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Thrombin induces nestin expression via the transactivation of EGFR signalings in rat vascular smooth muscle cells

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ABSTRACT

Regulation of nestin gene expression is largely unknown despite that it is widely used as a progenitor cell marker. In this study, we showed that nestin expression is regulated by the thrombin-mediated EGFR transactivation in serum-deprived primary cultures of rat vascular smooth muscle cells (VSMCs). This resulted from the direct binding of thrombin to PAR-1 rather than indirectly affecting through the binding to thrombomodulin, as demonstrated by thrombomodulin RNAi. In this process, the PAR-1-induced c-Src plays a critical role through two routes; one was the direct intracellular phosphorylation of EGFR and the other was the extracellular activation of the MMP-2-mediated shedding of HB-EGF. The transactivated EGFR then led to the downstream Ras–Raf–ERK signaling axis, but not the p38 or JNK pathways. In addition, the EMSA experiment showed that the transcriptional factor Sp1 is critical for the thrombin-induced nestin expression in rat VSMCs. Furthermore, RNAi of nestin attenuated the thrombin-induced cell proliferation, indicating that thrombin-induced nestin expression and cell proliferation share the same EGFR transactivation mechanism. This study also suggested that nestin may play an important role in cell proliferation induced by the thrombin-mediated EGFR transactivation.

1. Introduction

Nestin is a type IV IF protein mainly expressed in muscle and neural progenitors, as well as in their immature descendants during development [1–3]. The expression of nestin is generally ceased in mature cells, but resumes following injuries [4–6]. Regarding its functions, Chou et al. [7] demonstrated that nestin promotes cell proliferation by enhancing the phosphorylation-dependent disassembly of vimentin during mitosis. Furthermore, a recent nestin RNAi study revealed that transient expression of nestin promotes the proliferation of repopulating mesangial cells following mesangial injury in the anti-Thy1 nephritis model [8]. These findings imply that the re-expression of nestin appears to be responsible for the promotion of cell proliferation during the post-injury phase. However, nestin expression in SMCs, particularly in VSMCs during development and following injuries remains unknown. In addition, although nestin has been widely used as a marker for muscular and neural progenitor cells, its regulatory mechanism remains elusive and has been the focus of recent researches. We recently showed that nestin is expressed in primary cultures of rat vascular smooth muscle cells (VSMCs) which represent the synthetic phenotype, and its expression is diminished as these cells re-differentiate into the contractile phenotype after serum deprivation [9]. The phenotypic modulation of VSMCs from a differentiated phenotype to a reactive state that de-differentiates is a prominent characteristic in vascular disease and balloon injury [10–12]. To decipher the mechanisms of this phenotypic switch, we identified epidermal growth factor (EGF) as an inducer of nestin expression and this induction is mainly mediated through the ERK-dependent pathway in rat VSMCs [9].

It has been well-established that EGFR may be utilized by G-protein-coupled receptors (GPCRs) as the intermediate signaling protein and this process is termed EGFR transactivation [13–15]. Kalmes et al. [16] revealed that the EGFR phosphorylation and ERK activation in response to thrombin, a GPCR ligand, are abrogated by the inhibition of EGFR using its specific inhibitor AG1478, indicating that EGFR transactivation may play an important role in the GPCRs-

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induced ERK cascade. GPCRs transactivate EGFR through at least two mechanisms. One mechanism involves the activation of membrane-bound matrix metalloproteinases (MMPs) that catalyze the extracellular shedding of heparin-binding EGFs (HB-EGFs), with its shed products subsequently activating the EGFRs [14,15]. This activating mechanism is a widely accepted model for EGFR transactivation in several cell systems including VSMCs, and is termed "triple-membrane-passing-signaling" in such system. The other mechanism involves the activation of intracellular c-Src that leads to the phosphorylation and activation of EGFR [15,17]. This interaction allows GPCRs to make use of the pathways downstream of EGFR in order to influence cell function such as ERK activation, cell proliferation and protein synthesis [13,14].

Thrombin, a multifunctional serine protease generated at the site of vascular injury, is a potent stimulator of platelet aggregation promoting blood coagulation [18]. It is also known to act as a growth factor for VSMCs via the protease-activated receptor (PAR), which is a member of the seven-transmembrane GPCRs [19,20]. Activation of PARs is achieved when the extracellular NH2 terminus of the receptor is cleaved by the proteolytic activity of thrombin. The newly generated NH2 terminus then binds like a tethered ligand to the extracellular loop 2 of the receptor, leading to downstream signalings and other cellular effects [21]. Synthetic peptides mimicking the sequence of the tethered ligand can bypass the requirement of proteolytic activation directly binding to and activating the receptor. Four discrete types of PARs have been identified and characterized to date, PAR-1, PAR-2, PAR-3, and PAR-4 [20,21]. Thrombin can activate PAR-1, PAR-3, and PAR-4, whereas PAR-2 is mainly activated by trypsin [21]. It has been shown that the thrombin-activated PARs can transactivate EGFR to regulate several critical downstream signaling pathways such as ERK activation which promotes proliferation and other cellular functions in VSMCs [21,22].

Previous studies have reported that thrombin induces cell proliferation and the synthetic phenotype formation of VSMCs via EGFR transactivation [13,16]. In addition, our previous study revealed that nestin expression is regulated by EGF stimulation. Thus, to study whether nestin expression is regulated by the GPCR-induced EGFR transactivation, the primary cultures of rat VSMCs were used. Herein, we report that thrombin can up-regulate nestin expression through the PAR-1-induced EGFR transactivation, and this nestin expression appears to play an important role in cell proliferation stimulated by the thrombin-induced EGFR transactivation. This is the first report to show that the intermediate filament protein nestin can be regulated by thrombin via the EGFR tranactivation mechanism in rat VSMCs.

2. Materials and methods

2.1. Reagents

Thrombin, actinomycin D, LY294002, GW5074, U73122, U0126, SP600125, SB203580, and MTT reagent were purchased from Sigma-Aldrich (St. Louis, USA). PPACK, AG1478, MMP-2 inhibitor and PP1 were purchased from Calbiochem (Darmstadt, BRD). SCH79797 was purchased from TOCRIS (Missouri, USA). PAR-1 agonist (TFNNR-NH_2), PAR-2 agonist (SLIGRL-NH_2), PAR-3 agonist (TFRGAP-NH_2), and PAR-4 agonist (AYPGKF-NH_2) were purchased from MD Bio (Taipei, ROC). Mouse anti-nestin monoclonal antibody (clone 401) was purchased from PharMingen (San Diego, USA). Rabbit anti-αSMA antibody was purchased from Abcam (Cambridge, UK). Mouse anti-SM-MHC, rabbit anti-phospho tyrosine^{845} EGFR, rabbit anti-phospho tyrosine^{992} EGFR, mouse anti-ERK1/2, mouse-anti-phospho ERK1/2 and mouse anti-c-Src antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse anti-EGFR antibody was purchased from Sigma-Aldrich (St. Louis, USA). The secondary antibodies conjugated with AF488 or AF568 were purchased from Molecular Probes (Carlsbab, USA).

2.2. Rat aortic smooth muscle cell culture

Cell cultures of rat aortic VSMCs were prepared using an explant method as described previously [9] and those VSMCs at passages 4–10 were used in all experiments.

2.3. Intermediate filament-enriched preparations and whole cell extractions

IF-enriched preparations and whole cell lysates were isolated from rat VSMCs as described previously [9]. Protein concentration was measured by the Bio-Rad method using bovine serum albumin as standard reference.

2.4. Western blotting analysis

IF-enriched preparations or whole cell lysates were separated by 7.5–12.5% SDS–polyacrylamide gel electrophoresis and processed for immunoblotting as described previously [9]. The former was used to identify nestin, and the latter for αSMA, SM-MHC, p-EGFR, EGFR, p-ERK, ERK1/2, c-Src, and MMP-2.

2.5. Immunofluorescence microscopy

The frozen sections of embryos and postnatal carotid arteries were immunolabeled with antibodies to nestin (1:500, PharMingen, San Diego, USA), and αSMA (1:200, Abcam, Cambridge, UK) for the identification of muscular progenitor cells, and mature SMCs, respectively. DAPI was used for the counterstaining of nuclei. Preparations were examined with a Leica TCS SP5 confocal microscopy system, or with a Zeiss Axioplan2 photomicroscope equipped with epifluorescence optics.

2.6. RNA extraction and RT–PCR

Total RNA was isolated from carotid artery through an RNeasy® Mini kit (QIAGEN, Valencia, USA) and 1 μg of it was used in RT–PCR with specific sets of oligonucleotides: nestin (sense) 5′-GGAGCAGGAGAAGCAAGGT-3′; nestin (antisense) 5′-TGAGGGTTGTGGCTAAGGAG-3′; PAR-1 (sense) 5′-CTTCTCCGCCATCTTCTTTC-3′; PAR-1 (antisense) 5′-ACTCCGTTCCCATCACCTTG-3′; PAR-2 (sense) 5′-CCGAACGAAGAAGAAGCAC-3′; PAR-2 (antisense) 5′-GTAGGCAGACGCAGTAAGG-3′; PAR-3 (sense) 5′-CGAAGTGTCCCGAAGAAAG-3′; PAR-3 (antisense) 5′-GGCAAAGCAGATGGTGAA-3′; PAR-4 (sense) 5′-ACGCCTCACCACCATACTC-3′; PAR-4 (antisense) 5′-GTCCCCTTTCCCAACTCA-3′. The amplification was performed in 32 cycles at 95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s.

2.7. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Rat VSMCs were washed with cold PBS and suspended in buffer A containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, BRD) on ice for 15 min, and 6.25% Nonidet P-40 was then added. The extracts were centrifuged at 15,500 *g* for 2 min. The pellets were dissolved with buffer B containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, BRD) on ice for 15 min with intermittent mixing. The nuclear extracts were then centrifuged at 15,500 *g* for 5 min. The supernatant was collected for nuclear extract preparations. The EMSA were performed using a gel-shift kit (Panomics, Fremont, USA) according to the manufacturer's instructions. The double-stranded oligonucleotides containing the consensus sequences for AP-1 (5′-GATCAGCTTGATGATGAGTCAGCCCG-3′), Sp1 (5′-AATAGTGCCCCGCCCCGCCCCGCCC-3′), and Pit1 (5′-GCTGTCTTCCTGAATATGAATAAGAAATAAGCCA-3′) were end-labeled biotin (Panomics, Fremont, USA) and used as probes for EMSA.

2.8. Small interfering RNA knockdown assay

Rat VSMCs at 80% confluency were trypsinized and centrifuged. The harvested cells were resuspended in 100 μl of PBS and electroporated in the presence of 2.5 μg of nestin small interfering RNA (siRNA) (AAG AUG UCC CUU AGU CUG GAG), TM siRNA (GGA AGU AGG GUU UUG AUU Utt), MMP-2 siRNA (CAC CAU CGA GAC CAU GCG Gtt) (Amaxa Biosystems, Kölin, BRD), c-Src siRNA (GAA GCU GAG GCA UGA GAA Gtt), EGFR siRNA (GAG UCG GGC UCU GGA GGA Att), ERK-1 siRNA (UCC AAG GGC UAC ACC AAA Utt), ERK-2 siRNA (GUG CUG UGU CUU CAA GAG Ctt) (MDBio Inc., Taipei, ROC), or scramble negative control (Amaxa Biosystems, Kölin, BRD). These transfected cells were then plated to 96-well plates for detecting the cell proliferation or 6 cm-dishes for immunoblotting analyses. The effects of siRNAs were examined 72 h after transfection.

2.9. Proliferation assay

Rat VSMCs in a 96-well plate were subjected to the treatments of various concentrations of thrombin. To access the cell proliferation,

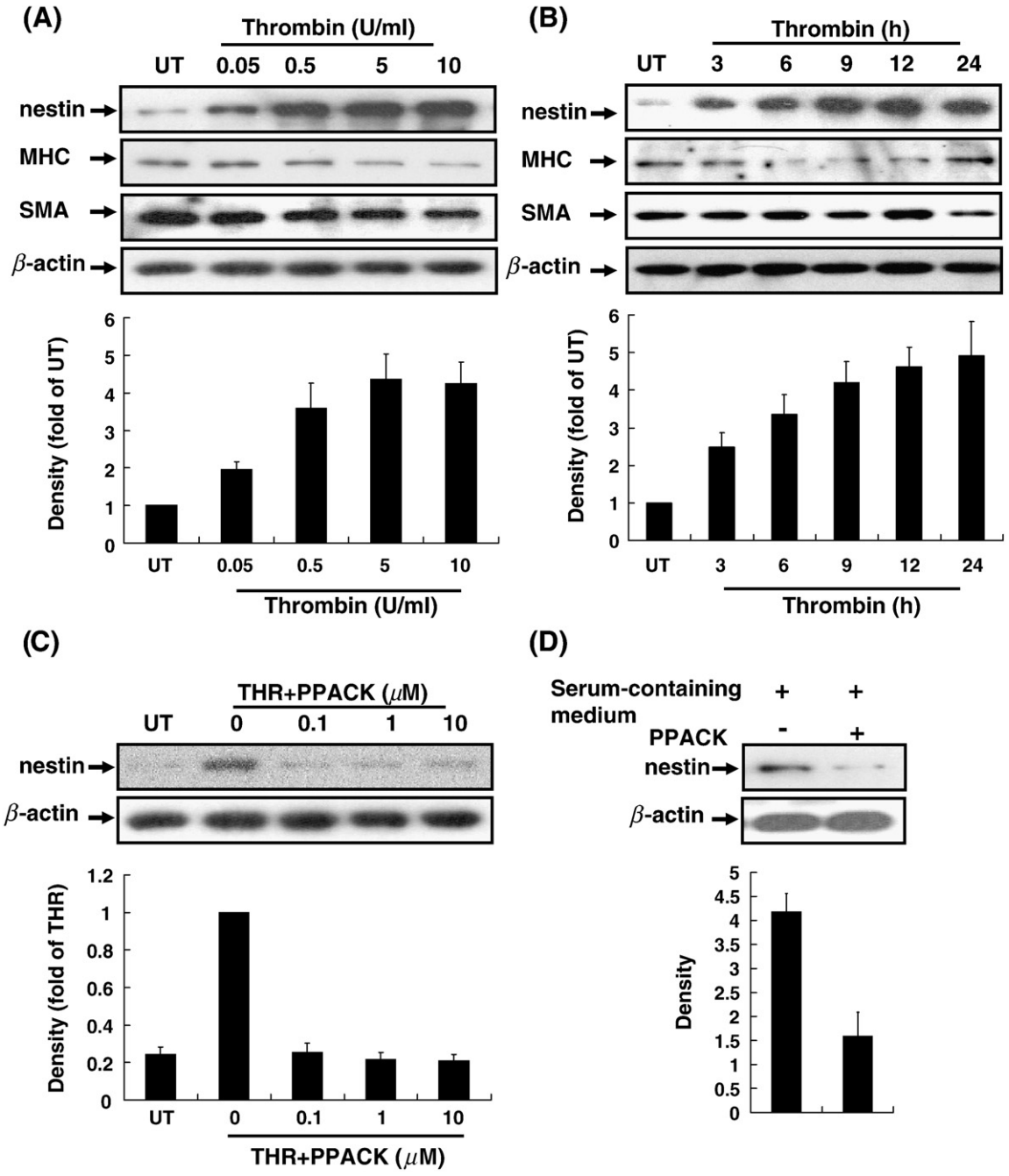

Fig. 1. Nestin expression is induced by thrombin stimulation in primary cultures of rat VSMCs. (A) Thrombin dosage responses after treatments of 24 h and (B) time course relationships with 0.5 U/ml of thrombin were tested on serum-deprived VSMCs. IF proteins (5 μg) were immunoblotted with anti-nestin and whole cell lysates (20 μg) with anti-αSMA or anti-SM-MHC. Data were shown as folds of untreated control (set at 1.0 as UT). (C) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 0.1, 1 and 10 μM each of PPACK and followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. (D) Rat VSMCs were cultivated in serum-containing medium with or without the treatment of 10 μM PPACK for 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin.

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. To breach the cells and to dissolve the formazan crystals, 100 μl of dimethyl sulfoxide was added to each well and the absorbance was then measured with a microplate reader at a wavelength of 595 nm.

3. Results

3.1. Thrombin induces nestin re-expression in serum-deprived rat VSMCs

We recently reported that nestin expression is regulated by EGF simulation [9]. To investigate whether EGFR transactivation is involved in nestin expression in rat VSMCs, several ligands of GPCRs, including thrombin, lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P) were applied to serum-deprived rat VSMCs. The results showed that thrombin significantly up-regulated nestin expression in a concentration-dependent manner, reaching a plateau at 0.5 U/ml (3.6 ± 0.7-fold increase) (Figs. 1A and 2A). However, this was not observed in LPA or S1P treatments (data not shown). In addition, the thrombin-induced nestin expression was time course-dependent, reaching a maximal level at 12 h and was sustained for 24 h (4.9 ± 0.9-fold increase) (Fig. 1B). Thus, a 24-h treatment with 0.5 U/ml thrombin was used as the positive control in subsequent experiments. To determine whether the thrombin-induced nestin expression is mediated through the proteolytic activity of thrombin, rat VSMCs were serum deprived for 2 days (Fig. 1C) then treated with a selective thrombin inhibitor PPACK for another 24 h. The results showed that PPACK abolished the thrombin-induced nestin re-expression starting at the concentration of 0.1 μM in serum-deprived rat VSMCs (Fig. 1C). Furthermore, PPACK abolished nestin expression even in VSMCs that are cultivated in serum-containing medium (Fig. 1D). These results indicated that thrombin plays a critical role in the regulation of nestin expression.

3.2. Thrombin induces nestin expression via de novo protein synthesis

To determine whether transcriptional and translational activities are needed for the thrombin-induced nestin expression, VSMCs were

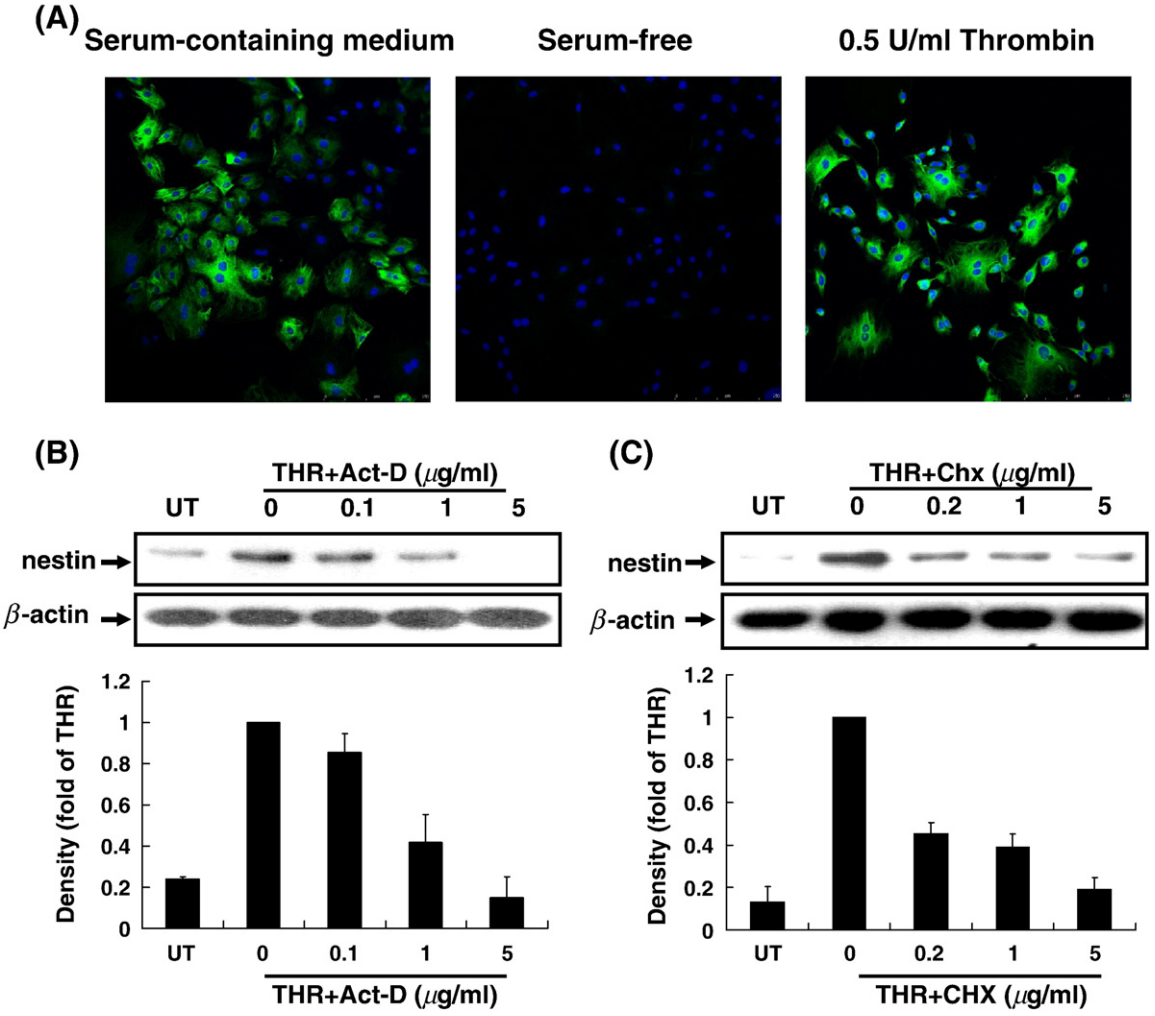

Fig. 2. Thrombin induces nestin expression via the *de novo* protein synthesis in rat VSMCs. (A) Rat VSMCs were cultivated in serum-containing medium (right panel), in serum-free medium (central panel), or in 0.5 U/ml thrombin (left panel). Nestin expression was then assessed by immunofluorescence microscopy. (B) and (C) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 0.1, 1 and 5 μg/ml of each of actinomycin-D (B), 0.2, 1 and 5 μg/ml of each of cycloheximide (C), followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin; Act-D, actinomycin-D; CHX, cycloheximide.

treated with actinomycin D (Act-D), a transcriptional inhibitor, or cycloheximide (CHX), an inhibitor of protein synthesis for 24 h. Both Act-D and CHX inhibited the thrombin-induced nestin expression in a concentration-dependent manner (Fig. 2B, C), indicating that the thrombin-induced nestin expression requires both *de novo* RNA and protein synthesis.

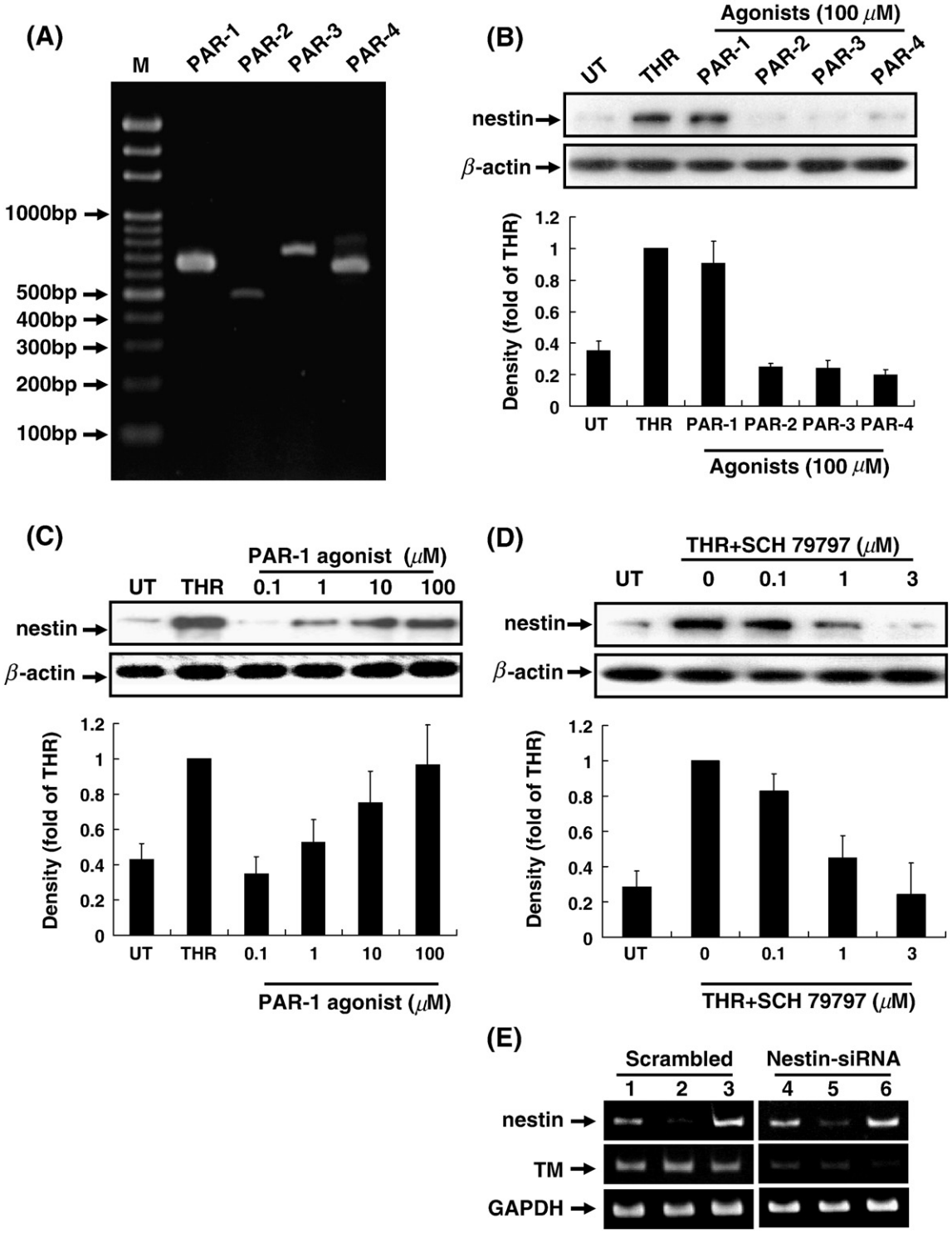

Fig. 3. Thrombin-induced nestin expression is mediated through PAR-1 in rat VSMCs. (A) The mRNA was extracted from rat VSMCs, and the expressions of PARs were then assayed by RT–PCR. Rat VSMCs were cultivated in serum-free medium for 2 days and followed by treatments with the following reagent sets for another 24 h: thrombin (0.5 U/ml), PAR-1 (100 μM), PAR-2 (100 μM), PAR-3 (100 μM), and PAR-4 (100 μM) agonists (B), or thrombin (0.5 U/ml), 0.1, 1, 10, 100 μM of each of the PAR-1 agonists (C). (D) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 0.1, 1 and 3 μM of each of SCH79797 and followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts. (E) Rat VSMCs were transfected with scrambled (lane 1–3) or TM siRNA (lane 4–6). Twenty four hours after transfection, rat VSMCs were cultivated in serum-free medium for 2 days (lane 2 and 5) and followed by treatments with 0.5 U/ml thrombin for another 24 h (lane 3 and 6), or cultivated in regular medium containing 10% FCS (lane 1 and 4). Nestin, TM and GAPDH expressions were then assessed by RT–PCR with a specific primer against nestin, TM and GAPDH, respectively. UT, untreated; M, marker; THR, thrombin.

3.3. Thrombin-induced nestin expression is mediated by PAR-1

By RT–PCR analysis, mRNAs of four subtypes PARs were present in rat VSMCs (Fig. 3A). To determine which PAR is involved in the thrombin-induced nestin re-expression, specific agonists of all four PARs were applied. Among them, only PAR-1 agonist induced nestin re-expression in serum-deprived rat VSMCs and its effect was similar to that of thrombin (Fig. 3B). PAR-1 agonist up-regulated nestin expression in a concentration-dependent manner, reaching a plateau at 100 μM, as demonstrated by immunoblotting analysis (Fig. 3C). Furthermore,

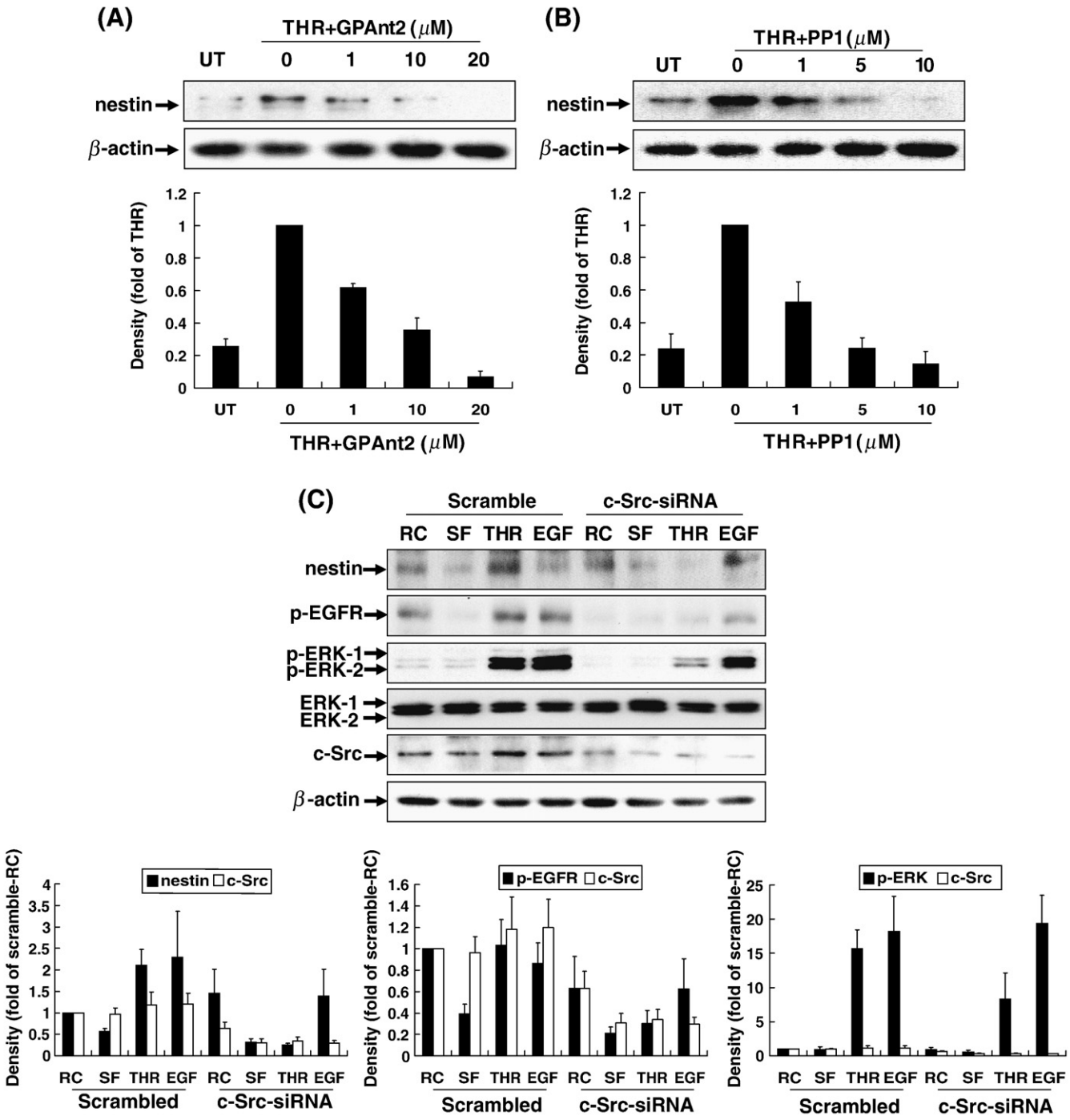

Fig. 4. Nestin expression induced by thrombin stimulation is mediated by G_i protein-coupled receptors and their downstream c-Src signaling in rat VSMCs. Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 1, 10 and 20 μM of each of GP antagonist-2 (A), or 1, 5 and 10 μM of each of PP1 (B), followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin were used as controls (set at 1.0) and data were shown as folds of it. (C) Rat VSMCs were transfected with scrambled, or c-Src siRNA. Twenty-four hours after transfection, rat VSMCs were cultivated in serum-free medium for 2 days, followed by treatments with thrombin (0.5 U/ml) or EGF (10 ng/ml) for another 24 h, or cultivated in regular medium containing 10% FCS. The nestin and c-Src expressions were then assessed by immunoblotting with anti-nestin and anti-c-Src, respectively. To determine the phosphorylations of EGFR and ERK, these transfected rat VSMCs were cultivated in serum-free medium for 2 days, followed by treatments with thrombin (0.5 U/ml) or EGF (10 ng/ml) for another 5 min. The EGFR and ERK phosphorylations were then assessed by immunoblotting with anti-phospho-EGFR and anti-phospho-ERK, respectively. The results were shown as means ± SEM ($n = 3$). Immunoblots were re-probed with anti-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin; GPAnt2, GP antagonist-2; RC, regular medium containing 10% FCS.

SCH79797, a specific PAR-1 antagonist, significantly prevented the thrombin-induced nestin expression in a concentration-dependent manner (Fig. 3D). It has been suggested that besides directly binding to PAR-1, thrombin can also implement its effects on PAR-1 through its binding to thrombomodulin (TM) [23]. To examine whether the PAR-1-induced nestin expression is mediated through the thrombin–TM interaction, specific TM siRNA was applied to thrombin-treated rat VSMCs. The RT–PCR experiments revealed that the depletion of TM failed to prevent the thrombin effect on nestin induction (Fig. 3E), indicating that TM is not involved in the thrombin-induced nestin expression. Taken together, these results indicated that thrombin-induced nestin expression is mainly through PAR-1 activation.

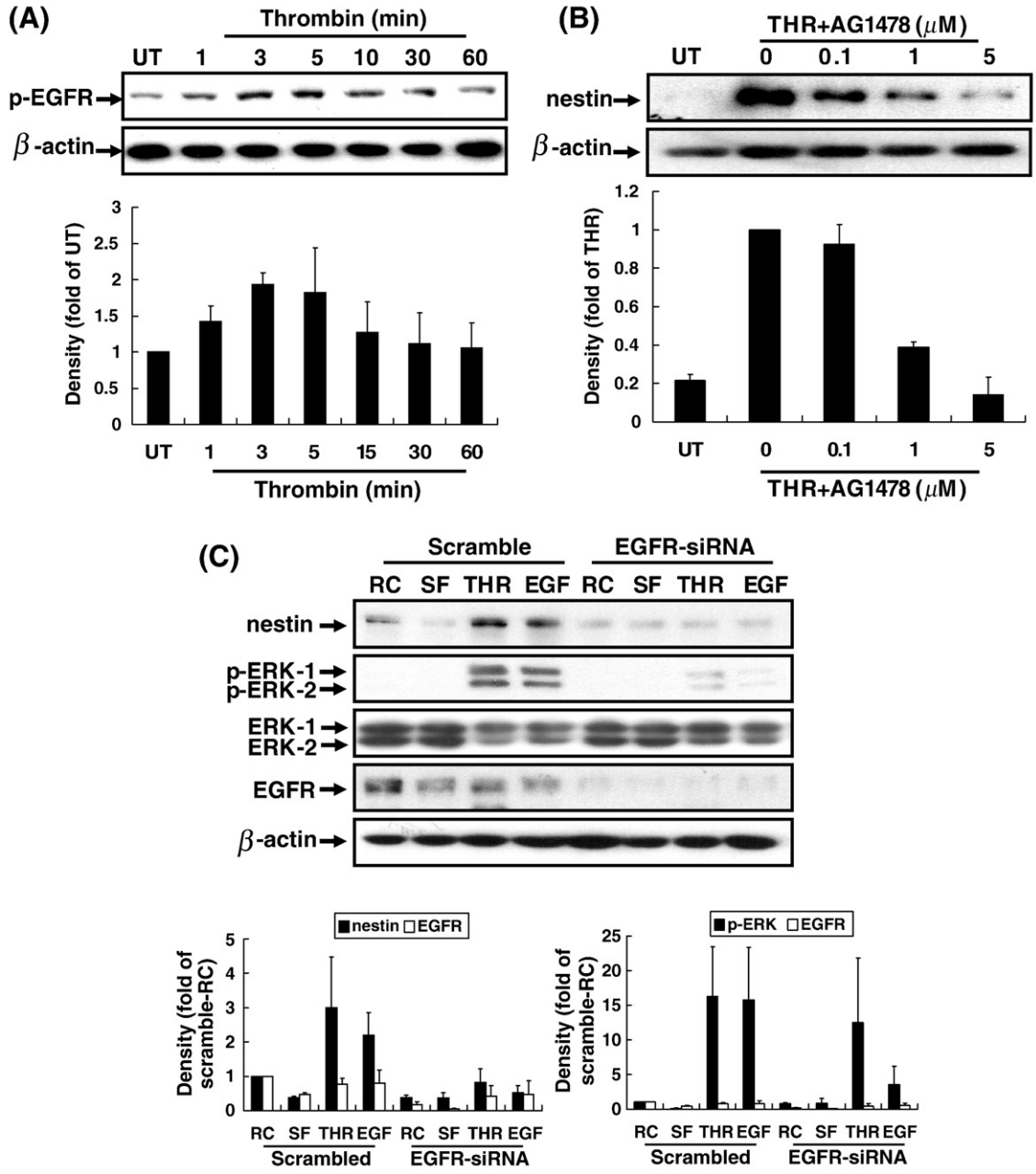

Fig. 5. Thrombin-induced nestin expression is mediated through the EGFR transactivation in rat VSMCs. (A) The serum-deprived rat VSMCs were treated with 0.5 U/ml for various time periods (1–60 min) and EGFR phosphorylation were then assessed by immunoblotting with anti-phospho-tyrosine^{845} EGFR. Cells without any treatment were used as controls (set at 1.0) and data were shown as a fold of it. (B) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 0.1, 1 and 5 μM of each of AG1478, followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin were used as controls (set at 1.0) and data were shown as folds of it. (C) Rat VSMCs were transfected with scrambled, or EGFR siRNA. Twenty-four hours after transfection, rat VSMCs were cultivated in serum-free medium for 2 days, followed by treatments with thrombin (0.5 U/ml) or EGF (10 ng/ml) for another 24 h, or cultivated in regular medium containing 10% FCS. The nestin and EGFR expressions were then assessed by immunoblotting with anti-nestin and anti-EGFR, respectively. To determine the ERK phosphorylation, these transfected cells were cultivated in serum-free medium for 2 days, followed by treatments with thrombin (0.5 U/ml) or EGF (10 ng/ml) for another 5 min. The ERK phosphorylation was then assessed by immunoblotting with anti-phospho-ERK. (D–F) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 1, 25 and 50 μM of each of GM6001 (D), 1, 10 and 20 μg/ml of each of heparin (E), or 1, 5 and 10 μM of each of HB-EGF neutralizing antibody (F), followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin; RC, regular medium containing 10% FCS; HB-EGF nAb, HB-EGF neutralizing antibody.

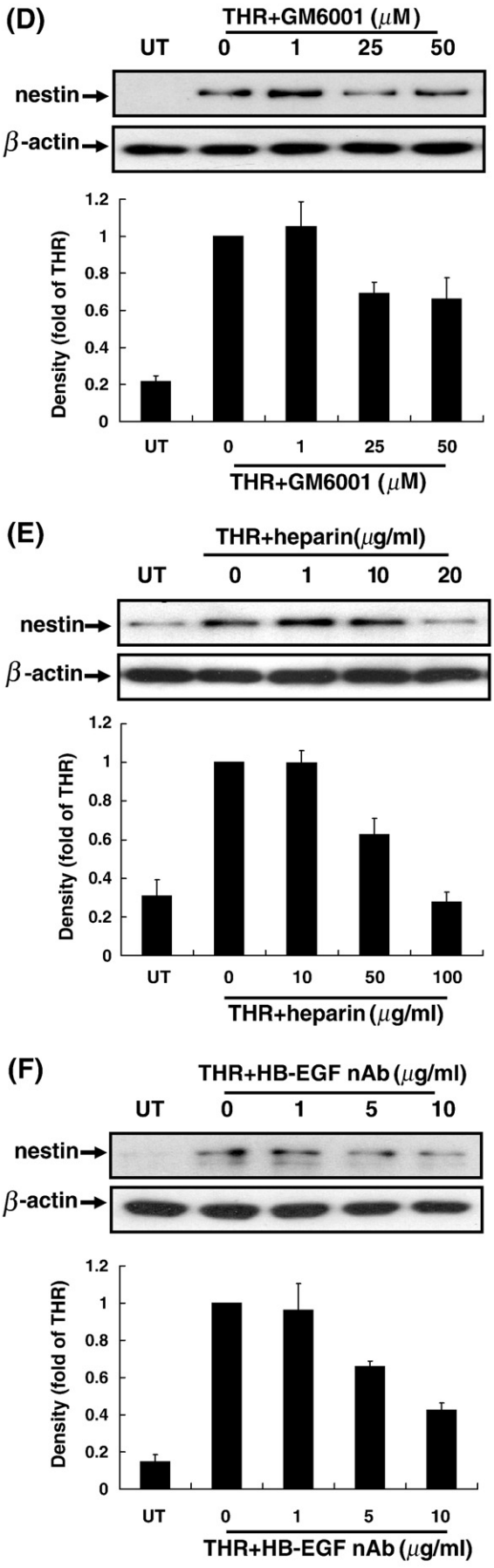

Fig. 5 (*continued*).

3.4. Thrombin induces nestin expression via the G_i protein/c-Src signaling pathway

Several studies have indicated that c-Src, a downstream molecule of Gi protein signaling, serves as a second messenger for the GPCR-induced EGFR transactivation [15,17]. In this regard, we first determine whether this G_i protein is required for the thrombin-induced nestin expression. As shown in Fig. 4A, treatment with GP antagonist-2, a selective G_i protein antagonist, inhibited the thrombin-induced nestin expression, implicating that the thrombin-induced nestin expression through PAR-1 is coupled to the G_i protein in rat VSMCs. Furthermore, PP1, a specific c-Src inhibitor, obviously inhibited the thrombin-induced nestin expression in a dosage-dependent manner and abolished the effect of thrombin at 10 μM (Fig. 4B). To further confirm whether the thrombin-induced nestin expression was mediated through the c-Src-dependent EGFR transactivation pathway, a specific c-Src siRNA was applied to thrombin-treated rat VSMCs. The results showed that the depletion of c-Src obviously inhibited EGFR phosphorylation, ERK activation, and nestin expression in thrombin-treated rat VSMCs, but not in EGF-treated cells (Fig. 4C). Taken together, these results indicated that the thrombin-induced nestin expression is mediated through c-Src signaling.

3.5. Thrombin induces nestin expression through the intracellular mechanism of EGFR transactivation

Two mechanisms of c-Src on GPCRs-induced EGFR transactivation have been reported, one is through the intracellular phosphorylation of EGFR at tryoine^{845} residue and the other one through the extracellular activation of MMPs [15,17,20,21,24,25]. We first examined whether the c-Src-mediated intracellular signaling mechanism is involved in EGFR transactivation in rat VSMCs. By using a specific antibody against the phospho-tryosine^{845} residue of EGFR, we showed that thrombin induced EGFR phosphorylation at tyrosine^{845} within 1 min and then declined after 5 min (Fig. 5A). To further determine whether EGFR transactivation was required for the thrombin-induced nestin expression, an EGFR-specific tyrosine kinase inhibitor AG1478 and its specific siRNA were used. As shown in Fig. 5, both AG1478 (Fig. 5B) and the EGFR-specific siRNA (Fig. 5C) inhibited the thrombin-induced nestin expression in rat VSMCs. The EGFR RNAi experiments also revealed that the depletion of EGFR significantly abrogated the thrombin effects on ERK phosphorylation (Fig. 5C). These results indicated that the thrombin-induced nestin expression was mediated through the intracellular mechanism of EGFR transactivation.

3.6. Thrombin induces nestin expression through the extracellular mechanism of EGFR transactivation

To determine whether the extracellular mechanism of the EGFR transactivation was involved in the thrombin-induced nestin expression, the MMP inhibitor GM6001 was applied to thrombin-treated rat VSMCs. The results revealed that at the concentrations of 25 and 50 μM, GM6001 attenuated the effects of thrombin on nestin expression (Fig. 5D). To determine whether the MMP-mediated shedding of HB-EGF was involved in the thrombin-induced nestin expression, heparin and the neutralizing antibody against HB-EGF were applied. As shown in Fig. 5, both heparin (Fig. 5E) and the neutralizing antibody against HB-EGF (Fig. 5F) inhibited the thombin-induced nestin expression in a concentration-dependent manner. In addition, HB-EGF treatment also up-regulated the expression of nestin in serum-deprived rat VSMCs (data not shown). It has been documented that MMP-2 is required for the ectodomain shedding of HB-EGF during the thrombin-mediated EGFR transactivation process in several cultured cells including VSMCs [14,26,27]. We examined the involvement of MMP-2 using the MMP-2 inhibitor and MMP-2 RNAi experiments. As shown in Fig. 6, both the MMP-2 inhibitor (Fig. 6A)

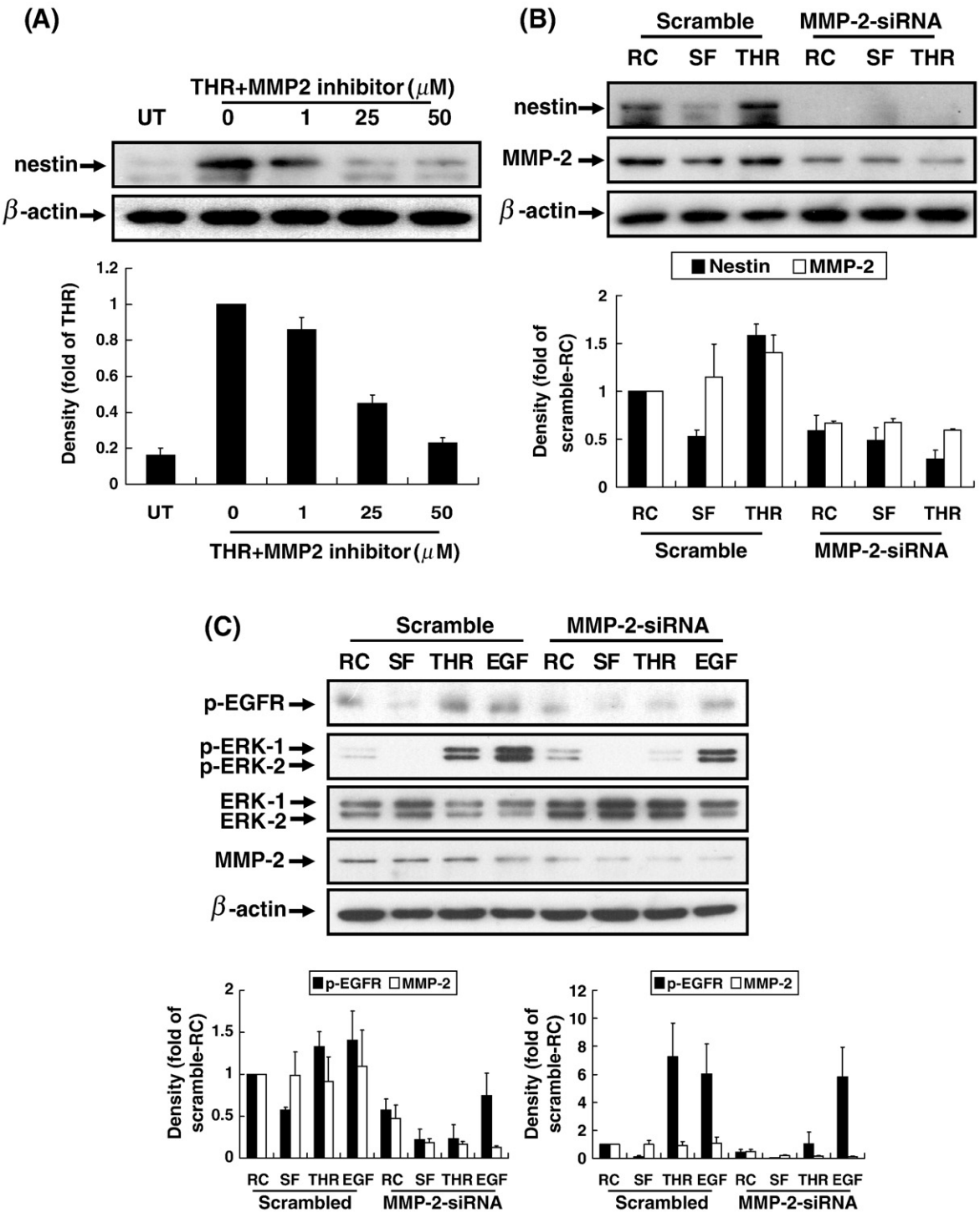

Fig. 6. Thrombin-induced nestin expression is mediated through MMP-2 in rat VSMCs. (A) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 1, 25 and 50 μM of each MMP-2 inhibitor, followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin were used as controls (set at 1.0) and data were shown as folds of it. (B) Rat VSMCs were transfected with scrambled, or MMP-2 siRNA. Twenty-four hours after transfection, rat VSMCs were cultivated in serum-free medium for 2 days, followed by treatments with 0.5 U/ml thrombin for another 24 h, or cultivated in regular medium containing 10% FCS. The nestin and MMP-2 expressions were then assessed by immunoblotting with anti-nestin and anti-MMP-2, respectively. To determine the phosphorylations of EGFR and ERK, these transfected cells were cultivated in serum-free medium for 2 days, followed by treatments with thrombin (0.5 U/ml) or EGF (10 ng/ml) for another 5 min. The EGFR and ERK phosphorylations were then assessed by immunoblotting with anti-phospho-EGFR and anti-phospho-ERK, respectively. The results were shown as means ± SEM ($n = 3$). Immunoblots were re-probed with anti-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin; RC, regular medium containing 10% FCS; SF, serum-free.

and its specific siRNA (Fig. 6B) significantly abrogated the thrombin-induced nestin expression in rat VSMCs. The MMP-2 RNAi experiment also showed that the downregulation of MMP-2 obviously abolished the phosphorylations of EGFR and ERK in response to the stimulation of thrombin, but not to the treatments with EGF (Fig. 6C). Taken together, these results indicated that the thrombin-induced nestin expression was also mediated through the MMP-2-mediated extracellular mechanism of EGFR transactivation.

3.7. Thrombin induces nestin expression through the Ras–Raf–ERK signaling axis

Previous reports have demonstrated that the stimulation of PAR-1 leads to rapid activation of Ras signalings such as mitogen-activated protein kinases (MAPK), ERK, p38 and JNK [24]. To examine the requirement of the Ras–Raf pathway, a Raf-1 inhibitor GW5074 was applied to thrombin-treated rat VSMCs. The results showed that GW5074 significantly prevented the thrombin-induced nestin expression in a concentration-dependent manner and totally abolished the

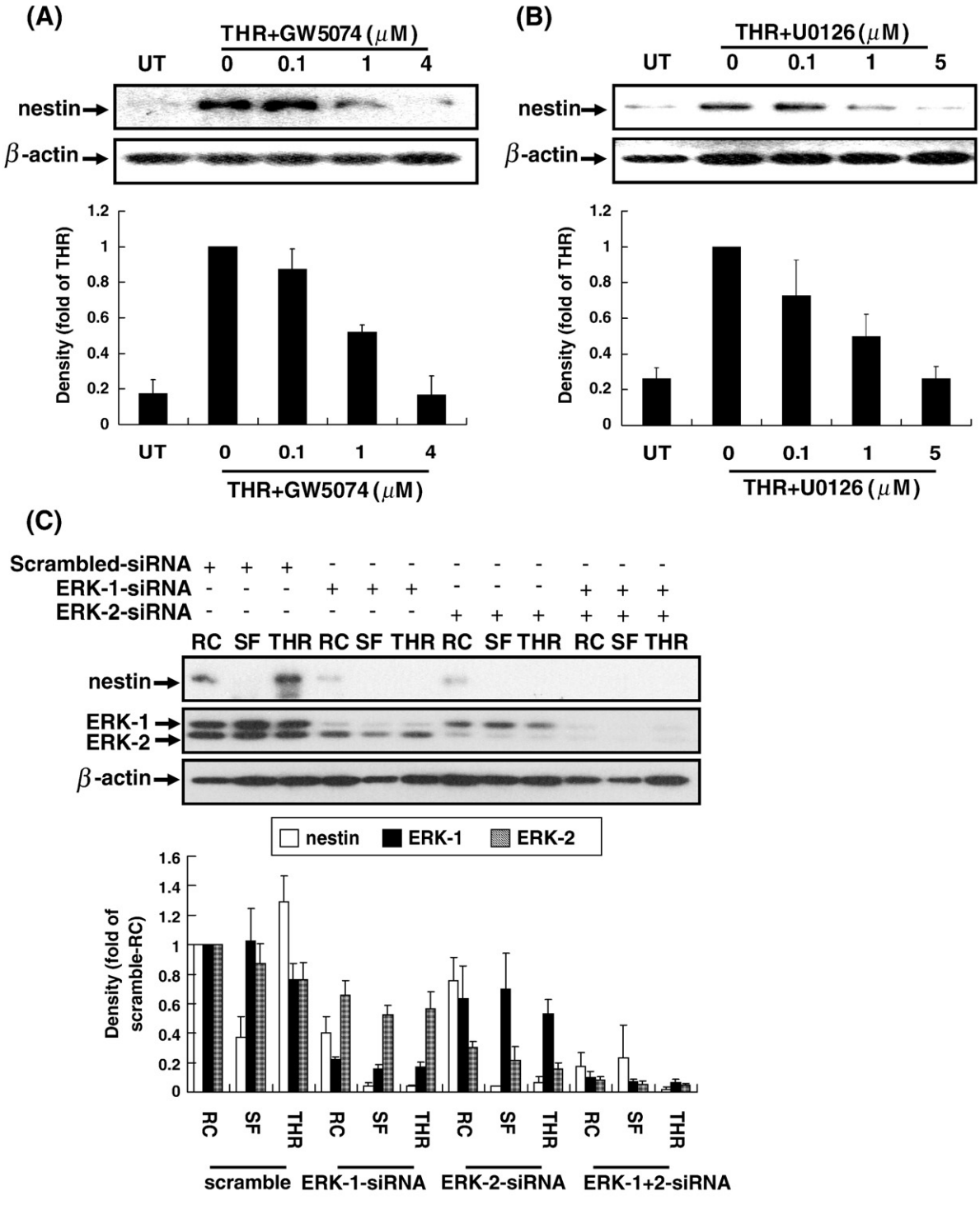

Fig. 7. Thrombin-induced nestin expression is mediated by the Ras–ERK, but not the p38 or JNK signaling pathway in rat VSMCs. Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 0.1, 1 and 4 μM of each GW5074 (A), 0.1, 1 and 5 μM of each U0126 (B), followed by cotreatments with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin were used as controls (set at 1.0) and data were shown as folds of it. (C) Rat VSMCs were transfected with scrambled, ERK-1 siRNA, ERK-2 siRNA or both ERK-1 and ERK-2 siRNA. Twenty-four hours after transfection, rat VSMCs were cultivated in serum-free medium for 2 days, followed by treatments with 0.5 U/ml thrombin for another 24 h, or cultivated in regular medium containing 10% FCS. The nestin and ERK1/2 expressions were then assessed by immunoblotting with anti-nestin and anti-ERK1/2, respectively. (D) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 1, 10 and 20 μM of each SB203580 (D), or 1, 10 and 20 μM of each SP600125 (E), followed by cotreatments with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts. UT, untreated; THR, thrombin.

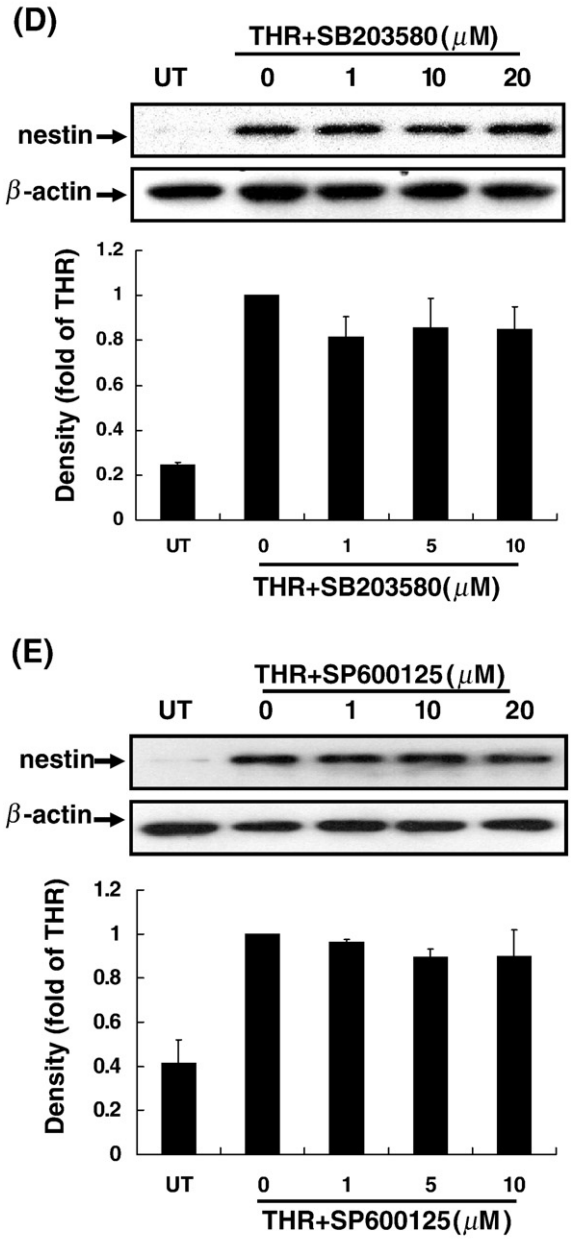

Fig. 7 (*continued*).

effect of thrombin at 4 μM (Fig. 7A). We found that ERK1/2 phosphorylation was induced by thrombin within 1 min, as demonstrated by immunoblotting (data not shown). To examine the role of ERK1/2 in the thrombin-induced nestin expression, the effects of U0126, a MEK-1 inhibitor that blocks ERK1/2 activation was examined. Treatments with U0126 suppressed the thrombin-induced nestin expression in a concentration-dependent manner and abolished the effect of thrombin at 5 μM (Fig. 7B). To further confirm the requirement of ERK in the thrombin-induced nestin expression, specific ERK-1 and ERK-2 siRNA were applied to thrombin-treated rat VSMCs. The results showed that depletion of either one of ERK-1 and ERK-2, or both of them abolished the thrombin-induced nestin expression in rat VSMCs (Fig. 7C). Roles of other MAPK pathways in the thrombin-induced nestin expression were also examined. As shown in Fig. 7, neither the inhibition of p38 (Fig. 7D) nor JNK (Fig. 7E) affected the thrombin-induced nestin expression. These results indicated that the thrombin-induced nestin expression is mediated through the Ras–Raf–ERK signaling axis, but not the JNK or p38 pathways.

3.8. ERK-activated Sp1 is involved in the thrombin-induced nestin expression

The nestin promoter was shown to contain several binding sites for various transcriptional factors (TFs) including AP-1, Sp1, and Pit-1 [28,29]. To determine which TFs were involved in the thrombin-induced nestin expression, we prepared nuclear extracts from thrombin-treated rat VSMCs and performed the EMSA experiment using the promoter binding site sequences for AP-1, Sp1 and Pit-1 as probes. Shifted bands were observed with the biotin-labeled Sp1 probe (Fig. 8A, lane 4 *versus* lane 3), but not with biotin-labeled AP-1 or Pit-1 probes (Fig. 8A, lane 1–2, and lane 5–6, respectively) following thrombin treatment. The shifted bands obviously competed with the 66-fold non-biotin-labeled Sp1 probe (Fig. 8A, lane 8). To further determine whether the thrombin-induced ERK activity is required for Sp1 binding ability, the MEK-1 inhibitor U0126 was applied. As shown in Fig. 8B, the thrombin-induced Sp1-DNA binding ability was significantly attenuated by the co-treatment with U0126 (lane 3 *versus* lane 2). To further confirm the Sp1 requirement in the thrombin-induced nestin expression, mitramysin A, a selective Sp1 inhibitor was used. The results showed that mitramycin A significantly abrogated the thrombin-induced nestin expression in a concentration-dependent manner and abolished the effect of thrombin at 500 nM (Fig. 8C). Taken together, these results indicated that thrombin induces nestin expression through the Sp1 binding activity.

3.9. Nestin expression plays an important role in cell proliferation induced by the thrombin-mediated EGFR transactivation

It was well known that thrombin induces cell proliferation through the EGFR transactivation in VSMCs [21,22]. Our above experiments showed that thrombin induces nestin expression also through the EGFR transactivation. Chou et al. reported that nestin may promote cell proliferation by enhancing the disassembly of vimentin IFs during mitosis [7]. In this regard, RNAi of nestin was carried-out to investigate the inter-relationship between nestin expression and cell proliferation induced by the thrombin-mediated EGFR transactivation in rat VSMCs. As shown in Fig. 9, nestin expression was significantly decreased by nestin RNAi (Fig. 9A) and the thrombin-induced cell proliferation was apparently attenuated in these nestin depleted cells (Fig. 9B). These results confirmed that the nestin expression induced by thrombin treatments is indeed mediated via the EGFR transactivation mechanism which appears to be the same as that involved in the thrombin-induced cell proliferation. Additionally, the results also showed that nestin expression plays an important role in the thrombin-induced cell proliferation. To further confirm that the EGFR transactivation system existed in the thrombin-induced nestin expression in rat VSMCs, nestin siRNA was transfected into EGF-treated rat VSMCs and cell proliferation was then examined by MTT proliferation assay. As shown in Fig. 9C, the EGF-stimulated cell proliferation effect was obviously attenuated by the depletion of nestin expression. To further confirm these, the expression of PCNA, a DNA-encircling homotrimer that is important for DNA replication was analyzed by immunoblotting. The results revealed that the expression of PCNA increased significantly (3.9 ± 1.2-fold increase), while this effect was not observed in nestin-depleted cells (1.3 ± 0.3-fold increase) following EGF treatment (Fig. 9D). Taken together, these results indicated that nestin expression is induced by thrombin via the same EGFR transactivation mechanism as in thrombin-induced cell proliferation and plays an important role in the cell proliferation induced by the thrombin-mediated EGFR transactivation (Fig. 10).

4. Discussion

The IF cytoskeleton is composed of more than 70 subunit proteins which are expressed in a tissue- and differentiation stage-specific

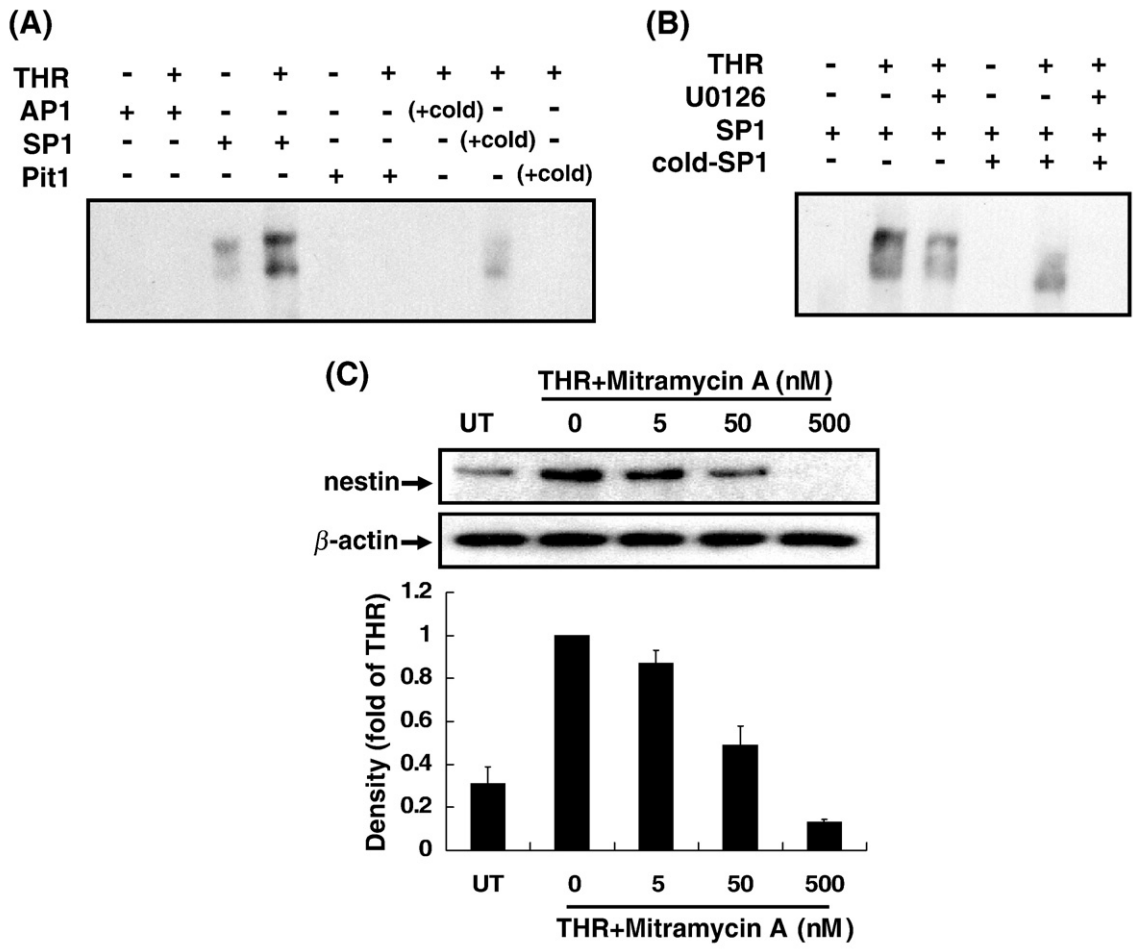

Fig. 8. Thrombin-induced nestin expression is mediated by ERK–SP1 in rat VSMCs. (A) Rat VSMCs were serum-deprived for 2 days, followed by the treatments with 0.5 U/ml thrombin for another 24 h and the nuclear proteins were then extracted. The potential TFs were determined by EMSA experiments using biotin-labeled promoter binding site sequences for AP-1, Sp1, and Pit-1 as probes (lane 1–6). The competition experiments were performed with a 66-fold excess of non-biotin-labeled AP-1, Sp1, and Pit-1 oligonucleotide probes (cold probes) in EMSA experiments (lane 7–9). (B) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only or with 5 μM U0126, followed by the treatments with 0.5 U/ml thrombin for another 24 h. Nuclear protein was then extracted and analyzed by EMSA using the biotin-labeled Sp1 oligonucleotide probe (lane 1–3). The specificity of Sp1-DNA binding ability was assessed by pre-incubating the nuclear extracts with a 66-fold excess non-biotin-labeled Sp1 oligonucleotide probe as competitor (cold-Sp1, lane 4–6). (C) Rat VSMCs were serum-deprived for 2 days, preincubated for 1 h with thrombin-only, 5, 50 and 500 nM of each mitramycin A, followed by the cotreatment with 0.5 U/ml thrombin for another 24 h. Nestin expression was then assessed by immunoblotting with anti-nestin. Cells treated only with thrombin 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-β-actin to ensure equal loading amounts.

manner [30]. Regarding their functions, they may provide mechanical support to cells [30], act as tissue-specific scaffolds to organize and modify signaling pathways for cell survival, migration, and sequester stress-activated kinases [31]. However, little is known about their gene regulation. We recently reported that nestin is regulated by an extracellular ligand EGF in rat VSMC [9]. In this study, we further showed that nestin can also be regulated by thrombin via the EGFR transactivation mechanism in rat VSMCs.

Previous studies have classified VSMCs into two groups, the contractile and the synthetic [32]. The phenotypic modulation from the contractile phenotype to the synthetic one is a hallmark of VSMCs following various cardiovascular diseases and balloon injury [10–12]. In contrast to the incapability of mature skeletal and cardiac muscle cells to proliferate, SMCs exhibit the ability to proliferate and the proliferation of tunica media VSMCs caused by the above phenotypic modulation leads to medial hyperplasia following injuries. Since the majority of previous studies focused on VSMCs in weeks after a lesion [12], the mechanisms underlying the de-differentiation and activation of VSMCs shortly following injury remain elusive. Thus, the identification of a proper marker corresponding to the early de-differentiation process is important. Nestin is a marker for muscle and neural progenitors, its expression is generally ceased in those mature cells but resumes in reactive astrocytes and muscle cells following injuries [1,2,4,5,9].

It is well established that primary cultures of VSMCs represent the synthetic phenotype [32] and re-differentiates into contractile ones following serum deprivation [33]. By using this cell culture system, we recently reported that nestin is expressed in primary cultures of VSMCs and its expression disappears in serum-deprived VSMCs [32]. In addition, we also demonstrated that the expression of nestin is regulated by EGF stimulation via EGFR activation [18]. These results suggested that nestin expression is closely associated with the contractile–synthetic transformation of VSMCs.

It is well-established that EGFR signaling is also activated by GPCR stimulation. This process is known as EGFR transactivation [13–15]. To verify whether nestin expression is also induced by the GPCR-mediated EGFR transactivation mechanism, serum-deprived rat VSMCs were challenged with thrombin, LPA, and S1P which were all reported to be involved in VSMC proliferation via the EGFR transactivation [20,22]. The results showed that only thrombin up-regulated nestin expression in rat VSMCs (Fig. 1). Besides the coagulant effects of thrombin [18], it is also acts as a potent mitogen for VSMCs by binding to PARs, its specific GPCRs [22,34,35]. In normal arteries, the expression of PARs is limited to the endothelial cells.

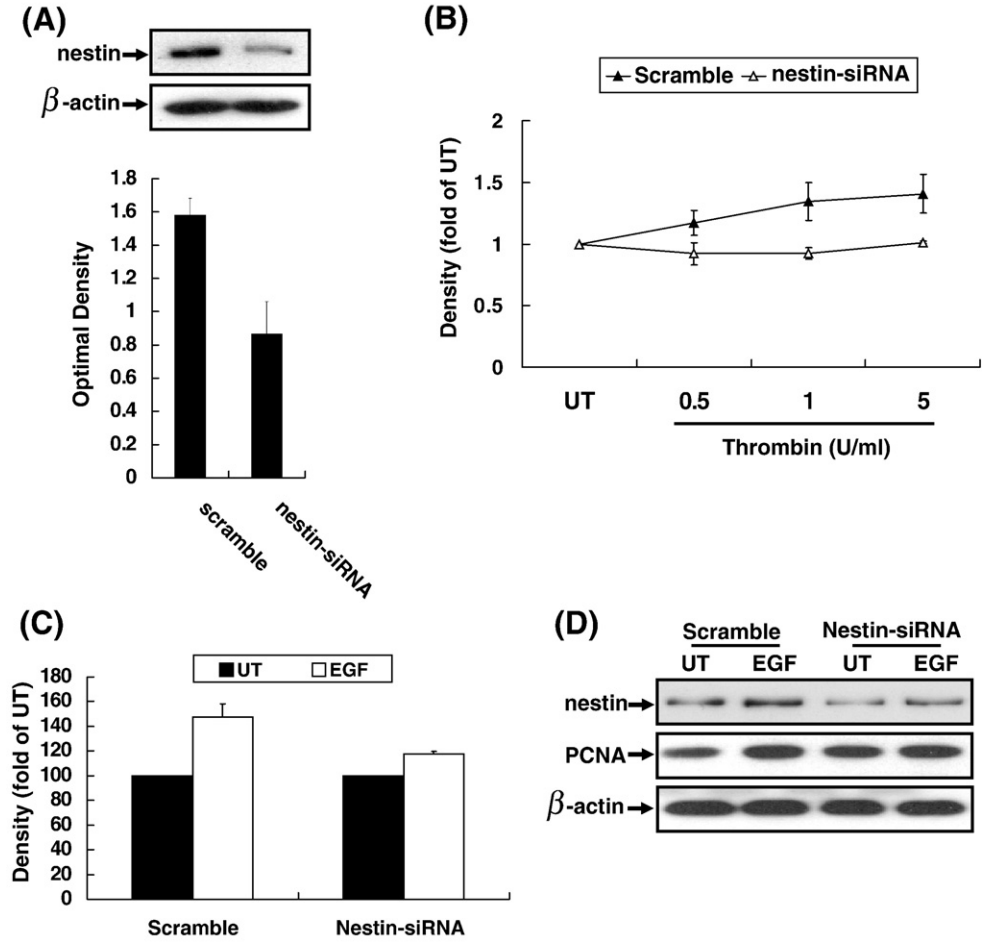

Fig. 9. The EGFR-mediated nestin expression upon thrombin stimulation participates in the cell proliferation of rat VSMCs. Rat VSMCs were transfected with scrambled (black bars) or nestin siRNA (white bars). (A) The nestin expression was assessed by immunoblotting with anti-nestin. Immunoblots were re-probed with anti-β-actin to ensure equal loading amounts. Two days after the transfection, these transfected rat VSMCs were treated with 0.5–5 U/ml thrombin (B) or 10 ng/ml of EGF (C) and (D) for another 24 h. The cell proliferation was then assessed by MTT analysis. Cells without treatments were used as controls (set at 1.0) and data were shown as folds of it. The results were shown as means ± SEM ($n=3$). (D) The nestin and PCNA expressions were assessed by immunoblotting with anti-PCNA. Immunoblots were re-probed with anti-β-actin to ensure equal loading amounts.

However, the expression of PAR-1 is up-regulated in VSMCs of the tunica media following balloon injury [36]. These results suggested that thrombin appears to contribute to the phenotypic modulation of VSMCs which characterizes the de-differentiation process. In this study, we showed that thrombin induces nestin expression in a dose- and time course-dependent manner (Fig. 1A, B) through PAR-1 (Fig. 2B). On the contrary, the downregulation of SM-MHC and αSMA upon thrombin stimulation that represent the synthetic phenotype. This is consistent to our previous report showing that nestin is a de-differentiation marker for synthetic VSMCs [9]. The above results indicated that thrombin can modulate the phenotype by regulating nestin expression in rat VSMCs.

Previous studies have revealed that the PAR-induced EGFR transactivation leading to proliferation is mediated by G_i protein signaling [13–15,17] and c-Src plays a pivotal role in this process [15,17,20,21,25]. In this study, we showed that the thrombin-induced nestin expression is indeed mediated through the PAR-1 coupling to G_i protein, as demonstrated by using its specific inhibitor GP antagonist-2 (Fig. 4A). Furthermore, treatments with c-Src inhibitor PP1 totally abolished the thrombin-induced nestin expression (Fig. 4B). Both intracellular and extracellular mechanisms are involved in the c-Src signaling leading to EGFR transactivation. For the intracellular signaling effect of c-Src, our results revealed that thrombin induces the c-Src-specific EGFR phosphorylation at tyrosine^{845} residue (Fig. 5A) and this effect was abolished by the treatment of PP1 (data not shown), indicating that c-Src indeed plays a critical role in the intracellular signaling mechanism.

Accumulating evidences have demonstrated that MMPs are the key mediators triggered by GPCRs to produce a second ligand which leads to EGFR transactivation [26,37]. In addition, the activity of MMPs is activated by c-Src [15,17,20,21,24,25]. Thus, to examine the c-Src-mediated extracellular signaling, the MMP inhibitor GM6001 was applied to thrombin-treated VSMCs. Our results showed that the thrombin-induced nestin expression is attenuated by the treatments of GM6001 (Fig. 5D). Furthermore, the thrombin-induced nestin expression is also attenuated by the inhibition of HB-EGF using its neutralizing antibody (Fig. 5F). These results indicated that the extracellular signaling mechanism is also required for the thrombin effects on nestin induction and suggested that HB-EGF is a second ligand for EGFR activation. It has been reported that the release of HB-EGF under pressure-induced myogenic tone and subsequent EGFR transactivation is significantly inhibited by challenging VSMCs with the MMP-2 inhibitor in mouse mesenteric resistance arteries [38]. This indicates that MMP-2 is responsible for shedding of HB-EGF during the EGFR transactivation. In this study, we used two kinds of inhibitory methods, its specific inhibitor and RNAi to block the MMP-2 activity, and showed that the thrombin-induced nestin expression is significantly abrogated by these MMP-2 inhibitions (Fig. 6). These results suggested that c-Src transmits two signalings in the thrombin-induced nestin expression, one intracellular and the other extracellular.

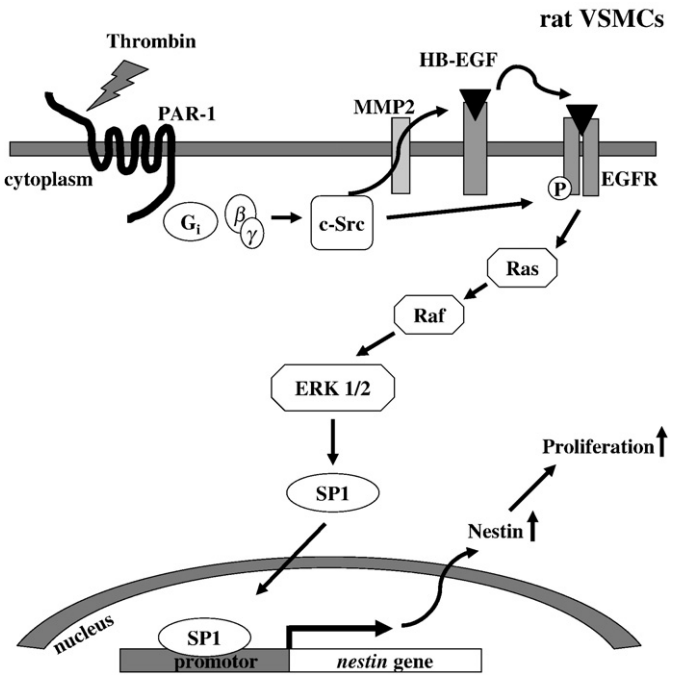

Fig. 10. Schematic representation of signaling pathways involved in the thrombin-induced nestin expression and proliferation in rat VSMCs. Thrombin binds to its receptor, PAR-1 coupled with G_i protein, resulting in the activation of Ras, Raf, ERK1/2, and Sp1 via a EGFR transactivation mechanism. The c-Src plays at least two roles in this process, one is the phosphorylation of EGFR at tyrosine^{845} and the other is the activation of the MMP-2/HB-EGF pathways. Nestin transcription is regulated by the ERK–Sp1 signaling axis. These signaling pathways contribute to the thrombin-induced cell proliferation in rat VSMCs.

It is well known that the EGFR–Ras–Raf–ERK signaling pathway plays an important role in the thrombin-induced proliferation [15,22,24]. Our recent study has also showed that the nestin expression induced by EGF is mediated through the Ras–Raf–ERK signaling pathway [9]. In this study, we showed that the thrombin-induced nestin expression is abolished by the treatment of Raf-1 inhibitor GW5074 and MEK-1 inhibitor U0126 (Fig. 7). These results confirmed that the thrombin-induced EGFR–Ras–Raf–ERK signaling axis is involved in both nestin expression and cell proliferation in rat VSMCs.

Although nestin has been widely used as a marker for muscular and neural progenitor cells, its gene regulation mechanism by transcriptional factors is unclear. By serial deletion of the nestin promoter, Cheng et al. showed that the minimal promoter of nestin gene resides in the region −11 to + 183 of the 5′-non-coding region [28]. The report also showed that two-adjacent Sp-1-binding sites are necessary for the activities of the nestin promoter in mouse embryonic carcinoma P19 cells, whereas AP-1 and AP-2 are not required, as demonstrated by EMSA experiments [28]. It has been reported that the POU-family transcriptional factors are essential for specific gene expression of CNS stem cells including nestin [29]. In this study, we identified that the nestin promoter contains several potential binding sites for transcriptional factors including a POU-family member Pit-1 using the TRANSFAC-TESS software (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Among these three TFs, our results showed that only the Sp1-DNA binding ability was increased upon thrombin treatments and that this binding ability was prevented by the ERK1/2 inhibition in rat VSMCs (Fig. 8). We also showed that the Sp1 activation is involved in the thrombin-induced nestin expression, as demonstrated by the application of a Sp1 inhibitor mitramycin A (Fig. 8C). It is interesting to note that the expression of Sp1, a zinc finger transcriptional factor, was increased in neointimal VSMCs in the carotid artery ligation model [39], indicating that Sp1 is implicated to maintain the synthetic phenotype of VSMCs. Taken together, our results showed that Sp1, but not AP-1 nor Pit-1 is required for the thrombin-induced nestin expression.

Since the thrombin-induced nestin expression and cell proliferation in VSMCs [15,22] are both mediated through the EGFR transactivation, RNAi of nestin were applied to delineate whether these two thrombin-induced effects share the same EGFR transactivation signaling mechanism. The results revealed that both the EGF- and the thombin-induced cell proliferation were attenuated in these nestin-depleted rat VSMCs (Fig. 9), indicating that they both were mediated through a related EGFR transactivation system. Regarding the functional role of nestin, previous studies showed that nestin plays an important role in cell proliferation by regulating the structural dynamics of vimentin IFs in BHK-21 cells [7]. It is interesting to note that BHK-21 cells appear to be smooth muscle progenitor cells in origin due to its expression of desmin and nestin [40,41]. Furthermore, a recent study has revealed that the knockdown of nestin using specific siRNA results in diminished proliferation of mesangial cells [8]. Consistently, our results revealed that nestin expression is important for the promotion of cell proliferation in rat VSMCs. Several studies have showed that the proliferation of VSMCs in the tunica media of injured carotid artery was attenuated by the blockage of EGFR using anti-EGFR [10], as well as in HB-EGF knockout mice [11]. Based on our above results, nestin may play an important role in the cell proliferation induced by the thrombin-mediated EGFR transactivation.

5. Conclusion

In this study, we revealed that the nestin gene in rat VSMCs is up-regulated by thrombin via a process that is dependent on activation of PAR-1 and c-Src followed by transactivation of MMP-2-mediated shedding of HB-EGF which leads to activation of EGFR. The transcriptional factor Sp1 is implicated in the final up-regulation of nestin gene expression. This process may be important for facilitating cell proliferation in rat VSMCs.

Acknowledgments

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