Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H₂O₂

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Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H₂O₂. Am J Physiol Cell Physiol 296: C711–C723, 2009. First published November 26, 2008; doi:10.1152/ajpcell.00442.2008.—NADPH oxidase (Nox4) generates reactive oxygen species (ROS) that are important for vascular smooth muscle cell (SMC) behavior, but the potential impact of Nox4 in stem cell differentiation is unknown. When mouse embryonic stem (ES) cells were plated on collagen IV-coated dishes/flasks, a panel of SMC-specific genes was significantly and consistently upregulated. Nox4 expression was markedly correlated with such a gene induction as confirmed by real-time PCR, immunofluorescence, and Western blot analysis. Overexpression of Nox4 specifically resulted in increased SMC marker production, whereas knockdown of Nox4 induced a decrease. Furthermore, SMC-specific transcription factors, including serum response factor (SRF) and myocardin were activated by Nox4 gene expression. Moreover, Nox4 was demonstrated to drive SMC differentiation through generation of H₂O₂. Confocal microscopy analysis indicates that SRF was translocated into the nucleus during SMC differentiation in which SRF was phosphorylated. Additionally, autosecreted transforming growth factor (TGF)-β–activated Nox4 and promoted SMC differentiation. Interestingly, cell lines generated from stem cells by Nox4 transfection and G418 selection displayed a characteristic of mature SMCs, including expression of SMC markers and cells with contractile function. Thus we demonstrate for the first time that Nox4 is crucial for SMC differentiation from ES cells, and enforced Nox4 expression can maintain differentiation status and functional features of stem cell-derived SMCs, highlighting its impact on vessel formation in vivo and vascular tissue engineering in the future.

NADPH oxidase; serum response factor; myocardin

METHODS AND MATERIALS

Materials. Antibodies against Nox4 for immunostaining and flow cytometric analysis (goat, N-15, sc-21860), serum-response factor (SRF, rabbit, G-20, sc-335), and myocardin (goat, N-16, sc-21559) were purchased from Santa Cruz Biotech. The antibody against Nox4 for Western blot analysis was obtained from Professor Ajay Shah’s laboratory (1). Antibody against phosphorylation SRF was purchased from Abcam, Cambridge, UK (Phospho S99, ab53130). Antibody against smooth muscle myosin heavy chain (SM-MHC) was from AbD Serotec (Rabbit, AHP1117). Antibodies against α-tubulin (mouse), histone 4 (Rabbit), and monoclonal anti-α smooth muscle actin (SMAα) (Clone 1A4, A5228) were from Sigma. All secondary antibodies were from Dako. All other materials used in this study were purchased from Sigma except those indicated.

ES cell culture and SMC differentiation. Detailed protocols for mouse ES cell (ES-D3 cell line, CRL-1934; ATCC, Manassas, VA)
culture and SMC differentiation were described in our previous studies (36, 37). Briefly, for SMC differentiation, undifferentiated ES cells were seeded on mouse collagen IV (5 μg/ml)-coated flasks or plates in differentiation medium [DM, MEM α-medium (GIBCO) supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 4–12 days before further treatment. The medium was refreshed every other day. For TGF-β1 treatment, the predifferentiated ES cells were cultured in serum-free TGF-β1 basal medium [MEM α-medium supplemented with 1% bovine serum albumin (BSA), 10 ng/ml insulin (Sigma), 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin] for 1 h, followed by the addition of different amounts (1, 2, 5, and 10 ng/ml) of TGF-β1 (Sigma) and further incubation for 1, 3, 6, or 24 h. For H2O2 treatment, the predifferentiated ES cells were cultured in serum-free H2O2 basal medium (MEM α-medium supplemented with 1% BSA, 10 ng/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 1 h, followed by the addition of different amounts of H2O2 (Sigma). In some cases, day 4 or day 5 of differentiating ES cells were incubated in serum-free H2O2 basal medium for 1 h and pretreated with 3,000 U/ml of catalase (Sigma) for 3–4 h followed by the addition of 3,000 U/ml of catalase (Sigma) for 3–4 h followed by the addition of 10 μM of H2O2 for a further incubation of 5 h.

**Immunoblotting.** Cells were harvested and lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 1 mM EDTA, pH 8.0) supplemented with protease inhibitors and 0.5% Triton X-100 for whole cell lysate, or with hypotonic buffer (10 mM HEPES-KOH, pH 7.2, 1.5 mM MgCl2, 10 mM KCl) and high-salt buffer (20 mM HEPES-KOH, pH 7.2, 25% glycerol, 1.5 mM MgCl2, 420 mM KCl, 0.2 mM EDTA) supplemented with protease inhibitors and 0.5% Nonidet P-40 for nuclear and cytoplasmic fractions. Forty micrograms of protein were separated by SDS-PAGE with 4–20% Tris-glycine gel (Invitrogen, Carlsbad, CA) and subjected to standard Western blot analysis.

**Indirect immunofluorescent assay.** Indirect immunofluorescent assay was performed as described before (36). Briefly, ES-derived SMCs or undifferentiated ES cells were labeled with isotype IgG control or SM α-MHC (AbD Serotec, AHP111), Nox4 (N-15, Santa Cruz), and SM-MHC antibodies for 30 min on ice, followed by incubation with appropriate secondary antibody conjugated with FITC (DAKO). Cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma) and examined with SP5 confocal microscope (Leica, Germany).

**Real-time RT-PCR.** Total RNA was extracted from cells using RNAeasy kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription was performed using an ImProm-II RT kit (Promega, Madison, WI) with RNase inhibitor (Promega) and random primers (Promega). Simultaneous RT reactions were performed without the addition of reverse transcriptase to control the possible transcription of contaminating genomic DNA. Primers were designed using Primer Express software (Applied Biosystems), and the published sequence for the mouse genes are available here (supplemental Table S1). Relative mRNA expression level was defined as the ratio of target gene expression level to 18S expression level with that of the control sample set as 1.0.

**Flow cytometric analysis.** The procedure used for flow cytometry was similar to that described previously (36). Briefly, the harvested cells were fixed, permeabilized with 0.1% Triton-X-100, and incubated in diluted serum for 20 min. The single-cell suspension was aliquoted and incubated with either isotype IgG control or SMα-MHC, Nox4, and SM-MHC antibodies for 30 min on ice, followed by incubation with appropriate secondary antibody conjugated with FITC (DAKO). Stained cells were suspended in FACS analysis buffer containing propidium iodide (PI) and analyzed with a FACS scan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). For apoptotic analysis, Annexin V staining was performed according to the manufacturer’s instructions (Bender Medsystems). Briefly, the cells were resuspended in 200 μl of binding buffer containing 5 μl of annexin V-FITC for 10 min at room temperature. After washing was completed, cells were incubated with 200 μl of binding buffer containing 10 μl of propidium iodide. After appropriate marking for negative and positive populations, the percentage of annexin V+/PI− or V−/PI+ cells was determined and compared with controls.

**Nucleofection.** For transient transfection, Nox4 plasmids pCDNA3.1-Nox4 (obtained from Professor Ajay Shah’s group) (1) were introduced into undifferentiated ES cells with mouse ES cell nucleofection kit (Amaza, VPH-1001) using nucleofector II (Amaza, Germany) according to the manufacturer’s instructions. In some cases, pmax-GFP (Amaza) was included as positive control to evaluate the transfection efficiency. An average of 70% of cells was transfected successful in
our study (data not shown). Transfected cells were plated in dishes coated with 5 μg/ml of collagen IV and cultured for 3–4 days in the DM. Total RNA and protein were harvested and subjected to real-time PCR analysis for gene expression and Western blot analysis for protein levels.

siRNA experiments. The Nox4 small interfering RNA (siRNA, sc-41587), TGF-β receptor 1 siRNA (sc-40223), and fluorescein-conjugated nontargeting control siRNA-C (sc-44240) were purchased from Santa Cruz Biotech. ES cells were cultured on collagen IV-coated six-well plates for 3–5 days, and 10 μl of 10 μM siRNA were introduced with siIMPORTER transfection reagents (Millipore) according to the protocol provided. Cells were harvested at 48 or 72 h after transfection, and real-time RT-PCR and Western blot analysis were performed.

Lucigenin-enhanced chemiluminescence assay. Cells were suspended in 400 μl buffer B (50 mM monobasic potassium phosphate, 1 mM EGTA, and 5.5 mM sucrose, pH 7.0) with 2 μl protease inhibitor cocktail [containing 4-(2-amino-ethyl)-benzenesulfonamide fluoride, pepstatin A, bestatin, leupeptin, and aprotinin] and homogenized on ice with a Polytron PT2100 Sonicator in 10-s bursts for three times. Ten micrograms of protein were added to a 96-well microplate to a total volume of 75 μl. NADPH (300 μM) was added as the substrate to each well. Superoxide production from each well was measured and expressed as mean arbitrary light units over 20–30 min. The enzymatic source of NADPH-stimulated superoxide production in undifferentiated and differentiating ES cells was determined using the following inhibitors: diphenylene iodonium (DPI), which inhibits all flavoproteins (10 μM); nitro-o-arginine methyl ester (l-NAME); nitric oxide synthase inhibitor (100 μM); rotenone, which is a mitochondrial respiratory chain complex I inhibitor (10 μM); thenoyltrifluoroacetone (TTFA), a mitochondrial respiratory chain complex II inhibitor (10 μM); allopurinol, an xanthine oxidase inhibitor (100 μM); and tiron, a superoxide scavenger (16 mM). All inhibitors were added 5 min before the start of the lucigenin-enhanced chemiluminescence assay.

Nox4-derived H2O2 measurement. The detailed measurement was described as previous study (29). Briefly, transfected cells with pcDNA3.1-Nox4 were treated with the H2O2-sensitive probe 5-(and-

Fig. 2. Nox4 expression was paralleled with SMC markers expression and translocated into the nucleus during SMC differentiation. Undifferentiated ES cells were plated in dishes coated with 5 μg/ml of collagen IV and cultured for 4, 8, and 12 days in the DM. Total RNA and protein from undifferentiated ES cells or differentiating ES cells were harvested and subjected to real-time PCR analysis with a set of specific primers for Nox4 (A) and Western blot analysis with specific antibodies for SMαA, smooth muscle myosin heavy chain (SM-MHC), and Nox4 (B). α-Tubulin was included as internal control. Double immunofluorescence staining was conducted on undifferentiated ES cells, days 4 and 8 differentiating ES cells with antibodies against Nox4 and SMC-specific markers (SMαA and SM-MHC). Isotype IgG substituted primary antibody as negative control during staining process (C–G). The data presented here were representative or an average of three independent experiments. *P < 0.05 vs. day 0.
6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (DCF, 5 mg/ml, Molecular Probes) for 30 min at 37°C in Krebs-Ringer bicarbonate solution (in mmol/l: 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, 25.0 NaHCO3, and 11.1 glucose). When analyzing the effect of H2O2-specific inhibitors, the cells were incubated with 3,000 U/ml of catalase for 3–4 h. After DCF incubation, the attached cells were harvested by trypsinization, washed in cold Krebs-Ringer solution, and analyzed by flow cytometry.

Enzyme-linked immunosorbent assay. Protein levels of TGF-β1 in the conditioned medium and cell lysate were determined using commercially available kits (Quantikine, R&D Systems). The concentration of TGF-β1 was determined by comparison with a standard curve, following manufacturer’s instruction. The final concentration of TGF-β1 in cell lysate was normalized to per microgram of protein.

Generation of stable long-term Nox4 gene modified ES-derived SMC cell lines. Plasmids pcDNA3.1-Nox4 (10 μg) (1) or empty vector pcDNA3.1 (10 μg) were introduced into 5 × 10^6 undifferentiated ES with mouse ES cell nucleofection kit as described above. Transfected cells were plated on collagen IV-coated dishes. After 2 days, cells were subjected to G418 selection. Stable colonies were isolated by gradually increasing the G418 concentration to 300 and 600 μg/ml of G418. After 2 wk in the presence of G418, an average

Fig. 3. Nox4 is essential for SMC differentiation from ES cells. Undifferentiated ES cells were nucleofected by nucleofector II with different amounts of Nox4 expression plasmids pcDNA3.1-Nox4. Nucleofected cells were plated in dishes coated with 5 μg/ml of collagen IV and cultured for 3–4 days in DM. Total RNA and protein were harvested and subjected to real-time PCR analysis for gene expression and Western blot analysis for protein levels (A). Appropriate amount of empty vector pcDNA3.1 were included as plasmid amount compensation. Nox4-specific small interfering RNA (siRNA) and random siRNA control were transfected into day 3 or 4 differentiating ES cells; after additional 2–3 days of culture, total RNA and protein were harvested and subjected to real-time PCR analysis and Western blot analysis (B). α-Tubulin was included as internal control. *P < 0.05 vs. control. The data presented here were representative or an average of six independent experiments.
of 160 colonies per dish were observed and given the stable transfection efficiency of 8 in $10^5$ cells. Chosen stable G418-resistant Nox4-positive colonies with good morphology were amplified and maintained in G418 medium for at least 1 mo.

**Contractility assays.** Agonist-induced contractile activity of the differentiated cells was assayed as described (38). Cells from stable long-term Nox4 gene-modified, ES-derived SMC cell lines were washed with PBS, stimulated with 1 mM carbachol or 40 mM KCl (Sigma-Aldrich, St. Louis, MO) in the DM, and monitored under the microscope up to 15 min. Images of the same field before and after carbachol or KCl treatment were snapped and compared.

**Statistical analysis.** Data expressed as means $\pm$ SE were analyzed with a two-tailed Student’s $t$-test for two-groups or one-way ANOVA for different groups. A value of $P < 0.05$ was considered statistically significant.

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**RESULTS**

Nox4 expression is upregulated during ES cell differentiation to SMCs. When ES cells were cultured on collagen-IV-coated plates for 4, 8, and 12 days, a panel of SMC marker genes such as SMαA, calponin, SM22α, and SM-MHCs were significantly upregulated, whereas pluripotent/undifferentiated stem cell-related gene expression, e.g., Nanog and Oct4, was downregulated as confirmed by real-time RT-PCR (Fig. 1). Increased mRNA for SMC marker genes was detected as early as day 1 (data not shown) and reached a significant level at day 4. These data indicate that collagen IV coating promotes ES cell differentiation to SMC lineage in the absence of leukemia inhibitory factor. Since ROS is an important second messenger

![Graph A](image1.png)

**Fig. 4.** Nox4 activates SMC-specific transcription factors. A: total RNA from undifferentiated ES cells or differentiating ES cells at day 4, 8, and 12 were subjected to real-time PCR to examine serum-response factor (SRF) and myocardin gene expression. *$P < 0.05$ vs. day 0. B: RNA and protein samples as Fig. 3A were subjected to real-time PCR analysis and Western blot analysis to examine gene expression and protein levels of SRF and myocardin. C: RNA and protein samples as Fig. 3B were subjected to real-time PCR analysis and Western blot analysis to examine gene expression and protein levels of SRF and myocardin. α-Tubulin was included as internal control in Western blot analysis. *$P < 0.05$ vs. control. The data presented here were representative or average of six independent experiments.
in cellular signaling processes, we wondered whether any member of Noxs, a major source for ROS production in vascular cells, was involved in ES cell differentiation. The real-time RT-PCR data shown in Fig. 2A indicate that Nox4, but not Nox1 (data not shown), expression was upregulated during ES cell differentiation. Western blot (Fig. 2B) and immunofluorescence analysis with confocal microscopy (Fig. 2C) confirmed highly produced Nox4 proteins. Double-immunofluorescence staining with Nox4 and SMC-specific markers (Fig. 2, C–G) indicates that ES-derived SMCs are Nox4 positive. Nox4 was mainly distributed in a perinuclear location in undifferentiated ES cells (Fig. 2D), gradually translocated to nucleus during differentiation at day 4 (Fig. 2E), and mostly shifted into the nucleus in the late stage of SMC differentiation (day 8, Fig. 2, F–G).

Nox4 is crucial for SMC differentiation. To test whether Nox4 is involved in SMC differentiation, Nox4 overexpression studies were conducted in the differentiating ES cells. Enforced expression of Nox4 induced all four SMC-specific marker genes expression (SMαA, SM22α, h1-calponin, and SM-myh11) in a dose-dependent manner (Fig. 3A). Similarly, Western blot analysis revealed the increased protein production in the cells overexpressing Nox4. Furthermore, when Nox4 siRNA knockdown was performed, downregu-
lated SMC marker gene expression at the protein and RNA levels (SMαA, SM22α, h1-Calponin, SM-Myh11) was observed (Fig. 3B). To confirm whether Nox4 expression is specific for SMC differentiation, gene “knockin” and “knockdown” experiments in the differentiating ES cells were performed. The chosen cell lineage markers based on our previous findings were analyzed with real-time RT-PCR. In additional, two genes, i.e., Alcam for hematopoietic progenitor and Dlx3 for trophoblast, were upregulated by Nox4 overexpression, whereas no genes determined was downregulated by Nox4 knockdown (see supplemental Fig. S1), indicating that Nox4 is favorable for SMC differentiation from ES cells under our culture condition.

Nox4 upregulates SRF and myocardin transcription factors. There is evidence that both SRF (23) and myocardin (20, 40) are essential transcription factors for SMC differentiation. We, therefore, detect gene expression of SRF and its coactivator myocardin during SMC differentiation with real-time PCR. As shown in Fig. 4A, both were significantly upregulated in the differentiating ES cells. To further investigate whether Nox4 could regulate SRF and myocardin gene expression, Nox4 overexpression and knockdown experiments were performed with Nox4 expression vector and siRNA. Overexpression of Nox4 upregulated SRF and myocardin gene expression as well as protein production in a dose-dependent manner (Fig. 4B), whereas Nox4 siRNA knockdown significantly decreased SRF

Fig. 5.—(Continued)
and myocardin expression at mRNA and protein level (Fig. 4C). These data suggest that Nox4 may mediate SMC differentiation through activation of SRF and myocardin.

Role of Nox4-induced ROS and H2O2 generation. To explore the role of ROS generation in SMC differentiation, the lucigenin-enhanced chemiluminescence assay was used to measure NADPH oxidase activity and ROS generation in the differentiating ES cells. The levels of ROS generation were significantly increased during SMC differentiation, peaking at day 8, which were completely ablated by flavoproteins-specific inhibitor diphenylene iodonium (DPI) and superoxide scavenger tiron, but not affected by other inhibitors, such as nicric oxide synthase inhibitor (1-NAME), mitochondrial respiratory chain complex I inhibitor (rotenone), mitochondrial respiratory chain complex II (TTFA), and xanthine oxidase inhibitor (allopurinol), indicating that the enzymatic source of ROS generation was originated from Noxs during SMC differentiation (Fig. 5A). Furthermore, overexpression of Nox4 in the differentiating ES cells increased H2O2 generation in a dose-dependent manner, which was ablated by H2O2-specific inhibitor catalase (Fig. 5B), whereas knockdown of Nox4 by siRNA decreased significantly ROS production in the differentiating ES cells (supplemental Fig. S2). These results suggested that Nox4-mediated SMC differentiation might occur through the generation of H2O2.

To investigate whether H2O2 is a direct mediator of SMC differentiation, exogenous H2O2 was added into cell culture, and RNA levels of SMC differentiation markers were determined by real-time PCR. Addition of H2O2 enhanced SmαA, SM22α, h1-calponin, and SM-myh11 gene expression, peaking at 10 μM of H2O2 (Fig. 5C). However, high concentration of H2O2 (100 μM) resulted in cell apoptosis (Fig. 5D). Additionally, exogenous addition of 10 μM of H2O2 significantly increased SMC-specific gene expression that was ablated by catalase at RNA and protein levels (Fig. 5, E and F). Taken together, these data strongly suggest that Nox4-generated H2O2 is an important mediator for SMC differentiation.

To explore Nox4-derived H2O2 in transcription factor activation, immunostaining was used to detect SRF expression during cell differentiation. SRF was expressed mainly in the cytoplasm in ES cells and gradually translocated into the nucleus during differentiation (Fig. 6A). When differentiating cells were treated with H2O2 in the absence or presence of catalase, the levels of both total SRF and phosphorylated SRF in the nucleus were upregulated by the addition of H2O2, which can be ablated by catalase (Fig. 6B). Total SRF in the cytosol was also decreased after catalase treatment (Fig. 6B). Moreover, myocardin was upregulated by H2O2 in the nuclear fraction (Fig. 6B).

TGF-β1 activates Nox4 in SMC differentiation. There is evidence indicating the role of TGF-β1 in SMC differentiation (4, 17, 26). To confirm the involvement of Nox4 in this process, we detected TGF-β1 mRNA level during ES cell differentiation. TGF-β1 mRNA were transiently upregulated during SMC differentiation, peaked at day 4, and rapidly returned to a baseline level (data not shown). ELISA analysis of TGF-β1 indicates that TGF-β1 proteins in the condition medium (Fig. 7A) and cell lysate (Fig. 7B) increased from day 2 of the culture and peaked at day 4. Addition of TGF-β1 resulted in marked increase in Nox4 expression followed by enhancement of SMC differentiation (Fig. 7, C and D). Knockdown of Nox4 with siRNA significantly ablated TGF-β1-induced Nox4 gene expression and SMC differentiation (Fig. 7E). We also found that TGF-β receptor 1, not receptors 2 and 3, was significantly activated by addition of TGF-β1 (Fig. S3). Data shown in Fig. 7F demonstrated that knockdown of TGF-β receptor 1 significantly decreased Nox4 activation and retarded SMC differentiation.

Nox4-expressing cell line maintaining SMC phenotype. To investigate whether Nox4 is required for maintenance of differentiated phenotype of ES-derived SMCs, cell lines stably expressed Nox4 were generated. Nox4-positive cell lines were gradually acquiring typical SMC morphology and maintaining high proliferative potential (Fig. 8A). Most Nox4-positive cell lines displayed a differentiated phenotype expressing higher levels of protein SM-MHC (Fig. 8B). Double-immunofluorescence staining revealed that Nox4 coexpressed and colocalized with SmαA and SM-MHC (Fig. 8C). When compared with transient Nox4 overexpression in the differentiating ES cells (supplemental Fig. S4) in which Nox4 was mainly localized in the nucleus, stable cell lines displayed that Nox4 was mainly localized in the cytoskeleton, indicating that enforced Nox4 associated with the cytoskeleton. FACS analysis revealed that the purity of Nox4-positive ES cell-derived SMCs was about 94.6% and 83.4% positivity for SmαA and SM-MHC, respectively (Fig. 8D). Importantly, Nox4-expressing cell lines were characterized with SMC contractibility in response to muscarinic agonist carbachol and KCl (Fig. 8E), further indicating the impact of Nox4 in maintenance of differentiated phenotype of ES cell-derived SMCs.

DISCUSSION

Exploring the molecular mechanism regulating stem cell differentiation is crucial for the basic and clinical medicine. In the previous study, we have established a simple, but effective, SMC differentiation model from ES cells in which protein collagen IV triggered SMC differentiation through integrin (α1, β1, and αv)-FAK/paxillin pathway (36). In the present study, we demonstrated for the first time that Nox4-derived H2O2 is crucial for SMC differentiation from ES cells. During ES cell differentiation, it was found that autocrine TGF-β1 activates Nox4, resulting in H2O2 generation which in turn upregulated SRF and its translocalization into the nucleus where it may form a complex with coactivator myocardin. Finally, we also generated several stable Nox4 overexpression cell lines, which resembled functional mature vascular SMC. Thus our findings have an impact on understanding the mechanisms of stem cell differentiation into SMCs and also on the application of Nox4-expressing SMC cell lines to vascular tissue engineering in the future.

It has been previously reported that TGF-β1 is a regulator for SMC differentiation (12, 26). Recent report indicates that TGF-β1 acts as a differentiating factor for cultured SMCs (12). TGF-β1 is also a key inducer for stem/progenitor cell differentiation toward myofibroblast (7) and SMCs (4, 17, 26). In the present study, the results indicate that Nox4 is a major participant linking TGF-β1 with the downstream transcription factor. Data from our real-time PCR and ELISA revealed that the mRNA and protein levels of TGF-β1 in conditioned medium...
and cell lysate were upregulated dramatically during SMC differentiation, which suggest that TGF-β1 has an important function in the Nox4-mediated SMC differentiation. Moreover, our further experiments showed that SMC differentiation from ES cells and Nox4 activation were significantly enhanced by short stimulation with TGF-β1 (peaked at 3 h) in the absence of serum, but the effects of TGF-β1 on Nox4 activation and SMC differentiation were ablated by knockdown of Nox4 signal, indicating that Nox4 is a mediator in SMC differentiation mediated by TGF-β1. Since only type 1 receptor for TGF-β1 has been activated upon stimulation of TGF-β1, the data of siRNA for the TGF-β receptor 1 imply the role of the TGF-β1 signal pathway in the SMC differentiation from ES cells. Taken together, these results strongly support the notion that Nox4 is a major player within TGF-β1 signaling cascade during stem cell differentiation toward SMCs.

SRF is a master regulator and transcription factor for SMC differentiation, which binds to CAréG element located within promoter-enhancer regions of SMC-specific genes to regulate the cell differentiation (23, 24, 39). SRF is expressed in all cell lineages but only activates transcription of SMC-restricted contractile genes in SMC. It is believed that SRF binds to SMC gene promoters and subsequently recruit their muscle-specific coactivator factor myocardin (3, 10, 22, 23). Myocardin is exclusively expressed on vascular and visceral SMC as well as cardiomyocytes and is a critical SRF coactivator in the transcriptional program regulating SMC differentiation (3, 10, 19, 40). Myocardin, which interacts with SRF through a basic and glutamine-rich domain near the NH2-terminus, increases SRF association with methylated histone and CAréG box chromatin during activation of SMC gene expression (9, 20, 35). In line with these findings, our data demonstrated that Nox4 mediates SMC differentiation through activation of SRF and myocardin. In the present study, we found that SRF was expressed mainly within the cytoplasm in undifferentiated ES cells but gradually translocated into nucleus during differentiation (Fig. 6A). In
addition, phosphorylated SRF signal was mainly detected in the nuclear fraction, and weak phosphorylated SRF signal in the cytosol fraction was only observed with catalase treatment, suggesting that endogenous and exogenous H2O2 leads to phosphorylation of SRF and drives the translocation into the nucleus, which can be inhibited by a H2O2 scavenger. Interestingly, SMC-related transcription factor myocardin was also upregulated by H2O2, and it was only detected in the nuclear fraction, which suggests that the nuclear localization of myocardin, similar to myocardin-related transcription factors (15), is important for regulation of SMC gene expression. These results indicate that SRF translocation into the nucleus maybe due to their phosphorylation by Nox4-derived H2O2 during SMC differentiation, suggesting that the interaction of SRF and myocardin might be essential for Nox4-mediated stem cell differentiation.

Another finding in the present study is that the intracellular localization of Nox4 might relate to their different function. During SMC differentiation, Nox4 is mainly localized in the nucleus (Fig. 2, F and G), whereas Nox4 in SMC-like cell lines stably expressing Nox4 is distributed in a cytoskeleton-related localization (Fig. 8C). The intracellular localization of Nox4 may influence its downstream targets. In the early stage of SMC differentiation, Nox4 localized in the nucleus may play a role in modulating SRF/myocardin and regulated SMC gene expression, whereas Nox4 may be localized adjacent to the cytoskeleton to maintain their functional characteristics (contractility or cytoskeletal rearrangement). This concept, at least in part, is supported by our data that Nox4 mainly localized in nucleus when Nox4 was transiently introduced by transfection (supplemental Fig. S4). Furthermore, Hillenski et al. (14) found that Nox4 localized in focal adhesions, actin

![Fig. 7. Transforming growth factor (TGF)–β1 promotes SMC differentiation through activation of TGF-β receptor 1 and Nox4. Data from ELISA measurement demonstrated that protein levels of TGF-β1 were increased in conditioned medium (A) and cell lysate (B) during SMC differentiation. Exogenous TGF-β1 enhances SMC differentiation as well as Nox4 activation in a dose-dependent (C) and time-dependent (D) manner. *P < 0.05 vs. control. Nox4 siRNA ablated the effects of TGF-β1 on Nox4 activation and SMC differentiation (E). *P < 0.05 (TGF-β1 vs. vehicle); #P < 0.05 (Nox4 siRNA vs. control siRNA). Blockdown of TGF-β receptor 1 inhibits Nox4 activation and SMC differentiation (F). *P < 0.05 vs. control. The data presented here were representative of an average of three independent experiments.](http://ajpcell.physiology.org/)

**Fig. 7.** Transforming growth factor (TGF)-β1 promotes SMC differentiation through activation of TGF-β receptor 1 and Nox4. Data from ELISA measurement demonstrated that protein levels of TGF-β1 were increased in conditioned medium (A) and cell lysate (B) during SMC differentiation. Exogenous TGF-β1 enhances SMC differentiation as well as Nox4 activation in a dose-dependent (C) and time-dependent (D) manner. *P < 0.05 vs. control. Nox4 siRNA ablated the effects of TGF-β1 on Nox4 activation and SMC differentiation (E). *P < 0.05 (TGF-β1 vs. vehicle); #P < 0.05 (Nox4 siRNA vs. control siRNA). Blockdown of TGF-β receptor 1 inhibits Nox4 activation and SMC differentiation (F). *P < 0.05 vs. control. The data presented here were representative of an average of three independent experiments.
fibers, as well as nuclei in mature SMCs, and proposed that the intracellular localization of Nox4 might be due to their plasticity and mediates their distinct function. Unlike either skeletal or cardiac muscle that are terminally differentiated, SMCs within adult animals retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental cues that normally regulate phenotype (25). However, further studies are still required to elucidate the exact molecular mechanisms by which different cellular Nox4 controls...
specific cell functions in mature vascular SMCs and stem cell-derived SMCs.

Vascular SMCs have a critical role in both physiological maintenance of the cardiovascular system in embryonic development and in the pathophysiology of vascular diseases in adults. Vascular SMC is also a major component in engineered vascular graft, particularly in the large vessels. In the present study, we established several SMC-like cell lines by stable expression of Nox4. Our Nox4-expressing cell lines with high purity and functional SMC characteristics also provide the possibility to generate SMCs from ES cells, which can be used as a source of cells for vascular engineering and repair of injured vessels, and highlight the importance of Nox4 in the generation of high purity of SMCs. Considering the limited life span of mature vascular SMC and the tendency of stem/progenitor-derived vascular SMC to dedifferentiate, our stable long-term Nox4 ES-derived SMC cell lines represent an advantage in the future vascular tissue engineering research field.

In summary, we demonstrated that Nox4 is crucial for SMC differentiation and required for maintenance of the differentiated phenotype of ES-derived SMCs. In the early stage of differentiation, differentiating ES cells can secrete TGF-β1 that activates Nox4 through activation of its receptor 1. Nox4 in cytoplasm could translocate into the nucleus and releases H₂O₂, which may upregulate SRF gene transcription and protein translation. Phosphorylated SRF binds to CArG elements within the promoter-enhancer region of SMC-specific genes and recruits coactivator myocardin and other transcription factors. The possible signaling pathway involved in SMC differentiation model is illustrated in supplemental Fig. S5. Thus our findings that Nox4-mediated stem cell differentiation via TGF-β1 initiated signal pathway provided the basic information for searching potential target for promoting/inhibiting stem cell differentiation toward SMCs.

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