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Oxidative stress in muscle progenitor cell function

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# Oxidative stress in muscle progenitor cell function

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An abstract of a dissertation submitted to the Faculty of the Graduate School of
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Abstract

Oxidative stress in muscle progenitor cell function
By Sukkyoo Lee

Muscle progenitor cells are essential for muscle development, growth and regeneration. Differentiation of muscle progenitor cells accompanies the complex molecular change and muscle progenitor cells are exposed to oxidative stress throughout the life span *in vivo*. In this dissertation, I investigated the effect of oxidative stress on muscle progenitor cells using primary myoblast cells isolated from transgenic mice. Gpx1-null myoblasts are globally defective in proliferation, differentiation and apoptosis during differentiation *in vitro*. In addition, oxidative stress increased by Gpx1 deletion in muscle tissue induced early senescence. I have also studied the role of mitochondrial SOD2 in muscle progenitor cells during aging. Sod2 overexpression preserves the function and relative abundance of mitochondria, but not muscle mass with aging in myoblasts. Data also suggest that the PI3K/Akt pathway is redox-regulated by oxidative stress. The fusion defect of $Sod2^{+/-}$ (young) during differentiation might result in part from this altered PI3K/Akt signaling pathway. In addition, the haplo-deficiency of SOD1 ($Sod1^{+/-}$) in myoblasts has the significantly decreased myotube formation compared to wild-type with the altered expression of HIF-1α and myogenin. Overall, the work characterizes the effect of oxidative stress in muscle progenitor cell function, and highlights the importance of oxidative stress during muscle differentiation.

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**List of Abbreviations**

bHLH: basic helix-loop-helix

BrdU: bromodeoxyuridine

CM-$H_2$DCFDA: carboxydichlorodihydrofluorescein diacetate

$C_p$R: crossing point for 18s ribosomal RNA as a control

$C_p$T: crossing point for target gene reaction

CuZnSOD: copper zinc superoxide dismutase

DAPI: 4'-6-diamidino-2-phenylindole

DM: differentiation media

DMEM: Dulbecco's modified Eagle medium

DP: differentiation potential

ECL: entactin-collagen-laminin

$E_R$: efficiency of primers for 18s ribosomal RNA as a control

$E_T$: efficiency of primers for target gene

FGF: fibroblast growth factor

GFP: green fluorescent protein

GM: growth media

Gpx1: glutathione peroxidase-1

GSH: glutathione

HIF: hypoxia inducible factor

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

PGC1α: peroxisome proliferator-activated receptor-γ-coactivator-1α

ROS: reactive oxygen species

TUNEL: terminal deoxynucleotidyl nick end labeling

# Chapter 1: Introduction

**An introduction to reactive oxygen species**

Oxygen ($O_2$) is essential for life as an electron acceptor and participates to produce ATP through oxidative phosphorylation. However, oxygen tension is about 21% in the atmosphere and reactive oxygen species (ROS) are produced as detrimental by-products during oxidative phosphorylation. Living organisms have developed anti-oxidant defense mechanisms comprised of enzymes and small molecules during evolution. The phylogenic study of superoxide dismutase (SOD) suggests that the evolutionary advent of SOD family occurred when the atmosphere became oxygen rich (about 1.5 billions year ago). The SOD family genes are conserved from yeast to humans during evolution (Landis and Tower, 2005).

**Free radical theory of aging**

Reactive oxygen species (ROS) are generally very small and highly reactive molecules partially reduced from oxygen ($O_2$). The primary cellular source for ROS generation has been proposed to be mitochondria. The free radical theory of aging was established (Harman, 1956). Mitochondria, cellular respiration sites as well as major sites of ROS generation, are proposed to be remnants of anaerobic bacteria based on phylogenic studies and morphology observations (Foth, 2007; Gabaldon and Huynen, 2007). Briefly, the mitochondrial oxidative phosphorylation system is composed of five protein-lipid enzyme complexes - NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:ferrycytochrome c

oxidoreductase (complex III), ferrocytochrome c:oxygen oxidoreductase (complex IV) and ATP synthase (complex V). Complex I, II, III and IV plus ubiquinone (Q) and cytochrome c compose the respiratory chain of mitochondria. Complex I catalyzes electron transfer from NADH or NADPH to ubiquinone homologs, ferricyanide and DNA. The reduction of ubiquinone is inhibited by rotenone, piericidin A, barbiturates and mecurials and complex I is a major source of superoxide production with complex ll and lll. The major component of complex II is succinate dehydrogenase (SDH), a membrane-bound enzyme of the citric acid cycle. Complex II catalyzes electron transfer from succinate to ubiquinone. The oxidized ubiquinone by complex I and II transfer electron to cytochorome c, a reaction catalyzed by complex III. The release of cytochorome c into the cytosol is one of the characteristics of intrinsic apoptosis. Complex IV is a multisubunit enzyme complex including cytochrome oxidase that catalyzes terminal transfer of electrons to oxygen. The formation of ROS is summarized as following chemical reactions.

(1) $O_2 + e^- \rightarrow \bullet O_2^-$ (superoxide anion radical)

(2) $\bullet O_2^- + e^- \rightarrow \bullet O_2^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)

(3) $H_2O_2 + e^- \rightarrow \bullet OH + OH^-$ (hydroxyl radical and hydroxide anion)

(4) $\bullet OH + e^- + H^+ \rightarrow H_2O$

(5) $H_2O_2 + Fe^{2+}$ $(Cu^+) \rightarrow \bullet OH + OH^- + Fe^{3+}$ $(Cu^{2+})$ (Fenton reaction)

Free radical formation in mitochondria is initiated with formation of superoxide anions ($\bullet O_2^-$) as by-products of respiration. Superoxide anions are further catalyzed to hydrogen peroxide by SOD2. Consequently, hydrogen peroxide is

catalyzed to water by catalase or Gpx enzyme family. The increase of ROS might result from the loss of cytochrome c or a defective respiratory chain as well as coenzyme Q in mitochondria (Kushnareva et al., 2002).

ROS are also produced by enzymes and small molecules in the cytosol. NADPH oxidase and xanthine oxidase family produce superoxide anions using oxygen as substrate following chemical reaction (1) (Linas et al., 1990; Cheng et al., 2001). The ROS-generative capacity of NADPH oxidases is the subject of much recent study. The catalytic subunit gp91phox (Nox2) of the NADPH oxidase in phagocytes produces superoxide anion ($O_2^-$) in response to microbes and immune response (Cheng et al., 2001). Various family members of NADPH oxidase have been identified in other cells and tissues, and are termed the Nox/Duox family. In humans, seven homologs in the Nox/Duox family have been identified, Nox1-5, Duox1 and Duox2 (Suh et al., 1999; Cheng et al., 2001; Shiose et al., 2001). Unlike mitochondrial generation of superoxide, Nox/Duox family generates superoxide anion in a highly regulated manner to perform a physiologic function, rather than being produced as by-products of normal cellular function. The Nox/Duox family is implicated in multiple biological responses such as phagocyte function in innate immunity (Cheng et al., 2001), proliferation (Arnold et al., 2001; Chamulitrat et al., 2003), apoptosis (Geiszt et al., 2003), receptor signaling (Lassegue et al., 2001; Mahadev et al., 2004) and biosynthesis of extracellular matrix (Edens et al., 2001). In smooth muscle, Nox2 and Nox4 have been known to increase superoxide anion production in human coronary atherosclerosis (Sorescu et al., 2008) and Nox1 overexpressing

transgenic mice have increased hypertension and cardiac hypertrophy (Dikalova et al., 2005), suggesting an important role for Nox1 in maintaining normal blood pressure.

All of the above-mentioned ROS differ in their activity. Hydroxyl radical (•OH) is known to be most reactive and destructive chemical among them. It is a strong oxidant capable of taking either an electron or a hydrogen from compounds and can be incorporated into an aromatic ring. Thus, the hydroxyl radical can damage directly all components of cells; DNAs, proteins, lipids, and amino acids. Hydroxyl radical can be formed by reactions (3) and (5) above. The fenton reaction (5) depletes transition metal ion such as $Fe^{2+}$ and Cu.

**Major antioxidant defense mechanism and implications in pathology**

In mammalian, the expression and activity of antioxidant enzymes are cell/tissue specific and subcellular localization of antioxidant enzymes are highly specialized. In addition to anti-oxidant enzymes, various small molecules such as vitamins function as antioxidants and contribute to regulating the redox-status. Thus, the picture of control of ROS production and redox-status in cells is exceedingly complicated and considerably more inter-connected than anticipated by Harman's original formulation, which emphasized mitochondria as a major site of ROS production. In mammals, multiple anti-oxidant enzymes and small molecules have been identified and their functions have been studied.

SODs are metalloenzymes that catalyze the dismutation of two superoxide anions ($O_2^-$) into hydrogen peroxide ($H_2O_2$) and $O_2$. Three types of SODs are

known in mammals. SOD1, also known as Cu, Zn-SOD, is dimeric, mostly abundant in the cytosol and requires Cu, Zn as cofactors. SOD1 is implicated in neurological disorders such as amyotrophic lateral sclerosis (ALS) (Bowling et al., 1993; Rossen et al., 1993). ALS is a clinically and genetically heterogeneous late-onset neurodegenerative disease accompanied with degeneration of cortical and spinal motor neurons. Mutations of Sod1 gene are found to cause ALS and more than 100 different Sod1 mutations have been identified to cause the similar phenotype of this disease (Beckman et al., 2002). Neuron-specific expression of mutant SOD1 was known to be sufficient to induce ALS-like phenotype supported by transgenic mice (Jaarsma et al., 2008). In addition, the number of neurons and axons in mutant SOD1 transgenic mice decreased and ubiquitin expression increased in the spinal neurons of these mice (Jaarsma et al., 2008; Wang et al., 2008).

SOD2, also known as Mn-SOD, is tetrameric, localized in mitochondria and requires Mn as a cofactor. SOD2 is the first anti-oxidant defense enzyme against ROS generated in mitochondria because SOD2 is strictly localized in mitochondria. Various pathologies are implicated with aberrant SOD2 expression including cardiomyopathy (Li et al., 1995), hemolytic anemia (Friedman et al., 2004), seizures (Liang et al., 2004), cancers (Connor et al., 2007), tumor metastasis (Nelson et al., 2003), Alzheimer's disease (Esposito et al., 2006), hypertension (Rodriguez-Iturbe et al., 2007) and aging (Kokoszka et al., 2001). SOD2 knock-out mice have similar phenotypes and are embryonically or

neonatally lethal depending on the mouse strain (Li et al., 1995; Huang et al., 2001).

SOD3, a secretory copper enzyme, also known as EC-SOD, is localized in the extracellular matrix and classified into A, B, and C depending on the affinity to heparin (Sandström et al., 1993). SOD3 is implicated mainly in cardiovascular disorders and ischemia because its role is modulating the levels of extracellular superoixde anion generated in the vasculature (Qin et al., 2005). SOD3 knock-out mice have increased cerebral infarct volume after ischemic injury (Sheng et al., 1999). SOD3 overexpressing transgenic mice have attenuated aneurysmal subarachnoid hemorrhage (SAH) which is known to be caused in part by the increased superoxide anion production (McGirt et al., 2002).

Glutathione peroxidase (Gpx) catalyzes the reduction of $H_2O_2$ or organic hydroperoxides to $H_2O$. Free glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine), is a cofactor in the reaction (for Gpx1-6), and is converted into glutathione disulfide (GSSG) during Gpx-mediated catalysis. Except for Gpx5 and Gpx6, the other Gpx enzymes also use selenocysteine (SeCys) as a cofactor. In mammals, the Gpx family includes six proteins with distinct but overlapping amino acid sequences, substrate specificity and subcellular localization. Cytosolic (Gpx1), gastrointestinal (Gpx2), plasma (Gpx3), phospholipids hydroperoxide (Gpx4), epididymal (Gpx5) and olfactory epithelium (Gpx6) have been identified as major sites of expression for the different family members (Brigelius-Flohe, 1999). Various pathologies are implicated as a consequence of changes in Gpx expression including colorectal cancer (Murawaki et al., 2008), atherosclerosis

(Espinola-Kein et al., 2007), hypertension (Kashyap et al., 2005), endothelial dysfunction (Galasso et al., 2006) and aging (Ji et al., 1990). In addition, Gpx4 knock-out is embryonic lethal in mice, and Gpx4 heterozygous knock-out mice have decreased survival when they are exposed to cytotoxic oxidative stress (Yant et al., 2003).

Catalase mediates the same reaction mediated by the Gpx family; dismutation of two $H_2O_2$ into $H_2O$ and oxygen. Most catalase is strictly localized in peroxysome in eukaryotic cells, and biogenesis of the peroxysome is also one of the critical antioxidant defense mechanisms. Aberrant change of catalase expression has been implicated in liver tumorigenesis (Litwin et al., 1999), colon carcinoma (Lauer et al., 1998), liver allograft rejection (Steinmetz et al., 1998) and ischemia-reperfusion injury (Singh, 1996). The important role of catalase during aging has been emphasized by transgenic relocalization and overexpression of catalase in mitochondria (Schriner et al., 2005). Transgenic mice overexpressing catalase in mitochondria showed decreased age-dependent arteriosclerosis and increased genomic stability with catalase activity increased up to 50-fold in mitochondria of cardiac and skeletal muscle (Schriner et al., 2005). The most striking result from these studies was increased lifespan (about 17-21%) of the mt-catalase transgenic mice compared to wild-type mice (Schriner et al., 2005). This result suggests that catalase might be key in oxidant-mediated regulation of longevity, but the expression of catalase in a place where it is not normally expressed may also mean that the longevity effect in not related to a normal physiologic role for catalase alone in longevity.

Small molecules also function as antioxidants. Glutathione (GSH) is the most abundant non-protein thiol in mammalian cells. The role of GSH is includes its function as a substrate for glutathione S-transferase, which catalyzes GSH conjugation and leads the detoxification of xenobiotic compounds, and for Gpx as noted earlier. GSH is oxidized to its disulfide form, GSSG after these enzyme reactions and the ratio of GSH/GSSG is a standard surrogate measure of intracellular redox status (Erden-Inal et al., 2002). GSH is implicated in many diseases for which oxidative stress plays an important role in diseases generation and progression. The importance of GSH as a ubiquitous antioxidant is now widely appreciated. For example, tumor progression increased with the decreasing ratio of GSH/GSSG and oxidative stress caused by tumor promoters was not decreased by the supplementing free GSH (Navarro et al., 1999).

Thioredoxin is a ubiquitous 12 kDa redox regulator with multiple biological functions. Reduced thioredoxin functions like a protein disulfide oxido-reductase. Oxidized thioredoxin is converted back to reduced form by thioredoxin reductase. Thioredoxin as well as GSH is one of the important thiol-disulfied based redox buffers in cells. The members of thioredoxin family are thioredoxin 1 (Trx1; cytosolic) and thioredoxin 2 (Trx2; mitochondrial). Thioredoxin has a growth factor effect on MCF-7 breast cancer cell by increasing cell proliferation when it is added into culture medium *in vitro* (Gasdaska et al., 1995) and functions as a signaling molecule by augmenting the expression of tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Schenk et al., 1996). Transgenic mice overexpressing Trx are protected against reperfusion injury in the brain (Takagi et al., 1999),

have increased the glucose metabolism (Umekawa et al., 2008), and extended life span (Mitsui et al., 2002).

**Roles of ROS during aging**

The detrimental effects of ROS produced by enzymes or non-enzymatic source like mitochondria will accumulate in cells over the life span and eventually result in the death of the organism. Consistent with this view, all cellular components - DNAs, proteins and lipids – have been shown to be progressively damaged directly by ROS. However, though lower organism lifespan has a clear connection to function of specific antioxidants (Sun and Tower, 1999; Sun et al., 2002; Sun et al., 2004), deletion of single antioxidants in mouse models has not resulted in a clear connection to lifespan (Van Remmen et al., 1999; Muller et al., 2006), likely because of complex interactions and redundancies in the antioxidant systems. Independent of longevity, detrimental effects of ROS are implicated in many diseases associated with aging (Finkel and Holbrook, 2000) including cancer (Klaunig and Kamendulis, 2004), atherosclerosis (Qin et al., 2005), aging (Finkel and Holbrook, 2000), and muscular atrophy (Pansarasa et al., 1999; Muller et al., 2007).

ROS also function as signaling molecules in normal (non-pathologic) processes resulting in various biological responses in cells. ROS promote cell proliferation (Foreman et al., 2003; Geiszt and Leto, 2004), skeletal muscle and differentiation (Franco et al., 1999; Li et al., 2006), apoptosis (Haendeler et al., 2002; Choi et al., 2006), receptor signaling (Choi et al., 2005; DeYulia et al.,

2005), migration (Chiarugi et al., 2003; Giannoni et al., 2005) and signaling cascades (Cho et al., 2004; Kamata et al., 2005). Currently, how these biological responses are regulated by the temporal and transient oxidative stress is not well-understood. However, for some of these functions, the redox-sensitive sites of proteins have been identified and studied in some detail (Aslund et al., 1999; Lee et al., 2002; Zhou et al., 2004; Ali et al., 2006; Bossis and Melchior, 2006; Kohli et al., 2007).

Several methods are currently applicable to measure specific ROS. First, electron spin trapping is applicable to detect superoxide anion or hydrogen peroxide. Mechanism of this method is using the reaction of unstable free radical with diamagnetic molecule (the spin trap) to form a relatively stable free radical. Radical adducts formed can be detected and the amount of the specific ROS can be quantitated. A vast majority of spin traps form radical adducts through the addition of the radical to the trap to form a nitroxide radical. Two major classes of traps are widely used such as nitrones and nitroso compounds. This method is fairly specific for quantitation of ROS using cells. For example, the inhalation of $H_2$ gas markedly suppresses brain injury by buffering the effects of oxidative stress and the decrease of hydroxyl radical is identified as target of $H_2$ gas by electron spin trapping (Ohsawa et al., 2007). Another example is that a time-dependent ROS production and hydrogen peroxide release from mitochondria of human hepatoblastoma cell line (HepG2/C3A) in response to palmitate is measured using electron spin trapping (Srivastava and Chan, 2007). Disadvantages of this method are low sensitivity, higher cost of equipment, and

possible artifacts due to difficulty of differentiating hydroxyl radical from hydroxyl-like species. Second, multiple fluorescent chemicals, which react with ROS, are available for use with either the fluorescence microscope or fluorometer. This method is easier to carry out than the spin trap method but more difficulty comes from unspecific reaction with heterogeneous radical-like compounds inside cells. A third method is to evaluate the overall redox-status inside cells because the increase or decrease of specific ROS eventually changes the redox-status in cells. As mentioned above, the ratio of GSH to GSSG is a standard parameter, for this type of measurement, and can be measured using colorimetric methods or high performance liquid chromatography. There may be difficulty in connecting the redox-status with the increase or decrease of ROS because of the complexity of ROS reactions in cells.

**Skeletal muscle development and regeneration**

Skeletal muscle comprises about 50% of the body mass and generates force through the contractile movement of myofibers. Muscle mass is maintained and regenerated to form myofibers through the activation of the quiescent satellite cells during the entire life span. At molecular level, there is a common mechanism underlying muscle differentiation between embryogenic myogenesis and adult myogenesis.

After fertilization, the zygote makes its way to the uterus, which takes three to four days in mice and five to seven days in humans. As it moves, the zygote divides. The first cleavage produces two identical cells and then divides

again to produce four cells. The cells divide further asynchronously to produce 8 cells, 16 cells, and so on (Gilbert, 2000). By the 16-cell stage, the compacted embryo is called a morula in which maternal influences such as mRNA and proteins in the oocyte cytoplasm are significantly decreased and the cells of the embryo adhere tightly each other. Cells have become specialized and form an outer rim of cells called the trophectoderm and an inner core of cells, the inner cell mass (Gilbert, 2000). The cells of the inner cells mass will give rise to all the tissues of the embryo's body as well as to nontrophoblast tissues that support the developing embryo. The cells of the inner cell mass and trophectoderm divide continuously and form a cavity called blastocoel (Beddington and Robertson, 1999). It is filled with a fluid secreted from trophectodermal cells. As a result of captivation (a process that occurs during the formation of the blastocyst and establishes the polarity of embryonic cells) and the separation and differentiation of the trophectoderm from the inner cell mass, morula becomes a blastocyst. The blastocyst will generate three primary germ layers - endoderm, mesoderm and ectoderm through a process called gastrulation (Gilbert, 2000). The mesodermal layer is created through gastrulation, and located between the ectoderm and the endoderm. The mesoderm is divided into the axial mesoderm (near notochord), intermediate mesoderm, paraxial mesoderm and the lateral plate mesoderm. Most skeletal muscles are derived from the paraxial mesoderm (Brand-Saberi et al., 1996). Somites (segmentally arranged blocks of mesoderm lying on either side of the notochord and neural tube during development of the vertebrate embryo) are formed through the separation of the paraxial mesoderm into two

cell clusters. Cells of ventral part of somites form sclerotome, which eventually forms the vertebrate ribs. This process is accompanied by down-regulation of Pax3 and Pax7, members of paired/homeodomain transcription factor family (Ben-Yair and Kalcheim, 2005; Gros et al. 2005; Esner et al., 2006). Cells from the dorsal part of somites will form dermomyotome and maintain expression of Pax3 and Pax7, with dermomyotome, a major source for the musculature (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). An epaxial and hypaxial parts of somites will be formed from dermis and generate the back muscle and limb muscle. The function of genes regulating developmental myogenesis have been elucidated using transgenic animals. Both of c-Met (the receptor for hepatocyte growth factor) and Pax3 knockout mice have impaired myogenic cell migration to the developing limb (Dietrich et al., 1999; Relaix et al., 2004). In addition, Pax3 is essential to muscle cell survival (Bober et al., 1994; Goulding et al., 1994). After the migration of cells at the edges of the dermomyotome, down-regulation of Pax3 follows, and the myogenic regulatory factors (MRFs: including myf5, MyoD, Mrf4 and myogenin) are up-regulated and regulate the differentiation of myoblasts (Tajbakhsh et al., 1996; Kassar-Duchossoy et al., 2004). Among four MRFs, myf5 and MyoD are suggested as the determination MRFs because they are upregulated earlier than Mrf4 and myogenin and required for turning on the expression of Mrf4 and myogenin during limb myogenesis. More significantly, double knock-out mice which have disrupted both myf5 and MyoD have failed to either produce or sustain myoblast population (Megeney and Rudnicki, 1995; Arnold and Brown,

1996; Yun and Wold, 1996). Compared to myf5 and MyoD, myogenin and Mrf4 are differentiation MRFs supported by finding that myogenin knock-out mice are highly deficient in muscle differentiation while myoblasts population is normal (Megeney and Rudnicki, 1995; Arnold and Brown, 1996).

Myogenesis in the adult continues postnatally during growth and developmental myogenesis is recapitulated in ongoing repair of skeletal muscle to maintain the muscle mass during the entire life span. Satellite cells are known as muscle stem cells which are located between the basal lamina and muscle fibers in muscle observed under electron microscopy (Mauro, 1961; Hawke et al., 2001). These cells are normally quiescent, but can be stimulated to enter cell cycle, proliferate, undergo self-renewal and differentiate into mature myofibers. Active proliferating muscle progenitor cells (differentiated from satellite cells) are also called myoblasts, and are committed to a muscle fate. Two factors that activate quiescent satellite cells into myoblasts have been identified; (1) hepatocyte growth factor (HGF) (Allen et al., 1995; Tatsumi et al., 1998), and (2) nitric oxide (NO) (Anderson, 2000; Tatsumi et al., 2002). Consistent with HGF as an activation factor, c-met (receptor of HGF) is identified to express in quiescent and activated satellite cells (Tatsumi et al., 1998). A burst of NO produced from nitric oxide synthase (NOS) within basal lamina of damaged muscle fiber is suggested as an initial signal for the activation (Anderson, 2000). The activation of satellite cells is enhanced in response to exercise damage or muscle degenerative diseases when the myoblasts are required for growth, repair or regeneration of muscle. The regulatory factors for the activation of satellite cells

are similar to embryonic myogenesis which is regulated by Pax3, Pax7 and Myf5 (Relaix et al., 2006). Quiescent satellite cells expressing Pax7 migrate into injury site and the up-regulation of either Myf5 or MyoD activates the proliferation of myoblasts (Braun et al., 1992; Rudnicki et al., 1993). Terminal differentiation of myoblasts is accompanied with the down-regulation of Pax3 and Pax7 and the up-regulation of myogenin and Mrf4 (Olguin et al., 2004; Relaix et al., 2006). Eventually, these myoblasts differentiate, fuse and become new myofibers or fuse to existing damaged myofibers and repair them. Consistent with embryo myogenesis as above-mentioned, myf5 and MyoD are suggested as the determination MRFs because they are essential for myoblast formation while myogenin and Mrf4 are required for terminal differentiation of myoblasts.

Sarcopenia (also known as muscle atrophy with aging), the loss of skeletal muscle mass and strength occurs during aging and decreases the performance of aged muscle. Sarcopenia is accompanied with both decreased regenerative potential and acceleration of existing muscle wasting. Impairment of the regenerative potential is known to be due to the decreased number and function of satellite cells during aging (Shefer et al., 2006). The mechanism of sarcopenia is complex and broad, and involves increased ROS, mutations in mitochondrial DNA and/or apoptosis (Papa and Skulachev, 1997; Pak et al., 2003). On the other hand, muscle wasting due to unweighting, cachexia or renal disease is accompanied by the activation of muscle wasting ligases such as atrogin-1 and MuRF-1 through signaling pathways such as PI3K-Akt signaling pathway (Sandri et al., 2004; Stitt et al., 2004). The question arises whether there is a different

mechanism between sarcopenia and muscle wasting or whether a universal mechanism governs both. This thesis will focus primarily on how oxidative stress affects differentiation of myoblasts, regeneration of muscle and signaling pathways.

**The source of ROS and its generation in myoblasts**

The decreased expression of various anti-oxidant enzymes has been reported as the source of oxidative stress in myoblasts (Van Remmen., 1999; Fulle et al., 2004). Among three types of SODs, SOD1 and SOD2 are identified to be expressed in both muscle and myoblasts (Van Remmen., 1999). SOD1 knock-out mice develop normally and exhibit about 17-20% lower muscle mass than wild-type mice and rapid decrease of muscle mass during aging (Muller et al., 2006). Myoblasts isolated from SOD1 knock-out mice rapidly died *in vitro* and myoblasts isolated SOD1 heterozygous knock-out had a significantly decreased myotube formation *in vitro* (Lee., Unpublished data).

SOD2 knock-out mice are embryonically or neonatally lethal depending on the mouse strain (Li et al., 1995; Huang et al., 2001). In muscle tissue, both of mitochondrial ROS production and SOD2 enzyme activity are known to increase during aging (Gianni et al., 2004). SOD2 heterozygote mice have a similar life span compared to wild-type. The levels of mRNA coding for the major antioxidant enzymes (CuZnSOD, catalase, and glutathione peroxidase) are not significantly altered in liver, kidney, heart, lung, or brain in the SOD2 heterozygote mice (Van Remmen et al., 1999). Interestingly, glutathione peroxidase activity and the ratio

of GSH/GSSG decrease approximately 50% in the muscle of SOD2 heterozygote mice. Myoblasts isolated from SOD2 heterozygous knockout mice had a severe defect in terminal differentiation to form myotubes *in vitro* (Lee, Unpublished data).

SOD3, also known as EC-SOD, mRNA of SOD3 has been identified to be expressed at the message level in muscle tissue (Oury et al., 1992; Folz and Crapo, et al., 1994), but its protein-level expression and role are still unclear. No data are concerning myoblast expression of SOD3.

In muscle, Gpx1 and Gpx4 are expressed (Zhang et al., 1989; Knopp et al., 1999). Gpx1 knock out mice develop normally, but are exquisitely sensitive to paraquat and diquat (Cheng et al., 1999). In Gpx1 knock-out mice, lipofuscin, a senescence marker, forms in muscle and connective tissue earlier than in wild-type (Lee et al., 2006). Gpx1 knock-out myoblasts have impaired terminal differentiation, which might result from decreased proliferation and increased apoptosis (Lee et al., 2006).

Gpx4 knock-out is embryonically lethal in mice, and Gpx4 heterozygous knock-out mice have decreased survival when they are exposed to cytotoxic oxidative stress (Yant et al., 2003). Gpx4 heterozygous myoblasts exhibit cell aggregations in growth medium *in vitro* (Lee, Unpublished data). Gpx enzyme activity in human satellite cells is known to decrease during aging (Fulle et al., 2007). No data are concerning myoblast expression of other Gpx enzymes.

Antioxidant activity of catalase in satellite cells derived from the elderly is significantly decreased compared to that in cells isolated from young individuals

(Fulle et al., 2007). In addition, cell membrane fluidity is considerably decreased in aged human satellite cells while basal $Ca^{2+}$ levels increase significantly in an age-dependent manner (Fulle et al., 2007). When myoblasts are exposed to higher oxidative stress such as $H_2O_2$, catalase activity increases about 4 to 5 fold compared to normal status (Franco et al., 1999). These data might suggest that there is a different regulation of catalase expression in response to acute and sustained oxidative stress.

Various family members of NADPH oxidase have been identified in muscle and muscle precursor cells. Among seven homologs in the Nox/Duox family, Nox2, Nox4, p22, p47 and p67 were detected in human and murine myoblasts (Mofarrahi et al., 2008). The Nox/Duox family is implicated in muscle differentiation. In skeletal muscle, Nox2 is known to stimulate muscle differentiation through activation of NF-κB/iNOS pathway, the downstream of PI3-kinase and p38 MAPK (Piao et al., 2005). Nox2 activity increases during muscle differentiation and treatment of C2C12 cells with siRNA against Nox2 decreased muscle differentiation. However, there is no effect on muscle differentiation by knocking down Nox1 (Piao et al., 2005). In smooth muscle, Nox2 and Nox4 are known to increase superoxide anion production in human coronary atherosclerosis (Sorescu et al., 2002), suggesting that up-regulation of Nox activity is implicated in cardiac disease.

The ratio of GSH/GSSG is a standard surrogate measure of intracellular redox status as noted earlier (Erden-Inal et al., 2002). In muscle, free GSH is known to decrease during exercise as it is used to counteract increased oxidative

stress (Viguie et al., 1993). GSH is depleted rapidly in clinically ill patients after many kinds of surgical procedures, trauma and age-related chronic diseases (Hammarqvist et al., 1997). *In vitro*, C2C12 cells treated with buthionine-sulfoximine (BSO) have impaired myogenesis due to the depletion of GSH by BSO (Ardite et al., 2004).

## Signaling pathways that participate in muscle differentiation and regeneration and their possible redox regulation

ROS mediates diverse biological responses through redox-sensitive signaling pathways in muscle differentiation and regeneration. In this section, IGF/PI3kinase/Akt, mitogen-activated protein kinase (MAPK) cascade, NFκB and HIF-1α signaling pathways will be discussed in terms of redox-regulation of these pathways specifically in muscle differentiation and regeneration.

### IGF-1/PI3kinase/Akt

Insulin-like growth factor 1 (IGF-1) expression is increased during muscle work and its delivery or overexpression results in muscle hypertrophy via autocrine and paracrine signaling (DeVol et al., 1990). Transgenic mice overexpressing IGF-1 under control of a muscle specific promoter increase muscle mass about two-fold (Musarò et al., 2001). When IGF-1 binds to IGF-1 receptor tyrosine kinase, the receptor trans-phosphorylates itself, resulting in subsequent phosphorylation of insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1 activates phosphatidylinositol 3-kinase (PI3K) and results

in the production of phosphatidylinositil-3,4,5-triphosphate ($PIP_3$) from phosphatidylinositil-4,5-bisphosphate ($PIP_2$). $PIP_3$ can be reversed to $PIP_2$ catalyzed by the $PIP_2$ phosphatase, PTEN. $PIP_3$ provides the docking site at the plasma membrane for the serine/threonine kinase, Akt. Akt is phosphorylated by PDK-1 at the membrane and activated for the phosphorylation of downstream target proteins which mediate cell proliferation, survival, protein synthesis and the activation of transcription factors. Three isoforms (Akt 1/PKBα, Akt2/PKBβ and Akt3/PKBγ) of Akt exist encoded by the different genes but have more than 85% amino acid sequence identity (Alessi and Cohen., 1998; Coffer et al., 1998; Nakatani et al., 1999). Akt1 knock-out mice are viable but smaller than wild-type littermates (Chen et al., 2001). However, when they were exposed to γ-irradiation, survival of Akt1 knock-out mice decreased compared to wild-type (Chen et al., 2001). Selective inhibition of Akt1 is known to block MyoD-mediated muscle differentiation in the transformed mesenchymal murine C3H10T1/2 fibroblasts (Wilson and Rotwein, 2007). However, the selective knockdown of Akt2 had no effect on myoblast survival and differentiation (Wilson and Rotwin, 2007). Interestingly, Akt2 knock-out mice have insulin resistance and diabetes mellitus and this phenotype is accompanied by expansion of beta cell mass in pancreatic islets (Cho et al., 2001), supporting a role for regulation of glucose metabolism by Akt2 (Ng et al., 2008; Tassi et al., 2008). The distinct phenotypes of Akt1 knock-out and Akt2 knock-out mice suggest that the selective activation of Akt1 might stimulate its downstream targets for protein synthesis without the disruption of the glucose homeostasis.

Two critical phosphorylation sites for the activation of Akt are threonine 308 (T308) and serine 473 (S473). These sites are known to be phosphorylated in response to growth-factor via PI3K in contrast to other constitutive phosphorylation sites such as serine 124 (S124) and threonine 450 (T450) of Akt (Bellacosa et al., 1998). Site-directed mutagenesis supports the idea that mutation of these phosphorylation sites into non-phosphorylatable sites (dominant negative) abolishes the activation of Akt in response to IGF-1 or insulin, whereas mutations to acidic residues (constitutive activation) render the kinase active independent of growth factors (Bellacosa et al., 1998). Each phosphorylation site might have a distinct role for the activation of downstream targets. Ablation of mTORC2, Akt S473 kinase, changed the phosphorylation status of FOXO3 but not TSC2 or GSK3β, substrates of Akt (Guertin et al., 2006). S473 phosphorylation of Akt results in 5 to 10-fold increase of Akt activity but is not required for the stimulation of Akt by insulin and IGF-1 (Alessi et al., 1996). In addition, there is no known signaling which causes T308 phosphorylation without S473 phosphorylation. The possible role of S473 phosphorylation dictating the choice of downstream target is suggested by a genetic study (Jacinto et al., 2006) but further work is required to understand the differential role of the two phosphorylations. The function of Akt is well characterized in cell growth and the activation of muscle wasting in muscle biology. mTOR complex 1 (mTORC1 or the mTOR-raptor complex) activated by phospho-Akt increases the translation initiation and ribosome biosynthesis in response to growth factors and nutrients (Wullschleger et al., 2006). Tumor cells, which grow aberrantly and activate the

PI3K-Akt pathway, show increased sensitivity to mTOR inhibitors, such as rapamycin, suggesting the importance of mTOR as one of the downstream target for Akt (Aoki et al., 2001). The substrates of mTOR, such as S6 kinases (S6K1-2) and eukaryotic initiation factor 4E (eIF4E)-binding protein1 (4E-BP1) might be affected by the activation of Akt through mTOR but it is not clear whether the substrates of mTOR are directly affected by the activation of Akt. In muscle, mTOR and S6K activation by the PI3K/Akt pathway is crucial for muscle growth (Bodine et al., 2001b; Rommel et al., 2001; Ohanna et al., 2005; Mieulet et al., 2007). Muscle hypertrophy indicated by about 10% more nuclei per myotube in response to IGF-1 can be abolished by treatment with the mTOR specific inhibitor, rapamycin, in C2C12 cells (Rommel et al., 2001). Muscle hypertrophy caused by *in vivo* transfection of constitutive active form of Akt into rat muscle is also blocked by the oral administration of rapamycin (Bodine et al., 2001b). These results support a model in which the activation of mTOR is crucial for muscle differentiation and growth. S6K1, a substrate of mTOR, is also critical for muscle cell growth supported by the finding that S6K1 knock-out mice have decreased muscle mass and muscle fiber size compared to wild-type, and S6K1 knock-out myoblasts are fusion-defective during terminal differentiation (Ohanna et al., 2005). At the molecular level, S6K1 knock-out myoblasts have decreased global protein synthesis without a change in levels of the transcription of muscle wasting genes such as Atrogin-1 and MuRF-1 (Mieulet et al., 2007).

FOXO proteins are transcription factors, downstream of Akt, that are involved in diverse biological functions such as cell proliferation, survival,

differentiation, and metabolism (Accili and Arden, 2004; Barthel et al., 2005). Three of the four FOXOs (1, 3a and 4a) are substrates of Akt and have PKB motifs at N- and C- terminal; FOXO6 lacks C-terminal PKB and is localized constitutively in the nucleus (Van der Heide et al, 2005). FOXO factors function as a convergence point for signaling in oxidative stress and growth factor stimulation. PI3K/Akt-dependent phosphorylation of FOXOs is crucial to the regulation of FOXOs and the phosphorylated FOXOs translocate from nucleus to cytosol (Takaishi et al., 1999; Bos et al., 2001; Burgering and Kops, 2002). Various target genes, which are induced by active FOXOs, have been identified including the cell cycle inhibitors, p15 (Katayama et al., 2007), p19 (Katayama et al., 2007), p21 (Nakae et al., 2003) and p27 (Motta et al., 2004), metabolic genes, G6pc (Housley et al., 2008) and Igfbp1 (Kim et al., 2003a), apoptotic mediators, Bim (Stahl et al., 2002; Sunters et al., 2003), Hid (Luo et al., 2007), antioxidant genes, Sod2 (Brunet et al., 2004), catalase (Alcendor et al., 2007), stress response gene, Gadd45α (Tran et al., 2002), autophagy mediators, Bnip3 (Mammucari et al., 2008), and LC3 (Mammucari et al., 2008) and muscle-specific atrophy genes, atrogin-1 (Sandri et al., 2004) and MuRF1 (Stitt et al., 2004).

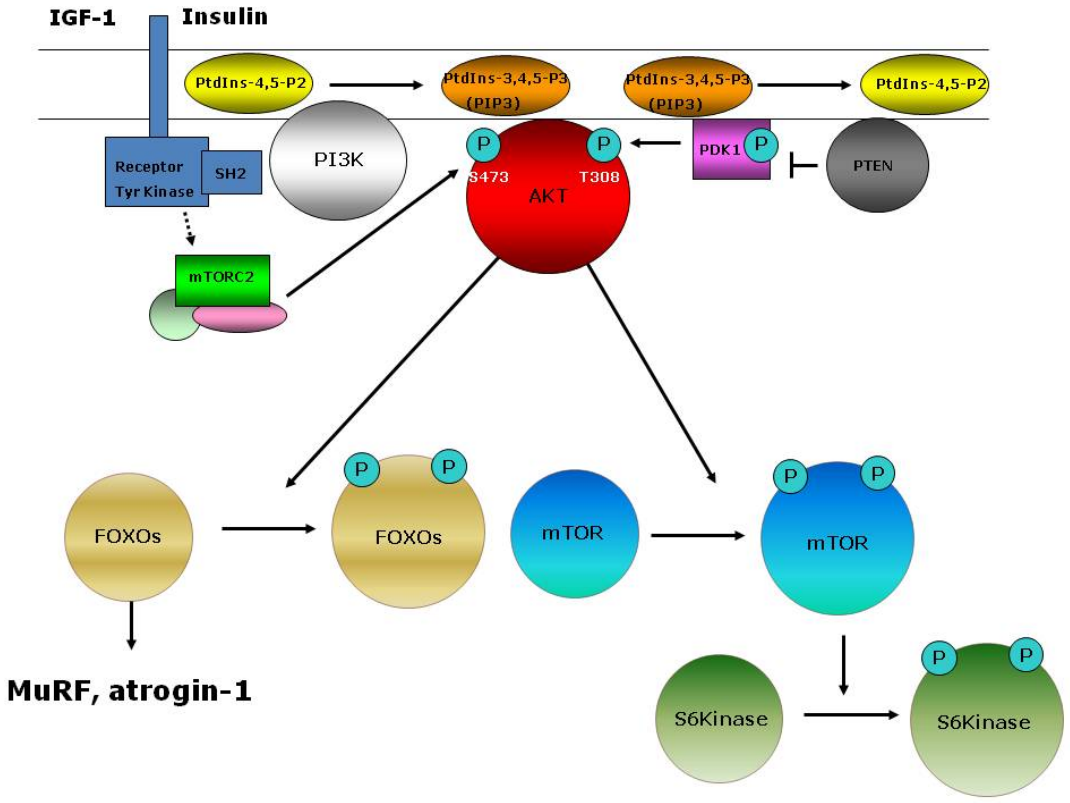
In muscle, the specific role of FOXOs is well characterized during myocyte differentiation (Wu et al., 2008) and muscle atrophy (Sandri et al., 2004; Stitt et al., 2004). Expression of constitutively active form FOXO1 inhibits muscle differentiation through the negative regulation of PI3K-Akt-mTOR (Wu et al., 2008). Transgenic mice overexpressing constitutively active FOXO1 have smaller muscle mass compared to wild-type controls and decreased levels of

mTOR, Raptor and the decreased phosphorylation of S6K, all known to be essential for protein synthesis (Southgate et al., 2007). Increased active FOXO proteins up-regulate muscle-specific ubiquitin ligases such as atrogin-1 (MAFbx) and MuRF1 *in vitro* and *in vivo* and muscle atrophy is accelerated through the inactivation of PI3K/Akt/FOXO pathway (Sandri et al., 2004; Stitt et al., 2004).

Redox regulation of PI3K/Akt pathway is supported by many studies (Deora et al., 1998; Niwa et al., 2003; Connor et al., 2005; Lu et al., 2007). $H_2O_2$-induced oxidative stress increases the phosphorylation of Akt at T308 and S473 in various cells *in vitro* (LaHair et al., 2006). In more detail, the phosphorylation of T308 or S473 in response to $H_2O_2$ is either dependent on PI3K or calcium/camodulin-dependent kinase (CaM-K) activity (LaHair et al., 2006). The downstream targets of Akt are also known to be regulated by oxidative stress. The phosphorylation and activity of S6K regulated by PI3K/Akt/mTOR pathway decreased by $H_2O_2$ treatment in rat hepatoma cell line H4IIE (Patel et al., 2002). FOXOs are activated to defend cells from oxidative stress by up-regulating anti-oxidant enzyme such as SOD2 (Brunet et al., 2004) and induce G2/M cell cycle arrest due to oxidative stress in C2C12 cells *in vitro* (Furukawa-Hibi et al., 2002). While many results support that PI3K/Akt pathway is redox-regulated, the target protein modified or activated by the oxidative stress is variable depending on the cell type and the concentration of ROS. Though exogenous administration of $H_2O_2$ activates PI3K-Akt pathway, the physiological levels of oxidants regulating the pathway are not known, and the mechanism of specific protein modifications by ROS are incompletely characterized.

**Figure 1.1**

**Major role of the PI3K-Akt pathway in muscle maintenance**



**MAPK cascade**

MAPK (mitogen-activated protein kinases) pathways are multi-layered signaling cascades. The MAPK family of proteins consists of four major subfamilies, p38 MAPK, ERKs (extracellular signal-regulated kinases), JNKs (c-Jun $NH_2$-terminal kinases) and ERK5 or big MAPK. The MAPK subfamily is composed of three sequentially acting kinases, MEKK (mitogen activated protein kinase kinase), MEK (mitogen activated protein kinase) and MAPK, resulting in the activation via the sequential phosphorylation of downstream kinases. The primary functions of the MAPK pathway are to regulate the function of proteins in the cytoplasm and the transcription of diverse genes involved in cell growth, proliferation, differentiation, metabolism, transcription, translation and remodeling. Though considerable detail is known about these pathways, the integration of their signaling into coherent cellular behavior is a complex process, requiring mathematical treatment (Poon and Ferrell, 2007). In general, ERKs regulate anabolic processes such as cell growth, survival and differentiation while p38 MAPK and JNKs modulate genes in response to stress such as oxidative stress. The functions of activated p38 MAPK, ERKs and JNK pathways are well characterized in myogenesis (Frost et al., 2006; Li and Johnson, 2006; Lluis et al., 2006; Strle et al., 2006; Yang et al., 2006; Perdiguero et al., 2007).

p38 MAPK is activated via phosphorylation by the sequential cascades of MAPKKK (TAK1, ASK1, DLK and MEKK4) and MAPKK (MKK3 and MKK6). In mammals, four isoforms of p38 have been identified: p38α, β, γ and δ. Among these, p38α and β are involved in muscle differentiation. The requirement of

p38α and β in myogenesis was initially supported by the finding that the treatment of C2C12 cells with SB203580, the specific inhibitor of both p38α and β, impairs the fusion of myoblasts and decreases muscle specific gene expression during differentiation (Jones et al., 2005). More convincingly, the myoblasts isolated from p38α knock-out mice have a phenotype of impaired fusion and differentiation compared to wild-type myoblasts and the cross-sectional fiber area of muscle of these mice is smaller than those of heterozygous p38α knock-out mice (Perdiguero et al., 2007). The molecular mechanisms underpinning p38α and β function during myogenesis have been recently further elucidated. Activated p38α and β directly phosphorylate myocyte enhancer factor (MEF) family members such as MEF2A and MEF2C during myogenesis thereby increasing the transcription of MEF-dependent muscle specific genes (Zetser et al., 1999; Wu et al., 2000). MEFs are co-factors for the MyoD family of bHLH genes hence the induction of MyoD-dependent transcription by p38 might be mediated through MEF (Wu et al., 2000).

The redox sensitivity of the p38 pathway has been widely described in various cell types. Briefly, the phosphorylation or the activity of p38 is increased in response to oxidative stress in mouse NIH 3T3 cells (Guyton et al., 1996), rat arterial smooth muscle cells (Ogura and Kitamura, 1998), H9c2 rat cardiac myoblasts (Aikawa et al., 1997; Zu et al., 1997), rat vascular smooth muscle cells (Ushio-Fukai et al., 1998) and rat cardiomyocytes (Clerk et al., 1998).

In the ERK pathway, Ras activates the serine/threonine kinase Raf by recruiting Raf to the membrane in response to extracellular signals transduced

via receptor tyrosine kinases, G-protein-coupled receptors (GPCRs) or integrins. The activated Raf phoshorylates and activates MEK. ERKs are activated by MEK and downstream targets of ERKs are, in turn, further regulated. The major functions of ERKs include regulation of cell cycle (Blalock et al., 1999; Malumbres et al., 2000; Chang et al., 2001), cell transformation (Davies et al., 2002; Fransen et al., 2004; Wan et al., 2004), apoptosis (Ley et al., 2003; Luciano et al., 2003; Harada et al., 2004) and response to oxidative stress (Buder-Hoffmann et al., 2001; Kim et al., 2001; Xiao et al., 2002). Two isoforms of ERKs, ERK1 (p44 MAPK) and ERK2 (p42 MAPK) exist in mammals. ERK1 and ERK2 are ubiquitously expressed and distinctive role for the two isoforms are not clearly understood. The function of the two isoforms might be different considering that ERK1 knock-out mice are viable and fertile while ERK2 knock-outs are embryonic lethal (Pages et al., 1999). In addition, ERK1 can be compensated by ERK2 (Pages et al., 1999), but ERK1 is unable to restore the function of ERK2. Interestingly, ERKs have dual functions, positive as well as the negative regulation of muscle differentiation depending on the extracellular signaling. MM14 mouse myoblasts treated with bFGF which stimulates proliferation and represses differentiation of myoblast results in activation of ERKs and inhibition of differentiation (Campbell et al., 1995). This effect may be mediated in part through cell cycle regulators interacting with myogenic pathways (Skapek et al., 1995). ERKs activated by cyclic mechanical stress decrease myogenic differentiation of adult bovine satellite cells (Kook et al., 2008). ERK2 has a positive effect on muscle differentiation supported by the finding that the

down-regulation of ERK2 using siRNA decreases terminal differentiation while siRNA of ERK1 has no significant effect on muscle differentiation (Li and Johnson, 2006).

Redox regulation of the ERK pathway is supported by studies using various cells. Oxidative stress caused by lactosylceramide stimulates the cell proliferation of smooth muscle cells via the activation of ERK1 (Bhunia et al., 1997). Apoptosis of cardiac myocytes and HeLa cells is attenuated by the activation of ERKs in response to $H_2O_2$, (Aikawa et al., 1997; Wang et al., 1998). Further work is still needed to understand precisely how the ERK pathway is redox-regulated in response to physiological oxidative stress *in vivo*, since most previous studies have focused on artificial *in vitro* conditions in which the oxidative stress is likely well above physiological levels.

The JNK pathway is one sub-group of MAPK cascades that is activated in response to cytokines and extracellular stress (Kyriakis and Avruch, 2001; Huwiler et al., 2004; Roux and Blenis, 2004). JNK is phosphorylated and activated by MKK4/7, MAPKK isoforms which are activated by MAPKKK in response to various stimuli. A major downstream target of the JNK pathway is AP-1 (activator protein-1) transcription factor, which is activated, in part, by the phosphorylation of c-Jun and other molecules (Angel et al., 1988). JNK pathway signaling is involved in multiple cellular functions including apoptosis, cell survival, oncogenic transformation, metabolism and aging. The activation of the JNK pathway balances cell survival versus apoptosis mediated in part JNK regulation in a short burst or more sustained manner depending on the stimuli, cell and

tissue type (Ventura et al., 2006). The inhibition of muscle differentiation caused by TNF-α, which decreases the transcription of IGF-1, is also mediated through the activation of JNK in C2C12 cells (Frost et al., 2003b; Strle et al., 2006) and TNF-α is a mediator of clinical muscle wasting. Inflammatory stimuli such as LPS (lipopolysaccharide, a bacterial product that causes toxicity in septic patients), which contributes to blood pressure instability with infection or injury, stimulates nitric oxide synthase-2 or IL-6 (interleukin-6) through JNK pathway in C2C12 cells (Frost et al., 2003a; Frost et al., 2004).

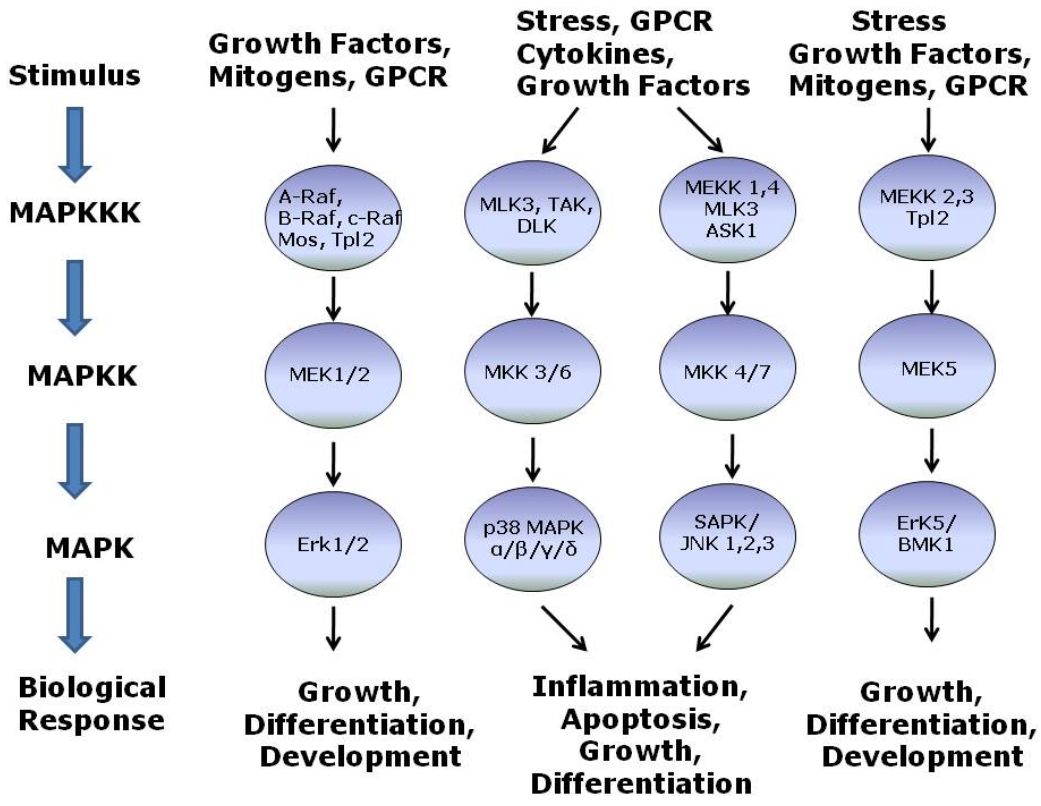
The redox regulation of the JNK pathway is supported by many studies. The phosphorylation and activity of JNKs increases in response to ROS (Guyton et al., 1996; Zhang et al., 1998). AP-1 and c-Jun, the downstream targets of JNK signaling, are also regulated by oxidative stress. Increased or decreased DNA binding of AP-1 occurs in response to redox change in various cells (Shono et al., 1996; Li et al., 1998; Mietus-Snyder et al., 1998). In addition, mRNA of c-Jun is increased due to oxidative stress through the JNK pathway in human fibroblasts (Collart et al., 1995), rat vascular smooth muscle (Rao et al., 1993; Zhang et al., 1998), and in human ML-2 leukemic cells and HeLa cells (Devary et al., 1991; Collart et al., 1995).

**NF-κB**

NF-κB (Nuclear factor-κB) is a family of dimeric transcription factors composed of p50/p105, p52/p100, c-Rel, Rel A and Rel B subunits. All of these

**Figure 1.2**

**MAPK signaling pathways**



share similarities in the Rel homology domain (RHD) of 300 amino acids at N-terminus. RHD includes sequences required for DNA binding, dimerization, nuclear localization and inhibitor factor binding. The NF-κB family is classified into two sub-families; the NF-κB (p105 and p100) and the Rel proteins (c-Rel, RelA and RelB). The NF-κB family is produced as precursor proteins (p105 and p100) and cleaved into mature, DNA-binding forms, p50 and p52 respectively, while the Rel proteins are not cleaved. All NF-κB and Rel proteins can form homo-dimers or hetero-dimers that have the ability to bind to specific DNA sequences, the κB sites. The affinity of every dimer is variable depending on the combination of subunits and each κB site. Inactive NF-κB is normally associated with I-κB in the cytosol, which contains multiple ANK domains for binding to the RHD of NF-κB. In response to extracellular stimuli, I-κB is phosphorylated by I-κB kinase (IKK) and this causes the proteolysis of IKK, the release and activation of NF-κB. Consequently, the activated NF-κB with post-translational modifications enters the nucleus and activates the downstream DNA target genes in association with other transcription coactivators such as CBP/p300 and HATs. The functions of NF-κB are myriad and it is one of the most studied molecules in biology and medicine. NF-κB is implicated in cancer development, immune responses, aging and muscular dystrophy (Perkins, 2003; Cai et al., 2004; Li et al., 2004; Salminen; 2008).

The role of NF-κB in muscle differentiation is still not clear. In C2C12 cells, insulin promotes adult myogenesis through activation of NF-κB, and the differentiation-impaired phenotype of Ras transfected C2C12 cells is restored in

response to insulin through the Akt/S6K/p38 MAPK pathway (Conejo et al., 2001; Conejo et al., 2002). These data suggest that activation of NF-κB promotes muscle differentiation. However, NF-κB expression decreases in C2C12 cells in response to differentiation medium and subsequently, results in the down-regulation and decreased DNA binding of AP-1 and Sp-1, which is reversed by the treatment of cells with cholera toxin and okadaic acid, inhibitors of muscle differentiation (Lehtinen et al., 1996). In addition, NF-κB inhibits muscle differentiation through silencing the myofibrillar gene by YingYang1 (YY1) (Wang et al., 2005). Moreover, myogenesis and cross sectional area of muscle fibers are increased in myoblasts from p65 knock-out mice (Bakkar et al., 2008). These data are hard to interpret, but a possible explanation might in the multiple targets of NF-κB which can be variably activated depending on a variety of circumstances, interconnecting with many other signaling pathways. Thus interpretation of the role of NF-κB in muscle differentiation will require careful analysis of activation of diverse downstream genes in response to distinct and well-controlled stimuli.

NF-κB is a classical example of a redox-sensitive factor. Redox regulation of NF-κB is mediated through the post-translational modification of NF-κB and IκB in response to various stimuli. IKK phosphorylates IκB at serine residues, which results in the release of NF-κB and activated NF-κB translocates into nucleus. Oxidative stressors such as $H_2O_2$ induce and prolong the activity of IKK, thereby increasing the phosphorylation of IκB with consequent activation of NF-κB (Jaspers et al., 2001; Kamata et al., 2002). This activation is attenuated by

NAC or PDTC (pyrrolidene-dithiocarbamate) anti-oxidant chemicals (Kaltschmidt et al., 1997; Kretz-Remy et al., 1998; Rahman et al., 2003).

**HIF-1**

HIF-1 (hypoxia-inducible factor-1) is a transcription factor responsible for the induction of genes in response to low oxygen tension (hypoxia), and is considered the master regulator of physiologic responses to oxygen levels. The concentration of oxygen in the atmosphere is 21%, while the oxygen at tissue *in vivo* averages about 2-3 % depending on the proximity to blood vessels and tissue types. HIF-1 is composed of two subunits; a hypoxia-inducible subunit, HIF-1α and a constitutively expressed subunit, HIF-1β also called aryl hydrocarbon receptor nuclear translocator (ARNT). Two isoforms of HIF-1α are also identified, HIF-2α and HIF-3α. HIF-2α and HIF-3α heterodimerize with HIF-1β similarly to HIF-1α and the function and expression of them generally overlap with HIF-1α. In normoxic oxygen conditions, HIF-1α is rapidly degraded by ubiquitin-mediated proteolysis though the transcription and synthesis of HIF-1α is not affected by the oxygen concentration, thus HIF-1α abundance is controlled by the degradation process. Hydroxylation of two proline residues and acetylation of a lysine residue are critical for the interaction with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Srinivas et al., 1999; Masson et al., 2001). During hypoxia, HIF-1α becomes stabilized (not degraded), enters the nucleus, dimerizes with HIF-1β and activates the downstream target DNAs that contain hypoxia response elements (HRE) in association with transcriptional coactivator,

CBP/p300. HIF-1α, through this transcriptional regulation, regulates a wide range of physiologic responses to oxygen insufficiency including erythropoiesis, angiogenesis, and upregulation of glycolysis. Under pathologic conditions in the hypoxic core of tumors, HIF-1α upregulation induces vasculogenesis which enhance tumor progression.

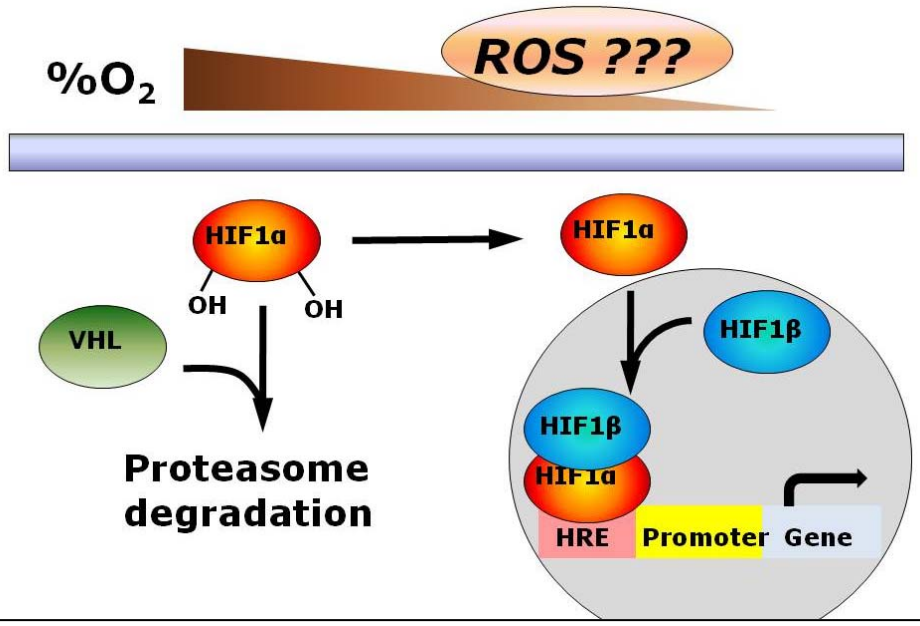
Diverse roles of HIF-1α in muscle differentiation have also been studied. HIF-1α is ubiquitously expressed in skeletal muscle tissue under normoxic and hypoxic conditions, as in all other tissues (Stroka et al., 2001). Hypoxia inhibits muscle differentiation in C2C12 cells through accelerated degradation of MyoD. Since MyoD feeds forward on other myogenic regulators, hypoxia results in a block in the normal accumulation of myogenic markers and prevents cell cycle withdrawal (Di Carlo et al., 2004). When rabbit primary myoblasts are exposed to 3% oxygen, HIF-1α is exclusively localized in nuclei rather than being degraded by ubiquitin-mediated proteolysis (Kubis et al., 2005). The activated HIF-1α in myoblasts due to 3% oxygen decreases the binding with Hsp90 (Kubis et al., 2005). These data indicate that the mechanism of activation of HIF-1α in muscle differentiation possibly depends on the affinity with Hsp90 which masks nuclear localization signal (NLS), in addition to control of HIF-1α by ubiquitin-proteasome degradation. However, HIF-1α is also regulated by the cell cycle, and is not detected in normoxic proliferating C2C12 cells and increases its expression during differentiation (Ono et al., 2006). More significantly, the knock-down of HIF-1α using siRNA inhibits C2C12 myoblast differentiation and HIF-1α expression increases in regenerating muscle *in vivo* (Ono et al., 2006). Taken

together, HIF-1α is one of the transcriptional factors crucial for the muscle differentiation.

HIF-1α is regulated by ROS as well as by oxygen tension. Treatment of cells with $H_2O_2$ or an oxidative stress-inducing chemical results in the stabilization of HIF-1α and the activation of the downstream target genes of HIF-1α regardless of oxygen concentration (Chandel et al., 2000; Duyndam et al., 2001; Mansfield et al., 2005). The knock-down of SOD2 also activates HIF-1α through the increase of superoxide production in MCF10A cells (Kaewpila et al., 2008). Furthermore, cells treated with anti-oxidants such as N-acetyl cysteine (NAC), glutathione, vitamins E and C significantly decrease the accumulation of HIF-1α and attenuate its activation in response to ROS (Shatrov et al., 2003; Rodriguez et al., 2005; Griguer et al., 2006).

**Figure 1.3**

**HIF-1α as a master regulator of oxygen sensing**



**The crosstalk between redox-regulated signaling pathways**

Redox-regulation of signaling pathways is complex and involves diverse changes of gene expression and post-translational modifications, as well as changes in subcellular localization of proteins. The effect of redox-regulation on signaling pathways is not uniform and depends on cell types and stimuli. Recent studies suggest that redox-regulated signaling pathways discussed above have considerable interconnection and crosstalk. For example, phosphatase such as protein phosphatase 2A (PP2A) and MAPK phosphatases (MKPs) mediates crosstalk between MAPK pathways (Liu and Hofmann, 2004; Wu and Bennett, 2005). In addition, NF-κB is regulated by Akt and p38 signaling in muscle differentiation (Conejo et al., 2002). Vascular endothelial growth factor (VEGF), one of the downstream targets of HIF-1α is regulated by peroxysome proliferators activated receptor-γ coactivator-1α (PGC-1α) in a HIF-1α independent manner (Arany et al., 2008). These interactions are certainly a small minority of all the relevant interactions. For a more complete understanding of the significance of these interactions, systems biology approaches are being increasingly applied for elucidating the crosstalk between signaling pathways (Bharucha et al., 2008; Dittrich et al., 2008; Varjosalo et al., 2008).

**The regulation of mitochondria in muscle differentiation**

Mitochondria are important sites for production of free radicals as by-products. In mammals, mitochondria encode 37 genes: 13 for proteins (polypeptides), 22 for transfer RNA (tRNA) and one each for the small and large

subunits of ribosomal RNA (rRNA). Mitochondria have their own DNAs, and can replicate independent of genomic DNA and cell cycle. However, many studies points to regulation of mitochondrial biogenesis by signaling from the nucleus (Puigserver et al., 1998; Wu et al., 1999; Lehman et al., 2000).

The mechanism of mitochondrial biogenesis is complex, including the coordinated expression of multiple nuclear-encoded genes. Several transcription factors are identified to have a crucial role in coordinating the expression of genes for mitochondrial biogenesis. Nuclear respiratory factor-1 (NRF-1) and NRF-2 regulate many genes involved in oxidative phosphorylation (Patti et al., 2003). Peroxysome proliferator activated receptors (PPARs) regulate genes involved in fatty acid oxidation (Gulick et al., 1994; Leone et al., 1999). Most studies about mitochondrial biogenesis emphasize PGC-1α as a master regulator of mitochondrial biogenesis (Puigserver et al., 1998; Wu et al., 1999; Lehman et al., 2000). PGC-1α induces expression of NRFs and NRFs in turn promote up-regulation of other mitochondrial transcription factors as well as many genes required for mitochondrial respiration (Wu et al., 1999).

Mitochondrial biogenesis is one of the important regulatory events in muscle differentiation. Stem cells are relatively deficient in mitochondria relative to differentiated muscle, so differentiation involves a relative increase of mitochondrial biogenesis over parenchymal cell expansion. Therefore, increased mitochondrial DNA (mtDNA) transcription and biogenesis are important parts of differentiation (Moyes et al., 1997; St John et al., 2005). Proliferating myoblasts have a lower mRNA level of PGC-1α, NRFs, citrate synthase and mitochondrial

transcription factor A (mtTFA) than differentiated myotubes *in vitro* (Duguez et al., 2002; Kraft et al., 2006). In addition, myogenin, one of the MRFs, is a specific transcriptional target regulated by mitochondrial activity during muscle differentiation (Rochard et al., 2000).

Mitochondrial biogenesis is also regulated by redox status in cells, and *vice versa*. Diverse diseases are caused by the excessive ROS produced in mitochondria and mitochondrial biogenesis is also regulated by ROS. PGC-1α is required to induce anti-oxidant enzymes such as Gpx1 and SOD2 and excessive oxidative damage in neurons is a feature of the pathology seen in PGC-1α knock-out mice (St-Pierre et al., 2006). The overexpression of PGC-1α promotes recovery after oxidant injury by increasing mitochondrial biogenesis and function (Rasbach and Schnellmann, 2007a). In addition, up-regulation of PGC-1α after oxidant injury is mediated by the activation of the p38 signaling pathway, also known to be redox-sensitive (Rasbach and Schnellmann, 2007b). These results indicate that bi-directional signaling exists between mitochondrial biogenesis and ROS and further studies are required to elucidate this mechanism.

**Oxidative stress in muscle during aging**

Loss of skeletal muscle mass, known as sarcopenia, is a progressive process leading to decreased strength associated with aging. In fact, the most conserved phenotype of aging across species is loss of or slowed motility. Muscle atrophy is the unintentional loss of muscle mass that results from many catabolic conditions (e.g. diabetes, cancer, sepsis, and aging). One of the major

factors contributing to muscle atrophy is oxidative stress. Skeletal muscle is exposed to higher oxidative stress than other tissue due to contractile activity of muscle and high levels of oxygen consumption. Aging and disuse-induced muscle atrophy are both accompanied by an increase in oxidative stress (Kondo et al., 1993; Lawler et al., 2003; Fulle et al., 2004). The increase in oxidative stress is most likely due to two factors: (1) altered electron transfer through the respiratory chain and (2) insufficient anti-oxidant defense mechanisms. Nonetheless, quantifying the loss of anti-oxidant defense mechanism with aging has not been straightforward. Many studies examining individual antioxidant pathways with aging show no dramatic changes in levels or activity of specific antioxidants (Pansarasa et al., 1999; Kim et al., 2003b). However, aging is systemically associated with worse pathology or survival upon insult with oxidants. The ultimate molecular mechanism underlying muscle atrophy involves an increase in protein degradation resulting in an imbalance between protein synthesis and degradation.

**Protein degradation in muscle atrophy; Ubiquitin ligases**

The ubiquitin-proteasome system is activated with muscle atrophy and responsible for degrading muscle proteins. The ubiquitin-proteasome system is composed of three major enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase. Ubiquitin conjugation occurs in serial reactions including these three major enzymes. Briefly, ubiquitin is activated by an E1 ubiquitin-activating enzyme using ATP as an energy source.

Ubiquitin is transferred from E1 to the active site cysteine of an ubiquitin-conjugating enzyme E2 via a trans(thio) esterification reaction. Finally, one of the hundreds of E3 ubiquitin-protein ligases, E3 enzymes function as the substrate recognition modules of the system, mediate the interaction with both E2 and substrate and prevent target proteins from being modified by nonspecific modification. Poly-ubiquitinated target protein is recognized by the 26S proteasome, unfolded, and degraded into the 20S proteolytic chamber using ATP. A single E1 is responsible for the activation of ubiquitin while 20-30 genes encode E2 in mammals. E3 ligases are more diverse and composed of three different families: N-end rule family, homologous to E6-AP carboxy terminus (HECT) domain family and ring finger family. In addition, E3 ligases usually form complexes composed of several subunits. Recently, the proteasome is understood to also provide diversity and specificity to protein targets for degradation by altering proteasome composition under certain conditions (Ferrington et al., 2001; Glickman and Raveh, 2005; Lecker et al., 2006; Hanna et al., 2007). Specificity and complexity of the ubiquitin-proteasome system are regulated depending on the combination of these diverse ubiquitins, three major enzymes and proteasome. During muscle atrophy, increased mRNAs of ubiquitin-proteasome system and ubiqiuitin conjugation have been reported using *in vitro* and *in vivo* models (Lecker et al., 1999; Jagoe et al., 2002; Stevenson et al., 2003; Lecker et al., 2004). In muscle, three E3 ligases are identified to be expressed: atrogin-1 (also known as MAFbx), MuRF1 and E3α-II (Bodine et al., 2001a; Gomes et al., 2001; Kwak et al., 2004). Atrogin-1 is one of the SCF

ubiquitin-ligases complexes and strongly induced in muscle in response to many catabolic states such as fasting, diabetes, tumor burden and renal failure (Gomes et al., 2001). MuRF1 is a ring finger family E3 ligases and up-regulated during muscle atrophy (Bodine et al., 2001a). More significantly, atrogin-1 knock-out mice and MuRF-1 knock-out mice have attenuated muscle loss, about 56% and 36% less than wild-type after denervation injury (Bodine et al., 2001a). In addition, the decrease of cross sectional area of muscle fibers after denervation is blocked in both atrogin-1 knock-out mice and MuRF-1 knock-out mice (Bodine et al., 2001a). E3α-II is one part of the ubiquitin-proteasome system, which is known to selectively degrade proteins with basic or large hydrophobic $NH_2$-terminal residues (Kwon et al., 1998). E3α-II is muscle-specific and up-regulated during cancer cachexia (Kwak et al., 2004). In addition, the overexpression of E3α-II in C2C12 cells increases ubiquitination of whole proteins *in vitro* (Kwak et al., 2004).

Oxidative stress also contributes to muscle atrophy. Increased production of ROS (Fagan et al., 1996; Barreiro et al., 2005; Muller et al., 2007) and decreased antioxidant gene expression (Muller et al., 2007) are tightly associated with muscle wasting. Treatment of C2C12 cells with $H_2O_2$ stimulates ubiquitin-proteasome system via up-regulation of E2 and E3 ubiquitin enzymes (Gomes-Marcondes and Tisdale, 2002; Li et al., 2003a). Lastly, anti-oxidant treatment of cells attenuates muscle wasting (Buck and Chojkier, 1996; Appell et al., 1997; Bianca et al., 2002). However, it is controversial whether muscle atrophy due to aging mostly depends on the activation of muscle specific E3 ligases (Clavel et al., 2006; Edström et al., 2006; Raue et al., 2007). The number of studies about

muscle atrophy due to aging is limited and more research is required considering aging is highly complex and includes diverse change of signaling pathways.

**Signaling pathway for protein degradation**

The activation of muscle-specific E3 ligases depends on several signaling pathways. The IGF-1/PI3K/Akt pathway regulates protein degradation as well as protein synthesis. The PI3K/Akt pathway increases protein synthesis via activation of mTOR, S6K, GSK3 and 4EBP-1 (Cross et al., 1995; Scott and Lawrence, 1998; Nave et al., 1999). Activation of PI3K/Akt also phosphorylates FOXO transcription factors and sequesters them outside the nucleus, thereby suppressing activity of FOXO transcription factors. Decrease of phosphorylation of Akt also increases the amount of FOXOs in the nucleus by decreasing the phosphorylation of FOXOs (Stitt et al., 2004). During muscle atrophy, FOXOs induce atrogin-1 and MuRF1 (Stitt et al., 2004). More significantly, FOXO3 binds directly to the 5' end of the atrogin-1 promoter region and induces transcription of atrogin-1 in response to catabolic conditions (Sandri et al., 2004). The induced atrogin-1 and MuRF1 function as E3 ligases and increase the ubiquitination of target proteins (Kwak et al., 2004). Recently, atrogin-1 was found to ubquitinate FOXO1 and FOXO3a and coactivate their transcriptional activity thereby inhibiting Akt-dependent cardiac hypertrophy (Li et al., 2007). This result suggests that there might be more unknown post-translational modifications of FOXOs and coactivation of FOXOs by atrogin-1. As atrogin-1 segregates Akt-

dependent cardiac hypertrophy, unknown interactions between downstream targets of PI3K/Akt might exist.

During aging, sarcopenia is a multifactor disorder and recent studies suggest aged muscle decreases the response of muscle protein synthesis to anabolic stimuli (Cuthbertson et al., 2005; Rasmussen et al., 2006; Drummond et al., 2008). The IGF-1/PI3K/Akt pathway is one of the most important pathways that function abnormally in muscle atrophy. Muscle protein synthesis in response to a mixture of amino acid and glucose is blunted significantly in healthy elderly humans (Volpi et al., 2000). The downstream targets of Akt such as mTOR, S6K and 4EBP-1 show decreased phosphorylation in response to essential amino acids in healthy elderly humans compared to younger subjects (Guillet et al., 2004; Cuthbertson et al., 2005). In addition, muscle of healthy elderly humans does not respond to insulin, and lower protein synthesis is observed in response to insulin compared to younger subjects (Rasmussen et al., 2006). These results suggest that the balance of protein synthesis and degradation in aged muscle favors degradation more than in young muscle.

Another signaling pathway involved in protein proteolysis in muscle is the NF-κB dependent pathway. NF-κB is up-regulated in muscle during exercise, immobilization and cytokines. NF-κB is activated in exercised muscle compared to sedentary rats and this exercise-induced NF-κB activation is blocked as much as 76% by inhibitors of p38 MAPK and ERK (Ji et al., 2004; Ho et al., 2005). Possible mechanisms involving ROS for the activation of NF-κB during intense acute exercise include increased glutathione oxidation, which results in the

increase of ROS which then mediates NF-κB activation (Sen, 1999). But at the opposite end of muscle activity, immobilization of muscle also activates NF-κB pathway (Hunter et al., 2002; Bar-Shai et al., 2005). Further, there is a difference in the pattern of NF-κB activation between young and aged muscle. In both young and aged rats, NF-κB is activated via an alternative pathway, which does not involve the activation of the ubiquitin-proteasome system (Bar-Shai et al., 2005). After more prolonged immobilization, the activation of NF-κB, in concert with the ubiquitin-proteasome system, is more predominant in aged muscle compared to the young (Bar-Shai et al., 2005). Anti-inflammatory stimuli such as glucocorticoids and pro-inflammatory TNF-α also activate the NF-κB pathway and increase protein degradation in muscle (Du et al., 2000; Li et al., 2003b). Dexamethasone, a glucocorticoid, induces muscle proteolysis *in vivo* (Chrysis and Underwood, 1999) and *in vitro* (Thompson et al., 1999), which suggests that increased levels of endogenous glucocorticoid may be involved in both disuse atrophy and cachexia, leading to protein degradation. Administration of TNF-α can cause cachexia (Fong et al., 1989), and the inhibition of TNF-α by torbafylline also prevents progression of muscle wasting (Combaret et al., 2002). The activation of NF-κB was more definitively shown to be required to mediate protein degradation in muscle using transgenic mice (Cai et al., 2004; Hunter and Kandarian, 2004). Briefly, transgenic mice with the activated IκB kinase β induce more MuRF-1 and accelerate the breakdown of protein in muscle (Cai et al., 2004). In addition, both of Nfkb1 and Bcl3 knockout mice demonstrate decreased skeletal muscle atrophy (Hunter and Kandarian, 2004). Nfkb1 encodes the p65

subunit of NF-κB and Bcl3 increases the NF-κB dependent transcription, demonstrating that disruption of NF-κB signaling can counteract muscle atrophy.

**Autophagy of muscle during aging**

Post-mitotic cells are unable to maintain their normal structure over long periods, and undergo age-dependent degeneration. After terminal differentiation accompanied by cell cycle withdrawal, post-mitotic cells including neurons and skeletal muscle undergo degeneration, leading to progressive organ dysfunction. Accumulation of post-mitotic cells, which function abnormally, is a hallmark of degenerative diseases, and degenerative diseases increase in frequency of aging. Autophagy is the process by which damaged organelles and aberrant cellular components, are delivered to lysosomes for degradation. Autophagy is activated in starvation or stress conditions, and serves a normal homeostatic function. Protein degradation of muscle during aging also depends in part on autophagy. The accumulation of autophagosomes in lysosomal protein deficiency and by the inhibition of lysosome function supports a model in which autophagy is constitutively active in muscle (Shintani and Klionsky, 2004). The autophagy of muscle is mainly regulated by FOXO3 (Mammucari et al., 2007; Zhao et al., 2007). Transfection of constitutively active FOXO3 increases protein proteolysis that is dependent more on lysosomal degradation than on proteasomal degradation, and up-regulates autophagy related genes (Mammucari et al., 2007; Zhao et al., 2007). FOXO3 controls the transcription of autophagy related genes such as Bnip3, Bnip3L, LC3b, Gabarapl1 and Atg12l

via binding to promoter regions (Mammucari et al., 2007; Zhao et al., 2007). Autophagy is independent of muscle-specific ubiquitin ligases and proteasome dependent proteolysis (Mammucari et al., 2007). In addition to the normal constitutive homeostatic function of autophagy, its activation is implicated in diverse pathological processes. A better understanding of FOXO3 and autophagy related genes during aging will be important to elucidate the mechanisms of age-related sarcopenia.

**Apoptosis of muscle during aging**

Apoptosis, also known as programmed cell death, is an important process by which the body eliminates unnecessary or damaged cells during development, and apoptosis is critical to maintain homeostasis in adults. Apoptosis is classified into extrinsic and intrinsic pathways, depending on the signaling mechanism that initiates the response. The extrinsic pathway of apoptosis is induced by death-inducing signals such as ROS and ligands for the death receptors. Activated death receptors, such as TNF-receptor (TNFR) superfamily, induce the activation of caspase-8, an initiator caspase and subsequently, result in the cascade of activation including caspase-3, -6 and -7, which are “executor” caspases. Caspase-3 is cleaved in response to pro-apoptotic signaling and leads to DNA fragmentation and death of mononucleated cells via the activation of caspase-activated DNase (Baker and Reddy, 1998).

The intrinsic pathway features mitochondria as central mediators of apoptosis. This pathway is usually initiated by internal cell stimuli including ROS

(Polack and Leeuwenburgh, 2001; Mayer and Oberbauer, 2003). In response to pro-apoptotic signaling, mitochondria release cytochrome-c into the cytosol where it forms part of the apoptosome in association with Apaf-1, caspase-9 and dATP. Subsequently, the apoptosome activates caspase-3. Another form of an intrinsic pathway involves the cascade of caspase activation such as caspase-12, -9 and -3 independent of mitochondrial release of cytochrome c. For example, the disruption of intracellular calcium homeostasis activates this pathway.

Muscle is composed of multinucleated myofibers and the myonuclear domain is the amount of cytoplasm supported by a single muscle fiber nucleus. Myonuclear domain, as far as it can be measured, is relatively constant, suggesting that at least two factors contribute to regulate myonuclear domain; (1) the incorporation of satellite cells into existing myofibers and (2) the loss of nuclei during muscle atrophy. Apoptosis is one of the mechanisms responsible for the loss of nuclei during muscle atrophy. Previous observations of the loss of nuclei of muscle involve chromatin condensation and DNA fragmentation, which are the characteristics of apoptosis (Allen et al., 1999; Dupont-Versteegden et al., 1999; Borisov and Carlson, 2000). In multinucleated cells, all nuclei are not transcriptionally active and apoptosis of one nucleus can occur within subdomains of cytoplasm without the involvement of other nuclei, suggesting that individual nuclei within muscle have different thresholds of susceptibility to apoptosis and/or local apoptotic signals are not uniformly distributed in cytosol of muscle (Newlands et al., 1998). In animal models of muscle atrophy, both the abundance of activated caspase-3 and caspase-3 enzymatic activity are

increased (Libera et al., 1999; Yasuhara et al., 2000; Leeuwenburgh et al., 2005). In addition, caspase-3 is also increased during apoptosis in myotubes *in vitro* (McArdle et al., 1999). However, other studies have not detected any change of caspase-3 using similar muscle atrophy models (Persinger et al., 2003; Tews et al., 2005). A few other caspases such as caspase-7, -8 and -9 have been studied. The amount of caspase-8 increases after muscle unloading or denervation (Alway et al., 2003). And caspase-7 appears to be induced in a fiber type specific manner in response to pro-apoptotic signaling (Tews et al., 2005). During muscle atrophy, Bcl-2, an anti-apoptotic protein, decreased and Bax, a pro-apoptotic protein, increased while there is no significant change of Bcl-xl, another anti-apoptotic Bcl-2 family member (Tews et al., 1997; Libera et al., 1999; Jin et al., 2001; Persinger et al., 2003).

The controversy surrounding the precise details of activation of the major apoptotic pathway during muscle atrophy has to do, in part, with the temporal relationship of apoptosis and muscle protein degradation. Apoptosis and protein degradation occur simultaneously and they are closely connected. For example, caspase-3 is an executor enzyme responsible for both breakdown of myofibril protein and apoptosis (Hasselgren and Fisher, 2001; Du et al., 2004). During aging, both the cross sectional area of muscle fibers and the myonuclear domain decrease in skeletal muscle. Accumulating evidence suggests that an aged-related acceleration of apoptosis might represent one of the major factors to drive age-related muscle atrophy. First, TUNEL-positive nuclei increase in an age-dependent manner in muscle (Strasser et al., 2000). Apoptosis is more common

in aged muscle than in young muscle, and this increase of apoptosis is related to the increase in relative abundance of active caspase-3, but not necessarily increased enzyme activity (Dirks and Leeuwenburgh, 2004). This finding raises another reason that it has been difficult to fully characterize apoptosis in muscle using traditional approaches that focus on activated caspase-3. Recent studies suggest that the unactivated form of caspase-3 plays a positive role in muscle differentiation (Moresi et al., 2008). Nonetheless, both of apoptosis-inducing factor (AIF) and caspase-12 increase in aged muscle, implying that the apoptotic pathway independent of mitochondria is involved in increased apoptosis of aged muscle compared to young muscle (Dirks and Leeuwenburgh, 2004; Baker and Hepple, 2006). Pertinent to aging, different muscle fiber types have different susceptibility patterns. The release of endonuclease G (Endo G) out of mitochondria increases in aged soleus muscle (Type I) while no change was detected in interstitial muscle (Type II) between young and aged mice (Dupont-Versteegden et al., 2006). In addition, the balance of pro-apoptotic and anti-apoptotic proteins in response to TNF-α also depends on fiber-type (Phillips and Leeuwenburgh, 2005). More research should be done to determine the role of the different apoptotic pathways in muscle during aging and the functional consequences of apoptosis in aged muscle.

**Mitochondria of skeletal muscle during aging**

Long-lived postmitotic cells such as skeletal muscle alter dramatically during aging because they can not prevent the accumulation of damaged

(unrepaired) subcellular structures and can not generate undamaged replacements. Mitochondria are important sites of ROS production as well as major sites for ATP production, and are significantly affected by aging. ROS are, in part, by-products of mitochondrial respiration. Thus, malfunction of this system is thought to increases ROS generation. Age-dependent dysfunction of mitochondria mostly results from altered function of mitochondrial proteins, accumulation of mutations in mitochondrial DNA (mtDNA), and dysregulation of mitochondrial biogenesis. Electron transfer activity through the electron transport chain decreases during aging. Enzymatic assays using isolated mitochondria from various tissues of rodents showed an age-related selective decrease in the enzymatic activity of complex I and IV while complex II and III are largely unaffected due to aging (Lenaz et al., 1997; Navarro et al., 2002). In human skeletal muscle, the decreased function of complex I, II and IV is due to decreased protein expression of these complexes (Boffoli et al., 1994). In murine skeletal muscle, respiratory control ratios, which are indicative of the coupling between respiration and oxidative phosphorylation, are about 21-40% lower in the aged muscle compared to the young (Chabi et al., 2008). The accumulation of mutated mtDNA increases during aging and the mutated DNA replaces normal DNA locally in atrophic segments of muscle fibers (Cao et al., 2001). The pathologic role of mutated mtDNA is not clear. However, the abundance of mutant mitochondria appears to increase over time since some mutated mtDNA may have a replicative advantage over normal mtDNA. In normal cells, mitochondria that have the damaged mtDNA are subject to degradation through

autophagy. Mitochondria which have mutated mtDNA may escape autophagy by increasing replication, a pattern seen in some tumors with increased mutated mtDNA (Hochhauser, 2000) and in human colonic crypt stem cells (Taylor et al., 2003). More significantly, transgenic mice which express an error-prone version of mtDNA polymerase γ accumulate mtDNA point mutations (Trifunovic et al., 2004). These mice exhibit an aged cellular phenotype with the severely impaired function of the respiratory chain (Trifunovic et al., 2005). However, in this study, ROS production is not affected by the increased mutation of mitochondrial DNA (Trifunovic et al., 2005). These data may suggest that the cytotoxic effect of ROS is the starting point for dysfunction of mitochondria during aging, and impaired mitochondria consequently contribute to the senescence of cells. With aging, the biogenesis of mitochondria is also impaired in skeletal muscle. Both PGC-1α and the primary mtDNA transcription factor, Tfam decrease at the protein expression level in aged murine skeletal muscle compared to the young (Chabi et al., 2008). In addition, mRNA of both PGC-1α and Tfam also decrease in the aged smooth muscle cells (Ungvari et al., 2008). These alterations suggest that both protein and mRNA level of genes involved in mitochondrial biogenesis decrease during aging and eventually contribute to the decreased function of these cells, and their increased susceptibility to stress.

**Aging markers in muscle**

It is widely accepted that there is no single specific aging marker for all tissues and cell types. In higher organisms, the degree of complexity is more

diverse than in lower organisms due to the need to maintain organs (and their interactions) generated during complex development. Furthermore, commonly used development models (yeasts, flies, and worms) and rodent models used to study aging are considerably less genetically diverse than humans. Aging patterns are also significantly affected by environmental factors, which are also more diverse than in model organisms. For this reason, markers of aging in humans will necessarily be multiple, and are incompletely understood.

One major marker of aging is the oxidative modification of DNA. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is formed when DNA is exposed to hydroxyl radicals. The action of ROS to form 8-OHdG occurs in both genomic DNA and mtDNA (Lopez-Torres et al., 2002; Wolf et al., 2002). The increase of 8-OHdG in skeletal muscle during aging is a feature of rodent (Wolf et al., 2005) and human aging (Gianni et al., 2004).

Oxidative stress-induced lipid peroxidation is another aging marker. Increased oxidative stress damages membrane-bound receptors or enzymes by lipid peroxdation. F2-isoprostanes, malondialdehyde (MDA), 4-hydroxy-2-none-nal (HNE) and low-density lipoproteins (LDL) are all affected by lipid peroxidation with aging. MDA and HNE in plasma increase with age in healthy humans starting at age 18 and continue to increase into old age (Gil et al., 2006). Oxidized LDL is implicated in various degenerative diseases such as diabetes mellitus and atherosclerosis (Steinberg et al., 1996). In skeletal muscle, HNE increased during aging accompanied with increased ROS and RNS (Barreiro et al., 2005; Braga et al., 2008).

Global protein modification by oxidant reactions is also characteristic of aging. Protein is carbonylated through metal catalyzed oxidative (MCO) reaction and carbonylation occurs on the amino-acid side chain, prolines, arginines, lysines, and threonines. Furthermore, protein glycation occurs by the reaction of carbonyl derivatives on lysine, cysteine, and histidine with reactive carbonyl compounds formed in carbohydrates (glycoxidation products), lipids, and advanced glycation end products (AGEs) (Nyström, 2005). The cross-linking of AGEs with peptides and proteins makes protein resistant to potentially reparative proteases and causes irreversible damage to various tissues (Schleicher et al., 1997; Westwood et al., 1997) including smooth muscle cells (Hattori et al., 2002). We have found that proteins in Gpx1 knock-out myoblasts are globally carbonylated due to the increased oxidative stress (Lee et al., 2005).

Lipofuscin collects in aged worms, crabs, and mammals with aging. Lipofuscin is a heterogeneous mixture of oxidized protein and lipids with lesser amounts of carbohydrates and metals that emit autofluorescence easily detectable using microscopy. Lipofuscin is likely formed by the consequence of unfinished cellular degradation processes, and the complex mixture of lipofuscin components is resistant to protease and lipase digestion. The resistance of lipofuscin to breakdown is due to the stable polymeric structure formed by the crosslinking between peptide amine groups and proteins (Kikugawa et al., 1989). Lipofuscin likely accumulates due to both dysfunction of lysosomes and accumulated ROS damage with aging. The lysosomal-mitochondria axis theory of postmitotic aging and cell death has been proposed based on findings that

autophagy is decreased in lipofuscin-contained postmitotic cells (Brunk and Terman, 2002). This theory proposes that futile attempts to degrade lipofuscin promote leakage of lysosomal enzymes outside the lysosome, such that lysosomes no longer contain the machinery to remove damaged mitochondria. Consequently, the increase in defective mitochondria and the surplus of lysosomal enzymes in the cytosol are critical factors accelerating functional cellular senescence. The age-dependent increase of lipofuscin is observed in brain cells, cardiac myotyctes, and skeletal muscle (Nakano and Gotoh, 1992; Jolly et al., 1995; Terman and Brunk, 1998) as well as other organs. Recently, we have found that increased ROS stress associated with Gpx1 deletion in mice results in premature deposition of lipofuscin in skeletal muscle of young Gpx1 knock-out mice (Lee et al., 2006).

In the case of mitotic cells, replicative senescence occurs after limited number of cell divisions. Cellular senescence results in growth arrest in G1 phase of the cell cycle (Pignolo et al., 1998). The shortening of telomeres is one of the factors that may contribute to the cellular senescence. Telomeres are specialized chromatin structure composed of TTAGGG repeats at the end of chromosome, generated by a reverse transcriptase, telomerase (Tert) at every cell division. In eukaryotes, telomere shortening occurs during aging due to the inability of DNA polymerase to copy the ends of chromosomes with fidelity (Bodnar et al., 1998). The dysfunction of telomeres results in a DNA damage response characterized by the formation of double strand DNA breaks containing the phosphorylated histone H2AX (Celeste et al., 2002; Herbig and Meier et al.,

2007). Recently, the decreased regenerative potential of stem cells (as well as better studied differentiated tissues) during aging has also been attributed to the shortening of telomeres. The specificity of telomere maintenance for ongoing proliferative potential was demonstrated in muscle satellite cells in which senescence was accompanied by telomere shortening, and could be rescued by overexpression of telomerase (Cudre-Mauroux et al., 2003; Zhu et al., 2007). However, the telomere length of skeletal muscle as a whole may not change as much during aging (Ponsot et al., 2008). These findings suggest that telomere shortening is not affected in postmitotic skeletal muscle with aging specifically because the cells are non-dividing.

Other well-known molecular markers of aging are cyclin-dependent kinase inhibitors, the INK4/ARF proteins. The INK4 class of cell cycle inhibitors is composed of $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INKd}$. These inhibitors are homologous to cyclin-dependent kinases, CDK4 and CDK6 and binding of INK4 to CDK4 and CDK6 inhibits phosphorylation of retinoblastoma (Rb) family members resulting in growth arrest of cells. The ARF family members, $p14^{ARF}$ and $p19^{ARF}$, bind to MDM2 protein, and MDM2 inactivates p53, a tumor suppressor. The importance of these proteins was initially identified in various tumors in which the locus of INK4a/ARF/INK4b is deleted or epigenetically modified to decrease their expression (Nairn et al., 1996). This decrease facilitates tumor cell division in an unregulated manner. Recent evidence has suggested that the increased expression of $p16^{INK4a}$ can be used as a biomarker of aging, supported by the increase seen in aged skin (Ressler et al., 2006),

cardiomyocytes (Chimenti et al., 2003; Torella et al., 2004) and kidney (Chkhotua et al., 2003). However, $p16^{INK4a}$ as a biomarker of aging in skeletal muscle or myoblasts has not been specifically addressed.

**Oxidative stress in muscle progenitor cell function**

Skeletal muscle mass is maintained and regenerated to form myofibers through the activation of the satellite cells during the entire life span. Skeletal muscle and muscle precursor cells are exposed to oxidative stress that accompanies contractile activity. During aging, the oxidative stress increases while the regenerative potential and the pool size of muscle precursor cells decrease, but the precise mechanism of the increased oxidative stress affecting adult myogenesis remains unclear. The oxidative stress broadly affects proliferation, apoptosis and differentiation of muscle precursor cells through regulating intracellular signaling pathways as well as by damaging cellular components directly. Finally, the decreased function of muscle precursor cells during aging contributes in part to muscle atrophy.

The research outlined in this dissertation was largely based on the role of anti-oxidant enzymes and ROS in myoblasts during proliferation, differentiation and aging. As outlined in Chapter 1, SOD2 is a mitochondrial enzyme that catalyzes dismutation of superoxide into hydrogen peroxide and $O_2$ and functions as the first line of defense against ROS produced in mitochondria. SOD2 is essential for the survival of the organism, supported by the lethality of homozygous knockout mice (Li et al., 1995; Huang et al., 2001). Gpx1 is a

cytosolic anti-oxidant enzyme that catalyzes the catabolism of hydrogen peroxide. Gpx1 is not essential for the organism to survive, since other enzymes have the same catalytic function. The central goal of the research outlined in this dissertation was to investigate the mechanisms by which the deficiency or overexpression of specific anti-oxidant enzymes contribute to muscle precursor cells. We formulated specific aims to address this goal in Chapter 3-5.

Gpx1 knock-out ($Gpx1^{-/-}$) mice are viable, develop normally, and have a similar life span compared to wild-type mice, due to the redundancy of other enzymes. However, they are less tolerant of a variety of oxidant stresses compared to wild-type mice. $Gpx1^{-/-}$ mice have decreased survival after parquet or diquat challenges (Lei, 2002) or systemic infections (Beck et al., 1998), and show increased organ damage after ischemia-reperfusion injuries (De Haan et al., 2003). A deficiency of human Gpx1 activity has been associated with myocardial diseases (Leopold and Loscalzo, 2005). However, $Gpx1^{-/-}$ myoblasts have not been studied. Thus, our first aim was **to test the hypothesis that the deficiency of Gpx1 in myoblasts affects proliferation, apoptosis and differentiation** (Chapter 3). To address this hypothesis, we first isolated and observed the phenotypes of $Gpx1^{-/-}$ versus wild-type myoblasts. We also examined ROS production, cell proliferation, apoptosis and terminal differentiation *in vitro*. Myoblasts from $Gpx1^{-/-}$ mice are defective in many aspects of muscle biology, and show increased intracellular ROS. However, we have not found any significant alteration in intracellular signaling pathways affected by the deficiency of Gpx1 during differentiation.

As a second aim, we have extended our research to the role of SOD2 in myoblasts during aging as described in Chapter 4. Multiple pathological abnormalities are seen in SOD2 knock-out mice including dilated cardiovascular structure (Li et al., 1995; Huang et al., 2001), increased lipid peroxidation (Li et al., 1995), severe anemia and degeneration of neurons (Lebovitz, 1996). However, the role of SOD2 in myoblasts or muscle differentiation has not been studied. Therefore, our second aim was **to hypothesis that Sod2 overexpression in myoblasts preserve mitochondrial function and mass thereby, maintain muscle differentiation with aging (Chapter 4).** To address this issue, we have studied young (3-4 weeks old) and aged (27-29 months old) myoblasts from Sod2 heterozygote knock-out ($Sod2^{+/-}$) and SOD2 overexpressing (Sod2Tg) mice. In addition, we tested the hypothesis that the PI3K/Akt pathway is affected by the deficiency or overexpression of SOD2 during differentiation. We were also interested in the effect of SOD2 on mitochondrial integrity because the subcellular localization of SOD2 is restricted to mitochondria. Our results demonstrated that PI3K/Akt pathway is affected by Sod2 deficiency, typically in young myoblasts, and this might be responsible in part for the fusion defect of $Sod2^{+/-}$myoblasts.

Our final aim was **to hypothesis that Sod1 haplo-deficiency in myoblasts altered oxygen sensing which contributes to defective muscle differentiation** (Chapter 5). Interestingly, $Sod1^{-/-}$ myoblasts cannot survive *in vitro*. However, $Sod1^{-/-}$ mice are viable. The mutation of SOD1 in mice is implicated in ALS (Bowling et al., 1993; Rossen et al., 1993) as described in

Chapter 1. SOD1 has a function equivalent to SOD2 except that SOD1 is most abundant in the cytosol. Towards this goal, we have exposed myoblasts cells to various oxygen concentrations. Our results demonstrate that the deficiency of SOD1 impairs muscle differentiation. In addition, HIF-1α signaling was altered by SOD1 deficiency. Thus, our data indicate that ROS also affect the oxygen sensing of myoblasts.

The research presented in this dissertation characterizes broadly the role of ROS during muscle differentiation. The effect of ROS on myoblasts differs depending on the enzyme deficiency. In addition, the alteration of signaling pathway and direct detrimental effect of ROS affect muscle differentiation.

Our data not only provide broad information about ROS involved in myogenesis but may also suggest an approach for the treatment of muscular diseases during aging.

# Chapter 2: Materials and Methods

**Animals**

All animal studies conformed to National Institutes of Health guidelines and were 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 on the C57BL/6 background (Ho, et al., 1997) and backcrossed for 10 generations onto a C57BL/6J background. Sod2 heterozygote and transgenic mice were generated on the CD1 background (Li, et al., 1995; Chen, et al., 1998) and were backcrossed for 10 generations onto a C57BL/6J background. Sod1 heterozygote knock-out mice were originally generated on the C57BL/6 background (Reaume et al., 1996) and backcrossed for 10 generations onto a C57BL/6J background. Mice between 3-4 weeks of age were used as sources of Gpx1 null myoblasts and Sod1 heterozygote myoblasts, respectively. Mice between 3-4 weeks of age and 27-29 months of age were used as sources of Sod2 heterozygote and transgenic myoblasts.

**Isolation and culture of myoblasts**

Myoblasts were isolated from age-matched mice at 3-4 weeks or 27-29 months of age using a modification of published protocols (Rando et al., 1994). After euthanasia, hind leg muscles were dissected from the bones and non-muscle tissue removed; muscle was minced, washed, then digested in 0.1% pronase (Calbiochem) for 1 hr at 37$^{o}$C. The muscle was repeatedly triturated, passed through a 100 μm nylon mesh, and myoblasts were separated on a Percoll (Amersham Biosciences) gradient. The cells were plated onto collagen I (Cohesion Technologies)-coated

polystyrene cell culture dishes (Corning Life Sciences) (Yablonka-Reuveni and Nameroff, 1990). During the first several passages of the primary cultures, myoblasts were enriched by pre-plating (Richler and Yaffe, 1970) 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 (Developmental Studies Hybridoma Bank) (Yablonka-Reuveni and Nameroff, 1990).

Myoblast expansion and prevention of differentiation were accomplished by culture in growth medium (GM): 20% fetal bovine serum (HyClone) supplemented with 10 ng/ml fibroblast growth factor-2 (Promega) in Ham's F-10 (Invitrogen) with 200 U/ml penicillin G and 200 g/ml streptomycin (Invitrogen). Differentiation was induced by switching myoblast cultures to ECL-coated dishes and differentiation medium (DM): Dulbecco's Modified Eagle Medium (Invitrogen) with 2% horse serum (HyClone) and penicillin/streptomycin. For the inhibition of the PI3K-Akt pathway, LY294002 (20 μM, Promega) was added to DM when differentiation was initiated and myoblasts were cultured for 5 days without the change of medium.

**Terminal Differentiation of skeletal muscle *in vitro***

Each day after incubation in differentiation medium, cultures were fixed with 1:1 methanol/acetone and immunostained for myosin heavy chain (MHC), a marker of terminal muscle differentiation. Primary MF 20 antibody (Developmental Studies Hybridoma Bank) was used at 1:50 dilution and Alexa

Fluor 568 goat anti-mouse secondary (Molecular Probes) at 1:400. At least 1,000 nuclei were counted for each sample from approximately 20 random fields. The fusion index was calculated as follows: [(Number of nuclei in MF 20-stained myotubes with ≥ 2 nuclei/ Total number of nuclei) × 100]. The differentiation potential (DP) was calculated as follows: [(Number of nuclei in MF 20-stained cells/Total number of nuclei) × 100].

**Proliferation of myoblasts**

Bromodeoxyuridine (BrdU) incorporation was used to assay proliferation. Myoblasts were plated in GM at a density of $2 \times 10^5$ cells/60 mm on 10% gelatin, ECL or collagen-coated dishes. The cells were incubated with 10 μM BrdU for 4 hours (44–48 h after plating), washed, and fixed in 4% paraformaldehyde (Mallinckrodt). 1/1000 dilution of rat monoclonal anti-BrdU antibody (Harlan Sera Labs) was used as previously reported (Csete et al, 2001) to label myoblasts that incorporated BrdU during the pulse. Total numbers of labeled and unlabeled cells (>3000 cells counted per condition) were used to construct 2×2 $\chi 2$ comparisons. Data were expressed as mean ± standard deviation from the duplicate experiments.

**Apoptosis of myoblasts**

Terminal deoxynucleotidyl nick end labeling (TUNEL) was used to compare myoblast apoptotic cell death rate in DM. Cells were seeded at a density of $10^5$ cells/6 cm plate in DM on ECL-coated plates. After 3 and 5 day

incubations, apoptotic cells were counted using DeadEnd™ Fluorometric TUNEL system (Promega). Nuclei were stained with DAPI (Vectashield). Data were expressed as mean ± standard deviation from the duplicate experiments.

**Analysis of lipid formation in myoblasts**

To evaluate the potential of myoblasts to accumulate lipid, a characteristic of myoblasts isolated from aged animals and cultured satellite cells in high $O_2$ conditions (Csete et al., 2001), myoblasts were incubated in medium that promotes adipogenesis: DMEM (and 10% fetal bovine serum and penicillin/streptomycin) with 10 g/ml insulin, 1M dexamethasone, and 0.5 mM isomethylbutylxanthine (additives all from Sigma-Aldrich). After 2 or 3 days the cultures were fixed with 10% formalin and stained for triacylglycerides with oil red O as previously reported (Csete et al., 2001).

**Real-time quantitative fluorescence PCR for myogenic bHLH gene expression**

RNA was isolated using RNeasy kits (Qiagen). cDNA was synthesized using the Superscript III first-strand synthesis system (Invitrogen) with 250 ng RNA input per reaction. Real-time PCR was performed on a LightCycler (Roche Applied Science) using primers specific for MyoD, myf5, mrf4, myogenin and 18S rRNA for internal control (Table 2.1). All cDNA was amplified using master SYBR green I (Roche) by incubation until the block reached 95$^{o}$C followed immediately by 40 cycles of: ramp to 95$^{o}$C then annealing temperature for 10 sec, then 72$^{o}$C

for 20 sec. The annealing temperatures of primers were shown in Table 2.1. Analysis of message abundance was based on Roche Applied Science, Technical Note No. LC 13/2001 as follows: Primer efficiency was measured using serial dilutions of cDNA. For quantitation, Crossing point ($C_t$), defined as the PCR cycle number that crosses an arbitrarily placed signal threshold was calculated. Gene expression was calculated using: $E_T^{CpT(C)-CpT(S)} X E_R^{CpR(C)-CpR(S)}$ [$E_T$ is Efficiency of target gene reaction; $E_R$ is Efficiency of reference gene reaction (here 18S ribosomal RNA)]; CpT(C) is crossing point of target gene reaction with control sample (wild-type proliferating myoblasts), CpT(S) is the crossing point of target gene reaction in the experimental samples; CpR(C) is the crossing point of 18S ribosomal RNA in control myoblasts; CpR(S) is crossing point of 18S ribosomal RNA in the experimental samples. Quantitative PCR results of wild-type proliferating myoblast cDNA at time zero were used for normalization of the other samples (normalized to a value of 1 for each gene).

Differentiation was performed in both 20% and 6% $O_2$, since myogenic gene expression is turned on earlier in differentiating satellite cells in lower $O_2$ conditions (Csete et al., 2001). For each assay two independent time courses were performed in duplicate, and the results were pooled for analysis.

**Cross sectional area of muscle fibers *in vivo***

Female Gpx1$^{-/-}$ and wild-type mice (23 weeks old, n=4 per group), age-matched mice wild-type and Sod2Tg mice at 3-4 weeks or 27-29 months of age (n=4 per group) were used to measure cross sectional muscle fiber area in

hindlimb muscles. After euthanasia, leg muscles were removed, weighed, fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, and sectioned (9-10 mm thick). The sections were stained with Mayer's hematoxylin and counterstained with eosin. Fibers were outlined on these stained sections and the areas of muscle fibers captured and calculated using Image J Software (http://rsb.info.nih.gov). Mean fiber areas were compared between groups using Student's t-test.

### *In vivo* muscle regeneration pilot studies

Female Gpx1$^{-/-}$ mice and their wild-type littermates, 15 weeks of age were used in these studies. Sterile cardiotoxin 2.5M (Calbiochem) in normal saline was injected into the anterior compartment (100 μl) and posterior compartment (200 μl) of the right hind limb. The anterior compartment consists predominantly of the tibialis anterior (TA) muscle while the triceps surae complex (gastrocnemius, soleus and plantaris muscles) resides in the posterior compartment. Equivalent volumes of normal saline were injected into the anterior and posterior compartments of the left hind limb, thus, each animal served as its own control. Mice were euthanized 14 days after cardiotoxin injection (2 mice/group). The skin was removed from the hind limbs, the muscle compartments removed, transected longitudinally, placed in 10% neutral buffered formalin and processed for paraffin embedding. The sections were stained with hematoxylin and eosin, and cross-sectional area of muscle fibers in transverse sections were measured using Image J Software. Regenerated fibers contain a centrally located nucleus,

and so are distinct from primary fibers in which nuclei are confined to the periphery of the fiber in cross-section. Cross-sectional fiber sizes of cardiotoxin-injected muscle of each genotype were compared to each other as the percentage.

**Lipofuscin accumulation in skeletal muscle**

Lipofuscin accumulates in all organs with aging, and is considered a hallmark of the aged phenotype in lower organisms as well as vertebrates (Burnk and Terman, 2002). (Unperturbed) hindlimb muscle sections were assayed for lipofuscin accumulation using Sudan Black staining. Sections were deparaffinzed then placed in 70% EtOH/PBS. Saturated Sudan Black solution was prepared by dissolving 0.5 g Sudan Black B (Sigma-Aldrich) in 50 mL of 70% EtOH and filtering two times through Whatman paper. Slides were incubated with Sudan Black solution for 20 hours at room temperature, rinsed once with 70% EtOH and developed in 70% EtOH for 10 minutes, until background was reduced. Slides were then washed in tap water for 10 minutes, dried and mounted in Permount (Fisher Scientific). Photomicrographs of the section were taken using light microscope and SimplePCI image system.

**Real-time PCR to determine relative mitochondrial abundance**

Mitochondrial abundance was estimated using quantitative PCR to compare the ratio of mitochondrial DNA to genomic DNA (Vaillant and Nagley, 1995). Total DNA was isolated from myoblasts using DNeasy kits (Qiagen). 100

ng of total DNA was used for real-time PCR. Four regions of murine mtDNA were selected for amplification, homologous to human mtDNA regions used in studies to quantitate human mitochondrial abundance (Vaillant and Nagley, 1995; Miller et al., 2003). Four sets of primers were used for the amplification of the specific regions of mtDNAs (Table 2.2). DNA sequences of 18S rRNA were amplified for quantitation of nuclear DNA abundance. Probes for each PCR template were generated using Vector NTI (Invitrogen). The 5' end of the probe was conjugated to 6-carboxyflourescein (FAM) and the 3' end was conjugated to 6-carboxytetramethylrhodamine (TAMRA). Real-time PCR was performed on a LightCycler (Roche Applied Science) using primers specific for four different regions of mtDNA and 18S rRNA (Table 2.2). Total DNA extracted from myoblasts was amplified using LightCycler Taqman Master (Roche Applied Science) by pre-incubation at 95$^{o}$ C for 10 min followed immediately by 45 cycles of: denaturing at 95$^{o}$ C for 10 sec, then the specific annealing temperature (Table 2.2) for 40 sec, then 72$^{o}$ C for 1 sec. The efficiency of primer sets and probes was measured using serial dilution of total DNA extracted from wild-type myoblasts. For quantitation, the same calculations of efficiency were used as noted above for quantitation of myogenic bHLH genes. Quantitative PCR results from wild-type proliferating myoblasts (normalized to 1) were used as the control comparison for the other samples. Data were expressed as mean ± standard deviation from the duplicate experiments.

**ROS measurements**

Relative amounts of intracellular ROS were measured using two methods. Carboxy-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA, Molecular Probes) fluorescence, where fluorescence is proportional to the amount of oxidative modification of the dye by hydroxyl radical (·OH) and peroxynitrite anion ($ONOO^-$). CM-$H_2$DCFDA fluorescence is probably the most widely used ROS assay. Relative amounts of mitochondrial superoxide production were also measured using MitoSOX (Molecular Probes) which fluoresces after selectively reacting with superoxide in mitochondria.

For intracellular ROS assays, myoblasts were seeded at a density of $10^6$ cells/6 cm plate in GM. After 3 day, cells were washed with phosphate buffered saline (PBS) three times and CM-$H_2$DCFDA (30 μM) was added for 90 min at 37$^o$C. Cells were washed with phosphate buffered saline (PBS) three times, resuspended in 200 μL of DMEM-F12 medium (without phenol-red and HEPES, Hyclone, Logan, UT) and loaded into black, flat-bottom 96 well plate. Fluorescence was measured (485/530 nm) using a microplate reader (Spectrofluor plus, Tecan, Boston, MA), and the results normalized to cell numbers quantitated by Cyquant assays (Invitrogen). Wild-type (young) cells treated with increasing concentrations of buthionine sulfoxide (which inhibits the rate-limiting glutathione synthesizing enzyme; γ-glutamylcysteine synthetase, Schafer and Buettner, 2001) were used for standard curve generation. Background fluorescence measured in wells without cells was subtrated from raw fluorescence measured for each data point. The measured fluorescence was

normalized to cell numbers and data are expressed as relative fluorescence units (RFU).

Mitochondrial superoxide was measured using the MitoSOX (Molecular Probes) with some the modification of the manufacturer's protocol. Myoblasts were seeded at a density of $10^6$ cells/6 cm plate in GM. After 3 days, cells were washed with phosphate buffered saline (PBS) three times and $10^5$ cells were resuspended in 200 µL of DMEM-F12 medium containing 10% FBS and MitoSOX (5 µM) and loaded onto black, flat-bottom 96 well plates. Wild-type (young) cells treated with various concentrations of rotenone were used for standard curve generation. Fluorescence was measured (535/590 nm) using a microplate reader every 1 min for 30 min and background fluorescence and normalization were handled as citied above for the CM-$H_2$DCFDA assays. Data were expressed as mean ± standard deviation from the duplicate experiments. Differences between mean relative fluorescence in each group were compared using Student's t-tests.

**Mitochondrial enzyme activity**

The mitochondrial fraction was extracted from myoblasts grown to confluency on collagen-coated 150 mm dishes using a commercial mitochondria extraction kit (Imgenex).

SOD2 enzyme activity was assayed using a commercial superoxide dismutase activity kit (Dojindo). Briefly, whole cell lysates were collected using the manufacturer's cell extraction buffer and protein concentration was

quantitated using micro-Bradford assays (Bio-Rad). Serial dilutions of lystates were mixed with the commercial master mix including WST-1 reagent, [WST-1 is reduced by oxygen radicals which are produced by the reaction between xanthine oxidase and hypoxanthine to produce a yellow dye, detected by absorbance at 450 nm (Peskin and Winterbourn, 2000)], xanthine oxidase and 2mM sodium cyanide (SOD1 inhibitor). The reaction was initiated by addition of xanthine solution into the wells and absorbance change was recorded at 450 nm every min for 30 min at room temperature. The SOD2 activities were calculated from the inhibition rate of xanthine oxidase reaction and expressed as the percentage. Data were expressed as mean ± standard deviation from the duplicate experiments. Differences between mean relative fluorescence in each group were compared using Student's t-tests.

Succinate dehydrogenase (SDH) activity assays were modified from published protocols (Rosen et al.,1987; Powell and Jackson, 2003). Briefly, cell lysates extracted from mitochondrial fractions were diluted to a concentration of 0.1 µg/ µL in 30 mM Tris-HCl (pH 8.0). 50 µL of the lysate was mixed with 190 µL of 30 mM Tris-HCl (pH 8.0), 5 µL of 1M sodium succinate (pH 7.4), and 4 µL of 4.65 mM 2, 6-dichloroindophenol (DCIP). DCIP reduction was initiated by addition of 6 µL of 6.5 mM phenazidine methosulfate and the decrease in absorbance at 600 nm was measured for 30 min at room temperature. For the calculation of SDH activity, molar extinction coefficient, ε = 21,000 was used and activity was expressed as mmol DCIP reduced·$min^{-1}$ ·mg $protein^{-1}$. Data were expressed as mean ± standard deviation from the duplicate experiments.

Differences between mean relative fluorescence in each group were compared using Student's t-tests.

Aconitase enzyme activity was measured using commercially available kits (Oxisresearch), modified for a microplate reader. Briefly, 50 µL of cytosolic or mitochondrial cell lysates (1µg/µL) was diluted with 20 µL of Tris-HCl (pH 7.4) buffer, mixed with 60 µL of trisodium citrate (pH 7.4; substrate) and 60 µL of NADP, and then loaded into 96-well plates. The reaction was initiated by the addition of 60 µL of isocitrate dehydrogenase at 37$^{o}$ C for 15 min protected from light. The absorbance change at 340 nm was recorded for 30 min at 37$^{o}$ C. For the calculation of aconitase activity, molar extinction coefficient, ε=6.220 X $10^{-3}$ mL/nmol was used. Data were expressed as mean ± standard deviation from the duplicate experiments. Differences between mean relative fluorescence in each group were compared using Student's t-tests.

**Western blots**

Cultured myoblasts were collected in M-PER extraction reagent (Pierce) with protease and phosphatase inhibitors. Micro Bradford reagent (Bio-Rad) was used to determine the protein concentration. Proteins (35 µg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with TBST (Tris-buffered saline-Tween-20; 25 mM Tris-HCl, pH 7.5, 1mM NaCl and 0.1% Tween-20) containing either 5% milk or 5% BSA (for phospho-antibodies) for 1 hr, then incubated with the primary antibody at 4$^{o}$ C overnight. The following primary antibodies were used for

western blotting: SOD2 (1:2000 dilution, Assay Designs); 1/1000 dilution of antibodies from Cell Signaling: PGC-1α, β-tubulin, total Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), total S6 kinase, phospho-S6 kinase (Thr389), total mTOR, phospho-mTOR (Ser2448), p44/42, phospho-p44/42, p38, phospho-p38 and GAPDH; total FoxO1 (Santa Cruz), phospho-FoxO1 (Invitrogen), HIF-1α (Sigma-Aldrich) and myogenin (Sigma-Aldrich). After three washes with TBST, blots were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase for 1 hr at room temperature. After three washes with TBST, membranes were developed using the enhanced chemiluminescent method (ECL, Pierce). Band intensity of each protein was analyzed using Image J Software and normalized to the band intensity of β-tubulin (loading control). Data were expressed as mean ± standard deviation from the duplicate experiments.

**Data analysis**

Results are expressed as mean ± standard deviation (S.D.) throughout. Statistical analyses were performed using Image J Software or Excel (Microsoft Office 2000). Unpaired Student's t-testing was used for continuous data and $Chi^2$ testing was used for categorical data, as noted in figure legends. For the correlation statistics, the coefficient of correlation (r) was calculated using Minitab software.

**Table 2.1**

**The sequences of primers for quantitation of myogenic regulatory factors (MRFs)**

| Primer Name | SEQUENCE (5' to 3') | Product size (bp) | Annealing Tm (°C) | Efficiency |
|---|---|---|---|---|
| MyoD | CCCCGGCGGCAGAATGGCTACG | 234 | 65 | 2.252 |
| | GGTCTGGGTTCCCTGTTCTGTGT | | | |
| myf5 | TGCCATCCGCTACATTGAGAG | 353 | 56 | 2.184 |
| | CCGGGGTAGCAGGCTGTGAGTTG | | | |
| mrf4 | ACCCTTACAGCTACAA | 215 | 50.4 | 2.043 |
| | AATACTGTCCACGAT | | | |
| myogenin | CCGTGGGCATGTAAGGTGTG | 198 | 63 | 1.982 |
| | TAGGCGCTCAATGTACTGGATGG | | | |
| DNA of 18s RNA | ACAGGTCTGTGATGCC | 234 | 55.2 | 2.023 |
| | ATCGGTAGTAGCGACG | | | |

**Table 2.2**

**The sequences of primers and probes for quantitation of mitochondrial DNA abundance**

| Dual DNA Probe | 5’ to 3’ (5'-FAM & 3'-TAMRA conjugated) |
|---|---|
| A3 probe | TAGAGAAGGTTATTAGGGTGGCAGAGCC |
| B1 probe | CCACTTCACTAACAATATTTCCAACCAACA |
| C3 probe | TATTAATCGCAGCTACAGGAAAATCAGCA |
| D2 probe | ATATATACACGCAAACGGAGCCTCAATATT |
| Probe for DNA of 18s RNA | CCCCATGAACGAGGAATTCCCAGTAAGT |

| Primer Name | SEQUENCE (5' to 3') | Product size (bp) | Annealing Tm (°C) | Efficiency |
|---|---|---|---|---|
| A3 | GTACGAAAGGACAAGAG | 288 | 54 | 1.956 |
| | CGTAGTTGTATGTACCC | | | |
| B1 | CTAACAGGATTCTTACCAAA | 250 | 54 | 1.881 |
| | CTAGGGGTAGGGTTAT | | | |
| C3 | TATTCTCCAACAACAACG | 238 | 54 | 1.836 |
| | CCGAGGCAAAGTATAG | | | |
| D2 | TTACGGGTGACTAATCC | 260 | 45 | 2.060 |
| | CTAGGGTTGTTCCAAT | | | |
| DNA of 18s RNA | ACAGGTCTGTGATGCC | 234 | 47 | 2.306 |
| | ATCGGTAGTAGCGACG | | | |

# Chapter 3: Glutathione-peroxidase-1 null muscle progenitor cells are globally defective

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## 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 (Ho et al., 1997), but are less tolerant of a variety of oxidant stresses compared to wild-type mice. They have decreased survival after paraquat or diquat challenges (Lei, 2002), or after systemic infections (Bech et al., 1998), and increased organ damage after ischemia-reperfusion injuries (De Haan et al., 2003). Recently, human deficiency of Gpx1 activity has been associated with endothelial dysfunction and increased risk of myocardial disease (Leopold and Loscalzo, 2005; Loscalzo et al., 2005).

Gpx1 expression varies in different organs and is substantially lower in skeletal muscle than in other organs such as the liver (Esposito et al., 2000). In a murine myoblast line, Gpx1 and other major antioxidant activities are progressively downregulated during muscle differentiation, increasing the susceptibility to oxidant stress (Franco et al., 1999). Muscle progenitor populations are certainly exposed to oxidant stress with aging and as a function of pathologies including muscular dystrophies (Disatnik et al., 1988), muscle atrophy with disuse (Lawler et al., 2002) or contraction-induced muscle injury (McArdle et al., 1999). 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.

## Results

### Early fusion of $Gpx1^{-/-}$ myoblasts

As small colonies of myoblasts were first developing after isolation, some of the $Gpx1^{-/-}$ myoblasts fused, despite the presence of high serum and FGF2 in the culture medium (Figure 3.1A). These prematurely fused myoblasts were not observed after passage 3. Fusion in GM was never observed in wild-type myoblasts (Figure 3.1B). As a consequence of fusion, multinuclear myotubes exit the cell cycle. Therefore, as cultures were passaged for expansion and analysis, the $Gpx1^{-/-}$ fused cells were lost.

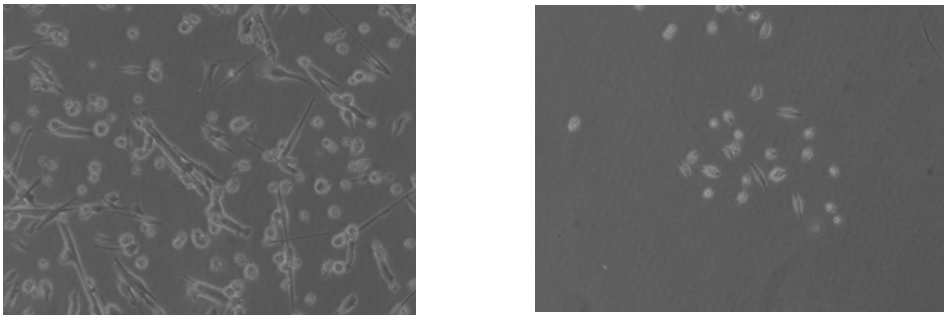
When Gpx1$^{-/-}$ myoblasts at passage 3 were induced to differentiate (in DM), they fused and became hypercontractile, again a feature never observed in the wild- type myoblasts. Without any stimulus, the myotubes spontaneously contracted constantly for more than a week in culture, a distinctly abnormal behavior. Videos of these contractile muscle cells can be viewed on the Free Radical and Biology website, doi:10.1016/j.freeradbiomed.2006.07.005.

The purity of the population of undifferentiated myoblasts was confirmed using anti-desmin immunostaining. Before differentiation, 98% of the Gpx1$^{-/-}$ cells were desmin-positive, and 93% of the wild-type myoblasts (stains not shown).

**Figure 3.1**

**Abnormal morphology of freshly isolated Gpx1$^{-/-}$ myoblast colonies**

**A. Gpx1$^{-/-}$ myoblasts** **B. Wild-type myoblasts.**



Myoblasts in GM containing bFGF and 20% serum.

(A) Abnormal fusion of Gpx1$^{-/-}$ myoblasts, a pattern never observed in wild-type cells in GM.

(B) Wild-type myoblasts do not fuse in GM.

**Intracellular oxidant stress in Gpx1$^{-/-}$ vs. wild-type myoblasts as a function of $O_2$ culture conditions**

Within genotypes, both wild-type and Gpx1$^{-/-}$ myoblasts accumulated significantly less intracellular ROS in 6% $O_2$ than in 20% $O_2$ as assessed by both CM-$H_2$DCFDA fluorescence and Amplex Red assays ($p<0.05$, Figures 3.2A and 3.2B). For Amplex Red, wild-type cells in 20% $O_2$ accumulated more intracellular hydrogen peroxide (1547 ± 92 RFU) than the same cells cultured in 6% $O_2$ (1228 ± 27 RFU), $p=0.027$ (Figure 3.2B).

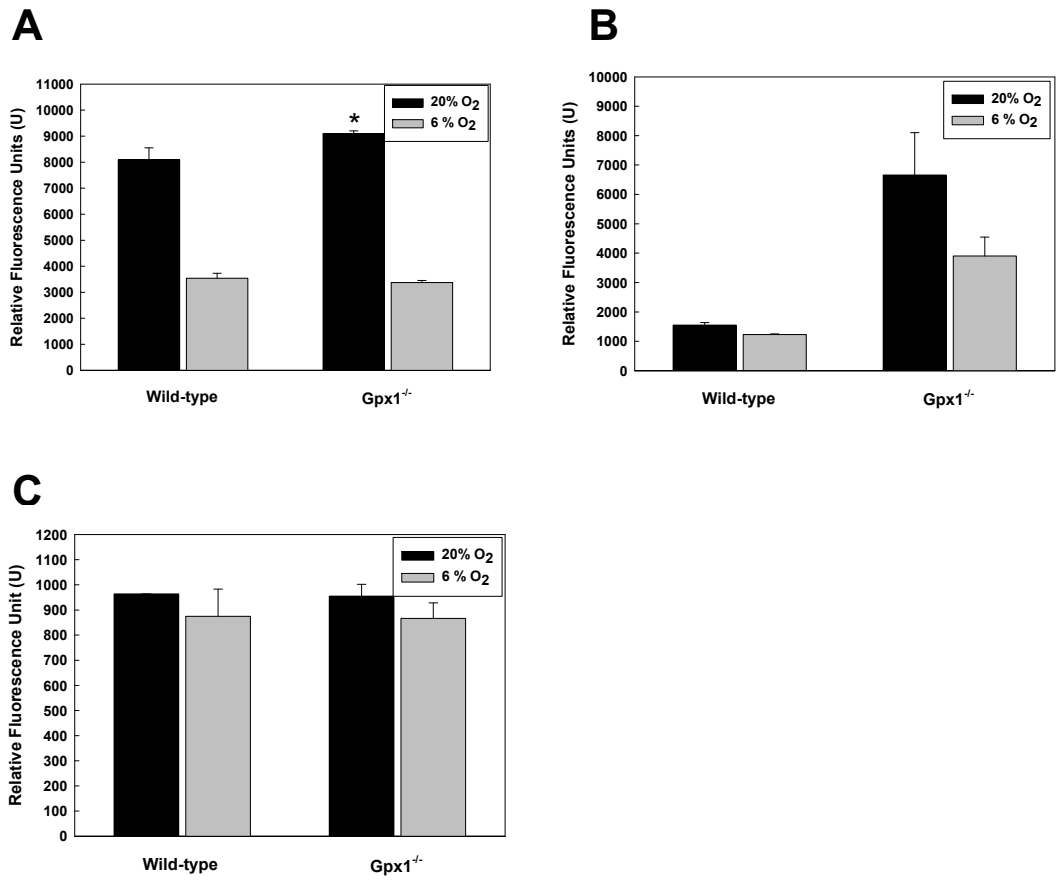
Gpx1$^{-/-}$ intracellular ROS were slightly elevated compared to wild-type myoblasts in 20% $O_2$ (9027 ± 103 vs. 8091 ± 462 RFU, $p=0.009$, $n=4$ per group) using CM-$H_2$DCFDA fluorescence. Intracellular ROS levels using this assay were similar between the two genotypes in 6% $O_2$ (Figure 3.2A). Using the assay more specific for hydrogen peroxide, Amplex Red, Gpx1$^{-/-}$ myoblasts accumulated significantly more intracellular $H_2O_2$ than wild-type myoblasts in both $O_2$ conditions (Figure 3.2B).

**Relative oxidant levels in culture medium of Gpx1$^{-/-}$ and wild-type myoblasts**

Extracellular peroxide concentrations were similar between the two myoblast genotypes and in both oxygen conditions (Figure 3.2C). Relative fluorescence U for wild-type cells in 20% and 6% $O_2$ were 964 ± 0 and 875 ± 109 U, and for Gpx1$^{-/-}$ myoblasts in 20% and 6% $O_2$, 955 ± 47 and 866 ± 62 U.

**Figure 3.2**

**Relative intracellular oxidant stress as a function of $O_2$ culture conditions and Gpx1 deletion**




(A) Myoblast intracellular oxidant stress after 3 days of culture was assessed by CM-$H_2$DCFDA fluorescence, and normalized to protein content. Both wild-type and $Gpx1^{-/-}$ myoblasts accumulate significantly less oxidant stress when cultured in 6% $O_2$ than when grown in 20% $O_2$ ($p<0.05$). Oxidant stress in $Gpx1^{-/-}$ myoblasts in 20% $O_2$ was significantly more than that in wild-type myoblasts in the same conditions (*$p=0.009$).

(B) Amplex Red assay reveals significantly more intracellular hydrogen peroxide in Gpx1$^{-/-}$ myoblasts compared to wild-type in both 6% $O_2$ and 20% $O_2$ conditions ($p=0.027$ and 0.038 respectively).
(C) Amplex Red assay reveals no significant difference of extracellular hydrogen peroxide in Gpx1$^{-/-}$ myoblasts compared to wild-type in both 6% $O_2$ and 20% $O_2$ conditions ($p>0.05$ for both conditions).

**The relative abundance of mitochodria in myoblasts**

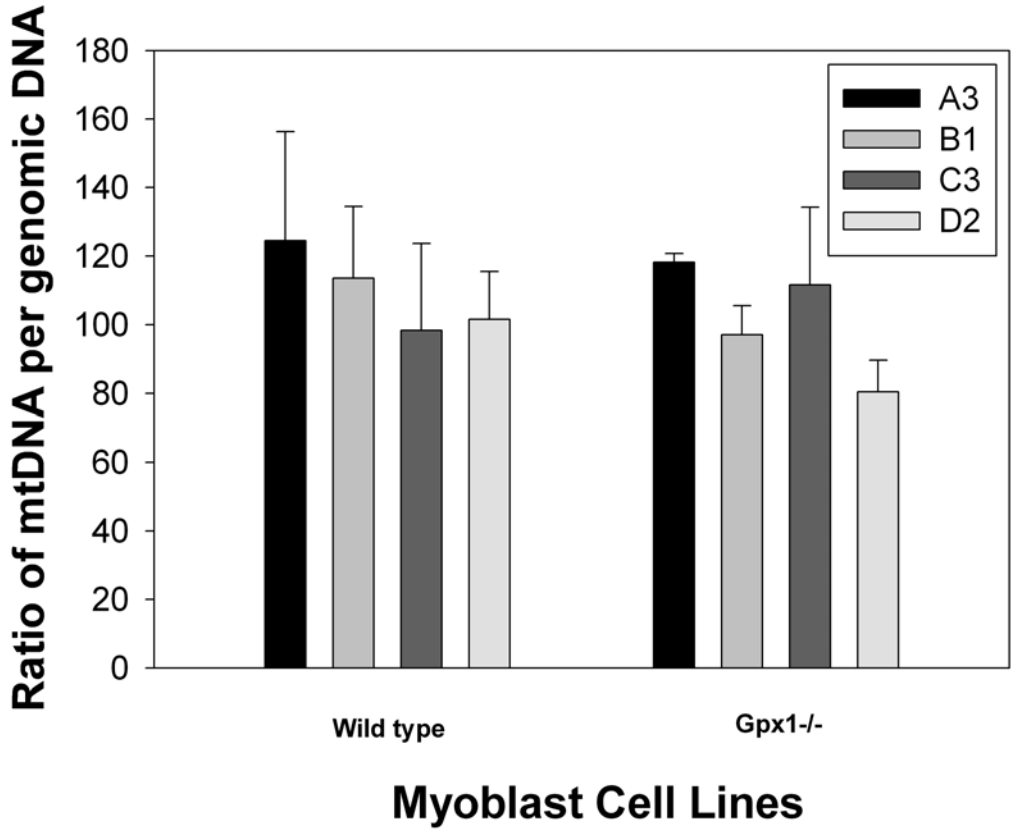
Four regions of mtDNA sequences were selected for qRT-PCR amplification (Miller et al., 2003; Vaillant and Nagley, 1995 to quantify the relative abundance of mitochondria (using mtDNA:genomic DNA) in myoblasts. These studies suggested that Gpx1$^{-/-}$ and wild-type myoblasts from young mice had similar mitochondrial abundance (Figure 3.3, $p>0.2$). Overall, there was not complete concordance of the ratio of mtDNA to genomic DNA using the different primer sets. However, the general pattern for each region was similar. Different efficiencies of the primer sets and probes in qRT-PCR reaction likely account for the differences in readout for each individual reaction.

**Young Gpx1$^{-/-}$ myoblasts accumulate oxidatively modified proteins**

Western blotting to demonstrate abundance of proteins with carbonyl modifications in Gpx1$^{-/-}$ myoblast lysates revealed protein bands at a wide range of molecular weights. By comparison, only very faint bands were visualized in lysates of wild-type myoblasts (Figure 3.4).

**Figure 3.3**

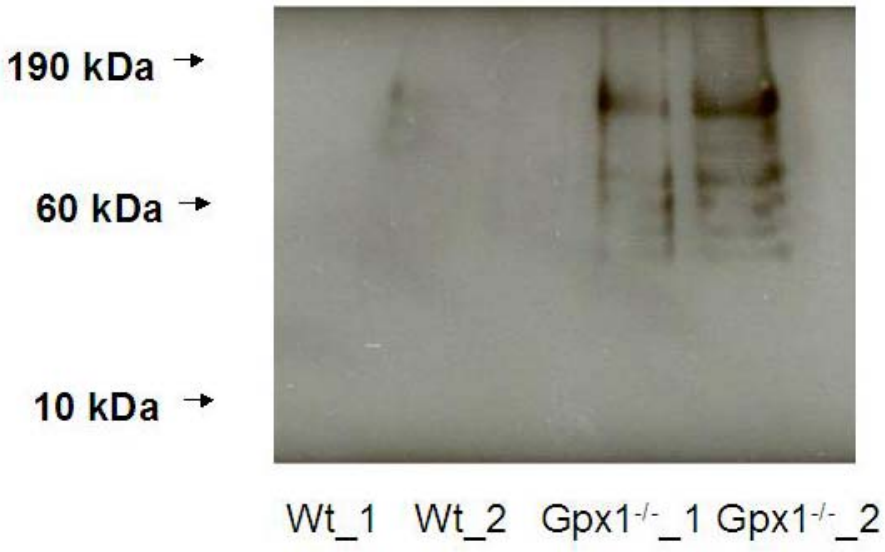
**The relative abundance of mitochondria in myoblasts was quantitated by qRT-PCR using amplification of mitochondrial genome and chromosomal genome regions.**




Overall, there was no significant difference in the ratio of mtDNA:genomic DNA in Gpx1$^{-/-}$ myoblasts compared to wild-type ($p>0.2$).

**Figure 3.4**

**Western blotting to detect carbonyl modification of intracellular proteins**




Lysates from Gpx1$^{-/-}$ myoblasts yielded distinct bands of proteins of various molecular weights with carbonylated side chains, visualized by western blotting. Wild-type myoblast lysates revealed only very faint bands, by comparison. Wild- type lanes are labeled Wt_1 and Wt_2, and Gpx1$^{-/-}$ myoblasts lanes, Gpx1$^{-/-}$_1 and Gpx1$^{-/-}$_2.

**Impaired proliferation of Gpx1$^{-/-}$ myoblasts**

BrdU uptake was used to quantitate proliferation of myoblasts on several different matrices, at 24 and 48 hours after plating (Table 3.1). On all matrices, a smaller percent of the Gpx1$^{-/-}$ myoblasts was actively proliferating compared to

**Table 3.1**

**Proliferation of myoblasts on various extracellular matrices**

| MARTIX | TIME | $Gpx1^{-/-}$ | WT | $Chi^2$ |
|---|---|---|---|---|
| **Collagen** | **24 hr** | **58.0 ± 17.1** | **66.8 ± 18.5** | **55** |
| | **48 hr** | **52.1 ± 16.1** | **65.5 ± 10.8** | **139** |
| **Gelatin** | **24 hr** | **47.2 ± 24.6** | **55.7 ± 28.2** | **117** |
| | **48 hr** | **48.5 ± 14.7** | **65.4 ± 14.5** | **213** |
| **ECL** | **24 hr** | **54.4 ± 19.7** | **76.4 ± 13.8** | **161** |
| | **48 hr** | **52.0 ± 12.6** | **73.7 ± 10.4** | **319** |

Data are expressed as the percentage of BrdU-immunolabeled cells after a 4-hour BrdU pulse $\pm$ S.D. for each matrix at 24 and 48 hours after plating. Total numbers of labeled and unlabelled cells (>3000 cells counted per condition) were used to construct 2 X 2 $Chi^2$ comparisons. For all times and matrices, p value was $\leq 0.001$ for $Gpx1^{-/-}$ compared to wild-type myoblasts. Wild-type myoblast proliferation was significantly greatest on ECL at both 24 hr ($p=0.01$) and 48 hr ($p=0.002$), whereas $Gpx1^{-/-}$ myoblasts proliferated best on collagen at 24 hr ($p=0.02$) but similarly on all matrices at 48 hr.

wild- types ($p<0.001$). $Gpx1^{-/-}$ myoblasts proliferated most on collagen I, and least on gelatin ($p=0.02$); proliferation of $Gpx1^{-/-}$ myoblasts on collagen vs. ECL and ECL vs. gelatin was similar at 24 hr. For wild-type myoblasts, proliferation on ECL was significantly better than on both gelatin and collagen I ($p<0.01$), and

proliferation on gelatin was significantly greater than on collagen (p=0.04). At 48 hours, there were no significant proliferation differences on the three extracellular matrix coatings for Gpx1$^{-/-}$ cells, whereas proliferation of wild-type cells on ECL remained significantly greater than on the other matrices (p= 0.002).

**Apoptosis of Gpx1$^{-/-}$ myoblasts in high and low oxidant stress conditions**

After three days in DM on ECL, a greater proportion of Gpx1$^{-/-}$ myoblasts in 20% $O_2$ underwent apoptosis vs. wild-type controls ($p<0.05$, Figure 3.5A). Apoptosis of Gpx1$^{-/-}$ vs. wild-type myoblasts in 6% $O_2$ was not significantly different. After 5 days, Gpx1$^{-/-}$ myoblasts underwent significantly more apoptosis than wild-type cells in both $O_2$ conditions ($p<0.05$).

The TUNEL assay was repeated in Gpx1$^{-/-}$ myoblasts maintained in the presence of the glutathione donor, N-acetylcysteine (NAC, 10 μM) or catalase (500 U/ml), in 20% $O_2$. At 3 days of culture in differentiation medium, only catalase mediated a significant decrease in the percentage of cells undergoing apoptosis (p=0.002). After 120 hours in the presence of either NAC (p=0.04) or catalase (p=0.03) apoptosis of Gpx1$^{-/-}$ myoblasts was significantly reduced (Figure 3.5B).

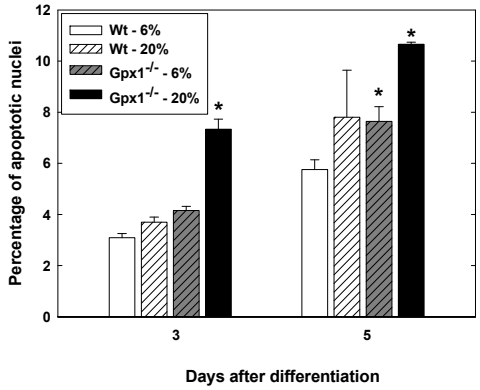
**Impaired terminal muscle differentiation of Gpx1$^{-/-}$ myoblasts**

The most striking difference between Gpx1$^{-/-}$ and wild-type myoblasts was in the formation of myotubes in culture (Figure 3.6). After 5 days in DM on ECL, less than 20% of Gpx1$^{-/-}$ myoblasts were incorporated into myotubes in both 20%

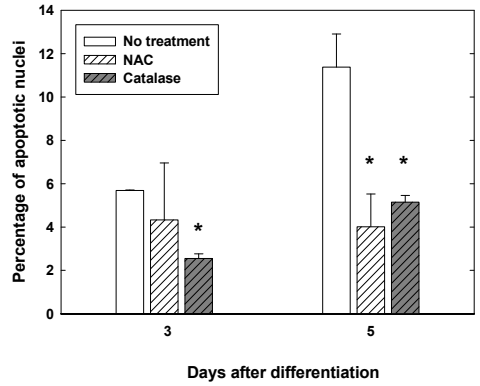
**Figure 3.5**

**Increased apoptosis of cultured Gpx1$^{-/-}$ myoblasts**

**A**




**B**




(A) After three days, Gpx1$^{-/-}$ myoblasts exhibited significantly more apoptosis than wild-type myoblasts in 20% $O_2$, but not in 6% $O_2$. At 5 days, the rate of apoptosis was greater in Gpx1$^{-/-}$ myoblasts than in wild-type cells in both $O_2$ conditions. (*$p<0.05$ vs. wild-type).

(B) Treatment of Gpx1$^{-/-}$ myoblasts with either NAC (10 μM) or catalase (500 U/ml) for 3 days reduced the percentage of cells undergoing apoptosis, but the reduction mediated by NAC was not statistically significant. Five days of treatment with either catalase ($p= 0.03$) or NAC ($p=0.04$) mediated a significant reduction in apoptosis of Gpx1$^{-/-}$ cells in differentiation medium.

and 6% $O_2$, whereas about 90% of wild-type nuclei were in myotubes in 6% $O_2$, and 80% were in myotubes in 20% $O_2$ ($p<0.05$ compared to $Gpx1^{-/-}$ for both). Futhermore, only rare myotubes with more than 3 nuclei were formed by $Gpx1^{-/-}$ myoblasts after 5 days of differentiation while the majority of wild-type myotubes contained 4 or more nuclei. Thus, mature myotube formation from $Gpx1^{-/-}$ myoblasts was completely absent.

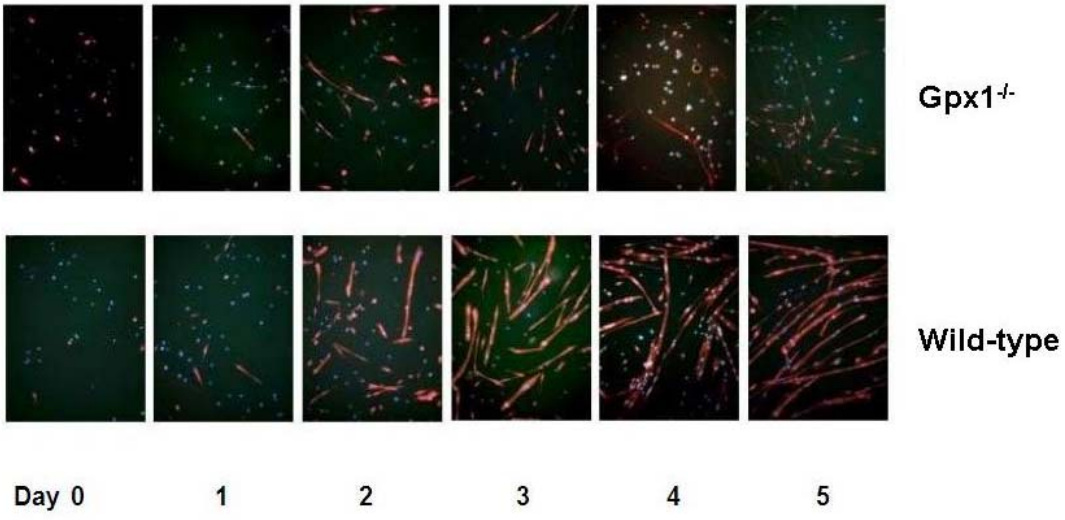
Differentiation of wild-type cells exposed to 2.5 μM $H_2O_2$ was also quantified in detail. Of note, when this low concentration of $H_2O_2$ was added to the myoblasts at the start of differentiation, the cells lifted off the plate. When $H_2O_2$ was added at 48 hours of culture in differentiation medium, the cells remained on the plate and differentiation could be assayed. The fusion indices from days 3 to 5 in $Gpx1^{-/-}$ myoblasts are virtually identical to those of wild-type cells treated with $H_2O_2$. The addition of $H_2O_2$ also significantly reduced the differentiation potential of wild-type cells (Figure 3.6B).

Differentiation, like proliferation, can be significantly altered by the extracellular matrix, (Adams and Watt, 1993) so differentiation studies were repeated on three different matrix coatings. Collagen I coating facilitated the formation of a few mature $Gpx1^{-/-}$ myoblasts with greater than 10 nuclei, whereas no myotubes of this size were formed on ECL or gelatin. The percentage of nuclei incorporated into mature myotubes (>4 nuclei) for $Gpx1^{-/-}$ was significantly lower than for wild-type nuclei on all matrices (p values all >0.2). In summary, no matrix facilitated the formation of mature myotubes to wild-type levels by $Gpx1^{-/-}$ myoblasts.

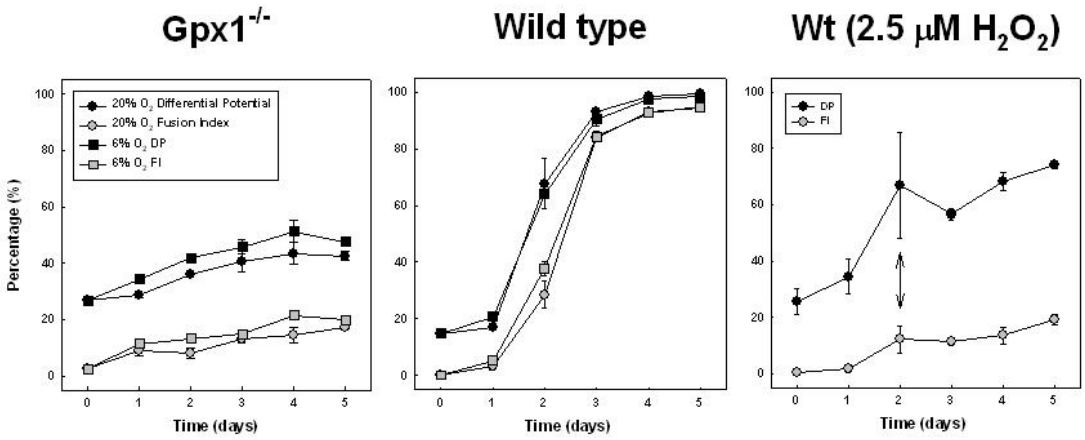
**Figure 3.6**

**Terminal differentiation of skeletal muscle *in vitro***

**A**




**B**



(A) Morphological differentiation of Gpx1$^{-/-}$ myoblasts. Myoblasts incubated in DM were fixed and stained with MF20 antibody recognizing myosin heavy chain (red) at 1-day intervals. From day 1 on, Gpx1$^{-/-}$ myoblasts morphologic differentiation, characterized by formation of multinucleate myotubes, was significantly reduced compared to wild-type.
(B) Quantitation of Gpx1$^{-/-}$ myoblasts vs. wild-type myoblasts vs. wild-type myoblasts with 2.5 μM $H_2O_2$. Differentiation potential (DP) was calculated as follows: [(Number of nuclei in MF 20-stained cells/Total number of nuclei) X 100]. Fusion index (FI) was calculated as follows: [(Number of nuclei in MF20-stained myotubes with ≥ 2 nuclei/Total number of nuclei) X 100]. Within genotype, $O_2$ conditions did not cause statistically significant differences in either DP or FI. Starting on day 2, wild-type myoblasts (center) formed considerably more myotubes than did Gpx1$^{-/-}$ myoblasts (left), and significant differences in both FI and DP persisted through day 5 ($p<0.05$ for days 2-5).

Various strategies directed at lowering oxidant stress were also used in an effort to improve differentiation of Gpx1$^{-/-}$ myoblasts. Lower (6%) $O_2$ conditions did not significantly enhance differentiation in either Gpx1$^{-/-}$ or wild-type cells compared to culture in 20% $O_2$ (Figure 3.6B). Treatment with the ROS scavenger phenyl-N-tert-butylnitrone (PBN, 1 mM) did not increase the number of mature myotubes (>4 nuclei) formed by Gpx1$^{-/-}$ myoblasts in DM (not shown). More nascent myotubes (2-3 nuclei) formed during differentiation of Gpx1$^{-/-}$ myoblasts

in the presence of catalase (500 U/ml) or NAC (10 μM), though not to wild-type levels (Table 3.2). At day 5, both NAC and catalase treatments resulted in formation of some mature myotubes (4-10 nuclei) whereas untreated Gpx1$^{-/-}$ myoblasts generated virtually none, but neither treatment influenced the inability of these cells to form very large myotubes (>10 nuclei, Table 3.2).

**Table 3.2**

**Antioxidant treatment improves differentiation capacity of Gpx1$^{-/-}$ myoblasts**

| Gpx1$^{-/-}$ | Time | 1 nucleus | 2-3 nuclei | 4-10 nuclei | > 10 nuclei | DP |
|---|---|---|---|---|---|---|
| No treatment | Day 3 | 86.7±2.8 | 13.8±2.8 | 0 | 0 | 13.3±2.8 |
| | Day 5 | 81.4±0.4 | 17.0±0.7 | 1.6±0.3 | 0 | 18.6±0.4 |
| NAC | Day 3 | 79.1±0.2 | 20.9±0.2 | 0 | 0 | 20.9±0.2 |
| | Day 5 | 69.1±2.2 | 25.9±1.2 | 4.9±1.0 | 0 | 30.9±2.2 |
| Catalase | Day 3 | 79.6±1.0 | 19.5±2.2 | 0.8±1.2 | 0 | 20.4±0.4 |
| | Day 5 | 72.5±0.4 | 22.4±1.0 | 5.0±1.5 | 0 | 27.5±0.4 |

Differentiation of Gpx1$^{-/-}$ myoblasts in the presence of either NAC (10 mM) or catalase (500 U/ml) improved formation of nascent myotubes with 2-3 nuclei but not significantly. Treatment with NAC or catalase did improve differentiation of Gpx1$^{-/-}$ myoblasts into mature myotubes (4-10 nuclei) but not nearly to wild-type levels. Neither treatment rescued the inability of Gpx1$^{-/-}$ myoblasts to generate very large myotubes (>10 nuclei).

High seeding density did significantly improve the formation of mature myotubes by $Gpx1^{-/-}$ myoblasts, to the same extent in 6% and 20% $O_2$. When initial plating density for differentiation was increased four-fold, 15.3 ± 0.2% of $Gpx1^{-/-}$ nuclei at day five were incorporated into myotubes with > 10 nuclei (highly significant since no large myotubes formed at lower seeding density). However, significantly more mature myotubes (>4 nuclei) were formed by wild-type cells seeded at low density than by $Gpx1^{-/-}$ myoblasts seeded at high density ($Chi^2$=14.7, $p<0.001$).

Co-cultured wild-type myoblasts (expressing GFP) significantly improved the differentiation of $Gpx1^{-/-}$ myoblasts in a dose-dependent way, that is, the higher the ratio of wild-type to $Gpx1^{-/-}$ cells in culture, the greater the number of $Gpx1^{-/-}$ nuclei incorporated into myotubes (Table 3.3). At even the lowest ratio of wild-type to $Gpx1^{-/-}$ cells (1:4), the incorporation of $Gpx1^{-/-}$ nuclei into mature myotubes was significantly greater for $Gpx1^{-/-}$ myoblasts alone under the same conditions (Table 3.3). These results suggest that secreted product(s) of myoblasts necessary for optimal differentiation may be missing or impaired in the $Gpx1^{-/-}$ myoblasts, since density (cell contact) was independently tested (above). Co-culture of wild-type cells with $Gpx1^{-/-}$ myoblasts reduced the number of large, mature myotubes generated compared to that of wild-type cells alone (Table 3.3). The majority of myotubes formed in all these experiments were mixed, suggesting that $Gpx1^{-/-}$ cells have not completely lost the capacity to fuse (with a normal target myoblast).

**Table 3.3**

**Deficient myotube formation of Gpx1$^{-/-}$ myoblasts is partially rescued by co-culture with wild-type myoblasts in a dose-dependent manner**

| TIME | Gpx1$^{-/-}$ : Wt = 1 : 4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 d | 1 nucleus | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 18.3±0 | | 62.9±0.3 | | 18.8±0.3 | | 1.2±0.5 | |
| | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt |
| | 10.2±0.7 | 8.1±0.7 | 32.9±2.2 | 30.0±1.9 | 9.2±1.3 | 10.1±1.1 | 0.7±0.3 | 0.5±0.2 |
| 5 d | 1 nuclei | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 13.4±1.5 | | 61.5±2.3 | | 25.1±0.8 | | 5.5±0.2 | |
| | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1$^{-/-}$ | Wt |
| | 8.0±0.2 | 5.4±1.3 | 33.6±2.5 | 28.0±0.2 | 12.8±0.5 | 12.3±1.3 | 2.9±0.1 | 2.5±0.2 |
| | Gpx1$^{-/-}$ : Wt = 1 : 1 | | | | | | | |
| 3 d | 1 nucleus | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 32.3±0.1 | | 53.9±3.1 | | 13.6±2.6 | | 1.0±0.5 | |
| | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt |
| | 21.2±1.1 | 11.1±1.0 | 30.5±1.8 | 21.9±0.8 | 6.7±0.7 | 7.0±1.9 | 0.4±0.2 | 0.6±0.7 |
| 5 d | 1 nuclei | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 19.1±1.2 | | 61.7±0.6 | | 18.4±0.6 | | 0.8±0 | |
| | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1$^{-/-}$ | Wt |
| | 11.8±0.3 | 7.3±0.9 | 30.8±5.3 | 31.0±6.9 | 8.6±3.6 | 9.9±2.9 | 0.4±0.2 | 0.3±0.1 |
| | Gpx1$^{-/-}$ : Wt = 4 : 1 | | | | | | | |
| 3 d | 1 nucleus | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 45.8±4.8 | | 47.6±4.4 | | 6.7±0.3 | | 0 | |
| | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt | Gpx1$^{-/-}$ | Wt |
| | 31.1±3.0 | 14.7±1.8 | 36.4±4.8 | 11.2±0.3 | 4.3±0.9 | 2.4±0.6 | 0 | 0 |
| 5 d | 1 nucleus | | 2, 3 nuclei | | 4 to 10 nuclei | | > 10 nuclei | |
| | 46.6±2.1 | | 44.3±0.8 | | 9.1±1.3 | | 0 | |
| | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1-/- | Wt | Gpx1$^{-/-}$ | Wt |
| | 28.4±2.5 | 18.2±0.4 | 27.2±1.6 | 17.1±0.8 | 4.6±1.2 | 4.5±0.1 | 0 | 0 |

Differentiation of mixed myoblast cultures, with co-cultured wild-type and Gpx1$^{-/-}$ myoblasts, showed that presence of wild-type myoblasts significantly improved the ability of Gpx1$^{-/-}$ cells to contribute to mature myoblast formation. The numbers of mature myotubes incorporating Gpx1$^{-/-}$ nuclei is significantly greater in all these mixed cultures than for Gpx1$^{-/-}$ cells alone (which can form no mature myotubes after passage 3 at plating density used for these studies).

**Expression of core regulators of myogenesis in Gpx1$^{-/-}$ myoblasts**

The myogenic basic helix-loop-helix (bHLH) transcription factors are core mediators of skeletal muscle development, commitment and differentiation (Berkes and Tapscott, 2005). A time course of message expression during differentiation was performed to determine whether the observed differentiation defect was due to downregulation of this family of genes in the Gpx1$^{-/-}$ myoblasts. Detailed quantitation of message abundance for MyoD, myf5, mrf4 and myogenin was performed using real-time quantitative fluorescence PCR (Table 3.4). These data revealed that the morphologic differentiation defect seen in Gpx1$^{-/-}$ myoblasts was not a “classical” muscle differentiation defect mediated by downregulation of myogenic transcription factors: All four genes are upregulated during early differentiation of Gpx1$^{-/-}$ myoblasts compared to wild-type (Table 3.4). The most aberrant difference in expression was the high (inappropriate) increase in bHLH expression in undifferentiated Gpx1$^{-/-}$ myoblasts compared to wild-type controls.

**Table 3.4**

**Expression of myogenic bHLH genes during myoblast differentiation**

**A. Myogenic bHLH relative message abundance during myoblast differentiation in 20% $O_2$.**

| Transcript | Genotype | Pre-diff | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|---|---|
| MyoD | $Gpx1^{-/-}$ | 4.4 ± 0.2 | 6.6 ± 0.8 | 18.8 ± 5.3 | 11.4 ± 5.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | wt | (1) | 4.0 ± 3.8 | 0.6 ± 0.3 | 0.5 ± 0.5 | 0.1 ± 0.0 | 0.2 ± 0.0 |
| myf5 | $Gpx1^{-/-}$ | 4.6 ± 0.7 | 6.6 ± 0.4 | 4.6 ± 1.5 | 2.2 ± 0.9 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | wt | (1) | 1.2 ± 0.8 | 1.5 ± 0.4 | 2.8 ± 0.5 | 1.4 ± 0.0 | 0.7 ± 0.1 |
| mrf4 | $Gpx1^{-/-}$ | 9.6 ± 2.3 | 8.2 ± 0.7 | 8.5 ± 3.9 | 5.7 ± 2.9 | 0.7 ± 0.4 | 0.2 ± 0.2 |
| | wt | (1) | 1.5 ± 0.1 | 1.5 ± 0.0 | 1.1 ± 0.2 | 0.9 ± 0.0 | 0.7 ± 0.1 |
| myogenin | $Gpx1^{-/-}$ | 1.5 ± 0.3 | 1.3 ± 0.3 | 14.1 ± 1.7 | 9.4 ± 4.0 | 0.1 ± 0.1 | 0.0 ± 0.0 |
| | wt | (1) | 1.1 ± 0.0 | 1.7 ± 0.3 | 2.7 ± 0.5 | 2.6 ± 0.6 | 1.6 ± 0.2 |

**B. Myogenic bHLH relative message abundance during myoblast differentiation in 6% $O_2$.**

| Transcript | Genotype | Pre-diff | Day1 | Day 2 | Day 3 | Day 4 | Day 5 |
|---|---|---|---|---|---|---|---|
| MyoD | $Gpx1^{-/-}$ | 4.4 ± 0.2 | 14.1 ± 7.6 | 7.3 ± 1.5 | 19.7 ± 10.3 | 0.1 ± 0.0 | 0.0 ± 0.0 |
| | wt | (1) | 1.3 ± 0.6 | 1.2 ± 0.5 | 2.1 ± 0.4 | 2.1 ± 0.1 | 1.2 ± 0.1 |
| myf5 | $Gpx1^{-/-}$ | 4.6 ± 0.7 | 8.3 ± 4.5 | 1.6 ± 0.4 | 7.9 ± 2.4 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | wt | (1) | 0.9 ± 0.6 | 0.6 ± 0.3 | 0.7 ± 0.9 | 0.7 ± 0.6 | 0.1 ± 0.0 |
| mrf4 | $Gpx1^{-/-}$ | 9.6 ± 2.3 | 12.2 ± 7.0 | 2.8 ± 0.4 | 11.4 ± 7.2 | 1.1 ± 0.1 | 1.2 ± 0.9 |
| | wt | (1) | 1.6 ± 0.2 | 1.3 ± 0.0 | 1.0 ± 0.0 | 0.9 ± 0.1 | 0.9 ± 0.1 |
| myogenin | $Gpx1^{-/-}$ | 1.5 ± 0.3 | 2.8 ± 1.4 | 3.6 ± 0.9 | 14.2 ± 5.3 | 0.3 ± 0.1 | 0.1 ± 0.1 |
| | wt | (1) | 1.3 ± 0.3 | 14.1 ± 4.4 | 9.4 ± 4.0 | 0.1 ± 0.2 | 0.0 ± 0.0 |

Real-time quantitative PCR time course of myogenic bHLH gene expression during differentiation of wild-type and Gpx1$^{-/-}$ myoblasts shows that myogenic gene expression is not repressed in the knockout cells. Two independent cultures were assayed, and each reaction was run in duplicate. Data are expressed as fold-increase $\pm$ S.D. of mRNA expression compared to undifferentiated wild-type levels for all genes.

**Evaluation of adipogenic potential**

As both high oxygen culture conditions and aging are associated with satellite cell (Csete et al., 2001) and myoblast adipogenesis (Taylor-Jones et al., 2001), myoblasts were cultured for 3 days in conditions known to promote adipogenesis, then stained with oil red O, to visualize lipid accumulation. No lipid droplets were seen with oil red O staining in either wild-type or Gpx1$^{-/-}$ myoblasts. However, a distinct morphologic change was noted in the Gpx1$^{-/-}$ myoblasts in adipogenic medium: Cells formed large, multinucleate three-dimensional clusters (Figure 3.7A).

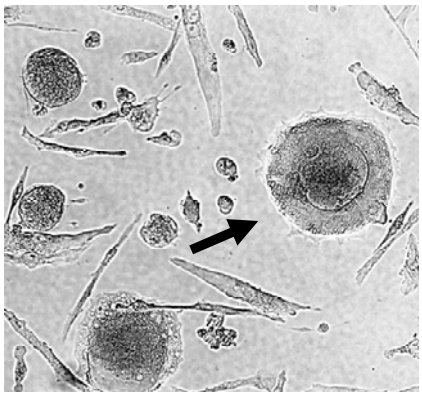
**Gpx1$^{-/-}$ muscle fibers are small and their regeneration may be impaired**

Cross-sectional areas of fibers of three different hindlimb muscles from young knockout animals and their wild-type littermates were measured. Despite similar body and muscle weights (not shown), Gpx1$^{-/-}$ fiber size was reduced significantly in all muscles examined compared to that of littermate wild-type controls, suggesting a muscle developmental defect. Cross-sectional area of

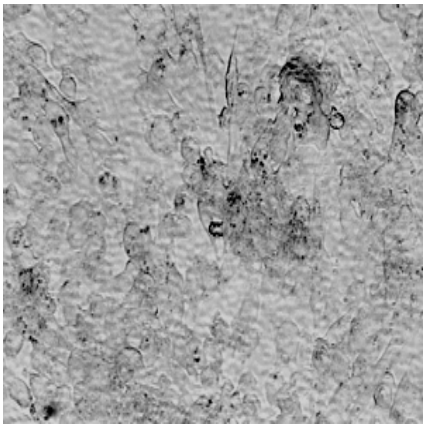
**Figure 3.7**

**Evaluation of adipogenic potential**

## A Gpx1$^{-/-}$



## B Wild-type



(A) Gpx1$^{-/-}$ myoblasts exposed to adipogenic medium (containing insulin, isobutylmethylxanthine and dexamethasone) rounded up into large balls, with obvious nuclear clusters (arrow).

(B) Wild-type myoblasts exposed to adipogenic medium did not accumulate lipid and remained in monolayers.

of tibialis anterior muscle fibers of 23 week old Gpx1$^{-/-}$ mice (n=4) were 83.9% of wild-type areas (1353 ± 221 vs. 1586 ± 34 μm2, p=1.4e$^{-05}$). Similarly, the cross-sectional areas of fibers from Gpx1$^{-/-}$ gastrocnemius muscles were 85.4% of wild-type fibers (p=2.5e$^{-08}$) and Gpx1$^{-/-}$ quadriceps fibers were 79.6% of wild-type area (p=4.9e$^{-08}$).

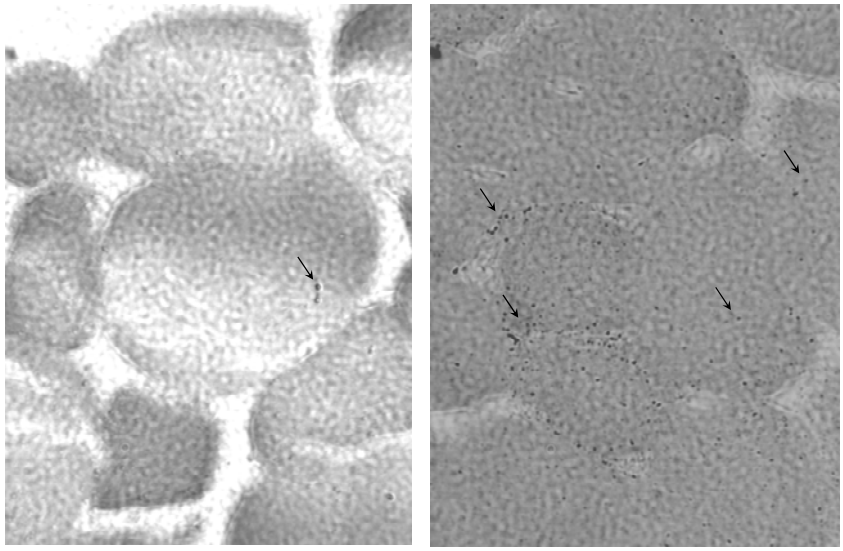
In a pilot study, fiber sizes of cardiotoxin-injected muscle (a standard model to induce muscle regeneration) were examined 14 days after injury, when regeneration is usually complete in wild-type mice. Only 2 mice per group were examined in this preliminary study. No residual foci of necrosis were identified in either group. The regenerated tibialis anterior fibers of Gpx1$^{-/-}$ mice were 68.9% the size of the regenerated wild-type fibers. Since this relative size of Gpx1$^{-/-}$ TA fibers after regeneration (69.8% of wild-type) was smaller than the relative size prior to injury (85.4% of wild-type), these preliminary data suggest that muscle regeneration may be impaired in Gpx1$^{-/-}$ mice. To fully evaluate this defect, serial regeneration assays will be required.

**Lipofuscin accumulates in skeletal muscle of young Gpx1$^{-/-}$ mice**

Young wild-type muscle contained very rare lipofuscin deposits, whereas age-matched (23 week old) Gpx1$^{-/-}$ muscle contained scattered small amounts of the pigment, in both the interior of the fiber and in the extracellular matrix surrounding fibers (Figure 3.8). The amount of lipofuscin in the Gpx1$^{-/-}$ muscle was significantly less than that observed in aged mouse muscle (data not shown) but is nonetheless an indication of pathology in young Gpx1$^{-/-}$ muscle.

**Figure 3.8**

**Lipofuscin staining (Sudan Black)**



Muscle cross-section from young wild-type and $Gpx1^{-/-}$ mice (94.5X magnification).

(A) Wild-type muscle contained very rare areas (dots) of lipofuscin (arrow).

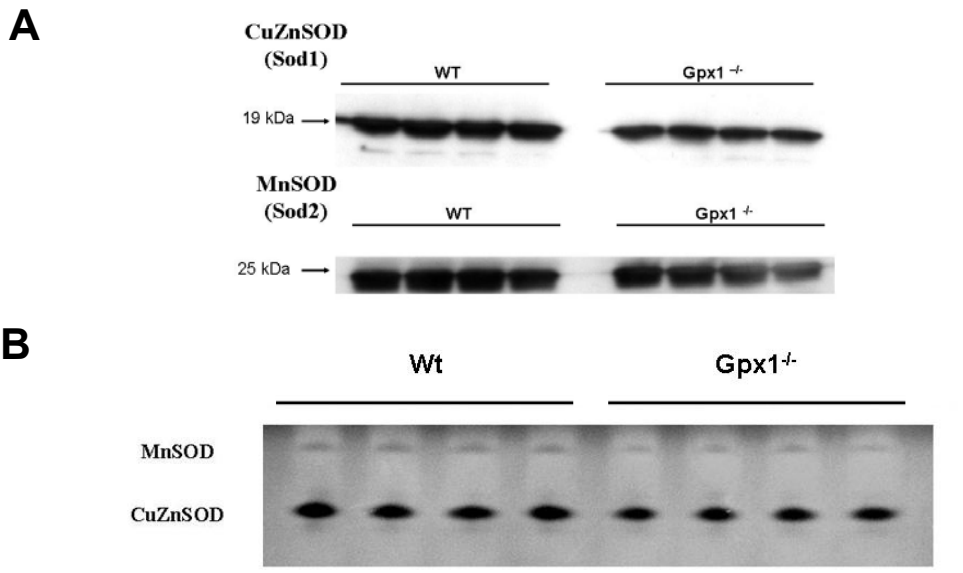
(B) $Gpx1^{-/-}$ muscle sections had scattered accumulation of lipofuscin both in the fiber (right 2 arrows) and especially in connective tissue surrounding the fibers (left 2 arrows).

**No compensatory feedback for SOD family in Gpx1$^{-/-}$ myoblasts**

Protein expression as well as enzyme activity of SOD family including SOD1 and SOD2 was not significantly in Gpx1$^{-/-}$ myoblasts compared to wild-type myoblasts (Figure 3.9). These data might suggest that no compensatory feedback for SOD1 and SOD2 by the deletion of Gpx1$^{-/-}$ in myoblasts.

**Figure 3.9**

**The expression and activity of SOD family MAPK in wild-type and Gpx1$^{-/-}$ myoblasts**




(A) Western blot of SOD family in wild-type and Gpx1$^{-/-}$ myoblasts (n=4). The protein expression level of SOD1 (19 kDa) and SOD2 (25 kDa) is not significantly different between wild-type and Gpx1$^{-/-}$ myoblasts.

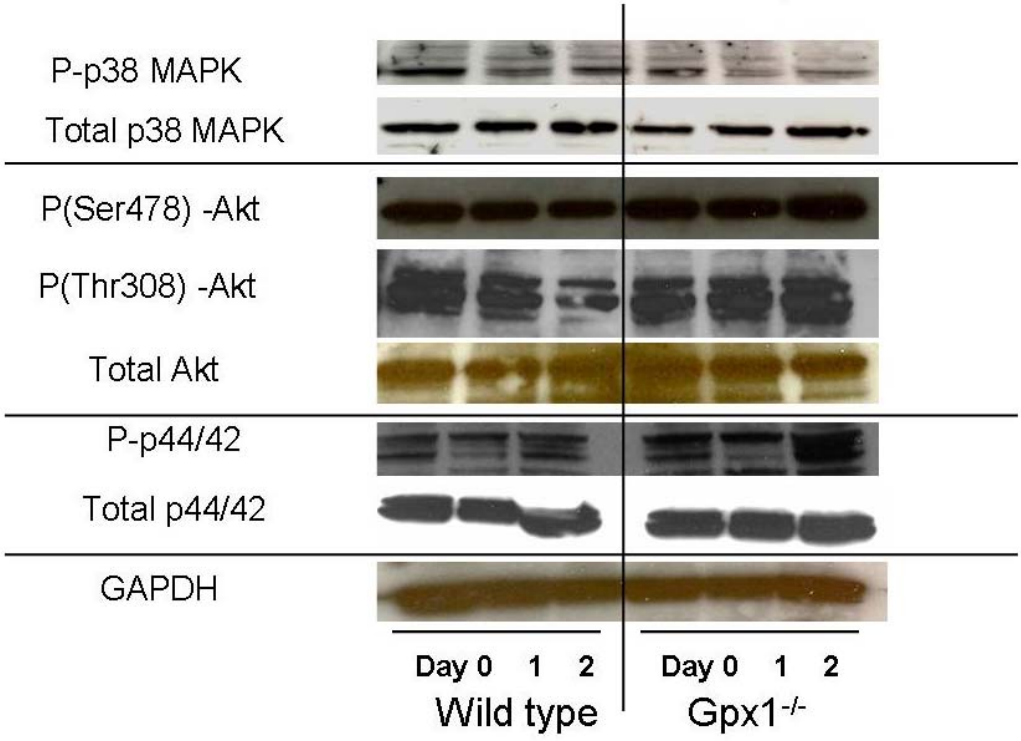
(B) Activity gel SOD family in wild-type and Gpx1$^{-/-}$ myoblasts (n=4). The enzyme activity of SOD1 (19 kDa) and SOD2 (25 kDa) is not significantly different between wild-type and Gpx1$^{-/-}$ myoblasts.

**Akt and MAPK signaling pathways are not altered in Gpx1$^{-/-}$ myoblasts**

Phosphorylation of several signaling pathways including Akt, p38 and p44/42 MAPK were not significantly altered in Gpx1$^{-/-}$ myoblasts compared to wild-type myoblasts (Figure 3.10).

**Figure 3.10**

**Akt and MAPK signaling pathways during differentiation in wild-type and Gpx1$^{-/-}$ myoblasts**




Phosphorylations of Akt, p38 MAPK and p44/42 were assayed using whole cell lysate for 3 days in DM. Overall there was no significant alteration of these pathways.

**Discussion**

Fundamental aspects of muscle progenitor cell biology were significantly perturbed by deletion of the antioxidant Gpx1: Significant changes were observed in proliferation, apoptosis, differentiation and myogeneic transcription factor expression *in vitro*. *In vivo*, muscle fiber size was significantly smaller in $Gpx1^{-/-}$ myoblasts than in wild-type mice, suggesting a developmental defect.

Proliferation was impaired both in qualitative and quantitative ways: $Gpx1^{-/-}$ myoblasts not only proliferated less than wild-type cells but also had a different optimal matrix for proliferation compared to wild-type. Given the central role of MAPK signaling in proliferation, we examined core MAPK pathway proteins. These data suggested that MAPK signaling was not different in cultured $Gpx1^{-/-}$ myoblasts from wild type myoblasts. The threshold for $Gpx1^{-/-}$ myoblasts apoptosis was also reduced in culture, especially in high oxidant stress conditions. These data are consistent with the previous reports of increased apoptosis in brains of $Gpx1^{-/-}$ mice after freeze-induced or focal ischemic infarcts (Crack et al., 2001; Flentjar et al., 2002). Two different antioxidant treatments reduced apoptosis, suggesting that increased apoptosis was in part due to oxidant stress. Although treatment with either NAC or catalase did not completely reverse the apoptosis pattern of the $Gpx1^{-/-}$ myoblasts , it is possible that multiple antioxidant strategies could compensate for loss of Gpx1. In addition, the deletion of Gpx1 had no effect on the relative abundance of mitochondria in myoblasts in muscle from young animals. Though mitochondria are the major sites of ROS production in cells, the increased oxidative stress due to the

deletion of Gpx1 does not seem to impact mitochondrial mass. These results contrast with those we found in myoblasts with genetic manipulation of superoxide dismutase (SOD2). In this case overexpression and underexpression of SOD2 in myoblasts from young mice were both associated with lower mt:genomic DNA ratios than found in young wild-type myoblasts. These results suggest that the local ROS levels inside mitochondria, rather than the ROS levels affected more globally by Gpx1 manipulation, are involved in maintenance of mitochondrial mass.

Formation of myotubes by progenitor $Gpx1^{-/-}$ myoblasts was distinctly impaired in culture. In general, $Gpx1^{-/-}$ myoblasts could form nascent (small) myotubes, but not large mature myotubes. There are distinct differences in the signals that mediate formation of nascent versus mature myotubes (Horsley et al., 2003). Given the defects in mature myotube formation, use of the $Gpx1^{-/-}$ model should provide valuable data to further characterize these signals that mediate the various stages of muscle differentiation.

The sensitivity of stem cell populations to stress as a function of their differentiation status is of considerable interest to developmental biologists. In conducting the differentiation assays we found that undifferentiated myoblasts were more sensitive to added $H_2O_2$ (lifted off the plate and died) than were differentiated myoblasts. These results differ from a report showing that differentiated myotubes are more sensitive to oxidant stress than undifferentiated myoblasts (Franco et al., 1999), however this latter report was conducted in transformed C2C12 myoblasts. The difference in oxidant stress (amount and

type) may also explain the discrepancy, and we are addressing the developmentally-regulated sensitivity to oxidant stress using other antioxidant deleted mice.

This inability of passaged Gpx1$^{-/-}$ myoblasts to form myotubes is not a classical differentiation defect: Expression of core regulators of muscle differentiation, the myogenic bHLH transcription factors myf5, MyoD, mrf4, and myogenin, was not reduced in Gpx1$^{-/-}$ vs. wild-type myoblasts. The abnormal, high levels of myogenic bHLH gene expression in Gpx1$^{-/-}$ myoblasts in GM suggest that regulation of these genes is impaired by deletion of Gpx1. Many other molecular pathways contribute to terminal muscle differentiation, including genes that control myoblast attraction, adhesion, alignment, and fusion and important molecular co-factors for myogenic bHLH genes. Although these and other factors could contribute to the aberrant differentiation phenotype of Gpx1$^{-/-}$ myoblasts, they were not examined in this study.

The experiments with co-cultured wild-type and Gpx1$^{-/-}$ myoblasts suggest that at least some of these necessary co-factors for differentiation (impaired in the knockout cells) are intrinsic to myoblasts. By the nature of the experiments only myoblast factors contributing to differentiation could be examined. In addition, the lack of lipid accumulation in Gpx1$^{-/-}$ myoblasts exposed to adipogenic media suggests that poor myotube formation was not due to a skewing of differentiation toward adipogenesis, a feature of myoblast aging (Taylor-Jones et al., 2002).

Some aspects of senescent muscle are recapitulated by deletion of Gpx1. The cultured Gpx1$^{-/-}$ myoblasts did not have a flattened, splayed appearance in culture as reported for Gpx1$^{-/-}$ fibroblasts (De Haan et al., 2004) or in general for senescent myoblasts. The progressive differentiation defect in culture, sensitivity of apoptosis and proliferation to oxidant stress, and the premature appearance of lipofuscin in Gpx1$^{-/-}$ muscle and protein carbonyls in cultured myoblasts suggest that this complex Gpx1$^{-/-}$ phenotype mimics some aspects of muscle senescence. Future studies will examine the effects of aging on the Gpx1$^{-/-}$ muscle stem cell population.

Overall, our studies suggest that Gpx1$^{-/-}$ muscle differentiation is very sensitive to oxidant stress. When first isolated, the myoblasts avidly formed contractile myotubes. But with only a few passages in culture, this ability was largely lost. Similarly, Gpx1$^{-/-}$ mice developed muscle that was normal in histologic appearance, but significantly smaller than wild-type fibers), and exhibited impaired ability to recover from a toxic injury.

Previous analysis of the colony of Gpx1$^{-/-}$ mice used in this study, and confirmed in our data, revealed similar activity levels of manganese superoxide dismutase (MnSOD), CuZnSOD and catalase as well as similarities in body weight as compared to wild-type mice (Van Remmen et al., 2004). The accumulation of intracellular hydrogen peroxide as well as evidence of accumulation of oxidative damage to proteins strongly suggest that there is no functional compensation for loss of Gpx1 in the muscle progenitor cells. This lack of compensation for Gpx1 deletion by other major antioxidant systems likely

contributes to the muscle phenotype described here. Other investigators have reported that Gpx1$^{-/-}$ mice were smaller than wild-type littermates, but this discrepancy may be due to strain background differences (129/Svs3 vs. C57Bl/6J in the current study) (Esposito et al., 2000).

High throughput studies will be necessary to determine the effects of Gpx1 deletion on expression of other genes, as well as the functional effects of oxidative changes to specific proteins. Our data indicate that wide-ranging changes in the cells at the message (myogenic bHLH genes are upregulated), protein (oxidative modifications) and lipid levels (accumulation of lipofuscin) contribute to an abnormal muscle progenitor phenotype. However, classically redox-sensitive signaling pathways such as Akt, p38 MAPK, and p44/p42 pathways are changed during differentiation in myoblasts with deletion of Gpx1.

Though Gpx1 is expressed in low levels in muscle, and though many other factors control muscle $H_2O_2$ homeostasis, deletion of Gpx1 results in globally defective muscle progenitor cells. To our knowledge, these are the first detailed analyses of stem or progenitor cells from mice with an antioxidant deletion. It is not yet known whether other stem cell populations are similarly affected by Gpx1 deletion, but our results suggest that antioxidant balance plays a critical role in maintaining adult muscle progenitor cell integrity.

# Chapter 4: Sod2 overexpression preserves myoblast mitochondrial mass and function, but not muscle mass with aging

**Data presented in this chapter are submitted to *Aging Cell* as:**

Sod2 overexpression preserves myoblast mitochondrial mass and function, but not muscle mass with aging

## Introduction

Superoxide dismutase-2 (SOD2 or MnSOD) is a mitochondrial matrix antioxidant enzyme that catalyzes dismutation of superoxide anion ($O_2^-$) to form hydrogen peroxide ($H_2O_2$) and oxygen. MnSOD is essential, as homozygous Sod2-knockout mice do not survive embryonic development (Li et al., 1995) or die shortly after birth (Huang et al., 2001) depending on genetic background. This lethality is associated with multisystem pathological abnormalities including dilated cardiomyopathy (Li et al., 1995; Huang et al., 2001), severe anemia, and degeneration of the central nervous system neurons (Lebovitz, 1996). Heterozygous Sod2 knockout mice have increased susceptibility to seizures (Liang et al., 2004) and decreased survival to superimposed severe oxidant stress (Asikainen et al., 2002). These animals show diffuse increases in oxidant damage at the level of lipid peroxidation (Strassburger et al., 2005), proteins (Friedman et al., 2004), and compromised mitochondrial function (Van Remmen et al., 1999; Melov et al., 1999; Kokoszka et al., 2001; Van Remmen et al., 2001; Strassburger et al., 2005). However, the decreased mitochondrial function of $Sod2^{+/-}$ animals is managed by compensatory antioxidant responses with aging, so that there is not a compounded oxidant damage with aging and loss of the enzyme (Mansouri et al., 2006), and the animals have a normal lifespan. Overexpression of Sod2 in mice leads to enhanced mitochondrial function (Silva, et al., 2005), and protection from various oxidant stressors including reperfusion injury (Suzuki et al., 2002), but no increase in lifespan.

Generally, studies of muscle in mice genetically manipulated for Sod2 expression have been done at the level of whole muscle, and the effects of Sod2 expression on muscle progenitor cell populations have not been studied. Skeletal muscle contains a resident stem cell population, the satellite cell, located between the basal lamina and plasma membrane of the muscle fiber (Mauro, 1961). Satellite cells are generally quiescent, but enter the cell cycle and become myoblasts when muscle is damaged. Myoblasts are the cellular source of muscle regeneration throughout life. This transient amplifying population of skeletal muscle proliferates as single cells, then fuses and forms myotubes *in vitro* and integrates into myofibers *in vivo* in response to appropriate differentiation cues. The numbers and capability of myoblasts to fuse and differentiate to myofibers generally decrease with chronic oxidative stress or aging (Lee et al., 2006; Shefer et al., 2006; Collins et al., 2007; Verdijk et al., 2007) which contributes to loss of regenerative capacity with aging and to age-related sarcopenia.

Here we explored the effects of MnSOD on myoblast function in cells derived from both young and old mice that under- or over-express the enzyme. These studies suggest that mitochondrial MnSOD levels significantly alter the phenotype of cultured myoblasts. Myoblasts that are deficient in MnSOD have significantly impaired proliferation and differentiation capacities, but these differences are blunted rather than amplified with aging. On the other hand, lifelong overexpression of MnSOD in myoblasts has some benefit, and preserves myoblast differentiation potential and mitochondrial mass, despite high levels of intracellular hydrogen peroxide.

## Results

### Phenotype of myoblasts with different doses of Sod2: Proliferation and terminal differentiation

BrdU uptake was used to quantify proliferation of cultured myoblasts (with a 4-hour pulse at 44-48 hours after plating). The highest percentage of proliferating cells was in myoblasts from young, wild-type mice. Surprisingly, both overexpression and reduced expression of MnSOD resulted in similar and significantly decreased proliferation of myoblasts from young animals (Figure 4.1A). In wild-type myoblast cultures, 65.5 ± 0.4% of cells were BrdU-immunoreactive compared to 44.3 ± 4.0% of Sod2Tg ($p=0.03$) and 56.8 ± 1.2% of $Sod2^{+/-}$ myoblasts from young mice ($p=0.01$).

Myoblasts from aged mice all showed significantly reduced proliferation compared to their young genotype-matched counterparts. For myoblasts from aged mice, the highest percentage of proliferating cells was again found in wild-type cells; both overexpression and reduced expression of MnSOD in myoblasts from aged mice resulted in similar and significantly decreased proliferation compared to the wild-type myoblasts (both $p=0.02$). These data suggest that evolved optimal levels of MnSOD are important in myoblast proliferation, and that proliferation is quite sensitive to changes of the enzyme function in either direction.

Gross morphologic defects in differentiation of young $Sod2^{+/-}$ myoblasts were apparent on examination of myosin heavy chain (MHC)-stained cells after 5 days of differentiation (Figure 4.1B). MHC is a marker of terminal differentiation

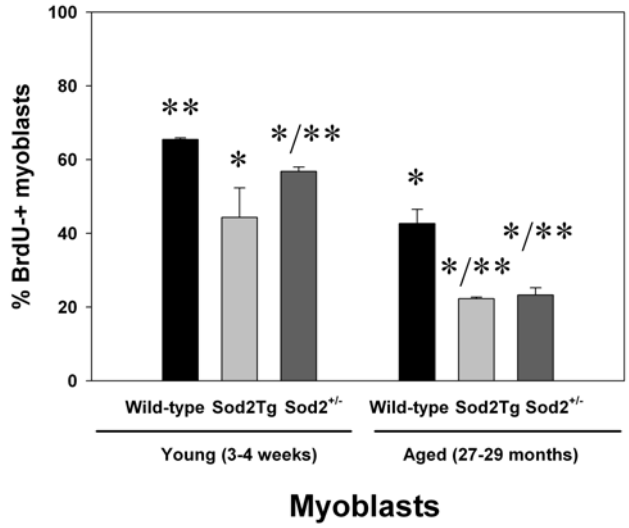
of skeletal muscle. Using fusion indices to quantitate this morphologic observation (Figure 4.1C), 93.8 ± 1.1% of wild-type cells were incorporated into myotubes after 5 days of differentiation vs. 82.1 ± 0.4% of Sod2Tg myoblasts (p=0.005) and only 35.9 ± 10.8% of $Sod2^{+/-}$ myoblasts (p=0.02 compared to wild-type, and p= 0.03 compared to Sod2Tg). The similar levels of expression of MHC at 5 days in wild-type and Sod2-Tg myoblasts (Figure 4.1D, p=0.19), compared with the difference in fusion index between these two cell types, suggests that the Sod2Tg cells were turning on MHC expression prematurely (before terminal fusion events) in myoblasts or in early myotubes with only two nuclei. In other words, the normal kinetics of muscle differentiation was disrupted as well as the efficiency of differentiation. Aged cells similarly appeared to turn on MHC inappropriately in immature cells, since the percentage of MHC-expressing cells (Figure 4.1D) is always higher the then percentage of MHC-expressing cells in myotubes (Figure 4.1C).

Aging was associated with significant reductions in myogenic differentiation of all three genotypes compared to the patterns seen in young myoblasts. $Sod2^{+/-}$ myoblasts from aged mice differentiated minimally, with only 7.2 ± 2.0% of cells incorporated into multinucleate myotubes after 5 days of differentiation (Figure 4.1C, p=0.03 compared to wild-type, and p=0.02 compared to old Sod2Tg cells). Only 32.1 ± 5.8% of wild-type cells from aged mice were incorporated in multinucleate myotubes after 5 days of differentiation (about 60% less than young wild-type). Sod2Tg myoblast fusion indices also decreased with aging to 62.1 ± 10.9%, but this was not a statistically significant drop from the

**Figure 4.1**

**Myoblast proliferation and differentiation as a function of Sod2 and aging**

**A**

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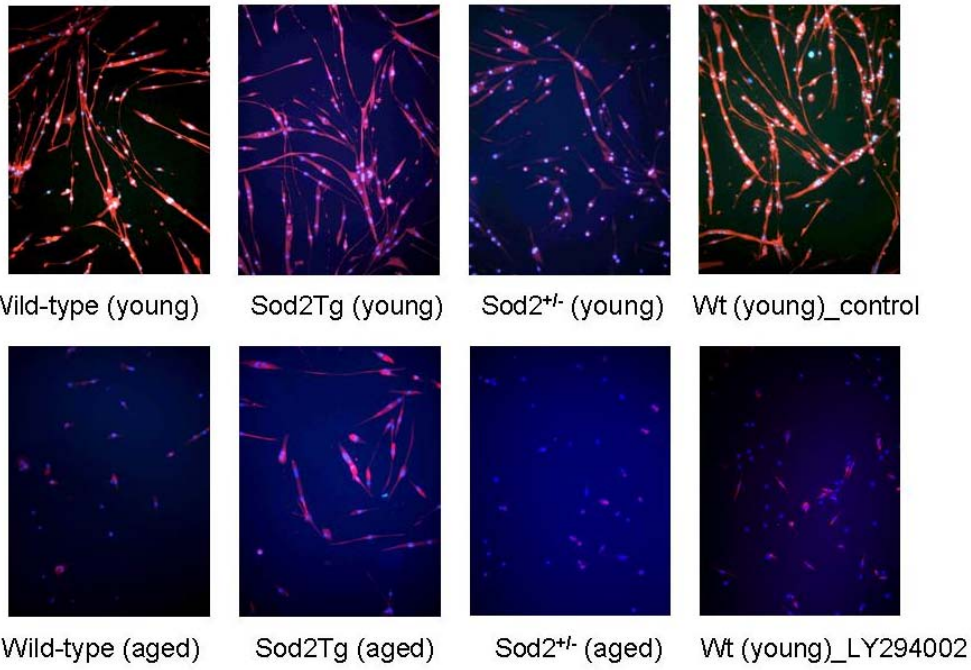
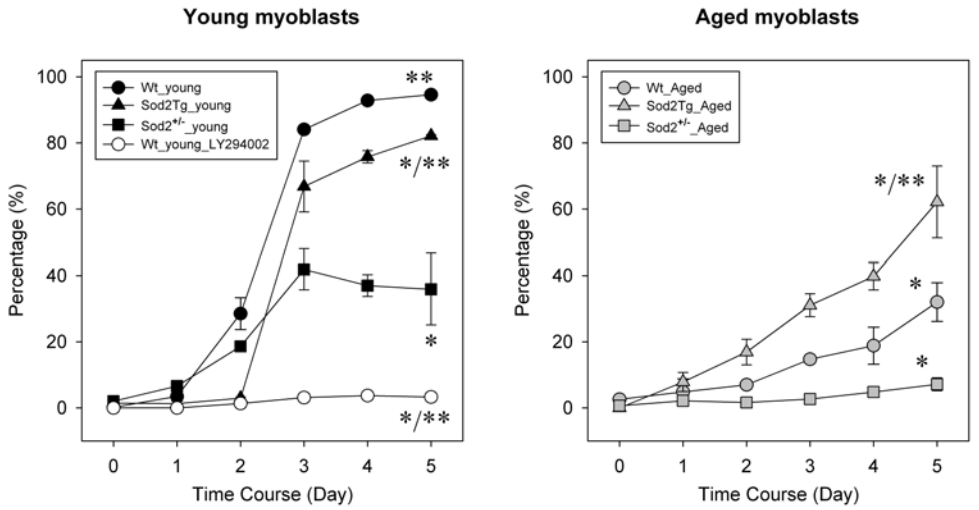


**C**


Young myoblasts
Aged myoblasts
Wt_young
Sod2Tg_young
Sod2+/-_young
Wt_young_LY294002
Wt_Aged
Sod2Tg_Aged
Sod2+/-_Aged
Percentage (%)
Time Course (Day)
**
*/**
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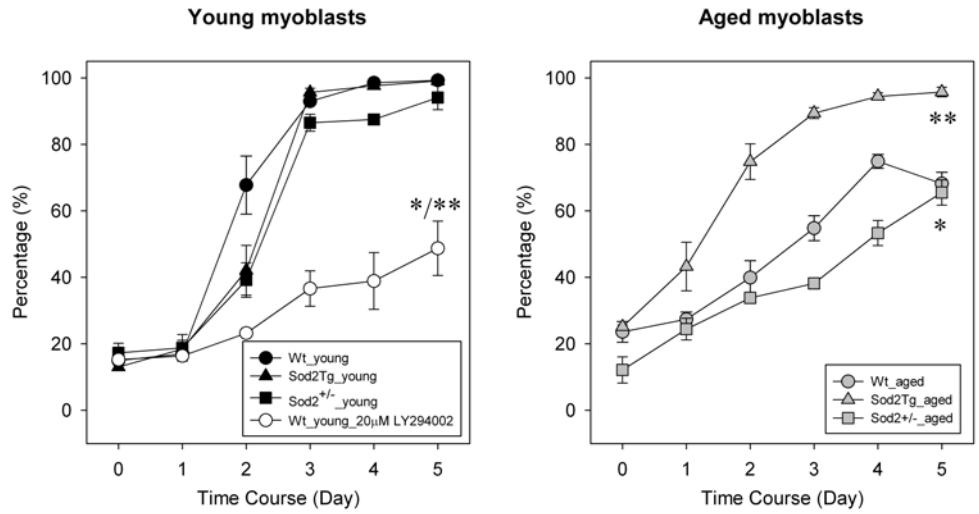
*/**
*
*

**D**


Young myoblasts
Aged myoblasts
Wt_young
Sod2Tg_young
Sod2+/-_young
Wt_young_20μM LY294002
Wt_aged
Sod2Tg_aged
Sod2+/-_aged
Percentage (%)
Time Course (Day)
*/**
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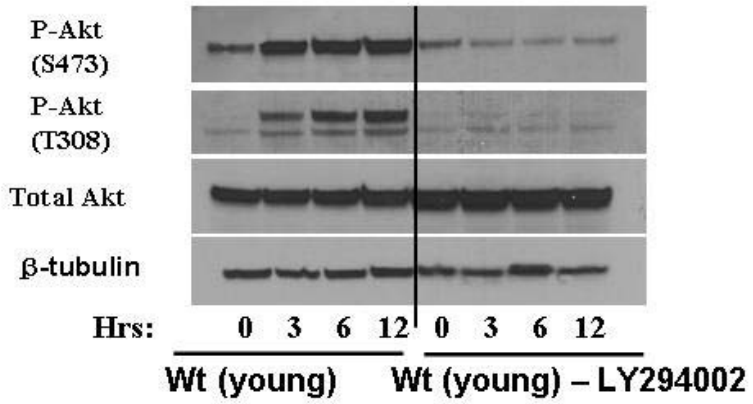
**E**


P-Akt
(S473)
P-Akt
(T308)
Total Akt
β-tubulin
Hrs: 0 3 6 12 0 3 6 12
Wt (young)
Wt (young) – LY294002

(A) Percentage of BrdU-labeled myoblasts after a 4 hr BrdU pulse (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).
(B) Differentiation assessed morphologically (fused cells) and by MHC staining (red). $Sod2^{+/-}$ myoblasts from young mice show obvious morphologic differentiation defects, with few and small fibers formed after 5 days of differentiation. Old Sod2Tg myoblasts differentiated better than other myoblasts. Treatment of young myoblasts with the PI3 kinase/Akt inhibitor, LY294002, confirms the importance of this signaling pathway in muscle differentiation.
(C) Fusion index [(# MHC-positive myotubes /Total # nuclei) × 100]. After 5 days, differentiation was most advanced in young, wild-type myoblasts (left) but best preserved with aging in Sod2Tg myoblasts (right). (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).
(D) Differentiation potential [(# of nuclei in MHC-stained myoblasts/Total # nuclei) × 100] confirms that overexpression of SOD2 over the lifetime of aged myoblasts preserved differentiation. (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).
(E) Akt is activated early in normal myoblast differentiation (left). For reference, pharmacologic blockade of LY294002 blocks Akt phosphorylation and morphologic differentiation.

young Sod2Tg myoblast differentiation pattern (p=0.12). Overexpression of MnSOD, then, was associated with relatively preserved differentiation capacity with aging, with more complete differentiation in the Sod2Tg myoblasts compared to aged wild-type myoblasts (p=0.01).

Phosphatidylinositol 3-kinase and serine kinase Akt/protein kinase B (PI3K/Akt) signaling pathways are well known as essential, positive regulators of muscle differentiation (Jiang et al., 1999; Tamir and Bengal, 2000), and pharmacologic inhibition of this pathway with the PI3K inhibitor, LY294002 turns off myoblast differentiation. LY294002 inhibits the phosphorylation of PI3K (Serra et al., 2007), and thereby inactivates Akt, one of the downstream targets of this pathway. Figure 4.1B illustrates that the profound differentiation defect of myoblasts from aged $Sod2^{+/-}$ mice is similar to that induced by 20 μM LY204002 during differentiation (where only about 3.3% of cells were incorporated into multinucleated myotubes). Confirmation that the early stages of differentiation (12 hours after mitogen withdrawal) are associated with phosphorylation of Akt is shown in Figure 4.1E western blots, contrasted with inhibited phosphorylation of Akt in myoblasts in the presence of LY294002.

**Akt is constitutively phosphorylated in aged wild-type, Sod2Tg, and $Sod2^{+/-}$ myoblasts**

Figure 4.2 shows western analysis of Akt phosphorylation patterns in differentiating myoblasts over a 4 day time course. Proliferating myoblasts from young mice of all three genotypes have very low levels of phosphorylated Akt (p-

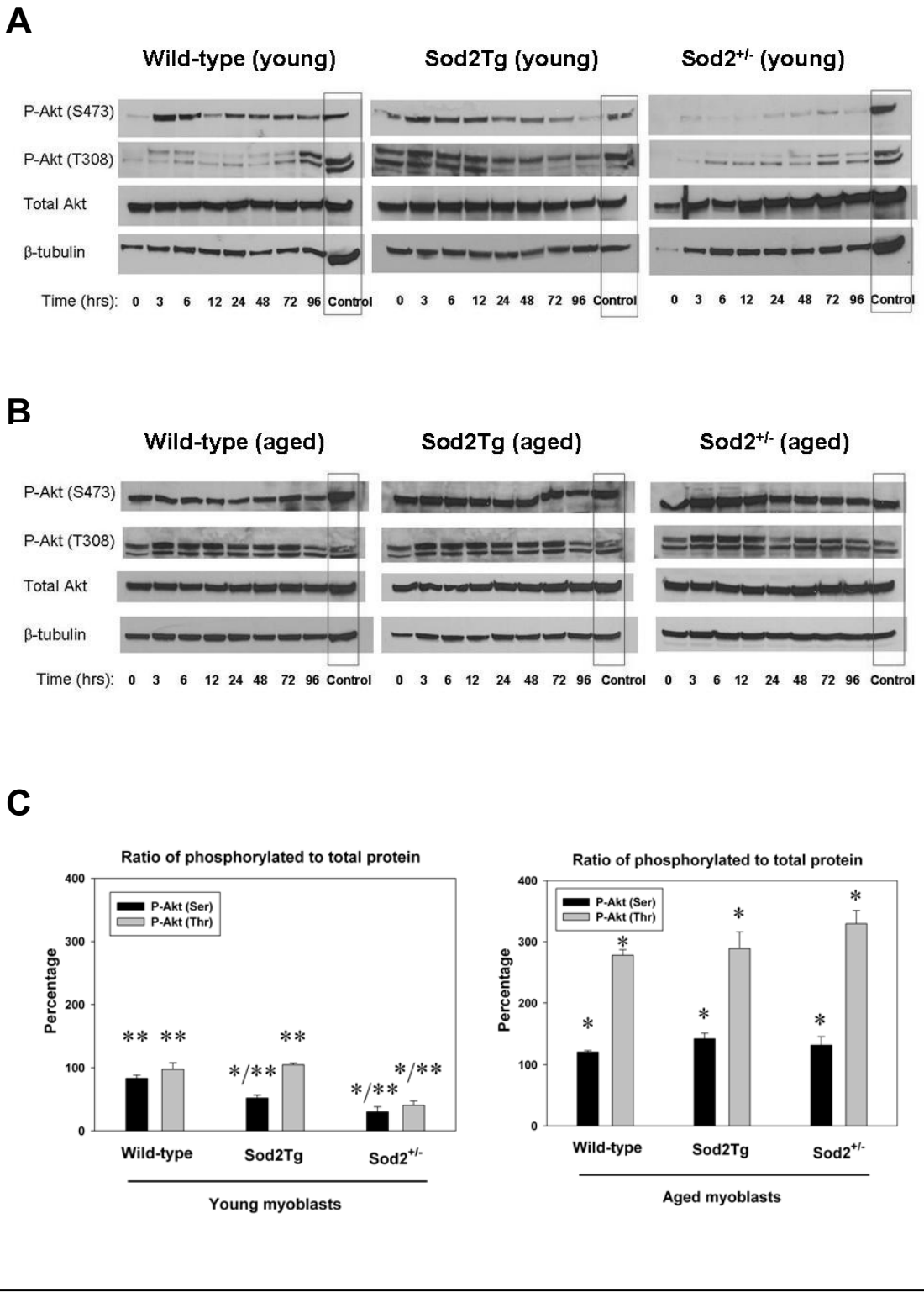
Akt) at serine 473 (0 time in Figure 4.2A), whereas aged, proliferating myoblasts of all three genotypes maintained in growth medium (0 time in Figure 4.2B) have considerable phosphorylation at that site. Activated Akt1 is required for myoblast proliferation (Heron-Milhavet et al., 2006), so these results suggest that the proliferative defects seen in myoblasts from aged mice and the young Sod2Tg and $Sod2^{+/-}$ myoblasts are not simply due to reduced capacity to phosphorylate Akt.

Withdrawal of mitogen (differentiation medium) from wild-type young myoblasts leads to early phosphorylation of Akt at serine 473, with a similar pattern in the young Sod2-Tg myoblasts, but S473 is not activated in $Sod2^{+/-}$ myoblasts at all during differentiation. These findings suggest that activation of Akt at this site may be an underlying defect leading to poor differentiation of the $Sod2^{+/-}$ myoblasts. Phosphorylation of Akt at threonine 308 occurs late in the differentiation time course in wild-type young myoblasts and is blunted in the $Sod2^{+/-}$ myoblasts. In the young Sod2-Tg myoblasts, Akt is phosphorylated at T308 before differentiation and the levels of phosphorylation fall during the differentiation time course. In general, the role of this phosphorylation site is less well studied in muscle differentiation.

The most striking finding in Figure 4.2B is the abundant and unchanging levels of p-Akt at both S473 and T308 in the aged myoblasts. In all three genotypes, Akt phosphorylation is insensitive to differentiation vs. growth conditions, suggesting a generalized dysregulation of Akt signaling.

**Figure 4.2**

**Activation of PI3K/Akt pathway during muscle differentiation**



(A) Phosphorylation of Akt at Ser473 and Thr308 during differentiation of young myoblasts. Boxed lanes are loading controls for each gel. P-Akt in $Sod2^{+/-}$ myoblasts is significantly lower than wild-type and Sod2Tg during muscle differentiation.
(B) Phosphorylation of Akt at Ser473 and Thr308 of aged myoblasts during differentiation. Globally, Akt in aged myoblasts is constitutively active regardless of genotype.
(C) The quantitation of western results shown in (B) (*$p<0.05$ compared to wild-type young; **$p<0.05$ compared to aged wild-type).

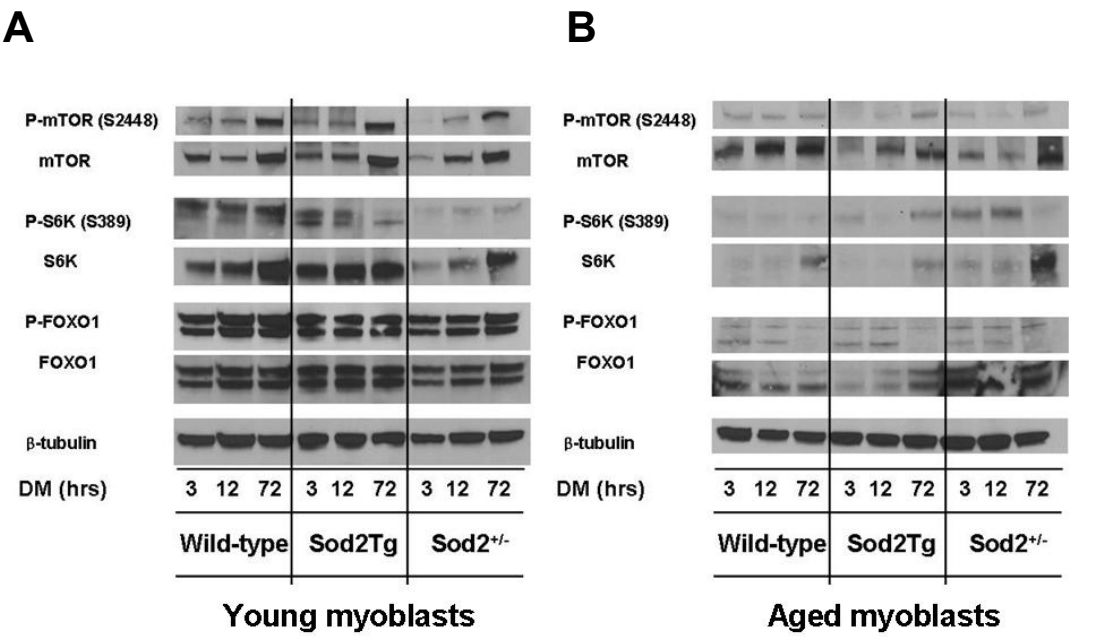
Downstream targets of the PI3K/Akt pathway are known to be important regulators of both protein synthesis and degradation. The activation of mTOR and S6K through PI3K/Akt are considered sufficient for protein synthesis (Bodine et al., 2001; Rommel et al., 2001; Mieulet et al., 2007; Ohanna et al., 2005). FOXOs are phosphorylated and inactivated through PI3K/Akt, keeping FOXOs sequestered outside the nucleus (Takaishi et al., 1999; Burgering and Kops, 2002). With this background in mind, we studied activation of these downstream targets starting at the time at which Akt S473 is phosphorylated during muscle differentiation *in vitro* in all the young myoblast genotypes. In myoblasts from young mice, phosphorylation of mTOR (at S2448) increased at the 72 hour time point, and both the transgenic and $Sod2^{+/-}$ young myoblasts showed a similar pattern (Figure 4.3A). Total mTOR also increased at the 72 hour time point, to similar amounts in all three genotypes. Though total mTOR levels increased in

myoblasts from aged mice during differentiation, phosphorylated mTOR remained at very low levels through differentiation in aged myoblasts of all three genotypes. The abundance of phosphorylated S6K at serine 389 increased slightly in differentiating young wild-type myoblasts. [The phosphorylated protein appeared as doublets in the western blots, as in other reports (Kuzman et al., 2007).] Phosphorylation of S6K S389 was decreased at 72 hours in the young Sod2Tg myoblasts (with the upper band barely detectable) and was barely detectable throughout differentiation in the $Sod2^{+/-}$ young myoblasts. Though factors other than Akt can impact activation of mTOR and S6K, these studies suggest that that the impaired activation of Akt in the $Sod2^{+/-}$ young myoblasts has more effect on S6K activation than on mTOR activation. Aging was associated with significant reductions in total S6K and in phosphorylated S6K in all genotypes. In particular, at the 72 hour time point, aged wild-type and $Sod2^{+/-}$ myoblasts had undetectable levels of the phosphorylated S6K, but the Sod2Tg aged myoblasts had a detectable band (Figure 4.3B).

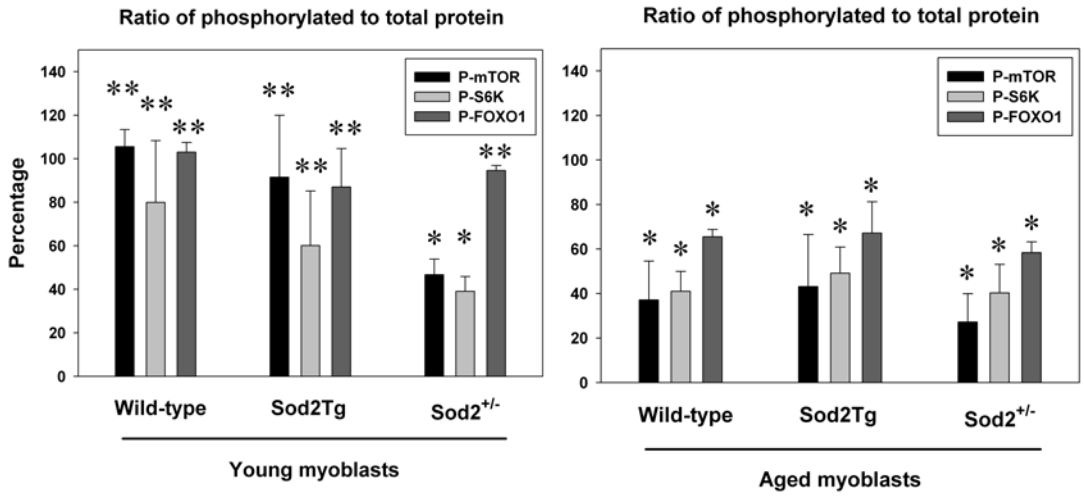
Total and phosphorylated FOXO1 levels were unchanged during differentiation in myoblasts from young mice, independent of genotype. Total levels of FOXO1 were less in aged wild-type and Sod2Tg myoblasts compared to young myoblasts, whereas total levels of FOXO1 were similar in aged and young $Sod2^{+/-}$ myoblasts. However, phosphorylated FOXO1 abundance was significantly reduced with aging (compared to young cells) across genotypes.

**Figure 4.3**

**Altered Akt signaling pathway during muscle differentiation**




**C**



(A) Phosphorylation of selected downstream targets of Akt: mTOR, S6K and FOXO1 in young myoblasts during differentiation reveals decreased activation of mTOR and S6K in young $Sod2^{+/-}$ myoblasts.
(B) Activation of mTOR and S6K in all aged myoblasts is significantly lower than in young wild-type myoblasts. There was no significant difference in these parameters between aged myoblasts as a function of Sod2 status.
(C) Quantitation of western blotting results (as in B) at then 72 hr differentiation time point (*$p<0.05$ compared to wild-type young; **$p<0.05$ compared to aged wild-type).

**Fiber cross-sectional area and muscle mass with aging**

Both protein synthesis defects and increased protein breakdown contribute to muscle atrophy with aging (Husom et al., 2004), which can be measured using the cross sectional area of muscle fibers. ($Sod2^{+/-}$ mice were not available for these studies.) As others have shown, the cross-sectional area of tibialis anterior muscle fibers fell dramatically with aging in both wild-type and Sod2Tg mice, but in both age groups the fiber size of Sod2Tg mice was greater. Mean area of fibers ($\pm$ S.D.) in young wild-type sections was 1315 ± 656 vs. 1498 ± 662 $\mu m^2$ in young Sod2Tg fibers ($p=2.4e^{-18}$). In old wild-type muscle, mean area of fibers was 512 ± 141 vs. 621 ± 149 $\mu m^2$ in old Sod2Tg fibers ($p=2.8e^{-42}$). Though the transgenic fibers were larger, the percentage loss of fiber area with aging in Sod2Tg and wild-type mice was identical.

Mean weight of gastrocnemius muscles in 5 month old wild-type male animals was 0.29 ± 0.01 vs. 0.28 ± 0.01 grams in the Sod2Tg, with no difference in the body weights of the mice. No difference in muscle weights emerged with aging: In 27 month old mice the weight of wild-type gastrocnemius muscles fell to 0.27 ± 0.01 g and transgenic muscle weighed 0.25 ± 0.01 grams.

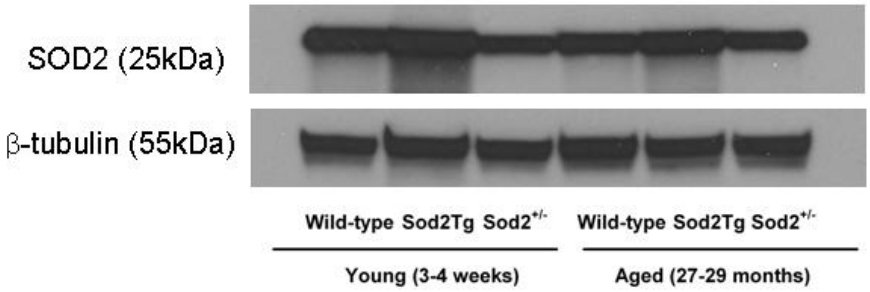
**MnSOD protein abundance and enzyme activity**

MnSOD in whole myoblast lysates was assessed using western blotting (Figures 4.4A & 4.4B) and enzyme activity was assayed in the various myoblasts as a function of aging (Figure 4.4C). In myoblasts from young mice, as anticipated, MnSOD was 1.3-fold more abundant in Sod2Tg myoblasts than in wild-type myoblasts ($p=0.04$), and in myoblasts from young $Sod2^{+/-}$ mice, MnSOD was 0.6 that of wild-type levels ($p=0.007$). This pattern of MnSOD expression in myoblasts is similar to that previously reported in total muscle lysates from the same mouse colony (Van Remmen, et al., 1999). In myoblasts from aged mice, MnSOD decreased in wild-type and Sod2Tg cells, but not significantly in the $Sod2^{+/-}$ myoblasts. Myoblasts from aged Sod2Tg mice had virtually the same amount of MnSOD as wild-type young myoblasts, though the decrease in protein was significant in the Sod2Tg myoblasts as a function of age ($p=0.04$). Absolute amounts of MnSOD in the aged myoblasts were greatest in the Sod2Tg ($p<0.05$ compared to the aged wild-type and to aged $Sod2^{+/-}$ myoblasts), but aged wild-type and $Sod2^{+/-}$ myoblasts had similar levels of MnSOD ($p=0.50$).

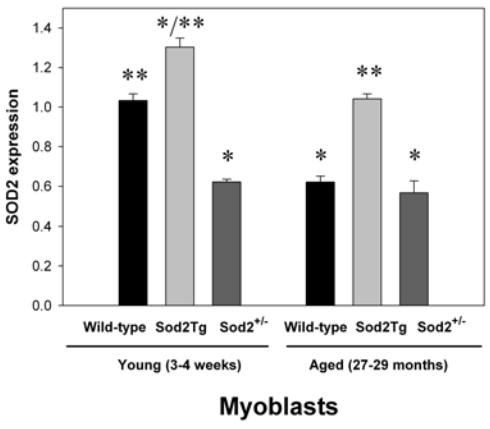
**Figure 4.4**

**SOD2 expression and enzyme activity levels**

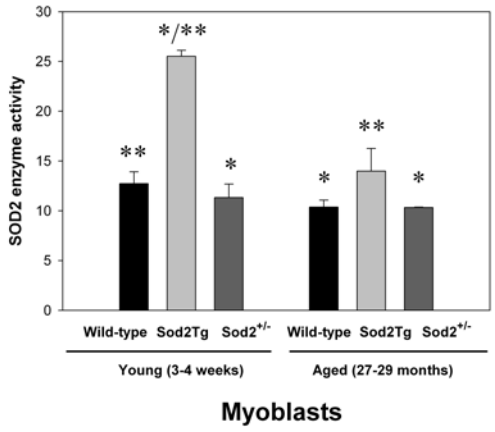
**A**




**B**




**C**




(A) Western blotting confirms that SOD2 protein levels are increased in the transgenic myoblasts and decreased in the Sod2$^{+/-}$ cells.

(B) Quantitation of western results from (A). Data are expressed as fold-change compared to wild-type (young) myoblasts (*$p<0.05$ compared to wild-type young; **$p<0.05$ compared to aged wild-type).

(C) SOD2 enzyme activity in myoblasts (*$p<0.05$ compared to wild-type young; **$p<0.05$ compared to aged wild-type).

MnSOD activity was significantly higher, as expected based on protein levels, in the young Sod2Tg myoblasts compared to wild-type and $Sod2^{+/-}$ myoblasts (Figure 4.4C), about 2-fold more than wild-type ($p=0.008$). MnSOD activity in $Sod2^{+/-}$ myoblasts was about 0.8 that of wild-type myoblasts ($p=0.012$). In aged myoblasts, the sustained protein-level expression of MnSOD in the Sod2Tg myoblasts looks at first not to be accompanied by an equivalent maintenance of enzyme activity (comparing Figures 4.4B and 4.4C). Given that the correlation between protein expression and activity of MnSOD was strong generally ($r^2=0.858$), these data suggest that the catalytic efficiency of the transgenic enzyme diminished with aging. However, in absolute terms, MnSOD activity in the old Sod2Tg myoblasts was equivalent to that of the young wild-type myoblasts, while aged wild-type or aged $Sod2^{+/-}$ myoblasts had significantly less activity than that of young wild-type myoblasts ($p=0.03$).

**Intracellular ROS levels in myoblasts as a function of Sod2 expression**

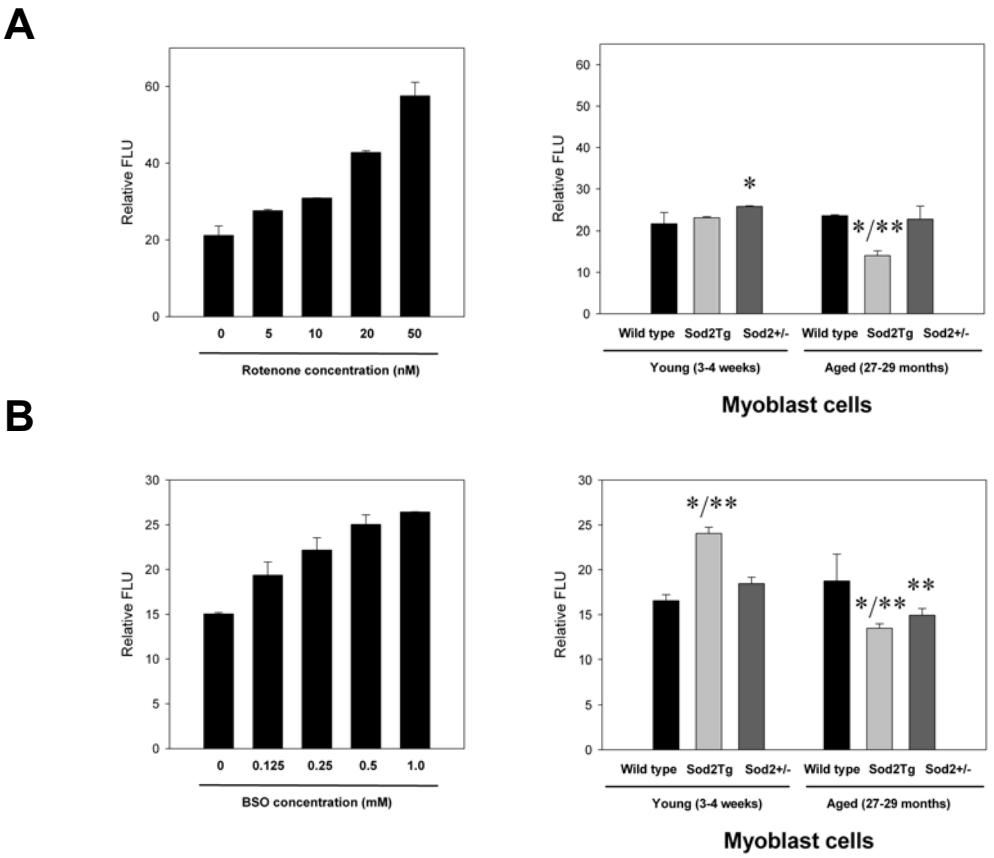
Superoxide anion in myoblasts was quantified using indirect fluorescence with MitoSox (Invitrogen). MitoSox is considered to be mitochondrion-specific: Reaction of superoxide in mitochondria with the dye causes fluorescence emission (Mukhopadhyay et al., 2007). As a reference for these reactions, a dose response curve of MitoSox fluorescence with increasing concentrations of rotenone was generated using young wild-type myoblasts (Figure 4.5A, left). Rotenone is a specific inhibitor of complex I and causes increased superoxide generation from mitochondria (Kushnareva et al., 2002). Myoblasts from young

wild-type mice and from young Sod2Tg mice generated equivalent amounts of superoxide, with $Sod2^{+/-}$ myoblasts from young mice generating about 20% more ($p=0.046$), (Fig 4.5A, right). Aged wild-type and aged $Sod2^{+/-}$ myoblasts did not have a significantly different pattern of superoxide levels than young genotype-matched myoblasts, but superoxide production of aged Sod2Tg myoblasts was about 40% less than that from young Sod2Tg myoblasts ($p=0.008$). Though myoblasts from young $Sod2^{+/-}$ mice exhibited the highest levels of superoxide of the young myoblasts, aged wild-type myoblasts exhibited similar superoxide levels to aged $Sod2^{+/-}$ myoblasts.

The most commonly used measure of intracellular ROS in cultured cells is indirect fluorescence proportional to oxidative reaction of the dye CM-$H_2$DCFH, which is very sensitive to peroxide levels. For CM-$H_2$DCFH assays of ROS, increasing concentrations of the glutathione scavenger, BSO (buthionine sulfoxide), were used as a reference (Figure 4.5B, left). Young wild-type and young $Sod2^{+/-}$ myoblasts had similar levels of reactive oxygen species (ROS) using this assay ($p=0.11$), but the myoblasts from young Sod2Tg mice contained significantly more ROS, likely reflecting excess $H_2O_2$ levels ($p=0.008$ compared to young wild-type), similar to wild-type cells treated with 0.5 mM BSO. With aging, intracellular ROS were not changed from young values in wild-type myoblasts ($p=0.42$). However, Sod2Tg and $Sod2^{+/-}$ myoblasts from aged animals had significantly less ROS than genotype-matched young myoblasts ($p<0.05$ for both). The pattern of increased ROS in young Sod2Tg myoblasts compared to the wild-type and $Sod2^{+/-}$ myoblasts was reversed with aging, and myoblasts

**Figure 4.5**

**Measurement of ROS in myoblasts**




(A) Superoxide in mitochondria measured using Mitosox. Left: For reference, wild-type (young) myoblasts were treated with the increasing concentration of rotenone. (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).

(B) Intracellular ROS including hydrogen peroxide ($H_2O_2$), hydroxyl radical (HO•) measured using CM-$H_2$DCFH fluorescence. Left: For reference, intracellular ROS of wild-type (young) myoblasts treated with increasing concentrations of BSO.

from aged Sod2Tg animals had similar ROS compared with aged wild-type myoblasts (p=0.135) and aged $Sod2^{+/-}$ myoblasts (p=0.152).

**The relative abundance of mitochondria in myoblasts**

Because MnSOD is exclusively a mitochondrial protein, we were interested in normalizing our results to some measure of mitochondrial abundance. Mitochondrial DNA and chromosomal DNA abundance were compared using quantitative PCR methods adapted from the literature. Four regions of mtDNA that have low susceptibility to mutation with aging were selected for qRT-PCR amplification (Vaillant and Nagley, 1995; Miller et al., 2003). The four different primer sets and probes used to amplify these regions are shown in Table 2.2. In general, the four probes generated very similar quantitative information, giving confidence in the approach (Figure 4.6). In myoblasts from young animals, wild-type cells had greatest density of mitochondria with $Sod2^{+/-}$ mitochondrial density intermediate and Sod2Tg lowest. (The three young groups are all significantly different from each other.) Among aged myoblasts, wild-type and $Sod2^{+/-}$ showed significantly decreased mitochondrial abundance compared to the young genotype counterparts. In myoblasts from aged wild-type mice the ratio of mitochondrial:genomic DNA was only 15% that of young wild-type myoblasts (0.14 ± 0.04 vs. 1.02 ± 0.14, p=0.014). With aging, $Sod2^{+/-}$ myoblasts also showed significantly decreased ratios of mitochondrial:genomic DNA (p=0.014 & p=0.05), similar to wild-type. Surprisingly, Sod2Tg myoblasts had an increased mitochondrial:genomic DNA

ratio with aging. Of the myoblasts from aged animals, Sod2Tg myoblasts had the most mitochondria, with a 2.5-fold increase in the mitochondrial:genomic DNA ratio from that of young Sod2Tg myoblasts ($p=0.03$).

**PGC1α, a controller of mitochondrial biogenesis, is not regulated in parallel to myoblast mitochondrial density in aged myoblasts**

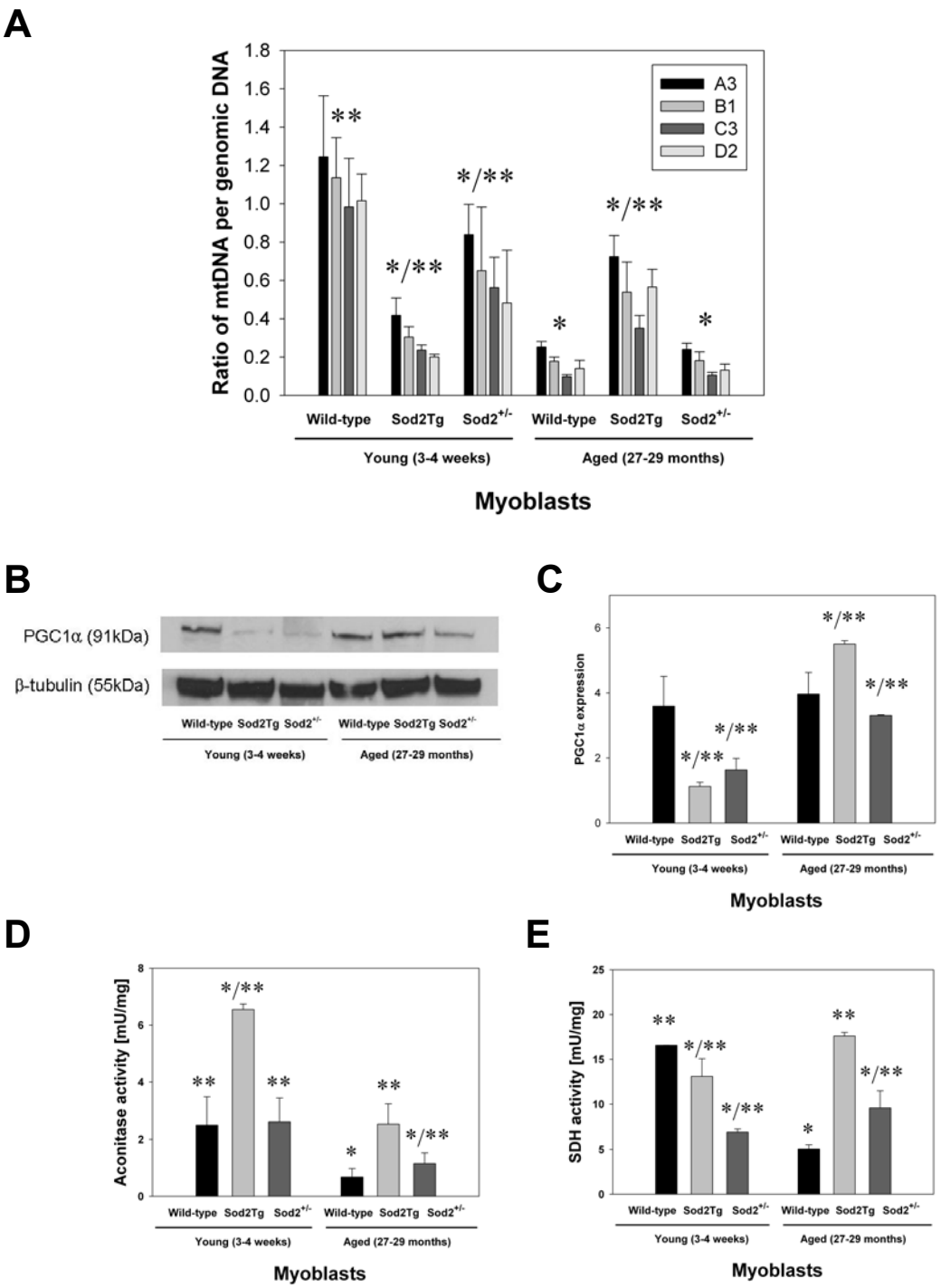
Peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC1α) levels in myoblasts were measured by western blotting, using whole cell lysates. PGC1α is upregulated by a variety of stressors including oxidant stress and promotes mitochondrial biogenesis (Puigserver et al., 1998; Wu et al., 1999; Lehman et al., 2000; Lin et al., 2002). In myoblasts from young mice, relative PGC1α levels followed the same pattern as the mitochondrial:genomic DNA ratios, with the least expression of PGC1α in young Sod2Tg myoblasts (Figure 4.6B). PGC1α expression increased with age in the Sod2Tg myoblasts, increased to a lesser degree in the $Sod2^{+/-}$ myoblasts, but was unchanged from young levels in wild-type myoblasts. The higher levels of PGC1α in $Sod2^{+/-}$ myoblasts were insufficient to maintain mitochondrial mass with aging, whereas the high levels of PGC1α in the Sod2Tg myoblasts were associated with maintained mitochondrial mass (Figure 4.6).

**Mitochondrial enzyme activity**

Myoblast mitochondrial function was assessed by measuring aconitase and SDH enzyme activity (Figure 4.6D & 4.6E). Aconitase is a specific target of

**Figure 4.6**

**Myoblast mitochondrial abundance and function as a function of SOD2 levels and aging**



(A) Mt:genomic DNA ratio assayed by qRT-PCR. For each condition, the four bars indicate results using four distinct primer sets. Normal myoblast aging is associated with significant loss of mitochondrial mass, which was blunted by SOD2 overexpression.
(*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).
(B) PGC-1α abundance (by western blotting) increases most dramatically with aging in the Sod2Tg cells.
(C) Quantification of PGC-1α western blots from (B) (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type).
(D) Aconitase activity (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type) is highest in Sod2Tg myoblasts.
(E) Succinate dehydrogenase activity (*p<0.05 compared to wild-type young; **p<0.05 compared to aged wild-type) does not correlate with aconitase activity, but is best preserved with aging in the Sod2Tg myoblasts.

oxidative damage (Fridovich, 2004). Aconitase activity appears to be preserved by overexpression of MnSOD in myoblasts from both young and aged mice, as the transgenic mice had highest levels in young and old groups. Aconitase activity fell significantly in all myoblasts, including in the Sod2Tg myoblasts, suggesting that the MnSOD levels in these aged myoblasts could not overcome accumulation of oxidant damage mediated by other age-related factors. Nonetheless, the aconitase activity in myoblasts from aged Sod2Tg was close to

that of young wild-type myoblasts (p=0.97). The tricarboxylic acid enzyme, succinate dehydrogenase (SDH) is also a target of oxidative stress. SDH activity assays showed that overexpression of MnSOD contributed to preserved SDH enzyme function with aging. The pattern of SDH activity in myoblasts from young animals did not, however, track with MnSOD levels.

**Discussion**

Myoblast function is critical for normal repair and replacement of skeletal muscle throughout life. These studies confirm that the normal aging phenotype of myoblast progenitors is characterized by loss of proliferation in response to mitogens in culture, and reduced ability to form myotubes when mitogens are withdrawn, both features thought to reflect the impaired repair capacity of skeletal muscle with aging. Similar loss of differentiation potential with aging in mice has been well-documented by many groups (Brack and Rando, 2007) and in human myoblasts (Bigot et al., 2008). In our studies in wild-type myoblasts, normal aging was also associated with loss of MnSOD activity and a corresponding increase in intracellular superoxide levels, consistent with the pattern of whole muscle in these animals (Mansouri et al., 2006). Recent work suggests that aged muscle does not necessarily produce more superoxide than young muscle (Close et al., 2007) but whole muscle and the myoblast compartment may not necessarily follow the same pattern. Our studies also suggest that the massive differentiation defects in young $Sod2^{+/-}$ myoblasts are not exaggerated with age despite the documented sensitivity of the $Sod2^{+/-}$ animals to oxidant stress — The

differences between wild-type and $Sod2^{+/-}$ myoblasts are generally not exacerbated as the animals age.

Interestingly, proliferation was impaired in both young Sod2Tg and $Sod2^{+/-}$ myoblasts compared to wild-type, and deteriorated with aging in both, suggesting that both excess superoxide and excess hydrogen peroxide negatively affect proliferative responses. Overexpression of MnSOD resulted in increased intracellular hydrogen peroxide similar to that seen in glutathione peroxidase-1 (Gpx1) knockout myoblasts that proliferate less in culture than wild-type myoblasts (Lee et al., 2006). Overexpression of MnSOD throughout the lifetime of the myoblast did not protect against aging-induced proliferative defects, as it did against aging-associated defects in differentiation. The relative preservation of morphologic differentiation in the Sod2Tg myoblasts despite elevated intracellular peroxide levels is distinct from the pattern seen in GPx1-null myoblasts (Lee et al., 2006) suggesting that $H_2O_2$ alone is not the major cause of the differentiation dysfunction seen in the Gpx1-null myoblasts.

Given the central importance of insulin signaling in muscle differentiation and maintenance, we examined downstream targets of insulin signaling that are particularly important for protein synthesis, muscle regeneration, and therefore maintenance of muscle mass. Akt is at the center of this pathway, activating mTOR and protein synthesis, and regulating the forkhead factors, which repress gluconeogenesis. Inactivation of PI3K/Akt pathway (decreased p-Akt) results in dephosphorylation of FOXO transcription factors, which activates muscle atrophy-inducing ubiquitin-ligases such as Atrogin-1 and MuRF1 (Sandri et al.,

2004; Stitt et al., 2004; Leger et al.,2006). FOXO transcription factors are also post-translationally regulated by oxidative stress (Brunet et al., 2004; Bakker et al., 2007). However, changes of these important signaling pathways have often been studied using immortalized cell lines (Sandri et al., 2004; Wilson and Rotwein, 2007; Diel et al., 2008) rather than primary myoblasts as used here, or in whole muscle (Gomes et al., 2001; Stitt et al., 2004; Leger et al., 2006).

The PI3K/Akt pathway was significantly altered by haplo-insufficiency of Sod2 in young myoblasts, but again these defects were not amplified with aging in the $Sod2^{+/-}$ myoblasts. The effects of aging on this pathway, with constitutive activation of Akt, apparently override the differences caused by reduced MnSOD levels. mTOR and S6K, downstream targets of Akt which are essential for protein synthesis, were also less phosphorylated during differentiation in $Sod2^{+/-}$ myoblasts compared to wild-type controls, suggesting that the signaling pathway is a target of superoxide that affects differentiation. However, an unexpected finding was that FOXO1 activation was not impacted by Sod2 expression, though it has been reported to be regulated in other contexts by oxidant signaling.

We used quantitative PCR to estimate mitochondrial mass, and were surprised to see significantly less mitochondrial:genomic DNA ratios in myoblasts from young Sod2Tg animals compared to wild-type. The aconitase and SDH activity levels were not reduced to the same degree as mitochondrial mass suggesting that mitochondrial function is enhanced by transgenic overexpression of MnSOD, at least in young animals. The most surprising finding in our studies was the suggestion that MnSOD overexpression may mediate preservation and

of mitochondrial mass with aging in the muscle progenitor population, an observation that deserves further study. This finding may simply be a reflection of the increased scavenging of superoxide locally in the mitochondria by the overexpressed MnSOD. The relatively high mitochondrial:genomic DNA ratios in the old Sod2Tg myoblasts was associated with relatively preserved mitochondrial function compared to the other genotypes with aging. Though the mitochondrial mass was preserved, whole muscle mass was not preserved in the transgenic animals. Nonetheless, the preserverd mitochondrial mass may have important effects on muscle and systemic metabolic function, and deserves further study.

Previous studies suggest that the activity of PGC-1α decreases in whole muscle during aging (Chabi et al., 2007). In contrast, we found that wild-type myoblasts have unchanged levels of PGC-1α with aging while both Sod2Tg and $Sod2^{+/-}$ myoblasts had elevated PGC-1α compared to their young counterparts, suggesting that PGC-1α is sensitive to both increased superoxide and increased hydrogen peroxide even in the context of the oxidized aged muscle environment. The differences in patterns of PGC-1α expression with aging in published reports and our data may be due to the differences between whole muscle vs. the progenitor population. Further study will be necessary to examine mitochondrial biogenesis vs. degradation as a function of aging and overexpression of MnSOD.

Use of primary myoblasts from the genetically manipulated animals allowed us to distinguish the effects of MnSOD levels on the progenitor cell population from those on the whole organ (muscle). However, a major limitation of these studies is that only cell-intrinsic effects can be measured, and we have

not yet examined the impact of altered MnSOD levels on the microenvironment of myoblasts. Nonetheless, overall our study suggests muscle differentiation is very sensitive to oxidant stress, both the oxidant stress of normal aging, and the specific stresses mediated by manipulating MnSOD levels. Further the results suggest that methods to enhance MnSOD function could benefit muscle regeneration in aged subjects, regeneration that is necessary to regain function and strength after trauma, surgery, or prolonged bedrest. The data suggest that MnSOD mediates its beneficial effects on muscle differentiation acutely at least in part through the Akt pathway, and chronically may also promote mitochondrial biogenesis.

# Chapter 5: Aberrant HIF-1α expression in response to oxygen in Sod1 heterozygote myoblasts

## Introduction

Superoxide dismutase-1 (Sod1 or CuZnSOD) is a ubiquitous, primarily cytosolic enzyme also localized to the intermembrane spaces of mitochondria, which catalyzes the dismutation of superoxide anion ($O_2^-$) into hydrogen peroxide ($H_2O_2$) and oxygen. Sod1 homozygous knockout ($Sod1^{-/-}$) mice develop normally and appear healthy at birth (Reaume et al., 1996). However, oxidative stress-induced damage results in multiorgan dysfunction and degenerative changes. On a B6 background, hepatocellular carcinoma is common by 20 months of age in $Sod1^{-/-}$ mice (Elchuri, et al., 2005).

Motor neurons are particularly susceptible to loss of CuZnSOD, and loss of muscle mass is thought to be largely secondary to distal axonal loss. On a B6 background, $Sod1^{-/-}$ mice have profound muscle loss, and more severe age-related sarcopenia than wild-type mice (Muller et al., 2006). On ultrastructural examination, skeletal muscle defects are subtle with mean fiber cross-sectional area preserved. However, fiber grouping of fast 2a fibers suggests injury secondary to denervation, and some muscles show evidence of rounds of degeneration/regeneration (Kostrominova et al., 2007).

Many defects in CuZnSOD-manipulated mice suggest that physiologic responses to hypoxia, coordinated by hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) are impaired. $Sod1^{-/-}$ mice, for example, have cerebral arteriolar hypertrophy (Baumbach et al., 2006), and disordered central control of hypoxic ventilatory responses (Cummings et al., 2008). Furthermore, endogenous VEGF protects against motor neuron loss in mouse ALS models (Lambrechts et al., 2003) that

have increased oxidant stress (Marden et al., 2007). For these reasons, we examined the effects of reduced Sod1 expression on myoblasts from CuZnSOD-deficient mice, with a focus on expression of HIF-1$\alpha$.

## Results

### $Sod1^{-/-}$ myoblasts do not survive in culture

Many attempts were made to culture myoblasts from $Sod1^{-/-}$ mice *in vitro*, but could not achieve survival despite culture in low oxygen conditions and supplementing the medium with NAC or catalase, and in spite of our extensive experience isolating myoblasts from other antioxidant-deficient mice (Lee et al., 2006). For this reason we conducted all the studies using myoblasts from $Sod1^{+/-}$ mice.

### Intracellular superoxide levels in $Sod1^{+/-}$ myoblasts

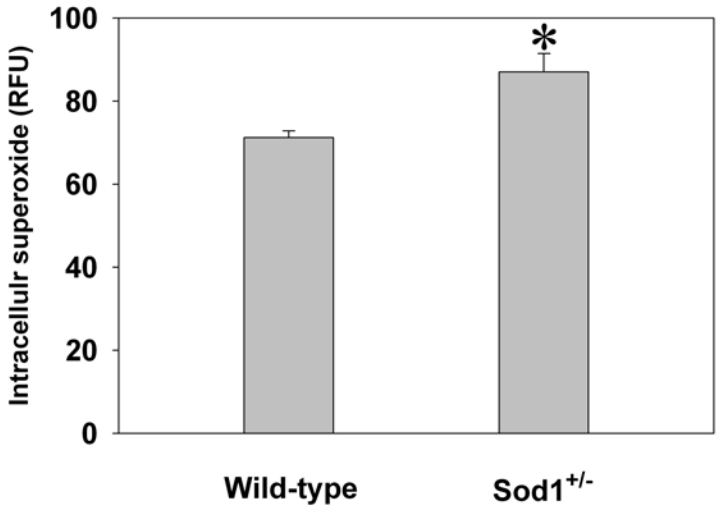
Intracellular superoxide was measured using fluorescence of the superoxide-sensitive dye, dihydroethidium (DHET). Superoxide production in $Sod1^{+/-}$ myoblasts was about 20% higher than in wild-type myoblasts mice ($p=0.03$, Figure 5.1).

### Morphologic differentiation of $Sod1^{+/-}$ myoblasts in various oxygen concentrations

Normal terminal differentiation of myoblasts in culture involves fusion of single-cell myoblasts into multinucleate myotubes, and coordinated upregulation

**Figure 5.1**

**Relative intracellular superoxide levels in Sod1$^{+/-}$ vs. wild-type myoblasts**




Intracellular superoxide anions were assayed using dihydroethidine (DHET) fluorescence. Superoxide was 1.2-fold more in Sod1$^{+/-}$ myoblasts than in wild-type cells.

of myosin heavy chain (MHC). When subjected to differentiation conditions, Sod1$^{+/-}$ myoblasts exhibited obvious defects compared to wild-type myoblasts in a wide range of ambient oxygen concentrations, with significantly less morphologic differentiation into myotubes (Figure 5.2). These morphologic observations are confirmed by calculating the fusion index (FI), which also showed that the optimal oxygen concentration for differentiation of wild-type and Sod1$^{+/-}$ myoblasts was different. FI of wild-type myoblasts was lowest in 0%

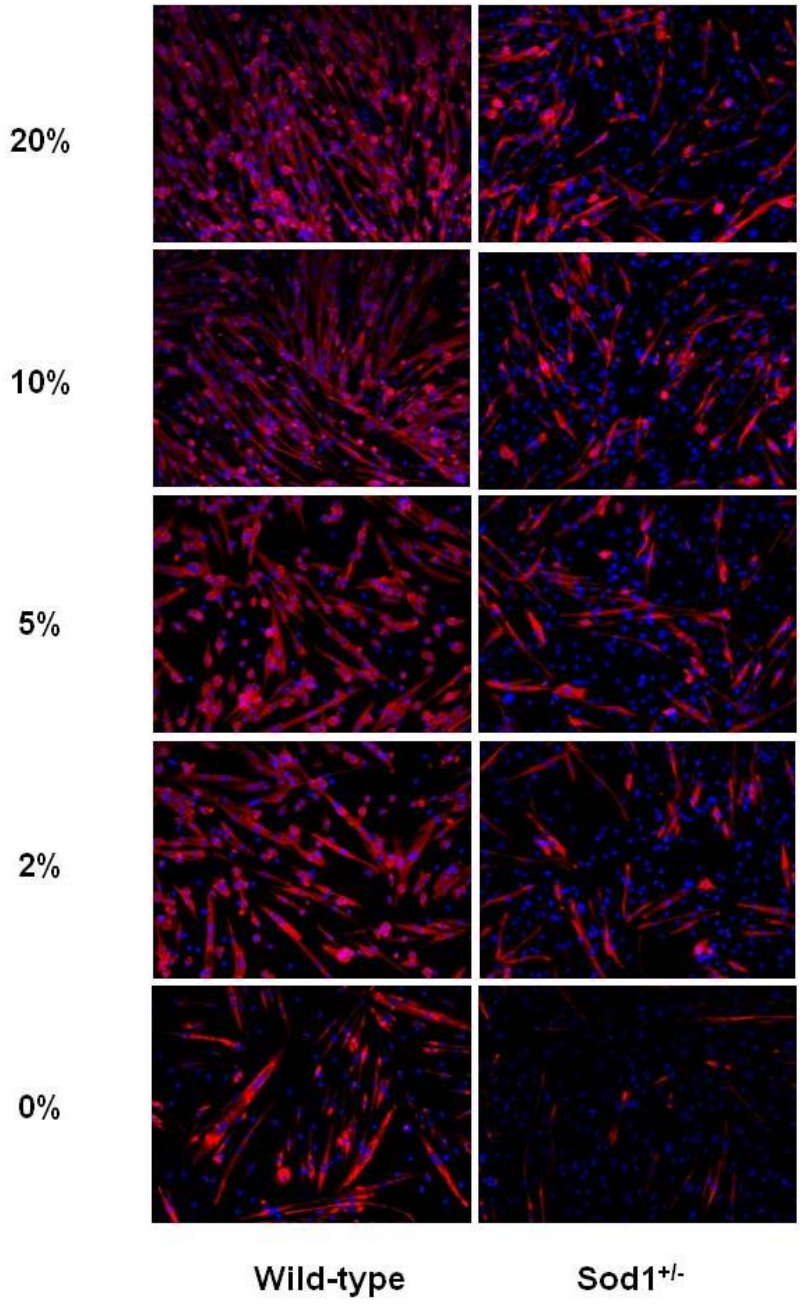
oxygen (23.6 ± 6.1%) but the survival of the cells in these conditions confirms other reports that normal myoblasts are hypoxia-tolerant. The highest FI in wild-type myoblasts was observed in 5% (approximate physiologic levels), 10% and 20% oxygen, with no significant difference over this range. $Sod1^{+/-}$ myoblasts had a FI of virtually zero in 0% oxygen (0.84 ± 0.19). $Sod1^{+/-}$ myoblast FI was highest in 10% and 20% oxygen, but only ~25% of cells were in differentiated myotubes at the end of 3 days of differentation. Most importantly, at all oxygen concentrations, fusion indices were reduced in $Sod1^{+/-}$ myoblasts compared to wild-type controls. At best FI of $Sod1^{+/-}$ myoblasts was only 35% that of wild-type cells (Figure 5.2B).

Differentiation potential (DP) is a complementary method of quantifying efficiency of differentiation, and DP of $Sod1^{+/-}$ myoblasts was also lower than that of wild-type cells over the entire oxygen range. The DP of wild-type myoblasts, as expected, was lowest in 0% oxygen. In these conditions 69.8 ± 5.6% of cells expressed MHC. Comparing this number to the FI in 0% oxygen, many more cells turned on MHC than were incorporated into myotubes, suggesting that MHC was prematurely turned on, not coordinated with fusion. DP of $Sod1^{+/-}$ myoblasts was lowest in 0% oxygen. The highest DP in wild-type myoblasts was observed in 5% oxygen (88.54 ± 0.49), and decreased gradually in 10% (82.49 ± 0.97, $p=0.018$ compared to 5%) and 20% oxygen (77.52 ± 0.27, $p=0.001$ compared to 5%). At best, DP of $Sod1^{+/-}$ was about 40% of that of wild-type (Figure 5.2C).

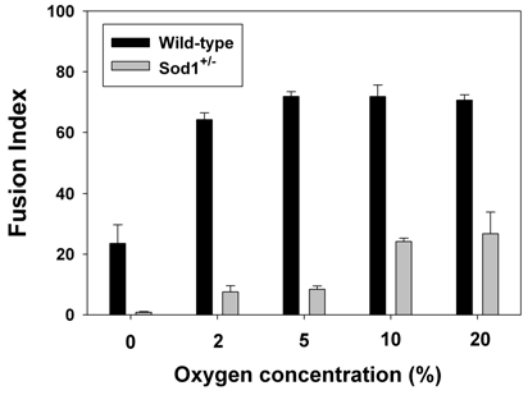
**Figure 5.2**

**Differentiation of myoblasts in different oxygen conditions**

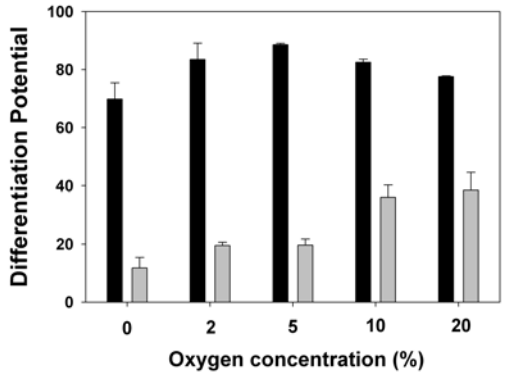
**A**



**B**




**C**




(A) Myoblasts were differentiated in various oxygen concentrations for 3 days *in vitro*. Immunostaining for myosin heavy chain (MHC, red) was used to mark differentiated cells. Blue is the nuclear stain, DAPI.

(B) Quantitation of fusion index (FI) using data derived from micrographs as in A. Fusion index (FI) = [(Number of nuclei in MF 20-stained myotubes with $\geq$ 2 nuclei/ Total number of nuclei) × 100]**.** At least 500 cells were counted in each of 2 experiments. Data are expressed as mean ± S.E.

(C) Differentiation potential (DP) was calculated as follows: [(Number of nuclei in MF20-stained cells/Total number of nuclei)] X100. Data are mean ± SE, n=2 for each genotype.

**Myoblast HIF-1α abundance in various oxygen conditions**

HIF-1α expression patterns were significantly different in wild-type vs. $Sod1^{+/-}$ myoblasts as a function of oxygen concentration at day 3 in DM (Figure 5. 3). Wild-type myoblasts had similar HIF-1α protein levels in 20% and 10% oxygen. In lower oxygen concentration, as expected, HIF-1α expression increased. In 5% oxygen, HIF-1α protein was about 40% more abundant in wild-type myoblasts than in 20% oxygen, and expression was highest in 2% oxygen. In the extreme hypoxic condition (~0% oxygen), HIF-1α expression of wild-type myoblasts was similar to that in 20% oxygen ($p=0.25$).

HIF-1α protein-level expression was higher in $Sod1^{+/-}$ myoblasts than in wild-type in all oxygen conditions except 0% oxygen. However, the expression pattern over the oxygen concentration range suggested dysregulation of HIF-1α expression in these cells. In general, higher oxygen concentrations were associated with higher HIF-1α expression, opposite of expected expression patterns that are central to physiologic responses to hypoxia.

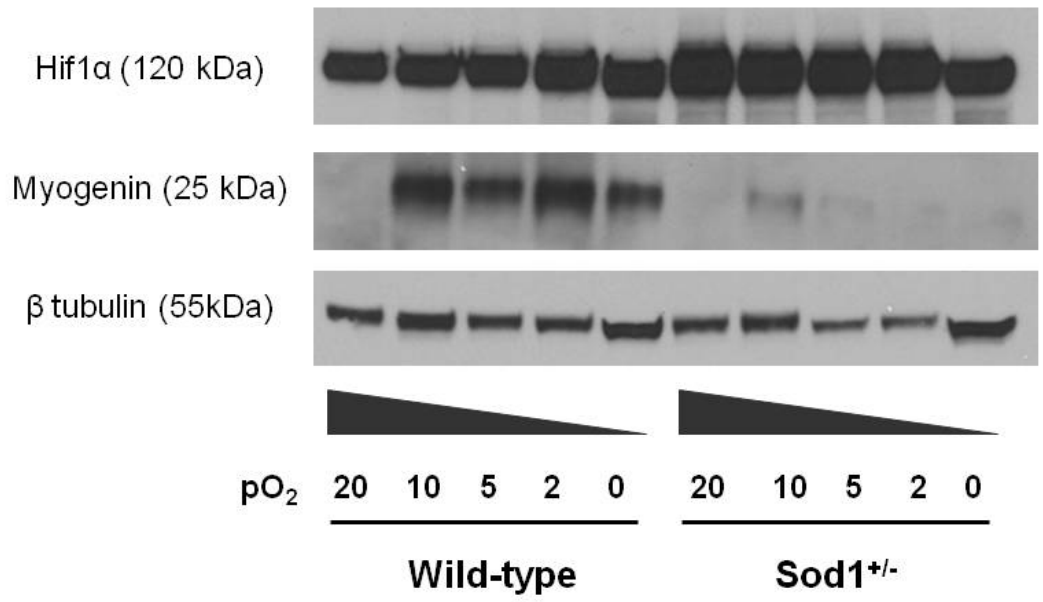
**Myogenin expression in various oxygen environments**

Myogenin is a member of the MyoD family of basic helix-loop-helix myogenic transcription factors and is up-regulated in the later phases of muscle differentiation. Previous work showed that myogenin mRNA levels in whole muscle of $Sod1^{+/-}$ mice (but not MyoD levels) were upregulated relative to wild-type muscle (Kostrominova et al., 2007). In contrast we found that myoblasts from $Sod1^{+/-}$ mice expressed significantly lower levels of myogenin protein overall

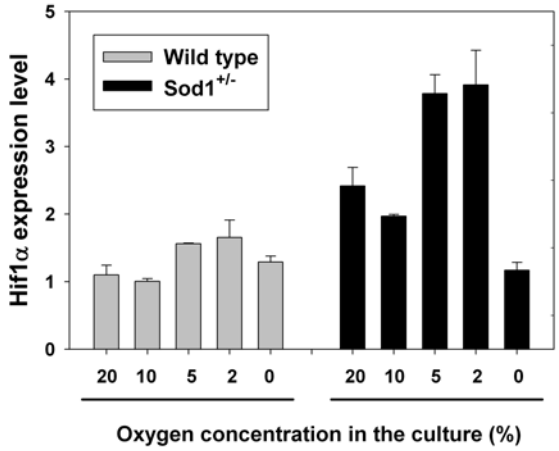
than the wild-type myoblasts (Figure 5.3). In wild-type myoblasts, myogenin expression was dramatically less in 20% oxygen than in lower oxygen concentrations after 3 days in differentiation conditions. Myogenin protein was only clearly detectable in $Sod1^{+/-}$ myoblasts in 10% oxygen. The generally lower levels of myogenin expression in $Sod1^{+/-}$ myoblasts may simply be a reflection of the poor differentation of these cells.

**Figure 5.3**

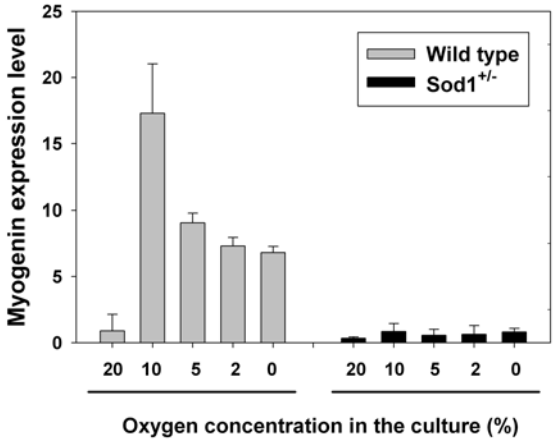
**HIF-1α and myogenin expression patterns are altered by loss of CuZnSOD**



**B**




**C**




(A) Western blots demonstrate that normal upregulation of HIF-1α in low oxygen conditions is lost in Sod1$^{+/-}$ myoblasts.

(B) Quantitation of HIF-1α expression in myoblasts after 3 days of differentiation. β-tubulin was used as a loading control. Data are mean (fold-change) ± S.E.

(C) Myogenin expression in Sod1$^{+/-}$ myoblasts is much lower than in wild-type myoblasts after three days of differentiation. β-tubulin was used as a loading control. Data are mean (fold-change) ± S.E.

**Discussion**

Differentiation of Sod1-deficient myoblasts was significantly impaired *in vitro*, suggesting an intrinsic effect of the induced oxidant stress on the progenitor compartment of muscle, in addition to the reported muscle loss secondary to oxidant-induced peripheral neuropathy (Chen et al., 2007). The reduced myogenin expression in $Sod1^{+/-}$ myoblasts is unlikely to contribute to the defect in differentiation, as recent studies suggest that postnatal muscle regeneration (and myoblast differentiation *in vitro*) is not dependent on myogenin expression, and other MyoD family members compensate for myogenin loss (Meadows et al., 2008). However, the reduced myogenin expression is consistent with our prior work in transformed myoblasts that showed reduced message-level expression of myogenin in 20% oxygen (vs. 6%) and dramatically reduced expression when the cells were exposed to 25 $\mu$M $H_2O_2$ (Hansen et al., 2007). The observed myogenin expression pattern in the $Sod1^{+/-}$ myoblasts was different from that reported in whole muscle of these mice (Kostrominova et al., 2007) but regulation of myogenin in myoblasts may be distinct from that in whole muscle.

Reduced differentiation potentials of the Sod1-deficient myoblasts is not surprising, as we have seen similar differentiation defects in Sod2-deficient (in preparation) and glutathione peroxidase-1 null myoblasts (Lee et al., 2006). Taken together these data suggest that the molecular controls over skeletal muscle differentiation and myoblast fusion are highly sensitive to increased superoxide and increased peroxide levels.

Dysregulation of HIF-1α expression in Sod1$^{+/-}$ myoblasts was unexpected. Regulation of HIF-1α expression as a function of oxygen conditions is well-studied, but regulation by reactive oxygen species is less well-characterized, though in conditions of generalized oxidant stress, HIF-1α expression is often increased (Chandel et al., 2000; Duyndam et al., 2001; Mansfield et al., 2005). It is more difficult to find literature in which superoxide regulation of HIF-1α has been assayed, and this literature does not yield a clear picture. In vascular cells exposed to superoxide, HIF-1α expression is reduced (Wellman et al., 2004). In the MCF cancer cell line, acute increases in superoxide result in amplified HIF-1α upregulation in hypoxic conditions (Kaewpila et al., 2008). These studies used relatively acute superoxide exposures. In our studies, chronic exposure of myoblasts to increased superoxide levels due to genetic manipulation of Sod1 led to a pathologic HIF-1α response to oxygen levels, and apparent inability of the cells to respond to hypoxic conditions. Although speculative, our results suggest that HIF-1α dysregulation should be studied in other organs affected by Sod1 deficiency, especially because HIF-1α is a master regulator of physiologic responses to oxygen and is central to regenerative processes (Liu et al., 2008). In particular, work in C2C12 myoblasts suggests that HIF-1α is expressed at very low levels while myoblasts are proliferating in culture, but is obligatorily up-regulated as the cells differentiate (Ono et al., 2006). These findings suggest that the abnormal regulation of HIF-1α in response to normal cues in the Sod1$^{+/-}$

myoblasts may be a factor in their defective differentiation especially since the efficiency of muscle differentiation is oxygen dependent (Yun et al., 2005).

# Chapter 6: Discussion

**Oxidative stress and muscle progenitor cell function**

ROS have been implicated in the pathophysiology of many diseases. However, the effect of ROS on muscle progenitor cells has not been extensively studied. The broad goal of this dissertation work was to provide greater insight into the role of ROS in every aspect of muscle progenitor cell biology using primary myoblasts isolated from genetically manipulated mice. The specific goals of this dissertation were to characterize the effect of anti-oxidant enzyme deficiency or overexpression on primary myoblast phenotype and gene expression patterns. The first aim of this dissertation was to characterize the effect of Gpx1 deficiency on myoblasts. The data presented in Chapter 3 support a role for global effects of ROS on progenitor cell function, as the deficiency of Gpx1 impairs all examined aspects of myoblast function and imposes an early senescence phenotype in whole muscle. We have shown that proliferation and differentiation are critically defective, and the threshold for apoptosis is low in cultured $Gpx1^{-/-}$ myoblasts from young animals. Additionally, the cross sectional area of muscle fibers of $Gpx1^{-/-}$ is smaller than wild-type and muscle accumulates lipofuscin in young $Gpx1^{-/-}$ mice. The data presented in Chapter 4 showed that Sod2 overexpression preserves myoblast mitochondrial mass and function, but not muscle mass with aging. We also demonstrated that there were downsides to Sod2 overexpression, likely because the overexpression of the enzyme results in increased intracellular $H_2O_2$ or decreased superoxide anions. Thus, although ROS in the form of superoxide is decreased, the overall balance of ROS in the cells is disrupted. As a result of this imbalance, we uncovered some impairment

of cellular function such as proliferation. The data presented in Chapter 5 indicate that Sod1 deficiency also impairs muscle differentiation, a common feature of antioxidant deletion. In preliminary studies, our results suggest that $Sod1^{+/-}$ myoblasts have a distinctly abnormal response to oxygen concentration, with disruption of HIF-1α expression responses that immediate many critical physiologic responses to oxygen. Overall, the data presented in this dissertation identify many and broad effects of ROS in muscle differentiation and highlight distinct effects of particular ROS specific to myoblasts. The implications of these studies for other progenitor populations can only be guessed, but these studies can serve as a basis for further studies of the effects of ROS generally on stem cell function.

**Redox regulation of proliferation**

Young $Gpx1^{-/-}$ myoblasts showed profoundly decreased the proliferation in culture. Because proliferation can be matrix dependent, we attempted to rescue the proliferation defect by exposing the cells to different matrices. None of the matrices afforded significant rescue, and proliferation was consistently impaired compared to wild-type myoblasts, suggesting that the increased intracellular $H_2O_2$ or product negatively regulates myoblast proliferation. Consistent with results in the $Gpx1^{-/-}$ myoblasts, Sod2 overexpression also increased the intracellular $H_2O_2$ level, and decreased the proliferation of myoblasts, confirming the sensitivity of proliferation of myoblasts to intracellular $H_2O_2$ levels. Although we did not compare the Sod2 overexpressing and Gpx-null myoblasts directly,

the assays conducted at different times comparing the cells to wild-type cells suggested that the two genetically manipulated cell types had a quantitatively similar decrease in proliferation, although the levels of intracellular $H_2O_2$ seemed to be higher in $Gpx1^{-/-}$ than Sod2Tg myoblasts. Proliferation of myoblasts was also sensitive to increased levels of superoxide. $Sod2^{+/-}$ myoblasts had similar levels of $H_2O_2$ but produced about 20% more mitochondrial superoxide compared with wild-type myoblasts. However, proliferation of $Sod2^{+/-}$ myoblasts was higher than that of $Gpx1^{-/-}$ and Sod2Tg cells, and although difficult to directly compare, the data suggest that proliferation of myoblasts may be more sensitive to $H_2O_2$ than to mitochondrial superoxide.

We, like others, have shown that proliferation of aged wild-type myoblasts is decreased (to about 65% that of young wild-type myoblasts) with aging. Both aged Sod2Tg and $Sod2^{+/-}$ showed decrements in proliferation compared to wild-type with aging. Notably, chronic Sod2 overexpression has no beneficial effect on the proliferation of aged myoblasts. Overall this pattern suggests that the complex accumulation of oxidative damage with aging contributes to a decreased ability to proliferate, but that a chronic decreased in superoxide does not improve the aging defect. However, chronically increased superoxide and hydrogen peroxide in the mutant myoblasts does not provide an enormous added burden compared to the complex changes that occur with aging.

**Redox regulation of differentiation of muscle progenitor cells**

Differentiation of cells accompanies cell cycle withdrawal both in development of many lineages and in differentiation of myoblasts in culture. Differentiating myoblasts *in vitro* undergo a requisite withdrawal from the cell cycle, before they fuse and turn on myogenic regulatory factors in a highly specific manner. Striking differentiation defects were seen in all the antioxidant gene-manipulated myoblasts. The most defective differentiation was in $Gpx1^{-/-}$ myoblasts, then Sod2Tg, then $Sod2^{+/-}$ and $Sod1^{+/-}$ compared to wild-type myoblasts. The severe differentiation defect observed in $Gpx1^{-/-}$ myoblasts was somewhat surprising, since Gpx1 is only one of a family of genes that have the same enzymatic function. The Gpx1-deletion defect might be partly attributable to dysregulation of myogenic transcription factors, since the temporal sequence of expression of these genes and their coordination is critical to normal differentiation. Our PCR data showed that the genes were not underexpressed, indicating that Gpx1-null cell failure to generate myotubes is not a classical differentiation defect. Rather it likely represents a fusion defect. This phenotype may relate back to the earliest observation of these cells after their isolation. We saw a clear premature fusion morphologic phenotype, with aberrant formation of myotubes in the presence of a rich mitogen medium. It is likely that the cells that fused could not be passaged (exited the cell cycle), while the population that could be passaged was defective in fusion (and were the ones we analyzed over time). Thus the deletion of Gpx1 may result in two phenotypes in myoblasts – one with premature, inappropriate fusion and one that cannot fuse. We did not observe this kind of heterogeneity of cell populations in the other myoblasts.

Interestingly, aged Sod2Tg myoblasts showed much more myotube formation than other myoblasts from aged mice. (As noted above, in contrast, Sod2 overexpression throughout the lifetime of the myoblast did not protect against aging-induced proliferative defects, as it did against aging-associated defects in differentiation.) The relative preservation of morphologic differentiation in the Sod2Tg myoblasts despite elevated intracellular hydrogen peroxide levels is distinct from the pattern seen in $Gpx1^{-/-}$ myoblasts (Lee et al., 2006) suggesting that $H_2O_2$ alone is not the major cause of the differentiation dysfunction seen in the $Gpx1^{-/-}$ myoblasts. It is possible that ROS generated in different subcellular localizations might have a distinct role in myoblasts during differentiation, and the overexpression of enzyme in the mitochondrial compartment over time may have a particularly beneficial effect on differentiation.

**Altered signaling pathways induced by oxidative stress in myoblasts**

ROS are well characterized for playing a dual role as both detrimental and beneficial, depending on the repertoire and amounts of the different species in the cell. We have shown that the disrupted optimal balance of anti-oxidants resulted in altered intracellular signaling as well as phenotypic changes. We postulated that ROS might change redox-sensitive signaling pathways which are known to function in muscle differentiation, and focused our analysis on specific candidate gene pathways.

First, the oxidative stress induced by Gpx1 deletion resulted in the up-regulation of transcription of MRFs. Decreased expression of MRFs was

previously described as underlying the classical model of myogenic differentiation defects (Berkes and Tapscott, 2005). In contrast to the classical differentiation defect, message-level expression of MRFs in $Gpx1^{-/-}$ myoblasts is up-regulated during differentiation *in vitro*. This may in itself be pathologic. Clearly, the high levels of MRFs in undifferentiated, proliferating myoblasts is abnormal, and the inability to augment this high level expression may play a role in the morphologic differentiation defect we saw. Additional work is needed to fully identify the expression, post-translational modification and interactions of MRFs affected by the deficiency of Gpx1.

Second, the PI3K/Akt pathway was significantly altered specifically in young $Sod2^{+/-}$ myoblasts. This inactivation of Akt was accompanied by less activation of mTOR and S6K compared to Sod2Tg and wild-type myoblasts. The differentiation defect observed in young $Sod2^{+/-}$ myoblasts likely arises in part through this altered Akt pathway. However, the phosphorylation of FOXO1 was not affected by the decreased Akt activation in $Sod2^{+/-}$ myoblasts. It is known that downstream targets of Akt including mTOR, S6K and FOXO1 can be phosphorylated by other kinases (Guertin et al., 2006; Deguil et al., 2008) and may explain these differences in activation of various downstream Akt signals. With aging, the relative Akt inactivation was not further amplified in the $Sod2^{+/-}$ myoblasts. The surprising effects of aging on this pathway, constitutive activation of Akt, apparently override the differences caused by decreased SOD2 levels. However, the downstream targets of Akt including mTOR, S6K and FOXO1 showed much less phosphorylation in all aged genotype myoblasts in spite of the

constitutive activation of Akt. Thus, aging superimposed a dysregulation of the normal relationship between Akt and its downstream targets, but our work did not address a cause for this dysregulation. Nonetheless, our results are consistent with previous reports support that aging is associated with decreased phosphorylation of S6K even with high activation of Akt in whole muscle (Li et al., 2003c).

We also showed that Sod1 haplo-deficiency in myoblasts may impose altered sensing of oxygen via the master regulator, HIF-1α. HIF-1α is the central molecule involved in physiologic responses to oxygen, and functions as a redox sensor (Chandel et al., 2000; Duyndam et al., 2001; Mansfiled et al., 2005). The $Sod1^{+/-}$ myoblasts have aberrantly increased HIF-1α expression in high oxygen concentrations compared to the normal pattern of high-level expression in low oxygen concentrations. In terms of phenotype, Sod1+/- myoblasts demonstrated severely defective differentiation in all oxygen concentrations compared to wild-type. With these preliminary data we cannot directly prove how the alteration of HIF-1α signaling contributes to the fusion/differentiation defect of $Sod1^{+/-}$ myoblasts. Nonetheless, the reversal of normal HIF-1α responsiveness to oxygen in $Sod1^{+/-}$ myoblasts is striking, and deserves further investigation as it may contribute to many physiologic problems in these mice.

Additionally, although mice with complete deletion of SOD1 ($Sod1^{-/-}$) are viable and develop normally (Matzuk et al., 1998), we were unable to isolate primary myoblasts despite multiple attempts, and despite our experience with other mutant myoblast populations. $Sod2^{-/-}$ deletions are lethal at embryonic or

neonatal stages (Li et al., 1995; Huang et al., 2001). But the viability of myoblasts in the Sod2 knockouts could not be evaluated, since we did not have access to these animals.

**Mitochondrial biology under oxidative stress**

Oxidative damage accumulation in mitochondria in skeletal muscle during aging is well documented including damage to mtDNA, increased carbonylation of proteins and membrane functional damage (Floyd et al., 2001; Stadtman, 2002; Sohal, 2002). However, the role of mitochondria in muscle progenitor cell function with aging is not well-studied. Because stem cells are usually quiescent in adults, energy requirements are low, and mitochondria are less abundant than in fully differentiated and metabolically more active cells. We have shown that the mitochondria mass is unaffected by $Gpx1^{-/-}$ deletion in young mice, in contrast to the significant change observed in both Sod2Tg and $Sod2^{+/-}$ in young myoblasts. These data suggest that the distinct catalytic function of SOD2 and Gpx1 or the subcellular localization of enzymes might be a crucial factor to regulate the mitochondrial mass in myoblasts. With aging, only Sod2Tg myoblasts preserved mitochondrial mass compared, in constrast to the significant decrease observed in wild-type and $Sod2^{+/-}$ myoblasts. (We did not have access to Gpx1-deleted, aged animals.) The expression of PGC1α, which is known to be sufficient for mitochondrial biogenesis, was also regulated by the overexpression or haplo-deficiency of SOD2. In contrast to the down-regulation of PGC1α in whole muscle with aging (Chabi et al., 2008), there was no significant change of PGC1α

expression in wild-type myoblasts during aging. In both Sod2Tg and $Sod2^{+/-}$, PGC1α expression was up-regulated with aging suggesting that PGC1α expression with aging at the whole muscle level is distinct from the regulation in the myoblast compartment.

**Maintenance of muscle mass *in vivo***

Muscle mass or single muscle fiber size is used for determining the effect of the oxidative stress (or other stresses) on muscle maintenance. Despite the fusion defect observed in $Gpx1^{-/-}$ myoblasts, there was no significant difference of total muscle weight in $Gpx1^{-/-}$ mice compared to wild-type, but these were young animals, and the defect may impose an effect on muscle over time. Originally, we anticipated that Sod2 overexpression would preserve muscle mass with aging. However, despite the preservation of mitochondrial mass and activity in Sod2Tg myoblasts with aging, this effect did not translate into improvement of muscle mass maintenance with aging. The major insights of this work as a whole: The overexpression of Sod2 did not yield a readout that was opposite to that of underexpression of Sod2. These results are understandable since the ROS generated by these manipulations are not just quantitatively different, but are qualitatively different as well.

Another major conclusion from our work is that overexpression of a single powerful antioxidant did not reverse many of the complex decrements in function associated with cellular aging. These results, though perhaps disappointing, are

not surprising given the large numbers of clinical studies in which single antioxidant supplementation did not reverse various features of senescence.

These data broadly suggest that many aspects of the mitochondria are altered due to oxidative stress, and may directly impact differentiation. Future directions including high throughput approach to profile the expression of protein and mRNA with mtDNA will be very informative about the role of mitochondria during muscle differentiation and regeneration. In addition, translational approaches to understand the role of mitochondria in muscle differentiation are required. For example, transplantation of myoblasts with enhanced functional mitochondria into mice models of muscle may be an interesting approach.

**Therapeutic implications**

The data presented in dissertation contribute to understanding the mechanisms of cell responses to altered redox status as well as detrimental effects of ROS in muscle differentiation during aging. This work has some potential therapeutic implications for maintaining regenerative potential of muscle progenitor cells with aging. Muscle loss with aging is traditionally thought to results in part from the decreased numbers and regenerative potential of satellite cells (Yablonka-Reuveni et al., 2006). The connection to oxidant stress is not direct and these losses are loosely attributed to oxidant damage, hormonal changes, and reduced motility associated with aging.

Importantly, our data suggest that Sod2 overexpression preserves the relative abundance of mitochondria, mitochondrial enzyme activity and the core

regulator of mitochondrial biogenesis in myoblasts with aging. And though these protective effects of Sod2 overexpression did not prevent muscle loss with aging, regenerative potential of muscle progenitor cells with aging was relatively preserved. Thus, overall the benefit of Sod2 overexpression may be considerable, if regeneration is improved after environmental stresses with aging. Chemical mimics of anti-oxidant enzymes have been developed and tested *in vitro* (Limoli et al., 2006; Day, 2008) and in pre-clinical models of aging associated diseases such as arthritis (Salvemini et al., 2001). Our work suggests that clinical application of Sod2 mimetics will require a careful assessment of the balance between reduction of stem cell proliferative potential vs. differentiation potential.

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