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IFNγ-mediated inhibition of cell proliferation through increased PKCδ-induced overexpression of EC-SOD

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Extracellular superoxide dismutase (EC-SOD) overexpression modulates cellular responses such as tumor cell suppression and is induced by IFNγ. Therefore, we examined the role of EC-SOD in IFNγ-mediated tumor cell suppression. We observed that the dominant-negative protein kinase C delta (PKCδ) suppresses IFNγ-induced EC-SOD expression in both keratinocytes and melanoma cells. Our results also showed that PKCδ-induced EC-SOD expression was reduced by pretreatment with a PKC-specific inhibitor or a siRNA against PKCδ. PKCδ-induced EC-SOD expression suppressed cell proliferations by the up-regulation of p21 and Rb, and the downregulation of cyclin A and D. Finally, we demonstrated that increased expression of EC-SOD drastically suppressed lung melanoma proliferation in an EC-SOD transgenic mouse via p21 expression. In summary, our findings suggest that IFNγ-induced EC-SOD expression occurs via activation of PKCδ. Therefore, the upregulation of EC-SOD may be effective for prevention of various cancers, including melanoma, via cell cycle arrest. [BMB Reports 2012; 45(11): 659-664]

INTRODUCTION

Reactive oxygen species (ROS) are important second messengers for the induction of various genes in a variety of physiological and pathological conditions. Mammals and most chordates express three distinct isoforms of superoxide dismutase (SOD). SOD1 (Cu/Zn-SOD) is located predominantly in the cytoplasm and contains copper and zinc, whereas SOD2 (Mn-SOD) is located in the mitochondrial matrix of cells and contains manganese in its active site. The third form of SOD, extracellular SOD (EC-SOD), contains copper and zinc in its active site and is present in extracellular matrix fluids, such as plasma, lymph, and synovial fluid, (1). Previous studies have revealed the key roles of SOD isozymes in various physiological and pathological processes (2). A number of studies have implicated SODs in the modulation of tumor cell proliferation and apoptosis (3, 4). Recently, adenoviral expression of EC-SOD was found to efficiently suppress the growth of melanoma and breast carcinoma cells (5, 6). Furthermore, it has been demonstrated that inflammatory cytokines such as IFN-γ, IL-4, and IL-1α, up-regulate the expression of EC-SOD protein and mRNA in human skin fibroblasts and vascular smooth muscle cell (7, 8). Conversely, EC-SOD is down-regulated by both TNF-α and TGF-β (7, 8). However, the specific molecular mechanisms remain to be investigated, particularly with regard to IFN-γ-induced EC-SOD expression.

IFNγ is implicated in a variety of biologic functions, including anti-tumor effects, induction of cell differentiation, and regulation of gene expression (9). The participation of protein kinase C (PKC) isoenzymes in the regulation of IFNγ-induced responses has been reported in various systems (10, 11). The PKC families, including cPKC (α, βI, βII, and γ), nPKC (δ, ε, η, and θ), and aPKC (ζ and λ/ι), are involved in the regulation of cell proliferation, differentiation, and apoptosis (12). PKCα and PKCε have been primarily associated with anti-apoptotic effects in various systems (13, 14) whereas PKCθ and PKCδ have been implicated in pro-apoptotic cascades (15, 16). Furthermore, it has been reported that the anti-tumor effect of IFNγ is via the PKCδ pathway (17). Thus, a reduction in the level of PKCδ protein or activity may be important for tumor formation.

IFNγ elicits PKCδ-dependent anti-tumor effects, and induces EC-SOD expression, which also has an anti-tumor effect. Therefore, we investigated the relationship between IFNγ, PKCδ, and EC-SOD on tumor cell suppression.We showed that up-regulation of EC-SOD by IFNγ is PKCδ-dependent and that the anti-tumor effect of PKCδ is mediated by the EC-SOD.

RESULTS

Up-regulation of EC-SOD expression by IFNγ

It has been reported that IFNγ induces EC-SOD expression (8). Because PKCδ is regulated by the IFN signaling pathway and is implicated in the regulation of cellular processes, including the cell cycle and apoptosis (17, 18), we hypothesized that IFNγ might influence EC-SOD expression by inducing PKCδ activation. We found that EC-SOD expression was up-regu-

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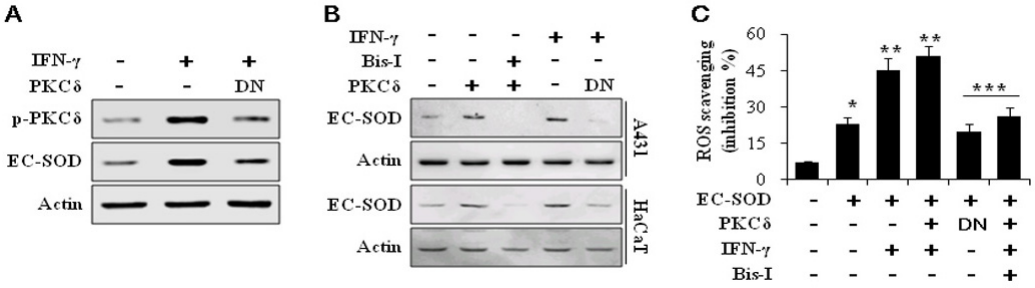


Fig. 1. EC-SOD expression is up-regulated by the IFNγ-PKCδ pathway. (A) A375 cells were transfected with either empty vector or dominant-negative PKCδ. The cells were stimulated with IFNγ, and the expression of phospho-PKCδ and EC-SOD was assessed by Western blot analysis. (B) A431 or HaCaT cells were transfected with wild-type or dominant-negative PKCδ, and the cells were incubated in the presence or absence of either Bis-I or IFNγ. EC-SOD expression was assessed by Western blot analysis. (C) A375 cells were transfected with EC-SOD and either wild-type or dominant-negative PKCδ, and the cells were cultured in the presence or absence of either Bis-I or IFNγ. EC-SOD enzyme activity was measured by ROS scavenging activity using WST-1 reagents as described in Materials and Methods. IFN-γ activated PKCδ and increased EC-SOD expression. EC-SOD expression was decreased after transfection with DN-PKCδ or treatment with Bis-I. IFN-γ and PKCδ increased the ROS scavenging activity of EC-SOD, and DN-PKCδ or Bis-I decreased this activity. Results are representative of three experiments, showing the mean ± SD, *P < 0.001 versus control group, **P < 0.001 versus EC-SOD-transfected group, ***P < 0.001 versus IFNγ-stimulated group.

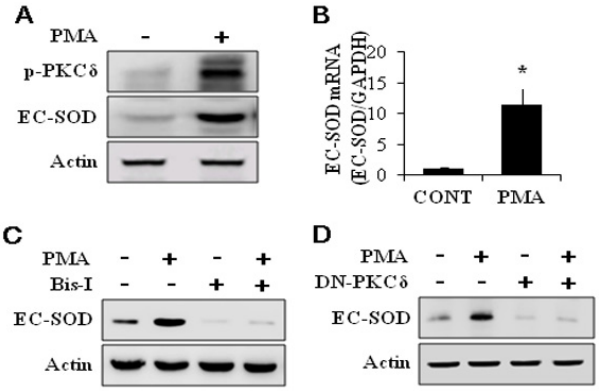


Fig. 2. PMA up-regulates EC-SOD expression. (A) A375 cells were stimulated with 40 nM PMA for 24 h, and the expression of phospho-PKCδ and EC-SOD was assessed by Western blot analysis. (B) EC-SOD mRNA expression was measured using quantitative real-time PCR. (C) A375 cells were stimulated with PMA in the presence or absence of Bis-I and EC-SOD expression was assessed by Western blot analysis. (D) A375 cells were transfected with dominant-negative PKCδ, and the cells were stimulated with PMA. EC-SOD expression was assessed by Western blot analysis. Results are representative of three experiments, showing the mean ± SD, *P < 0.001.

lated in IFNγ-treated melanoma cell lines (A375). To investigate the effects of IFNγ on EC-SOD gene expression, A375 cells were treated with exogenous IFNγ. The treatment drastically elevated the expression levels of EC-SOD protein; IFNγ-induced EC-SOD up-regulation was inhibited by transfecting dominant-negative PKCδ (DN-PKCδ) in these cells (Fig. 1A). We next examined the effect of IFNγ on EC-SOD expression in human squamous carcinoma cells (A431) and keratinocytes (HaCaT). EC-SOD expression was up-regulated in both cell lines after either IFNγ stimulation or PKCδ transfection; this up-regulation was suppressed following treatment with bis-indolylmaleimide-I (Bis-I; a PKC inhibitor) or transfection with DN-PKCδ (Fig. 1B). We investigated whether overexpressed EC-SOD was enzymatically active by measuring its ROS scavenging activity. The ROS scavenging activity of EC-SOD was increased after PKCδ transfection and/or IFNγ stimulation, and these activities were decreased upon treatment with Bis-I or transfection with DN-PKCδ in A375 cells (Fig. 1C). These results indicate that the up-regulation of EC-SOD expression is mediated by either the IFNγ/PKCδ or PKCδ pathway and the that effect is not cell-line dependent.

PKCδ-dependent EC-SOD up-regulation

PKCδ is activated by IFNγ through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and is associated with ROS production in a nicotinamide adenine dinucleotide phosphate oxidase-dependent manner in phagocytic cells (17, 19). Therefore, we postulated that PKCδ might up-regulate the expression of EC-SOD. To confirm this, we first stimulated A375 cells with phorbol 12-myristate 13-acetate (PMA) to activate PKCδ. EC-SOD expression could be detected in quiescent cells, whereas PMA stimulation dramatically increased the levels of EC-SOD protein and mRNA (Fig. 2A and B). However, EC-SOD expression in these cells was inhibited upon treatment with Bis-I and transfection with DN-PKCδ (Fig. 2C and D). These results suggest that PKCδ is an important mediator in the up-regulation of EC-SOD expression, because both IFNγ and PMA can activate PKCδ by distinct pathways.

To investigate the direct effect of PKCδ on EC-SOD expression, we determined EC-SOD mRNA levels by quantitative real-time RT-PCR after PKCδ transfection in A375 cells. We ob-

served that EC-SOD mRNA levels were markedly increased compared to empty vector- transfected cells, similar to levels observed after PMA stimulation (Fig. 3A). To better understand PKCδ-dependent EC-SOD up-regulation, we evaluated EC-SOD expression following treatment with the PKC inhibitor, Bis-I, in A375 cells transfected with EC-SOD and PKCδ. The up-regulated EC-SOD expression induced by transfection with EC-SOD and PKCδ was inhibited upon treatment with Bis-I (Fig. 3B). In addition, the up-regulated EC-SOD expression induced by PKCδ was decreased by transfection of PKCδ siRNA, and the ROS scavenging activity was decreased upon either transfection with DN-PKCδ or treatment with Bis-I (Fig 3C and D). The results shown in Fig. 3 clearly demonstrate that the up-regulation of EC-SOD expression is regulated by PKCδ and that overexpressed EC-SOD by PKCδ is enzymatically active.

Inhibitory effect of EC-SOD on cell proliferation

We further investigated the effect of EC-SOD on cell proliferation because previous studies stated that EC-SOD inhibits the growth of certain types of cancer cells (5, 6). To assess cell proliferation, we performed the trypan blue exclusion assay after transfection with either empty vector or EC-SOD plasmid in various cell lines. EC-SOD transfection or IFNγ stimulation decreased the number of viable cells in all of the tested cell lines, and this proliferation inhibitory effect was increased by treatment with IFNγ in EC-SOD-transfected cells (Fig. 4A). We used PI staining to determine whether the inhibited proliferation resulted from cell cycle arrest. Fig. 4B shows that the transfection of EC-SOD plasmid induced G0/G1 cell cycle arrest. To investigate whether apoptosis occurs after EC-SOD plasmid transfection, a Western blot analysis was done using A375 cells. The expression of p21 was up-regulated by EC-SOD, which inhibits cell cycle progression. Furthermore, EC-SOD suppressed the expression of cyclins A and D, which regulates cell cycle progression. On the other hand, the level of pro-apoptotic molecules, Bax, Bad, and cleaved caspase-3, was not changed by the transfection of EC-SOD plasmid (Fig. 4C). Treatment with IFNγ also increased p21 and Rb but decreased Cyclin A and D like EC-SOD plasmid transfection, confirming that the anti-tumor effect of IFNγ might be mediated by EC-SOD (Fig. 4D). These results suggest that EC-SOD inhibits cell proliferation through cell cycle arrest by up-regulating the cyclin-dependent kinase (CDK) inhibitor protein and down-regulating cell cycle progression proteins without affecting apoptotic signaling.

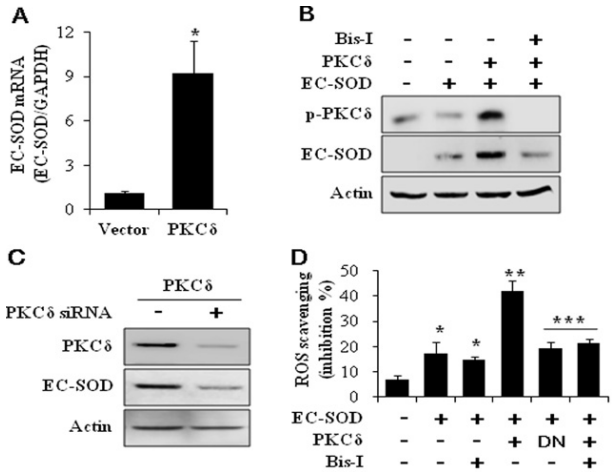


Fig. 3. EC-SOD expression is up-regulated by PKCδ. (A) A375 cells were transfected with either empty or PKCδ vector, and EC-SOD mRNA expression was measured using quantitative real-time PCR. (B) A375 cells were transfected with EC-SOD and PKCδ, and the cells were incubated with or without Bis-I for 24 h. The expression of phospho-PKCδ and EC-SOD was assessed by Western blot analysis. (C) PKCδ-transfected A375 cells were transfected with PKCδ siRNA for 24 h, and the expression of PKCδ and EC-SOD was assessed by Western blot analysis. (D) A375 cells were transfected with EC-SOD and either wild-type or dominant-negative PKCδ, and then incubated in the presence or absence of Bis-I for 24 h. EC-SOD enzyme activity was measured by ROS scavenging activity using WST-1 reagents. Results are representative of three experiments, showing the mean ± SD, *P < 0.001 versus control group, **P < 0.001 versus EC-SOD-transfected group, ***P < 0.001 versus both EC-SOD and PKCδ-transfected group.

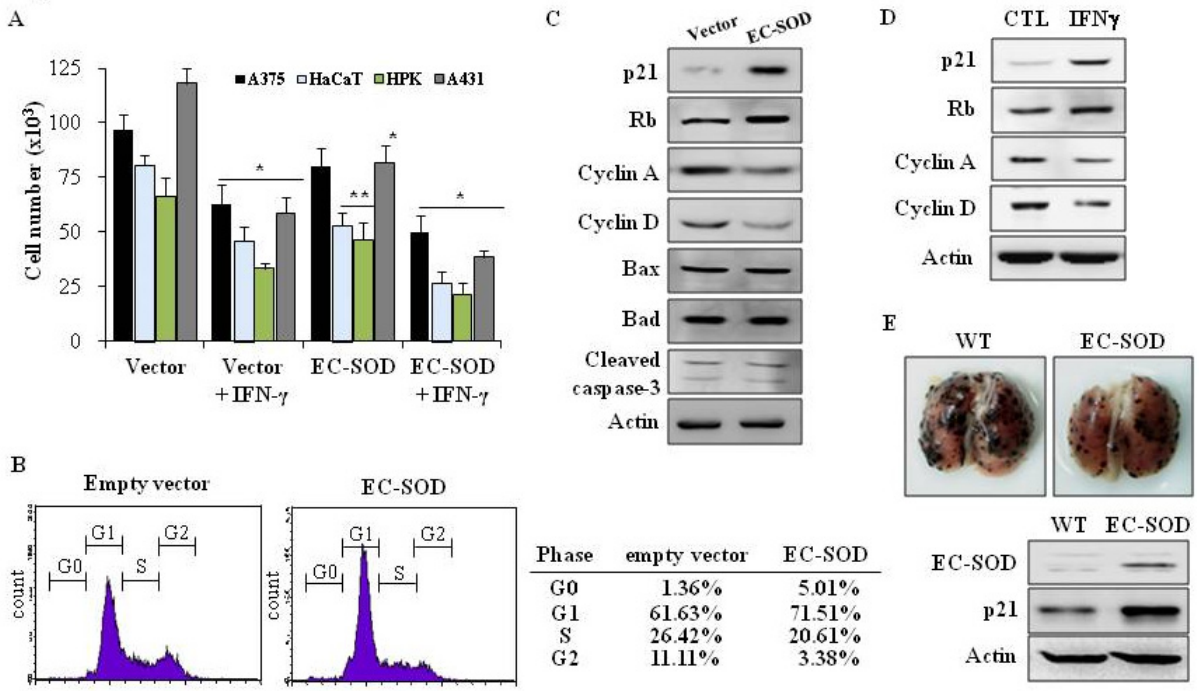
Inhibitory effect of EC-SOD on melanoma tumorigenesis

To evaluate the effects of EC-SOD on tumor cell growth *in vivo,* we injected B16F10 mouse melanoma cells into the tail veins of EC-SOD transgenic mice, and quantified the number/size of metastatic melanoma nodules on the surface of the lungs. On day 14 following B16F10 injection, nodule growth drastically decreased in the lungs of the EC-SOD transgenic (EC-SOD TG) mice. However, the number of melanoma nodules in EC-SOD-TG mouse lungs did not significantly differ from the control mice. Moreover, the expression of p21 was increased in EC-SOD TG mice (Fig. 4E). Therefore, we concluded that EC-SOD mediates its inhibitory effects on cell proliferation and tumorigenesis via cell cycle arrest by up-regulating p21 expression.

DISCUSSION

EC-SOD is generated by many tissues, playing a significant role in vascular pathologies, hypertension, and metastases (1, 20). Overexpressed EC-SOD inhibits tumor formation and cell proliferation in various types of cancer cells (6, 21), suggesting that EC-SOD may function as a new type of tumor suppressor.

It has been observed that EC-SOD levels are significantly elevated in IFNγ-treated fibroblasts (7, 8), and IFNγ has been implicated in the regulation of PKCδ as a tumor suppressor. These findings imply that downstream substrates of PKCδ could be targets for therapeutic intervention in cancer (17,18, 22). We have explored the hypothesis that PKCδ communicates with cell growth inhibition machinery via the activation of EC-SOD. We showed that the EC-SOD overexpressed by



Phase	empty vector	EC-SOD
G0	1.36%	5.01%
G1	61.63%	71.51%
S	26.42%	20.61%
G2	11.11%	3.38%

Fig. 4. EC-SOD inhibits cell proliferation and melanoma tumorigenesis. (A) Various cells (5×10^4 cells per well in 12 well plate) were transfected with either empty vector or EC-SOD plasmid, and the cells were cultured in the presence or absence of IFNγ for 24 h. Viable cells were counted using the trypan blue exclusion assay. (B) A375 cells were transfected with either empty or EC-SOD vector for 24 h. PI staining and FACS analysis was performed to determine whether the inhibited proliferation resulted from cell cycle arrest. Expression of EC-SOD induced G_0/G_1 cell cycle arrest. (C) A375 cells were transfected with either empty or EC-SOD vector for 24 h, and the cells were assessed by Western blot analysis for the indicated molecules. (D) A375 cells were treated with IFNγ for 24 h and then analyzed by Western blot analysis. Treatment with IFNγ increased p21 and Rb but decreased Cyclin A and D like EC-SOD plasmid transfection. (E) B16F10 cells were injected into the tail veins of wild-type and EC-SOD TG mice, and tumor nodules were counted on day 14 in the mouse lungs (upper panel). Lysates of mouse lungs were assessed by Western blot analysis (lower panel). Results are representative of three experiments, showing mean ± SD, *P < 0.001; **P < 0.05.

PKCδ inhibits cell proliferation through cell cycle arrest. We further determined that p21 levels were significantly elevated in EC-SOD-over-expressed cells, whereas cyclin D levels were decreased. In addition, EC-SOD suppressed melanoma growth via an increase in the expression of p21 in EC-SOD TG mice.

p21 has been found to be a major target of p53; it was initially thought that p21 would be of similar importance in human cancers. Additionally, overexpression of p21 inhibits cancer cell proliferation in mammalian cells and can inhibit all cyclin-CDK complexes. Accordingly, it is possible to develop reagents targeting p21 to achieve beneficial effects in cancer therapy. Although the significance of p21 has been investigated in several different cancers (lung, gastric, head and neck, and ovarian), clinical effects have been mixed. Several anticancer agents such as statin (23), cisplatin (24), and NS-398 (25) and B_lapachone (26) exhibit profound anti-proliferative effects via p21 induction and are being investigated for their anti-tumorigenic activities. However, these DNA-damaging agents have major side effects on proliferating tissues and may lead to secondary cancer due to an induction of further mutations in precancerous and healthy cells. Therefore, greater understanding of the molecular mechanisms of EC-SOD should help improve anti-tumor efficacy and decrease the side effects of cancer therapy. Recently, we determined that EC-SOD might bind to kinases. Thus, it is possible that EC-SOD modulates gene expression by regulating kinase activity. Much further research is needed to address this last question.

In summary, we identified the mechanisms by which PKCδ inhibits cell proliferation and melanoma tumorigenesis. EC-SOD was up-regulated in a PKCδ-dependent manner and inhibited cell proliferation via cell cycle arrest through the p21 pathway. Therefore, our findings regarding PKCδ-induced EC-SOD expression and its downstream signaling cascade might be used to identify potential targets for cancer therapy.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine se-

rum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Gibco BRL (Rockville, MD, USA). Anti-EC-SOD antibody was purchased from AbCAM (Cambridge, UK). The anti-phosphorylated PKC, anti-cyc A, anti-cyc D, anti-Bax, anti-Bad and anti-Caspase-3 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Furthermore, the anti-RB and anti-p21 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Western blotting luminal reagent was purchased from Santa Cruz Biotechnology (SantaCruz, CA, USA).

Cell culture

The cell lines (HaCaT, A375, and A431) were grown as monolayer cultures in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% (v/v) CO_2 atmosphere. The cells were seeded at 1.0×10^6 cells per 100-mm plate.

Transfection

Transient transfections were performed using a Qiagen kit from Qiagen (Santa Clara, CA, USA) according to the manufacturer's protocol. The transfections were allowed to proceed for 24 h, and the transfected cells were washed with 4 ml phosphate buffered saline (PBS) and stimulated with IFNγ. The cells were continually cultured in DMEM and finally harvested. The PKCδ plasmid was provided by Jae-Won Soh (Inha University, Korea)

ROS scavenging effect

The EC-SOD plasmid was transfected into 293E cells with or without the PKCδ plasmid. After 24 hours of transfection, the supernatant was collected, and the activity of EC-SOD was determined using the water-soluble tetrazolium WST-1 reagent (Dojindo, Japan) according to the manufacturer's instructions to monitor the ROS scavenging effect.

Western blot analysis

Western blot was performed as previously described (27). Briefly, cells were lysed with radioimmunoprecipitation assay buffer (2 mM EDTA, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM sodium vanadate, 10 mM NaF, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail) and harvested immediately. The samples were loaded onto sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes obtained from Millipore (Bedford, MA, USA). After membranes were blocked, they were incubated with specific primary antibodies overnight at 4°C with gentle agitation. The membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Bands were detected using ECL Plus Western blotting detection reagents from Amersham Biosciences Co (Piscataway, NJ, USA).

Flow cytometry

For cell cycle analysis, subconfluent A375 cells were transiently transfected with the empty vector or EC-SOD plasmid. After 24 hours of transfection, the cells were harvested, fixed, permeabilized, stained with propidium iodine (PI), and then subjected to flow cytometry (Becton Dickinson FACScan, San Jose, CA) to evaluate the fluorescence intensity as previously described (28).

Animal studies

C57BL6 and EC-SOD transgenic mice were used for experiments. Generation of EC-SOD transgenic mice was previously described (29). The animals were housed and maintained in a barrier facility at the Institute for Animal Studies, School of Medicine, Catholic University of Korea. All animal protocols used in this study were approved by the Catholic Research Institute of Medical Science Committee for Institutional Animal Care and Use. All mice were maintained under a 12-h light/dark cycle, and the animal had *ad libitum* access to food and water. Melanoma cells (5×10^5 cells in 100 μl PBS) were injected intravenously into the tail vein of the mice. Lung specimens were collected at various time points (8, 12, and 14 days) after injection, and the effects of EC-SOD on the growth of melanoma nodules were investigated.

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