Reactive Oxygen Species Generated by NADPH Oxidase 2 and 4 Are Required for Chondrogenic Differentiation*

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Although generation of reactive oxygen species (ROS) by NADPH oxidases (Nox) is thought to be important for signal transduction in nonphagocytic cells, little is known of the role ROS plays in chondrogenesis. We therefore examined the possible contribution of ROS generation to chondrogenesis using both ATDC5 cells and primary chondrocytes derived from mouse embryos. The intracellular level of ROS was increased during the differentiation process, which was then blocked by treatment with the ROS scavenger N-acetylcysteine. Expression of Nox1 and Nox2 was increased upon differentiation of ATDC5 cells and primary mouse chondrocytes, whereas that of Nox4, which was relatively high initially, was decreased gradually during chondrogenesis. In developing limb, Nox1 and Nox2 were highly expressed in prehypertrophic and hypertrophic chondrocytes. However, Nox4 was highly expressed in proliferating chondrocytes and prehypertrophic chondrocytes. Depletion of Nox2 or Nox4 expression by RNA interference blocked both ROS generation and differentiation of ATDC5 cells, whereas depletion of Nox1 had no such effect. We also found that ATDC5 cells depleted of Nox2 or Nox4 underwent apoptosis. Further, inhibition of Akt phosphorylation along with subsequent activation of ERK was observed in the cells. Finally, depletion of Nox2 or Nox4 inhibited the accumulation of proteoglycan in primary chondrocytes. Taken together, our data suggest that ROS generated by Nox2 or Nox4 are essential for survival and differentiation in the early stage of chondrogenesis.

ROS are generated in cells by several pathways. The major sources of intracellular ROS production include mitochondria, various metabolic and detoxifying enzymes, and Nox. Nox isoforms generate ROS in a variety of cells and tissues in response to stimulation with various growth factors or cytokines (9). To date, Nox isoforms include Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 (10). Although they share structural features, each member of the Nox family is thought to play a specific biological role depending on the type of cell or tissue (11, 12). The classic Nox isoform Nox2 (also known as gp91phox) was first identified in phagocytes (8, 13). Nox isoforms including Nox2 have been shown to be expressed in a wide variety of nonphagocytic as well as phagocytic cells and to function in signal transduction (8, 11).

The receptor activator of nuclear factor-κB ligand (RANKL) induces osteoclast differentiation in a manner dependent on Nox-derived ROS (14, 15). The production of superoxide by Nox4 in osteoclasts also contributes to bone resorption (16). Furthermore, intracellular ROS were found to inhibit the proliferation of immature chondrocytes and promote the induction of chondrocyte hypertrophy in cells derived from ataxia telangiectasia mutated knock-out mice, whereas ROS produced by the up-regulation of Nox1 expression were found to be associated with chondrocyte differentiation (17).

The role of ROS generation in chondrogenesis remains to be fully characterized. We show here that intracellular ROS generated by Nox2 and Nox4 are required for differentiation of ATDC5 cells and primary chondrocytes.

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[3] The abbreviations used are: ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DPI, diphenyleneiodonium chloride; NAC, N-acetylcysteine; Nox, NADPH oxidase; qPCR, quantitative PCR.
EXPERIMENTAL PROCEDURES

ATDC5 Cell Culture—ATDC5 cells were obtained from the Riken Cell Bank (Tsukuba Science City, Japan) and were cultured in a 1:1 (v/v) mixture of DMEM and F-12 (Invitrogen) medium supplemented with 5% FBS (Invitrogen), human transferrin (10 µg/ml; Sigma), and 3 x 10^-8 M sodium selenite (Sigma), as described previously (18). The initial cell density was 6 x 10^4 cells/well in six-well plates (Nunc, Rockilde, Denmark). For induction of differentiation, confluent cells (culture day 0) were cultured in medium supplemented with bovine insulin (10 µg/ml; Sigma). The cells were maintained at 37 °C under a humidified atmosphere of 5% CO2, and the medium was replaced every other day.

Staining with Alcian Blue—The cells were stained with Alcian Blue 8G (Sigma) as described previously (19). Images of stained cells were recorded using Olympus CKX41. Stained cells were extracted with 6 M guanidine hydrochloride. The absorbance of the extracted dye was measured at 655 nm.

Isolation of RNA and RT-PCR Analysis—Total RNA was extracted from cells with TRIzol reagent (Invitrogen), and 1 µg portions of RNA were subjected to RT-PCR using oligo(dT) primer and SuperScript® III reverse transcriptase (Invitrogen). RT-PCR analysis of the resulting cDNA preparation was performed using a PCR Premix, Sapphire (Super Bio Co., Seoul, Korea), and the following primers (forward and reverse, respectively): Col2a1, 5'-AGG CAA GAG C-3', 5'-CAA TAA TGG GAA GCC CCA CTC TG-3'; Col1a1, 5'-CAT CAG AAG AAC GGA AAA C-3', 5'-GCT TCA CCA TGT TTT GCG CCC CAC ATG TA-3'; p22phox, 5'-TCA CAC CCC TCC TTC ACC TCC ACC TCT GCC GAT GTG ATG AGA GAA CAA GGA CCA GC-3'; 5'-CCG GCT TGT CTT CA-3'; Nox1, 5'-GTG CAA CAC ACG GGT ACC ACC ACC CTG GAG TGG GTG TAG TG-3'; Nox2, 5'-ATT GGG CCG TGC CCG CCA CAG GAT GGT TAT TCC ATG ACC TTA-3'; Nox4, 5'-CCC AGG GCC ACC ACT GCT TTT TTT TGT TTT GCC CCC CAC ATG TA-3'; siRNA-2, 5'-AGG AAU UUG GUG UUG AUA A for Nox2; and siRNA-1, 5'-AUA AUU ACA CCA GGA AAG GCC GAA CAA GGA AAG GCC CAA-3'. The abundance of each mRNA was normalized using a corresponding amount of 18S rRNA, and the normalized values were then expressed relative to that of the control sample. All of the reactions were performed in triplicate.

RNA Interference—Stealth siRNAs specific to mouse Nox1, Nox2, or Nox4 were obtained from Invitrogen; their sequences were: siRNA-1, GCU AUG GAG UUU UAC CGC AGG AAU U, and siRNA-2, GCA ACA UUG CUC GUC AUG CAG CAU U for Nox1; siRNA-1, GAA ACU ACC UAA GAU AGC AGU UGA U, and siRNA-2, GGC CUA AGU AUA AUU AUA CUC GAA for Nox2; and siRNA-1, AAG CAU UUG GUG UCC ACU AUU A, and siRNA-2, GGA GAA CAA GAA GGU UGG AUA A for Nox4. A Stealth negative control siRNA was also obtained from Invitrogen. ATDC5 cells were plated in 60-mm dishes and maintained in a 1:1 (v/v) mixture of DMEM/F-12 supplemented with 5% FBS and antibiotics until ~60% confluence was achieved. The cells were then transfected with siRNAs (100 nM) for 6 h using Lipofectamine 2000 (Invitrogen) in OPTI-MEM medium (Invitrogen). The medium was then replaced with normal culture medium.

Immunoblot Analysis—The proteins were isolated from ATDC5 cells and primary chondrocytes. The cells were then lysed as described previously (20). The following primary antibodies were used in this study: collagen type II (Chemicon, Temecula, CA), N-cadherin (Sigma), Sox9 (Chemicon), phosphorylated Akt1, Akt, ERK, phosphorylated ERK, cleaved caspase 3 (Cell Signaling Technology, Inc., Danvers, MA), p22phox (Santa Cruz Biotechnology, Santa Cruz, CA), Nox1, Nox2, Nox4 (kind gifts from Y. S. Bae), actin, vimentin, fibronectin, and β-actin (Sigma). The protein bands were detected by enhanced chemiluminescence reagents (Thermo Fisher Scientific Inc., Waltham, MA).

ROS Measurement—Intracellular ROS concentration was measured using 2',7'-dichlorofluorescin diacetate (DCFH-DA) (21). The fluorescence intensity of DCF was measured using a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Apoptosis Assay—For quantification of apoptosis, a FITC-conjugated annexin V and propidium iodide assay (BD Biosciences) was performed according to the manufacturer’s instructions. Analysis of FITC and propidium iodide fluorescence intensities was performed with a FACScan flow cytometer. A total of 10,000 events/sample was acquired.

Immunohistochemistry—For histological examination, the samples were obtained from the forelimbs of CD1 mouse (Orient Bio, Sung-Nam, Korea) embryos at 16.5 days postcoitum. The samples were immediately fixed in 4% (w/v) paraformaldehyde, dehydrated with graded ethanol, and embedded in paraffin. Tissue samples were cut into 5-µm-thick slices. According to the manufacturer’s protocol (CHEMICON IHC Select® immunoperoxidase secondary detection system), the sections were treated with sodium citrate buffer for antigen retrieval, followed by protein blocking to reduce nonspecific binding. The sections were then immunostained with antibodies (Nox1, Nox2, Nox4, and collagen type II in 2% BSA). Control was immunostained with control rabbit IgG in 2% BSA. After immunostaining, the sections were counterstained with hematoxylin. The sections were stained with 0.1% safranin O and 0.03% Fast Green as previously described.
cells were treated with H2O2, the expression of chondrogenic differentiation of ATDC5 cells. When ATDC5 were blocked by NAC. Together, these results suggest that ROS increased the mRNA expression of collagen type II (Col2a1), collagen type X (Col10a1), and aggrecan (Agc1), which are major chondrogenic marker genes, in a time-dependent manner (Fig. 1B). The effects of insulin on these genes were also blocked by NAC. Together, these results suggest that ROS generated in response to insulin stimulation were required for chondrogenic differentiation of ATDC5 cells. When ATDC5 cells were treated with H2O2, the expression of Sox9, Col2a1, and Col10a1 was also increased in a time-dependent manner for 48 h (supplemental Fig. S1). This phenomenon shows that additional ROS indeed accelerated chondrogenesis. This result is consistent with that of a previous report (17). We next measured the intracellular level of ROS during insulin-induced chondrogenesis in ATDC5 cells. Flow cytometric analysis of cells loaded with the ROS-sensitive indicator DCFH-DA revealed that the intracellular ROS concentration increased within 2 min of cell exposure to insulin (data not shown) and remained high throughout chondrogenesis, even after 10 days (Fig. 1C). To identify the source of ROS produced in response to insulin, we examined the effects of various inhibitors. Similar to NAC, diphenyleneiodonium chloride (DPI), a flavoprotein inhibitor, and apocynin, a Nox inhibitor (9), blocked the insulin-induced accumulation of ROS. In contrast, rotenone and antimycin, which are inhibitors of mitochondrial complexes I and III, respectively (25), had no such effect (Fig. 1D). Although rotenone reduced the basal level of ROS, the generation of ROS by insulin treatment was not affected. These results suggest that Nox was the source of ROS generated in association with insulin-induced chondrogenesis in ATDC5 cells.

Differential Expression of Nox during Differentiation of ATDC5 Cells and Primary Chondrocytes—To determine which members of the Nox family might be responsible for insulin-induced ROS generation in ATDC5 cells, we examined the abundance of various Nox mRNAs by RT-PCR analysis. The genes for Nox1, Nox2, and Nox4, as well as those for the cytosolic components Rac1, p40phox, p47phox, and p67phox were expressed in ATDC5 cells (Fig. 2A). In contrast, expression of Nox3 was only slightly expressed in cultures at day 10, and Nox5 was not detected at all (data not shown). Nox5 was previously shown to not be expressed in mouse (9, 26).

The mRNA levels of Nox1, Nox2, p22phox, p40phox, p47phox, and p67phox were increased during insulin-induced differentiation of ATDC5 cells, with Rac1 slightly increased (Fig. 2A). In contrast, the amount of Nox4 mRNA, which was relatively high at day 0, was decreased in association with chondrogenic differentiation. These changes in Nox1, Nox2, and Nox4 gene expression were confirmed by real-time qPCR analysis (Fig. 2C). The protein expression of Nox1, Nox2, Nox4, and p22phox was also analyzed by immunoblotting (Fig. 2B).

Using a micromass culture system, we also examined the mRNA expression of Nox during chondrogenesis. Primary chondrocytes derived from mouse embryonic limb buds were prepared as described under “Experimental Procedures.” RT-PCR analysis revealed that the mRNA levels of Nox1 and Nox2 were increased in association with chondrogenesis. However, the expression of Nox4 was decreased during chondrogenesis (Fig. 2D). The protein expression of Nox1, Nox2, and Nox4 was determined by immunoblotting (Fig. 2E). These results are consistent with those obtained using ATDC5 cells.

Expression of Nox1, Nox2, and Nox4 in Developing Cartilage—To examine how Nox is expressed in chondrocytes in vivo, we carried out immunostaining of forelimbs isolated from mouse E16.5 using antibodies against Nox1, Nox2, or Nox4. As a positive control, collagen type II was also determined. To distinguish morphologically distinct chondrocyte populations, the sections were stained with safranin O/Fast Green. Consistent with previous reports (27, 28), proliferating chondrocytes, prehypertrophic chondrocytes, and hypertrophic chondrocytes were detectable in the limbs at this stage (Fig. 3A). Collagen type II was detected in proliferating and prehypertrophic chondrocytes (Fig. 3B). Both Nox1 and Nox2 were detected in prehypertrophic and hypertrophic chondrocytes (Fig. 3, D and E). In the case of Nox1, it seems to have been slightly more expressed in prehypertrophic chondrocytes than in hypertrophic chondrocytes. The Nox4 signal was more strongly detected in proliferating chondrocytes and prehypertrophic chondrocytes than in hypertrophic chondrocytes (Fig. 3F). Further, expression of Nox was de-

**RESULTS**

**ROS Generation Is Accompanied by Insulin-induced ATDC5 Cell Differentiation**—To induce chondrogenesis, confluent ATDC5 cells (day 0) were exposed to insulin. The cells were cultured up to 10 days and then stained with Alcian Blue to detect the accumulation of sulfated proteoglycans as a marker of chondrogenic differentiation (24). Exposure of cells to insulin resulted in a time-dependent increase in the intensity of Alcian Blue staining. However, this effect of insulin was blocked in the presence of N-acetylcysteine (NAC), a ROS scavenger (Fig. 1A). RT-PCR analysis revealed that insulin increased the mRNA expression of collagen type II (Col2a1), collagen type X (Col10a1), and aggrecan (Agc1), which are major chondrogenic marker genes, in a time-dependent manner (Fig. 1B). The effects of insulin on these genes were also blocked by NAC. Together, these results suggest that ROS generated in response to insulin stimulation were required for chondrogenic differentiation of ATDC5 cells. When ATDC5 cells were treated with H2O2, the expression of Sox9, Col2a1, and Col10a1 was also increased in a time-dependent manner for 48 h (supplemental Fig. S1). This phenomenon shows that additional ROS indeed accelerated chondrogenesis. This result is consistent with that of a previous report (17). We next measured the intracellular level of ROS during insulin-induced chondrogenesis in ATDC5 cells. Flow cytometric analysis of cells loaded with the ROS-sensitive indicator DCFH-DA revealed that the intracellular ROS concentration increased within 2 min of cell exposure to insulin (data not shown) and remained high throughout chondrogenesis, even after 10 days (Fig. 1C). To identify the source of ROS produced in response to insulin, we examined the effects of various inhibitors. Similar to NAC, diphenyleneiodonium chloride (DPI), a flavoprotein inhibitor, and apocynin, a Nox inhibitor (9), blocked the insulin-induced accumulation of ROS. In contrast, rotenone and antimycin, which are inhibitors of mitochondrial complexes I and III, respectively (25), had no such effect (Fig. 1D). Although rotenone reduced the basal level of ROS, the generation of ROS by insulin treatment was not affected. These results suggest that Nox was the source of ROS generated in association with insulin-induced chondrogenesis in ATDC5 cells.

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detected in the perichondrium (27), surrounded by a layer of fibroblast-like cells.

Nox2 and Nox4 Are Required for ROS Generation in ATDC5 Cells—To investigate the relative roles of Nox1, Nox2, and Nox4 during the chondrogenic differentiation of ATDC5 cells, we transiently introduced two different types of siRNA for each enzyme into the cells. The expression of siRNA-1 and siRNA-2 was reduced by over 50% as demonstrated by RT-PCR and immunoblotting. (Fig. 4, A and B). However, siRNA-2 of each enzyme was more effective, which prompted us to use siRNA-2 in the following experiments. We then performed FACS analysis after knockdown of Nox genes. Whereas cells transfected with control or Nox1 siRNAs experienced a marked increase in intracellular ROS levels 2 days after insulin exposure, those transfected with Nox2 or Nox4 siRNAs did not (Fig. 4C). Furthermore, the basal ROS level in cells transfected with Nox4 siRNA was reduced by 75% compared with that in cells transfected with control siRNA. However, the basal ROS level was not affected by knockdown of Nox2, probably because of the presence of Nox4. As shown in Fig. 2, Nox4 was highly expressed in non-stimulated cells, but Nox2 was not. This suggests that Nox4 was largely responsible for basal ROS production in ATDC5 cells. In Nox4 knockdown cells at day 2, the ROS level was increased over 2-fold compared with that at day 0 because of the presence of Nox2. However, the ROS level was not sufficient for differentiation. Further, the ROS level was not increased in Nox2 knockdown cells at day 2 compared with that at day 0. At day 2, the level of Nox4 had decreased by over 50% (Fig. 2C). Therefore, Nox4 might not be able to overcome
the decrease in ROS by Nox2 knockdown. These results indicate that Nox2 and Nox4, but not Nox1, worked cooperatively for ROS generation by changing their expression during the early phase of insulin-induced differentiation of ATDC5 cells.

**Depletion of Either Nox2 or Nox4 Induces ATDC5 Cell Apoptosis—**Nox2- or Nox4-depleted ATDC5 cells appeared to be dying during chondrogenesis (data not shown). To examine whether or not these cells underwent apoptosis, the cells were stained with FITC-annexin V and propidium iodide. Upon insulin exposure for 2 days, the frequency of apoptosis was markedly increased in ATDC5 cells depleted of Nox2 or Nox4 compared with cells transfected with control or Nox1 siRNAs (Fig. 5A). Western blot analysis revealed that expression of cleaved caspase 3 was increased by Nox2 or Nox4 knockdown in differentiated ATDC5 cells (Fig. 5B). We next tested whether or not activation of the protein kinases Akt and ERK is related to the apoptosis observed in cells depleted of Nox2 or Nox4. Intracellular MAPKs and Akt are the major oxidative stress-sensitive signal transduction pathways (29). Akt is a key regulator of cell survival in response to growth factor stimulation (30) and promotes matrix synthesis and chondrocyte survival (31). We thus examined the phosphorylation of Akt on Ser374. Immunoblot analysis revealed that phosphorylation of Akt was greatly diminished in insulin-treated cells depleted of Nox2 or Nox4 compared with that in insulin-treated cells transfected with control or Nox1 siRNAs.

**FIGURE 2. Differential expression of Nox during differentiation of ATDC5 cells and mouse primary chondrocytes.** A and C, ATDC5 cells were cultured in the presence of insulin for up to 10 days, after which the expression of Nox1, Nox2, Nox4, Rac1, p22phox, p40phox, p47phox, p67phox, and 18S was examined by RT-PCR (A) or real time qPCR analysis (C). Quantitative data are the means ± S.E. from three independent experiments. Significant differences are compared with day 0 (***, p < 0.001; *, p < 0.05). B, lysates of cells cultured for the indicated times were subjected to immunoblot analysis with antibodies against Nox1, Nox2, Nox4, p22phox, and α-tubulin. D, primary chondrocytes were obtained from mouse embryos at 11.5 days postcoitum and cultured for up to 9 days. Total RNA isolated from primary chondrocytes was subjected to RT-PCR analysis for Nox1, Nox2, Nox4, and β-actin mRNA. The data are representative of three independent experiments. E, lysates of cells cultured for the indicated times were subjected to immunoblot analysis with antibodies against Nox1, Nox2, Nox4, and β-actin.

**FIGURE 3. Immunohistochemical analysis of Nox expression in limbs from mouse embryos.** Sections of ulna and radius from mouse embryos at 16.5 days postcoitum were immunostained (brown) with antibodies against rabbit IgG (A), collagen type II (B), Nox1 (D), Nox2 (E), and Nox4 (F), respectively. Sections were stained with safranin O/Fast Green (C) and then counterstained with hematoxylin (purple). P, proliferating chondrocytes; PH, prehypertrophic chondrocytes; H, hypertrophic chondrocytes. Bar, 50 μm.
According to previous reports (31–33), the balance between phosphatidylinositol 3-kinase-Akt and MEK-ERK activity plays a vital role in chondrocytes. Interestingly, phosphorylation of ERK in cells transfected with Nox2 or Nox4 siRNA was increased compared with that in cells transfected with control or Nox1 siRNA. These results suggest that ROS generation by Nox2 or Nox4 plays a role in the survival of ATDC5 cells.

Nox2 and Nox4 Are Required for Differentiation in ATDC5 Cells and Primary Chondrocytes—The roles of Nox2 and Nox4 in chondrogenesis were further confirmed by determining the expression of chondrogenic marker proteins in Nox1-, Nox2-, or Nox4-depleted cells. First, ATDC5 cells were transfected with control, Nox1, Nox2, or Nox4 siRNA and then exposed to insulin for 2 days for differentiation. The specific depletion of each Nox enzyme by its corresponding siRNA was verified by RT-PCR and Western blot analysis. Further, the expression of marker proteins such as Sox9, collagen type II, and N-cadherin was decreased in cells depleted of Nox2 or Nox4. In contrast, depletion of Nox1 did not inhibit differentiation but rather appeared to promote it (Fig. 6, A and B).

As described above, Nox2 and Nox4 play an important role in the differentiation of ATDC5 cells. To further confirm the role of Nox2 and Nox4 in chondrogenesis, we also introduced the same siRNAs used in ATDC5 cells into primary chondrocytes by RNAi, as described under “Experimental Procedures.” The depletion of Nox2 or Nox4 was analyzed by RT-PCR and immunoblotting. Expression of marker proteins, Sox9, and collagen type II was diminished in Nox2- or Nox4-depleted cells (Fig. 6, C and D).

To exclude off-target effects, we investigated the effects of differentiation using siRNA-1 and siRNA-2 of both Nox2 and Nox4 in ATDC5 cells and primary cells. We performed real-time qPCR analysis for expression of Col2a1 mRNA. Both siRNA-1 and -2 inhibited ~50% of the expression of Col2a1 (Fig. 6E). As shown in Fig. 6F, knockdown of two different types of Nox2 or Nox4 reduced the accumulation of proteoglycan by over 50% (Fig. 6F, left panel) in a micromass culture of primary chondrocytes at day 3. The accumulated proteoglycan was stained with Alcian Blue, which was then extracted and measured for its absorbance at 655 nm for quantification (Fig. 6F, right panel).

Sox9 is a master regulator of cartilage formation (1, 34). Adhesion molecules such as N-cadherin are important for the condensation of chondroprogenitor mesenchymal cells (35, 36). These results show that Nox2 and Nox4 were important...
FIGURE 6. Differentiation of ATDC5 and primary chondrocytes depleted of Nox. A, ATDC5 cells transfected with control, Nox1, Nox2, or Nox4 siRNAs for 48 h were cultured in the presence of insulin for 2 days. Total RNA isolated from cells was subjected to RT-PCR analysis for Nox1, Nox2, Nox4, Col2a1, and β-actin mRNA. All of the data are representative of three independent experiments. B, lysates of cells treated as in A were subjected to immunoblot analysis with antibodies against N-cadherin, collagen type II (Col II), Sox9, Nox1, Nox2, Nox4, and β-actin. C, primary chondrocytes were transfected with control, Nox2, or Nox4 siRNAs for 6 h and then cultured in differentiation medium for 3 days. Total RNA isolated from the cells was subjected to RT-PCR analysis for Sox9, Col2a1, Nox2, Nox4, and 18S mRNA. D, lysates of cells treated as in C were subjected to immunoblot analysis with antibodies against Sox9, collagen type II, Nox2, Nox4, and α-tubulin. E, ATDC5 cells and primary chondrocytes were transfected with Nox2 siRNA-1 or -2 or Nox4 siRNA-1 or -2 and then cultured as in A and C. Expression of Col2a1 was analyzed by real time qPCR. Quantitative data are the means ± S.E. from three independent experiments. Significant differences are compared with control siRNA (***, p < 0.001; *, p < 0.05). F, primary chondrocytes were treated as in E and stained with Alcian Blue (left panel). Bar, 200 μm. The dye was extracted from stained cells and measured at 655 nm (right panel). Significant differences are compared with control siRNA treatment (***, p < 0.001; **, p < 0.01).
for the condensation and differentiation of ATDC5 cells and primary chondrocytes.

DISCUSSION

We investigated the association of ROS generated by Nox and chondrogenesis in ATDC5 cells. Among Nox family members, Nox1, Nox2, and Nox4 were found to be expressed in ATDC5 cells during chondrogenesis (Fig. 2). Depletion of either Nox2 or Nox4, but not that of Nox1, inhibited ROS generation, suppressed differentiation, and induced apoptosis in ATDC5 cells (Figs. 5 and 6). These results suggest that ROS generation by Nox2 and Nox4 was required for chondrogenic differentiation of ATDC5 cells.

A previous study on ATDC5 cells revealed a role for ROS in chondrocyte hypertrophy and showed that induction of chondrogenic differentiation is associated with the up-regulation of Nox1 gene expression (17). We have now shown the expression of Nox1 along with that of Nox2 and Nox4 in ATDC5 cells as well as in primary chondrocytes isolated from mouse embryonic limb buds (Fig. 2). Using RNA interference, we also found that Nox2 and Nox4 were required in the early phase of chondrogenic differentiation in ATDC5 cells. The level of intracellular ROS was increased early and maintained during ATDC5 cell differentiation. The expression of the Nox1 and Nox2 genes was increased as differentiation progressed, whereas that of the Nox4 gene, which was initially high, was gradually decreased during differentiation (Fig. 2). By immunohistochemical analysis of developing limbs obtained from mouse embryos, we found that both Nox1 and Nox2 were strongly expressed in prehypertrophic and hypertrophic chondrocytes, whereas the Nox4 signal was observed in proliferating chondrocytes and prehypertrophic chondrocytes (Fig. 3). We also found that Nox2 and Nox4 were important for the condensation and differentiation of primary chondrocytes derived from mouse embryos (Fig. 6).

Nox1 did not appear to contribute to ROS generation during the early phase of ATDC5 cell differentiation, suggesting that the level of intracellular ROS was maintained by Nox2 and Nox4 and that the relative importance of these enzymes increases and decreases, respectively, with changes in their relative levels of expression. It was recently shown that Nox2 and Nox4 each compensate for the deficiency of the other in lung endothelial cells (37).

Down-regulation of Nox4 expression is associated with adipocyte differentiation (38), and Nox4 has been proposed to act as a switch that controls the transition between proliferation and differentiation of preadipocytes (39). Our results now suggest that increasing the expression of Nox1 and Nox2 and decreasing the expression of Nox4 are characteristics of chondrogenesis. Depletion of Nox2 or Nox4 resulted in the down-regulation of Sox9 and collagen type II expression in ATDC5 and primary chondrocytes (Fig. 6). Sox9 is an essential chondrogenic transcription factor, and N-cadherin is an important downstream target of Sox9 that modulates cell adhesion (40). Signaling by Rac1, a member of the Rho family of small GTPases, is also thought to regulate N-cadherin expression during chondrogenesis (20). Rac1 is also an important cytosolic activator of Nox enzymes (9, 12). Although our results suggest that Nox1 does not contribute to the early phase of differentiation in ATDC5 cells, observations that Nox1 expression gradually increased as differentiation progressed suggest that Nox1 may play a role in the later stages of differentiation.

We found that apoptosis was induced in ATDC5 cells depleted of Nox2 or Nox4 (Fig. 5). Apoptosis, a redox-regulated event, is triggered by disruption of the extracellular matrix in adherent cells. Matrix-cell contacts result in the generation of ROS as well as activation of pro-survival signaling (41). Nox2- or Nox4-depleted cells also did not undergo condensation or differentiation. Suppression of ROS generation by depletion of Nox2 or Nox4 inhibited the insulin-induced phosphorylation of Akt. Further, ERK was phosphorylated in Nox2 or Nox4 knockdown cells. It is reported that a balance between phosphatidylinositol 3-kinase-Akt and MEK-ERK activity regulates chondrocyte matrix synthesis, and this balance is modulated by ROS (33). ROS generated by Nox have been shown to be important for the survival, proliferation, and differentiation of various cell types, including pancreatic adenocarcinoma, glioma, endothelial, vascular smooth muscle, and embryonic stem cells (37, 42–46).

Although no bone abnormalities have been reported in Nox1−/− or Nox2−/− mice (14, 47, 48), it is clear that Nox-derived ROS are important for chondrogenesis. Nox2 and Nox4 were expressed in converse fashion (Fig. 2), but knockdown of each exerted a dramatic reductive effect on ROS generation even after 2 days of chondrogenesis (Fig. 4). This critical effect of Nox2 and Nox4 was also observed in the suppression of apoptosis (Fig. 5). However, expression of marker proteins was more reduced in Nox4 knockdown cells than in Nox2 knockdown cells (Fig. 6). This implies that Nox4 was important for the basal state, whereas Nox2 was important for differentiation. Thus, both Nox2 and Nox4 worked cooperatively for ROS generation by changing their expression. In summary, our findings suggest that ROS generated by both Nox2 and Nox4 played key roles in the early stage of chondrogenic differentiation in ATDC5 cells and primary chondrocytes.

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