Balance between S-nitrosylation and denitrosylation modulates myoblast proliferation independently of soluble guanylyl cyclase activation

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ABSTRACT

Nitric oxide (NO) contributes to myogenesis by regulating the transition between myoblast proliferation and fusion through cGMP signaling. NO can form S-nitrosothiols (RSNO), which control signaling pathways in many different cell types. However, neither the role of RSNO content nor its regulation by the denitrosylase activity of S-nitrosoglutathione reductase (GSNOR) during myogenesis is understood. Here, we used primary cultures of chick embryonic skeletal muscle cells to investigate whether changes in intracellular RSNO alter proliferation and fusion of myoblasts in the presence and absence of cGMP. Cultures were grown to fuse most of the myoblasts into myotubes, with and without S-nitrosocysteine (CysNO), 8-Br-cGMP, DETA-NO, or inhibitors for NO synthase (NOS), GSNOR, soluble guanylyl cyclase (sGC), or a combination of these, followed by analysis of GSNOR activity, protein expression, RSNO, cGMP, and cell morphology. Although the activity of GSNOR increased progressively over 72 h, inhibiting GSNOR (by GSNOR inhibitor – GSNORi – or by knocking down GSNOR with siRNA) produced an increase in RSNO and in the number of myoblasts and fibroblasts, accompanied by a decrease in myoblast fusion index. This was also detected with CysNO supplementation. Enhanced myoblast number was proportional to GSNOR inhibition. Effects of the GSNORi and GSNOR knockdown were blunted by NOS inhibition, suggesting their dependence on NO synthesis. Interestingly, GSNORi and GSNOR knockdown reversed the attenuated proliferation obtained with sGC inhibition in myoblasts, but not in fibroblasts. Hence myoblast proliferation is enhanced by increasing RSNO, and regulated by GSNOR activity, independently of cGMP production and signaling.
INTRODUCTION

Myogenesis is the process of muscle fiber formation, which involves the commitment of cells from the paraxial mesoderm, followed by mononucleated myoblast proliferation, differentiation, and fusion, culminating in formation of mature multinucleated myotubes (26). This process is controlled by a complex network of signal transduction pathways that are regulated by the intracellular redox environment (16). Among the free radicals that can alter the intracellular redox state, nitric oxide (NO) has a pivotal role in the proliferation and differentiation of endothelial cells (40), HeLa cells (3), fibroblasts (36), hepatocytes (15), satellite cells (20), primary culture of myoblasts (52), and in ovo myogenesis (12). Particularly in myoblasts, the mechanisms by which NO acts during the transition between myoblast proliferation and fusion have been reported to be regulated by a transient production of cGMP (27).

NO is a messenger molecule involved in several physiological and pathophysiological pathways. In skeletal muscle, NO can be produced by the three known nitric oxide synthase (NOS) isoforms, but the neuronal NOS isoform (NOS1) is the major source of NO, particularly during contractions (42). There are several targets for NO and its derivatives in cells, such as the soluble guanylate cyclase (sGC; which is responsible for enhancing the intracellular cGMP production), and cysteine thiol groups. The latter are targets of a process known as S-nitrosylation, which can modify either low-molecular-weight molecules or proteins, thereby producing low-molecular-weight S-nitrosothiols (lmw-RSNO) or S-nitrosoproteins (Ptn-SNO), respectively.
The most abundant low-molecular-weight thiol in cells is GSH, which can react with NO (or its derivatives), forming S-nitrosoglutathione (GSNO) (32). It has been proposed that the main mechanism of protein S-nitrosylation \textit{in vivo} is the transfer of the nitroso group from GSNO to protein thiols (23), but the level of protein S-nitrosylation depends on the finely tuned equilibrium between S-nitrosylation and denitrosylation (i.e., the removal of the nitroso group from thiols), which has been suggested to regulate several intracellular signaling pathways. There are several different enzymes able to denitrosylate S-nitrosothiols (RSNO), known as denitrosylases (4). Among those, the NADH-dependent S-nitrosoglutathione reductase (GSNOR) has been shown to be an important denitrosylase, one that can directly control the intracellular levels of GSNO, and ultimately the levels of Ptn-SNO (4).

Recently, the function of intracellular levels of RSNO during skeletal muscle development was tested in animals when the gene for GSNOR was ablated (35). GSNOR deficiency in adult mice increased intracellular Ptn-SNO, concomitant with less muscular mass, altered mitochondrial morphology, decreased signals for muscle regeneration and higher signaling for muscle atrophy compared to wild-type mice (35). Although these authors have demonstrated the consequences for muscle phenotype when there is dysregulation between S-nitrosylation and denitrosylation, little is known about the importance of this balance, particularly the role of GSNOR, during myogenesis.

In this study, we tested the hypothesis that denitrosylation by GSNOR plays an important role in balancing the stimulatory roles of NO during \textit{in vitro} proliferation and differentiation of chick embryonic skeletal muscle cells in primary cultures, composed primarily of myoblasts and
fibroblasts. We also tested whether the effects of changes in intracellular S-nitrosothiols on myoblast proliferation are due to sGC activation.
MATERIALS AND METHODS

Reagents

GSH, GMP, GDP, GTP, cGMP, paraformaldehyde, penicillin-streptomycin, HgCl₂, EDTA, Tris, DMSO, NADH, N-nitro-L-arginine methyl ester hydrochloride (L-NAME), sildenafil citrate salt, 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and a protease inhibitor cocktail (Cat#04693116001) were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaNO₂ was purchased from ACROS (Pittsburgh, PA, USA). Diethylenetriamine NONOate (DETA-NO) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). 3,4-Dimethoxyphenylglyoxal hydrate (DMPG, Cat# L20279) was from Alfa Aesar (Ward Hill, MA). GSNOR inhibitor (3-[1-(4-acetyl-phenyl)-5-phenyl-1H-pyrrole-2-yl]propanoic acid, also called C3 (47)) (GSNORi) was purchased from Chemical Diversity Research Institute (ChemDiv, Moscow Region, Khimki, Russia). This compound (GSNORi) was identified by Sanghani et al. (47) as GSNOR-specific, and it has been used systematically to inhibit GSNOR in vitro (11, 22) as well as in vivo (19, 30). Odyssey blocking buffer (Cat#927-40003) was from LI-COR (Lincoln, NE, USA). All cell culture reagents were from Invitrogen (São Paulo, Brazil).

Antibodies and probes

Rabbit polyclonal anti-desmin (Cat# D-8281, 1:50) and mouse monoclonal anti-α–actinin (Cat# A-7811, 1:50) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-α-tubulin (Cat# 05-829, 1:1,000) was purchased from Millipore (Temecula, CA, USA), polyclonal anti-GSNOR (Cat# 11051-1-AP, 1:800) from Proteintech Group (Chicago, IL,
USA), polyclonal anti-NOS1 (Cat# 8309, 1:1,000) from Santa Cruz Biotechnology (Dallas, Texas, USA), anti-IgG from mouse (Cat# 610-113-121, 1:10,000) or rabbit (Cat# 611-113-122, 1:10,000) were obtained from Rockland (Gilbertsville, PA, USA), Alexa Fluor 488-goat anti-mouse IgG (Cat# A-11029, 1:100) and Alexa Fluor 546-goat anti-rabbit IgG antibodies (Cat# A11035, 1:100), and DNA-binding probe DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Cat# D-3571, 1:2,000) were purchased from Molecular Probes (USA).

**Cell cultures**

All experimental procedures with chicken embryos were approved by the Ethics Committee for Animal Care and Use in Scientific Research from the Federal University of Rio de Janeiro (Protocol#: DAHEICB 004). Primary cultures of myogenic cells were prepared from breast muscles of 11-day-old chick embryos (34). Cells were grown in 2 ml of MEM (Minimum Essential Medium) supplemented with 10% horse serum, 0.5% chick embryo extract, 1% L-glutamine and 1% penicillin-streptomycin, at an initial density of 7.5 x 10^5 cells/35 mm culture dishes on 22-mm aclar plastic coverslips (Pro-Plastics Inc., USA) previously coated with rat tail collagen. Cells were grown in a humidified 5% CO₂ atmosphere at 37°C and were fed daily with fresh culture medium. Myogenic cultures were harvested after 24, 48 and 72 h.

**Cell culture treatments**

For experiments shown in Figures 2 to 5, cells were grown for 24 h, followed by 48 h with either DMSO (vehicle), or 1, 5, or 10 µM GSNORi (solubilized in DMSO), or 1 mM L-NAME (NOS inhibitor, solubilized in H₂O) + DMSO, or 10 µM GSNORi with 1 mM L-NAME. The medium was changed every 24 h with treatment addition. The final concentration of DMSO
for all groups was 0.16%. A control group without vehicle was included to evaluate whether
0.16% DMSO could affect cell culture viability and proliferation.

In another group of experiments (Fig. 6), primary cultures of myogenic cells were grown
for 24 h, followed by treatment with 100 μM CysNO during an additional 48 h (72 h total).
CysNO was added every 24 h when medium was changed. There was no DMSO present in these
experiments.

In the experiments of Figures 7 to 8, cells were grown for 24 h, followed by 48 h in the
presence of either DMSO (vehicle), or 10 μM DETA-NO (NO donor, solubilized in alkaline
H₂O), or 10 μM 8-Br-cGMP (a cell permeable cGMP analog, solubilized in H₂O), or 10 μM
GSNORi, or 10 μM ODQ (sGC inhibitor, solubilized in DMSO), 100 μM CysNO, or a
combination of these. When cells were treated with either ODQ + 8-Br-cGMP, ODQ + GSNORi
or ODQ + CysNO, there was a 10 min incubation with ODQ in order to inactivate sGC prior to
8-Br-cGMP, GSNORi or CysNO addition (50). All groups contained 0.32% DMSO. There was
also a control group without vehicle. The medium was changed every 24 h with treatment
addition.

In the experiments which GSNOR was knocked down (Fig. 10-12), cells were grown for
24 h, followed by transfection with different siRNA sequences to target GSNOR or to target
eGFP (see details below) in the absence and presence of 10 μM ODQ or 1 mM L-NAME, for an
additional 24 h (48 h total). Also, separate samples were treated with 10 μM GSNORi or 0.16%
DMSO (vehicle) instead of siRNA transfection for 24 h. All samples contained 0.16% DMSO.

**GSNOR knockdown**
Design and selection of all siRNA sequences, specific for GSNOR of *Gallus gallus* (mRNA sequence obtained from NCBI Reference Sequence: NM_001031152.2) were performed as described in Possidonio et al (43). For control siRNA (non-specific), we decided to target the enhanced Green Fluorescent Protein (eGFP), also used in (43). The three sequences of siRNA that were selected to target GSNOR, and the sequence to target eGFP are shown in Table 1. They were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) as duplex siRNAs.

For the siRNA transfection procedure, primary cultures of skeletal muscle cells from chick embryos were initially plated at a density of 1 x 10^5 cells/well in a 24-well plate, onto 22 mm Aclar plastic coverslips which were previously coated with rat-tail collagen. After 24 hours, cultures were transfected with either 5, 50, or 100 nM total siRNA for GSNOR or eGFP, previously diluted in siRNA medium (minimum essential medium, 10% horse serum, 1% L-glutamine; with no antibiotics and no chick embryo extract, final volume of 175 µL) and incubated for 5 min. In separate tubes, 3 µL Lipofectamine 3000 transfection reagent (Invitrogen, Cat# L3000015) was diluted into 12 µL siRNA medium and incubated for an additional 5 min. The tubes containing siRNA and lipofectamine were mixed (final volume of 190 µL), incubated for 20 min, diluted with 800 µL of siRNA medium (final volume of ~1 mL) and divided equally between two wells containing cells (each siRNA sequence and concentration was transfected in duplicate). Cells were cultured for the next 24 h prior to analysis.

**MTT assay in cell cultures**

In order to detect cytoplasmic soluble and mitochondrial-bound oxidoreductase activities (5), which are commonly used as a measure of cell viability (21), cells were cultured for 24 h in 94-well plates (7.5 x 10^5 cells), followed by 48 h treatments with the compounds mentioned
above. The medium was removed and cells were incubated with MTT (dissolved in 0.5% PBS (w/v)) for 2 h at 37°C in a humidified chamber. After MTT incubation, supernatants were discarded, and 100% DMSO (v/v) was added to each well to dissolve the intracellular formazan crystals formed during the reduction of MTT by the oxidoreductases. Absorbance was measured at 550 nm. Each sample was analyzed in quintuplicate.

**Synthesis of S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO)**

GSNO and CysNO were prepared immediately before each experiment by combining equimolar concentration of GSH or cysteine (acidified in 0.1 N HCl) and NaNO₂ (38). After 2 min, reaction was stopped by adding 50 mM HEPES and titrating the solution with KOH to pH 7.0.

**GSNOR activity in cell lysates**

The activity of GSNOR was determined by measuring the NADH-dependent GSNO consumption in fresh cell lysates, according to Liu et al. (28) with some modifications. Initially, cell cultures at different time-points of cell plating were lysed using 20 mM Tris-HCl pH 8, 0.5 mM K₂EDTA, 0.1% NP-40, 1 mM PMSF and 1% protease inhibitor cocktail (Cat#04693116001, Sigma-Aldrich), and centrifuged at 1,000 x g for 3 min at 4°C. The lysate supernatant (0.3 mg/ml) was incubated with 300 µM NADH and 300 µM GSNO in 20 mM Tris-HCl pH 8, 0.5 mM K₂EDTA, for 2.5, 5, 10, and 20 min in absence or presence of 10 µM GSNORi at room temperature. Reaction was interrupted by precipitating protein with 10% trichloroacetic acid (TCA) and remaining GSNO was measured by the absorbance at 340 nm. In order to convert the changes in absorbance at 340 nm to GSNO concentration, a standard curve of GSNO (0-200
µM) was performed in the same experimental buffer + 10% TCA, but without cell lysate. Each experiment was analyzed in triplicate.

Measurement of S-nitrosothiols

Total S-nitrosothiols (from lmw-RSNO and Ptn-SNO) were measured using the Griess/Saville method (48), which uses HgCl₂ to decompose SNO groups and produce NO₂⁻, as in Nogueira et al. (38). Briefly, the supernatant of a cell lysate was mixed with an equal volume of freshly prepared Griess reagent (0.05% N-[1-naphthyl]ethylenediamine dihydrochloride and 0.5% sulfanilamide in 2.5% orthophosphoric acid) for 20 minutes in presence or absence of 3 mM HgCl₂. Absorbance of each sample was measured at 540 nm. The amount of RSNO was obtained from the difference between the absorbance values with and without HgCl₂, using a standard curve of NaNO₂. Each experiment was analyzed in triplicate.

Measurements of cGMP

Cell cGMP production was measured by a reverse-phase HPLC-based fluorescence method to detect cGMP after derivatization by the fluorogenic reagent 3,4-dimethoxyphenyl)glyoxal (DMPG) for guanine-related compounds DMPG in cell lysates as described by Soda et al. (51).

Primary cultures of skeletal muscle cells from chick embryos were initially plated at a density of 7.5 x 10⁵ cells/well in a 35-mm well plate which was previously coated with rat-tail collagen. After 24 h, cells were treated with either vehicle (DMSO), ODQ (10 µM), DETA-NO (10 µM), GSNORi (10 µM), or GSNORi + ODQ for an additional 24 h (total 48 h). In order to avoid cGMP consumption by phosphodiesterases, all treatments contained 10 µM sildenafil.
(specific PDE5 inhibitor). Cells were washed twice with 1 mL PBS, then scraped into 100 µL of 50 mM HCl, followed by boiling for 3 min. The lysate was centrifuged (10 min; 10,000 xg), and the supernatant (containing nucleotides) was lyophilized and stored at -80°C for later analysis. On the day of measurement, the powder was resuspended in 50 µL of 30 mM sodium phosphate (pH 6.0) followed by the addition of 25 mM DMPG (10% DMSO final) for derivatization. DMPG was solubilized in 100% DMSO right before use. Derivatization of guanines was performed at 37°C for 20 min, followed by injection of 20 µL of the mixture into a Prominence Ultra-Fast Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with a CBM-20A system controller, a LC-20AT pump, a RF-20AXS fluorescence detector (12 µL volume), and a sample injection valve with a 20 µL loop. An octyl silica column (C8; Luna Phenomenex®, 10 nm; 10 µm, 10 cm × 0.46 mm I.D) was employed. Samples were eluted using a two-step gradient of acetonitrile in aqueous mobile phase containing 50 mM sodium phosphate pH 6.0 and 3% (v/v) tetrahydrofuran at a flow rate of 1 ml/min. In the first 5 min of elution, 5% (v/v) acetonitrile was employed, followed by 9% (v/v) acetonitrile on the last 15 min of elution. All elution procedure were performed at room temperature. The HPLC system was interfaced with a computer equipped with LC Solutions (v.2.1) software (Shimadzu, Kyoto, Japan).

In order to ascertain the position of a cGMP peak during the elution (Fig. 9A) as well as the relationship between fluorescence and the amount of cGMP injected (Fig. 9B and 9C), a standard mixture of nucleotides (GTP + GDP + GMP, 25 µM each) containing different amounts of cGMP (1.25 - 25 µM) was derivatized with DMPG for 20 min at 37°C. After derivatization, 20 µL of the mixture was injected into the HPLC system, and elution was performed as described above. The amount of cGMP accumulated in cells for each treatment (Vehicle, ODQ, GSNORi, GSNORi + ODQ) was determined by converting the fluorescence area under the
cGMP peak using the relationship between fluorescence and the amount of cGMP injected for each individual experiment.

**Immunofluorescence and digital image acquisition**

Chick myogenic cells were rinsed with PBS and fixed with either 4% paraformaldehyde in PBS for 10 min at room temperature or with methanol for 10 min at 4°C. They were then permeabilized with 0.5% Triton-X 100 in PBS 3 times for 10 min each. The same solution was used for all subsequent washing steps. Cells were incubated with primary antibodies for 1 h at 37°C, then washed for 30 min and incubated with Alexa Fluor-conjugated secondary antibodies for 1 h at 37°C, and nuclei were labeled with DAPI (0.1 μg/ml in 0.9% NaCl). Cells were mounted in Prolong Gold solution (Invitrogen, Brazil) and examined with an Axiovert 100 microscope (Carl Zeiss, Germany) or with a laser scanning confocal microscope (TCS SP5 AOBS, Leica, Japan). Image processing and stack projections were performed using Fiji software (based on ImageJ software, http://imageJ.nih.gov/ij/). Control experiments with no primary antibodies showed only a faint background staining (data not shown).

**Identification and quantification of myoblasts, fibroblasts and myotubes in culture**

Immunofluorescence images of 24-h and 72-h chick myogenic cultures stained for α-actinin, desmin (muscle-specific markers) and DAPI (nuclear dye) were acquired from each culture condition.

Number of nuclei in culture was counted manually using DAPI fluorescence. To distinguish between myoblasts and fibroblasts in culture, morphology and intensity of DAPI fluorescence appearing in mononucleated cells were evaluated (39, 41, 43). This method has
been proven to be adequate for distinguishing between the two mononucleated cell types in culture by comparison with automated quantification of nuclear area (41) and with muscle-specific protein labeling (43), as well as electron microscopy (53). As exemplified in Fig. 1C (arrows), in DAPI-labeled chick myogenic cultures, muscle fibroblasts have large, flattened, pale nuclei whereas myoblasts have small, round, bright nuclei (41). On average at 24 h, myoblasts made up 80% of each culture and fibroblasts comprised 20%. In order to define cell types in the present investigation, a myoblast was defined as a mononucleated cell that was positive for desmin staining, and a myotube was considered a muscle cell (positive for sarcomeric α-actinin labeling) containing three or more nuclei (43). Desmin and α-actinin fluorescence were used because they have stronger signals in differentiated myotubes (41) compared to myoblasts, thereby distinguishing multinucleated cells (differentiated myotubes) from mononucleated cells (myoblasts and fibroblasts). Also, the immunofluorescence of these proteins was used to calculate the myotube width in the regions of greater width for each myotube, for all myotubes in each microscope field. Myoblast fusion index was calculated by dividing the number of nuclei within myotubes by the total number of nuclei per microscopic field.

These measurements were performed using the software ImageJ, as described in (9). At least 5 different fields for each experimental condition were used to measure number of nuclei, myotube widths and myoblast fusion index.

SDS-PAGE and immunoblotting

Chick myogenic cells were grown for 24, 48 and 72 h. Cultures were then quickly washed in ice-cold PBS. Fifty μL of ice-cold sample buffer (4% SDS, 20% glycerol, 0.2 M DTT,
125 mM Tris-HCl pH 6.8) were added to the cultures and cells were scraped off the dish with a plastic cell scraper. Cell extracts were recovered in a tube, centrifuged at 1000 xg for 3 min at room temperature, and boiled for 5 min. Protein concentration was determined using the Pierce kit (Cat#23227; Rockford, IL, USA). Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels. Following electrophoresis (SDS-PAGE), proteins were transferred to polyvinyl difluoride (PVDF) membranes. The proteins immobilized on the membranes were immediately blocked for 1 h at room temperature in Odyssey blocking buffer. The membranes were then incubated for 12 h at 37°C with primary antibodies (in Odyssey blocking buffer). After 5 washes in phosphate-buffered solution supplemented with 0.1% (v/v) Tween-20 (5 min each), the membranes were incubated for 1 h at 37°C with CY5.5-conjugated secondary antibodies (in Odyssey blocking buffer), and washed again as described above. The bands were visualized using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA) and analyzed using ImageJ with data obtained from three independent experiments.

**Statistical analysis**

Data are expressed as mean ± SEM and represent at least three separate experiments performed in triplicate. For comparisons between multiple groups, one-way ANOVA was followed by the Tukey test. For single comparisons between two groups, Student’s t-test was used. Analyses were carried out using PRISM 4® software (GraphPad, La Jolla, CA) and P<0.05 was considered to represent a significant difference.
RESULTS

Identification of GSNOR in chick myogenic cells

In order to investigate whether GSNOR was present in primary cultures of skeletal muscle cells from chick embryos, we determined the GSNOR activity (Fig. 1A), or detected GSNOR and NOS1 by immunoblotting (Fig. 1B) and by immunofluorescence (Fig. 1C) at different times (24, 48 and 72 h) after plating cells. The enzymatic activity of GSNOR was statistically increased during the time course of cell differentiation (Fig. 1A). In order to test whether the increased NADH-dependent GSNO reduction activity detected during the time course of cell plating was from GSNOR, cell lysates were incubated with a specific GSNORi (10 µM) prior to GSNOR activity measurements (47). The GSNOR activity was inhibited by 95 ± 5 % (Fig. 1A) compared to the 72-h time point without GSNORi. Therefore, the data suggest that chick myogenic cell differentiation is accompanied by an increase in GSNOR activity.

Although the activity of GSNOR increased during muscle differentiation, there was no significant change in GSNOR content over the same time course in the immunoblotting experiments (Fig. 1B). The content of NOS1 was also not statistically altered during the course of muscle cell differentiation, but there was a trend toward lower values during cell plating in 2 out of 3 experiments (Fig. 1B).

In order to investigate whether GSNOR and NOS1 detected in immunoblotting experiments were present specifically in myoblasts and/or fibroblasts, 24 h cultures (containing mainly proliferating myoblasts and fibroblasts) or 72 h cultures (containing mostly multinucleated myotubes and few mononucleated cells) were labeled in vitro for GSNOR, NOS1 and nuclei (DAPI), followed by immunofluorescence microscopy. As shown in Figure 1C,
GSNOR and NOS1 were detected in all three cell types, myoblasts (mb), fibroblasts (f) and myotubes (mt).

Content of total S-nitrosothiols during chick myogenic differentiation

Although the activity of GSNOR was increased during chick muscle cell differentiation, the amount of total RSNO was also significantly increased (in pmol RSNO/mg protein: 7.0 ± 0.1, 9.1 ± 0.4, and 11.5 ± 2.1 for 24 h, 48 h, and 72 h, respectively; P<0.05, Fig. 2A). In order to determine whether GSNOR participates in controlling the total RSNO content, primary cultures of chick skeletal muscle were treated for 48 h with DMSO (vehicle), or GSNORi, or L-NAME (a general NOS inhibitor), or L-NAME + GSNORi (total cell plating of 72 h). The treatment with GSNORi led to a significant increase in total RSNO (~4.5x increase compared to vehicle, P<0.001, Fig. 2B). The presence of L-NAME produced a trend toward a decrease (not statistically significant) in total RSNO content (8.3 ± 0.8 vs. 4.3 ± 3.5 pmol RSNO/mg protein for vehicle vs. L-NAME, respectively, P>0.05). However, when L-NAME + GSNORi were both present, the increase in RSNO detected with GSNORi was completely abolished (P<0.05, Fig. 2B). There was no change in MTT-formazan absorbance with any of the treatments (data not shown). These data suggest that the content of total RSNO in primary cultures of chick skeletal muscle is regulated by the combined action of GSNOR and NOS activity.

The role of NOS and GSNOR in chick myogenic cell growth and differentiation

In order to understand the NOS and GSNOR contributions to cell growth and differentiation in skeletal muscle, we analyzed by immunofluorescence (as illustrated in Fig. 3A) and phase-contrast microscopy (as illustrated in Fig. 3B) the effects of GSNORi, L-NAME, or L-
NAME + GSNORi for 48 h (total cell plating of 72 h) in chick muscle cell cultures. The number and morphology of nuclei in mononucleated and multinucleated cells, as well as the distribution of desmin or α-actinin in myotubes, were analyzed by immunofluorescence microscopy (see Methods). Phase-contrast microscopy was also used to analyze the effects of the different treatments on cell culture confluence. The mean data obtained from 4 different preparations like that of Figure 3 are shown in Figure 4.

To determine whether the inhibition of GSNOR or NOS would affect cell proliferation, the total number of nuclei, as well as the number of nuclei present only in mononucleated cells (primarily myoblasts and fibroblasts) and in multinucleated cells (primarily myotubes), were counted (Figure 4A).

In this experiment, the incubation of cells with vehicle (0.16% DMSO) did not alter the total number of nuclei or the number of nuclei in either mononucleated or multinucleated cells (data without vehicle not shown). However, when cells were incubated for 48 h with GSNORi (which produced an increase in total RSNO in cells, as shown in Fig. 2B), there was a significant increase in the total number of nuclei (17 ± 3% increase vs. vehicle, P<0.05, Fig. 4A). When these cells were discriminated as mononucleated (myoblasts and fibroblasts) and multinucleated (myotubes, see Methods for details), there was an increased number of nuclei only in mononucleated cells (49 ± 1% increase vs. vehicle, P<0.001, Fig. 4A), but not in multinucleated cells.

To determine whether the increase in mononucleated cells when GSNOR was inhibited was from enhanced proliferation of fibroblasts or myoblasts, mononucleated cells were distinguished by the differences in nuclear shape and DAPI fluorescence emission intensity between the two cell types (41). The number of fibroblasts (obtained from the number of
fibroblast nuclei) and myoblasts (obtained from the number of myoblast nuclei), was statistically increased by 38 ± 2% and 103 ± 14%, respectively, during GSNORi incubation (P<0.001, Fig. 4B). Therefore, the inhibition of GSNOR stimulates proliferation of both cell types in cultures of skeletal muscle from chick embryo.

In order to evaluate whether NOS activity has a role in the proliferating chick myogenic cells when GSNOR is inhibited, L-NAME was used to block NO production, thereby blocking the increase in intracellular RSNO (Fig. 2B). The treatment with L-NAME did not alter either the total number of nuclei or the number of nuclei in mononucleated or multinucleated cells (Fig. 4A). In addition, the incubation with L-NAME + GSNORi produced results similar to L-NAME alone (i.e., no statistical significance vs. vehicle; Fig. 4A). These data suggest that absence of NOS activity blunts the stimulatory effect of GSNOR inhibition by blocking the increase in intracellular RSNO.

Although inhibiting GSNOR enhanced proliferation in chick fibroblasts and myoblasts, the number of nuclei within myotubes was not altered, which suggests that fewer mononucleated cells fused into myotubes. Figure 4C shows the fusion index, which is determined by dividing the number of nuclei within myotubes by the total number of nuclei in the microscopic field. The inhibition of GSNOR produced a statistically significant decrease in myoblast fusion (23 ± 3% decrease vs. vehicle, P<0.01, Fig. 4C). There was no change in fusion index when either L-NAME or L-NAME + GSNORi was present.

After the in vitro treatments the number and width of myotubes, as identified by desmin and α-actinin immunolabeling (i.e., desmin was distributed throughout the sarcoplasm and α-actinin in Z-lines in sarcomeres for all treatments (Fig. 3A)), were not changed by the presence of GSNORi (Fig. 4D and Fig. 4E). However, the presence of L-NAME or L-NAME + GSNORi
statistically decreased the number of myotubes (21 ± 11% and 36 ± 7% decrease vs. vehicle, respectively, P<0.05, Fig. 4D). Although L-NAME or L-NAME + GSNORi produced an increase in myotube width in 3 out of 4 primary culture preparations vs. vehicle, there was no statistical significance (P>0.05, Fig. 4E). We infer that inhibition of GSNOR diminishes myoblast fusion to myotubes without affecting their morphology, but inhibition of NOS activity leads to an increase in myotube width in culture.

The increased number of myoblasts was proportional to the inhibition of GSNOR

In order to determine whether the increased number of myoblasts obtained during treatment with GSNORi was correlated to the degree of GSNOR inhibition, GSNOR activity was measured in cell lysates and myoblast number was determined in cultured cells, after incubating, respectively, lysates and cells with different GSNORi concentrations.

The incubation of cell lysates from 72-h cultured cells with 1 or 5 or 10 µM GSNORi produced a dose-dependent decrease in GSNOR activity (Fig. 5A). Maximal inhibition and the GSNORi concentration which produced half-maximal inhibition, determined by a 2-parameter hyperbolic equation, were 84 ± 7 % and 0.8 ± 0.3 µM GSNORi, respectively.

Also, treating 24-h cultured cells with the same GSNORi concentrations for 48 h, produced a stepwise increase in myoblast number (35 ± 6 % for 1 µM, 45 ± 13 % for 5 µM, and 81 ± 5 % for 10 µM GSNORi, Fig. 5B). Although the increase in myoblast number was statistically significant after 1 and 5 µM GSNORi treatments, statistically significant decrease in fusion index was only detected with 10 µM GSNORi incubation (data not shown). When the dose-dependent effect of GSNORi on GSNOR activity was plotted and compared with the
effects of GSNORi on number of myoblasts (Fig. 5C), there was a positive correlation between GSNOR inhibition and myoblast proliferation.

Treatment with S-nitrosocysteine (CysNO) reproduces the effects of GSNOR inhibition on myoblast and fibroblast proliferation and differentiation

To ascertain whether the effects observed with GSNORi were due to the increase in intracellular RSNO, we treated chick myogenic cultures for 48 h with 100 µM CysNO, which is able to enter the cells (7). The treatment with CysNO induced an increase of total RSNO by 105 ± 34% (in pmol RSNO/mg protein: 9 ± 1 and 18 ± 3 for control and CysNO, respectively; P<0.05, Fig. 6A). There was also an increase in the total number of nuclei (15 ± 4%, P<0.01), and in the number of nuclei in mononucleated cells (43 ± 4%, P<0.01, Fig. 6B), but there was no change in the number of nuclei in multinucleated cells (myotubes) when CysNO was present. The numbers of fibroblasts and myoblasts were both increased with CysNO (35 ± 6% and 92 ± 11%, respectively, P<0.01, Fig. 6C). Fusion index was statistically decreased after treatment with CysNO (23 ± 2% decrease, P<0.01, Fig. 6D). These results suggest that the S-nitrosylating agent CysNO produces the same proliferating effect on mononucleated cells (fibroblasts and myoblasts) as does inhibiting GSNOR.

The effects of sGC inhibition on cell proliferation and myoblast fusion

The role of NO in myogenesis has been attributed to its ability to activate sGC, which increases the production of cGMP, and initiates cGMP-dependent intracellular signaling (sGC/cGMP pathway) (12, 17, 27).
In order to determine whether NO-regulated myogenesis in our primary culture of skeletal muscle cells is also regulated by sGC/cGMP pathway, 24-h cultured cells were incubated for 48 h with 10 µM ODQ, an inhibitor of sGC (50) in absence and presence of 10 µM 8-Br-cGMP, a membrane permeant cGMP analog (total of 72 h of cell plating). The treatment with ODQ reduced the number of nuclei in cell culture for both multinucleated (17 ± 7 %) and mononucleated (fibroblasts and myoblasts) cells (24 ± 1 %) (Fig. 7A), but did not produce cell metabolic dysfunction since an MTT assay did not show any change with ODQ (data not shown). This is expected since it has already been shown that ODQ inhibits (through blockage of cGMP-induced signaling) skeletal muscle development and diminishes muscle mass in chick embryos (12). The presence of 8-Br-cGMP completely abolished the inhibitory effect of ODQ (Fig. 7A), confirming the role of ODQ in inhibiting sGC thereby decreasing cell proliferation. Also, providing exogenous NO to 24-h cultured cells with the slow NO donor DETA-NO (10 µM) for 48 h led to an increase in mononucleated cells (22 ± 4 % for fibroblasts and 55 ± 10 % for myoblasts), which was completely abolished by ODQ (Fig. 7B). DETA-NO treatment slightly decreased the fusion index (8 ± 3 %, P<0.05, Fig. 7C), an effect that was also blocked with ODQ. These data suggest that NO directly regulates myogenesis in primary cultures of skeletal muscle from chick embryos by activating sGC/cGMP pathway.

Nonetheless, the effects on myogenesis of increasing RSNO by either inhibiting GSNOR or providing CysNO may be mediated by "nonclassical mechanisms" (32) such as post-translational modifications, and not by the classical sGC/cGMP pathway. To investigate whether the effects of GSNOR inhibition or CysNO treatment on cell proliferation and fusion were mediated by sGC activation, chick myogenic cells were incubated for 48 h with either GSNORi
or CysNO to increase intracellular RSNO in the absence or presence of 10 µM ODQ (total of 72 h of cell plating).

As also shown in the experiments of Figure 7, ODQ reduced the number of nuclei in cell culture for both mononucleated and multinucleated cells (Fig. 8A). However, when GSNORi was applied following a 10-min incubation with ODQ, there was a small, but statistically significant, increase in the number of nuclei only in mononucleated cells ($P<0.05$, Fig. 8A).

When fibroblasts were eliminated from the calculation, the increase in mononucleated cells (i.e., myoblasts) was quite striking (cf. ODQ vs. ODQ + GSNORi, $P<0.01$, Fig. 8B, myoblasts). Interestingly, it was not different from the effect of GSNORi alone (i.e., GSNORi vs. ODQ + GSNORi, Fig. 8B myoblasts). In order to pharmacologically increase intracellular RSNO in cells, CysNO was applied to the culture following the 10-min incubation with ODQ. This treatment did not statistically change the number of nuclei in mononucleated cells compared to ODQ alone ($P=0.08$, Fig. 8A), but there was a trend toward an increase (4 in 5 experiments showed a 25% increase in nuclei number). However, when only myoblasts were analyzed, CysNO completely reversed the inhibitory effect of ODQ on the number of nuclei (cf. ODQ vs. ODQ + CysNO, Fig. 8B myoblasts). This did not occur for fibroblasts. Therefore, these data suggest that the increase in nuclei number due to GSNOR inhibition or CysNO incubation occurred specifically in myoblasts, and was independent of sGC activation.

The enhanced proliferation of myoblasts obtained with GSNORi treatment (Fig. 8B) was accompanied by a modest decrease in myoblast fusion to myotubes (14 ± 1% decrease, *$P<0.01$, cf. Vehicle vs. GSNORi, Fig. 8C). With ODQ incubation, the decreased myoblast proliferation (~23% decrease, Fig. 8B) was accompanied by a small increase in fusion index, but it was not statistically different from vehicle (9 ± 3%, Fig. 8C). However, when ODQ was applied 10 min
before GSNORi treatment, the decrease in myoblast fusion was not present, which suggests that although myoblast proliferation was not dependent on sGC activation, the decrease in myoblast fusion with GSNORi treatment was dependent on sGC activation. Interestingly, treating these cell cultures with CysNO (but with 0.32% DMSO added compared to no DMSO addition in the experiments shown in Fig. 6) did not change the fusion index (Fig. 8C). We infer that different mechanisms are involved in activating cell proliferation and controlling myoblast fusion.

**Measurements of cGMP accumulation in cell cultures**

The enhanced proliferation of myoblasts obtained when both ODQ + GSNORi were present in cell cultures could be due to GSNORi-dependent sGC activation, which would increase cGMP in cells even when ODQ was present. Therefore, cGMP accumulation in cells was measured after treating 24-h cultured cells with either DMSO (vehicle), or 10 μM ODQ, or 10 μM DETA-NO, or 10 μM GSNORi, or GSNORi + ODQ for 24 h. As shown in Figure 9E, treating cells with GSNORi produced an evident increase in cGMP accumulation. This was expected since sGC can be S-nitrosylated by S-nitrosothiols, thereby transiently activating sGC (45). However, when cells were treated with GSNORi + ODQ, the stimulatory effect of GSNORi on cGMP accumulation was diminished (Fig. 9E).

The effect of sGC inhibition on myoblast proliferation (i.e., less myoblast proliferation) was overcome by GSNOR pharmacological inhibition (i.e., enhanced myoblast proliferation), which was not accompanied by an increase in cGMP accumulation. Therefore, these data suggest that the enhanced proliferation of myoblasts by GSNORi was likely due to activation of an alternative pathway, independent of the classical sGC/cGMP pathway.
**Effects of GSNOR knockdown on myoblast proliferation and fusion**

Although the inhibitor of GSNOR used in the present work has been claimed to be specific for GSNOR (alcohol dehydrogenase class III, also known as ADH5) within the alcohol dehydrogenase family (22, 47), it is necessary to compare the results from GSNORi to transient knockdown of GSNOR by using siRNA technology. We selected three sequences of siRNA that should be able individually to target a separate region of the full mRNA for translation of GSNOR (Table 1). Twenty-four-hour cultured cells were transiently transfected with one of the three different siRNA sequences for GSNOR or a control non-specific siRNA (eGFP) (see Methods for details) for 24 h, followed by cell lysis or immunostaining with fluorescent probes.

The siRNA transfection with sequences targeting mRNA for GSNOR produced a smaller amount of GSNOR protein, without reducing the loading control protein α-tubulin, as detected by immunoblotting (Fig. 10A). GSNOR was detected after transfection with siRNA targeting mRNA for eGFP. The sequences selected to knock down GSNOR produced a decrease in cell lysate GSNOR activity (decrease in activity was 48 ± 10 % for sequence #1, 50 ± 17 % for sequence #2, and 40 ± 18 % for sequence #3) (Fig. 10B), but only sequence #1 and #2 showed a statistical decrease in GSNOR activity (P<0.05, Fig. 10B). In order to ensure that cellular GSNOR function was impaired after GSNOR knockdown, total intracellular RSNO content was measured in cell lysates. Cells transfected with siRNA GSNOR seq#1 produced an increase in total cell RSNO (75 ± 9 vs. 11 ± 5 pmol RSNO.mg protein\(^{-1}\) for siRNA GSNOR seq#1 vs. siRNA eGFP, respectively; Fig. 10C), comparable to the effect of GSNORi treatment in non-transfected cells (89 ± 13 vs. 9 ± 6 pmol RSNO.mg protein\(^{-1}\) for GSNORi vs. vehicle, respectively; Fig. 10C). Therefore, the sequences of siRNA chosen to target the mRNA for GSNOR effectively able to produce an increase in RSNO by knocking down GSNOR.
Regarding the effect of knockdown on myogenesis, primary cultures of skeletal muscle from chick embryos were examined after 24 h of transfection with siRNA for eGFP or GSNOR seq#1. For comparison with the data of Figures 4 and 8, some cultures were also incubated with either L-NAME (1 mM) or ODQ (10 µM) during transfection. For each experiment, three groups of cell cultures were not transfected with siRNA, but were treated with either vehicle, GSNORi (10 µM) or ODQ for 24 h.

As shown in Figure 11, transfecting cells with siRNA targeting eGFP did not alter the density of nuclei and myotubes in culture (48 h) compared to control (vehicle), but there was a clear reduction in cell density during ODQ treatment. The transfection procedure targeting GSNOR induced an increase in the density of nuclei and myotubes, except when ODQ was also present.

The quantification of nuclei from similar experiments is shown in Figure 12. In cell cultures which were not transfected with siRNA (i.e., Non-transfected cells in Fig. 12), GSNORi enhanced proliferation by 41 ± 2 % and ODQ decreased proliferation by 28 ± 2 % in both types of mononucleated cells (Fig. 12B). Also, GSNORi produced a decrease in fusion index by 14 ± 1 % (Fig. 12C).

In cell cultures which were transfected with siRNA sequences (siRNA eGFP or siRNA GSNOR seq#1, Fig. 12), there was an increase in number of nuclei in fibroblasts (31 ± 6 % increase) as well as in myoblasts (45 ± 9 % increase) only when the siRNA GSNOR seq#1 was applied, but not when siRNA eGFP was applied (†P<0.05, cf. siRNA GSNOR vehicle vs. siRNA eGFP vehicle, Fig.12B). The increase in cell proliferation was blocked when L-NAME was present for both types of mononucleated cells (Fig. 12B, dark gray bars vs. black bars). Blocking sGC activity with ODQ (white bars in Fig. 12B) inhibited cell proliferation when siRNA eGFP
was transfected for both types of mononucleated cells, and also in fibroblasts when cell cultures were transfected with siRNA GSNOR (Fig. 12B). Interestingly, the myoblasts continued to proliferate in the presence of ODQ in cultures transfected with siRNA GSNOR (Fig. 12B). Moreover, siRNA GSNOR, but not siRNA eGFP, decreased fusion index (25 ± 3 % decrease), as also occurred in GSNORi-treated cells (Fig. 12C). The diminished fusion index was not present with L-NAME or ODQ (Fig. 12C). The results obtained with siRNA GSNOR seq#1 (sGC-dependent fibroblast proliferation and sGC-independent myoblast proliferation) were also obtained when cell cultures were transfected with siRNA GSNOR seq#2 and seq#3 (data not shown). Therefore, the knockdown of GSNOR confirmed the stimulatory role of increased intracellular RSNO on myoblast proliferation independent on cGc/cGMP pathway.
Nitric oxide (NO) has been shown to be an important regulator of proliferation and differentiation in skeletal muscle progenitor cells (i.e., myoblasts and satellite cells); it also participates in muscle repair (2, 20). The mechanism by which NO contributes to myogenesis and satellite cell activation has until now been attributed solely to the increase in cGMP production by sGC activation, through protein kinase G (PKG) downstream signaling (12, 17) (Fig. 13, in black). However, there is evidence that the effects of NO on muscle development are also mediated by protein S-nitrosylation (14, 31) and denitrosylation (24, 35).

The present investigation was designed to explore the importance of total intracellular RSNO and to determine whether the equilibrium between S-nitrosylation and denitrosylation regulates myogenesis in primary cultures of skeletal muscle progenitor cells from chick embryos. These cultures contain both myoblasts and fibroblasts, reproducing an environment that is similar to the *in-vivo* condition. These cells provide a robust model for studying the different steps during myogenesis (12, 27, 34, 41, 44), since the myoblasts proliferate before they withdraw from the cell cycle to fuse, forming myotubes (1), whereas fibroblasts will only continuously proliferate (27). We examined the impact of increasing intracellular RSNO content, either by inhibiting GSNOR (pharmacologically or by knocking down GSNOR translation) or by treating cells with CysNO, on the proliferation and differentiation of these cells in culture. Also, we tested whether these changes were mediated through activation of sGC. The data show that increasing intracellular RSNO during myogenesis stimulates cell proliferation in both myoblasts and fibroblasts, and diminishes myoblast fusion. Interestingly, there is clear evidence that RSNO-induced enhancement of myoblast proliferation is independent of sGC activation (Fig. 13, in red).
It has been demonstrated that the intracellular content of Ptn-SNO is regulated not only by the bioavailability of NO, but also by the removal rate of low-molecular-weight RSNO (e.g., GSNO and CysNO) which can serve as intermediates in protein S-nitrosylation (4). While the literature assigns great importance to the effects of S-nitrosylation on protein function, little is known about regulation of the denitrosylation or the physiological role of the balance between S-nitrosylation and denitrosylation in skeletal muscle (35). Denitrosylation has been shown to be mostly dependent on the activity from both GSNOR and the thioredoxin system (4). In particular, GSNOR, which is present in skeletal muscle (35) and many other tissues (29), has been demonstrated to have a central role in regulating intracellular GSNO, thereby indirectly controlling Ptn-SNO formation (4). Although GSNOR knockout mice show smaller fibers than in wild-type mice (35), the role of GSNOR in muscle development is not completely understood. Here, the in-vitro activity of GSNOR increased during cell plating but the expression did not, raising the possibility that GSNOR may be positively modulated during myogenesis by a post-translational modification such as S-nitrosylation (i.e., as detected in mice; (8)), but so far we have not been able to determine why GSNOR activity progressively increased during cell plating. Despite this increase, it did not prevent the progressive accumulation of total intracellular RSNO (an increase of ~60%) over the 72 h of cell culture, but when GSNOR was inhibited there was an even greater increase in total RSNO (7- to 8-fold), which is a hallmark of GSNOR inhibition in cells and tissues (22, 29, 37). Therefore, these results suggest that there is a growing requirement for GSNO denitrosylation during myoblast differentiation.

During the proliferative step of myogenesis, the synthesis of NO is enhanced in myoblasts (27) and in satellite cells (2), then it is decreased during the differentiation and fusion steps (27). When NO production is impaired -- either by NOS inhibition (17) or by a
pathophysiological condition such as Duchenne muscular dystrophy (13) -- proliferation of muscle progenitor cells is significantly depressed. Moreover, skeletal muscle repair following cardiotoxin-induced muscle injury, is significantly impaired in GSNOR knockout mice (35). Together with the data from the present investigation, these observations strengthen the hypothesis that there is a finely tuned balance of the NO metabolism (and S-nitrosylation/denitrosylation equilibrium) in muscle progenitor cells that may be critical for muscle development and repair. Since cells contain multiple intracellular targets for NO such as metalloproteins (e.g., sGC) and cysteine thiols (e.g., GSH) (25) (Fig. 13, in red), it is not yet clear whether changes in intracellular RSNO caused by GSNOR activity will in fact be responsible for regulating myoblast proliferation and fusion. There are a few reports showing that Ptn-SNO participates in the activation of satellite cell proliferation, through histone deacetylase (HDAC-2) S-nitrosylation (14), and it can inhibit myotube formation through myogenin S-nitrosylation (31). Our experiments favor a positive role for S-nitrosylation, since myoblast proliferation and intracellular RSNO were both enhanced by GSNORi and GSNOR knockdown, and both were blunted by NOS inhibition. The correlation between myoblast proliferation and intracellular RSNO was confirmed by the incubation of cells with CysNO, a lmw-RSNO that is capable of being transported into cells (including skeletal muscle cells (38)) and increasing the intracellular RSNO pool. Thus these data suggest that the intracellular RSNO equilibrium, controlled by GSNOR activity and the NO production from NOS, has an important role in the transition between cell proliferation and fusion during skeletal muscle development.

In myoblasts, this transition has been attributed to a transient activation of sGC, which increases the intracellular levels of cGMP, thereby activating its downstream signaling, particularly through PKG (10, 17, 27) (Fig. 13, in black). The present investigation confirmed
that NO/sGC/cGMP signaling enhances proliferation of both fibroblasts and myoblasts. Providing NO through DETA-NO decomposition enhanced cell proliferation, which was blocked during sGC inhibition (ODQ treatment, a specific inhibitor for sGC (50)). Also, the cGMP analog, 8-Br-cGMP, restored the inhibited cell proliferation obtained with ODQ. However in our experiments, an intriguing difference between myoblasts and fibroblasts appeared when cultures were incubated with ODQ, before GSNORi or CysNO treatment. Cell proliferation decreased (~30%) and this was independent of cell type (i.e., fibroblasts or myoblasts). However, when GSNOR was inhibited at the same time, by incubating with GSNORi or knocking down GSNOR by siRNA technology, the capacity for proliferation was restored -- but only in myoblasts, not in fibroblasts. Similar results were obtained when CysNO was added instead of GSNORi (to directly increase the intracellular pool of S-nitrosothiols). Thus we demonstrate for the first time that myoblast proliferation occurs without activation of sGC, and that enhanced proliferation is not followed by enhanced fusion. Furthermore, this mechanism seems to be mediated specifically by an increase in the intracellular pool of S-nitrosothiols (i.e., RSNO-induced myoblast proliferation). Fibroblasts, on the other hand, resemble other cells in that they seem to need sGC activation for RSNO-induced proliferation to proceed.

Interestingly, pharmacological GSNOR inhibition caused an increase in cGMP accumulation in cell cultures, which was diminished by ODQ. The GSNORi-induced cGMP accumulation could be due to an increase in NO release from the increased intracellular RSNO pool by intracellular flavoproteins (46), which would directly activate sGC. Another possibility is that sGC could be S-nitrosylated during GSNORi treatment. It has been shown that sGC, a protein containing an unusually large number of cysteines, can have its catalytic activity modulated by post-translational modifications (for review, see (6)). sGC can be S-nitrosylated on
several cysteine residues, inhibiting sGC activation by NO (33, 49), but has also been shown that low levels of sGC S-nitrosylation can in fact activate the enzyme (18). Thus it is not yet established whether the effects of S-nitrosylation on sGC activity are positive or negative. We speculate that the increased fibroblast proliferation detected with GSNORi treatment might be due to an activation of sGC by an increase in the intracellular pool of RSNO, which could augment NO release or lead to S-nitrosylation of sGC, and this is blocked by ODQ treatment. However in myoblasts, the GSNORi (and GSNOR knockdown)-induced cell proliferation was detected during ODQ treatment, which prevented GSNORi-induced cGMP accumulation.

The effects on myogenesis detected by changing the RSNO equilibrium could be due to GSNO activating cell proliferation, or to SNO transfer from GSNO to an intermediate protein that activates the cell proliferation program (dashed red lines in Fig. 13). There are several signaling proteins that may be targets for S-nitrosylation interfering with the cell cycle, proliferation and differentiation, such as p21Ras (3, 40), myogenin (31), and HDAC-2 (14). Among these, p21Ras is the best characterized signaling molecule in which S-nitrosylation activates cell proliferation in endothelial cells (3, 40). However, additional investigation would be required to establish which pathway is activated when GSNORi is inhibited.

These results point to RSNO metabolism as a new avenue for investigating pharmacological treatments that target muscle progenitor cells to alleviate symptoms of muscle development disorders. Manipulating GSNOR to upregulate the transition between proliferation and fusion may be a way to promote muscle repair, particularly under conditions where there is a large loss of muscle volume, such as in sarcopenia and after severe trauma.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTION:

A.M.S.Y., M.T.C.A., L.P.R, A.C.P., and C.P.S. performed experiments; A.M.S.Y., L.P.R., M.F., C.F.F., M.M.S., C.M., and L.N. designed research; A.M.S.Y., M.M.S., C.M., and L.N. analyzed data, prepared the figures and wrote the manuscript.
**Table 1.** List of siRNA sequences for chick GSNOR and eGFP.

<table>
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<th>Sequence Name</th>
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<th>Strand Guide</th>
<th>Strand Passenger</th>
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FIGURE LEGENDS

Figure 1. GSNOR activity is increased during chick myogenesis. Primary cultures of embryonic chick skeletal muscle were grown for 24, 48 or 72 h, followed by cell lysis and measurements of GSNOR activity (A), as well as GSNOR and NOS1 immunodetection by Western blotting (B) and immunofluorescence (C). A) GSNOR activity (see Methods for details) was measured on cell lysates obtained after 24, 48 and 72 h of cell growth. At 72 h of cell growth, a second set of sample lysates were treated with 10 μM GSNORi and GSNOR activity was measured. *P<0.01 vs. 24 h, **P<0.01 vs. 48 h, †P<0.001 vs. untreated 72-h, n=4; B) Immunodetection of GSNOR or NOS1 and α-tubulin in cell lysates (top), and the densitometry ratio of the bands (bottom); n=4. Data are presented as mean ± SE; C) Epifluorescence of cell cultures after 24 and 72 h of cell growth, when antibodies for GSNOR (red), NOS1 (green) and nuclear dye DAPI (blue) were used. Scale bars represent 50 μm. Legend: f, fibroblast; mb, myoblast; mt, myotube.

Figure 2. Total S-nitrosothiols in cell lysates increase with muscle differentiation and are regulated by NOS and GSNOR activities. A) Total S-nitrosothiols (RSNO) normalized to the amount of protein in cell lysates were measured by the Griess-Saville method. *P<0.05 vs. 24 h, n=4; B) Measurements of RSNO after 72 h cell plating, including 48 h of treatment with vehicle (DMSO), GSNORi, L-NAME (NOS inhibitor), or L-NAME with GSNORi. *P<0.001 vs. vehicle, L-NAME, and L-NAME with GSNORi, n=4; Data are presented as mean ± SE.

Figure 3. Immunofluorescence and phase-contrast microscopy of chick myogenic cells treated with GSNOR inhibitor or NOS inhibitor or both for 48 h (total of 72 h cell growth). Primary cultures of chick skeletal muscle cells were grown for 72 h (untreated) or 24 h followed by 48 h with vehicle (DMSO), GSNORi, L-NAME, or L-NAME with GSNORi. The cultures were analyzed by epifluorescence for desmin (red), α-actinin (green), and for the nuclear dye DAPI (blue) (A) and by phase-contrast microscopy (B). In B some myotubes are colored in green to show morphology. Scale bars represent 50 μm.
Figure 4. Inhibition of GSNOR enhances chick mononucleated cell proliferation and decreases myoblast fusion. Data were calculated from phase contrast microscopy (for the number and width of myotubes) and immunofluorescence microscopy (number of nuclei) of the cell cultures after 72 h of cell plating (48 h of treatment), as exemplified in Figure 3. A) Total number of nuclei per microscopic field, and the number of nuclei detected in mononucleated cells (fibroblasts and myoblasts) and in multinucleated cells (myotubes) after each treatment. All data were normalized to an untreated (control) group, set to 100%. *P<0.05 (for total nuclei) or P<0.001 (for mononucleated cells) vs. vehicle, L-NAME, and L-NAME with GSNORi, n=4; B) Number of fibroblasts or myoblasts per microscopic field after each treatment, normalized to their control groups. *P<0.001 vs. vehicle, L-NAME, and L-NAME with GSNORi, n=4; C) Fusion index, which is determined by dividing the number of nuclei within myotubes by the total number of nuclei per microscopic field, normalized to their control groups. †P<0.01 vs. vehicle, L-NAME, and L-NAME with GSNORi, n=4; D) Number of myotubes per microscopic field after each treatment. *P<0.05 vs. vehicle, or GSNORi, one-way ANOVA, n=4; E) Myotube width, n=4. Data are presented as mean ± SE.

Figure 5. Enhanced myoblast proliferation is proportional to GSNOR inhibition. Primary cultures of skeletal muscle from chick embryo were plated for 24 h, followed by incubation with different concentrations of GSNORi for 48 h. A) Dose-dependent effects of GSNORi on GSNOR activity in cell lysates (n=5); B) Dose-dependent effects of GSNORi on myoblast proliferation (n=3); C) Relationship between GSNOR inhibition by GSNORi (Data in A) and myoblast proliferation (Data in B). Legend: Open circles, no GSNORi (vehicle); closed circles, 1 µM GSNORi; closed triangles, 5 µM GSNORi; closed squares, 10 µM GSNORi. Mean ± SE. *P<0.01 vs. vehicle (i.e., 0 GSNORi), one-way ANOVA, repeated measures, Tukey posttest.

Figure 6. Exogenous S-nitrosocysteine (CysNO) enhances chick mononucleated muscle cell proliferation and decreases myoblast fusion. Primary cultures of chick skeletal muscle cells were grown for 24 h and then treated for 48 h with 100 µM CysNO once a day (total cell growth of 72 h). A) Total RSNO measured in cell lysates. *P<0.05 vs. control (untreated cells), n=4; B-D) Another set of primary cultured cells were grown and treated as in (A), followed by immunofluorescence staining for desmin, α-actinin and the nuclear dye DAPI. Images were
quantified using ImageJ Fiji software; B) Total number of nuclei, number of nuclei in mononucleated cells and in multinucleated cells. *P<0.01 vs. control, n=4; C) Number of fibroblasts and myoblasts. *P<0.01 vs. control, n=4; D) Fusion index, which is determined by dividing the number of nuclei in myotubes by the total number of nuclei per microscopic field. *P<0.01 vs. control, n=4. Data are presented as mean ± SE.

Figure 7. Myoblast and fibroblast proliferation are enhanced by nitric oxide due to activation of cGMP signaling. A) Effects of 48 h of ODQ (10 µM) in absence or presence of 8-Br-cGMP (10 µM) on cell proliferation. Mean ± SE, n=4 preparations. *P<0.05 vs. vehicle and †P<0.05 vs. ODQ, one-way ANOVA, repeated measures, Tukey post-test. B-C) Effects of incubation for 48 h with DETA-NO (10 µM) in the absence or presence of ODQ (10 µM) on myoblast and fibroblast proliferation (B) and myoblast fusion (C). Mean ± SE, n=5 preparations. *P<0.05 vs. vehicle, §P<0.05 vs. DETA-NO, †P<0.05 vs. vehicle on myoblast fusion, one-way ANOVA, repeated measures, Tukey post-test.

Figure 8. Inhibiting soluble guanylyl cyclase (sGC) does not affect the enhanced myoblast proliferation or reduced fusion induced by S-nitrosothiols. Data were calculated from phase contrast microscopy (for the number of myotubes) and immunofluorescence microscopy (number of nuclei) of the cell cultures. A) Total number of nuclei per microscopic field, and number of nuclei detected in mononucleated cells (fibroblasts and myoblasts) or in multinucleated cells (myotubes) after each treatment. All data were normalized to the untreated group; B) Number of fibroblasts or myoblasts per microscopic field after each treatment, normalized to their untreated groups; C) Fusion index (number of nuclei within myotubes divided by the total number of nuclei per microscopic field. *P<0.01 vs. vehicle; †P<0.01 vs. GSNORi; ‡P<0.05 vs. ODQ; §P<0.01 vs. CysNO; NS, not statistically different; n=5. Data are presented as mean ± SE.

Figure 9. cGMP accumulation in primary cultures of skeletal muscle from chicken embryos. Cyclic GMP (cGMP) from cell lysates was derivatized by DMPG, followed by elution of reverse-phase HPLC and fluorescence was measured. A) Representative fluorescent chromatograms of a fixed concentration of a mixture of guanosine nucleotides (GTP, GDP,
GMP; 500 pmol each) containing different concentrations of cGMP (25-500 pmol); B) Representative superimposed peaks of cGMP (peak was between 11-13 min of elution); C) Fluorescence area under the cGMP peak vs. the amount of cGMP injected into the HPLC (n=5 experiments, Mean ± SE); D) Representative chromatograms near the cGMP peak of the primary cultures of skeletal muscle from chicken embryos (peak was between 8-14 min of elution), in which cells were previously treated with vehicle (DMSO), or ODQ, or DETA-NO, or GSNORi, or ODQ + GSNORi for 24 h; E) Histograms (absolute and normalized to the maximal cGMP added) for cGMP concentrations after each cell treatment. Mean ± SE, n=6.

**Figure 10. Transfecting primary cultures of muscle cells from chicken embryo with siRNA sequences resulted in loss of GSNOR protein amount and activity, and increased total intracellular RSNO.** Primary cultures of skeletal muscle cells were transfected with 100 nM siRNA sequences (three sequences for GSNOR and one for eGFP, as a control) for 24 h (Total cell plating of 48 h). A) Cells were lysed and centrifuged, followed by electrophoresis (4-15% SDS-PAGE, 38 µg total protein each lane) and Western blotting. GSNOR and α-tubulin were detected by polyclonal antibodies; B) GSNOR activity was measured in lysates from siRNA-transfected and non-transfected cells. In non-transfected cells, GSNOR activity was measured in the absence (vehicle) and in the presence of 10 µM GSNORi. Mean ± SE, n=5 preparations. *P<0.05 vs. Vehicle, †P<0.05 vs. GSNORi, one-way ANOVA, repeated measures; C) Total RSNO measured in cell lysates from transfected (siRNA eGFP and siRNA GSNOR seq#1) and non-transfected cells (Vehicle and GSNORi). Mean ± SE, n=5. *P<0.05 vs. Vehicle, †P<0.05 vs. siRNA eGFP, one-way ANOVA, repeated measures.

**Figure 11. Immunofluorescence in chick myogenic cells treated with ODQ, or transfected with siRNA targeting eGFP or GSNOR.** Primary cultures of chick skeletal muscle cells were grown for 24 h followed by 24 h with either vehicle (DMSO), ODQ, or transfection with siRNA for eGFP or for GSNOR (sequence #1). ODQ was added right after the transfection procedure. The cultures were analyzed by epifluorescence for α-actinin (lefthand panels) and the nuclear dye DAPI (middle panels). In the righthand panels both stains are merged. Scale bars represent 50 µm.
Figure 12. Knockdown of GSNOR enhances myoblast proliferation independent of sGC activity. Effects of siRNA sequences to target either GSNOR translation or eGFP (as a control) for 24 h (Total cell plating of 48 h) on myoblast and fibroblast proliferation. In cell cultures not transfected with siRNA sequences, cells were treated for 24 h with either DMSO (0.16 %; vehicle), or GSNORi (10 µM) or ODQ (10 µM). In cell cultures transfected with siRNA sequences, cells were also treated with either DMSO (0.16 %; vehicle), or ODQ (10 µM), or L-NAME (1 mM), added right after the transfection procedure. A) Fibroblast and myoblast nuclei obtained from immunofluorescence of DAPI; B) Fusion index. Mean ± SE, n=5. *P<0.05 vs. Vehicle, §P<0.05 vs. L-NAME, †P<0.05 vs. siRNA eGFP with no treatment (vehicle), one-way ANOVA, repeated measures.

Figure 13. Scheme of the proposed role of intracellular S-nitrosothiols in myoblast proliferation. In black is shown the classical mechanism of NO activation of myoblast proliferation through cGMP production and signaling. In red is shown the hypothesis tested in this work, either by inhibiting GSNOR with GSNORi, by increasing intracellular RSNO with CysNO, by knocking down GSNOR with siRNA technology, by inhibiting NOS1 with L-NAME, or by inhibiting sGC with ODQ. Legend: L-AT, L-amino acid transporter; PKG, protein kinase G.
REFERENCES


Figure 1

A

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<th>Cell</th>
<th>GSNOR activity (nmol GSNO/mg protein⁻¹.min⁻¹)</th>
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<tr>
<td>Plating</td>
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<tr>
<td>24 h</td>
<td>26 ± 7</td>
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<td>51 ± 10†</td>
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<tr>
<td>72 h</td>
<td>78 ± 11**</td>
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<tr>
<td>72 h + 10 μM</td>
<td>4 ± 3†</td>
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B

IB: GSNOR
IB: α-tubulin

IB: NOS1
IB: α-tubulin

C

GSNOR
DAPI

NOS1
DAPI

Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

A

B

C

Cell lysate GSNOR activity (nmol GSNO·mg⁻¹·min⁻¹)

pmol RSNO / mg protein

Figure 10
Figure 11

- siRNA GSNOR seq#1 + ODQ
- siRNA GSNOR seq#1 ODQ
- siRNA eGFP
- Vehicle
Figure 12

### A

**Fibroblasts**

- Non transfected cells
- siRNA eGFP
- siRNA GSNOR seq #1

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<th>Number of nuclei (% of Control/field)</th>
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<tr>
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**Myoblasts**

- Non transfected cells
- siRNA eGFP
- siRNA GSNOR seq #1

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### B

**Non transfected cells**

- Vehicle
- GSNORi
- ODQ

**Fusion index (% of Control)**

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<th>Fusion index (% of Control)</th>
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**Transfected cells**

- Vehicle
- L-NAME
- ODQ

**Figure 12**
Plasma membrane

Nucleus

Arginine → Citrulline

NO

cGMP

sGC

PKG

Myoblast proliferation

GSNO

GSNOR

GSH

Cys

CysNO

Ptn-SNO

O2

GSH

CysNO

Cys

GSSG

GSNOR

Ptn-SH

L-NAME

ODQ

L-AT

CysNO

GSNO

L-NAME

CysNO

Cys

GSH

Ptn-SH

Ptn-SNO

ODQ

GSNORi

or

GSNOR knockdown

Nucleus

Myoblast proliferation

Figure 13