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Thyroid hormone induces artery smooth muscle cell proliferation: discovery of a new TRα1-Nox1 pathway

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Abstract

Thyroid hormone (T3) can stimulate protein synthesis and cell growth. NOX1 is a mitogenic oxidase. The aim of this study was to test a novel hypothesis that T3 induces artery smooth muscle cell proliferation by up-regulating NOX1. Immunofluoresence confocal microscopy was used to visualize the sub-cellular localization of NOX1 and TRα1 in rat aorta smooth muscle (RASM) cells. Optical sectioning showed that TRα1 and NOX1 co-localized around the nucleus. T3 promoted RASM cell proliferation as determined by the fact that T3 significantly increased the number of cytokinesis cells, proliferating cellular nuclear antigen (PCNA) and smooth muscle α-actin (SM α-actin). T3 increased NOX1 expression at both the transcription (mRNA) and translation (protein) levels as evaluated by RT-PCR and Western blot, respectively. T3 also significantly increased the intracellular ROS production based on the oxidation of 2',7'-dichlorodihydrofluoresein (H_2DCF) to a fluorescent 2',7'-dichlorofluoresein (DCF). RNAi silence of TRα1 or NOX1 abolished T3-induced intracellular ROS generation and PCNA and SM α-actin expression, indicating that TRα1 and NOX1 mediated T3-induced RASM cell proliferation. Notably, RNAi silence of TRα1 blocked the T3-induced increase in NOX1 expression, whereas silence of NOX1 did not affect TRα1 expression, disclosing a new pathway, *i.e.* T3-TRα1-NOX1-cell proliferation. TRα1 and NOX1 co-localized around the nucleus. T3 induced RASM cell proliferation by up-regulating NOX1 in a TRα1-dependent manner.

Keywords: thyroid hormone • TRα1 • NOX1 • ROS • smooth muscle cell • proliferation

Introduction

Thyroid hormone (T3) influences a variety of physiological processes, including cell growth and metabolism in mammals. T3 has been shown to be a powerful inducer of cardiomyocyte hypertrophy [1] and cardiac hypertrophy [2, 3]. Indeed, thyroid hormone enhances protein synthesis in the heart [4, 5]. T3 has strong mitogenic effect and can induce cell proliferation in rats [6, 7]. It is not known, however, if T3 induces artery smooth muscle cell (SMC) proliferation. The actions of thyroid hormone are primarily mediated by thyroid hormone receptors (TRs) (genomic effect), which belong to the family of nuclear hormone receptors containing a DNA-binding and a ligand-binding domain [8, 9]. There are two major TR isoforms encoded on separate genes, designated as TRα and TRβ, encoded on human chromosomes 17 and 3, respectively [10]. Alternative splicing of the initial RNA transcript of TRα gene generates two mature mRNA that encode two proteins: TRα1 and c-erbAα2 (TRα2). The latter cannot bind T3 because it contains a 122-amino acid caboxy terminus that replaces a region in TRα1, which is critical for thyroid hormone binding [11]. There are also two TRs derived from the TRβ gene. This gene contains two promoter regions each of which is vital for the transcription of an mRNA coding for a distinctive protein [12]. TRβ1 mRNA is highly expressed in liver, whereas TRβ2 mRNA is highly expressed in the anterior pituitary [13]. In present study, we demonstrated that TRα1 expressed in rat aorta smooth muscle (RASM) cells. The first objective of this study was to determine if T3 induces RASM cell proliferation in a TRα1-dependent manner. We hypothesize that T3 induces RASM cell proliferation *via* TRα1.

NOX1, a novel homologue of gp91, is highly expressed in colon epithelium [14–16]. It is also expressed in a variety of other cell types, such as endothelial cells [17, 18] and vascular smooth muscle cells (VSMC) [19–21]. Platelet-derived growth

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factor (PDGF), prostaglandin F2α, angiotensin II, phorbol ester and ATF-1 induce NOX1 expression in VSMC [14, 22–24]. NOX1 was implicated in the pathogenesis of atherosclerosis, hypertension and restenosis after angioplasty because it mediates the proliferation and hypertrophy of VSMC [25]. Reactive oxygen species (ROS), generated by NADPH oxidases, are implicated in mitogenic signalling in cancer [26–28]. ROS generation is generally a cascade of reactions that starts with the production of superoxide. Superoxide production was decreased significantly in NOX1-deficient mice [29]. ROS avidly interact with some intracellular and intra-nuclear molecules such as proteins and DNAs. Through such interactions, ROS may irreversibly alter the transcriptional and translational processes of target genes. Consequently, ROS have been increasingly identified as major contributors to cell proliferation.

It would be interesting and important to know if NOX1 mediates T3-induced RASM cell proliferation. Thus, the second objective of this study was to test a novel hypothesis that T3-induced RASM cell proliferation is mediated by up-regulating NOX1 *via* TRα1. To better understand the functional interaction of TRα1 and NOX1, the sub-cellular localization of TRα1 and NOX1 and their possible co-localization in RASM cells were evaluated in this study.

Methods

Cell culture

RASM cells (cell line) (ATCC, Manassas, VA, USA) were cultured in DMEM (Cell Signaling, Danvers, MA, USA) supplemented with 10% foetal bovine serum (FBS, ATCC), 100 μg/ml of streptomycin (Sigma) and 100 U/ml of penicillin (Sigma-Aldrich, Atlanta, GA, USA) at 37°C, 5% CO_2.

Cell treatment

Following the initial culture, RASM cells were incubated with DMEM medium containing 2% FBS and thyroid hormone (T3, 0, 1.5, 7.5, 75 nM) (Sigma) for 40 hrs. The cells were harvested for further studies. Control cells were treated with vehicle. Vehicle for thyroid hormone was 1 ml of NaOH (1N) and 49 ml of DMEM medium. The solution was filtered with 0.2-μm filter.

Confocal immunofluorescence microscopy

RASM cells were fixed with 3% paraformaldhyde (in PBS) for 10 min. at room temperature. The cells were permeabilized using 0.1% Triton X-100 (in PBS). Goat anti-NOX1 (1:100, Santa Cruz, Santa Cruz, CA, USA) and rabbit anti-TRα1 (1:50, Santa Cruz) antibodies were used for revealing the localization of NOX1 and TRα1 proteins, respectively. TRITC-labelled donkey anti-goat and FITC-labelled chicken anti-rabbit secondary antibodies (Santa Cruz) were then supplied. Signals were captured by immunoflurescence confocal microscopy (LEICA).

Western blot analysis of NOX1, NOX2, SM α-actin, proliferating cellular nuclear antigen (PCNA) and TRα1

The procedure for Western blotting was described in our previous studies [30, 31]. Briefly, the equal amount of protein was loaded in 10% Tris-HCl gel followed by electronic transfer. After blocking with 10% milk (in TBS-T), the membranes were incubated with antibodies (diluted in 5% milk/TBS-T) against NOX1 (Santa Cruz, 1:750), NOX2 (BD Transduction Laboratories Inc, Mississauga, ON, Canada, 1:1000), TRα1 (Santa Cruz, 1:500), α-actin (Abcam, Cambridge, MA, USA, 1:2000) and PCNA (Abcam 1:5000) at 4°C overnight. The membranes were incubated with HRP-conjugated secondary anti-goat, anti-mouse or anti-rabbit antibodies (1:2000–1:5000) for 1 hr at room temperature. Target protein expression was normalized with the expression of β-actin, which was served as an internal control. For this purpose, a parallel gel with identical samples was run and the Western blot analysis was performed with the β-actin monoclonal antibody (Abcam, 1:20,000).

RNA isolation and semi-quantitative RT-PCR of NOX1

Semi-quantitative RT-PCR was performed as described in our previous study [32]. Briefly, total RNA was prepared by using Trizol Reagent (Invitrogen, Carslbad, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was determined by optical density reading at 260 and 280 nm and verified by ethidium bromide staining. The RNA samples selected showed two clear bands of 18 S and 28 S corresponding to ribosomal RNAs for further RT-PCR analysis. Two μg of total RNA were mixed with OligodT_{20} (Invitrogen) in the presence of 10 μl dNTP for 1 hr at 55°C. Three μl of the complementary DNA (cDNA) obtained from RT step were amplified by PCR (Invitrogen, 2.5XPCR mix) in a thermal cycler (Eppendorf, Hamburg, Germany). The specific primers for NOX1 (Accession number: NM_053683): sense 5′-TTT CCT AAA CTA CCG ACT C-3′; anti-sense 5′-GTG CGA CAA CGG ACT ATC-3′. The amplification program involved denature at 94°C for 45 sec., primer annealing at 55°C for 1 min. and extension at 68°C for 1 min. Amplification was allowed to proceed for 30 cycles. PCR products were analysed by 2% agarose gel electrophoresis stained with ethidium bromide. Rat β-actin (Accession number: NM_031144) primers were used as an internal control (sense 5′GAG GGA AAT CGT GCG TGA C-3′, anti-sense 5′CTG GAAGGT GGA CAG TGA G-3′).

TRα1-SiRNA transfection and treatment with T3

Rat TRα1 (Accession number: NM_ 001017960) siRNA was designed, sythesized and annealed in Ambiom (Austin, TX, USA). The sequences are sense 5′-GAA CCU CCA UCC CAC CUA Utt-3′ and anti-sense 5′-AUA GGU GGG AUG GAG GUU Ctt. The scrambled siRNA was purchased from Ambiom (Cat#: AM4611) as a control. SiRNA for GAPDH labelled with Cy3 (Ambiom, Cat #: AM4649) was used as a monitor of transfection efficiency. siRNAs (30 nM) were used for transfection with SiPORT Neo^{TM} reagent according to the instruction (Ambiom, Cat#: AM4510). After 24 hrs of transfection, the medium was refreshed with DMEM containing 2% FBS with or without T3 (7.5 nM) and incubated for 40 hrs.

Construction of adenovirus carrying NOX1-shRNA (Adv.Nox1-shRNA)

Rat NOX1 (Accession number: NM_053683) shRNA was designed by using shRNA designer software (Dharmacon, Chicago, IL, USA), synthesized and annealed in IDTDNA company. The sequences are sense, 5′-ACT ACC GAC TCT TCC TCA C-3′; hairpin, TCC AAG AGA; anti-sense, 5′-GTG AGG AAG AGT CGG TAG T-3′. The shRNA was inserted into RNAi-Ready-pSIREN-Shuttle vector (BD Biosciences, San Jose, CA, USA). The fragment of pU6 promoter with the sequence of NOX1-shRNA was released by I-Ceu I and PI-SceI restriction enzymes. The fragment was then inserted into Adeno-X expression system (BD Biosciences). Adv.Scrambled-shRNA (Adv.Sc-shRNA) was also constructed as a control construct. The packaging, confirmation, amplification and titre of recombinant adenoviruses were described in our previous study [30, 31]. Purification of adenoviruses was performed according to the manufacturer's instruction (BD, Biosciences, Cat # 631532).

Introduction of Adv.NOX1-shRNA and treatment with T3

RASM cells (1×10^6) were sub-cultured in DMEM containing 10% FBS, 100 ug/ml of streptomycin (Sigma) and 100 U/ml of penicillin (Sigma). The cells were then incubated with Adv.NOX1-shRNA or Adv.Sc-shRNA in 5 ml of FBS-free DMEM for 4 hrs. After incubation, 5 ml of DMEM containing 20% FBS was added and incubated for 20 hrs. The medium was then refreshed with DMEM containing 2% FBS with or without T3 (7.5 nM) and incubated for 40 hrs.

Evaluation of intracellular ROS production

RASM cells were pre-treated with Adv.NOX1-shRNA or Adv.Sc-shRNA, and TRα1-siRNA or Sc-siRNA. The determination of intracellular oxidant production was based on the oxidation of 2',7'-dichlorodihydrofluoresein (H_2DCF) to 2',7'- dichlorofluoresein (DCF) (Sigma) [33]. Briefly, the cells were incubated with H_2DCF (20 μM, in PBS) at 37°C for 20 min. H_2DCF was removed and rinsed with PBS for 3 times. The signal was captured with FITC filter using Leica TCS NT Confocal microscopy. The average intensity values were measured at 400× magnification in three randomly chosen fields of each 10–15 cells from three samples.

Immunocytochemical (IHC) staining of PCNA

The cells were harvested and fixed with 3% paraformaldhyde (in PBS) and further permeabilized using 0.1% Triton X-100 (in PBS). The cells were then incubated with rabbit polyclonal anti-PCNA antibody (1:500, Abcam) at 4°C overnight. The slides were washed with PBS for 5 min. (3 times) before it was incubated with goat anti-rabbit IgG-HRP (1:1000, Santa Cruz) at room temperature for 1 hr. The slides were then washed with PBS for 5 min. (3 times). The brown colour was developed with DAB substrate for 5 min. at room temperature. The slides were rinsed with water and counterstained with haematoxylin. The positive PCNA staining showed in brown colour in the nucleus.

Statistical analysis

All data are expressed as mean ± S.E.M. Data were analysed using a one-way ANOVA or an unpaired Student's t-test. A probability value with $P \leq 0.05$ was considered to be statistically significant.

Results

TRα1 and NOX1 co-localized in RASM cells

To determine the association of TRα1 and NOX1 in RASM cells, we evaluated the sub-cellular localization of TRα1 and NOX1. TRα1 was predominately localized adjacent to the nuclear periphery (Fig. 1A). NOX1 was mainly localized around the nucleus and some were found in plasma membrane and submembrane-vesicle-like structure (Fig. 1B). Clearly, TRα1 and NOX1 co-localized around the nucleus (Fig. 1D).

Thyroid hormone (T3) selectively increased NOX1 expression

The effect of T3 on NOX1 mRNA and protein expression was evaluated by Western blot and RT-PCR, respectively, in RASM cells. T3 (0, 1.5, 7.5, 75 nM) increased NOX1 expression in a dose-dependent manner (data not shown). T3 (7.5 nM) significantly increased NOX1 expression at both translation (protein) (Fig. 2A and B) and transcription (mRNA) levels (Fig. 2C and D). In contrast, T3 did not change the expression of NOX2 ($gp91^{phox}$), a membrane-bound NADPH oxidase, in RASM cells (Fig. 2E). β-actin mRNA and protein expression were not changed by T3. Thus, T3 selectively up-regulated NOX1 in RASM cells.

T3 increased RASM cell proliferation

After RASM cells were treated with T3 for 40 hrs, the cytokinesis was evaluated by counting the number of dividing cells. T3 significantly increased the number of cells undergoing cytokinesis (Fig. 3A and B). T3 did not change RASM cell size. RASM cell proliferation was further evaluated by Western blot analysis of PCNA and SM α-actin. T3 increased PCNA and SM α-actin expression significantly (Fig. 3C–E). T3 did not alter β-actin (internal control) expression.

RNAi silence of TRα1 blocked T3-induced ROS production

The role of TRα1 in mediating T3-induced ROS generation was evaluated by RNAi silence of TRα1. TRα1-siRNA significantly

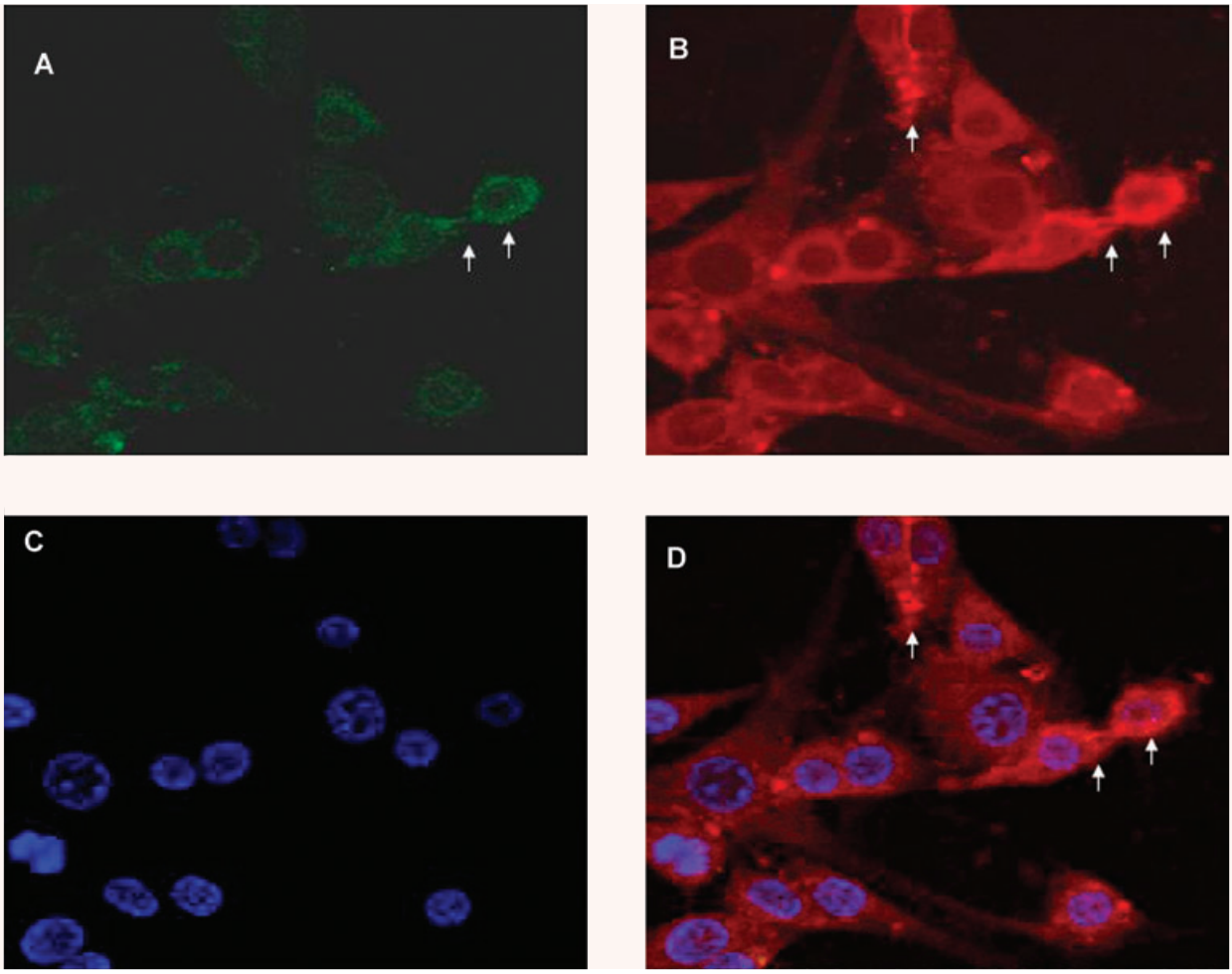

Fig. 1 Co-localization of NOX1 and TRα1 in RASM cells. RASM cells were fixed and then permeabilized using Triton X-100. Goat anti-NOX1 and rabbit anti-TRα1 antibodies were used for detecting NOX1 and TRα1 proteins, respectively. TRITC-labelled anti-goat and FITC-labelled anti-rabbit antibodies were then supplied. Signals were detected by immunoflurescence confocal microscopy. (**A**) TRα1 (green) localizes predominately at nuclear periphery. (**B**) NOX1 (red) localizes to the nuclear membrane and in the plasma membrane. (**C**) DAPI (blue) showed nuclear staining. (**D**) Merged A, B and C. Double arrows showed nuclear membrane staining of TRα1 and/or NOX1. Single arrow showed plasma membrane and submembrane-vesicle-like structure. $\times$630.

decreased intracellular ROS production compared with cells treated with Sc-siRNA (Scrambled-siRNA) (Fig. 4A and B), indicating that the minimal T3 level (physiological concentration) in the FBS induces ROS production. T3 increased intracellular ROS generation significantly in cells treated with Sc-siRNA. In contrast, T3 failed to increase intracellular ROS significantly in cells pre-treated with TRα1-siRNA, indicating that RNAi silence of TRα1 abolished T3-induced ROS production. TRα1-siRNA significantly decreased TRα1 expression, indicating that TRα1 was effectively silenced (Fig. 4C and D). T3 increased TRα1 expression significantly in cells pre-treated with Sc-siRNA, indicating that T3 up-regulated TRα1. T3 failed to increase TRα1 expression in cells pre-treated with TRα1-siRNA (Fig. 4C and D), suggesting complete inhibition of TRα1. In contrast, β-actin was not altered by TRα1-siRNA or T3 (Fig. 4D).

RNAi silence of TRα1 abolished T3-induced increases in NOX1, SM α-actin and PCNA expression

TRα1-siRNA significantly attenuated NOX1 expression compared with cells treated with Sc-siRNA (Fig. 5A and D), indicating that the minimal T3 level (physiological concentration) in the FBS regulates NOX1 expression. Notably, T3 failed to increase NOX1 expression in cells pre-treated with TRα1-shRNA, indicating that

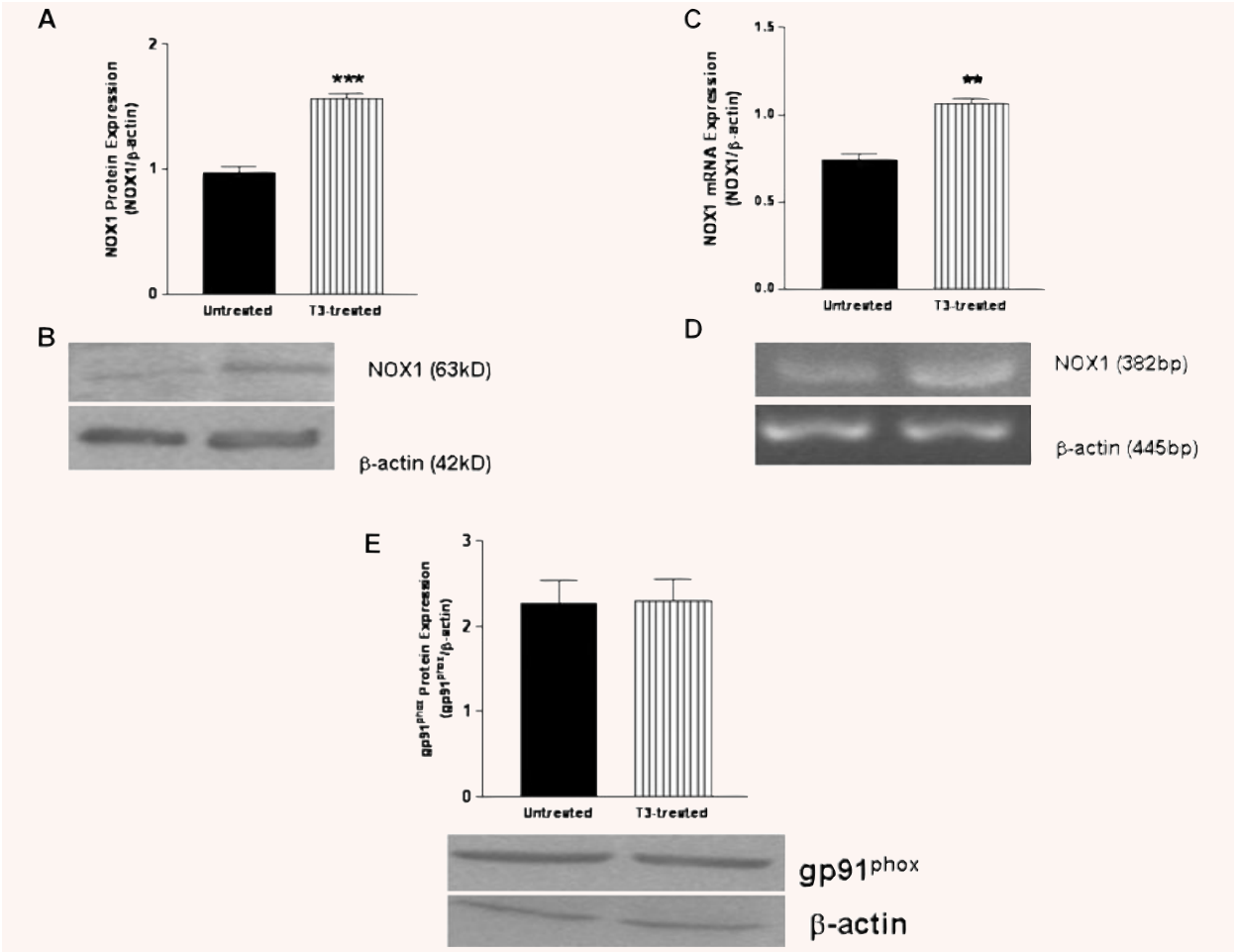

Fig. 2 Thyroid hormone (T3) up-regulated NOX1 expression in RASM cells. RASM cells (1×10^6/dish) were seeded in DMEM medium containing 10% foetal bovine serum, 100 U of penicillin and 100 μg of streptomycin at 37°C, 5% CO_2 for 24 hrs. The cells were then incubated with DMEM containing thyroid hormone (7.5 nM), 2% foetal bovine serum, 100 U of penicillin and 100 μg of streptomycin for additional 40 hrs. Following incubation, RASM cells were harvested for Western blot and RT-PCR. (**A** and **B**) NOX1 protein expression level. (**C** and **D**) NOX1 mRNA expression. (**E**) NOX2 protein expression. The results were expressed as the total counts of CNT (density [CNT*mm^2] times area [mm^2]). Equal amounts of protein loading were confirmed with β-actin. The error bars represent the standard errors from three experiments (**A**, **C** and **E**). $***P < 0.001$, compared with untreated cells.

RNAi silence of TRα1 abolished the T3-induced increase in NOX1 expression. Similarly, TRα1-siRNA also significantly decreased SM α-actin and PCNA expression and abolished T3-induced increases in SM α-actin and PCNA expression (Fig. 5B–D). These data suggest that TRα1 lies in the upstream of NOX1 in TRα1-NOX1 pathway. TRα1-siRNA did not affect β-actin expression.

RNAi silence of NOX1 abolished T3-induced ROS production

To determine the role of NOX1 in T3-induced ROS generation, the effect of NOX1-shRNA on T3-induced intracellular ROS production was evaluated by using H_2DCF staining. NOX1-shRNA significantly decreased intracellular ROS level compared with cells treated with Sc-shRNA (Fig. 6A and B). Notably, NOX1-shRAN abolished T3-induced intracellular ROS production. NOX1-shRNA significantly decreased NOX1 protein expression compared with cells treated with Sc-shRNA (Fig. 6C and D), indicating effective silence of NOX1. NOX1-shRNA also blocked T3-induced up-regulation of NOX1.

RNAi silence of NOX1 abolished T3-induced increases in SM α-actin, PCNA and NOX1 expression but did not alter TRα1 expression

RNAi silence of NOX1 by NOX1-shRNA did not affect TRα1 expression and the T3-induced increase in TRα1 expression in

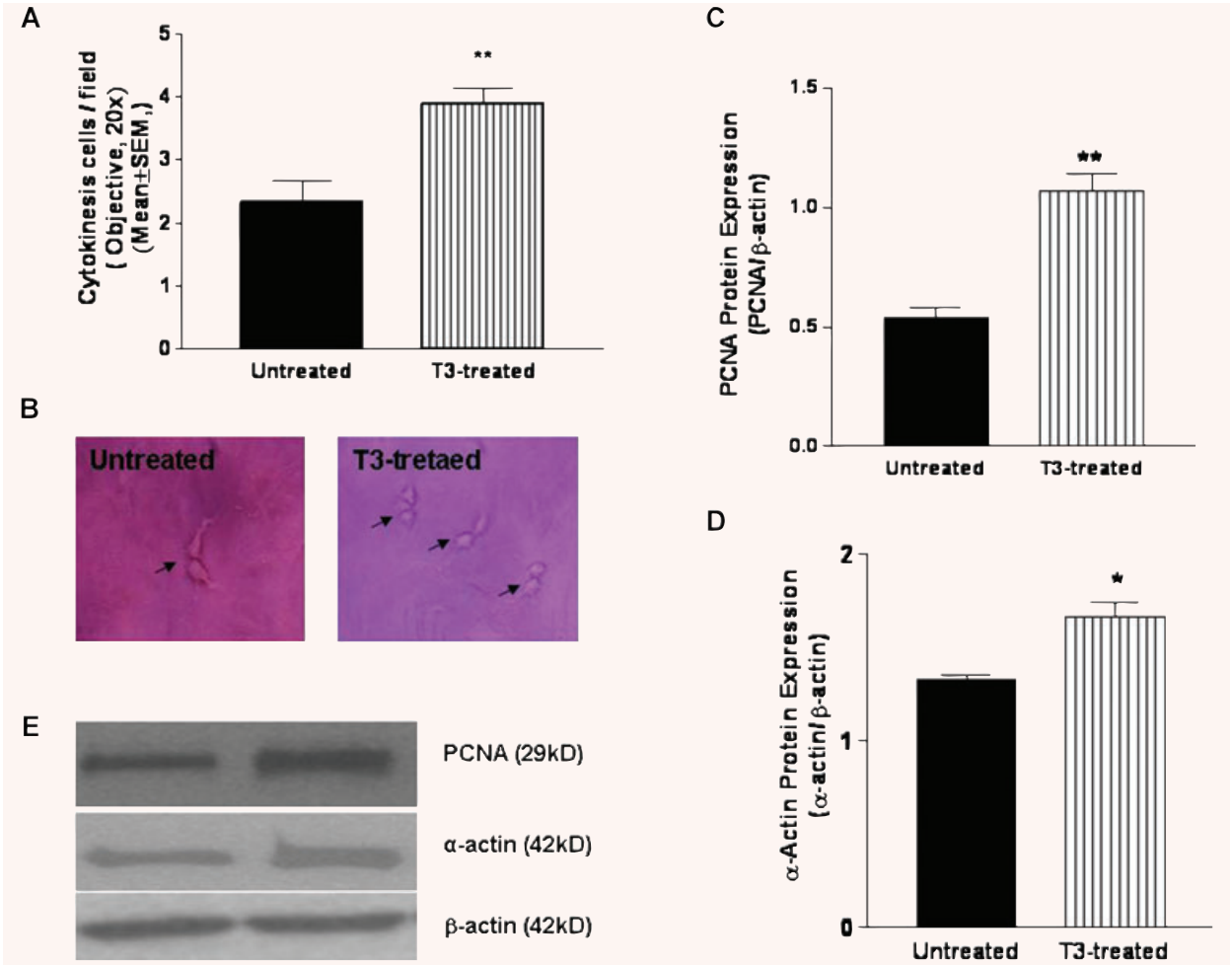

Fig. 3 Thyroid hormone (T3) promoted RASM cell growth and proliferation. RASM cells (5×10^4/dish) were seeded in DMEM medium containing 10% foetal bovine serum, 100 U of penicillin and 100 μg of streptomycin at 37°C, 5% CO_2 for 24 hrs. The cells were then incubated with DMEM containing T3 (7.5 nM), 2% foetal bovine serum, 100 U of penicillin and 100 μg of streptomycin for additional 40 hrs. The data were collected by viewing 9 different fields for each group. (**A**) The cells undergoing cytokinesis were counted under the microscope with 20× objective. (**B**) Arrows indicated cytokinesis (dividing cells); 1, un-treated cells; 2, T3-treated cells. RASM cells with the same treatment were harvested with whole cell lysis buffer for measuring PCNA (**C**) and α-actin (**D**) using Western blot. β-actin was used as an internal control. (**E**) Representative Western blots of PCNA (~29 kD) and α-actin (~42 kD). The error bars represent the standard error from three experiments (A, C and D). $*P < 0.05$, $**P < 0.5$ compared with untreated cells.

RASM cells (Fig. 7A and D), suggesting that NOX1 lies in the downstream of TRα1. NOX1-shRNA significantly decreased SM α-actin protein expression and also abolished the T3-induced increase in SM α-actin protein expression (Fig. 7B and D), indicating that NOX1 may mediate T3-induced SM α-actin protein synthesis. RNAi silence of NOX1 significantly decreased PCNA protein expression and abolished the T3-induced increase in PCNA expression (Fig. 7C and D), indicating that NOX1 may mediate T3-induced VSMC proliferation. The internal control β-actin was not affected by RNAi knockdown of NOX1 or T3 (Fig. 7D).

RNAi silence of NOX1 abolished the T3-induced increase in PCNA-positive cells

Figure 8 showed IHC analysis of PCNA expression in RASM cells. The positive PCNA staining showed in brown colour in the nucleus (Fig. 8A). RNAi silence of NOX1 significantly decreased the percentage of PCNA-positive cells and abolished the T3-induced increase in PCNA-positive cells (Fig. 8A and B), indicating that NOX1 may mediate T3-induced VSMC proliferation. RASM cells that were not incubated with anti-PCNA antibody showed no PCNA

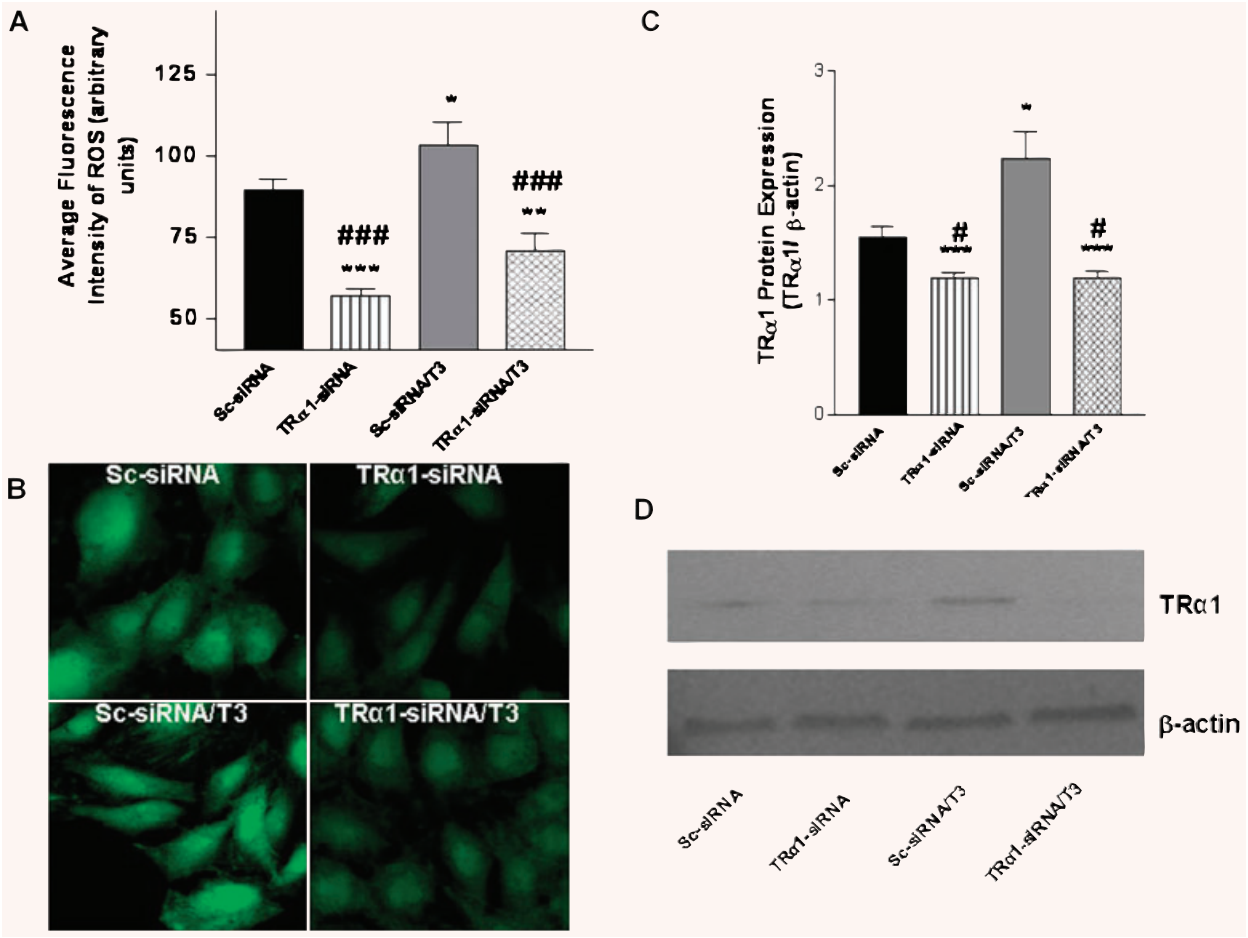

Fig. 4 TRα1-siRNA blocked T3-induced intracellular ROS production. RASM cells were transfected with scrambled-siRNA (Sc-siRNA) or TRα1-siRNA (60 nM) for 24 hr and then incubated with T3 (7.5 nM) for additional 40 hrs. (**A**) Quantification of intracellular ROS production. Ten to 15 cells were measured and the average fluorescence intensity values were calculated and presented as the mean ± S.E.M. (**B**) Representative fluorescence images (original magnification, 400×). (**C**) Quantification of TRα1 protein expression normalized with β-actin. (**D**) Representative Western blots of TRα1 (~48 kD) and β-actin (~42 kD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* the Sc-siRNA group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *versus* Sc-siRNA/T3 group.

staining (negative control) (Fig. 8C), indicating specificity of anti-PCNA antibody.

Discussion

We demonstrated, for the first time, that T3 promoted SMC proliferation. This finding reveals that T3 can regulate arterial SMC cytokinesis and proliferation. This study further demonstrated that T3-induced cell proliferation may be mediated specifically by TRα1 receptors based on the fact that RNAi inhibition of TRα1 abolished T3-induced cell proliferation.

NOX1 is a mitogenic oxidase (mox-1) that is responsible for cell proliferation and transformation [14]. NOX1-generated ROS serve as intracellular signals that increase DNA replication and cytokinesis [14]. Indeed, ROS activate many downstream signalling molecules, such as mitogen-activated protein kinase (MAPK), protein tyrosine kinases and transcription factors [34], leading to α-actin synthesis and SMC proliferation and migration. The present study discloses, for the first time, that NOX1 is mainly localized around the SMC nucleus membrane, a unique subcellular localization that facilitates its function, *i.e.* inducing mitogenesis and cell proliferation. This study further revealed that NOX1 was co-localized with TRα1 around the nucleus, providing a basis for the functional interaction between TRα1 and Nox1. T3 gets into the cells and binds to TRα1 receptors in the nuclear periphery. One of the most exciting findings is that T3 up-regulated NOX1 expression and increased intracellular ROS production, which can be abolished by RNAi inhibition of TRα1, suggesting that T3 up-regulated NOX1 *via* TRα1. Thus, NOX1 lies in the downstream of TRα1.

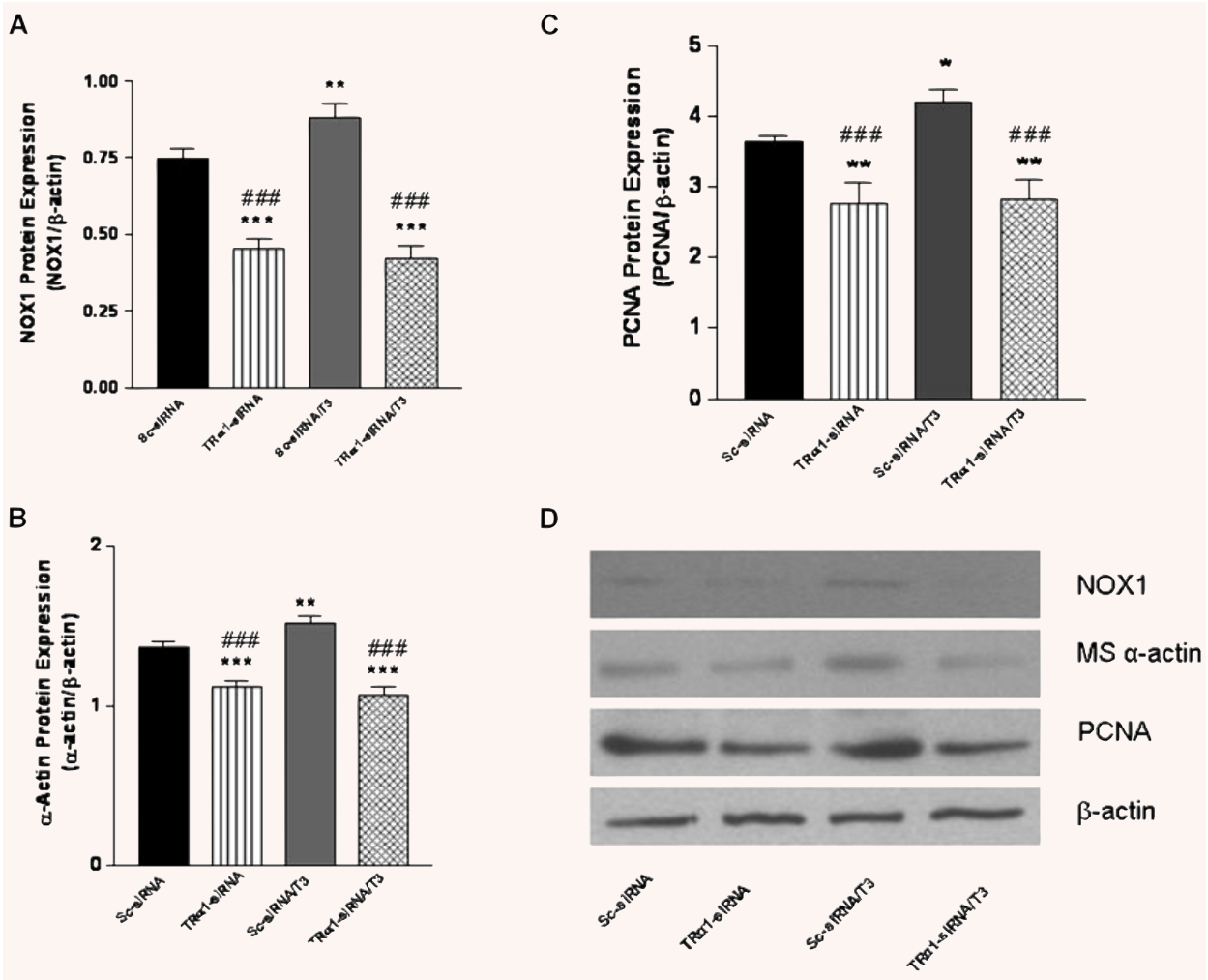

Fig. 5 TRα1-siRNA down-regulated NOX1, PCNA and α-actin expression. RASM cells were transfected with Sc-siRNA or TRα1-siRNA (60 nM) for 24 hrs and then incubated with T3 (7.5 nM) for additional 40 hrs. The cells were harvested and lysed with whole cell lysis buffer. Protein expression was measured by Western blot. (**A**) Quantification of NOX1 protein expresion. (**B**) Quantification of smooth muscle cell (SMC) α-actin expression. (**C**) Quantification of PCNA expression. (**D**) Representative Western blots of NOX1 (63 kD), SMC α-actin (42 kD) and PCNA (29 kD). Data are mean ± S.E.M. The error bars (A, B and C) represent the standard error from three experiments normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* the Sc-siRNA group; $^{\#\#\#}P < 0.001$ *versus* the Sc-siRNA/T3 group.

On the other hand, RNAi silence of NOX1 abolished T3-induced increases in ROS production, SM α-actin expression and cell proliferation, suggesting that NOX1 is essential to the T3-induced proliferating effects. RNAi knockdown of NOX1, however, did not affect TRα1 expression. Therefore, this study reveals a new pathway mediating T3-induced proliferation in artery SMCs, *i.e.* T3-TRα1-NOX1-SMC proliferation.

T3-induced ROS production was solely mediated by NOX1 since RNAi silence of NOX1 completely abolished T3-induced ROS production. Indeed, T3 did not affect expression of NOX2 (gp91phox), a membrane-bound NADPH oxidase [35], probably because T3 mainly binds to its nuclear receptor (TRα1).

It is noted that T3 also up-regulated TRα1 expression, which apparently challenges the traditional receptor theory that an increase in ligand down-regulates its receptors. The mechanism by which this occurs needs to be further investigated but it is not due to T3-induced activation of NOX1 or subsequent ROS production because RNAi inhibition of NOX1 did not affect T3-induced up-regulation of TRα1 expression.

PCNA is involved in DNA replication and is necessary for adequate leading strand synthesis, acting as the auxiliary protein of DNA polymerase δ [36, 37]. The staining pattern of the S-phase-related PCNA resembles the topographical patterns of DNA replication sites [38]. Thus, PCNA has been used as a marker for cells

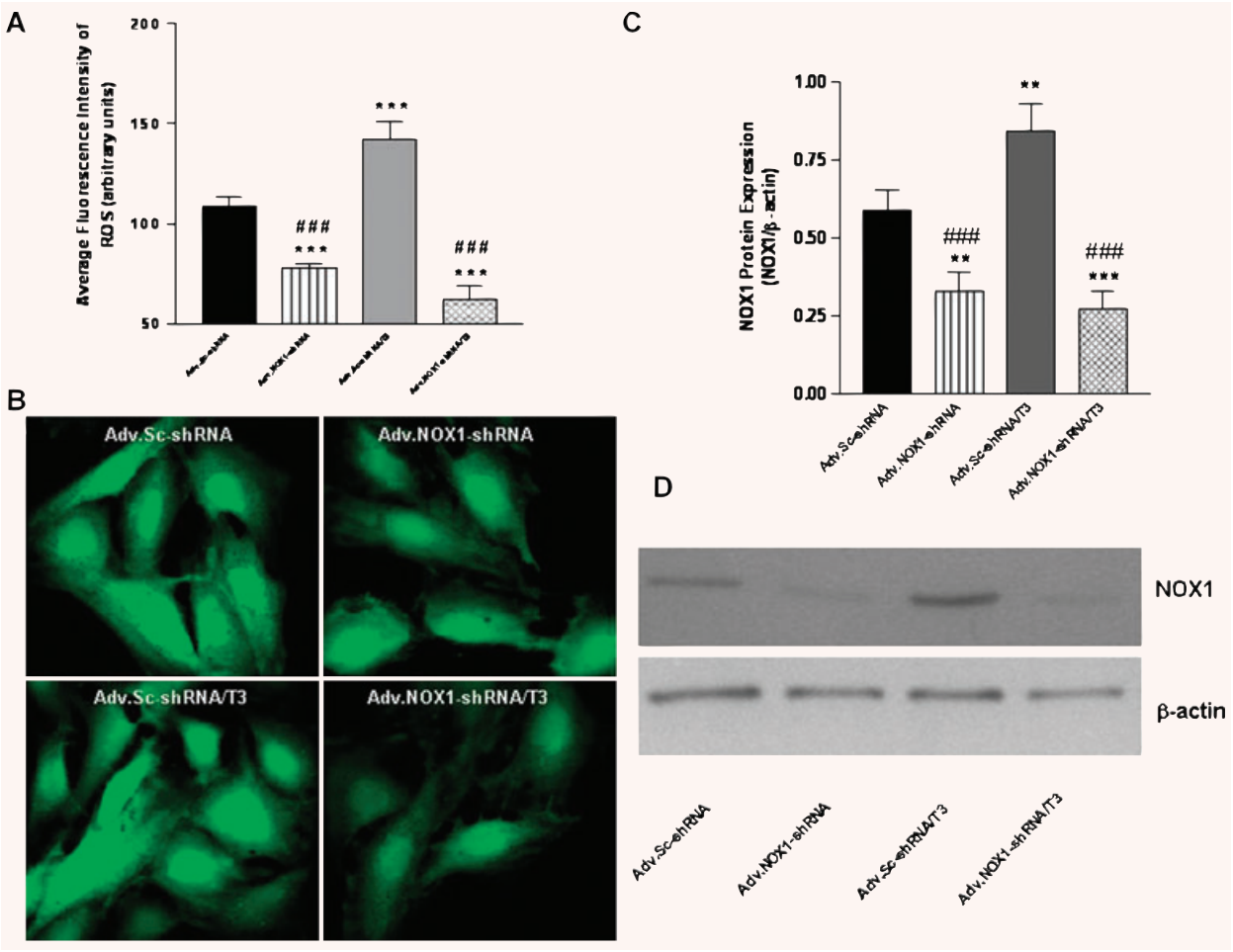

Fig. 6 Adv.NOX1-shRNA blocked T3-induced intracellular ROS production. RASM cells were seeded in 100 mm dishes (1×10^6, for Western blot) or 6-well plate (1×10^4, for intracellular ROS measurement) and incubated with DMEM containing 10% foetal bovine serum (FBS), 100 ug/ml of streptomycin and 100 U/ml of penicillin at 37°C, 5% CO2 for 12 hrs. The medium was replaced with Adv.NOX1-shRNA or Adv.Sc-shRNA (100 MOI) in 5 ml of FBS-free DMEM medium and incubated for 4 hrs. Five ml of DMEM containing 2% FBS was added and incubated for 20 hrs. T3 was then added and incubated for additional 40 hrs. (**A**) Quantification of intracellular ROS production. Ten to 15 cells were measured and the average fluorescence intensity value was calculated and presented as the mean ± S.E.M. (**B**) Representative fluorescence images (Original magnification, 400×). (**C**) Quantification of NOX1 protein expression level normalized with β-actin. (**D**) Representative Western blots of NOX1 (~63 kD) and β-actin (~42 kD). The error bars (A and C) represent the standard error from three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* the Adv.Sc-shRNA group; $^{\#\#\#}P < 0.001$ *versus* the Adv.Sc-shRNA/T3 group. Adv.Sc-shRNA, adenovirus with Scrambled shRNA; Adv.NOX1-shRNA, adenovirus with NOX1 shRNA; Adv.Sc-shRNA/T3, adenovirus with Scrambled shRNA plus T3; Adv.NOX1-shRNA/T3, adenovirus with NOX1 shRNA plus T3.

actively replicating DNA. SM α-actin is an index of cell proliferation or growth. Expression of SM α-actin is regulated by hormones and cell proliferation. Growth factor stimulation of dissociated SMCs causes proliferative growth with loss of contractility [39]. In the adult, SMC is a terminally differentiated cell that expresses cytoskeletal marker proteins like SM α-actin and SM myosin heavy chains, and contracts in response to chemical and mechanical stimuli. However, SMC is able to revert to a proliferative state equivalent to that seen during vasculogenesis in the foetus [40].

Several studies have shown that thyroid hormone (T3) is able to promote or inhibit cell proliferation in a cell type-dependent manner [41]. Premature elevations of thyroid hormone may lead to a 'mature' cardio-phenotype. Thyroid hormone stimulates maturation of ovine foetal cardiomyocytes in culture by decreasing their proliferation capacity [42]. Thyroid hormone may suppress DNA synthesis and cell proliferation in the adult VSMC (primary culture) [43, 44]. Treatment of human papillary and follicular thyroid cancer cells with T3 resulted in enhanced cell proliferation, measured by PCNA [45]. Additional experiments showed that DNA synthesis was induced as early as 2 days after T3 treatment [46]. The present study revealed that T3 selectively up-regulated NOX1, the key factor that mediated T3-induced SMC proliferation in

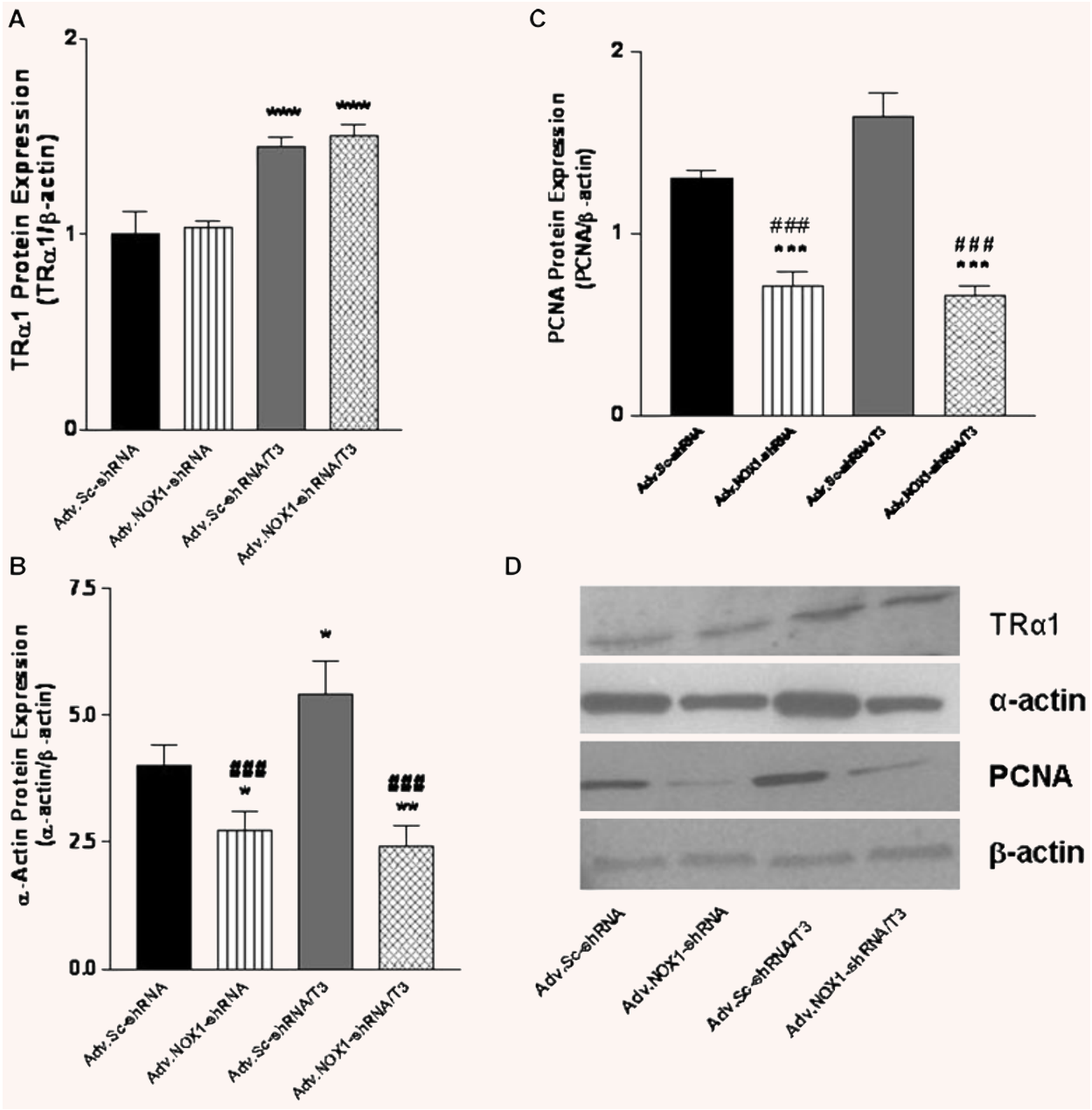

Fig. 7 Adv.NOX1-shRNA down-regulated PCNA and α-actin but not TRα1 expression. RASM cells (1×10^6) were incubated with DMEM containing 10% FBS, 100 ug/ml of streptomycin and 100 U/ml of penicillin at 37°C, 5% CO_2 for 12 hrs. The medium was removed and Adv.NOX1-shRNA or Adv.Sc-shRNA (100 MOI) was added in 5 ml of FBS-free DMEM medium and incubated for 4 hrs. Five ml of DMEM containing 20% FBS was added and incubated for 20 hrs. T3 (7.5 nM) was added and incubated for additional 40 hrs. The cells were harvested and lysed with whole cell lysis buffer. The protein expression was measured by Western blot. (**A**) Quantification of TRα1 protein expression level. (**B**) Quantification of SM α-actin protein expression level. (**C**) Quantification of PCNA expression. (**D**) Representative Western blots of TRα1 (48 kD), SM α-actin (42 kD), PCNA (29 kD) and internal control β-actin. The error bars (A, B and C) represent the standard error from three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* the Adv.Sc-shRNA group; $^{\#\#\#}P < 0.001$ *versus* the Adv.Sc-shRNA/T3 group.

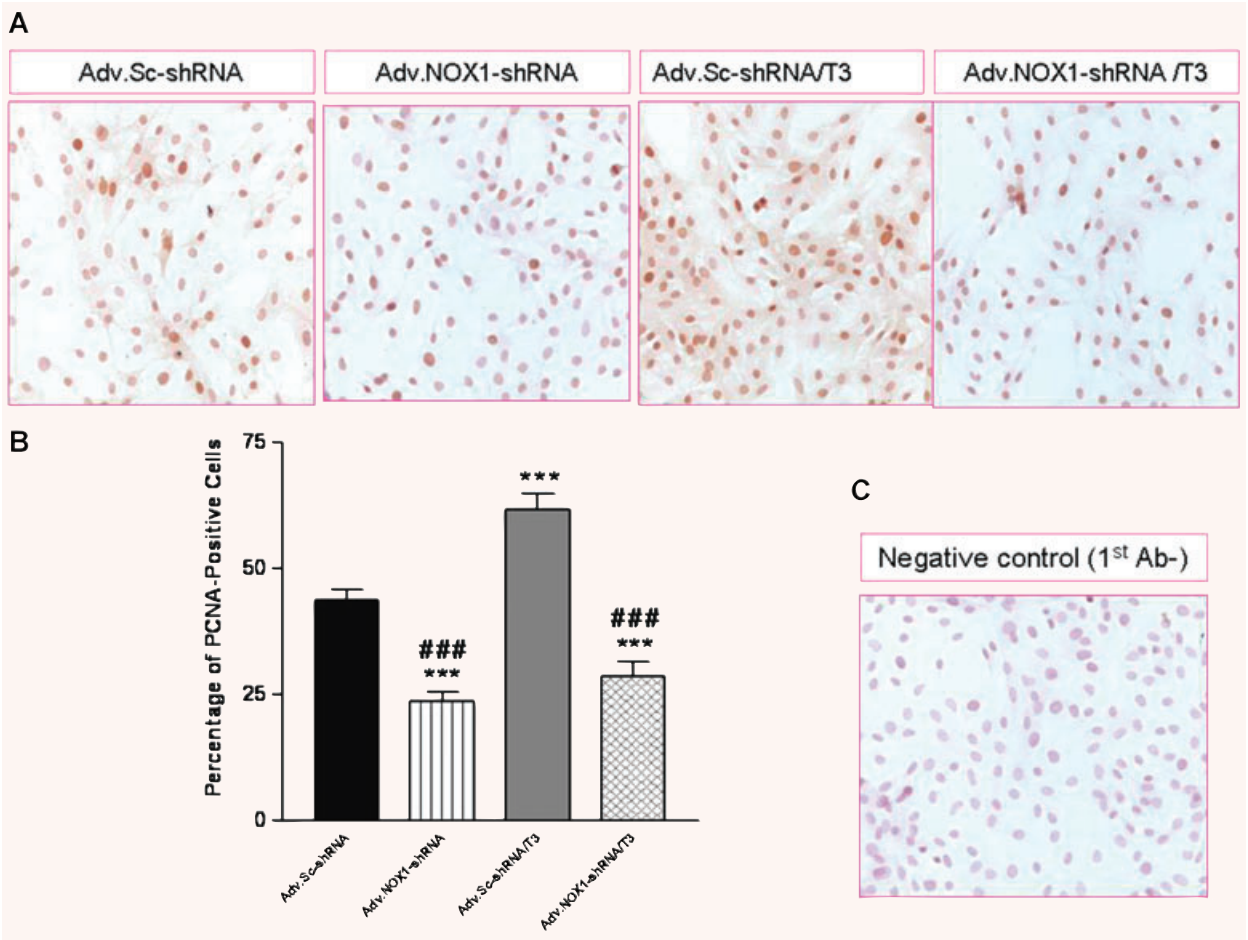

Fig. 8 Adv.NOX1-shRNA abolished the T3-induced increase in PCNA-positive cells. (**A**) Immunocytochemical analysis of PCNA expression in cells treated with Adv.Sc-shRNA, Adv.NOX1-shRNA, Adv.Sc-shRNA/T3 and Adv.NOX1-shRNA/T3, respectively. Brown colour indicates PCNA staining (20×). (**B**) The percentage of PCNA-positive cells in the four treatment groups. The percentage of PCNA-positive cells was calculated in a total of 200 RASM cells randomly selected. (**C**) Negative control (cells that were not incubated with anti-PCNA antibody) showed no PCNA staining, indicating specificity of anti-PCNA antibody. The error bars represent the standard error from three experiments. $^{***}P < 0.001$ *versus* the Adv.Sc-shRNA group; $^{###}P < 0.001$ *versus* the Adv.Sc-shRNA/T3 group.

RASM cell line (secondary culture). This finding may lead to a new understanding on how T3 promotes cell proliferation.

There are several studies reporting subcellular localization of NOX1. In keratinocytes, there was a weak cytoplasmic and a strong nuclear staining [47]. One study in VSMC suggests an endoplasmic reticulum (ER) pattern [48]. Another report describes that NOX1 is co-localized with caveolin in punctate patches on the surface and along cellular margins [49]. In contrast to neutrophils, VSMCs can use NOX1 rather than NOX2 (gp91phox) as a catalytic centre in the p22phox-based oxidase at or near the plasma membrane and submembrane vesicular structures in unstimulated cells [50]. NOX1 mediates NADPH-dependent production of ROS in RASM cells stimulated with angiotensin II and PDGF [23]. The present results indicated that NOX1 is predominately localized around the nucleus and co-localized with TRα1 in RASM cells. The requirement of NOX1 for T3-induced TRα1-specific SMC proliferation is novel for a nuclear hormone receptor.

In summary, T3 induced cell cytokinesis and proliferation *via* TRα1 receptors in RASM cells. TRα1 and NOX1 co-localized around the nucleus. T3 up-regulated NOX1 resulting in increased intracellular ROS. NOX1 was essential to T3-induced cell proliferation. The present study reveals a new intracellular pathway that mediates T3-induced cell proliferation, *i.e.* T3-TRα1-NOX1-cell proliferation.

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