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Original Contribution

# Glutathione-peroxidase-1 null muscle progenitor cells are globally defective

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**Abstract**

Mice lacking glutathione peroxidase-1 (Gpx1) have decreased resistance to systemically administered oxidants as well as infections, and sustain increased damage after ischemia–reperfusion injuries. However, stem or progenitor cell function in these animals has not been studied. We characterized patterns of proliferation, apoptosis, and differentiation of primary muscle progenitor cells (myoblasts) from $Gpx1^{-/-}$ mice. Myoblasts are the transit amplifying compartment of skeletal muscle. All aspects of myoblast biology are negatively affected by deletion of Gpx1. In particular, passaged, proliferating $Gpx1^{-/-}$ myoblasts, when induced to differentiate into fused multinucleated myotubes, show significant impairment, and form only a few immature myotubes. This defect occurs despite increased expression of the core regulators of muscle differentiation, the myogenic basic helix-loop-helix (bHLH) transcription factors, in the $Gpx1^{-/-}$ myoblasts. Furthermore, $Gpx1^{-/-}$ myoblasts exhibited decreased proliferation and increased apoptosis compared to wild-type cells. In vivo, muscle fiber areas are decreased in $Gpx1^{-/-}$ vs wild-type mice. These data suggest that Gpx1 is important for adult muscle progenitor cell function at many levels, is necessary for integrity of muscle differentiation, and that quiescent resident stem cell populations may be particularly vulnerable to peroxide-mediated damage.


*Keywords:* Glutathione peroxidase-1; Myoblast; Myotube; Peroxide; Reactive oxygen species; Skeletal muscle

## Introduction

Glutathione peroxidase-1 (Gpx1) is a highly conserved selenoprotein antioxidant which reduces hydrogen peroxide ($H_2O_2$) and hydroperoxides in cytosol and mitochondria using two molecules of glutathione (GSH). Mice in which Gpx1 is depleted by targeted genetic deletion develop normally [1], but are less tolerant of a variety of oxidant stresses compared to wild-type mice. They have decreased survival after paraquat or diquat challenges [2], or after systemic infections [3], and increased organ damage after ischemia–reperfusion injuries [4]. Recently, human deficiency of Gpx1 activity has been associated with endothelial dysfunction and increased risk of myocardial disease [5].

*Abbreviations:* bHLH, basic helix-loop-helix; BrdU, bromodeoxyuridine; CM-$H_2$DCFDA, carboxydichlorodihydrofluorescein diacetate; CpR, crossing point for 18S ribosomal RNA control; CpT, crossing point for target gene reaction; CuZnSOD, copper zinc superoxide dismutase; DAPI, 4′-6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; DP, differentiation potential; ECL, entactin-collagen-laminin; $E_R$, efficiency of ribosomal RNA PCR; $E_T$, efficiency of target gene PCR; FGF, fibroblast growth factor; GFP, green fluorescent protein; Gpx1, glutathione peroxidase-1; GSH, glutathione; MHC, myosin heavy chain; MnSOD, manganese superoxide dismutase; NAC, *N*-acetylcysteine; PBN, phenyl-*N*-*tert*-butylnitrone; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl nick end labeling.

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Gpx1 expression varies in different organs and is substantially lower in skeletal muscle than in other organs such as the liver [6]. In a murine myoblast line, Gpx1 and other major antioxidant activities are progressively downregulated during muscle differentiation, increasing the susceptibility to oxidant stress [7]. Muscle progenitor populations are certainly exposed to oxidant stress with aging and as a function of pathologies including muscular dystrophies [8], muscle atrophy with disuse [9], or contraction-induced muscle injury [10]. For these reasons, we characterized the phenotype of primary myoblasts isolated from mice with deletion of Gpx1.

Myoblasts are the proliferating progenitor cell population resident in skeletal muscle, derived from muscle satellite stem cells. Under appropriate cues myoblasts differentiate into multinuclear (fused) myotubes in vitro, and muscle fibers in vivo. These studies are the first to address the effects of Gpx1 deletion on any stem or progenitor population, and suggest that virtually all aspects of progenitor cell function are negatively impacted by loss of Gpx1 function. The most striking phenotype of $Gpx1^{-/-}$ myoblasts is a rapid loss of ability to fuse into myotubes with passage in culture, despite high levels of expression of transcription factors that promote muscle differentiation. These studies suggest that peroxide homeostasis may play an important role in long-term maintenance of muscle, and that GPx1 is important for normal function of muscle progenitors during oxidant stresses.

## Materials and methods

### *Isolation and purification of myoblasts*

All research and animal care conformed to National Institutes of Health guidelines and was conducted after approval of the Emory University and South Texas Veterans Health Care Institutional Animal Care and Use Committees. Mice with deletion of Gpx1 were originally generated by Ho et al. [1] and backcrossed for 10 generations onto a C57Bl/6J background. Myoblasts were isolated from 3- to 4-week-old mice using a modification of published protocols [11]. After euthanasia, leg muscles were dissected from the bones and nonmuscle tissue was removed; muscle was minced, washed, and then digested in 0.1% pronase (Calbiochem, La Jolla, CA) for 1 h at 37°C. The muscle was repeatedly triturated and passed through a 100-μm nylon mesh, and myoblasts were separated on a Percoll (Amersham Biosciences, Piscataway, NJ) gradient. The cells were plated onto collagen I (Cohesion Technologies, Palo Alto, CA)-coated polystyrene cell culture dishes (Corning Life Sciences, Acton, MA) [12]. During the first several passages of the primary cultures, myoblasts were enriched by preplating [13] to remove fibroblast contaminants. To confirm morphologic assessment of myoblast purity, the cultures were stained with anti-desmin (expressed by myoblasts but not fibroblasts) using antibody D3 [12] (developed by Donald A. Fischman, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA).

### *Myoblast cell culture conditions*

Myoblast expansion and prevention of differentiation were accomplished by culture in growth medium (GM): 20% fetal bovine serum (HyClone, Logan, UT) supplemented with 10 ng/ml fibroblast growth factor-2 (FGF2, Promega, Madison, WI) in Ham's F-10 (Invitrogen, Carlsbad, CA) with 200 U/ml penicillin G, 200 μg/ml streptomycin (Invitrogen). To quantify proliferation on different extracellular matrices, collagen I (as above), entactin-collagen-laminin (ECL, Upstate Biotechnology, Waltham, MA), or 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) was used to coat plates.

Differentiation was induced by switching myoblast cultures on ECL-coated dishes to differentiation medium (DM): Dulbecco's modified Eagle medium (DMEM, Invitrogen) with 2% horse serum (HyClone) and penicillin/streptomycin. At the time of differentiation, the cells were cultured in 20% $O_2$ for high oxidative stress conditions or in 6% $O_2$ to lower oxidative stress [14], with $CO_2$ kept constant at 5% in both. Cells in lower $O_2$ conditions were manipulated in a customized gas-controlled work area (Coy Labs, Grass Lake, MI) and incubated in modular gas-tight chambers (Billups-Rothenberg, Del Mar, CA).

### *Intracellular ROS measurement*

Relative amounts of intracellular ROS between culture conditions were measured using carboxydichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA, Molecular Probes, Eugene, OR), which fluoresces after oxidative modification. For these assays, myoblasts were seeded at a density of $2 \times 10^5$ cells/6 cm plate in GM. After 2 h, GM was replaced by DM. After 3 days, CM-$H_2$DCFDA (30 μM) was added for 90 min at 37°C in the dark. Cells were washed with phosphate-buffered saline (PBS) and lysed in a buffer of 50 mM Tris, 150 mM NaCl, 0.02% $NaN_3$, 0.1% SDS, 1.0% NP-40 (all from Sigma-Aldrich). Fluorescence was measured (485/530 nm) using a microplate reader (Synergy-HT, Biotek, Woburn, MA), and the results were normalized to protein concentrations. Protein concentrations were measured from an aliquot of lysed cells using a bicinchoninic acid assay (Pierce, Rockford, IL) using the manufacturer's protocol.

### *Extracellular ROS measurements*

Relative amounts of extracellular $H_2O_2$ between culture conditions were measured using Amplex Red hydrogen peroxide/peroxidase assay kits (Molecular Probes). Myoblasts were seeded and cultured as above for 3 days. Media were harvested and centrifuged before analysis, and results calculated using a standard curve generated by serial dilution of $H_2O_2$. Fluorescence was measured using the same equipment as above but at 560/590 nm, and normalized to protein content.

### *Proliferation of myoblasts*

Bromodeoxyuridine (BrdU) incorporation was used to assay proliferation. Myoblasts were plated in GM at a density of