influence of gonadal female hormones. In this study, we demonstrate that female astrocytes in vitro are a high glutamate-uptake phenotype that removes more glutamate and protects motoneurons more against glutamate than male ones. We also demonstrate that such a difference in astrocytic function does not depend on gonadal female hormones but depends rather on a local cell-autonomous mechanism (s).

Materials and Methods

All of the animals used in this study were obtained, housed, cared for, and used in accordance with the guidelines of the Universities of Yamanashi and Hiroshima.

Cell Culture

The culture of spinal astrocytes was prepared as described previously (Shibata et al. 2011) with minor modifications. The spinal cord was removed from neonatal male or female Wistar rats. Male and female rat pups were distinguished by the larger genital papilla and longer ano-genital distance in male versus female pups. To remove serum-derived hormones, charcoal-stripped fetal bovine serum was used. The culture of rat motoneurons was prepared as described previously (Nishijima et al. 2001).

Measurement of Glutamate Uptake

Glutamate clearance was measured as previously described (Sato et al. 2003).

Ca^{2+} Imaging in Single Motor Neurons

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) were measured by the fura-2 method as previously described (Koizumi et al. 2003). The amplitude of the high K^+-evoked $[Ca^{2+}]i$ elevation in motoneurons seeded on either male or female astrocytes was used as an index of neuronal function (Koizumi et al. 1994).

Chemicals

DL-*threo*-β-Benzyloxyaspartic acid (TBOA) and Dihydrokainate (DHK) were purchased from TOCRIS Bioscience (Bristol, UK). Anti-neurofilament H non-phosphorylated (SMI-32) antibody was from COVANCE Japan Co. Ltd (Tokyo, Japan). All other reagents were from Sigma-Aldrich Japan (Tokyo, Japan).

Statistical Analysis

Experimental results are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t* test. One way analyses of variance (ANOVA) followed by Tukey test were applied for multiple comparisons. The differences between means were considered to be significant when the *p* values were less than 5 %.

Results

Female astrocytes (grey columns) cleared significantly larger amounts of glutamate than male ones (open columns) both at 30 and 60 min (Fig. 1). At 30 min, female astrocytes showed two times higher glutamate uptake activity. When extracellular Na^+ was removed, the glutamate clearance disappeared almost completely (data not shown), indicating that the extracellular glutamate was uptaken by Na^+-dependent glutamate transporter(s). To identify the predominant glutamate transporter(s) of cultured astrocytes, we co-applied 0.3 mM TBOA, an inhibitor of both GLAST and GLT-1 (Shimamoto et al. 2004) or 1 mM DHK, a selective inhibitor of GLT-1 (Johnston et al. 1974) with glutamate. TBOA dramatically inhibited the glutamate uptake in both male and female astrocytes, whereas DHK showed only slight inhibition or no effect, suggesting that GLAST was dominant in both cultures.

We then investigated whether such sex-dependent differences in glutamate uptake might affect neuronal damage/death induced by exogenously applied glutamate. Since the glutamate clearance by spinal astrocytes greatly affects the survival of motoneurons (Jimonet et al. 1999),

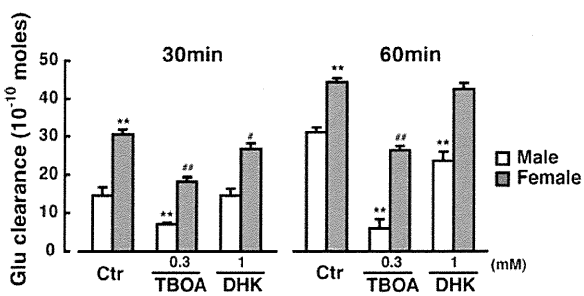

Fig. 1 Differences in glutamate (Glu) clearance in female and male astrocytes. The uptake activity of female (*grey columns*) and male (*open columns*) astrocytes, 30 and 60 min after incubation with glutamate in the absence (Ctr) and the presence (TBOA or DHK) of inhibitors of glutamate transporters. Female astrocytes uptook higher amounts of glutamate than male ones at both time periods (Ctr). DHK had no or only a slight effect on the glutamate uptake, but TBOA significantly decreased the glutamate clearance both in male and female astrocytes

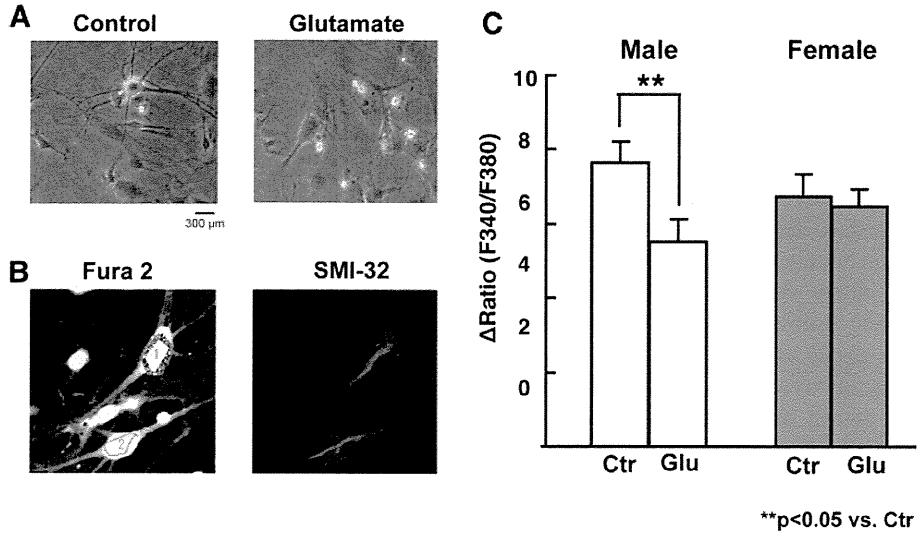

Fig. 2 Potent protection of motoneurons by female astrocytes. **a** Phase-contrast images of motoneurons seeded on male astrocytes, showing the effects of glutamate-treatment. *Left panel*, healthy motoneurons show phase bright morphology; *right panel*, motoneurons damaged by glutamate seeded on male astrocytes, show dark, flattened shape. **b** Fura-2 fluorescent images and immunostaining by SMI-32 antibody. *Left panel*, fura-2 fluorescence; *right panel*, immunocytochemical images of anti-SMI32 antibody of the motoneurons after Ca^{2+} imaging experiments. **c** The high K^+-evoked increase in $[Ca^{2+}]i$ in motoneurons after treatment of cells with glutamate (100 μM, 30 min, and then 24 h washout). Changes in $[Ca^{2+}]i$ in cells were expressed as Δ ratio of F340/F380. The high K^+-evoked increase in $[Ca^{2+}]i$ in motoneurons seeded on male astrocytes was decreased by glutamate but not in those seeded on female astrocytes

we used motoneurons cultured on either male or female spinal astrocytes. Figure 2a shows phase-contrast images of motoneurons, showing the effects of glutamate-treatment. Healthy motoneurons show phase bright morphology (left panel), but when damaged, they show a dark, flattened shape. Motoneurons were stimulated with glutamate (100 μM) for 30 min, and then washed-out and further incubated with glutamate-free medium for 24 h. The fraction of motoneurons with phase bright morphology was dramatically decreased by the glutamate-treatment. For quantitative analysis, we employed a high K^+-evoked increase in $[Ca^{2+}]i$ in neurons (Koizumi et al. 1994). The treatment with glutamate (100 μM, 30 min, and then 24 h washout) significantly decreased the high K^+-evoked responses in motoneurons on male astrocytes, whereas it had almost no effect on the $[Ca^{2+}]i$ responses in motoneurons on female astrocytes (Fig. 2c). After the Ca^{2+} imaging experiments, cells were stained with anti-SMI-32 antibody to confirm that the cells of interest were motoneurons (Fig. 2b, right).

Discussion

In this study, we demonstrated that (1) female astrocytes cleared more glutamate by GLAST than male ones; (2) spinal female astrocytes showed stronger protective action against glutamate-evoked neuronal damage in motoneurons than male ones; and most importantly, (3) these characteristic features of female astrocytes were not necessarily dependent on gonadal female hormones, since astrocytes were obtained separately from either female or male pups and cultured in the absence of sex hormones. Although differences in the vulnerability to several types of brain insults between female and male brains are often explained by the fact that gonadal female hormones act on and protect neurons, it is unlikely that such differences are entirely related to the hormonal effects on neurons. Thus, our present results could be novel and important as that we have shown that such sex differences could be explained by (i) functional differences in astrocytes but not neurons, and that (ii) these differences in astrocytic functions do not totally depend on gonadal sex hormones but, presumably, depend on the property of XX versus XY chromosomes, by which each astrocyte is transformed into a distinct phenotype in a cell-autonomous mechanism, although we must await further studies to clarify the detail molecular mechanisms.

As for the peripheral sex hormone-independent mechanisms, extragonadal production of E2 may be involved. E2 can be synthesized locally from testosterone by the aromatase cytochrome P450 in the CNS. In an experimental stroke model, mice with targeted deletion of *cyp*19, which codes for aromatase P450, showed more severe brain injury

than wild-type litter mates (McCullough et al. 2003), suggesting that aromatase and extragonadal E2 play an important role in protection of the brain. It should be noted that astrocytes express aromatase P450, and more importantly, that the expression of P450 is higher in female astrocytes than that in male astrocytes (Liu et al. 2007). These findings suggest that female astrocytes locally produce more estrogen than male, thereby leading to higher expression of glutamate-transporters. The higher capacity to clear glutamate causes the female astrocytes to have higher neuroprotection against glutamate. However, further study is required to clarify this issue.

Spinal astrocytes in vivo express more GLT-1 than GLAST. It is well known that, similar to cultured astrocytes obtained from the hippocampus or cortex, GLT-1 expression becomes less dominant by cultivation. If cultured with neurons, or in the presence of several factors such as cAMP-forming reagents or β-lactam antibiotics, GLT-1 expression is increased (Rothstein et al. 2005). When spinal astrocytes were co-cultured with motoneurons, it is possible that GLT-1 was upregulated contributing to the clearance of glutamate in female astrocytes. Thus, we do not exclude the involvement of GLT-1 in the higher uptake of glutamate in female astrocytes.

Taken together, we demonstrated that spinal astrocytes obtained from female pups showed higher glutamate uptake activity and more intensive neuroprotection against glutamate than those obtained from males. The effect was independent of gonadal female hormones, suggesting that astrocytes have cell-autonomous regulatory mechanisms by which they transform themselves into less vulnerable phenotypes.

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Reactive oxygen species mediate adipocyte differentiation in mesenchymal stem cells

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ABSTRACT

Aims: Mesenchymal stem cells (MSC) have the potential to differentiate into various cell lineages, including adipocytes and osteoblasts. The formation of adipose tissue involves the commitment of MSC to the preadipocyte lineage and the differentiation of preadipocytes into mature adipocytes. In the present study, we investigated the involvement of reactive oxygen species (ROS) in adipocyte differentiation from MSC.
Main methods: ROS signaling was evaluated by the effects of antioxidant N-acetyl-L-cysteine (NAC) or shRNA against NAD(P)H oxidase in the multipotent mesenchymal stem cell line 10T1/2 cells. Intracellular ROS was measured using an H_2DCF dye.
Key findings: We found that NAC blocked adipocyte differentiation in MSC. An H_2DCF assay revealed that differentiation-inducing agents induced ROS generation. These data suggest that ROS is involved in adipocyte differentiation in MSC. Next, we examined the source of ROS. Knockdown of NAD(P)H oxidase 4 (Nox4) by RNA interference inhibited ROS production and adipocyte differentiation by differentiation-inducing agents. Furthermore, treatment with NAC blocked the transcriptional activation of CREB, and the expression of dominant-negative mutants of CREB inhibited adipocyte differentiation.
Significance: The findings suggest that the increase in the intracellular ROS level via Nox4 mediates adipocyte differentiation through CREB in MSC. This data will provide new insight into the drug development for obesity.

Introduction

Obesity is the major risk factor for the development of type 2 diabetes, heart disease, hypertension, and stroke (Rexrode et al., 1996). It is caused due to a positive energy balance in the body that results in an increase in adipose tissue by an increase in either the number or the size of adipocytes (Spiegelman and Flier, 2001). Adipocytes could arise from mesenchymal stem cells (MSC) that have the potential to differentiate into various cell lineages, including adipocytes and osteoblasts (Pittenger et al., 1999). Thus, further understanding of the mechanism underlying adipocyte differentiation from MSC might facilitate the development of novel targets for new drugs in order to modify the in vivo function of adipocytes.

As determined using the murine preadipocyte cell line 3T3-L1, adipocyte differentiation in both preadipocytes and mature adipocytes is a well-defined process that occurs via a series of transcriptional events that involve several key transcription factors. Differentiation is initiated by the induction of the CCAAT/enhancer-binding protein (C/EBP) β that in turn induces C/EBPα and nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) (Rosen et al., 2000). Once expressed, C/EBPα and PPARγ enhance each other's production and are involved in terminal differentiation. In addition to the C/EBPs and PPARγ, cAMP response element-binding protein (CREB) has also been implicated as an early regulator of the adipocyte differentiation program. A previous study showed that CREB overexpression in 3T3-L1 preadipocytes promotes the expression of adipocyte markers and the accumulation of triglycerides (Reusch et al., 2000). Adipogenic agents induced the phosphorylation of Ser133 CREB, thereby increasing its capacity to activate adipocyte differentiation-related transcription.

In contrast to differentiation of preadipocytes to mature adipocytes, adipocyte differentiation of MSC has not been entirely elucidated. Although 3T3-L1 preadipocytes are an excellent model cell system with which to elucidate the terminal adipocyte differentiation program, a more primitive cell line should be useful in investigating earlier mechanisms in adipocyte development. In particular, 10T1/2 cells, which are derived from C3H mouse embryos (Reznikoff et al., 1973), have been widely used as a mesenchymal stem cell line for studies on stem cell differentiation (Pinney and Emerson, 1989; Taylor and Jones, 1979).

Reactive oxygen species (ROS), such as superoxide anions ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2), are produced by the NAD(P)H oxidase

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enzyme complex in phagocytic cells (e.g., macrophages and neutrophils) (Babior et al., 2002). Growing evidence suggests that ROS production is not restricted to phagocytic cells, and these species function as important secondary messengers in intracellular signal transduction pathways for several functions such as proliferation and differentiation (Sundaresan et al., 1995; Nishio and Watanabe, 1997; Sauer et al., 2000). Nonphagocytic ROS are produced by an enzyme that shows a high degree of homology with the phagocytic NAD(P)H oxidase and is now believed to belong to the Nox family, which comprises Nox1, Nox2 (gp91), Nox3, Nox4, and Nox5. ROS generation during adipogenesis in 3T3-L1 preadipocytes has already been reported (Lee et al., 2009). However, the mechanisms by which ROS affect MSC are largely unknown.

In the present study, we demonstrated that ROS mediate adipocyte differentiation in MSC. Furthermore, we show that the ROS generated via Nox4 play a key role in adipocyte differentiation.

Materials and methods

Materials

We obtained N-acetyl-L-cysteine (NAC) from Kanto Chemical Co. (Tokyo, Japan). 4′-hydroxy-3′-methoxyacetophenone (apocynin) was from Calbiochem (La Jolla, CA, USA). Anti-PPARγ polyclonal antibody (H-100) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CREB polyclonal antibody was from Upstate Biotechnology (Charlottesville, VA, USA). Anti-Prx II polyclonal antibody was from LabFrontier (Seoul, Korea). All other reagents were of analytical grade and obtained from commercial sources.

MSC culture

Rat bone marrow-derived mesenchymal stem cells were isolated by the previous method with slight modifications (Azizi et al., 1998). Eight-week-old Sprague–Dawley rats (Tokyo Laboratory Animal Science, Tokyo, Japan) were sacrificed, and their femurs and tibiae were carefully cleaned of the adherent soft tissue. The marrow was harvested by inserting a syringe needle (23 gauge) into one end of the bone and flushing with Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and were seeded in DMEM containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 100 U/ml penicillin (Gibco BRL), and 100 μg/ml streptomycin (Gibco BRL). After the cells were allowed to adhere to culture dishes for 3 days, the nonadherent cells (such as hematopoietic stem cells) were removed by changing the medium and the adherent cells were maintained in culture. The medium was changed every 3 days. All the procedures involving animals were in accordance with the guidelines of National Defense Medical College. The 10T1/2 cell line (clone 8; Japanese Cancer Research Resources Bank) was cultured in basal medium Eagle (BME; Gibco BRL, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Adipocyte differentiation

Adipocytes were differentiated as described previously with a slight modification (Cheng et al., 2003). Briefly, the cells were cultured to confluence in a normal growth medium. At 2 days post-confluence, the medium was changed to differentiation medium supplemented with 10% FBS, 5 μg/ml insulin, 0.1 μM dexamethasone, and 50 μM indomethacin. After 3 days, the medium was changed back to the normal growth medium, and the cells were further cultured for 4 days.

Oil red-O staining

Oil red O-staining was carried out as described previously (Green and Kehinde, 1975). The cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min. After they were washed twice with PBS, the cells were stained with 0.3% oil red-O solution (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min and visualized by standard light microscopy (Olympus IX70; Olympus, Okaya, Japan). Staining was quantified by extracting oil red-O from the stained cells with isopropanol and determining the absorbance of the solution at 518 nm using a Hitachi U-3010 spectrophotometer (Tokyo, Japan).

Measurement of intracellular ROS

In a 96-well plate (Perkin-Elmer Life Sciences, Wellesley, MA, USA), cells were seeded at a density of 1×10^4 cells per well, and they were allowed to adhere overnight. The cells were then incubated at 37 °C in serum-free medium containing 10 μM 2′,7′-dichlorofluorescein (H_2DCF; Molecular Probes, Eugene, OR, USA). After they were washed with PBS, the cells were incubated in the differentiation medium. The change in fluorescence was measured using a Fusion™ Multilabel Reader (Perkin-Elmer) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Cell lysis and immunoblotting

Cell lysates were prepared as described previously (Kanda and Watanabe, 2005). Briefly, the cells were lysed in a buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM $Na_4P_2O_7$, 20 mM NaF, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, and 10 μg/ml aprotinin] on ice. After incubation on ice for another 30 min, the lysed cells were centrifuged at 4 °C for 20 min at $15000\times g$, and the supernatant was collected. The protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Samples were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and they were electrophoretically transferred (15 V, 90 min) onto polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by a semi-dry blotting apparatus (ATTO, Tokyo, Japan). The membranes were blocked in 5% skimmed milk in PBS containing 0.2% Tween 20 (PBS-T) for 1 h at room temperature and were incubated with specific antibodies overnight at 4 °C. The blots were then washed with PBS and incubated with peroxidase-conjugated secondary antibodies (1:1000; Cell Signaling Technology) for 1 h at room temperature. They were washed with PBS-T, and peroxidase activity was detected by measuring enhanced chemiluminescence (ECL Detection Kit; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Plasmids and retrovirus

Plasmids encoding dominant-negative mutant of CREB were kindly provided by Dr. Goodman (Oregon Health and Science University, Portland, OR, USA). The plasmids were transfected into 10T1/2 cells by using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's protocol. Prx II retrovirus were previously reported (Choi et al., 2005).

Short hairpin RNA

pSuper.retro.neo (OligoEngine, Seattle, WA, USA) was used as the mammalian RNA interference expression vector. It was linearized with *Hin*dIII and *Bgl*II, and the annealed 64-nucleotide oligos (synthesized by Genosys) including the Nox4 sequence (GCTGTCCCTAAACGTTCTACT) was ligated into the linearized vector. The resulting positive clones of the recombinant expression vector were identified by *Eco*RI and *Hin*dIII digestion. Cells transfected with

Lipofectamine 2000 were selected with G418 (1 mg/ml), and polyclonal cells were used in the experiments. Empty vector was used as the control.

RNA isolation and real-time PCR

Total cellular RNA was extracted using the ISOGEN reagent (Nippon Gene, Tokyo, Japan). Reverse transcription was performed in a 20-μl reaction volume comprising 1 μg total RNA, 1× reaction buffer, 0.5 mM dNTP mixture, 2.5 μM oligo(dT)$_{20}$ primer, 40 U RNase OUT, and 200 U Superscript III reverse transcriptase (RT; Gibco). Real-time Polymerase chain reaction (PCR) was performed with the cDNA samples and SYBR PCR mixture (Takara) using an ABI PRISM 7900 sequence detector (Applied Biosystems). The results are expressed in $2^{-\text{deltaCt}}$ using β-actin as a reference. The sequences of the primers used in real-time PCR are as follows: rC/EBPα (forward, 5′- GAATCTCC-TAGTCCTGGCTC-3′; reverse, 5′-GATGAGAACAGAACGAGTAC-3′); rC/EBPβ (forward, 5′-GCCACGGACACCTTCGAGG-3′; reverse, 5′-CGGCTCCGCCTTGAGCTGC-3′); rPPARγ (forward, 5′-GCGGAGATCTC-CAGTGATATC-3′; reverse, 5′-TCAGCGACTGGGACTTTTCT-3′) rβ-actin (forward, 5′-AGGGAAATCGTGCGTGAC-3′; reverse, 5′-CGCTCATTGCC-GATAGTG-3′); mNox4 (forward, 5′-TGTTGGGCCTAGGATTGTGTT-3′;

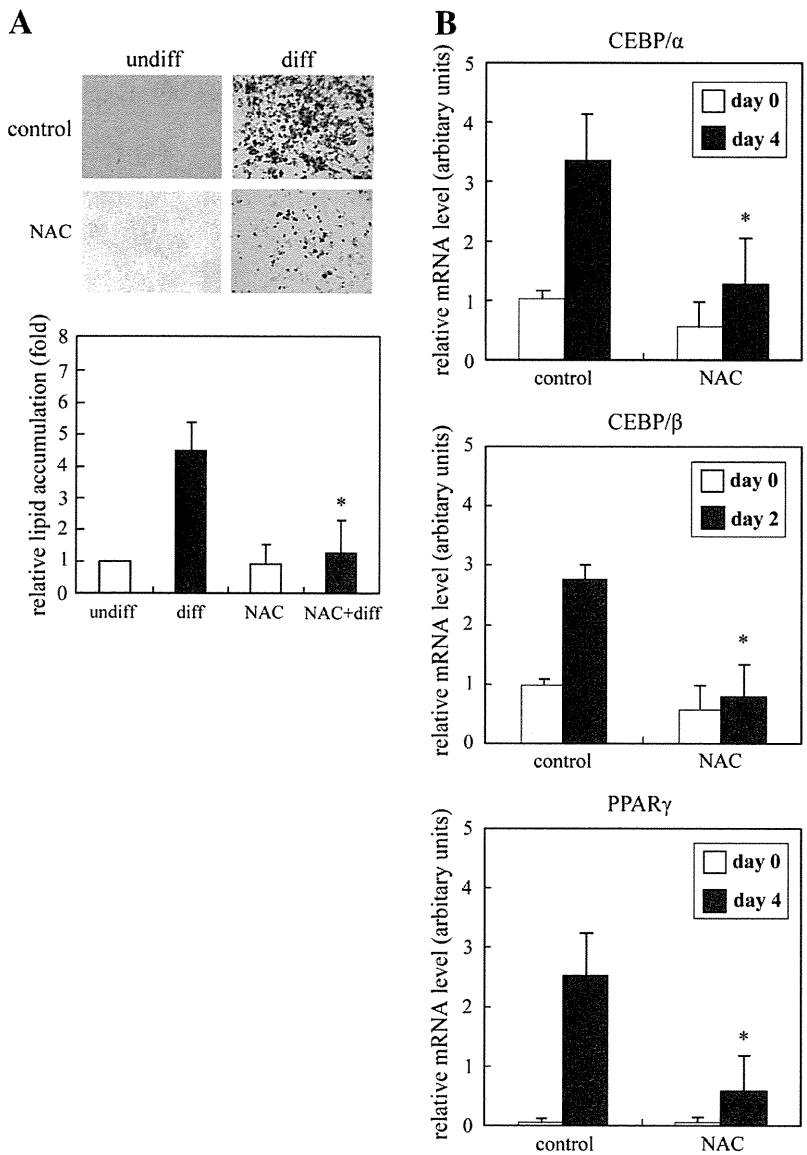

Fig. 1. ROS-mediated adipocyte differentiation in MSC. (A) Rat bone marrow-derived MSC were pretreated with or without NAC (5 mM), and then differentiation was induced for 14 days. The cells were fixed and stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance was measured at 518 nm. *P<0.05 vs diff. (B) Expression of adipogenesis markers were measured by real-time PCR using rat bone marrow-derived MSC with or without NAC. Open column indicates mRNA level at day 0. Closed column indicates mRNA level or at day 2 (C/EBPβ) or day 4 (C/EBPα and PPARγ) in the presence of differentiation-inducing agents. *P<0.05 vs control day 2 or day 4. (C) 10T1/2 cells were pretreated with or without NAC (5 mM), and then differentiation was induced for 7 days. The cells were fixed and stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance was measured at 518 nm. *P<0.05 vs diff. (D) Expression of adipogenesis markers were measured by real-time PCR using 10T1/2 cells with or without NAC. Open column indicates mRNA level at day 0. Closed column indicates mRNA level or at day 2 (C/EBPβ) or day 4 (C/EBPα and PPARγ) in the presence of differentiation-inducing agents. *P<0.05 vs control day 2 or day 4. (E) 10T1/2 cells were loaded with H_2DCF (10 μM) and incubated with differentiation-inducing agents for the indicated times. *P<0.05 vs time 0. (F) 10T1/2 cells were pretreated with NAC (5 mM) for 30 min, loaded with H_2DCF, and incubated with differentiation-inducing agents for 30 min. Intracellular ROS production was detected by measuring the fluorescence of H_2DCF as described in the Materials and methods section. *P<0.05 vs diff.

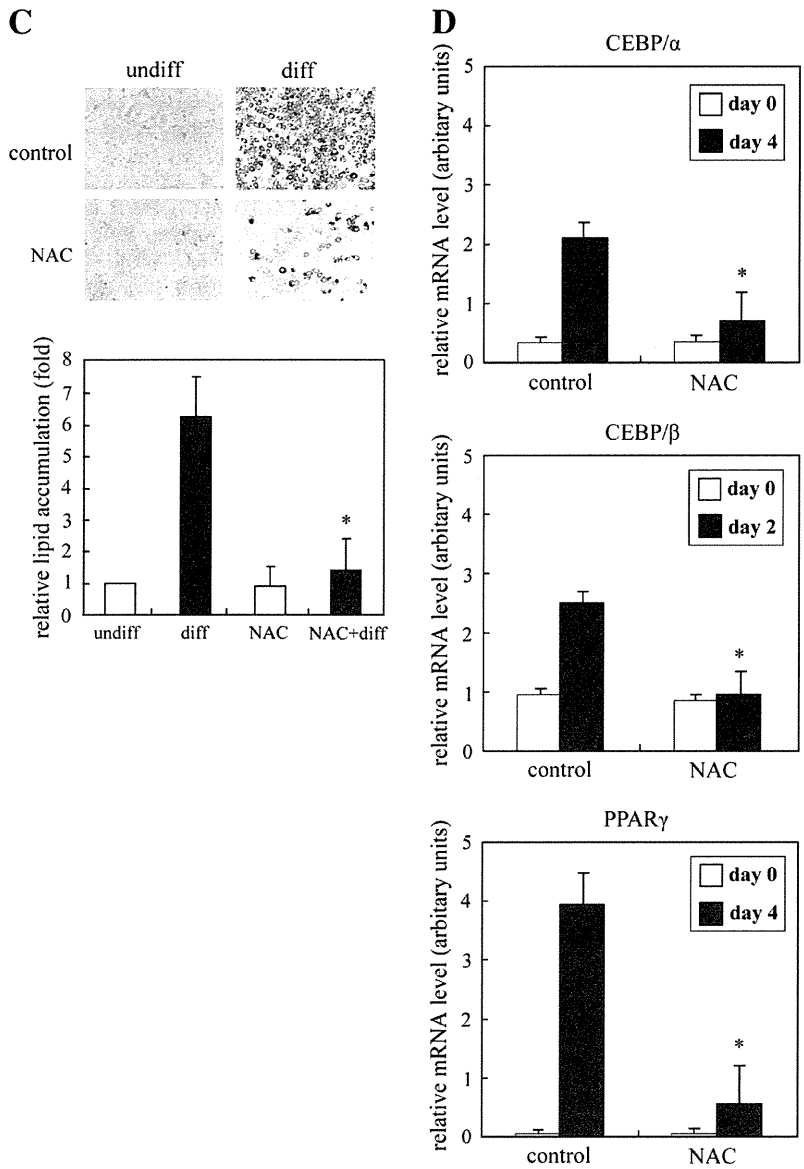

Fig. 1 (*continued*).

reverse, 5′-AGGGACCTTCTGTGATCCTCG-3′); mNox2 (forward, 5′-ACTCCTTGGGTCAGCACTGG-3′; reverse, 5′-GTTCCTGTCCAGTTGTCTTCG-3′); mC/EBPα (forward, 5′-GAACAGCAACGAGTACCGGGTA-3′; reverse, 5′-GCCATGGCCTTGACCAAGGAG-3′); mC/EBPβ (forward, 5′-CCAGAA-GAAGGTGGAGCAACTG-3′; reverse, 5′-TCGGGCAGCGTCTTGAAC-3′); mPPARγ (forward, 5′-AGTCTGCTGATCTGCGAGCC-3′; reverse, 5′-CTTTCCTGTCAAGATCGCCC-3′) mβ-actin (forward, 5′-ACCACACCTTCTA-CAATGAG-3′; reverse, 5′-AGCACAGCCTGGATGG-3′).

PCR analysis

PCR was performed using the 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) in a 100-μl reaction volume comprising 1 μl RT product, 1× PCR buffer, 0.5 μM each of the sense and antisense primers, and 2.5 U long and accurate Taq (Sigma). The following primers were used: Nox4 (forward, 5′-GAAGCCCATTTGAGGAGTCA-3′; reverse, 5′-GGGTCCACAGCAGAAAACTC-3′). The cycling conditions were as follows: 1 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, followed by 7 min at 72 °C.

Luciferase assay

pCRE-Luc (Stratagene, La Jolla, CA, USA) was transfected into 10T1/2 cells by FuGENE6. The cells were lysed 2 days after transfection, and luciferase activity was determined using the luciferase assay system (Promega, Madison, WI, USA), according to the manufacturer's protocol. To normalize the transfection efficiency, the pRL-CMV plasmid was co-transfected in all the experiments.

Statistical analysis

Data represent the mean ± S.D. of at least three separate experiments. The mean values were compared with control by using an unpaired Student's *t* test using Microsoft Excel-based application. A value of P less than 0.05 was considered statistically significant.

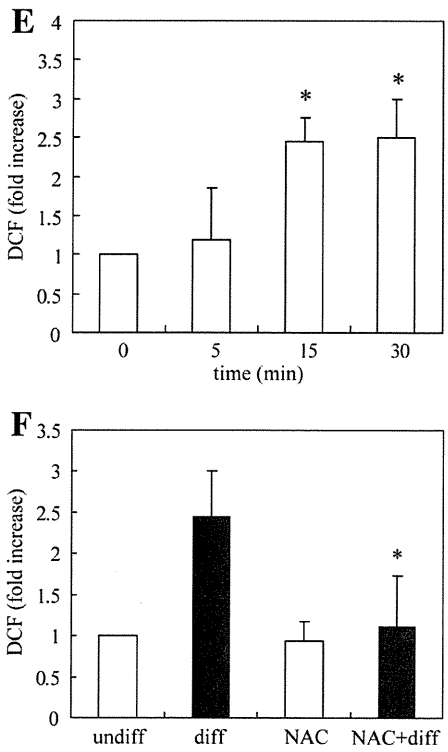

Fig. 1 (*continued*).

Results

Effects of antioxidants on adipocyte differentiation

To investigate the role of ROS on adipocyte differentiation from MSC, we first tested the effects of an antioxidant on adipocyte differentiation. Since MSC in mice has been shown to be more difficult than that in rat, due to the unwanted growth of non-MSC during the expansion in culture (Lindolfo Da Silva and Nance, 2003), we used rat bone marrow-derived MSC. As shown in Fig. 1A, treatment with differentiation-inducing agents induced formation of fat droplets, which triglycerol is stained by oil red-O. The antioxidant NAC inhibited the lipid accumulation in the cells. To confirm the effect of NAC, we performed real-time PCR for C/EBPα, C/EBPβ and PPARγ, which are well-known adipocyte markers. As shown in Fig. 1B, NAC inhibited the induction of C/EBPβ (at day2), C/EBPα and PPARγ (at day4) in rat bone marrow-derived MSC with the differentiation-inducing agents. Treatment with NAC for 30 min had little effects on the expression of the markers. Since primary MSC are heterogeneous, we then used murine mesenchymal stem cell line 10T1/2, which is widely used as a model of adipogenesis from MSC and do not require post-confluence mitosis (Cho and Jefcoate, 2004), for further analysis of the differentiation mechanism from MSC. Consistent with the data using primary cells, similar inhibitory effects of NAC on differentiation were observed in 10T1/2 cells (Fig. 1C and D). Thus, the observed inhibitory effects of antioxidants on adipocyte differentiation in both primary and 10T1/2 cells suggest that ROS might function during the early stage of adipocyte differentiation in MSC.

ROS generation by differentiation-inducing agents

Next, we examined the level of ROS in 10T1/2 cells by using the ROS-reactive dye H_2DCF. As shown in Fig. 1E, treatment with differentiation-inducing agents for 30 min induced an increase in the fluorescence intensity of H_2DCF. When the cells were treated with differentiation-inducing agents for 1 h, some cells underwent cell death (data not shown). Since this might be due to the cytotoxicity, ROS production was measured for 30 min. Moreover, this increase was abolished by NAC (Fig. 1F), suggesting that the fluorescence intensity was derived from ROS. NAC alone did not inhibit the basal intensity. These data suggest that ROS are generated after the induction of differentiation in 10T1/2 cells.

Effect of peroxiredoxin on adipocyte differentiation

To further confirm the involvement of ROS in adipocyte differentiation, we studied the effect of the overexpression of H_2O_2-scavenging enzyme peroxiredoxin II (Prx II) (Choi et al., 2005). As shown in Fig. 2A, retroviral transduction of Prx II inhibited lipid accumulation. Retrovirus against an empty vector did not affect lipid accumulation. Expression level of endogeneous Prx II was not affected during differentiation. In addition, retroviral transduction of Prx II decreased the fluorescence intensity of H_2DCF (Fig. 2B), confirming the activity of Prx II. When 10T1/2 cells were treated with 100 μM H_2O_2, the cells did not induce lipid accumulation (data not shown), suggesting that ROS is necessary, but not sufficient in 10T1/2 cells. In contrast to differentiation, cell growth was not inhibited by Prx II expression in the presence or absence of differentiation-inducing agents (Fig. 2C), suggesting that ROS are involved in differentiation, not proliferation.

Inhibition of NAD(P)H oxidase impairs adipocyte differentiation

Subsequently, we investigated the source of ROS in adipocyte differentiation. Because NAD(P)H oxidase is considered to generate low levels of ROS, we suspected that it plays a role in ROS production and adipocyte differentiation in 10T1/2 cells. To examine the involvement of NAD(P)H oxidase in adipocyte differentiation, we tested the effect of apocynin, a selective inhibitor of NAD(P)H oxidase, on adipocyte differentiation in 10T1/2 cells. As shown in Fig. 3A, apocynin abolished lipid accumulation. In contrast, L-NAME (an NO inhibitor) or allopurinol (a xanthine oxidase inhibitor) did not inhibit lipid accumulation. To further elucidate the role of NAD(P)H oxidase in adipocyte differentiation, we studied the expression level of the NAD(P)H oxidase family. Since NAD(P)H oxidase isoforms Nox2 and Nox4 are detected by real-time PCR in 10T1/2 cells, we checked their expression levels during differentiation. As shown in Fig. 3B, Nox4 expression was significantly decreased after differentiation. In contrast, Nox2 expression was not changed during differentiation. To assess the involvement of Nox4 in adipocyte differentiation, we silenced its expression by using the RNA interference. Nox4 knockdown was confirmed by RT-PCR and an H_2DCF assay (Fig. 3C, D). A significant decrease in lipid accumulation was observed in the Nox4-silenced cells, whereas normal lipid accumulation was observed in the cells that were transfected with a control vector (Fig. 3E). These data suggest that the ROS produced by Nox4 mediate adipocyte differentiation in 10T1/2 cells.

Effect of CREB on adipocyte differentiation

We examined the molecular mechanisms by which ROS regulate adipocyte differentiation. Since CREB is known to regulate adipocyte differentiation in 3T3-L1 preadipocytes (Reusch et al., 2000), we studied its effects on 10T1/2 cells. As shown in Fig. 4A, the expression of dominant-negative mutants of CREB (DN-CREB) inhibited lipid accumulation. Differentiation-inducing agents stimulated the transcriptional activation of CREB, while antioxidants (NAC or apocynin) suppressed the activation (Fig. 4B). Furthermore, treatment with H_2O_2 induced CREB transcriptional activation, confirming the presence of a signaling

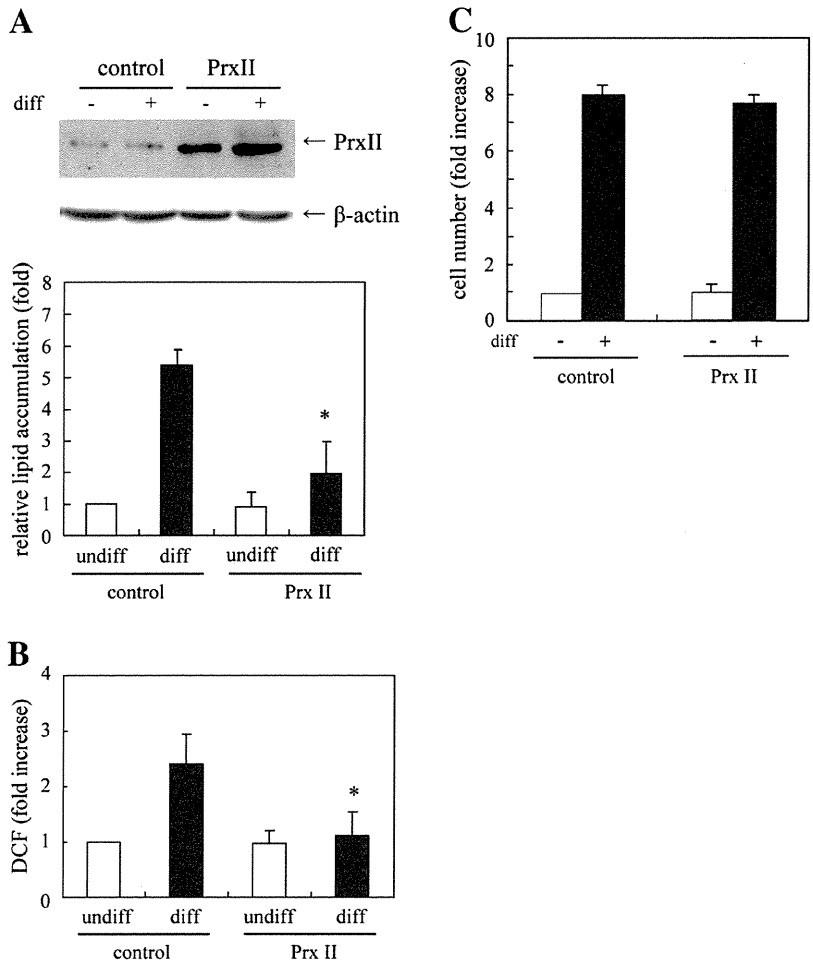

Fig. 2. Effect of peroxiredoxin II on adipocyte differentiation in 10T1/2 cells. (A) 10T1/2 cells were transduced with control or peroxiredoxin II (Prx II) retrovirus and were then incubated with differentiation-inducing agents for 7 days. Expression of Prx II was analyzed by immunoblotting with anti-Prx II antibody (upper panel). After the cells were stained with oil red-O, the oil red-O was extracted with isopropanol, and absorbance at 518 nm was measured (lower panel). *P<0.05 vs control diff. (B) 10T1/2 cells transduced with Prx II were loaded with H_2DCF and incubated with differentiation-inducing agents for 30 min. Intracellular ROS production was detected by measuring the fluorescence of H_2DCF as described in the Materials and methods section. *P<0.05 vs control diff. (C) 10T1/2 cells transduced with control or Prx II retrovirus were incubated with or without differentiation-inducing agents. After 3 days, cell number was determined using a hemocytometer.

pathway from ROS to CREB. These data suggest that adipocyte differentiation via ROS is mediated by CREB in 10T1/2 cells.

Effect of MAPK on adipogenesis

Previous studies using 3T3-L1 preadipocytes have reported that MAPK plays a role in adipocyte differentiation (Sale et al., 1995). Therefore, we examined the contribution of the MAPK pathway to adipocyte differentiation in 10T1/2 cells. We tested the effect of U0126, which is a potent and selective inhibitor of MAPK/ERK kinase (MEK; an upstream ERK kinase). As shown in Fig. 5A, U0126 decreased lipid accumulation by approximately 35%. PD98059, another MEK inhibitor, yielded similar results. Differentiation-inducing agents induced ERK phosphorylation, while the MEK inhibitors completely inhibited the ERK phosphorylation (Fig. 5B). Since ROS have been shown to induce heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) production (Zhang et al., 2004), we studied the effects of AG1478 (a selective EGF receptor kinase inhibitor) and CRM197 (a toxin that inhibits HB-EGF production) on differentiation. Both inhibitors did not inhibit lipid accumulation. As shown in Fig. 5C, NAC inhibited the phosphorylation of ERK by differentiation-inducing agents, suggesting the presence of a signaling pathway from ROS to ERK. These results suggest that the ROS-ERK pathway partially mediates adipocyte differentiation in 10T1/2 cells.

Discussion

In the present study, we demonstrated that ROS play an important role during the early stage of adipocyte differentiation in MSC. We showed that adipocyte differentiation is impaired in the presence of ROS scavengers or RNA interference against Nox4. Furthermore, we showed that CREB act downstream of ROS in adipocyte differentiation.

We showed that ROS are related to differentiation, not proliferation, by using the antioxidant NAC (Fig. 1) and Prx II retrovirus (Fig. 2). NAC inhibited the expression of adipocyte markers including C/EBPα, C/EBPβ and PPARγ. This is consistent with a previous report showing that ROS enhance C/EBPβ activation in 3T3-L1 cells (Lee et al., 2009). Since H_2O_2 induced CREB transcriptional activation (Fig. 4B) and CREB has been shown to regulate C/EBPβ (Reusch et al., 2000), it is possible that ROS act at an upstream of CREB, and subsequently induce C/EBPβ expression in MSC. Inconsistent with our observations, recent study using the 3T3-L1 preadipocytes has shown that ROS stimulate mitotic clonal expansion during adipogenesis in 3T3-L1 preadipocytes (Lee et al., 2009). The

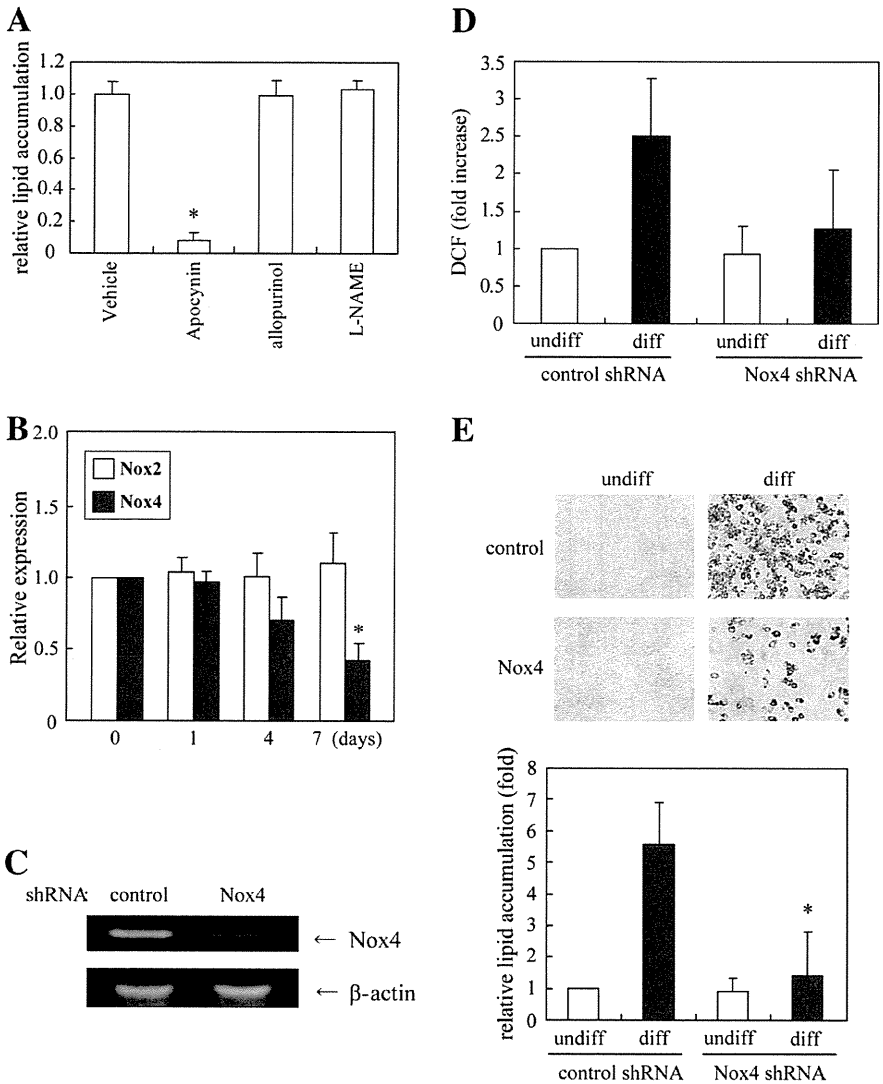

Fig. 3. Role of Nox4 in adipocyte differentiation in 10T1/2 cells. (A) 10T1/2 cells were pretreated with apocynin (1 μM), alluprinol (50 μM), L-NAME (50 μM) or vehicle and incubated with differentiation-inducing agents for 7 days. After fixation, the cells were stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance at 518 nm was measured. *P<0.05 vs vehicle. (B) After 10T1/2 cells were incubated with differentiation-inducing agents for the indicated times, total RNA was isolated and Nox2 and Nox4 were amplified using specific primers. *P<0.05 vs day 0. (C) Forty-eight hours after the cells were transfected with shRNA against Nox4 or control shRNA, total RNA was isolated, and Nox4 was amplified using specific primers. (D) The shRNA-transfected cells were loaded with H_2DCF and incubated with differentiation-inducing agents for 30 min. Intracellular ROS production was detected by measuring the fluorescence of H_2DCF, as described in the Materials and methods section. (E) The shRNA-transfected cells were incubated with differentiation-inducing agents for 7 days. After the cells were fixed, they were stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance was measured at 518 nm. *P<0.05 vs control diff.

discrepancy might be due to the differences of cell type or the stage of differentiation. 10T1/2 cells that we used here do not require post-confluence mitosis, whereas 3T3-L1 cells require one or two cell divisions before differentiation (Cho and Jefcoate, 2004; Tang et al., 2003). In addition, since 10T1/2 cells are considered to be multi-potent and more primitive than 3T3-L1 preadipocytes. Taken together, ROS could act at not only the mitotic clonal expansion but also the early differentiation stage.

We demonstrated NAD(P)H oxidase as the source of ROS in MSC by a pharmacological approach. Among the NAD(P)H oxidase family, our data showed that Nox4 was down-regulated after induction of differentiation in MSC and Nox4 shRNA inhibited lipid accumulation (Fig. 3). Consistent with our results, several reports have shown that Nox4 is down-regulated in mature adipocytes (Mouche et al., 2007; Schröder et al., 2009). Taken together, Nox4 is considered to play an important role in adipogenesis form MSC. Since Nox4 contains multiple transmembrane segments and has been reported to localize in the endoplasmic reticulum and nucleus (Chen et al., 2008; Kuroda et al., 2005), ROS generated via Nox4 could diffuse in the cells and function as secondary messengers. The intracellular localization of Nox4 in MSC remains to be determined.

We further demonstrated that the generated ROS induce differentiation via CREB in MSC. CREB has been identified as a transcription factor of adipocyte differentiation in 3T3-L1 preadipocytes (Reusch et al., 2000). Thus, it may also functional in MSC, which are more primitive than preadipocytes. Many CREB targets have already been identified (Shaywitz and Greenberg, 1999). MAPK is one of the candidates for differentiation. MAPK has been shown to induce

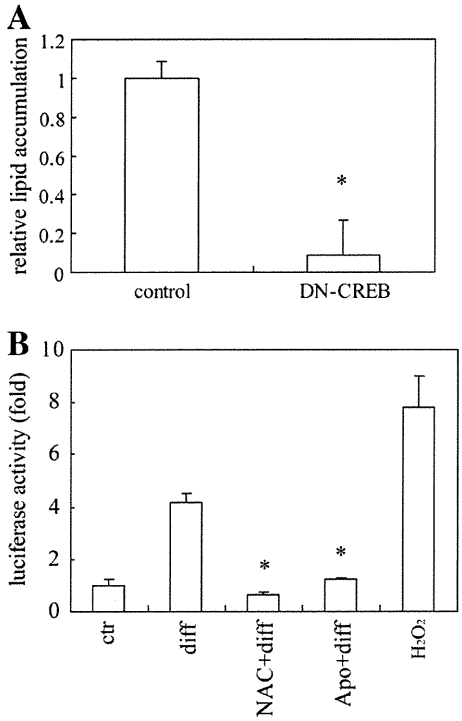

Fig. 4. Effects of antioxidants on transcriptional activation of CREB in 10T1/2 cells. (A) 10T1/2 cells were transfected with dominant-negative mutants of CREB (DN-CREB) or control vector. The cells were incubated with differentiation-inducing agents for 7 days. The cells were fixed and stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance was measured at 518 nm. *P<0.05 vs control. (B) 10T1/2 cells were transiently transfected with a CRE-luciferase construct. After pretreatment with or without ROS scavengers (NAC, 5 mM or Apocynin, 1 μM) for 30 min, the cells were incubated with differentiation-inducing agents or H_2O_2 (1 mM) for 1 h. Cell lysates were then analyzed for luciferase activity. pRL-CMV vector was co-transfected to normalize transfection efficiency. *P<0.05 vs diff.

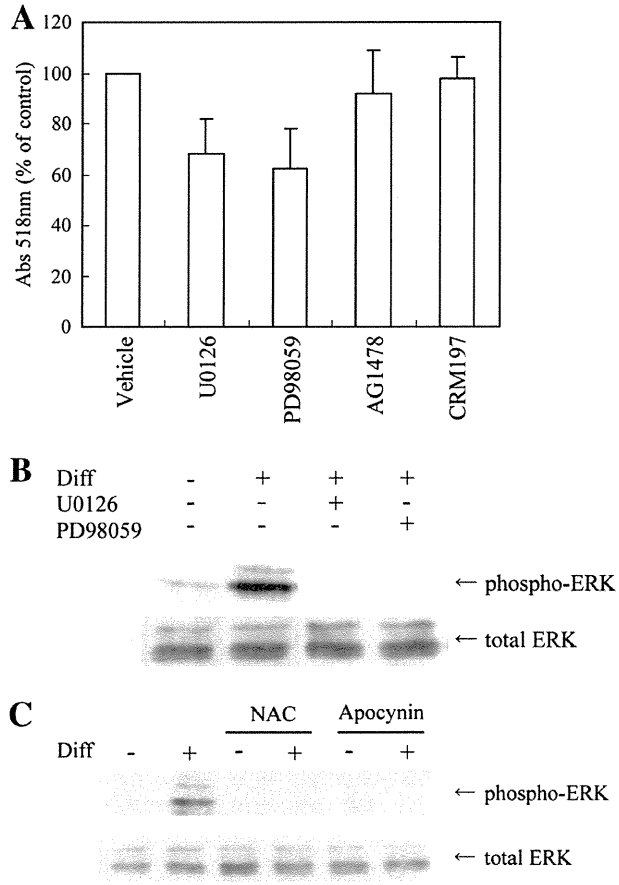

Fig. 5. Effects of kinase inhibitors on adipocyte differentiation in 10T1/2 cells. (A) 10T1/2 cells were pretreated with PD98059 (20 μM), U0126 (1 μM), AG1478 (10 μM), CRM197 (10 μM), or vehicle for 30 min and then incubated with differentiation-inducing agents for 7 days. After the cells were fixed, they were stained with oil red-O. The oil red-O was extracted with isopropanol, and absorbance was measured at 518 nm. (B) 10T1/2 cells were pretreated with MEK inhibitors (PD98059, 20 μM or U0126, 1 μM) or vehicle for 30 min and then treated with differentiation-inducing agents for 10 min. ERK phosphorylation was detected by immunoblotting with the anti-phospho-ERK or anti-ERK antibody. (C) 10T1/2 cells were pretreated with ROS scavengers (NAC, 5 mM or Apocynin, 1 μM) for 30 min or vehicle and then treated with differentiation-inducing agents for 10 min. ERK phosphorylation was analyzed by immunoblotting with the anti-phospho-ERK or anti-ERK antibody.

adipocyte differentiation in 3T3-L1 cells and acts downstream of ROS (Sale et al., 1995). However, its effect is only limited in 10T1/2 cells (Fig. 5). These data suggest the presence of other CREB targets in MSC. PGC-1, a regulator of mitochondrial biogenesis, is another good candidate which acts at a downstream of CREB. Oxidative stress has been shown to activate the PGC-1 promoter via CREB in 10T1/2 cells (St-Pierre et al., 2006). We are currently conducting studies to examine the involvement of PGC-1 in adipocyte differentiation.

In addition to MSC, several reports demonstrate the role of ROS in other kinds of stem cells. For example, Nox1 stimulates RANKL-induced osteoclast differentiation in bone marrow monocyte-macrophage lineage cells (Lee et al., 2005). Ito et al. revealed that ROS determine the lifespan of hematopoietic stem cells (Ito et al., 2006). Thus, stem cell differentiation utilizes the common route of ROS generation, and the NAD(P)H oxidase family appears to be a key regulator in this differentiation. Future studies will determine the common mechanisms by which ROS initiate stem cell differentiation.

Conclusions

We demonstrated that ROS mediate adipocyte differentiation using rat bone marrow-derived mesenchymal stem cells and murine multipotent mesenchymal stem cell line 10T1/2 cells. In addition, we identified that NAD(P)H oxidase family Nox4 is responsible for production of ROS at the early stage of differentiation in 10T1/2 cells. Furthermore, we showed that CREB act downstream of ROS in adipocyte differentiation. Thus, Nox4–ROS–CREB signaling provides a unique pathway to understand the early stage of adipocyte differentiation. The activity of Nox4 is a potential therapeutic target for anti-obesity drug discovery.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Role of α7-Nicotinic Acetylcholine Receptor in Normal and Cancer Stem Cells

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Abstract: The α7-nicotinic acetylcholine receptor (α7-nAChR) is widely known as a neurotransmitter receptor in nervous systems. α7-nAChR is also present in a variety of non-neuronal tissues, where it has been implicated in the regulation of essential cellular functions including proliferation, survival, differentiation and communication. We have recently found in breast cancer that α7-nAChR is involved in the proliferation of cancer stem cells, which constitute a minor subpopulation responsible for tumor development and metastasis. Since growing evidence suggests that α7-nAChR is present not only in mature tissues and organs but also in undifferentiated stem cells and progenitor cells, α7-nAChR emerges as a key mediator in the regulation of self-renewal and differentiation. We provide here an overview of the recent works on the expression and function of α7-nAChR in normal and cancer stem cells, and their relevance to disease-related cellular dysfunction. Understanding the role of α7-nAChR in stem cells would be of great interest for its application potential in drug discovery and in regenerative medicine.

Keywords: α7-nicotinic acetylcholine receptor, breast cancer, cancer stem cell, nicotine, stem cells.

INTRODUCTION

Acetylcholine (ACh) is known as one of the most important neurotransmitters in nervous systems. The effect of ACh is mediated through two types of membrane ACh receptors (AChR): the G protein-coupled muscarinic AChR and the nicotinic AChR (nAChR). nAChR are ligand-gated ion channels and can be activated by nicotine as agonist. They are plasma-membrane receptors composed of a hetero- or homo-pentamer of five subunits enclosing a central ion channel. The nAChR that contains α7 subunits (α7-nAChR) presents the distinctive features to be homomeric and to be selectively permeable to Ca^{2+} [1]. The specific expression and function of α7-nAChR can also be revealed by selective antagonists such as α-Bungarotoxin (α-BTX). α7-nAChR is one of the dominant nAChR in brain and has been implicated in several nervous and cognitive functions of significance in neurodegenerative disorders.

Recent studies have also accumulated evidence that AChR are expressed and functional in a wide diversity of non-neuronal mammalian cells and tissues [2]. α7-nAChR has been demonstrated to be involved in a number of basic cellular processes including proliferation, differentiation, survival and intercellular communication. There is growing interest in modulating the function of AChR, especially of α7-nAChR, for the study and treatment of both neuronal and non-neuronal diseases. It is also especially noteworthy that α7-nAChR has been suggested to be involved in the stimulation of cancer development, which provides a direct link between cancer risk and exposure to nicotine [3, 4]. nAChR were found to be expressed in human lung cancer and to regulate proliferation and apoptosis [5, 6]. Since then, α7-nAChR has been implicated in various cellular processes regulating tumor growth and survival, as reviewed in other chapters of this issue of Current Drug Targets.

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Stem cells are undifferentiated cells defined by two fundamental characteristics. First, they are able to self-renew, that is to sustain themselves for long periods without losing their properties through mitotic cell division. Second, they can be induced under certain conditions to proliferate and differentiate into a variety of mature cells, allowing the maintenance and repair of various tissues throughout life. Increasing evidence also pointed to a relationship between the mechanisms regulating the development of stem cells and those of tumor cells. Cancers are composed of heterogeneous cell populations proliferating uncontrollably with the risk of invading adjacent tissues and of spreading to other tissues by metastasis. The "cancer stem cell hypothesis" stipulates that a minor subpopulation of cells, characterized by their stem cell-like abilities, is able to initiate and maintain new tumors by reconstituting the whole cancer cellular heterogeneity, whereas the remaining bulk of the tumor lacks this potential [7, 8]. Cancer stem cells were first defined in acute myeloid leukemia by the demonstration that only a small subset of hematopoietic stem cell-like population is necessary and sufficient to allow serial leukemic transplantations in immunodeficient mice [9, 10]. Cancer stem cells were identified some years later in human breast cancer [11], followed rapidly by other types of solid tumors [8]. Although the clinical relevance of cancer stem cells still remains an issue and controversies have been raised in some types of tumors such as colon and brain cancers due to the difficulties in accurately defining and targeting the stem cell fraction [12, 13], accumulating evidence in various tissues including breast and lung cancers has demonstrated correlations between enrichment of cancer stem cells and tumor progression stage, enhanced tumorigenic and metastatic potential, and resistance to chemotherapies [8, 13-15].

As α7-nAChR appears implicated in major cellular processes in diverse tissues, considering its relevance to stem cells would be of particular interest to appreciate its role in tissue homeostasis and the consequences of nicotine exposure. Indeed, as summarized in Table **1**, several works have

Table 1. Expression of α7-nAChR in Stem Cells

Stem/Progenitor cell types	Evidence for the presence of α7-nAChR	Suggested function of α7-nAChR	Refs.
Embryonic stem cells			
Mouse ESC	RT-PCR, intracellular Ca^{2+} response	Inhibition of proliferation, survival	[24]
Human ESC	RT-PCR, microarray, immunostaining	Proliferation, differentiation	[21, 22]
P19 embryonic carcinoma	RT-PCR, immunostaining, intracellular Ca^{2+} response	Inhibition of proliferation	[25, 26]
Hematopoietic stem/progenitor cells			
Mouse bone marrow stem cells	Staining with labeled α-BTX, immunostaining	Proliferation, mobilization, communication with niche cells	[36]
Mouse myeloid, erythroid, lymphoid progenitor cells	Staining with labeled α-BTX, effects of α7-nAchR knock-out	Proliferation , survival	[34, 35]
Mesenchymal stem cells			
Human bone marrow stem cells	RT-PCR, immunostaining, intracellular Ca^{2+} response, inhibition by α-BTX	Proliferation, apoptosis, inhibition of chemotaxis	[41, 42]
Neural stem/progenitor cells			
Early embryonic mouse cerebral cortex	Immunostaining, intracellular Ca^{2+} response, membrane potential response	Proliferation, differentiation	[47]
P19-derived progenitor cells	RT-PCR, immunostaining, intracellular Ca^{2+} response	Proliferation, differentiation	[25, 26]
Adult rat hippocampal progenitor cells	RT-PCR, staining with labeled α-BTX	Apoptosis	[51]
Adult mouse hippocampal progenitor cells	Staining with labeled α-BTX, effects of α7-nAchR knock-out	Proliferation, survival	[52,53]
Cancer stem cells			
MCF-7 breast cancer cell line	RT-PCR, inhibition by α-BTX	Proliferation, survival	[76]

reported the expression of α7-nAChR in different categories of stem cells and have suggested its involvement in their regulation. Most interestingly, recent works in our laboratory demonstrated that α7-nAChR is involved in cancer stem cell proliferation in breast cancer, shading a new light on the mechanisms underlying tumor development and progression in presence of nicotinic agonists. Here we review the multiple facets of the expression and function of α7-nAChR in stem cells, including embryonic, hematopoietic, mesenchymal and neural stem cells, as schematically illustrated in Fig. (**1**). Then we focus on the most recent findings in breast cancer stem cells and on the insights these data may provide for understanding disease mechanisms and for developing therapeutic strategies.

EMBRYONIC STEM CELLS

Embryonic stem cells (ESC) are undifferentiated cells originally derived from the inner cell mass of blastocysts. They can be maintained *in vitro* and they are characterized by their pluripotency, meaning that they can be induced to differentiate into any cell lineage of all three embryonic germ layers, as opposed to more restricted somatic stem cells. Since the first successful establishment of human ESC [16, 17], research in this field has been the focus of considerable interest for developmental studies as well as for its huge therapeutic application potential [18].

ESC have been shown to be able to reconstitute a fully functional cholinergic system upon differentiation into ESC-derived neurons, hematopoietic and endothelial cells [19-22]. Interestingly, several members of the cholinergic signaling system, including AChR and enzymes for ACh synthesis (choline acetyltransferase) and degradation (acetyl- and butyryl-cholinesterases), have been demonstrated to be already expressed in undifferentiated ESC [23]. Expression analyses have confirmed the presence of nAChR in mouse ESC [24], undifferentiated human ESC [22] and human embryoid bodies [21]. The type of the nAChR subunits that were detected in ESC depended on the experimental conditions, but α7-nAChR was reliably found to be expressed in all of these studies [21, 22, 24]. Remarkably, α5, α7 and α9-nAChR in human ESC were shown to be significantly higher expressed in undifferentiated ESC than after initiation of differentiation, which suggests the possible importance of these receptors in regulating ESC in its undifferentiated state [22].

The function of nAChR has been demonstrated in mouse ESC by monitoring the increase of intracellular Ca^{2+} concentration in the presence of ACh or nicotine [24]. In contrast, muscarine did not have any effect on intracellular Ca^{2+} [24], suggesting that muscarinic AChR might not be fully functional in mouse ESC despite being transcriptionally expressed [23]. ESC proliferation was found to be decreased by exposure to nicotine [24] and by treatment with a butyryl-cholinesterase inhibitor, which increases the cholinergic signal by inhibiting ACh degradation [23]. In addition, ESC viability was increased by exposure to nicotine [24]. These results suggest that nAChR are involved in modulating ESC

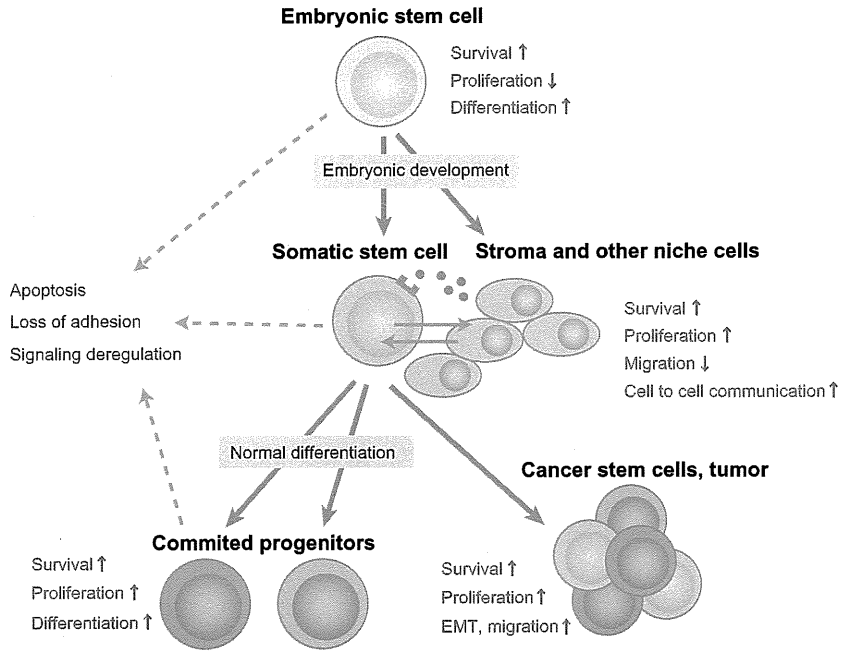

Fig. (1). Overview of the involvement of α7-nAChR in the regulation of essential biological processes in stem cells.

proliferation and promoting survival. In parallel, investigations in mouse P19 embryonic carcinoma cells showed that the nAChR-dependent increase of intracellular Ca^{2+} inhibits proliferation [25, 26]. Indeed, P19 cell proliferation was decreased in a dose-dependent fashion by nicotine exposure but not by muscarine, and the effect was blocked by the nAChR antagonist mecamylamine and the extracellular Ca^{2+} chelatant EGTA [25]. Since α7-nAChR was reported to be the most expressed nAChR in P19 cells [26] and that muscarinic receptors do not appear functional in mouse ESC and P19 cells, it would be tempting to suggest that α7-nAChR is the main AChR involved in the regulation of ESC proliferation and survival.

Since ESC express functional receptors as well as enzymes for the synthesis or degradation of their ligand, these might be required in some local intercellular signal pathway controlling the earliest stages of embryonic development. The concentration of nicotine in the plasma of chronic smokers has been reported to range from 10 nM to 10 μM [27]. It would be of interest to determine if exposure to nicotine in this range of concentrations could perturb the nAChR-mediated signaling system and consequently affect embryonic development *in vivo*. Initiation of embryonic differentiation can be studied to some extent in embryoid bodies, which are formed by aggregation of ESC in spheroid clusters when cultured in non-adherent conditions. Human embryoid bodies have been shown to express nAChR, especially α3 and α7. Their stimulation by nicotine at 10 nM induced a rapid phosphorylation of mitogen-activated protein kinases (MAPK) [21], which activate a highly conserved pathway known to stimulate growth and differentiation. Furthermore, when embryoid bodies were induced to differentiate into hematopoietic lineages, nicotine-treated embryoid bodies contained a higher number of hemangioblasts than non-treated ones. Thus nAChR appear involved in controlling proliferation and cell fate determination at the onset of differentiation. ESC has been also used as a model system to study the adverse effects of prenatal nicotine absorption [28], which might explain some of the pregnancy complications and fetal defects at early stages associated with maternal smoking [29]. Exposure of human ESC to nicotine at doses above 1.8 μM was shown to inhibit cell adhesion, alter cell morphology and induce apoptosis, an effect blocked by addition of the nAChR antagonist d-tubocurarine [28]. Exposure to nicotine was also found to decrease the expression of several known markers of pluripotency including OCT-4 [28], the amount of which should be tightly controlled for proper maintenance of ESC self-renewal and lineage commitment [30].

Thus, the function of α7-nAChR in ESC appears to be important in regulating proliferation, survival, and differentiation, most likely using ACh as one of the signaling molecules orchestrating embryonic development. Exposure to nicotine might directly affect the embryo *in vivo*, since many of these effects were observed at concentrations of nicotine comparable to those in the plasma of cigarette smokers.

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSC) are multipotent cells that are present throughout the adult life to allow the regeneration of blood cells. They are the best characterized somatic stem cells so far, as they can be identified by immunophenotyping and by assaying their ability to reconstitute all blood-cell types, including myeloid and lymphoid lineages, which

allows for instance to entirely repopulate the bone marrow of lethally irradiated animals [31]. Somatic stem cells are known to be maintained in specific cellular microenvironments referred to as niches. The cross-talk of signals between stem cells and niche constituents is believed to be essential for the proper control of their function. HSC are mainly located in the bone-marrow niche [32], where they are maintained in a relatively quiescent state. HSC can also exit the bone marrow by a process of mobilization, migrate through the systemic circulation and reintegrate again the bone marrow niche or facultative niches in other tissues such as the spleen or liver. This system of HSC trafficking and homing to the appropriate niche allows a fast proliferation and distribution for maintenance of blood cell homeostasis and immune surveillance [33].

The role of nAChR in hematopoiesis has been of particular interest because of the concerns about the effects of smoking on cardiovascular diseases and deficits in immune and inflammatory responses. Knockout and chimera mice experiments showed that α4β2 and α7-nAChR are required for survival and proliferation of B lymphocytes precursors [34]. Myeloid and erythroid progenitor cells also express α7-nAChR and the number of progenitor cells was found to be reduced in α7-nAChR knockout mice [35]. Thus, α7-nAChR seems to play a role in all blood-cell lineages at different stages of the hematopoietic development. Regarding undifferentiated HSC, a recent report has demonstrated that HSC purified from mouse bone marrow also express α7-nAChR as shown by binding of labeled α-BTX [36]. Exposure of mice to nicotine has been shown to increase the number of circulating white blood cells, in correlation with an increase of both the frequency and number of HSC in the bone-marrow. This effect might be mediated by a direct action of nicotine via α7-nAChR. On the other hand, nicotine also induced an increase of the plasma levels of soluble Kit ligand (sKitL or stem cell factor), a marker of stem cell activation [36]. This suggests that HSC mobilization and proliferation can be also stimulated by an indirect effect via the production of sKitL by the stroma cells of the HSC supportive niches. Another series of studies has also shown that nicotine induces concerted responses in both HSC and niche stroma cells. Nicotine interfered with HSC homing into the bone marrow by arresting HSC on the endothelium via nAChR [37] and by decreasing the expression of the CD44 adhesion molecule in bone stroma cells [38] and of β2-integrin in HSC [39]. Exposure of mice to nicotine during gestation was also found to affect hematopoiesis and HSC homing during fetal and newborn development, an effect accompanied by a perturbation of the profile of cytokines and chemokines released in the niche, as well as the expression of corresponding receptors in HSC [21].

These results strongly suggest that α7-nAChR and the cholinergic system plays a role in regulating HSC either by acting directly on HSC by nAChR-mediated effects, or by affecting the surrounding niche cells, or most likely by a synergy between both effects. Interfering on the signaling balance by excessive exposure to nicotine might induce disease-related consequences such as fetal and postpartum developmental defects [21], increased number of white blood cells, which has been shown to be a risk factor for heart diseases [36], and pathologic extramedullary hematopoiesis [39].

MESENCHYMAL STEM/STROMAL CELLS

Mesenchymal stem cells (MSC) are multipotent non-hematopoietic stromal cells found especially in the bone marrow and originating from the mesodermal germ layer. They can differentiate into various cell lineages, including osteoblasts, chondrocytes and adipocytes depending on the appropriate stimuli, and they modulate immune and inflammatory responses. Their great potential for clinical applications, by virtue of their relative ease of culture and their capacity to migrate to sites of injury for damage repair, has been the object of intense investigation in recent years [40].

The expression of α7-nAChR in human MSC has been confirmed by flow-cytometry [41] and by confocal microscopy [42]. Other nAChR subunits such as α3, α5, β2 and β4 were also reported to be expressed in MSC [41, 42] but they were not always detected, probably depending on the experimental conditions and the intrinsic heterogeneity of MSC [42]. Human MSC have been shown to respond to nicotine (1μM) by an increased influx of Ca^{2+} and by activation of the MAPK pathway [41]. Further confirmation of the involvement of α7-nAChR has been provided by the observation of a similar increase in MAPK phosphorylation in mouse C3H10T1/2 mesenchymal cells overexpressing α7-nAChR [41]. The activation of the MAPK pathway suggests a role in the regulation of proliferation and differentiation of MSC. However, the exposure of MSC to high concentrations of nicotine over the threshold of 1μM has also been shown to induce cell death by apoptosis [42], so that any putative effect of nicotine on proliferation might be possibly masked by its toxic effect in these experimental conditions. A moderate dose of nicotine (less than 1μM) has been shown to inhibit chemotaxis *in vitro*, which has been confirmed by *in vivo* observations of a defect in the migration of MSC to the bone marrow and spleen of mice injected with nicotine [42]. The induction of apoptosis and the inhibition of chemotaxis were blocked by the specific antagonist α-BTX, which suggests the involvement of α7-nAChR in mediating these effects. These findings could be of importance for the regulation of survival and homing of MSC systemically administrated in therapeutics applications.

NEURAL STEM/PROGENITOR CELLS

Neural stem cells (NSC) are defined by their ability for self-renewal and differentiation into all cell types of the nervous system, including neurons, astrocytes and oligodendrocytes. In the adult mammalian brain, neurogenesis has been demonstrated to occur in the subventricular zone (SVZ) and in the dentate gyrus (DG) of the hippocampus [43]. NSC and their derived progenitor cells can be enriched and propagated *in vitro* by their ability to form floating aggregates called neurospheres [44]. NSC can also be isolated from the developing brain of the embryo, where they proliferate to generate the complete nervous system of the organism. Understanding the control of NSC differentiation by neurotransmitters such as ACh is the focus of growing interest with the prospects for developing cell therapies for neurodevelopmental and neurodegenerative diseases [45]. nAChR are among the major players of neurotransmission and recent advances have shown their involvement in diverse other functions such as neuroprotection and regulation of

neurogenesis, which makes them a target of choice in therapeutic applications against nervous-system disorders [46]. The role of α7-nAChR in the human central nervous system being extensively reviewed in other chapters of this issue of Current Drug Targets, we provide here a summary restricted to the recent findings on its expression and functions in neural stem/progenitor cells.

The involvement of α7-nAChR in the early development of the nervous system has been analyzed by characterizing NSC in embryonic/fetal organs. The early embryonic mouse cerebral cortex at embryonic day 10 (E10) consists of a large majority of neural stem and progenitor cells as identified by stem cell-specific markers [47]. Dissociated cells from the early embryonic mouse cerebral cortex at E10 have been shown to express α3, α4, and α7-nAChR by immunofluorescence staining. The presence of functional receptors that respond to ACh and nicotine has been demonstrated by patch-clamp recordings. The response to nicotine was blocked by the α7-nAChR antagonist methyllycaconitine [47]. Interestingly, higher levels of expression were observed at early embryogenesis (E10) than at later stages (E18). The kinetics of ACh-induced cytosolic Ca^{2+} response showed a faster decay at later stages, suggesting that α7-nAChR is involved in early development and that its function might change with embryonic age [47]. The function of nAChR was also examined in P19 murine embryonic carcinoma cells induced to differentiate into neurons by retinoic acid treatment, which is commonly used as a model of neuronal development. As we have previously described for ESC cells, P19 cells express functional nAChR, which mediate an inhibition of proliferation by nicotine [25, 26]. Remarkably, the expression of several nAChR, including α7-nAChR, decreased in P19-derived neuronal progenitor cells and then increased again in mature neurons [25]. At the same time, muscarinic receptors, which were not functional in undifferentiated embryonic cells, were shown to become functional in progenitor cells. Both nicotinic and muscarinic receptors appeared to stimulate proliferation and differentiation by some mechanism involving Ca^{2+} mobilization from intracellular stores, which was suggested to override the inhibitory effects mediated by nAChR in undifferentiated cells [25]. This is consistent with the idea that the function of nAChR might change depending on the cellular state and on the presence of other factors acting in concert, notably of muscarinic AChR, which have also been reported as regulators of neuronal stem cell proliferation [48, 49].

In the adult central nervous system, several studies reported various effects of nicotine exposure on neural stem and progenitor cells, with some discrepancies regarding the suggested role of α7-nAChR in inducing apoptosis or on the contrary in promoting survival and proliferation. Exposure to nicotine induced a decrease of neurogenesis and cell death in the rat hippocampal DG *in vivo* [50], as well as in experiments using isolated primary progenitor cells or the rat HC2S2 immortalized hippocampal progenitor cells [51]. The expression of α7-nAChR was confirmed in hippocampal cells and the induction of apoptosis in undifferentiated progenitor cells was blocked by α-BTX and dependent on the increase of intracellular Ca^{2+} concentrations [51]. In contrast, apoptosis was not induced in differentiated HC2S2 cells, despite their expression of α7-nAChR. The greater sensitivity of undifferentiated progenitor cells to calcium toxicity was suggested to be due to the lack of calbindin D28K, which is expressed at much higher levels in differentiated HC2S2 cells and may provide a protective effect by calcium buffering [51].

As opposed to the induction of apoptosis, a protective effect of nicotine has been described in the mouse DG and the rat SVZ. α7-nAChR was found to be expressed in the neuronal progenitor cells of the mouse DG and in a subpopulation of progenitor cells derived from the SVZ [52, 53]. In the DG, neural progenitor cell survival was shown to be promoted when intracellular ACh was increased by a long-term treatment of mice with the cholinesterase inhibitor donepezil [53]. Mice heterozygous for α7-nAChR also showed a reduced cell proliferation in the DG, suggesting that the expression levels of α7-nAChR might be important in regulating proliferation [52]. Nicotine-induced proliferation of progenitor cells was also reported in the rat SVZ. [54]. The positive effect on proliferation was accompanied by an increase in the local expression of fibroblast growth factor (FGF-2) and of the corresponding FGF receptor (FGFR-1). A proposed model is that nicotine promotes the creation of a micro-environment favorable to proliferation in the SVZ by stimulating the release of growth factors in the stem cell niche and the coordinated expression of the appropriate receptors on neural progenitor cells [54].

Thus, the response of neural stem and progenitor cells to nicotine appears to differ depending on the cell type, the tissue localization, the animal species and the experimental conditions including the concentrations and the mode of administration of nicotine. Further investigation would be necessary to determine the extent of the contribution of α7-nAChR in stem cell proliferation and survival in the DG and SVZ.

CANCER STEM CELLS

Cancer stem cells (CSC) have been proposed to constitute a specific cellular subpopulation that is responsible for tumor progression and metastasis. Like somatic stem cells, CSC can be selected on the basis of a combination of cell surface markers such as $CD44^{+}/CD24^{-}$ in breast cancer [11] and $CD133^{+}$ initially used in brain [55]. Aldehyde dehydrogenase (ALDH), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, is another marker with increased activity in stem cells, which has been successfully used in recent years to identify a subpopulation enriched in CSC and correlated to a poor prognosis in breast and lung cancer patients [56, 57], increased metastasis [58, 59] and chemotherapy resistance [60]. The demonstration of CSC properties can be performed by *in vivo* observation of tumor growth after serial transplantation of a small amount of selected cells. CSC population can also be characterized by the selective formation of spheres [61], reminiscent of neurospheres formed by NSC, when cultured *in vitro* in non-adherent conditions. The maintenance of CSC has been shown to depend on their stem cell-like capacity for self-renewal, and major developmental signaling pathways such as Notch, Hedgehog and Wnt have been implicated in their viability and proliferation [62-64]. Notably, regarding breast cancer, overexpression of Notch in transgenic mice was shown to promote mammary tumor development [65],

aberrant Notch activation was reported in various subtypes of breast carcinoma [66] and the coexpression of Notch-1 and one of its ligand Jagged-1 was correlated with poor prognosis [67].

A number of studies have provided evidence that nicotine, although not carcinogenic in itself, can stimulate the development and progression of cancer. Research on the effects of nicotine has been especially active in the field of lung cancers, which could be initiated by other carcinogens of tobacco smoke, then maintained and stimulated by exposure to nicotine [68]. Breast cancer risk has been also associated to tobacco smoking by epidemiologic studies [69, 70] and clinical reports indicating that smoking enhance breast cancer mortality and metastasis from breast to lung [71, 72]. However, it is only recently that experimental data have suggested direct links between breast cancer and nicotine exposure. Nicotine has thus been shown in human MCF-7 breast cancer cells to induce a resistance to the anti-cancer drug doxorubicin [73] and to promote their migration by activation of protein kinase C (PKC) [74]. Nicotine also promotes proliferation, invasion and epithelial-to-mesenchymal transition (EMT), which is a process associated to the enhancement of tumor cell migration and metastasis, by calcium and Src dependent pathways in various cell lines expressing α7-nAChR, including the human MCF-7 and MDA-MB-468 breast cancer cells [75].

In a recent report from our laboratory, we have demonstrated for the first time that nicotine increases breast CSC population via α7-nAChR [76]. A possible model for the process inferred from our results is illustrated in Fig. (**2**). We showed that exposure to nicotine, at concentrations comparable to those reported in the plasma of cigarette smokers, increased in a dose-dependent manner both the frequency and the absolute number of CSC identified as the

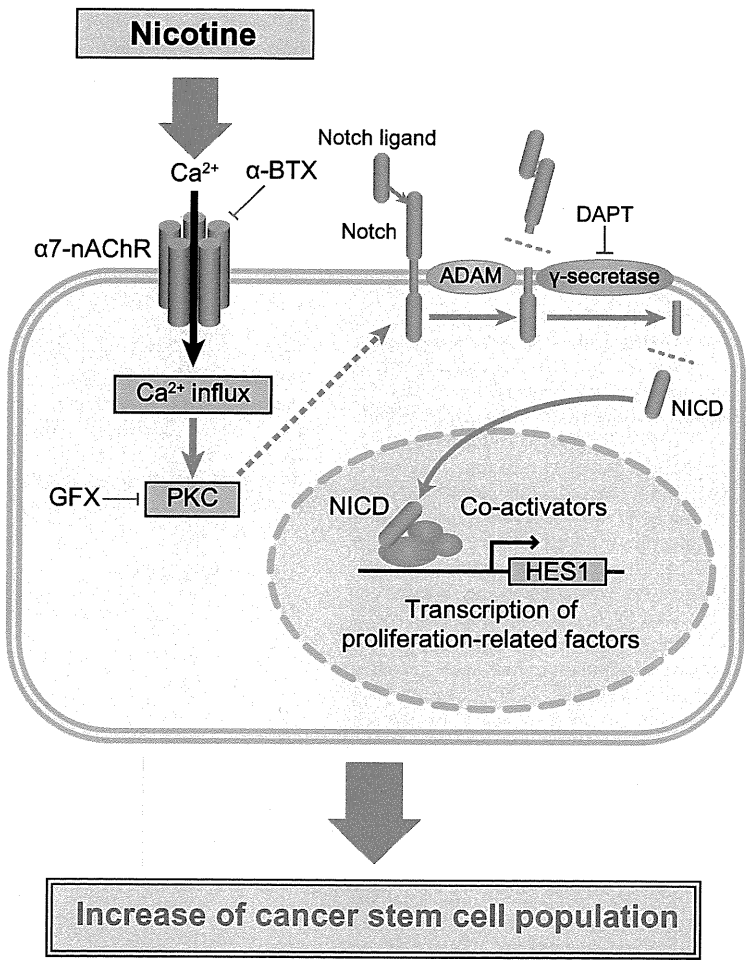

Fig. (2). Proposed model for cancer stem cell proliferation via α7-nAChR. The binding of nicotine to α7-nAChR induces an increase of the intracellular Ca^{2+} influx and the consequent activation of calcium-dependent signaling molecules such as protein kinase C (PKC) and activate the Notch signaling pathway. In the presence of notch ligands, Notch is cleaved by ADAM and by γ-secretase to release the Notch intracellular domain (NICD). NICD translocates to the nucleus and activates the transcription of downstream regulatory factors such as HES1, thereby promoting stem-cell proliferation and self-renewal. The inhibitors α-BTX, DAPT and GFX allow to target specific molecules of this pathway as indicated.

ALDH-positive fraction in MCF-7 cells. The increase of a population with stem-cell like properties was confirmed by observing a corresponding increase in mammosphere formation. The proliferation of ALDH-positive cells was blocked by α-BTX, suggesting that the effect was dependent on α7-nAChR. The increase of the ALDH-positive population was also reproduced when the MCF-7 cells were treated with the α7-nAChR-specific agonist PHA543613 (PHA). We confirmed the expression of α7-nAChR by RT-PCR in non-treated MCF-7 cells [76] and in both ALDH-positive and negative populations (unpublished data). Furthermore, we investigated the involvement of stem-cell related pathways, and we showed that nicotine induced an expression of the Notch target gene HES1. The increase in HES1 expression was blocked by DAPT, which prevents Notch signaling by inhibiting the cleavage of activated Notch receptor by γ-secretase. The nicotine-induced increase of ALDH-positive population was blocked by DAPT and by the PKC inhibitor GF109203X (GFX). This suggests that the Notch-Hes1 pathway is involved in the increase of CSC population and that it acts downstream of α7-nAChR and PKC. The mechanisms underlying the α7-nAChR-mediated activation of Notch have not been fully investigated yet, but PKC activity has been shown to induce the membrane translocation in glioblastoma of the ADAM protease [77], which is required for the cleavage of Notch receptors. CSC proliferation might be therefore induced by the upregulation of Notch signaling via the α7-nAChR-mediated PKC activation.

CSC maintenance and proliferation have been correlated to EMT [78], activation of anti-apoptotic pathways [79, 80] and increase of chemoresistance [60]. Our results indicating the involvement of CSC in nicotine-dependent breast carcinogenesis appear therefore in concordance with the previously mentioned other studies showing various effects of nicotine in breast cancer [73-75]. Our results also suggest that α7-nAChR is the main receptor controlling CSC self-renewal, although we do not exclude the contribution of other nAChR subunits, since it was reported that in some breast cancer cell lines, nicotine-induced tumor growth could be mediated by α9-nAChR [81]. It is noteworthy that α7-nAChR has been shown to be expressed in many different types of cancer cells in various tissues, including in particular non-small cell lung carcinoma (NSCLC) [3]. Tumorigenic ALDH-positive population has been recently identified and related to poor prognosis in lung cancers [57] and its maintenance shown to be dependent on Notch signaling in NSCLC [82]. α7-nAChR is already being considered as a target for the treatment of NSCLC and mesothelioma [83, 84]. Therefore, it would be also of great interest to consider the effects of α7-nAChR on CSC populations in other types of tumor such as NSCLC. The finding that α7-nAChR might be involved in CSC maintenance and proliferation sheds a new light on the effects of nicotine on tumor progression and opens the way to the development of new therapies to eradicate CSC by targeting α7-nAChR or the downstream signaling pathways.

CONCLUSIONS

A growing body of evidence suggests that α7-nAChR plays a key role in the regulation of stem cells. As summarized in Fig. (**1**), current data point to the prominent involvement of α7-nAChR in the control of maintenance, proliferation, and stem cell fate determination. Cellular signals mediated by α7-nAChR have been shown to promote survival and to limit proliferation of undifferentiated ESC, whereas they stimulate proliferation and differentiation of more developed somatic stem cells including hematopoietic and neuronal progenitors. Several works reviewed in this issue demonstrated that in the presence of an appropriate agonist, namely Ach in physiological conditions, α7-nAChR activates developmental signaling pathways such as MAPK and Notch. α7-nAChR-mediated effects also appeared to depend on the state of the cell itself, including the presence or absence of other receptors and factors modulating the cytosolic Ca^{2+} response. Thus, α7-nAChR possibly plays a central role in integrating intra- and extra-cellular information through the cholinergic signaling to coordinate stem cell maintenance and differentiation. To this effect, α7-nAChR has also been shown to enhance the cross-talk between stem cells and their niche to finely tune cellular development, as best demonstrated in experiments on hematopoietic or neural progenitor cells treated with nicotine *in vivo*. This also implies that stem cells are sensitive to environmental change through α7-nAChR, and in several experimental systems, the disruption of the signal balance was shown to induce detrimental effects such as apoptosis or anomalous differentiation and migration. Finally, the involvement of α7-nAChR in the promotion of stem cell maintenance and proliferation is of particular significance with regard to CSC in breast cancer, as this might explain nicotine-induced tumor progression and metastasis. The finding that α7-nAChR plays a major role in the regulation of various types of stem cells provides important insights for the prospects of developing new therapeutic strategies. The wide distribution of α7-nAChR in the organism and their involvement in a variety of functions imply that the risks for unexpected side effects must be carefully assessed. A precise evaluation of risk factors and of drug delivery systems would be required to validate α7-nAChR as target in therapeutic applications.

ABBREVIATIONS

ACh	=	Acetylcholine
AChR	=	Acetylcholine receptor
ALDH	=	Aldehyde dehydrogenase
α-BTX	=	α-bungarotoxin
CSC	=	Cancer stem cells
DG	=	Dentate gyrus
EMT	=	Epithelial-to-mesenchymal transitions
ESC	=	Embryonic stem cells
GFX	=	GF109203X
HSC	=	Hematopoietic stem cells
MAPK	=	Mitogen-activated protein kinases
MSC	=	Mesenchymal stem cells

nAChR	=	Nicotinic acetylcholine receptor
NSC	=	Neural stem cells
NSCLC	=	Non-small cell lung carcinoma
PHA	=	PHA543613
PKC	=	Protein kinase C
RT-PCR	=	Reverse transcription polymerase chain reaction
sKitL	=	Soluble Kit ligand
SVZ	=	Subventricular zone.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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