*J. Biochem.* 2009;146(3)307–315
doi:10.1093/jb/mvp070

# Nestin Serves as a Prosurvival Determinant that is Linked to the Cytoprotective Effect of Epidermal Growth Factor in Rat Vascular Smooth Muscle Cells

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Received April 14, 2009; accepted April 16, 2009; published online May 18, 2009

**Nestin is an intermediate filament protein mainly expressed in muscle and neural progenitors. Recently, we reported that nestin is expressed in rat vascular smooth muscle cells (VSMCs), disappears after serum-deprivation and then is re-expressed again following EGF stimulation. As the function of nestin in VSMCs remains unknown, its anti-apoptotic function was investigated in this study. We first showed that cell viability of nestin-depleted cells following $H_2O_2$ treatments decreased by nestin RNAi. Further DNA laddering analysis and flow cytometry results demonstrated that this loss of cell viability was mediated through apoptosis. In addition, caspase-9, caspase-3 and PARP were activated in nestin-depleted VSMCs following $H_2O_2$ treatments, indicating that nestin has an upstream inhibitory effect on caspase activation. It is well known that EGF serves as a survival factor in rat VSMCs. Here, we show that the cytoprotective effect of EGF was prevented by nestin RNAi. In addition, the inhibition of Cdk5 prevented Bcl-2 phosphorylation and enhanced $H_2O_2$-induced caspase-3 activation as well as subsequent DNA fragmentation. Taken together, these results provide evidence for another cytoprotective role of EGF in that it is mediated through its stimulation of nestin expression which leads to the prevention of caspase activation by Cdk-5-induced Bcl-2 phosphorylation in rat VSMCs.**

**Key words: nestin, apoptosis, EGF, caspase, CDK5, vascular smooth muscle cells.**

Abbreviations: CNS, central nervous system; IF, intermediated filament; VSMCs, vascular smooth muscle cells; EGF, epidermal growth factor; RNAi, RNA interference.

Nestin is a type VI intermediate filament (IF) protein mainly expressed in myogenic and neural stem cells, as well as in their immature descendants during development (*1–3*). Nestin expression is generally ceased in mature cells, but resumes following injuries (*4–6*). In skeletal muscle cells, it was reported that after shearing or in situ injuries, nestin reappears in myocytes of rat gastrocnemius muscle and its expression reaches maximal level at 3–5 days post-injury, then become downregulated thereafter (*6*). In the central nervous system (CNS), nestin reappears in reactive astrocytes immediately adjacent to wound regions during the early stages of CNS injury response (*1–5*). As a member of the IF cytoskeleton, nestin is generally assumed to function as a mechanical integrator to cells (*7*) by assembling with other IFs such as vimentin and α-internextin which cannot form filaments by itself (*8*). Furthermore, nestin has been shown to play an important part in the regulation of the structural dynamics of vimentin IFs through vimentin phosphorylations during mitosis in BHK cells (*9*). Besides, a recent nestin RNAi study has revealed that knockout of nestin obviously attenuated the proliferation of mesangial cells following mesangial injuries in the anti-Thy1 nephritis model (*10*), indicating that nestin plays an important role in the promotion of cell proliferation. Other than providing mechanical support and promoting proliferation, nestin was recently reported to exhibit cytoprotective functions in ST15A neural stem cells, by acting as a scaffold for Cdk5 wherein it prevents Cdk5-dependent apoptosis by the sequestering of Cdk5/p35 complexes (*11*). These results implied that during the early post-injury phase, the re-expression of nestin may play an important role in maintaining cell survival and subsequently promoting cell proliferation. Regarding the expression of nestin in VSMCs, our recent report revealed that nestin is expressed in the primary cultures of rat VSMCs and this expression of nestin ceases following serum deprivation, as correlated to the VSMC phenotypic switch (*12*). These findings raised our interest to investigate whether

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or not nestin is involved in the cytoprotective function of VSMCs.

Studies have indicated that epidermal growth factor (EGF) serves as a surviving factor in rat VSMCs (*13*, *14*). By using the dominant negative mutant of Ras, Miyamoto *et al*. (*13*) revealed that EGF failed to protect rat VSMCs against apoptosis. Later, a recent study conducted by Ying *et al*. (*14*) showed that the inhibition of ERK by U0126 significantly abolished the anti-apoptotic function of EGF. These results indicated that the cytoprotective effect of EGF is mainly mediated *via* the Ras-ERK signalling pathway in rat VSMCs. Over the years, two major routes, the caspase-8-dependent and the caspase-9-dependent pathways have been described for the caspase-3-related apoptosis (*15*). Oxidative stress (e.g. $H_2O_2$)-induced apoptosis in many cultured cells including VSMCs usually involves the caspase-9 pathway (*16*, *17*). In our recent report, we showed that EGF upregulates nestin expression *via* the Ras-ERK-dependent pathway in rat VSMCs (*12*). Thus, the present study aims to investigate the cytoprotective function of nestin in rat VSMCs and the involvement of caspases in this cytoprotection effect. Moreover, whether EGF serves as a surviving factor for rat VSMCs through the induction of nestin expression was also examined.

## MATERIALS AND METHODS

*Reagents and Antibodies*—EGF, propidium iodide and MTT reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). A mouse anti-nestin monoclonal antibody (clone 401) was purchased from PharMingen (San Diego, CA, USA). Rabbit anti-cleaved caspase-9, caspase-3 and PARP polyclonal antibodies were purchased from Cell signalling (Beverly, USA). Mouse anti-Bcl-2 and rabbit anti-Cdk5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-phosphoserine was purchased from Calbiochem (La Jolla, CA, USA).

*Rat Aortic Smooth Muscle Cell Culture*—Cell cultures of rat aortic VSMCs were prepared using an explant methodas described previously (*12*) and those VSMCs at passages 4–10 were used in all experiments.

*Whole-Cell Extractions and Western Blotting Analysis*—Whole-cell lysates were isolated from rat VSMCs as described previously (*12*). Protein concentration was measured by the Bio-Rad method using bovine serum albumin as standard reference. Thirty micrograms of whole-cell lysates were separated by 7.5–15% SDS–polyacrylamide gel electrophoresis and processed for immunoblotting.

*Small Interfering RNA Knockdown Assay*—Rat VSMCs at 80% confluency were trypsinized and centrifuged. The harvested cells were re-suspended in 100 μl of PBS and electroporated in the presence of 2.5 μg nestin small interfering RNA (AAG AUG UCC CUU AGU CUG GAG) or scrambled negative control (Amaxa Biosystems, Kölin, BRD). These transfected cells were then plated onto 96-well plates to detect cell viability or onto 6-cm dishes for immunoblotting, DNA fragmentation analysis and flow cytometry. The effects of siRNAs were examined 72 h after transfection.

*Cell Viability Assay*—Rat VSMCs in a 96-well plate were subjected to treatments of $H_2O_2$. To access the viability of cells, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent dissolved in PBS (0.04 mg/ml) was added to each well and incubated for 4 h. One hundred microlitres of dimethyl sulphoxide was also added to breach the cells and to dissolve the formazan crystals, and the absorbance was then measured with a microplate reader at the wavelength of 595 nm.

*DNA Laddering Analysis and Flow Cytometry*—Genomic DNA was isolated from rat VSMCs using a genomic DNA isolation reagent kit (Genomarker, Taipei, ROC) and then separated by electrophoresis on a 1.5% agarose gel for 2 h at 50 V. The gel was stained with ethidium bromide and photographed with an ultraviolet gel documentation system. For flow cytometry, floating and adherent VSMCs were collected and then fixed in 70% ethanol at 4°C. Following the incubation in PBS containing 40 μg/ml of propidium iodide and 100 μg/ml of RNase A for 1 h at room temperature in the dark, stained nuclei were analysed on a FACScan machine (BD Biosciences, CA, USA).

*Immunoprecipitation*—Whole-cell lysates (200 μg) were incubated for 24 h with 1 μl of anti-Cdk5 or anti-Bcl-2 antibodies, followed by adding 20 μl of agarose protein A/G beads for another 2–4 h at 4°C. The immunoprecipitated complexes were then assayed by immunoblotting with anti-nestin, anti-Bcl-2, or anti-phosphoserine antibodies.

*Statistic Analysis*—All data were presented as means ± SE or as proportions when compared to the untreated samples. Two-tailed *t*-tests and ANOVA, followed by the Bonferroni procedure, were used to test the differences in continuous variables. $P < 0.05$ was considered statistically significant.

## RESULTS

*Nestin Plays a Role in Maintaining Cell Survival in Rat VSMCs*—It has been reported that by acting as a scaffold for the Cdk5/p35 complexes, nestin serves as a prosurvival determinant in ST15A cells (*11*). To determine the cytoprotective role of nestin in rat VSMCs, these cells were transfected with scrambled and nestin-siRNA, and then treated with various concentrations of $H_2O_2$. As shown in Fig. 1A, nestin expression was significantly depleted by nestin RNA interference (RNAi). Cell viability slightly decreased among these nestin-depleted cells (by 24.22% ± 4.2%), and was almost completely abolished in response to treatment with 250 μM $H_2O_2$ (70.5% ± 4.3% *vs*. 24.1% ± 4.2% decreases, $n = 3$, $P < 0.05$), as demonstrated by the MTT assay. Treatments with 250 and 500 μM $H_2O_2$ revealed almost identical results in the loss of cell viability as shown as in nestin-depleted rat VSMCs (Fig. 1B), suggesting that nestin may be an essential participant in maintaining of cell survival under treatment wtih 250 μM $H_2O_2$. In order to determine whether the loss of cell viability is mediated by apoptosis, a DNA laddering analysis and flow cytometry were used to examine the two hallmarks of apoptosis in rat VSMCs following treatment with various concentrations of $H_2O_2$. Consistent with findings

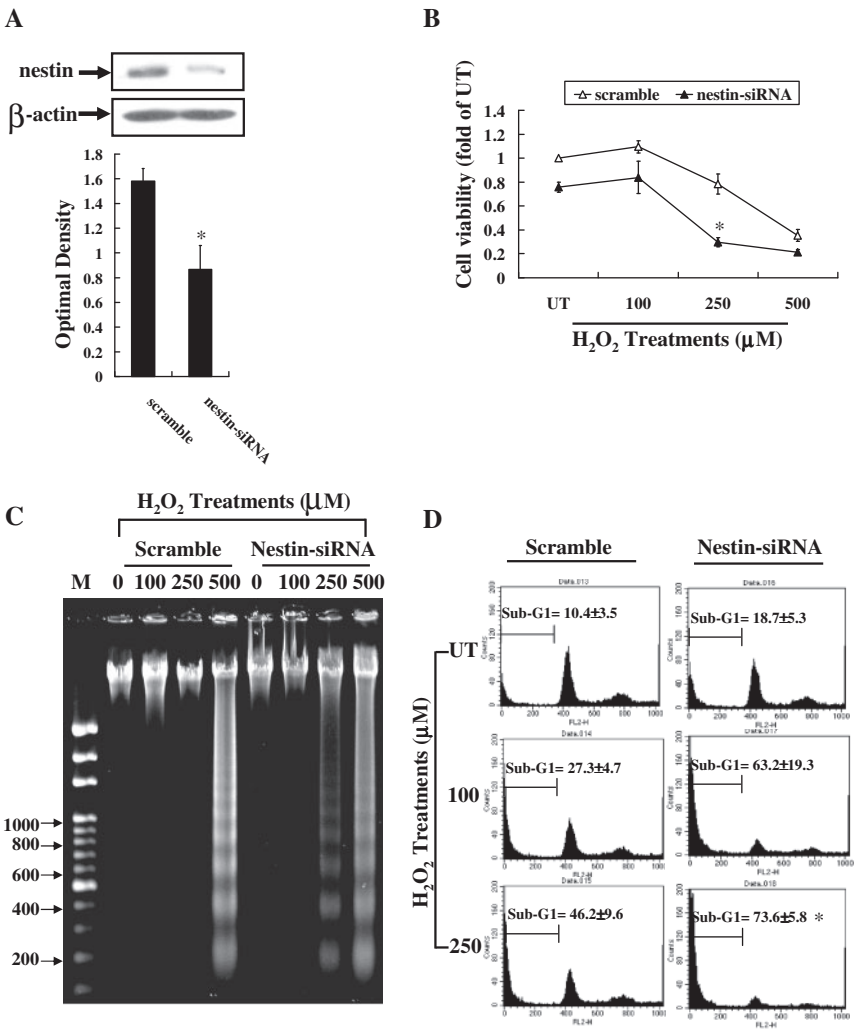


Fig. 1. **Nestin attenuates $H_2O_2$-induced apoptosis in rat VSMCs.** Rat aortic VSMCs were transfected with scrambled or nestin siRNA, and then seeded in a 6-cm dishes (A, C and D) or a 96-well plates (B). (A) IF-enriched preparations were isolated from these cells, then immunoblotted with nestin to ensure siRNA transfection efficiency, and re-probed against β-actin to show equal loading amounts. (B–D) Transfected cells were treated with 100, 250 or 500 μM of $H_2O_2$ for 24 h. Cell viability was assessed by an MTT assay (B). Apoptotic effects were tested by DNA laddering analysis (C) and flow cytometry (D). Results are shown as multiples of the scrambled siRNA control (set to 1.0). The data are shown as the mean ± SE ($n = 3$; $^*P < 0.05$ *vs*. the scrambled siRNA group). SF, serum-free; UT, untreated; M, marker.

from the MTT assay, both DNA laddering and flow cytometric results showed that nestin RNAi leads to DNA fragmentation (Fig. 1C) and also to an increase in the apoptotic sub-G1 population (Fig. 1D). Each of these two nestin RNAi effects reached a prominent level under the treatments with 250 μM $H_2O_2$. Taken together, the above results clearly indicated that the downregulation of nestin enhances the $H_2O_2$-induced apoptotic cell death in rat VSMCs.

*Nestin Attenuation of Rat VSMC Apoptosis is Caspase-Dependent*—Recent studies demonstrated that the $H_2O_2$-induced apoptosis is mediated by mitochondrial-dependent pathways via caspase-3 activation in VSMCs (*16*, *17*). In this study, antibodies against the active forms of caspase-9 and caspase-3 were applied to rat VSMCs following treatments with 250 and 500 μM $H_2O_2$. Results showed that while $H_2O_2$ at 250 μM failed to induce caspase-9 activation in scramble-transfected rat VSMCs, it successfully triggered caspase-9 activation in nestin-depleted rat VSMCs (Fig. 2). We then assessed the involvements of caspase-3 in nestin's protective effect. Results showed that the active form of caspase-3 was detected in nestin-depleted rat VSMCs, but not in scrambled-transfected cells under treatment with 250 μM $H_2O_2$ (Fig. 2). To further determine whether caspase-3 activity is involved in the nestin protection phenomenon of rat VSMCs under 250 μM $H_2O_2$ treatment, an antibody against poly[ADP-ribose] polymerase (PARP), a substrate of caspase-3, was used. Results showed that high expression of the cleaved form of PARP was observed in nestin-depleted rat VSMCs upon stimulation with 250 μM $H_2O_2$ (Fig. 2). Taken together, the above observations indicate that nestin attenuates the $H_2O_2$-induced apoptosis through caspase-3 activation.

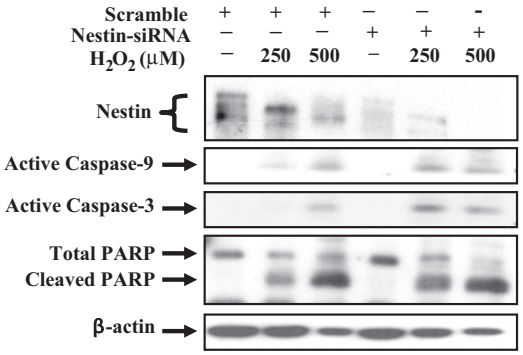


Fig. 2. **Nestin attenuates $H_2O_2$-induced apoptosis though caspase-dependent pathways.** Rat aortic VSMCs were transfected with scrambled or nestin siRNA, then seeded in 6-cm dishes, followed by treatment with 250 or 500 μM of $H_2O_2$ for 24 h. Whole-cell lysates were extracted from these cells, then immunoblotted with nestin, caspase-9, caspase-3 and PARP to detect these protein expressions and re-probed against β-actin to show equal loading amounts.

*The Cytoprotective Effect of EGF is Largely Mediated through the Induction of Nestin Expression in rat VSMCs*—To examine whether the EGF-induced nestin expression protects rat VSMCs against $H_2O_2$-induced apoptosis, various concentrations of $H_2O_2$ were applied to EGF-treated cells. As shown in Fig. 3A, nestin expression significantly increased following treatment with 10 ng/ml EGF. The MTT analysis results showed that cell viability significantly decreased in a dose-dependent manner following $H_2O_2$ treatment of rat VSMCs cultivated in serum-free media. However, this phenomenon was obviously reversed by EGF stimulation in treatment with 100 and 250 μM $H_2O_2$ (Fig. 3B). To determine whether this loss of cell viability is mediated through apoptosis, a DNA laddering analysis and flow cytometry were carried out. Consistent with the MTT observations, DNA laddering and flow cytometric results clearly indicated that the treatments of EGF not only led to a decrease of DNA fragmentation (Fig. 3C), but also

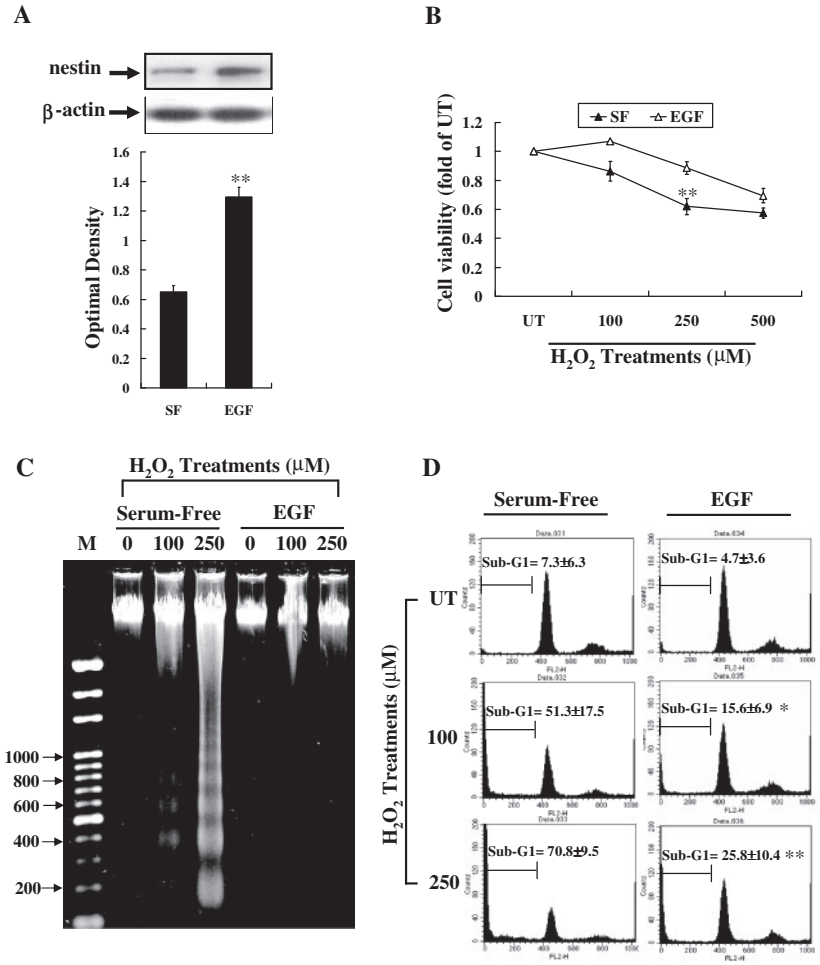


Fig. 3. **EGF protectes rat VSMCs from $H_2O_2$-induced apoptosis.** Rat aortic VSMCs were serum deprived for 2 days, followed by treatment with 10 ng/ml of EGF for another 24 h. (A) IF-enriched preparations were isolated from these cells, then immunoblotted with nestin to ensure the EGF effect on nestin expression, and re-probed against β-actin to show equal loading amounts. These cells were treated with 100, 250 or 500 μM $H_2O_2$ for 24 h. Cell viability was assessed by an MTT assay (B). Apoptotic effects were tested by DNA laddering analysis (C) and flow cytometry (D). Results are shown as multiples of the scrambled siRNA control (set to 1.0). Data are shown as the mean ± SE ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$ *vs.* the scrambled siRNA group). SF, serum-free; UT, untreated; M, marker.

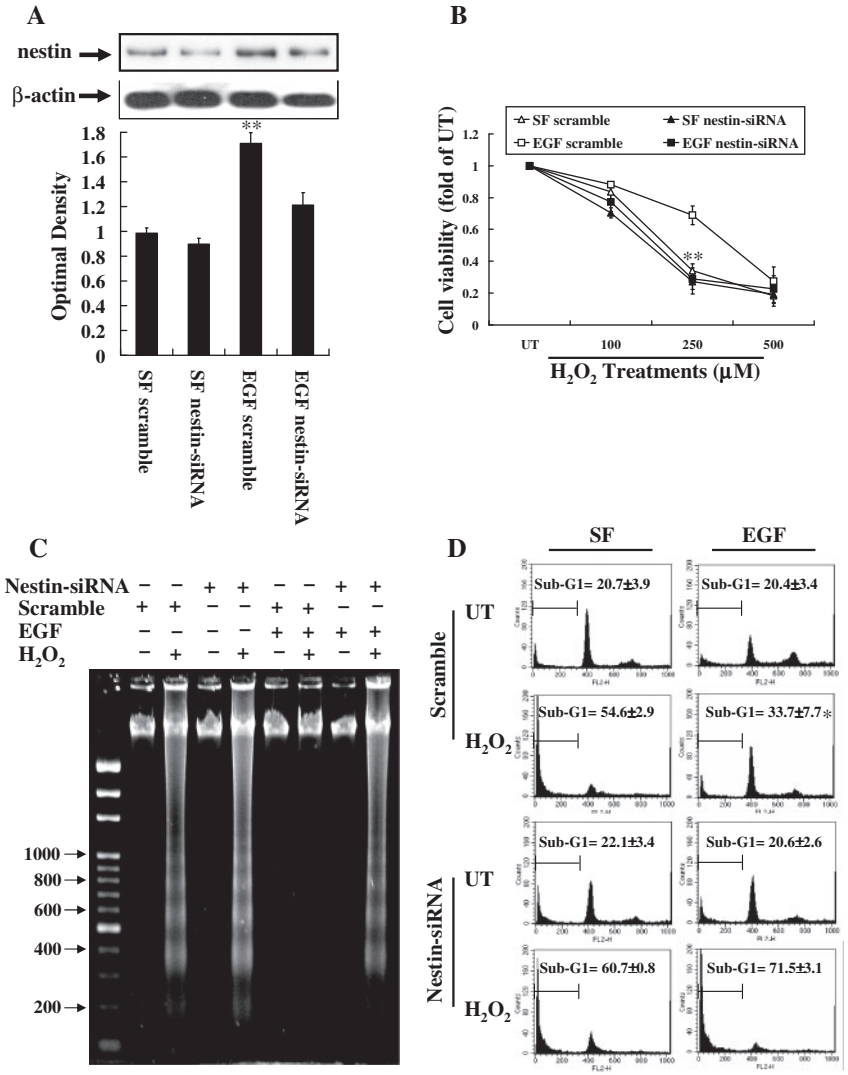


Fig. 4. **EGF prevents cell death through the stimulation of nestin.** Serum-deprived rat VSMCs were treated with 10 ng/ml EGF for 24 h, followed by the transfection with scrambled or nestin siRNA, and then seeded in 6-cm dishes (A, C and D) or 96-well plates (B). (A) IF-enriched preparations were isolated from these cells, and then immunoblotted with nestin to ensure siRNA transfection efficiency. (B) Cell viability was assessed by MTT assay. Apoptotic effects were tested by DNA laddering analysis (C) and flow cytometry (D). Cells were transfected with scrambled siRNA as the control (set to 1.0), and data are shown as multiples of that. Results are shown as the mean ± SE ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the scrambled siRNA group). SF, serum-free; UT, untreated; M, marker.

attenuated the apoptotic sub-G1 population increase which was initially induced by $H_2O_2$ (Fig. 3D).

To elucidate whether or not nestin is involved in the cytoprotective function of EGF, apoptotic events were examined using EGF-treated rat VSMCs transfected with nestin-siRNA. As shown in Fig. 4A, nestin expressions were depleted in both serum-free and EGF-treated VSMCs when transfected with nestin RNAi, but not in EGF-treated VSMCs transfected with scrambled siRNA. The viability of those cells was then assessed by an MTT analysis in EGF-containing medium. $H_2O_2$-induced loss of cell viability was detected at a concentration of 250 μM in these nestin-depleted VSMCs, but not in nestin expressing cells (Fig. 4B). Cell viability was reduced to the basal level in all VSMCs under treatment with 500 μM $H_2O_2$ (Fig. 4B). Thus, treatment with 250 μM $H_2O_2$ was used to examine whether the EGF-induced nestin expression is responsible for the prevention of apoptosis. Consistent with observations of the MTT analysis, the 250 μM $H_2O_2$-induced DNA fragmentation phenomenon (Fig. 4C) and an apoptotic sub-G1 population increase (Fig. 4D) were detected in those nestin-depleted, but not in those EGF-treated VSMCs transfected with scrambled siRNA. Based on the observations of MTT analysis (Fig. 4B), DNA laddering analysis (Fig. 4C) and flow cytometry (Fig. 4D), the results showed that the protection effects of EGF against $H_2O_2$-induced apoptosis were alleviated by the knockdown of nestin even in the presence of EGF. These results indicate that the cytoprotective function of EGF largely depends on its stimulating effects on nestin.

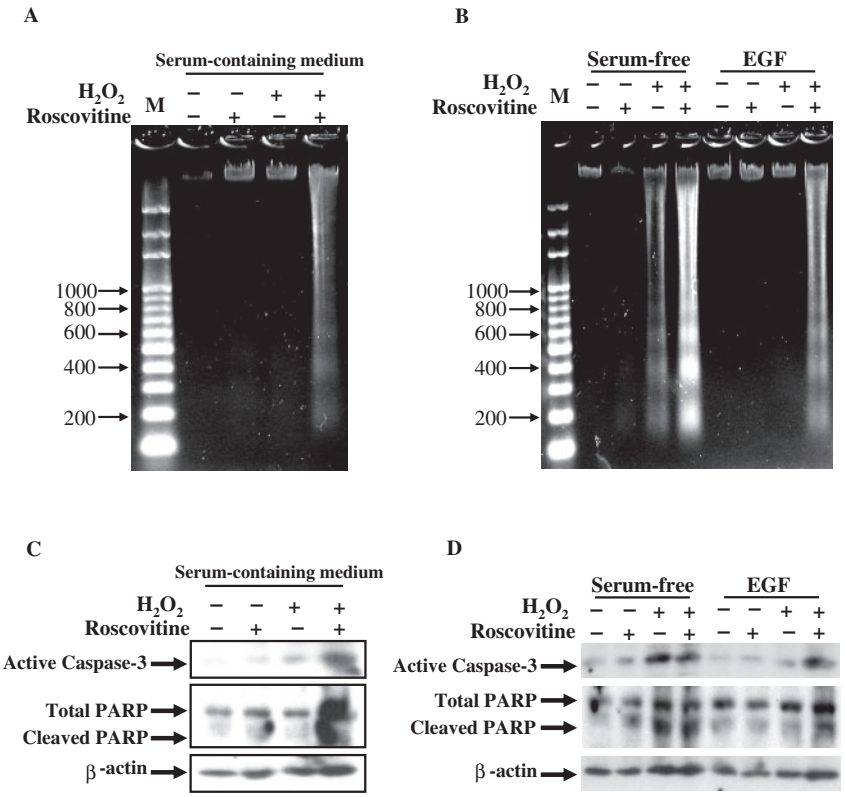


Fig. 5. **Cdk5 attenuates $H_2O_2$-induced apoptosis though caspase-dependent pathways.** Rat aortic VSMCs were pre-incubated for 1 h with 50 μM roscovitine, followed by co-treatment with 250 μM $H_2O_2$ for another 24 h. Apoptotic effects were evaluated by a DNA laddering analysis (A). The expressions of caspase-3 and PARP were assayed by immunoblotting with anti-caspase-3 and anti-PARP antibodies, and the immunoblots were re-probed against β-actin to show equal loading amounts (C). Serum-deprived rat VSMCs were treated with 10 ng/ml EGF for 24 h, followed by pre-incubation for 1 h with 50 μM roscovitine and then co-treatment with 250 μM $H_2O_2$ for another 24 h. Apoptotic effects were evaluated by a DNA laddering analysis (B). The expressions of caspase-3 and PARP were assayed by immunoblotting with anti-caspase-3 and anti-PARP antibodies, and the immunoblots were re-probed against β-actin to show equal loading amounts (D). M, marker.

*The Cdk5-Induced Bcl-2 Phosphorylation is Involved in the Cytoprotective Effect of Nestin in Rat VSMCs*— Previous studies reported that the cytoprotective function of nestin is mediated through its interaction with Cdk5 in ST15A cells (*11*). To examine whether Cdk5 is involved in the anti-apoptotic effect of nestin, the selective Cdk5 inhibitor roscovitine was applied to $H_2O_2$-treated rat VSMCs. As shown in Fig. 5, the inhibition of Cdk5 by roscovitine obviously enhanced 250 μM $H_2O_2$-induced DNA fragmentation (Fig. 5A), caspase-3 activation and PARP cleavage (Fig. 5C). To determine whether the cytoprotective function of EGF is also mediated through Cdk5, 50 μM roscovitine was applied to EGF-treated rat VSMCs. Results showed that the protective effects of EGF against $H_2O_2$-induced DNA fragmentation (Fig. 5B), caspase-3 activation (Fig. 5D) and PARP cleavage (Fig. 5D) were all abolished by the treatment with roscovitine. These results indicate that Cdk5 plays a prosurvival role in $H_2O_2$-induced apoptosis that is linked to the cytoprotective effect of EGF in rat VSMCs.

Recently, Cheung *et al*. (*18*) revealed that Bcl-2 phosphorylation by Cdk5 is important for the anti-apoptotic property of Bcl-2 and that this phosphorylation contributes to Cdk5-mediated maintenance of neuronal survival. This suggests that the Cdk5-mediated phosphorylation of Bcl-2 may play an important role in the cytoprotective function of rat VSMC nestin. To verify this possibility, we first examined whether nestin, Cdk5 and Bcl-2 were localized to the same sub-cellular fractions. As shown in Fig. 6A, Cdk5 was co-immunoprecipitated with nestin and Bcl-2, indicating that Cdk5 is associated with nestin and Bcl-2 endogenously. To examine whether Bcl-2 phosphorylation is involved in the cytoprotective function of nestin, Bcl-2 phosphorylation was monitored in nestin-depleted rat VSMCs. Results showed that the phosphorylation of Bcl-2 significantly decreased following 250 μM $H_2O_2$ treatment in nestin-depleted rat VSMCs, but not in nestin-expressing cells (Fig. 6B). We next investigated whether the phosphorylation of Bcl-2 by Cdk5 is involved in the prevention of $H_2O_2$-induced apoptosis. This was accomplished by applying the selective Cdk5 inhibitor roscovitine to $H_2O_2$-treated rat VSMCs. Results showed that Bcl-2 phosphorylation was slightly attenuated by treatments with roscovitine alone, but was abolished by co-treatment with roscovitine and $H_2O_2$ (Fig. 6C) which led to cell apoptosis (Fig. 5A). To determine whether the anti-apoptotic effect of EGF is also mediated through the same mechanism, Bcl-2

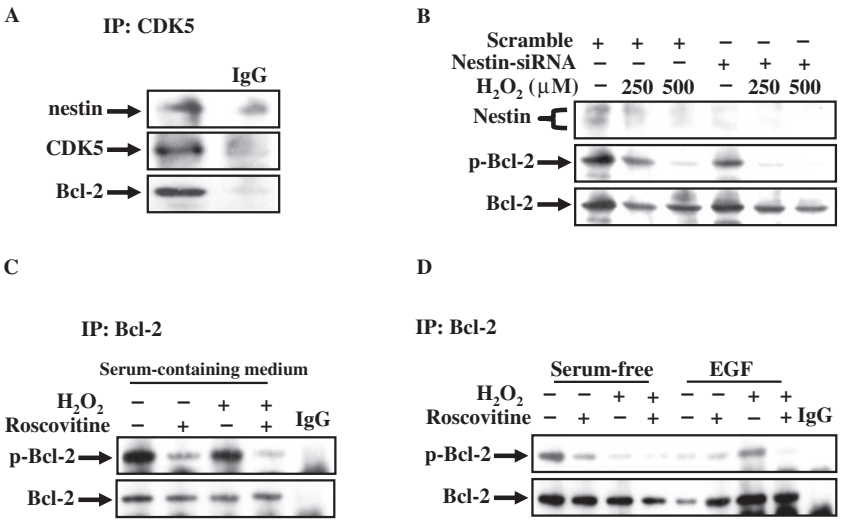


Fig. 6. **Cdk5-induced Bcl-2 phosphorylation is involved in the cytoprotective function of nestin in rat VSMCs.** (A) Whole-cell lysates were immunoprecipitated with anti-Cdk5. The expressions of nestin, Cdk5 and Bcl-2 were then assayed by the respective immunoblotting with anti-nestin, anti-Cdk5 and anti-Bcl-2. (B) Rat aortic VSMCs were transfected with scrambled or nestin siRNA, then seeded in 6-cm dishes, followed by treatment with 250 or 500 μM of $H_2O_2$ for 24 h. Whole-cell lysates were extracted from these cells, followed by immunoprecipitation with anti-Bcl-2 and then immunoblotting with phosphoserine to detect Bcl-2 phosphorylation. The immunoblots were re-probed against Bcl-2 to show equal loading amounts. (C) Rat aortic VSMCs were pre-incubated for 1 h with 50 μM roscovitine, followed by co-treatment with 250 μM $H_2O_2$ for another 24 h and whole-cell lysates were extracted. The phosphorylation of Bcl-2 was assayed by immunoprecipitation with anti-Bcl-2, followed by immunoblotting with anti-phosphoserine and the immunoblots were then re-probed with anti-Bcl-2 to ensure equal loading amounts. (D) Serum-deprived rat VSMCs were treated with 10 ng/ml EGF for 24 h, followed by pre-incubation for 1 h with 50 μM roscovitine and co-treatment with 250 μM $H_2O_2$ for another 24 h. The phosphorylation of Bcl-2 was assayed by co-immunoprecipitation with anti-Bcl-2 and anti-phosphoserine, and the immunoblots were then re-probed with anti-Bcl-2 to ensure equal loading amounts. M, marker.

phosphorylation was monitored following roscovitine treatments in EGF-treated rat VSMCs. As shown in Fig. 6D, Bcl-2 phosphorylation was abolished following a 250-μM $H_2O_2$ treatment in rat VSMCs cultivated in serum-free media, but not in those cultivated in EGF-containing media. These results indicate that Bcl-2 phosphorylation is responsible for the anti-apoptotic effect of EGF in rat VSMCs. Moreover, the maintenance of Bcl-2 phosphorylation by EGF following $H_2O_2$ treatment was reversed by its co-treatment with roscovitine (Fig. 6D). Taken together, these results indicate that the cytoprotective function of nestin is dependent on Cdk5-mediated phosphorylation of Bcl-2 in rat VSMCs.

## DISCUSSION

It has been well established that nestin is expressed in skeletal and cardiac muscle cells during development, disappears when these cells reach their mature state and then reappears in these cells following injuries (*2*, *3*, *6*, *19*). Our recent study reported that nestin is expressed in primary cultures of rat VSMCs and disappeared following serum deprivation-induced synthetic-contractile transition (*12*). Regarding the function of nestin, other than providing mechanical support, it has been reported that nestin is also involved in the promotion of cell proliferation in BHK cells and the prevention of apoptosis in ST15A cells (*9*, *11*). It is interesting to note that due to its expression of desmin and nestin, BHK cells appear to be smooth muscle cells in origin (*20*, *21*). Since the anti-apoptotic function of nestin in muscle cells remains unknown, in this study we first investigated its cytoprotective role in primary cultures of rat VSMCs using nestin RNAi. Results of DNA laddering analysis and flow cytometry following $H_2O_2$ treatments showed that nestin expression exhibits an anti-apoptotic function in rat VSMCs (Fig. 1).

Previous studies showed that for oxidative stress (e.g. $H_2O_2$)-induced apoptosis, caspase-9, but not caspase-8 serves as an initiator that leads to the activation of downstream effecter caspase-3 in most cell types examined, including rat VSMCs (*16*, *17*). In 2006, Sahlgren *et al*. (*11*) reported that knockdown of nestin by RNAi enhances the apoptotic activity *via* the cleavage of PARP, a substrate of caspase-3, following $H_2O_2$ treatments in ST15A cells, suggesting that caspases are involved in the cytoprotective function of nestin. In this study, we directly examined the caspase expression following $H_2O_2$ treatments in rat VSMCs, and the immunoblotting results revealed that the activated caspase-9, caspase-3 and PARP (Fig. 2), as well as the subsequent apoptotic events (Fig. 1B–D) were observed in nestin-depleted rat VSMCs under 250 μM $H_2O_2$ treatments. These results indicated that the cytoprotective function of nestin is caspase dependent.

Regarding the mechanisms of nestin's cytoprotective effects, Sahlgren *et al*. (*11*) revealed that nestin serves as a prosurvival determinant in ST15A neural stem cells as it sequesters and stabilizes the Cdk5/p35 complexes by binding them in the cytoplasm, thus reducing the

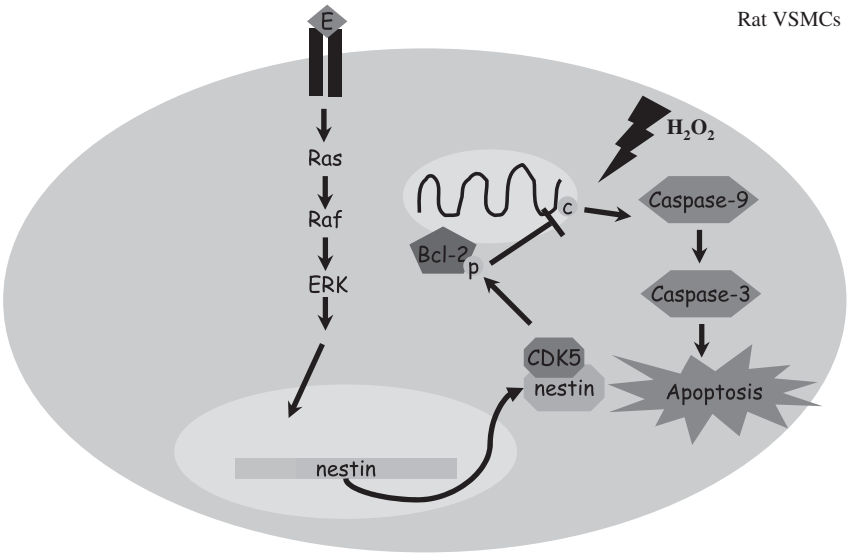


Fig. 7. **Schematic representation of signalling pathways involved in the activation of EGFR-induced anti-apoptosis *via* the induction of nestin in rat VSMCs.** EGF binds to EGFR which leads to the activation of Ras, Raf and ERK1/2, resulting in the induction of nestin in rat VSMCs. This is based on our previously reported data (Huang *et al*., 2008). These signalling pathways also contribute to the cytoprotective function of EGF in rat VSMCs. Regarding the prosurvival role of nestin, it is mediated through the Cdk5-induced Bcl-2 phosphorylation that leads to prevention of upstream caspase-9 activation in rat VSMCs.

generation of Cdk5/p25 from the breakdown of Cdk5/p35. The decrease in Cdk5/p25 was reported to attenuate neural cell death in response to injury (*22*, *23*). Cagnon *et al*. (*24*) revealed that the inhibition of Cdk5/p25 by the Cdk5-specific inhibitor, roscovitine, prevents caspase-3 activation following ammonia treatments in rat telencephalon cells, suggesting that Cdk5/p25 is an upstream positive regulator of caspase-3. Recently, it was shown that Cdk5/p35 is a new Bcl-2 kinase and this Bcl-2 phosphorylation contributes to Cdk5-mediated neuronal survival (*18*). These results corresponded well with our present study showing that Cdk5-mediated Bcl-2 phosphorylation protected rat VSMCs from $H_2O_2$-induced apoptosis (Fig. 6). In addition, the phosphorylation of Bcl-2 significantly decreased in nestin-depleted rat VSMCs following $H_2O_2$ stimulations (Fig. 6B). Taken together, these results indicate that the anti-apoptotic function of nestin is mediated through Cdk5-induced Bcl-2 phosphorylation which leads to inhibition of caspase-9 activation.

It is well known that EGF possesses a cytoprotective function following various apoptotic stimuli (*13*, *14*, *25*, *26*). In rat VSMCs, Miyamoto *et al*. (*13*) reported that Ras signalling is required for the anti-apoptotic effect of EGF, as demonstrated by use of the dominant negative form of Ras. Furthermore, Ying *et al*. (*14*) indicated that the cytoprotective effect of EGF against proteasome inhibition-induced mitochondrial-dependent apoptosis is mediated through the ERK signallings in rat VSMCs. Recently, we reported that EGF induces nestin expression via the Ras-ERK signalling axis in rat VSMCs (*12*). Herein, we showed that the anti-apoptotic activity of EGF under $H_2O_2$ treatments was abolished by the RNAi knockdown of nestin in rat VSMCs (Fig. 4). In summary, these results indicated that the EGF-induced nestin expression and its cytoprotective function share the same signalling pathways. In addition, it revealed that nestin may play a critical role in the cytoprotective function of EGF in rat VSMCs.

In conclusion, this is the first report to show that nestin plays an anti-apoptotic function via the prevention of caspase activation in rat VSMCs, as well as this effects link to the cytoprotective role of EGF (Fig. 7).

## FUNDING

Grant NSC96-2752-B-006-003-PAE, NSC96-2752-B-006-004-PAE and NSC96-2752-B-006-005-PAE.

## CONFLICT OF INTEREST

None declared.

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