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Post-mitotic role of nucleostemin as a promoter of skeletal muscle cell differentiation

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ABSTRACT

Nucleostemin (NS) is a nucleolar protein abundantly expressed in a variety of proliferating cells and undifferentiated cells. Its known functions include cell cycle regulation and the control of pre-rRNA processing. It also has been proposed that NS has an additional role in undifferentiated cells due to its downregulation during stem cell differentiation and its upregulation during tissue regeneration. Here, however, we demonstrate that skeletal muscle cell differentiation has a unique expression profile of NS in that it is continuously expressed during differentiation. NS was expressed at similar levels in non-proliferating muscle stem cells (satellite cells), rapidly proliferating precursor cells (myoblasts) and post-mitotic terminally differentiated cells (myotubes and myofibers). The sustained expression of NS during terminal differentiation is necessary to support increased protein synthesis during this process. Downregulation of NS inhibited differentiation of myoblasts to myotubes, accompanied by striking downregulation of key myogenic transcription factors, such as myogenin and MyoD. In contrast, upregulation of NS inhibited proliferation and promoted muscle differentiation in a p53-dependent manner. Our findings provide evidence that NS has an unexpected role in post-mitotic terminal differentiation. Importantly, these findings also indicate that, contrary to suggestions in the literature, the expression of NS cannot always be used as a reliable indicator for undifferentiated cells or proliferating cells.

Introduction

Nucleostemin (NS) is a nucleolar protein that regulates cell proliferation [1,2]. Up- and downregulation of NS result in cell cycle arrest, primarily through interactions with the tumor suppressor protein p53. Several reports have also suggested that NS might serve as a marker for an undifferentiated or dedifferentiating state. NS is highly expressed in neural stem cells, embryonic stem cells and cardiac stem cells and downregulated during differentiation [2,3]. The downregulated NS can be upregulated in the regions surrounding cardiac infarction sites. NS level is also downregulated during the differentiation of bone marrow stem cells during their differentiation into chondrocytes, adipocytes or osteocytes [4]. During regeneration of a newt lens, NS accumulates in the dedifferentiating pigmented epithelial cells two days before they reenter the cell cycle [5]. Additionally, NS is expressed in degenerating multinucleated muscle fibers during limb regeneration in a newt before formation of the blastema, a multipotent stem cell-like population [5]. However, we have recently found that NS has another function as a regulator of pre-rRNA processing and consequently ribosome synthesis [6]. This finding prompted us to hypothesize that NS might be also expressed in non-proliferating cells, including terminally differentiated cells, as long as they are actively synthesizing proteins. We tested this hypothesis using the differentiation process of skeletal muscle cells as an experimental model.

Myogenic stem cells, called satellite cells [7], are mitotically quiescent in adult muscle and their protein synthesis level is relatively low. However, they will initiate proliferation and enhance protein synthesis upon stimulation by weight bearing or through damage. The progeny of activated satellite cells, now called myoblasts, undergo multiple rounds of cell division prior to terminal differentiation and formation of multinucleated myotubes by cell fusion [7]. Nuclei in myotubes are generally post-mitotic. During maturation myotubes continuously enlarge through additional cell fusion as well as increased cytoplasmic volume per nucleus, result-

Abbreviations: APC, allophycocyanin; C_T, threshold cycle; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle Medium; dpc, days post coitum; FACS, fluorescent activated cell sorting; FBS, fetal bovine serum; FGF, fibroblast growth factor; FSC, forward scatter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCK, muscle creatine kinase; MHC, sarcomeric myosin heavy chain; NS, nucleostemin; PBS, phosphate buffered saline; PE, phycoerythrin; qRT-PCR, quantitative reverse transcription PCR; SEM, standard error of the mean; SSC, side scatter.

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ing in functional myofibers with the capability of contraction. Myotube formation and myofiber maturation are characterized by a striking increase in protein synthesis. For instance, chicken embryonic myotubes increase their protein amount per nucleus more than tenfold in ten days [8]. Mouse myotubes derived from C2C12 cells increase their protein synthesis rate fourfold in two days [9]. Thus, skeletal myogenesis provides an experimental model in which a radical increase of protein synthesis can be separated from cell proliferation. Taking advantage of this feature in skeletal muscle cells, in the current work we uncovered a unique expression pattern of NS and its role during skeletal myogenesis.

Materials and methods

Myoblast culture and preparation of muscle sections. $p53^{+/-}$ mice, homozygous dystrophin gene mutant ($DMD^{mdx\text{-}5Cv}$) mice and wild-type BALB/c and C57BL/6 mice were purchased from Jackson Laboratory. $p53^{-/-}$ mice were generated by crossing $p53^{+/-}$ parent mice. Satellite cell-derived myoblasts were isolated from the hind limbs of two-month-old wild-type BALB/c mice, $p53^{-/-}$ mice and $DMD^{mdx\text{-}5Cv}$ mice [10]. The myoblasts were maintained in collagen-coated dishes in myoblast growth medium consisting of HAM's F-10 medium supplemented with 20% fetal bovine serum (FBS) and 5 ng/ml basic fibroblast growth factor (FGF) (R&D Systems). To induce differentiation of myoblasts, the culture medium was replaced with differentiation medium that contained Dulbecco's Modified Eagle Medium (DMEM) with 5% horse serum on Day 0. The cells were harvested on Day 0 (before switching to the differentiation medium), 1, and 3 for Western blotting and immunostaining. Frozen sections were prepared from the tibialis anterior muscle of fetal and two-month-old C57BL/6 mice and $DMD^{mdx\text{-}5Cv}$ mice. The protein amount in the Day 0, 1, and 3 cells was measured with a Quant-iT Protein Assay kit (Invitrogen). Single myofibers were isolated from the extensor digitorum longus muscle prepared from one to two-month-old wild-type BALB/c mice by digestion as previously described [11].

Fluorescent activated cell sorting (FACS). Satellite cells were isolated from the hind limb skeletal muscle of one to two-month-old mice [12]. The sources of the antibodies are listed in Supplementary Table 1. Sorting gates were strictly defined based on single antibody-stained control cells, as well as the forward scatter (FSC) and side scatter (SSC) patterns of satellite cells. After the FSC/SSC gating the triple-negative cells for CD45- phycoerythrin (PE), CD31-PE and Sca-1-PE were gated out. Lastly, double-positive cells for integrin α7-Alexa 488 and integrin β1-APC were sorted to enrich for satellite cells [13].

Knockdown of NS. Myoblasts were transfected with NS or control siRNA using Lipofectamine 2000 (Invitrogen) on Day −3, −2, and −1 while cultured in the growth medium. The culture medium was replaced with the differentiation medium on Day 0 and the cells were harvested on Day 0 (before exposed to the differentiation medium), 1, and 3 for immunostaining and Western blotting. The sequences of NS siRNA and control siRNA were described before [6].

Metabolic labeling of protein with ^{35}S methionine. The abovementioned Day 0 myoblasts were washed and cultured in methionine-free DMEM containing 5% dialyzed FBS for 1 h. After addition of L-[^{35}S] methionine (GE Healthcare) to a final concentration of 15 μCi/ml, the cells were incubated for an additional 4 h. Whole cell extracts prepared from these cells were resolved by SDS–PAGE at 2×10^5 nuclei equivalent per well and analyzed by autoradiography of dried gels.

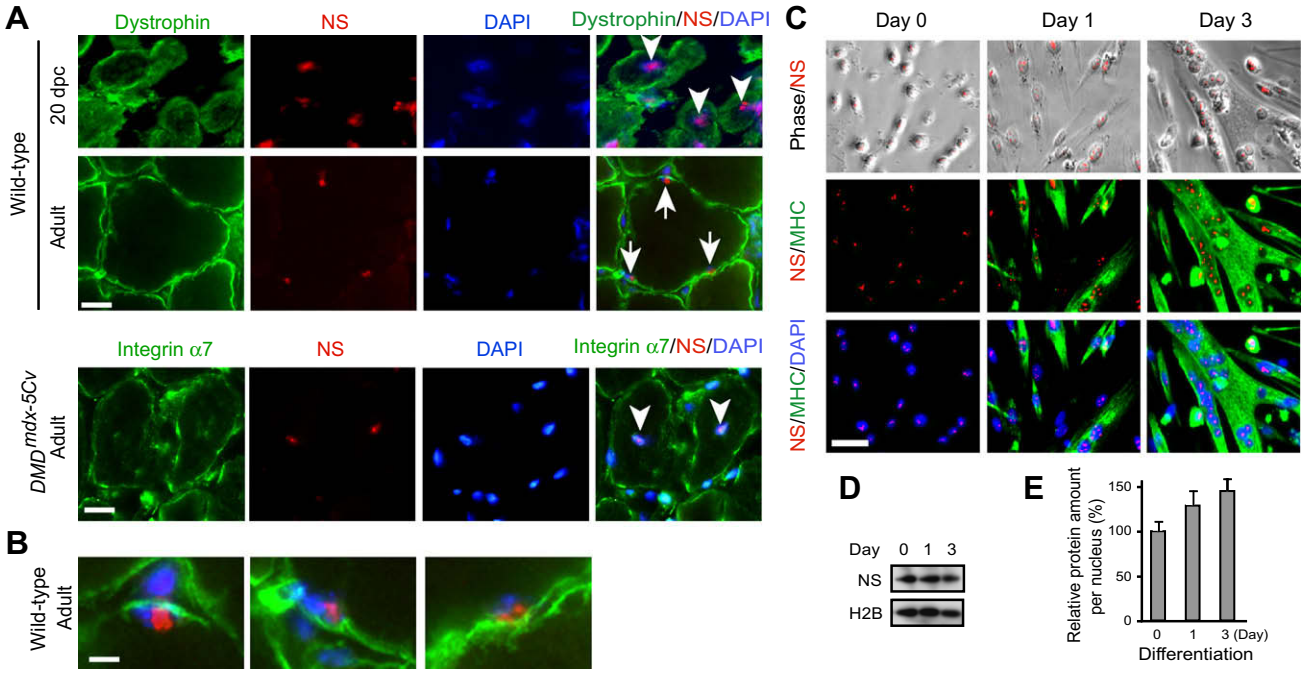

Fig. 1. Expression of NS during differentiation of skeletal muscle cells. (A) and (B) Immunofluorescence staining of sections prepared from the tibialis anterior muscle of 20 dpc and adult wild-type mice and an adult $DMD^{mdx\text{-}5Cv}$ mouse. The sections were double immunostained with a combination of anti-dystrophin antibody and anti-NS antibody or with a combination of anti-integrin α7 antibody and anti-NS antibody. DNA was counterstained with DAPI. Arrowheads indicate NS signals in centrally located nuclei in myofibers. The areas denoted by arrows in the adult wild-type mouse section were enlarged threefold in (B) to demonstrate the presence of NS-positive nuclei inside the plasma membrane of myofibers. Bar, 30 μm in (A) and 10 μm in (B). (C) Expression pattern of NS during differentiation of mouse myoblasts *in vitro*. Bar, 40 μm. (D) Myoblasts (Day 0) and myotubes on Day 1 and 3 were applied for Western blotting with anti-NS antibody. Histone H2B was monitored as a loading control. Whole cell extract corresponding to 2×10^5 nuclei was loaded in each well. (E) The total amount of cellular protein was compared among the muscle cells harvested on Day 0, 1, and 3. Protein amount in 1×10^6 muscle cells was divided by the number of DAPI-stained nuclei to determine the normalized protein amount per nucleus. The relative protein amount per nucleus on each day was calculated by using the normalized protein amount in the Day 0 cells as 100%. Results obtained from three sets of experiments are presented as mean + SEM.