1) • Reactive oxygen species (ROS)

Abstract
Background/Aims: Fractionated ionizing radiation (FIR) is an anti-cancer protocol widely applied for the treatment of diverse types of cancers to reduce damage to normal cells. However, cancer cells receiving multiple irradiations at low doses during FIR, often develop resistance to the therapy exhibiting malignant features including epithelial to mesenchymal transition (EMT). The present study has been performed to elucidate the mechanism of FIR-induced EMT signaling pathways and to identify a molecular target for radioresistance modulated by suppressors of cytokine signaling (SOCS)1. ***Methods:*** Colorectal cancer cell lines received FIR with a daily dose of 2 Gy for 3 days. Generation of intracellular reactive oxygen species (ROS) and its role in EMT signaling induced by FIR were analyzed in SOCS1 over-expressing and knock-down cells. ROS were measured by DCF fluorescence using flow cytometry. Expression levels of EMT markers and signaling molecules were analyzed by Western blotting and confocal microscopy. ***Results:*** FIR induced ROS and changes in EMT markers including down-regulation of E-cadherin with up-regulation of Twist and Snail. Pretreatment of anti-oxidant N-acetyl cysteine (NAC) abrogated the FIR-induced ROS generation and EMT response. Mechanistic studies indicated that the FIR-induced ROS-mediated EMT signaling proceeded through Akt→Src→Erk pathways. In accordance with the anti-ROS function, SOCS1 blocked the FIR-induced EMT and the associated signaling pathways through thioredoxin (Trx1) up-regulation. This is evidenced by the observation that Trx1 ablation in SOCS1 over-expressing cells negated the inhibitory action of SOCS1 by restoring the FIR-induced ROS and EMT markers. In addition, we have obtained data supporting that the FIR-induced ROS is derived from functional mitochondria and NADPH oxidases (Nox), which are both down-

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regulated by SOCS1. ***Conclusion:*** The results demonstrate that ROS signal acts as a mediator of the FIR-induced EMT. The data also suggest a potential anti-tumor function of SOCS1 by blocking the FIR therapy-induced resistance through the counter-regulation of ROS generating and scavenging systems.

Introduction

Colorectal cancers (CRC), the malignant tumors in the inner wall of the colon and the rectum, pose a leading cause of cancer-related deaths worldwide [1]. Most common treatments for the CRC malignancy include surgery, chemotherapy, radiation therapy, and combination therapy [1, 2]. While tumor size and lymph node metastasis are key pathological factors to predict the treatment outcome of CRC, the extramural venous invasion is also considered important [3]. For invasion, epithelial to mesenchymal transition (EMT) occurs through the orchestrated series of events involving alterations in cell-cell and cell-extracellular matrix interactions [4]. During the cancer-associated EMT, epithelial cells lose their polarities and intercellular adhesion, and then gain fibroblast-like properties, which endows cells with ability to migrate and invade into adjacent tissues [5]. Transcription factors such as Snail, Twist, and ZEB-1 have been reported as major activators of EMT through the suppression of E-cadherin expression and the promotion of migration properties [6].

Reactive oxygen species (ROS) are oxygen-containing radical ions or molecules that have a single unpaired electron, which exhibit high reactivity with a very short half-life. While the cellular ROS levels are kept in balance with biochemical anti-oxidants under a physiological state, excess ROS induced upon oxidative stress may act as important mediators of cancer initiation and development [7, 8]. This is due to their ability to damage DNA and induce mutations resulting in the gain of function for oncogenes or the loss of function for tumor suppressors [9]. In cancer cells, ROS level is likely promoted further by high metabolic activity, increased cellular receptor signaling, mitochondrial dysfunction, oncogene activation, and increased activity of oxidases [10, 11]. ROS are also shown to affect the EMT process of both normal and cancer cell types [12, 13]. As a mechanism of ROS-induced EMT in colon cancer cells, we have recently reported that hydrogen peroxide-induced EMT is mediated by Src-dependent activation of Snail [13].

Suppressor of cytokine signaling 1 (SOCS1) is a member of SOCS family proteins, which was initially identified as a negative regulator of the Jak/Stat pathway. SOCS1 participates in the regulation of multiple cellular functions including proliferation, differentiation and apoptosis, affecting tumor cell growth [14, 15]. The promoter hyper-methylation and the resulting transcriptional repression of SOCS1 gene are common in various tumors. In human hepatocellular and colorectal carcinomas, for example, SOCS1 gene was found highly methylated in the promoter. Treatment with methyl transferase inhibitors induced SOCS1 expression and reduced proliferation of cancer cells, suggesting the anti-tumor function of SOCS1 [16]. In addition, SOCS1 reduced the metastatic progression of colon cancer cells by preventing EMT with the induction of E-cadherin expression [17]. More recently, it is demonstrated that the EMT-suppressing function of SOCS1 is mediated by inhibition of Src, leading to Nrf-2 activation, an anti-oxidant transcription factor. The subsequent induction of thioredoxin significantly reduced ROS, which is a potent EMT inducer in colon cancer cells [13].

Ionizing radiation (IR) is a well-established adjuvant therapy for diverse cancers, typically administered in fractionated low doses to reduce side effects of damage to normal cells in adjacent tissues. However, such fractionated ionizing irradiation (FIR) therapy has been reported to induce invasiveness of cancer cells through EMT activation [18]. Furthermore, cancer stem cells with self-renewal and differentiation capacity may overcome the damage from FIR, leading to radioresistance and cancer recurrence [19, 20]. Studies conducted on the mechanism of radioresistance suggested the role of AMPK activity in metastatic colon cancers [21]. In addition, the Akt activation has been implicated in the inhibition of cell death noted upon chronic low dose irradiation [22] and in the PTEN deficiency - mediated EMT

response of esophageal cancer cells by FIR [23]. To date, however, the molecular mechanisms by which FIR induces EMT and the signaling pathways involved have not been clearly revealed.

IR is shown to induce ROS by up-regulation of mitochondrial electron transport chain (ETC) function [24], and by the activation of NADPH oxidases (Nox) producing superoxide anions from oxygen using NADPH as electron donors [25]. As SOCS1 exerted anti-ROS and anti-EMT effect [13, 26], we have investigated the role of ROS signal in the FIR-induced EMT response and the regulatory action of SOCS1 by examining its effect on the FIR-mediated EMT signaling of colorectal cancer cells. The results of our study indicate that FIR triggers the generation of intracellular and mitochondrial ROS to mediate EMT signaling through Akt→Src→Erk pathways, and that SOCS1 represses the ROS-mediated EMT through the up-regulation of ROS-scavenging as well as the suppression of ROS-generating systems in cancer cells.

Materials and Methods

Cell culture and radiation treatment

Human colorectal cancer cell lines, HCT116/p53 wt ($p53^{+/+}$), HCT116/p53 null ($p53^{-/-}$), HT29, SW480 and SW620 (from KCLB and ATCC) were maintained in DMEM supplemented with 10 % FBS (Hyclone Laboratories, South Logan, UT) and 100 mg/mL streptomycin. Cells grown in 60 mm-diameter dishes were irradiated with γ-rays using a Gamma cell 1000 Elan system (MDS Nordion Inc, Ontario, Canada) at varying doses as indicated. The optimal FIR condition for EMT induction was determined as irradiation of 2 Gy per day for 3 consecutive days. Treatments of anti-oxidants and inhibitors including NAC, diphenylene iodonium (DPI), PP1, LY294002, PD98059, SB203580, SP600125 (Sigma-Aldrich, St. Louis, MO) were done 1 h prior to the exposure of cells to the last irradiation at indicated concentrations.

Gene transfection

Cells in 500 μl serum-free DMEM were mixed with 5 μg each of pcDNA-HA, HA-SOCS1, shRNA control vectors or shRNAs targeting SOCS1, Src, or Trx1 as described [13]. The mixture was transferred into a 0.4 cm electrode gap cuvette and transfection was performed with five pulses of 240 V for 5 ms using Gene Pulser X cell electroporation system (Bio-Rad, Herbercules, CA).

Western blot and densitometry analysis

Whole cell lysates were prepared using the T-per lysis buffer. Lysates were clarified at 13,500 rpm for 20 min at 4°C. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was probed with anti-pS-Akt, anti-Akt, anti-pY-Src, anti-pY-FAK, anti-p(Y/S)-Erk, anti-Erk, anti-p-JNK, anti-Jnk, anti-p-p38, anti-p38 anti-E-cadherin, anti-Snail, anti-Twist, anti-Trx1 (Cell Signaling Technology, Beverly, MA), anti-vimentin, anti-TrxR1, anti-SOD1, anti-SOCS1(Santa Cruz Biotechnology, Santa Cruz, CA), or anti-c-Src antibodies (Abcam, Cambridge, MA) using an ECL system (Thermofisher Scientific, Waltham, MA). The intensity of protein bands was subject to the densitometry analysis using the ImageJ software.

Analysis of intracellular and mitochondrial ROS levels by FACS

For the analysis of intracellular ROS levels, cells were stained with 1 μM H_2DCF-DA for 20 min before the end of the incubation and DCF fluorescence was measured at 480 nm/530 nm using the FACSCalibur flow cytometry system equipped with CELLQuestpro software (BD Bioscience). Mitochondrial ROS was measured using Mitosox Red at 5 μM at 510 nm/580 nm. In addition, Mitotracker green and Mitotracker red dyes (Thermofisher Scientific) were used to determine total and functional mitochondria with ETC levels, respectively.

Immunofluorescence Analysis

Fix-permeabilized cells were stained with indicated primary antibodies for SOCS1, E-cadherin, or vimentin, followed by incubation with fluorescence-conjugated secondary antibodies (Alexa-488, Alexa-594:

Molecular probe, Eugene, OR). Nuclear staining was performed with Hoechst 33342 (Molecular probe). After extensive washing, cells were analyzed by using a confocal microscope (LSM 710 Meta DuoScan, Carl Zeiss Micro Imaging GmbH, Germany).

Cell death measurement

Cell death was measured using an apoptosis/necrosis detection kit (BD Pharmingen, San Diego) according to the manufacturer's protocols and flow cytometry. Both early apoptotic (Annexin V-positive, PI-negative), late apoptotic (Annexin V-positive and PI-positive), and necrotic (Annexin V-negative and PI-positive) cells were included in cell death determinations.

Statistical Analysis

Experiments were performed at least in three independent sets. The values are presented as means ± SE. Statistical significance was determined by a Student-t-test. A value of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ was considered statistically significant.

Results

Low dose fractionated ionizing radiation (FIR) treatment induced intracellular ROS generation and EMT in colorectal cancer cells in NAC-sensitive manners

The ROS-inducing capacity of IR is thought to play a role in the development of malignant cell types with EMT induction associated with radioresistance of cancer cells [27, 28]. To investigate the effect of IR on ROS generation leading to EMT in colorectal cancer cell lines, different doses and administration frequencies of IR were tested. First, upon the treatment of HCT116/p53 wt cells with gamma irradiation at a single dose of 1 to 10 Gy, intracellular ROS was significantly increased at 2 Gy in 30 min (170 % increase over untreated control, Fig. 1A). At this high ROS-inducing dose, no apparent cell death was noted by 24 h as compared with a prominent cell death induced from 5 Gy (5 % vs 105 % increase in cell death by 2 Gy vs 5 Gy, Supplementary Fig. S1A and S1B – for all supplementary material see www.cellphysiolbiochem.com). Antioxidant NAC pretreatment promoted cell death induced at 5 Gy and blocked the increase in ROS generation (20 % increase in cell death with negation of ROS generation, Supplementary Fig. S1B and S1C), which suggested a protective role of ROS in these cells receiving apoptosis-inducing radiation. Then, the ROS-generating capacity of low dose radiations delivered in fractions and the role of ROS in EMT induction were investigated. Notably, the fractionated irradiation of 2 Gy for 3 consecutive days (refereed as fractionated ionizing radiation: FIR) resulted in a further elevation of ROS (220 % increase over untreated control), which was blocked by the NAC pre-treatment. Kinetic analysis has shown that in these FIR-treated cells, ROS levels increased in 10 min, reached to the peak by 30 min, and declined thereafter (Fig. 1B). Under this condition, a prominent induction of the EMT markers such as up-regulation of Snail and Twist with down-regulation of E-cadherin, was noted along with the activation of signaling pathways associated with EMT including Akt, Src, and MAPKs by 2 h. Importantly, NAC pretreatment substantially reduced the levels of FIR-induced EMT markers and the associated signaling mediators, suggesting a critical role of ROS in the FIR - induced EMT response in colorectal cancer cells (Fig. 1C and 1D).

While the induction of ROS associated with EMT was seen until 24 h after FIR (Supplementary Fig. S1D and S1E), a maximal response for ROS generation was obtained at 30 min and the activation of EMT signaling pathways was observed by 2 h (Fig. 1B and 1C). Thus, these conditions were employed in the experiments followed to reveal the mechanism of FIR-induced upstream signaling pathways mediated by ROS leading to EMT. The induction of ROS generation and the activation of kinases leading to EMT response by FIR observed in HCT116/p53 wt were also seen in other colorectal cancer cell lines such as HCT116/p53 null ($p53^{-/-}$), HT29 (p53 mt) and SW480 (p53 mt) as shown in Supplementary Fig. S2 and S3. The data suggest that the observed FIR-induced response is p53-independent and may represent a common feature in cells derived from colorectal cancers.

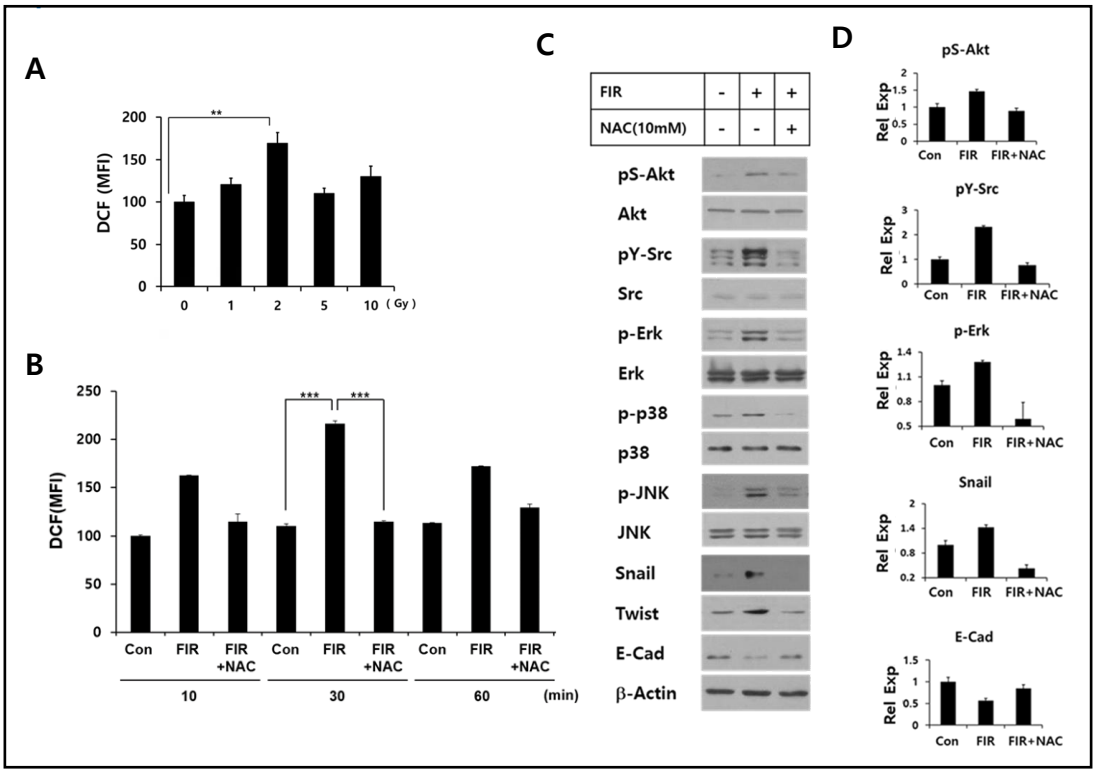

Fig. 1. FIR up-regulates intracellular ROS which mediates EMT signaling by FIR in colorectal cancer cells. (A) HCT116 $p53^{+/+}$cells were irradiated with indicated doses using the gamma irradiator, and the intracellular ROS levels were determined at 30 min by H_2DCFDA staining as described in the text. (B) HCT116 p53 +/+ cells received fractionated irradiation (FIR) administered at 2 Gy per day for three consecutive days. NAC pretreatment was done 1 h prior to the last irradiation and the ROS levels were determined at indicated time points after irradiation. Results show data (mean + SE) obtained from 3 experiments performed in triplicate wells (**, $p \leq 0.01$; ***, $p \leq 0.005$). (C) Cells received FIR as described in panel B (2 Gy for 3 days) were incubated for 2 h after the last irradiation, and subjected to Western blot to analyze EMT markers and signaling molecules. Results show representative blot data obtained from multiple experiments. (D) Densitometry analysis of immunoblots was performed to show the relative expression (Rel Exp) of pS-Akt, pY-Src, and pS/Y-Erk levels normalized to Akt, Src, and Erk levels, respectively. For example, the ratio of pS-Akt over Akt levels in control cells was taken as 1. Likewise, E-cad and Snail expression levels were normalized to beta-actin, and the expression ratio for each protein over beta-actin in the untreated control was taken as 1. Results show data (mean + SE) obtained from 3 experiments.

EMT induced by FIR proceeds through Akt→Src→Erk signaling pathways in ROS-dependent manners

Having observed the activation of Akt, Src and MAPKs during the FIR-induced EMT response in Fig. 1, the hierarchy of the kinases involved in EMT induction was studied to delineate the FIR-induced EMT signaling pathways. Upon treatment of PI3K/Akt inhibitor, LY294002, the levels of FIR-induced pY-Src and p-Erk were diminished, and the FIR-induced changes in EMT markers including Snail, vimentin and E-cadherin were all reversed (Fig. 2A and Fig. 2D). On the other hand, a Src inhibitor PP1 did not affect the level of pS-Akt, but blocked the FIR-induced pY-Src and p-Erk levels, indicating that Akt is acting upstream of Src and Erk (Fig. 2B). To confirm the role of Src in ROS-mediated EMT signaling, the levels of EMT markers and Akt activation were also examined using the Src knock-down cells. The shSrc-introduced cells exhibited persistently elevated E-cadherin and reduced Snail expression levels (Supplementary Fig. S4). While NAC blocked the FIR-induced pS-Akt, pY-Src, and p-Erk levels, Src deficiency reduced p-Erk with no effects on p-Akt level, suggesting that Akt→Src →Erk

pathways operate in the FIR-induced EMT signaling (Fig. S4), which are all dependent on ROS (Fig. 1D). Moreover, pretreatment of Erk inhibitor, PD98059 reduced the Snail expression and restored the FIR-induced down-regulation of E-cadherin without changes in activities of Akt and Src, supporting that Erk is acting in downstream signaling of Akt and Src (Fig. 2C). Although a modest activation of p38 and Jnk was induced by FIR, treatments with their respective inhibitors SB203580 and SP600125 had no significant effects on the expression levels of FIR-induced EMT markers, suggesting that p38 or Jnk may not be involved in the FIR-induced EMT signaling (Fig. 1C and data not shown). In other colorectal cancer cell lines examined for ROS generation, the ROS-dependent Akt, Src, and Erk pathways leading to EMT features were also observed (Supplementary Fig. S3).

Repression of the FIR-induced ROS generation and EMT marker expression in colorectal cancer cells by SOCS1

We have previously shown that SOCS1 reduced hydrogen peroxide-induced intracellular ROS levels and repressed EMT [13]. Thus it was of interest to examine whether such effect of SOCS1 is observed during the FIR-induced regulation of ROS and EMT response. To do this, the SOCS1 over-expressing HCT116 cells established by gene transfection were used. As compared to mock cells, HCT116/SOCS1 cells exhibited significantly reduced basal intracellular ROS levels, which were kept low with or without FIR treatment (Fig. 3A). The anti-EMT function of SOCS1 is clearly seen by the immunoblot analysis. The SOCS1 over-expressing

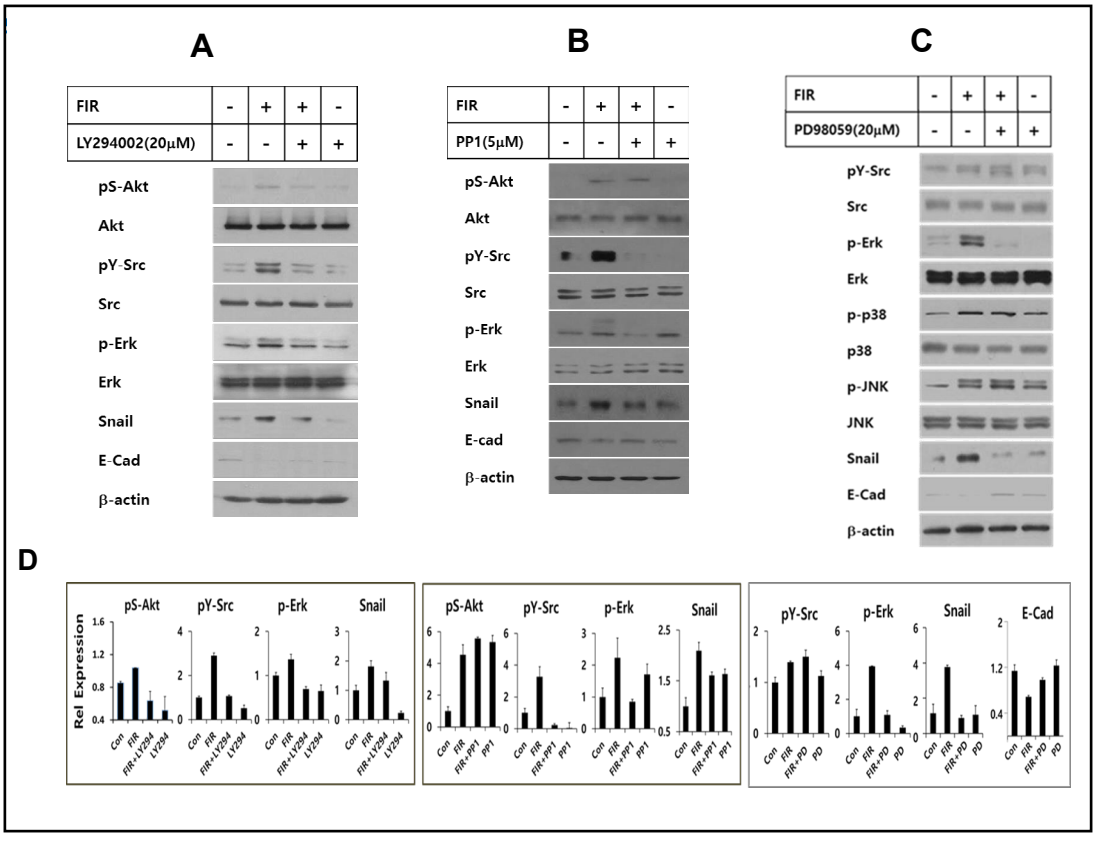

Fig. 2. FIR-induced EMT signaling proceeds through Akt → Src → Erk pathways. HCT116 $p53^{+}/^{+}$ cells were irradiated with 2 Gy per day for 3 days in the presence or absence of kinase inhibitors: (A) Akt inhibitor LY294002, (B) Src inhibitor, PP1, and (C) Erk inhibitor, PD98059 at indicated concentrations treated 1 h prior to the last irradiation. Expression levels of signaling molecules and EMT markers were analyzed by Western blot as described in Fig. 1. A representative blot is shown. (D) Quantitative data obtained from the densitometry analysis of multiple blots are expressed as described in Fig. 1.

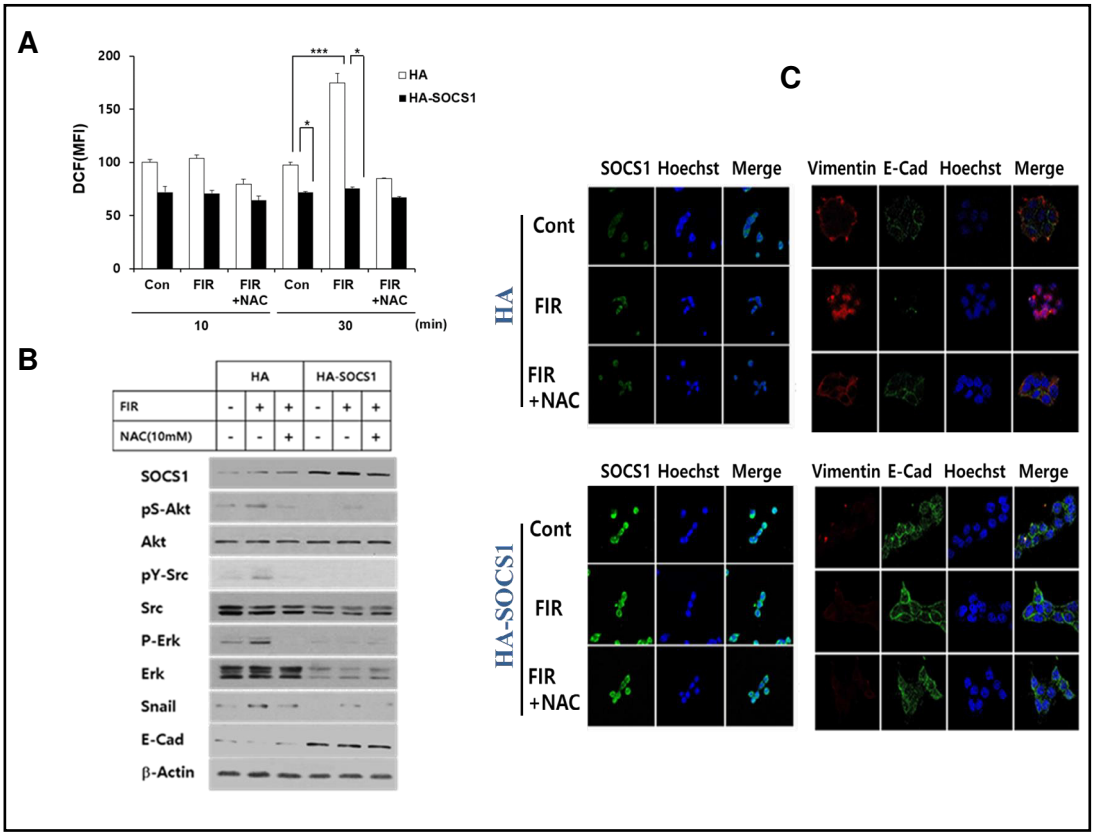

Fig. 3. SOCS1 over-expressing cells exhibited dampened response to FIR in ROS and EMT induction. HCT116 $p53^{+/+}$ cells transfected with pcDNA/HA-SOCS1 (HA-SOCS1) or mock (HA) cells received FIR in the presence or absence of NAC. (A) Intracellular ROS levels were determined by FACS analysis. Results show representative data of independent experiments performed in triplicate wells (*, $p \leq 0.05$; ***, $p \leq 0.005$). (B) The cells treated above were analyzed for the expression levels of EMT markers and signaling molecules by Western blot. The densitometric analysis data are shown in Fig. S5. (C) Immunofluorescence staining was performed as described in the text to show the expression of SOCS, vimentin and E-cadherin levels in mock (HA) vs HA-SOCS1 cells.

cells exhibited a dampened response in the FIR-induced activation of EMT signaling molecules, such as pS-Akt, pY-Src, p-Erk, and Snail. In contrast, E-cadherin levels down-regulated by FIR in mock cells were restored upon SOCS1 transfection (Fig 3B, Supplementary Fig. S5). The immunofluorescence analysis provided supporting evidence for the up-regulation of epithelial marker (E-cadherin) and down-regulation of mesenchymal marker (vimentin) in SOCS1 over-expressing cells (Fig. 3C). Effects of anti-oxidant NAC further suggested the operation of ROS-dependent Akt/ Src / Erk pathways leading to FIR-induced EMT response as observed in Western or confocal analysis (Fig. 3).

To confirm the ROS-suppressive and EMT-inhibitory function of SOCS1, the same parameters were examined in SOCS1 knock-down cells. HCT116/shSOCS1 cells exhibited consistently high basal levels of ROS than mock (sh) cells (Fig. 4A). It was noted that FIR induced a significant increase in ROS levels both in mock and shSOCS1 cells in 10 min, which declined thereafter. As compared to mock cells, a prominent up-regulation of EMT signaling molecules was seen in shSOCS1 cells including pS-Akt, pY-Src, p-Erk and Snail in both control and FIR-treated conditions (Fig. 4B and Supplementary Fig. S5). These elevated EMT markers in shSOCS1 cells were subject to a decrease upon NAC treatment, suggesting that increased ROS levels in SOCS1-ablated cells are responsible for the promotion of EMT.

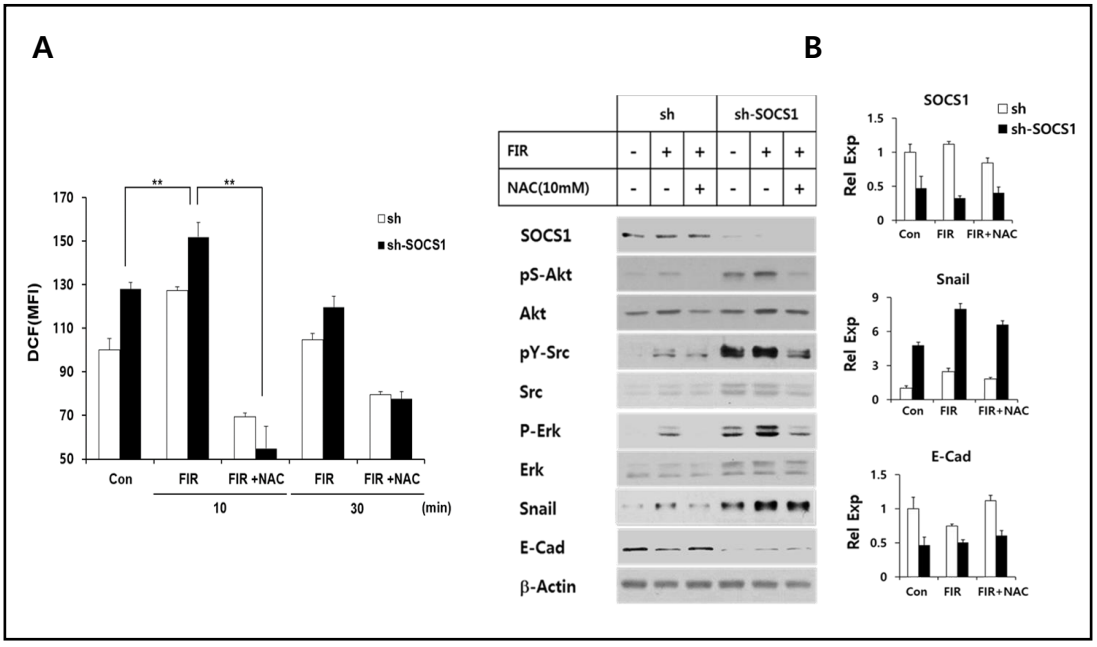

Fig. 4. SOCS1 knock-down promoted ROS levels and the FIR-induced Akt/Src/Erk signaling leading to EMT. (A) HCT116 p53 $^{+/+}$ cells with sh or shSOCS1 received FIR treatment in the presence and absence of NAC, and ROS levels were analyzed at indicated time points as described. Results show representative data of independent experiments performed in triplicate wells (*, $p \leq 0.05$; **, $p \leq 0.01$). (B) After receiving FIR, sh or sh-SOCS1 cells were subjected to Western blot to analyze the protein levels of EMT signaling molecules and phenotypic markers. The quantitated relative expression levels for Snail, E-cad and SOCS1 normalized to beta actin were obtained by densitometry analysis.

Critical function of Trx1 in the SOCS1-mediated inhibition of ROS and EMT signaling induced by FIR

The above data strongly suggest the down-regulation of EMT by SOCS1 is exerted by targeting the FIR-induced ROS. To investigate the mechanism by which ROS dysregulation occurs by SOCS1, the expression profile of anti-oxidant enzymes was analyzed. As shown in Fig. 5A, FIR treatment induced a transient up-regulation of thioredoxin 1(Trx1), but not of the other anti-oxidant enzymes, such as, thioredoxin reductase 1(TrxR1) and superoxide dismutase 1(SOD1). Upon SOCS1 over-expression, the basal level of Trx1 increased (Fig. 5A), suggesting that the consistent low levels of intracellular ROS in SOCS1 over-expressing cells may be, at least in part, due to the up-regulation of Trx1.

To find out whether the elevated expression of Trx1 is responsible for reduced intracellular ROS levels and inhibition of EMT upon SOCS1 over-expression, the effect of shTrx1 introduction into the HCT116/SOCS1 cells was examined. Indeed, Trx1 knock-down negated the SOCS1-induced ROS repression as seen with the elevation of both the basal and the FIR-induced ROS levels (Fig. 5B). Furthermore, Trx1 depletion abrogated the SOCS1-induced anti-EMT effect, as shown by the recovery of Src activity and Snail expression with suppression of E-cadherin levels (Fig. 5C). The result supports the notion that Trx1-mediated ROS scavenging action is responsible for the inhibitory effect of SOCS1 on the FIR-induced EMT response.

Role of mitochondria and NADPH oxidases in the FIR-induced ROS and EMT response

IR is reported to induce ROS by up-regulation of mitochondrial electron transport chain (ETC) function and also by the activation of NADPH oxidases in lung carcinoma and endothelial cells [24, 25]. In colorectal cancer cells under study, we have observed that FIR induced a prominent increase in mitochondrial ROS as assessed by Mitosox staining (Fig. 6A and 6B).

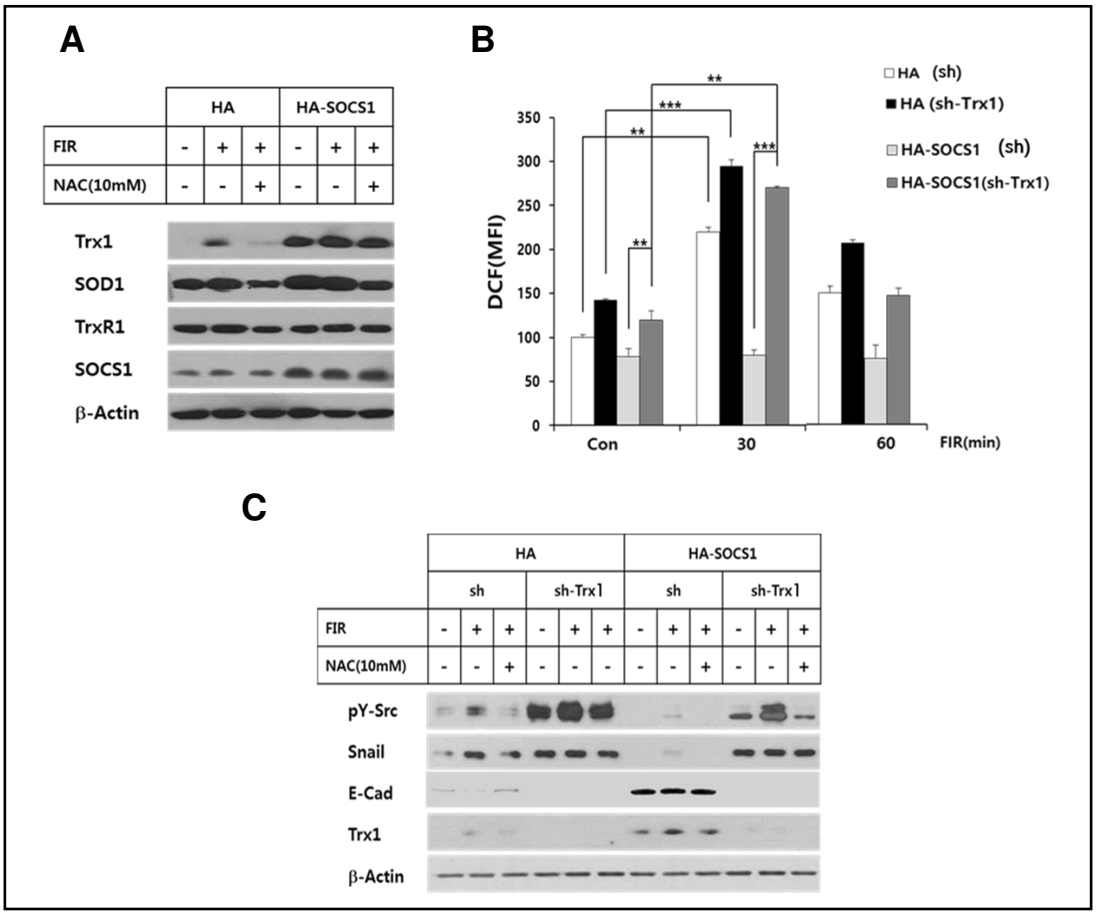

Fig. 5. Role of Trx1 in SOCS1-mediated reduction in ROS and EMT signaling induced by FIR. (A) HA vs HA-SOCS1 HCT116 $p53^{+/+}$cells treated with FIR were analyzed for expression levels of antioxidant proteins by immunoblot. (B) The HA vs HA-SOCS1 cells were additionally transfected with sh control or shRNA against Trx1, and analyzed for intracellular ROS levels after FIR treatment at indicated time points (**, $p \leq 0.01$; ***, $p \leq 0.005$). (C) The shTrx1-transfected cells were analyzed for EMT marker expression levels upon FIR treatment.

The increase in mitochondrial ROS appears through the up-regulation of functional mitochondria as analyzed by Mitotracker Green staining total mitochondria and Mitotracker Red staining mitochondria with ETC in cells receiving FIR (Fig. 6C and 6D). Cells over-expressing SOCS1, exhibited reduced production of ROS from mitochondria upon FIR treatment as compared with mock cells, along with modestly decreased levels of both total and functional mitochondria. In contrast, shSOCS1 cells were found to have different features, displaying increased basal levels for Mitosox and Mitotracker staining (Supplementary Fig. S6).

Next, to study the role of Nox in the FIR-induced ROS generation, the effect of DPI, an inhibitor of Nox and Duox [29] was examined. DPI effectively attenuated the intracellular ROS levels induced by FIR (Fig. 7A) with a selective inhibition of FIR-induced activation of Akt and Src as well as down-regulation of Snail levels (Fig. 7B). Unlike the universal suppressive effect of NAC on the FIR-activated signaling enzymes, the FIR-induced MAPKs such as Erk and Jnk were not repressed but rather enhanced by DPI. This indicates that the regulation of MAPKs by DPI is likely through multiple pathways, not being solely dependent on the Nox-ROS generation circuit. Together the results suggest that both mitochondria-derived and Nox-generated ROS contribute to the FIR-induced cellular ROS pool, which is the target of SOCS1. The ROS-mediated EMT signaling through the activation of Akt and Src is likely subject to regulation by SOCS1.

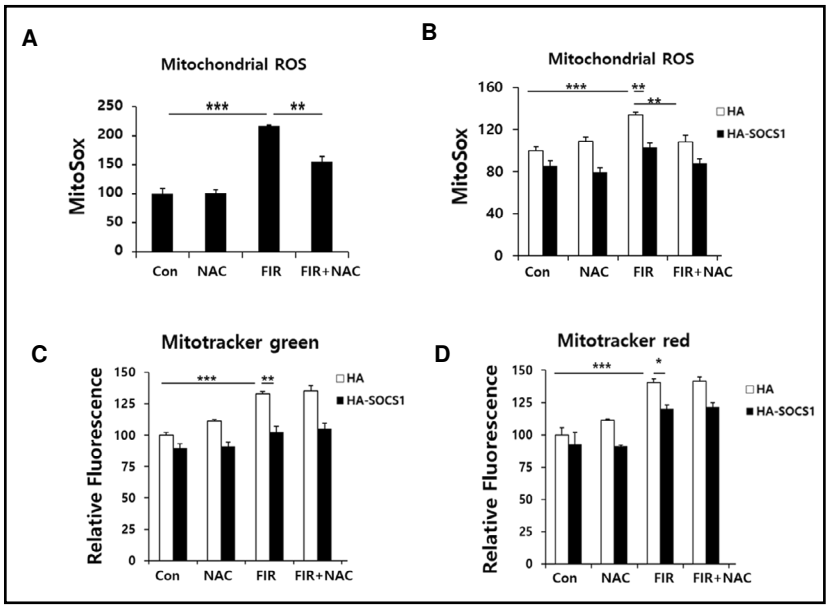

Fig. 6. Induction of mitochondrial ROS by FIR which is suppressed by SOCS1. (A) HCT116 $p53^{+/+}$ cells received FIR treatment were analyzed for the mitochondrial ROS levels at 30 min post irradiation by Mitosox staining as described in the text. (B) The HA vs HA-SOCS1 cells were compared for mitochondrial ROS generation upon FIR treatment as in panel A. (C and D) These HA & HA-SOCS1 cells were analyzed for total mitochondria as well as functional mitochondria by staining with Mitotracker green and Mitotracker red dyes, respectively. Data were obtained from 3 independent experiments performed in triplicate wells (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$).

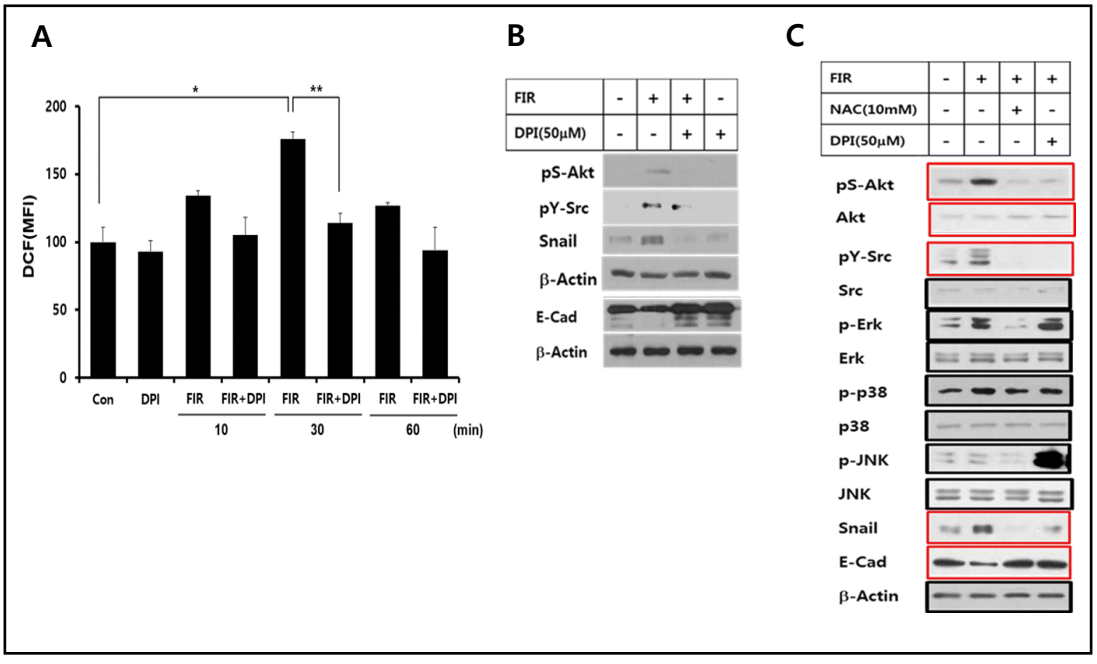

Fig. 7. Effects of Nox inhibitor on the FIR-induced ROS and EMT signaling. (A) HCT116 $p53^{+/+}$ cells received FIR with or without Nox inhibitor DPI treated 1 h prior to the last irradiation. Intracellular ROS levels were determined by FACS analysis for indicated time points. Results show representative data of independent experiments performed in triplicate wells (*, $p \leq 0.05$; **, $p \leq 0.01$). (B and C) Cells pretreated with DPI or NAC were analyzed by immunoblotting for the expression of EMT markers and associated signaling molecules as described.

Discussion

Over the years, fractionated ionizing radiation (FIR) administered at multiple low doses, has been established as a standard radiotherapy for solid cancers. This protocol is thought to cause less damage to normal cells by allowing DNA repair function better retained in normal cells, and thus expected to exert more selective killing of cancer cells [30]. However, a growing body of evidence indicates that FIR therapy induces radioresistance and may not be effective for controlling metastatic cancers of certain types. While the mechanisms of such radiotherapy-induced resistance remain largely unclear, the role of EMT for promotion of invasiveness and malignancy has been suggested in cancer resistance and recurrence after the radiation therapy in several cancer models [18-20]. The present investigation has thus focused on the elucidation of molecular mechanisms of FIR-induced EMT and the associated signaling pathways, which could be used to develop strategies to overcome the therapy-induced resistance.

We have first analyzed the role of ROS signal in the FIR-evoked intracellular signaling pathways leading to EMT, and then examined the regulatory function of SOCS1 on the FIR-induced EMT. Treatments of colorectal cancer cells with FIR at 2 Gy for 3 days induced a substantial increase in intracellular ROS levels in 30 min leading to EMT marker up-regulation as early as 2 h, which was sustained up to 24 h (Fig. 1 and Supplementary Fig. S1). Through a series of pathway inhibitor experiments, it was proposed that the EMT signaling induced by FIR proceeded through Akt→Src→Erk in ROS-dependent manners (Fig. 2). In a previous study both Src and Erk are shown to be the target of ROS activation during EMT induction [13]. In addition, the role of ROS in Akt activation under FIR has been suggested through the ROS-mediated PTEN inhibition and PI3K up-regulation associated with EMT in esophageal cancer cells [23]. The Akt activation under FIR has been also reported as an indirect response to GM-CSF induction noted after 24 h of FIR treatment leading to the invasiveness of lung cancer cells [18]. The results from the present study using colorectal cancer cells, however, indicate that the FIR-induced ROS mediates Akt activation by 2 h. The pretreatment of NAC blocked Akt activity induced by FIR (Fig. 1), whereas Akt-inhibiting LY294002 did not suppress the FIR-induced ROS generation (data not shown). These data suggest that FIR-mediated intracellular changes induce ROS generation, which leads to Akt activation and the downstream signaling pathways including Src and Erk for EMT. While Src-mediated activation of Akt through tyrosine phosphorylation has been reported in several cancer models, studies regarding the Akt-dependent Src regulation are very limited [31-33]. As no direct actions of Akt through the serine/threonine phosphorylation of Src are known, the observed Src activation downstream of Akt during the FIR-induced EMT signaling is likely an indirect event involving other mediators. In this regard, a recent report described both positive and negative regulation of Src activity by Akt in the endothelial barrier maintenance function via the cross-talk of TGF-beta and VEGF signaling, where an indirect regulation of Src by Akt has been proposed [34].

Our data also support the role of mitochondria and Nox enzymes, two major cellular ROS-generating systems for ROS induction upon FIR treatment. As shown in Fig. 6, FIR induced increases in mitochondrial ROS under the conditions applied to mediate EMT with substantial up-regulation of total mitochondrial mass (shown as Mitotracker green positivity) as well as mitochondria with functional ETC (shown as Mitotracker red positivity). In addition, the FIR-induced ROS was potently suppressed by treatment with a classic Nox inhibitor DPI, which then led to the inhibition of the FIR-induced EMT signals targeting Akt, Src, and Snail (Fig. 7). Drugs such as rotenone perturbing mitochondrial ETC [35] potently up-regulated intracellular ROS levels in cancer cells but did not induce EMT, supporting the role of functional or intact mitochondria for the induction of ROS-mediated EMT response under FIR (data not shown).

SOCS1 exerted anti-ROS and anti-EMT function in leukemic and colon cancer cell lines [13, 26]. In accordance with these actions, SOCS1 over-expressing cells exhibited dampened response to FIR for the induction of ROS and EMT marker expression with substantially in-

creased E-cadherin and reduced Snail levels (Fig. 3). A significant inhibition of pS-Akt, pY-Src and pS/Y-Erk was also noted in SOCS1-transfected cells. On the contrary, these EMT signal-associated parameters were all elevated in SOCS1-ablated cells with increased ROS levels under the basal and FIR-treated conditions (Fig. 4).

To investigate the mechanism by which SOCS1 suppresses the FIR-induced ROS levels and the ROS-mediated EMT signaling, we have examined the ROS-scavenging as well as ROS-generating systems as a target of SOCS1 action. In contrast to high basal levels of anti-oxidant enzymes such as SOD1 and TrxR1, HCT116/ p53 wt cells exhibited a relatively low level of Trx1 expression, which is selectively up-regulated by SOCS1 (Fig. 5A). Then, the role of Trx1 in both the ROS- and EMT-suppressing action of SOCS1 was assessed by Trx1 gene knock-down. Indeed, Trx1 gene ablation by shTrx1 caused ROS elevation in mock cells and a complete restoration of the SOCS1-mediated suppression of ROS levels accompanied with recovery of EMT response (Fig. 5B and 5C). As for the effect of SOCS1 on the ROS-generating system, the FIR-induced increase in mitochondrial ROS and in mitochondria with functional ETC was significantly reduced upon SOCS1 over-expression (Fig. 6). On the other hand, the basal level of functional mitochondria was substantially up-regulated in SOCS1-ablated cells (Supplementary Fig. S6).

As Nox enzymes have been implicated in the ROS-mediated Src activation and EMT induction in colon and breast cancer cells [36, 37], we then examined changes in Nox levels induced by SOCS1 to regulate FIR-induced ROS. Nox enzymes exist in multiple isoforms in cell-type specific manners, and transcriptional regulation of Nox isoforms occurs upon diverse cellular stimuli affecting the intracellular redox balance [38]. While Nox1 is thought to play a role in the oncogene-activated cancer cell malignancy, its expression levels were not readily detectable in colorectal cancer cells used in our study. Instead, the regulation of most of Nox isoforms was clearly seen in leukemic T cells, in which we have previously observed the ROS-suppressing function of SOCS1 [26]. It is noted that expression levels of 5 Nox isoforms (Nox 1, 3, 5 and Duox 1, 2) were down-regulated in cells over-expressing SOCS1 (Supplementary Fig. S7). Together our data suggest that anti-ROS function of SOCS1

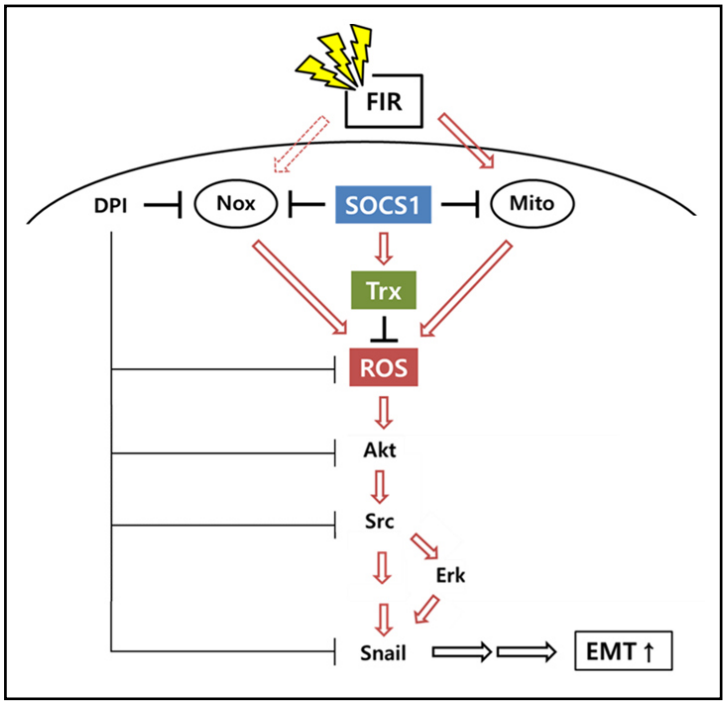

Fig. 8. A model for the FIR-induced ROS-mediated EMT signaling pathways and the inhibitory action of SOCS1 through the regulation of ROS-generating and ROS-scavenging systems. FIR triggers the induction of intracellular ROS derived from the action of Nox and functional mitochondria. ROS activates signaling pathways leading to EMT response through the Akt/Src/Erk pathways, resulting in increase in Snail with decrease in E-cadherin expression levels. SOCS1 suppresses ROS-mediated signaling through the induction of anti-oxidant factor Trx1 as well as through the down-regulation of Nox and mitochondria. The model depicts the critical role of ROS during the FIR therapy-induced EMT response and suggests the mechanism of anti-EMT function of SOCS1 by targeting both ROS-scavenging and ROS-generating systems to overcome the FIR-induced radioresistance associated with EMT.

involves both the up-regulation of ROS-scavenging enzyme as well as the down-regulation of ROS-generating systems, which in turn leads to anti-EMT response against FIR.

ROS are known to play diverse roles in cell survival, growth, and malignancy of tumor cells [10-12]. Among them, the ROS generated at physiological levels by radiations at low or intermediate doses likely acts to mediate the survival signal pathways to reduce cell death under genotoxic stress as shown in colorectal cancer cell systems (Supplementary Fig. S1). Our data further demonstrate that ROS acts as a key signaling molecule mediating the FIR-induced EMT response through the activation of Akt /Src/Erk pathways. While both the Nox-derived and mitochondria-derived ROS contribute to the FIR-triggered cellular ROS pool, SOCS1 reduces the FIR-induced ROS levels by suppressing these ROS-generating systems as well as up-regulating an anti-oxidant enzyme Trx1. Such bimodal ROS down-regulation exerted by SOCS1 may constitute a molecular mechanism of its anti-EMT action in colorectal cancer cells receiving FIR (Fig. 8). The present study thus proposes a potential anti-tumor effect of SOCS1 by blocking the therapy-induced resistance in certain invasive tumor models. Studies are now in progress to examine the vivo effects of SOCS1 in the FIR-induced EMT and radioresistance. Using nude mice models, the growth of tumors generated from implantation of SOCS1-transfected cells (HCT116/HA-SOCS1) vs mock cells (HCT116/HA) and their EMT responses with invasive properties induced under FIR therapy are under evaluation.

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Author Contributions

Sumin Kim conducted most of the experiments described in the text, including the irradiation, cell culture, gene transfection, Western and FACS analysis.

Seol-Hee Kim participated in the initial stage of this study including construction of sh-SOCS1 and performed qRT-PCR.

Choong-Eun Lee designed the study, supervised the experiments, analyzed the data with interpretation, and wrote the manuscript.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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