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Epidermal growth factor up-regulates the expression of nestin through the Ras-Raf-ERK signaling axis in rat vascular smooth muscle cells

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ABSTRACT

The contractile-synthetic phenotypic modulation of vascular smooth muscle cells (VSMCs) is a key event during atherosclerosis progression. Although many studies have reported possible cytokines and growth factors implicated to this process, the critical factors affecting the VSMC phenotype remain unclear due to the lack of early de-differentiation marker identifications. In this study, we showed that nestin, an intermediate filament protein, is expressed in primary cultures of rat VSMCs representing the synthetic phenotype and its expression is diminished as these cells re-differentiate after serum deprivation. However, the regulation of nestin expression was never reported despite its common usage as an early differentiation marker. Herein, we showed that nestin expression is regulated by epidermal growth factor (EGF) via *de novo* RNA and protein synthesis. Furthermore, signaling analyses revealed that the EGF-induced nestin re-expression is mediated through the activation of the Ras-Raf-ERK signaling axis. This is the first report to show that nestin expression is regulated by an extracellular signaling molecule.

Vascular smooth muscle cells (VSMCs) exhibit various phenotypes during different developmental and pathological states. Previous studies have classified VSMCs into two groups, contractile and synthetic [1,2]. In mature arteries, VSMCs exist in a differentiated state with contractile abilities [2]. In response to vascular injuries, VSMCs undergo a phenotypic modulation which involves a de-differentiation process. It has been shown that many genes are differentially expressed in the contractile and synthetic SMCs. In order to provide insights into the processes underlying SMC de-differentiation, several attempts were made to characterize these phenotype-specific genes. The alteration of these gene profiles throughout the de-differentiating process can be modulated by growth factors such as epidermal growth factor (EGF) [3]. Since the majority of previous studies concentrated on VSMCs in weeks after lesion [4], the early phenotypic modulation mechanism underlying VSMC de-differentiation shortly following injury remains elusive. Thus, identification of a proper marker corresponding to the early de-differentiation process would shed new light on investigations in this area.

Nestin is a 240 kDa intermediate filament (IF) protein specifically expressed in myogenic and neural stem cells [5–7]. It is also known as IFAP-70/280kD for hamster nestin [8,9]. Nestin expression was primarily studied in the central nervous system (CNS) where it is transiently expressed in CNS stem cells [5]. Nestin reappears immediately adjacent to the wound during the early stages of CNS injury response [5–7,10,11]. Regarding the nestin expression in muscle cells, Sejersen and Lendahl [7] showed that nestin is transiently expressed in developing skeletal muscle cells of rat thighs during embryogenesis and is markedly down-regulated as these cells differentiate. Furthermore, Vaittinen et al. [12] reported that nestin reappears in myoblasts of rat gastrocnemius muscle 6 h after shearing or in situ injury, reaches maximal level at 3–5 days post-injury, and become down-regulated thereafter. Although nestin has been widely used as a marker for muscular and neural progenitor cells, its regulation by extracellular ligands has never been reported. Thus, the present study investigated nestin expression and the regulatory signaling of its re-expression following serum deprivation in primary cultures of rat aortic VSMCs.

Materials and methods

Immunofluorescence microscopy. The cells were fixed with cold methanol on ice and then immunolabeled with antibodies

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to nestin (1:500, PharMingen, San Diego, USA), smooth muscle-α actin (αSMA) (1:200, Abcam, Cambridge, UK), and smooth muscle myosin heavy chain (SM-MHC) (1:400, Santa Cruz Biotechnology, Santa Cruz, USA). DAPI was used for the counterstaining of nuclei. Preparations were examined with Zeiss Axioplan2 photomicroscope equipped with epifluorescence optics (Carl Zeiss, Oberkochen, BRD).

Rat aortic smooth muscle cell culture. Cell cultures of rat aortic VSMCs were prepared using an explant method [13] and those VSMCs at passages 4–10 were used in all experiments. Double-labeling immunofluorescence microscopy with different combinations of nestin, αSMA, and SM-MHC were carried out on 300 cells to identify the phenotype of the primary cultures and to verify the percentage of VSMCs in them.

Intermediate filament-enriched preparations. IF-enriched preparations were isolated from rat VSMCs as described previously [8] with a few modifications. In brief, cells were lysed with an extraction buffer containing 1% Triton X-100, 20% glycerol, 10% DMSO, 2 mM EGTA, 100 mM $MgCl_2$, 100 mM NaCl, 20 mM sodium acetate, and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, BRD), pH 6.5. The extracts were centrifuged at 21,460*g* for 15 min. The pellets were dissolved with urea buffer (8 M urea, 10 mM Tris–HCl, 15 mM 2-mercaptoethanol, pH 7.5) for 30 min, then centrifuged at 21,460*g* for 15 min. The supernatant was collected for IF-enriched preparations.

Whole cell extraction. Whole cell lysates were obtained as described previously [14]. Protein concentration was measured by the Bio-Rad method.

Western blotting analysis. IF-enriched preparations or whole cell lysates were separated by SDS–polyacrylamide gel electrophoresis and processed for immunoblotting as described previously [14]. The former was used to identify nestin and vimentin, and the latter for αSMA or SM-MHC.

Results

Nestin expression in primary cultures of rat aortic VSMCs

Previous studies indicated that cultured VSMCs displayed a synthetic phenotype and re-differentiate into the contractile type following serum deprivation [2]. In this study, primary cultures of VSMCs were prepared from adult aorta. When cultured in regular medium containing 10% FCS, over 98% of cells were αSMA^+ while less than 3% of those cells were SM-MHC^+ and no SM-MHC-IR was observed in αSMA^- cells (Fig. 1A), indicating that majority of cells were VSMCs and these VSMCs exhibit the synthetic phenotype. Double-labeling with anti-nestin and anti-αSMA showed that majority of αSMA^+ VSMCs were $nestin^+$, and nestin-IR was not detected in αSMA^- cells (Fig. 1B). In addition, few $nestin^+$ cells exhibited faint SM-MHC-IR based on anti-nestin and anti-SM-MHC double-labeling (Fig. 1C). Three days following serum deprivation, nestin-IR diminished significantly (Fig. 1E and F). Meanwhile, SM-MHC-IR increased markedly in αSMA^+ VSMCs (Fig. 1D). It is interesting to note that the α-actin-containing stress fibers became prominent under serum deprivation (Fig. 1D and E). These results were further confirmed by immunoblotting analyses. Time course experiment showed that nestin expression level reduced progressively and reached to its basal level 3 days after serum deprivation while SM-MHC level increased (Fig. 2A). Taken together, these results indicated that nestin was expressed in primary cultures of VSMCs which exhibit the synthetic phenotype and its expression was diminished markedly as VSMCs re-differentiate into the contractile phenotype following serum deprivation.

EGF regulates nestin re-expression in serum-deprived rat VSMCs

To identify factors capable of inducing nestin re-expression in serum-deprived VSMCs, we examined the effects of potential mediators [15,16] including EGF, fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor-BB (PDGF-BB), and interleukin 1-β (IL 1-β). Among them, EGF was the most effective in inducing nestin re-expression (3.3 ± 0.6-fold increase) (Fig. 2B). We next examined the dose and time course responses on nestin expression towards EGF treatments. Nestin expression was stimulated by EGF in a concentration-dependent manner, reaching a plateau at 10 ng/ml (2.2 ± 0.3-fold control) (Fig. 2C). In addition, EGF-induced nestin expression was time-dependent, reaching a maximal level at 12 h and sustained for 24 h (3.0 ± 0.4-fold increase) (Fig. 2D). Thus, a 24-h treatment with 10 ng/ml EGF was used as positive control in subsequent experiments.

To ascertain the specificity of EGF effects on nestin re-expression, serum-deprived VSMCs were treated with various concentrations of EGF neutralizing antibodies (0.1–5 μg/ml). The immunoblotting (Fig. 3A) and immunofluorescence (Supplementary Fig. 1D) results showed that EGF neutralizing antibody at 1 and 5 μg/ml abolished EGF-induced nestin re-expression. It has been reported that VSMCs express all four types of EGFRs, ErbB1–4. However, among these receptors, EGF only binds to ErbB1 [17]. Hence, an ErbB1-specific tyrosine kinase inhibitor, AG1478 was used to examine its effects on EGF-induced nestin expression. As shown by immunoblotting (Fig. 3B) and immunofluorescence (Supplementary Fig. 1E) analyses, AG1478 inhibited the EGF-induced nestin re-expression in a dose-dependent manner and abolished EGF effect at the concen-

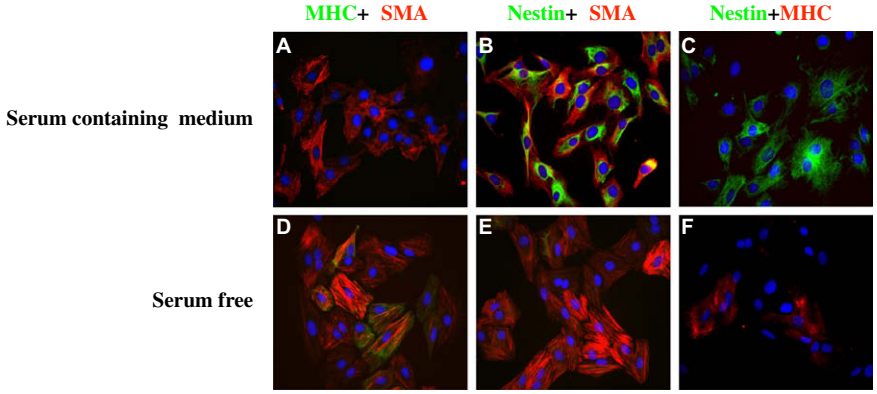

Fig. 1. Nestin expression in primary cultures of rat VSMCs. Primary cultures of rat VSMCs were cultivated in serum-containing medium (A–C) or in serum-free medium (D–F). Merged images of SM-MHC and αSMA were shown in (A,D), those of nestin and αSMA in (B,E), and those of nestin and SM-MHC in (C,F). Nuclei were counterstained by DAPI.

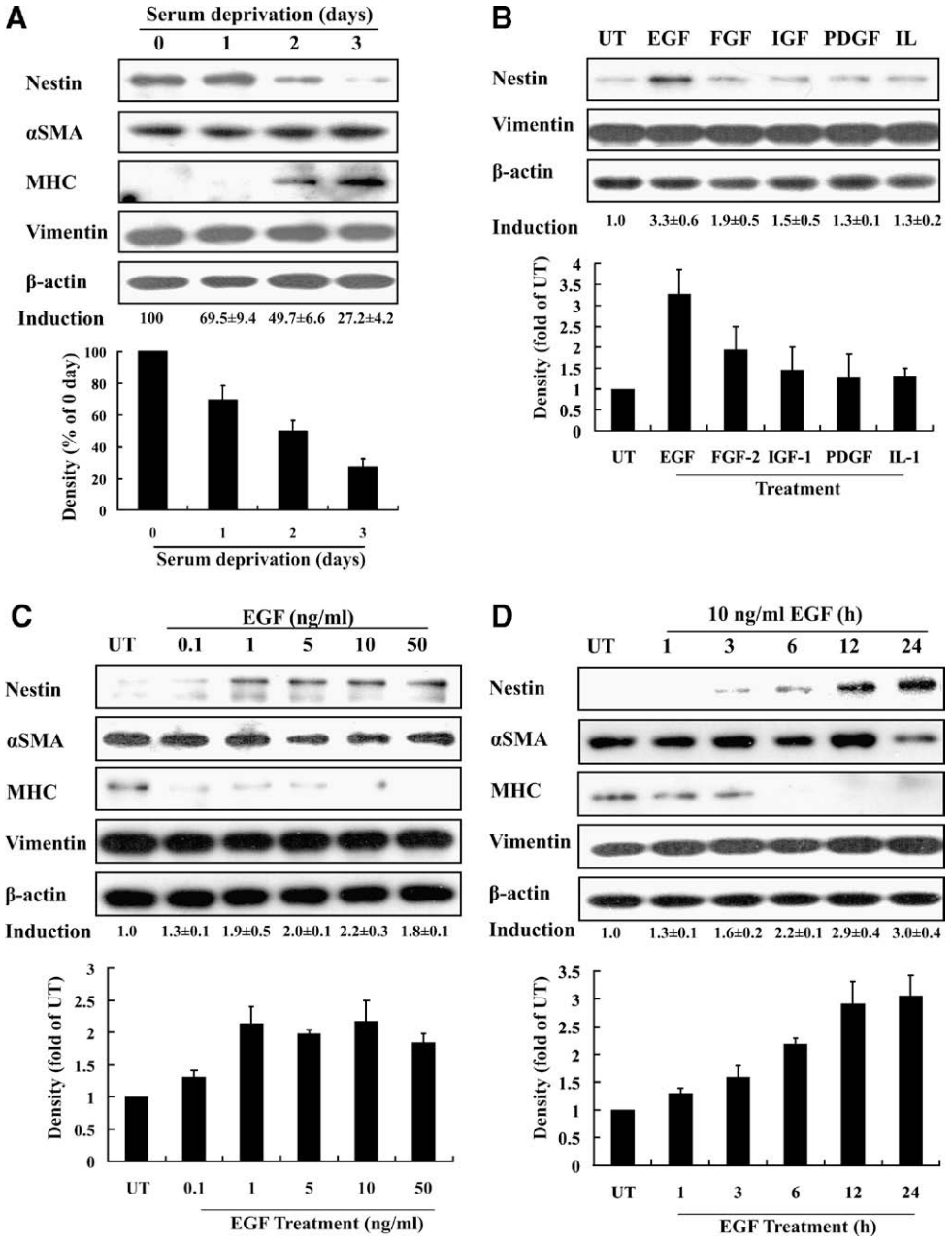

Fig. 2. Nestin expression induced by EGF stimulation in rat VSMCs. (A) Primary cultures of rat aortic VSMCs were cultivated in serum-free medium for different durations (0–3 days). Data were shown as percentages of the serum-containing medium control (set at 100 as 0 day) and results are shown as means ± SEM ($n = 4$). (B) Identification of factors regulating nestin expression in primary cultures of rat VSMCs. Primary cultures of rat aortic VSMCs were cultivated in serum-free medium for 2 days and followed by the treatments for another 24 h with the following reagents: EGF (10 ng/ml), FGF-2 (10 ng/ml), IGF-1 (200 ng/ml), PDGF (10 ng/ml), and IL-1β (10 ng/ml). Nestin expression was then assessed by immunoblotting. Data were shown as folds of untreated control (set at 1.0 as UT) and results were shown as means ± SEM ($n = 4$). EGF dosage responses after treatments for 24 h (C) and time course relationships with 10 ng/ml of EGF (D) were tested on serum-deprived VSMCs. IF proteins (5 μg) were immunoblotted with anti-nestin and whole cell lysates (20 μg) with anti-αSMA or anti-SM-MHC. Immunoblots were re-probed with anti-vimentin and anti-β-actin to ensure equal loading amounts. UT, untreated.

tration of 5 μM. These data indicated that EGF stimulates nestin re-expression via the EGFR activation.

EGF induces nestin expression via de novo protein synthesis

To determine whether transcriptional and translational activities are needed for the EGF-induced nestin expression, VSMCs were pretreated for 1 h with actinomycin D (Act-D), a transcriptional inhibitor, or cycloheximide (CHX), an inhibitor of protein synthesis. Both Act-D and CHX inhibited the EGF-induced nestin expression in a concentration-dependent manner (Fig. 3C and D), indicating that the EGF-induced nestin expression requires both *de novo* RNA and protein synthesis.

EGF-induced nestin expression is mediated by Ras-Raf-ERK dependent pathways

It is known that EGFR stimulates mitogenesis mainly through the PLCγ-PKC, PI3 K-Akt, and Ras-Raf pathways [17]. To determine the signaling pathways involved in the EGF-induced nestin expression, the effects of specific pharmacological inhibitors was examined. We first examined the involvement of PLCγ-PKC pathway by applying

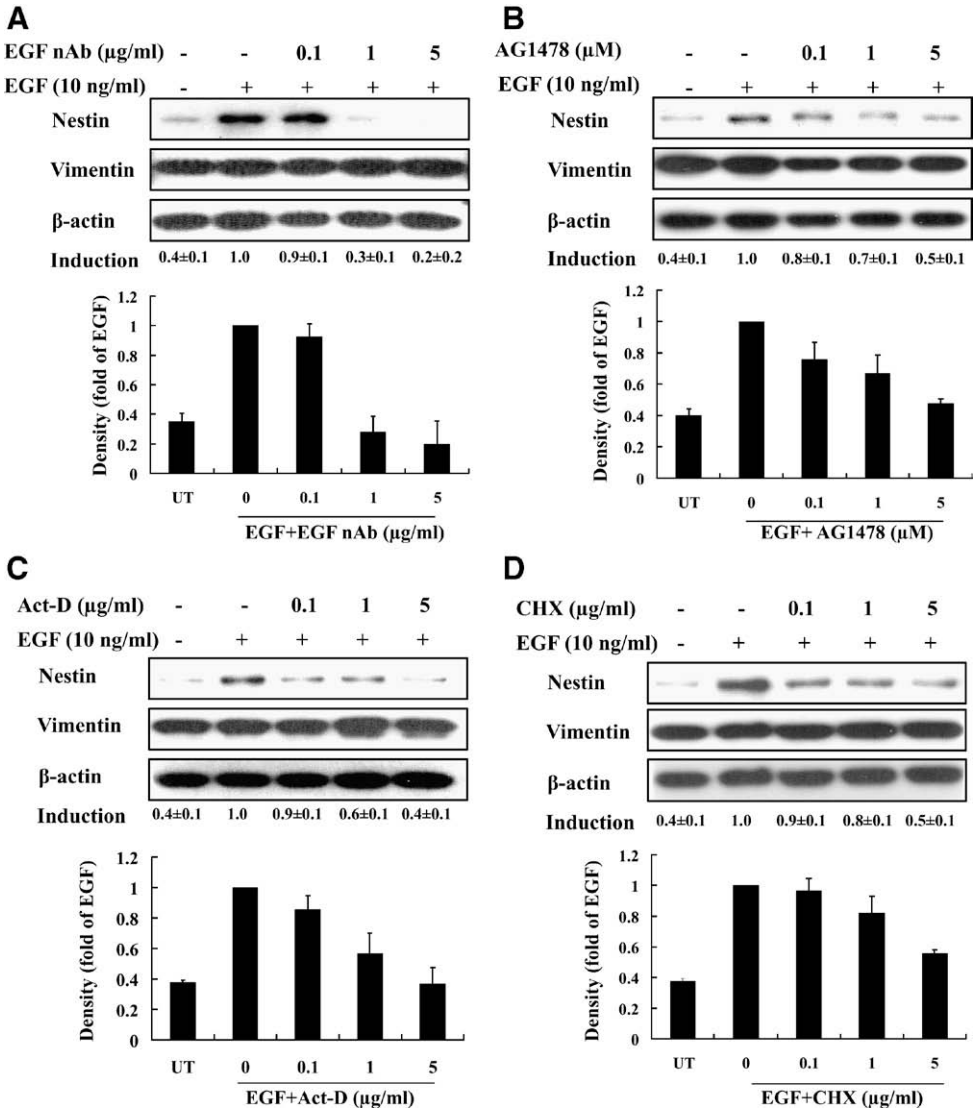

Fig. 3. EGF-induced nestin re-expression via *de novo* protein synthesis in rat VSMCs. Rat aortic VSMCs were serum-deprived for 2 days, preincubated for 1 h with different dosages of various reagents and followed by the cotreatment with 10 ng/ml EGF for another 24 h, and nestin expression were then assessed by immunoblotting with anti-nestin. Reagents used for preincubation and cotreatments were 0.1, 1, and 5 μg/ml of each of EGF neutralizing antibody (A), actinomycin D (C), cycloheximide (D), or 0.1, 1, and 5 μM of AG1478 (B). Cells treated only with EGF were used as controls (set at 1.0) and data were shown as folds of it. The results were shown as means ± SEM ($n = 3$). Immunoblots were re-probed with anti-vimentin and anti-β-actin to ensure equal loading amounts. UT, untreated; EGF nAb, EGF neutralizing antibody; Act-D, actinomycin D; CHX, cycloheximide.

a PLCγ inhibitor (U73122) and two PKC inhibitors (GF109203X and calphostin C) to EGF-treated rat VSMCs. As shown in Fig. 4A, Supplementary Fig. 2A and B, neither treatment affected the EGF-stimulated nestin expression in VSMCs. Similarly, inhibition of the PI3K pathway by its inhibitor LY294002 also did not attenuate the EGF-induced nestin expression (Fig. 4B). In contrast, treatment with a Raf-1 inhibitor, GW5074, inhibited EGF-induced nestin expression in a concentration-dependent manner, with the EGF effects abolished at the concentration of 3 μM (Fig. 4C).

It is well documented that mitogen-activated protein kinases (MAPK), ERK, p38, and JNK are major mediators of Ras-activated mitogenesis [18]. We found that ERK1/2 phosphorylation was induced by EGF within 1 min (data not shown). To examine the role of ERK1/2 in the EGF-induced nestin re-expression, the effects of U0126 which blocks ERK 1/2 activation was examined. Treatment with U0126 suppressed EGF-induced nestin expression in a concentration-dependent manner and abolished EGF effects at the concentration of 10 μM, as shown by immunoblotting (Fig. 4E) and immunofluorescence microscopy (Supplementary Fig. 1G). The roles of other MAPK pathways in the EGF-induced nestin expression were also examined. As shown by immunoblotting analyses (Supplementary Fig. 2C and D) and immunofluorescence microscopy (Supplementary Fig. 1H and I), neither the inhibition of p38 or JNK affected the EGF-induced nestin expression. These results indicated that EGF induces nestin re-expression through the Ras-Raf-ERK, but not the PLCγ-PKC, PI3K, JNK, or p38 pathways.

Discussion

The IF cytoskeleton is composed of more than 70 subunit proteins which are expressed in a tissue- and differentiation stage-specific manner [19]. It is widely known that the main function of IFs is to provide mechanical support to cells of multicellular organisms [19]. Intriguingly, recent studies have revealed a non-mechanical cytoprotective role of IFs in which they could act as tissue-specific scaffolds to organize and modify signaling pathways for cell survival, migration, and the sequestering

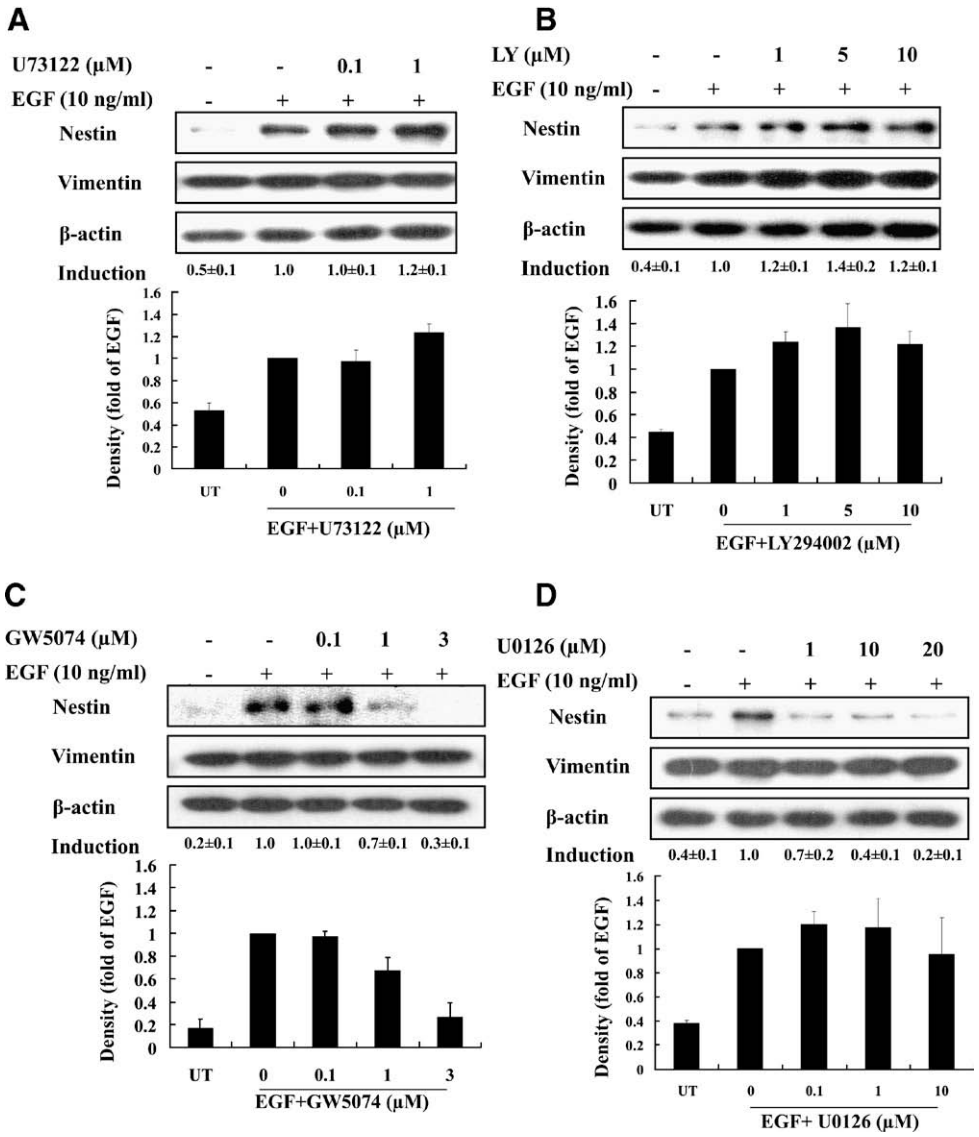

Fig. 4. EGF-induced nestin expression through Ras-Raf-ERK dependent pathways in rat VSMCs. Rat aortic VSMCs were serum-deprived for 2 days, preincubated for 1 h with different dosages of various reagents and followed by the cotreatment with 10 ng/ml EGF for another 24 h, and nestin expression were then assessed by immunoblotting with anti-nestin. Reagents used for preincubation and cotreatments were EGF only, 0.1 and 1 μM of U73122 (A), 1, 5, and 10 μM of LY294002 (B), 0.1, 1, and 3 μM of GW5074 (C), or 1, 10, and 20 μM of U0126 (D). Data were shown as folds of EGF only cells (set at 1.0). Immunoblots were re-probed with anti-vimentin and anti-β-actin to ensure equal loading amounts. UT, untreated; LY, LY294002.

of stress-activated kinases [20]. Nestin is a type IV IF protein mainly expressed in muscle and neural progenitors, as well as in their immature descendants during development [5–7]. Nestin expression is generally ceased in mature cells, but resumes following injuries [10–12]. Although nestin has been widely used as a marker for muscular and neural progenitor cells, its role and function remain largely unknown and has been a focus of recent researches. Previous studies have shown that nestin plays an important role in regulating the structural dynamics of vimentin IFs through vimentin phosphorylations during mitosis [21]. Furthermore, a recent nestin RNAi study has revealed that transient expression of nestin promotes the proliferation of repopulating mesangial cells following mesangial injury in the anti-Thy1 nephritis model [22]. In addition, nestin was indicated to have cytoprotective functions in a neural stem cell line, ST15A, by acting as a scaffold for Cdk5 wherein it prevents Cdk5-dependent apoptosis by the sequestering of Cdk5/p35 complexes [23]. In addition, our preliminary nestin RNAi data revealed that the knockdown of nestin expression increased cell mortality following H_2O_2 treatment and attenuated the EGF-induced proliferation in rat VSMCs (unpublished data).

Based on the observations on morphology and the loss of contractile proteins, it has been shown that cultured VSMCs display the synthetic phenotype [2]. Our present study further showed that nestin is indeed expressed strongly in primary cultures of αSMA^+ VSMCs, while faint SM-MHC-IR was detected only in less than 3% of these cells. Han et al [24] showed that serum deprivation results in cell re-differentiation with increased expression of VSMC-specific contractile proteins such as SM-MHC, αSMA, and SM22. In addition, our results showed that nestin-IR was diminished whereas SM-MHC-IR was augmented upon serum deprivation (Figs. 1 and 2A). Furthermore, serum deprivation also markedly induced the re-organization of actin filaments into stress fibers (Fig. 1D and E) which provides another indicator for the differentiation state of the contractile VSMC [25]. These results suggest that nestin is a de-differentiation marker for the synthetic SMCs.

Identification of factors that induce nestin re-expression is important for understanding the early phase of atherosclerotic

progression. However, regulators of nestin expression have never been reported. Previous studies have suggested that due to their stimulatory effects on contractile-synthetic transformation features such as proliferation and migration in VSMCs, several growth factors such as EGF, FGF-2, IGF-1, PDGF-BB, and IL-1β may act as proatherogenic factors [15,16]. Herein, these growth factors were applied to investigate their effects on nestin re-expression. Results illustrated that among those potential mediators, only EGF significantly induced nestin re-expression in serum-deprived rat VSMCs (Fig. 2B). The fact that the EGF-induced nestin expression was abolished by EGF neutralizing antibody further confirmed EGF effects on nestin expression (Fig. 3A). The development of atherosclerosis involves a multistep process regulated by multiple factors. In this study, the results showed that among all factors examined, nestin expression is regulated mainly by EGF.

Previous studies have shown that the EGFRs have been identified immunocytochemically on primary cultures of rat VSMCs [17]. In this study, we found that EGFR1 was also expressed on rat VSMCs as demonstrated by RT-PCR and immunoblotting (data not shown). Treatments of AG1478 abolished the EGF-induced nestin re-expression, confirming the involvement of EGFR in this process. It is interesting to note that EGF promotes de-differentiation of primary cultures of visceral and aortic SMCs [3]. It has also been shown that the blockage of EGFR inhibits VSMCs proliferation in the tunica media at 2 days upon balloon injury of carotid artery, and also inhibits intimal hyperplasia at 14 days post-injury [26]. Based on the fact that nestin may facilitate proliferation, EGF-stimulated nestin expression may also play an important role in the subsequent VSMC proliferation and neointima formation.

In the present study, we showed that nestin re-expression in serum-deprived rat VSMCs was induced through the EGF-ErbB1 interaction and the subsequent intracellular signalings involving the Ras, Raf, MEK, and ERK, but not the PLC, PI3K, p38, or JNK pathways. The PI3K/PKB(Akt) pathway is involved in the maintenance of the differentiated phenotype of VSMCs, whereas the ERK and p38MAPK pathways are responsible for inducing the de-differentiation of VSMCs in primary cultures [3]. In agreement, our results showed that the ERK pathway plays an important role in the growth factor-induced VSMC de-differentiation.

In conclusion, the present study showed that nestin is expressed in primary cultures of synthetic VSMCs, and its expression is closely associated with the contractile-synthetic transformation of VSMCs. Furthermore, nestin disappeared in serum-deprived VSMCs. In addition, this study showed that EGF acts as a regulator of nestin expression via ErbB1 activation in VSMCs. This is the first to provide evidence that nestin expression can be modulated by an extracellular signaling molecule.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.143.

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