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# Protection of Mouse Embryonic Stem Cells from Oxidative Stress by Methionine Sulfoxide Reductases

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## 1. Introduction

Methionine sulfoxide reductases (Msr) belong to a family of enzymes that includes one MsrA and three MsrBs (MsrB1, MsrB2 and MsrB3) [Zhang et al., 2010, 2011]. We have identified all four enzymes expressed in mouse embryonic stem cell cultures. In addition, we have found a truncated form of MsrA transcript that could have easier access to oxidize methionine residues on proteins than the longer form of the MsrA protein, thus possibly providing an evolutionary selective advantage [Jia et al., 2011].

In this chapter, we will review and summarize the findings from our recent studies. We have stated previously [Zhang et al., 2010, 2011] that the vital cellular functions of the Msr

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family of enzymes are to protect cells from oxidative damage by enzymatically reducing the oxidized sulfide groups of methionine residues in proteins from the sulfoxide form (-SO) back to sulfide thus restoring normal protein functions as well as reducing intracellular reactive oxygen species (ROS). Studies have been performed on the Msr family genes to examine the regulation of gene expression. Using real-time PCR, we have shown that expression levels of the four Msr family genes are under differential regulation by anoxia/reoxygenation treatment, acidic culture conditions and interactions between MsrA and MsrB. Results from these *in vitro* experiments suggest that although these genes function as a whole in oxidative stress protection, each of the Msr genes could be responsive to environmental stimuli differently at the tissue level [Zhang et al., 2011]. We have further shown that one member of the Msr gene family, methionine sulfoxide reductase A (MsrA) can reduce methionine sulfoxide residues in proteins formed by oxidation of methionine by reactive oxygen species (ROS). Msr is an important protein repair system that can also function to scavenge ROS. Our studies have confirmed the expression of MsrA in mouse embryonic stem cells (ESCs) in culture conditions [Zhang et al., 2010]. A cytosol-located and mitochondria-enriched expression pattern has been observed in these cells. To confirm the protective function of MsrA in ESCs against oxidative stress, a siRNA approach was used to knockdown MsrA expression in ESCs. MsrA siRNA treated cells showed less resistance than control ESCs to hydrogen peroxide treatment. Overexpression of MsrA gene products in ES cells showed improved survivability to hydrogen peroxide treatment. This indicates that MsrA plays an important role in cellular defenses against oxidative stress in ESCs. Thus, Msr genes may provide a new target in stem cells to increase their survivability during therapeutic applications [Zhang et al., 2010].

We confirmed that oxidative stress was induced by exposing cells for increasing time periods to anoxia and reoxygenation in a hypoxia chamber [Zhang et al., 2010, 2011]. The expression of MsrA mRNA and protein expression decreased after 4 hours of oxygen depletion. Localization of MsrA proteins in the cytosol and mitochondria of mouse ESCs was evaluated by confocal microscopy and showed a differential distribution with more concentrated levels in the mitochondria. In further studies, knockdown of MsrA expression in mouse embryonic stem cells using siRNAs reduced resistance of the cells to H<sub>2</sub>O<sub>2</sub> mediated oxidative stress. In these studies we also found that overexpression of an MsrA-eGFP fusion protein in mouse ESCs provided additional protection against H<sub>2</sub>O<sub>2</sub> induced oxidative stress [Zhang et al., 2011].

Based on our studies, we believe that by overexpressing MsrA in stem cells, we can provide significant protection against harsh environments such as ischemia/reperfusion, for example during stroke or heart attack. Currently new approaches to treat disease, such as neurodegenerative disease or ischemia/reperfusion-induced brain or heart damage, are being developed using adult or embryonic stem cell transplantation. One severe limitation in using these stem cells is the low survival of the cells after surgery partly due to high levels of oxidative stress. Results of our recent studies [Zhang et al., 2010, 2011; Jia et al., 2011] suggest a very promising approach to solving this problem by enhancing the resistance of stem cells to oxidative damage after transplantation into a hostile environment caused by ischemia-reperfusion. It will be important to further investigate the differentiation capability of stem cells overexpressing MsrA. Due to the antioxidant actions of these

proteins and their reparative properties in recovering target protein function and indirect control of cellular reactive oxygen species (ROS) levels, we believe that overexpressing Msr genes in stem cells could reduce ROS damage to the cells without losing the cells' sensitivity to ROS as a differentiation signal. Thus, the Msr gene family could potentially serve a key role in engineering stem cells to obtain higher resistance to oxidative damage, while retaining the cells' potential for differentiation into adult cell types [Zhang et al., 2010].

## 2. Background and overview of oxidative stress

ROS are chemically-reactive molecules containing molecular oxygen. They are free radicals, containing an unpaired shell electron: singlet oxygen, superoxide radical, superoxide anion, hydrogen peroxide and hypochlorite ion. ROS are formed by an incomplete one-electron reduction of oxygen. The other non-oxygen chemical radicals are also highly chemically reactive, however, ROS are most abundant in biochemical reactions. They are capable of damaging all bio-molecules: lipids, proteins and nucleic acids.

There are several cellular organelles that generate ROS. Mitochondria are the energy powerhouses of cells producing the major biological energy carrier ATP. In mitochondria, certain enzymes of the cycle transfer electrons from the substrates to carriers such as NAD(P) and FAD which bring electrons to the electron transport (or respiratory) chain. The electron transport chain is generally described as four protein complexes that include NADH oxidase, succinic dehydrogenase, cytochrome c reductase and cytochrome c oxidase; ATP synthase is sometimes referred to as a fifth complex. A small percentage of electrons leak from complex I or III to oxygen resulting in the formation of superoxide anion radicals instead of water. Superoxide anion radicals may spontaneously, or in the presence of metals, turn into hydrogen peroxide and further into a hydroxyl radical: the most chemically active reactive oxygen species.

There are defensive enzymes that are capable of catalyzing chemical reactions of these reactive species to less harmful molecules. Protein oxidation potentially compromises many protein functions, including inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity which then interrupts normal cell functions and induces significant biological damage. Cells have multiple antioxidant systems to scavenge free radicals, including superoxide dismutases (SODs), peroxidases, and catalases. SODs catalyze the dismutation of superoxide anion radicals into molecular oxygen and the less toxic hydrogen peroxide. Peroxidases are a group of enzymes that catalyze the conversion of multiple peroxides (including hydrogen peroxide) into hydroxides. Catalase is an enzyme responsible for conversion of hydrogen peroxide to water and oxygen. The other enzymes, including methionine sulfoxide reductases, reduce oxidized protein molecules.

Methionine sulfoxide reductase is an important enzyme which catalyzes the reduction of free and protein-bound methionine sulfoxide to methionine (Fig. 1). Depending on the nature of the oxidizing species, methionine may undergo a two-electron oxidation to methionine sulfoxide or a one-electron oxidation to the methionine radical cation. This reduction is a repair mechanism for oxidatively damaged proteins. There are two enzymes responsible for reduction of oxidized methionine in proteins: Methionine sulfoxide reductase (Msr) A and B. Most organisms, from bacteria to humans possess MsrA and MsrB

genes. The MsrA and MsrB genes exhibit no sequence similarity. MsrA and MsrB genes in several bacterial species are clustered as operons or are fused with each other via connecting domains [Lowther et al., 2002]. In eukaryotes, the MsrA and MsrB genes are typically single.

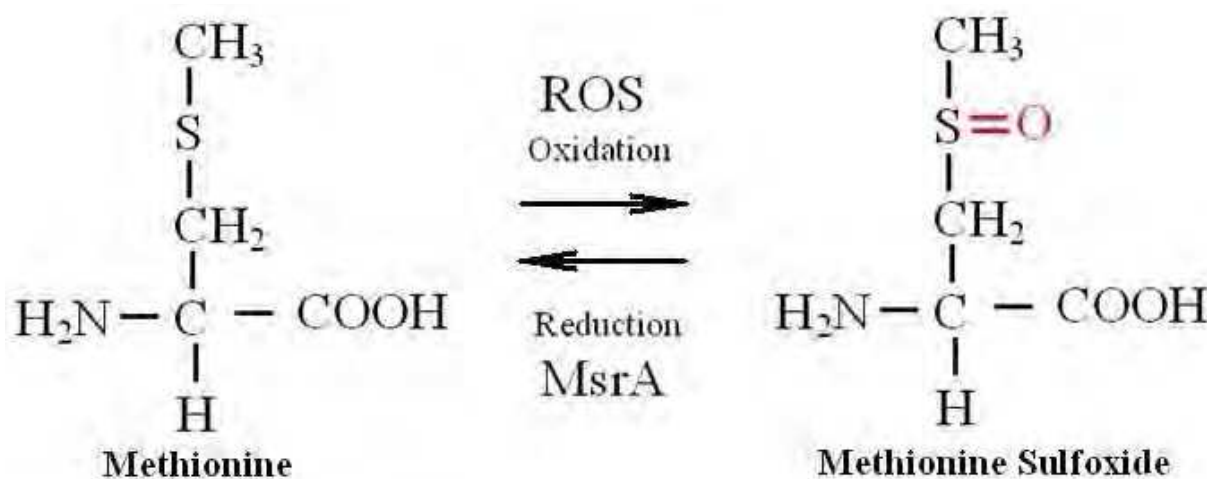


Fig. 1. Methionine Sulfoxide Reductase reduces oxidized methionine.

In mammals, three MsrB genes (MsrB1, B2 and B3) and one MsrA gene have been identified. MsrB1 is distributed in the cytosol and nucleus of a cell while MsrB2 is localized in mitochondria [Kim and Gladyshev, 2004]. In humans there are four alternatively spliced isoforms of MsrB3 [Ahmed et al, 2011]. MsrB3 is targeted to the endoplasmic reticulum (ER) and mitochondria [Kim and Gladyshev, 2004].

Although only a single MsrA gene is found in mammals, the corresponding protein is localized in multiple cellular compartments [Kim and Gladyshev, 2005]. Studies on human MsrA gene structures have identified two distinct putative promoters that generate three transcripts. The main MsrA transcript (MsrA1, GenBank: mouse, NM026322; human, NM012331) has been translated into the longest protein. The long MsrA transcript consists of six exons separated by introns (Fig. 2). In the first exon, MsrA has a mitochondrion localization signal peptide (Fig. 3) that targets it to mitochondria [Kim & Gladyshev, 2005]. There is an alternative splicing form of MsrA (S) with short exon 1 missing a mitochondrion localization signal peptide (Fig. 2 and 3) (GenBank: mouse, AK018338; human, AY690665). MsrA (S) is targeted to the cytosol and nucleus. Expression of the MsrA (S) was mostly found in the brain, especially in an early developmental stage.

Alternative forms without exon 3 were identified (Figure 2, GenBank: mouse, BC014738; human, CK819754) which do not have enzymatic activity. MsrA shortform (-3) is present in the cytosol and nucleus. An additional alternative form has also been detected, in which exon 6, the last exon, is replaced with a segment of an unknown gene [GenBank: CD 365491]. This form shared the first exon with the mitochondrial MsrA form, but it is probably catalytically inactive due to an absence of the last exon which contains two cysteine residues critical for catalysis [Kim & Gladyshev, 2005].

Deletion of the fifth exon (113bp) was found in the smaller MsrA form that generates a frame shift in the sixth exon directly attached to the fourth exon thereby forming a premature stop codon (Figure 2). A c-terminal truncated form of MsrA also has been cloned



from mouse ESCs due to the skipping of exon 5 and subsequent frame shift in exon 6, also generating a premature stop codon (Figure 2). The total length of the truncated form protein is 148 amino acids, compared to the full length long form protein that has 233 amino acids; both contain a mitochondrial signal peptide at the N-terminus. The truncated form still retains the GCFWG functional motif (catalytic active site) but contains neither of the two cysteines at the c-terminus. The truncated protein shows a different subcellular localization and altered response to anoxia/reoxygenation. These different methionine sulfoxide reductases exhibit different substrate specificities. They may target different methionine residues depending on the protein sequence. Due to the functional importance of the two c-terminal cysteines in the redox reaction, it is likely that the enzyme activity of the variants will be dramatically decreased.

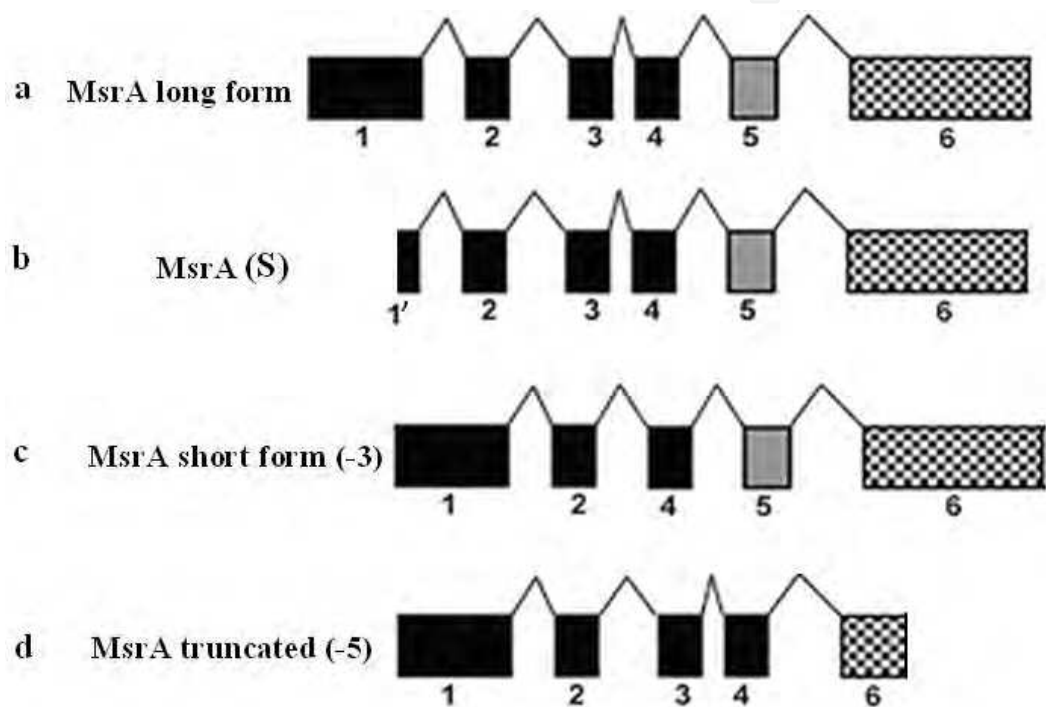


Fig. 2. Alternative splicing isoforms of MsrA gene. a. The long form of MsrA has six exons. b. The one short form MsrA (S) has a short exon 1. c. The short form lacks exon 3. d. The truncated form lacks exon 5, and exon 6 is truncated. (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

Mouse MsrA	<div><i>MLSASRRALQLLS</i> -- <i>SANPVRR</i></div> MGDSASKVISAEELPGRTEPIPVTA <sup>↓</sup> KHHVSGNRTVEP 58
Mouse MsrA (S)	-----MSKA <sup>↓</sup> KHHVSGNRTVEP 16
Human MsrA	<div><i>MLSATRRACQLLLHSLFPVPR</i></div> MGNSASNIVSPQEALPGRKEQTPVAA <sup>↓</sup> KHHVNGNRTVEP 60
Human MsrA (S)	-----MCSE <sup>↓</sup> PKHHVNGNRTVEP 17

Fig. 3. Variants of mouse and human MsrAs. The mitochondrial signal peptide is indicated in italic letters in the box. The arrows show the last amino acid residue of exon 1.

### 3. Methionine sulfoxide reductase

#### 3.1 Protection of embryonic stem cells

As described earlier, methionine sulfoxide reductase A (MsrA), a member of the Msr gene family, can reduce methionine sulfoxide residues in proteins formed by oxidation of methionine by reactive oxygen species (ROS). Msr can also function to scavenge ROS [Levine et al., 1996]. We have confirmed the expression of MsrA in cultured mouse ESCs with a mitochondria-enriched expression pattern [Zhang et al., 2010].

It is well known that oxidative stress plays a key role in cellular injury of patients suffering acute ischemia/reperfusion of multiple tissues including acute myocardial infarction (AMI, heart attack) or chronic insufficient blood perfusion (ischemia) caused by atherosclerosis or vascular stenosis [Zhang et al., 2010]. During oxidative stress, excess reactive oxygen species (ROS) induce damage to proteins, lipids, and nucleic acids that can lead to cell death [Honig & Rosenberg, 2000; Boldyrev et al., 2004; Onyango et al., 2005]. Stem cell transplantation and subsequent differentiation into mature tissues may be able to repair cells and tissues lost to oxidative stress. Cells have multiple anti-oxidation mechanisms to protect against the oxidative insults induced by ischemia/reperfusion [Boldyrev et al., 2004]. Enzymes including superoxide dismutase, catalase, and glutathione peroxidase scavenge the superoxide anion and  $H_2O_2$  to prevent ROS-induced damage. Cells that are modified to over-express such genes are expected to be more resistant to oxidative stress [Blass, 2001]. However, if the levels of ROS become too low, this could have a deleterious effect because ROS are required as signaling molecules to promote differentiation of ESCs, for example into cells of the cardiovascular system [Boldyrev et al., 2004; Sauer et al., 2000; Thiruchelvam et al., 2005; Wo et al., 2008]. Thus, reducing ROS inside cells by using direct anti-oxidants such as vitamin E could interfere with normal cell function. Clearly, this also could interfere with stem cell therapy since the ROS level must be balanced so that cells can undergo normal differentiation without resulting in damage or cell death. Thus, if indeed this hypothesis is correct, then an indirect mechanism to reduce ROS involving the Msr pathways would be advantageous for stem cell therapy by retaining normal ROS basal levels.

ESCs, with their remarkable property of totipotency, have the potential to produce new adult cells to replace those in damaged tissues. Tissue damage and cell death can be caused by reperfusion and ischemia from an accumulation of free radicals in the cells which could result in oxidation and functional impairment directly or through signal transduction pathways such as c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) [Ueda et al., 2002]. In patients suffering from myocardial infarct or stroke, the infarcted organ is stressed from acidosis due to lack of an adequate blood supply resulting in hypoxia. Kubasiak et al. [2002] have proposed that hypoxia and acidosis are the two conditions that can dramatically induce cell death in cardiomyocytes when present simultaneously. Improvement in the survival of transplanted stem cells in harsh hypoxic and acidic environments is a critical issue for the success of therapeutic tissue repair. Stem cells under conditions of oxidative stress and in acidic environments are an area that requires further study.

The Msr system [Weissbach et al., 2002] is an important self-defense mechanism that protects cells against free radical damage. Proteins, lipids, nucleic acids and other cellular

components can be oxidized by free radicals leading to impaired cellular function and/or cell death. Methionine (met), either as a free amino acid molecule or as a peptide, can be oxidized to methionine sulfoxide (Met-(O)). This kind of structural change can cause impaired function of a variety of proteins [Abrams et al., 1981; Taggart et al., 2000; Jones et al., 2008; Shao et al., 2008]. There is a family of enzymes, encoded by methionine sulfoxide reductases (Msr) genes that can reduce Met-(O) to Met. MsrA and MsrB genes have been identified that are specific for reducing the epimers, Met-(O)-S and Met-(O)-R [Weissbach et al., 2002]. Both MsrA and MsrBs have been implicated in the protection of cells against oxidative damage that in turn have been attributed to MsrA and MsrB genes, suggesting that they may be involved in various age related diseases [Gabbita et al., 1999; Pal et al., 2007; Brennan & Kantorow, 2008; Kim & Gladyshev, 2005].

With respect to protective mechanisms against oxidative damage, it will be important to determine whether the local environment conditions within damaged tissues modulate Msr expression in adjacent stem cells. Cross talk between family members is not well understood and it is not clear whether altered expression levels of one enzyme in the Msr family affects other(s), whether there might exist a co-regulation between different Msr genes; or what controls Msr gene expression. In recent studies, we have described how individual Msr genes respond to varying regulatory processes [Zhang et al., 2010, 2011; Jia et al., 2011]. An acidic culture environment and depletion of oxygen affect Msr gene expression, at the mRNA level; the most significant response was observed in MsrB3, indicating a non-housekeeping activity for this particular gene [Zhang et al., 2011]. Interestingly, MsrB3 showed downregulation of transcription concomitant with MsrA mRNA knockdown by MsrA-specific siRNA [Zhang et al., 2011].

Free radical damage to cellular components, including proteins, is commonly observed under disease conditions and in aging processes. One of the cellular defense mechanisms to reduce oxidation of proteins, thus reducing oxidative stress and restoring their functions, relies on the methionine sulfoxide reductase (msr) family of genes in mammals. Although both MsrA and MsrB genes conduct the redox reactions using a similar chemical reaction, MsrBs convert the R epimer of Met-(O) (Met-R-(O)) back to methionine while MsrA reduces the S epimer of Met-(O) (Met-S-(O)) [Kryukov et al., 2002; Kim & Gladyshev, 2005]. MsrBs localize in different cellular compartments; the MsrB1 protein is cytosolic and nuclear while the MsrB2 is localized in the mitochondria. Human MsrB3, by alternative first exon splicing, produces two forms which targets the endoplasmic reticulum (ER) and the mitochondria of the cell [Kim & Gladyshev, 2004, 2005, 2006].

Even though there is only a single MsrA gene in mammals, its corresponding protein is found in multiple cellular compartments [Vougier et al., 2003]. It turns out that there are two distinct putative promoters of the MsrA gene that generate three transcripts. MsrA1 transcript forms the longest protein that targets the mitochondria. MsrA2 and MsrB3 are formed from a second promoter and localize in the cytosol and nuclei [Lee et al., 2006; Pascual et al., 2009]. Two novel splice forms: MsrA2a and MsrA2b were found recently in rat smooth muscle cells [Haenold et al., 2007]. Alternative splicing occurred in the second exon with the MsrA2, a functional isoform. Thus, alternative promoters and alternative splicing both contribute to the variety of MsrA isoforms that are responsible for methionine sulfoxide reduction in the different cellular compartments.



Currently, most studies on MsrA isoforms emphasize the 5' terminus where different isoforms for mitochondrial signal peptide are present and dictates where protein products are localized within the mitochondria [Kim & Gladyshev, 2006; Lee et al., 2006; Haenold et al., 2007]. There is evidence that transcripts of MsrA from alternative splicings at the 3' end of the MsrA gene are present in the mammalian EST database, however, no detailed studies on these transcripts have been reported [Kim & Gladyshev, 2006].

In our most recent studies in cultured mouse embryonic stem cells, we discovered an MsrA transcript from alternative splicing at the 3' end, that skips exon 5, and produces a shortened isoform with a truncated protein product containing the conserved catalytic active site [Jia et al., 2011]. We performed studies on this truncated isoform's expression pattern under normal culture conditions and in response to oxygen depletion/reoxygenation conditions in mouse embryonic stem cells. The MsrA/B system does not function to eliminate ROS directly, but rather to reverse its damaging effects in the cells. Moreover, methionine is oxidized by ROS in proteins, causing the formation of the R (Met-R-O) and S (Met-S-O) epimers of methionine sulfoxide (met-O) and potential diminution of protein function. MsrA and MsrB are able to reduce protein bound Met-S-O and Met-R-O, respectively, and restore their function. MsrA has been shown to reduce Met-S-O in several oxidized proteins. Scavenging ROS by the Msr system may result from methionine residues in proteins acting as catalytic anti-oxidants. Strong support for a scavenger role of the Msr system is derived from our recent studies on the overexpression of the Msr system, or knocking out the Msr system in cultured cells [Zhang et al., 2010, 2011]. Overexpression of MsrA using adenovirus delivery significantly lowers the hypoxia-induced increase in ROS ordinarily induced by hypoxia and promotes cell survival of PC12 cells in culture [Yermolaieva et al., 2004]. The siRNA knockdown of MsrA and MsrBs in human lens cells also reveals that the MsrA/B system has a protective influence on hydrogen peroxide-induced cellular injury [Kantorow et al., 2004; Yermolaieva et al., 2004].

MsrB is the only Msr family gene whose protein can reduce the R form of methionine sulfoxide unlike the MsrA that is able to reduce the S form of methionine sulfoxide [Kim & Gladyshev, 2004, 2005]. In our studies using siRNA to knockdown MsrA expression and MsrA-GFP fusion protein overexpression, we demonstrated that MsrA plays an important role in protecting stem cells against oxidative damage which has important implications in stem cell therapy.

### **3.2 MsrA expression patterns in mouse ESCs under differing levels of oxidative stress**

We have confirmed MsrA gene expression in mouse embryonic stem cells and have performed studies on MsrA gene expression during hypoxia/reoxygenation using real-time RT-PCR for mRNA transcription, as well as Western blotting with anti-MsrA antibody for MsrA protein expression. This work was published originally in detail in our recent paper [Zhang et al., 2010] and will be summarized below.

Using real-time RT-PCR, mRNA transcription level regulation of the MsrA gene under different levels of oxidative stress was analyzed. Oxidative stress levels were induced by exposing cells to increased time periods of anoxia (in 90% N<sub>2</sub>, 5% H<sub>2</sub> and 5% CO<sub>2</sub>) and reoxygenation combinations in a hypoxia chamber. It was found that the MsrA mRNA

levels decreased with increased exposure to hypoxic and reoxygenation treatments. After 4 hours of oxygen depletion, decreases of MsrA mRNA could be observed; however, the original expression level returned by 8 hours of reoxygenation. One explanation for this observation is that it was a transient, instant response of stem cells to oxygen depletion, since mRNA transcription reductions were apparent for MsrA, MsrBs and MMP9 (Matrix Metalloproteinase-9) genes.

Mouse embryonic stem cell protein samples were collected after the above treatments. Proteins from each sample were loaded in equal amounts onto 4-12% gradient NuPAGE polyacrylamide gels (Invitrogen, Carlsbad, CA). Anti-MsrA antibodies (Abcam, Cambridge, MA) were used in Western blot analysis that showed a gradual decrease of the MsrA protein, with extending treatments of anoxia/reoxygenation, consistent with the mRNA level results.

### 3.3 Laser confocal microscopic analysis of MsrA protein localization in mouse ESCs

A MsrA-eGFP fusion plasmid was constructed in the pEGFP-N1 vector (Clontech, CA) and transfected into the plasmids into ES cells. 48 to 72 hours after transfection, the ES cells were observed in order to study the subcellular localizations of MsrA in the expression of MsrA-eGFP fusion proteins [Zhang et al., 2010]. Cells then were incubated with MitoTracker (Molecular Probes, WI) for mitochondrial staining before fixation and later mounted in DAPI to stain the nuclei. Confocal microscopy demonstrated a strong colocalization of the green fluorescence signals (MsrA-eGFP) with red MitoTracker signals (mitochondria). Also observed was a general cytosol distribution of MsrA-eGFP outside of the nuclei and what appeared to be between mitochondria. Cytosolic localization with concentrated mitochondrial localization of MsrA-eGFP was not seen in stem cells transfected with the pEGFP-N1 vector control. GFP (eGFP) expression in stem cells showed signals in both nuclei and cytosolic domains suggesting nonspecificity (Figure 4).

### 3.4 Knock down of MsrA expression using siRNAs reduced resistance of mouse embryonic stem cells to H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress

To determine whether MsrA can protect stem cells from oxidative stress, we designed and commercially synthesized three siRNAs for MsrA knockdown experiments (Invitrogen, CA). A negative control siRNA and a FITC-labeled siRNA also were synthesized. Using FITC-labeled siRNA, we optimized transfection conditions so that nearly 100% of the stem cells were transfected with siRNA [Zhang et al., 2010].

#### 3.4.1 Real-time RT-PCR confirms knock down of the MsrA mRNA

siRNA transfection in stem cells was performed and total RNA collected at one, two and three days after transfection. The siRNA designed for the MsrA gene (168) and a negative control siRNA, with randomized sequence (882c), were transfected into the stem cells using identical methods. Real-time RT-PCR experiments were conducted with MsrA specific primers designed against the c-terminal end coding sequence, in order to avoid the alternative spliced 5' end transcripts [Kim & Gladyshev, 2006]. Comparing MsrA mRNA levels after MsrA specific siRNA transfections with the negative control siRNA, we found that one siRNA, from the three designed in our laboratory reduced the mRNA level of the MsrA in the stem cells to only 20% of the control cells (Figure 5A). The reduced levels of MsrA mRNA were maintained for at least 72 hours post-transfection [Zhang et al., 2010].

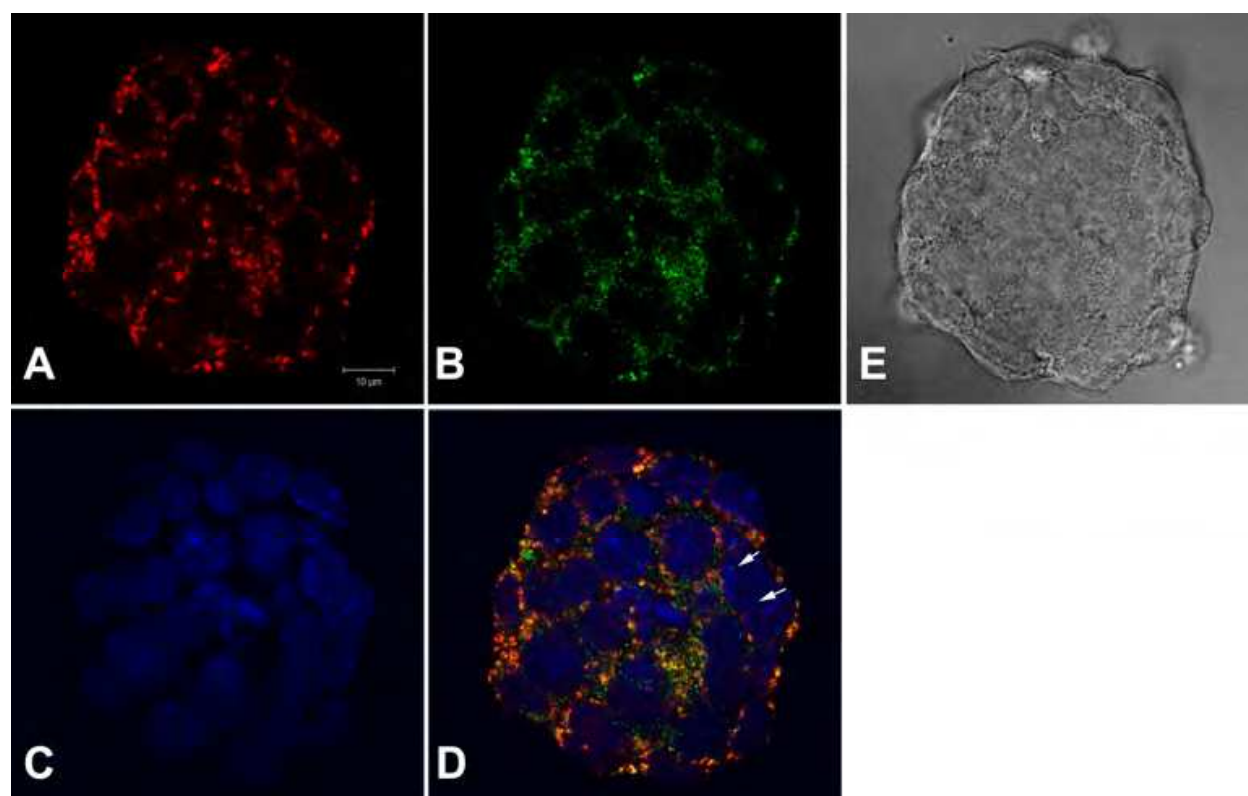


Fig. 4. Confocal microscopy of the subcellular localizations of MsrA-eGFP fusion protein in mouse embryonic stem cells after pEGFP-N1-MsrA fusion expression plasmid transfection for 3 days. A: Stem cells scanned for Mitotracker staining for mitochondria. B: Green fluorescence shows the MsrA-eGFP fusion protein. C: DAPI staining shows nuclei. D: Merged image of A–C showing that MsrA-eGFP localizes in both mitochondria and cytosol, occasionally in nuclei (arrows). E: Phase contrast image of a colony of stem cells. Scalebar is 10  $\mu$ m. (Reprinted with permission of the authors from Zhang et al., 2010 J. Cell. Biochem. 111(1):94-103).

### 3.4.2 Western blots confirm knock down of the MsrA proteins

Protein samples from stem cells were collected post-siRNA transfection at 24 hours, 48 hours and 72 hours. Western blotting experiments were conducted using anti-MsrA antibody which binds to a 24kD MsrA protein band (Figure 5B). Western membranes were stripped and rehybridized with  $\beta$ -actin antibody to normalize the MsrA concentrations in each sample (Figure 5D). From the Western blotting data as well as the densitometry of blots (Figure 5E) scanned by a gel imaging system (Alpha Innotech Corp., San Leandro, CA), we unequivocally demonstrate a significant reduction of MsrA protein 24-hours after transfection of MsrA-specific siRNA in the stem cells as compared to the negative control siRNA. It is notable that the MsrA protein levels quickly return to normal levels on the second day after transfection (Figure 5B and 5E), in spite of the mRNA levels still remaining low (see Figure 5A). We also detected a faint band corresponding to MsrA protein dimers (~50kD) from Western blotting after the protein samples were run in reducing polyacrylamide gels (Figure 5B). To verify the existence of the dimer form of the MsrA protein in mouse embryonic stem cells, we repeated the same Western blotting protocol but

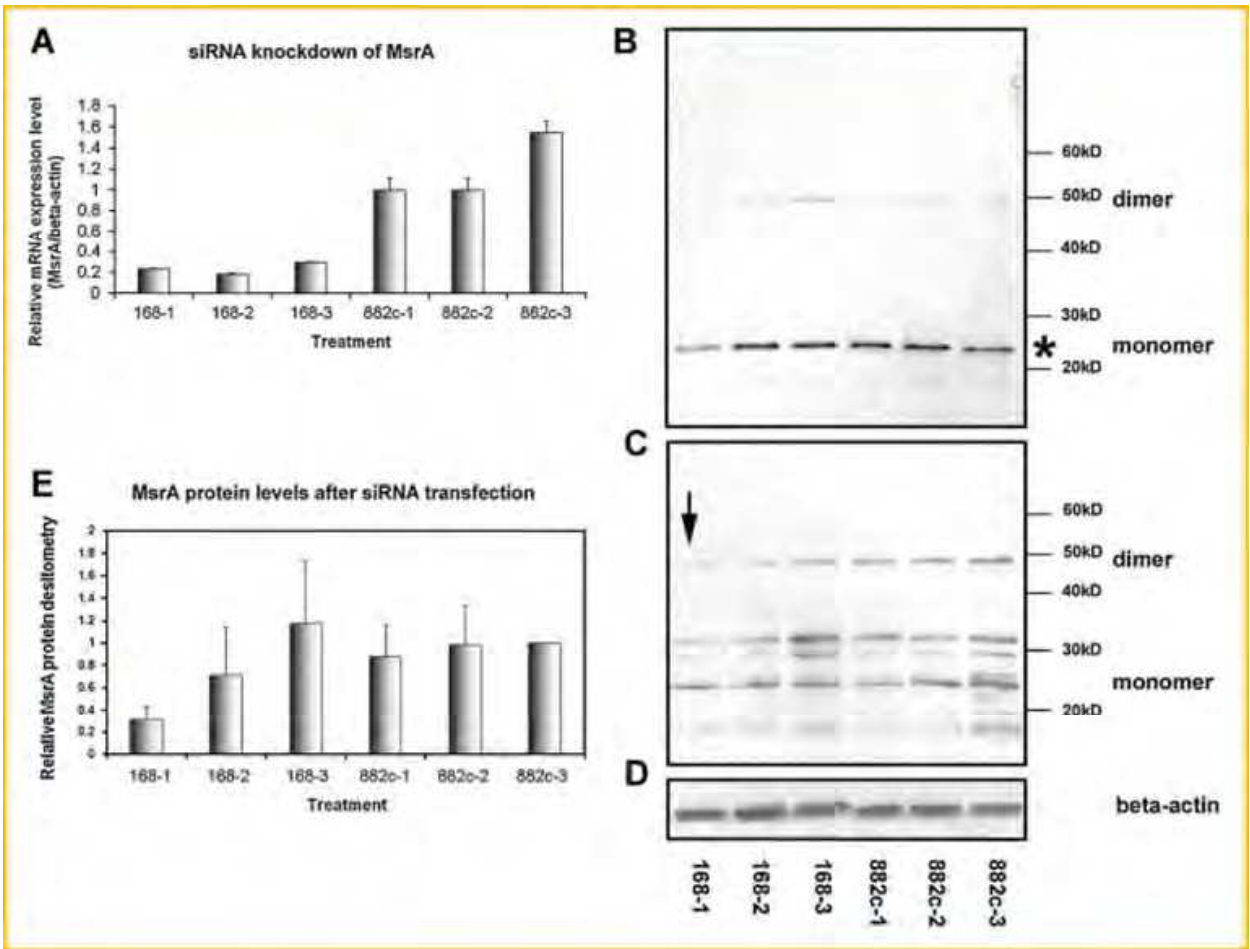


Fig. 5. A: Confirmation of the knockdown of MsrA mRNA by MsrA-specific siRNA transfection using real-time RT-PCR. MsrA-specific siRNA (168) successfully reduces MsrA mRNA levels to only ~20% compared to the negative control siRNA (882c) transfected cells 1 day post-transfectionally. mRNA levels remain low even 3 days after transfection. 168-1 stands for MsrA-specific siRNA (168) transfection for 1 day. B: Confirmation of the knockdown of MsrA protein by MsrA-specific siRNA transfection using Western blot analysis. Western blotting was done with protein samples from stem cells transfected with MsrA-specific siRNA (168) or negative control siRNA (882c) for various days (days 1, 2, and 3) in a reducing PAGE gel. C: Western blot after running the same protein samples as in panel B in a non-reducing PAGE gel. Arrow points indicate significantly reduced MsrA dimers after 1 day transfection of MsrA-specific siRNA (168). D: The same blot as used in B was reprobed for  $\beta$ -actin protein showing equal loading of protein samples in each lane. E: Densitometry studies after scanning the monomer bands shown in panel B (shown with an asterisk) a confirmed significantly decreased expression level of MsrA after 1 day transfection of MsrA-specific siRNA compared to the negative control (168-1 vs. 882c-1). Data were pooled from four separate experiments. 168-1 and 882c-1 stand for transfection of MsrA-specific siRNA (168) or a negative control siRNA (882c) for 1 day. (Reprinted with permission of the authors from Zhang et al., 2010 J. Cell. Biochem. 111(1):94-103).

used a non-reducing PAGE. It clearly shows that the dimer form is present in significant amounts in the stem cells (Figure 5C). These data suggest that it is the dimer form, instead of



monomer form, that is reduced after siRNA knockdown of the MsrA mRNA (arrow, Figure 5C) [Zhang et al., 2010]. Densitometry in Figure 5E illustrates the mean values using data pooled from four separate experiments with reducing PAGE (i.e., from four blots as shown in Figure 5B). Only densitometry of the monomer forms (indicated by \* in Figure 5B) after reducing PAGE was performed and shown in Figure 5E.

### **3.4.3 Stem cells show a lowered resistance to H<sub>2</sub>O<sub>2</sub> treatments after MsrA siRNA transfection**

After examining the effects of siRNA in the MsrA knockdown experiments, we transfected the siRNA into mouse ES cells and treated the cells with hydrogen peroxide to evaluate their resistance to hydrogen peroxide after maximal MsrA protein downregulation after 24 hours. Hydrogen peroxide was diluted into the culture medium at differing concentrations and incubated with the cells overnight prior to MTT assays to compare cell numbers in each survival group. Results clearly showed a significantly reduced resistance of stem cells to hydrogen peroxide treatment with less cell survival after MsrA knock down (Figure 6). Importantly, differences, between MsrA-specific siRNA and the negative control siRNA transfected cells were most significant at the higher hydrogen peroxide concentrations used (i.e., between 100  $\mu$ M and 300  $\mu$ M). No differences were observed at lower concentrations when cells begin to die (i.e., between 25  $\mu$ M to 50  $\mu$ M) [Zhang et al., 2010].

## **4. Overexpression of MsrA-eGFP provides additional protection against hydrogen peroxide–induced oxidative stress to mouse embryonic stem cells**

We performed experiments in which MsrA-eGFP fusion constructs were transfected into cultured stem cells for two days [Zhang et al., 2010]. With the confirmation of expression of fusion proteins by the appearance of green fluorescence under a fluorescence microscope, hydrogen peroxide at a concentration of 75  $\mu$ M was added to the medium for 2 hours before the cells were incubated with propidium iodide to stain for non-living cells. At 75  $\mu$ M concentration, about 50% of the cells survived from previous serial dilution assays (Figure 6). In further studies (illustrations not included in this review – see Zhang et al., 2011), cells were trypsinized and flow cytometry was performed to compare the differences of the cell death ratios in cells with or without MsrA-eGFP fusion protein expression [Zhang et al., 2010]. Results from flow cytometry confirm the expression of the MsrA-eGFP fusion protein, when comparing the cell numbers of EGFP channels (excitation wavelength at 488 nm) between transfected cells and nontransfected cells [Zhang et al., 2010]. Based on cell flow cytometry, the cells were subdivided into four groups: 1. dead cells with no eGFP expression; 2. dead cells with eGFP or MsrA-GFP expression; 3. live cells with no eGFP expression; 4. dead cells with eGFP or MsrA-GFP expression. These experiments showed that of those cells expressing MsrA-GFP fusion protein only 8.67% were dead cells [Zhang et al., 2010]. However, in cells expressing eGFP only, 12.14% of the cells show propidium iodide positive staining (dead). Therefore MsrA-eGFP protected the cells from hydrogen peroxide-mediated oxidative damage, and thus, decreased cell death by 28.6% compared to the eGFP control there are no significant differences in cell death ratios among



nontransfected cells within DMEM medium (no DNA transfection), eGFP transfected cells and MsrA-eGFP transfected cells without hydrogen peroxide treatment [Zhang et al., 2010].

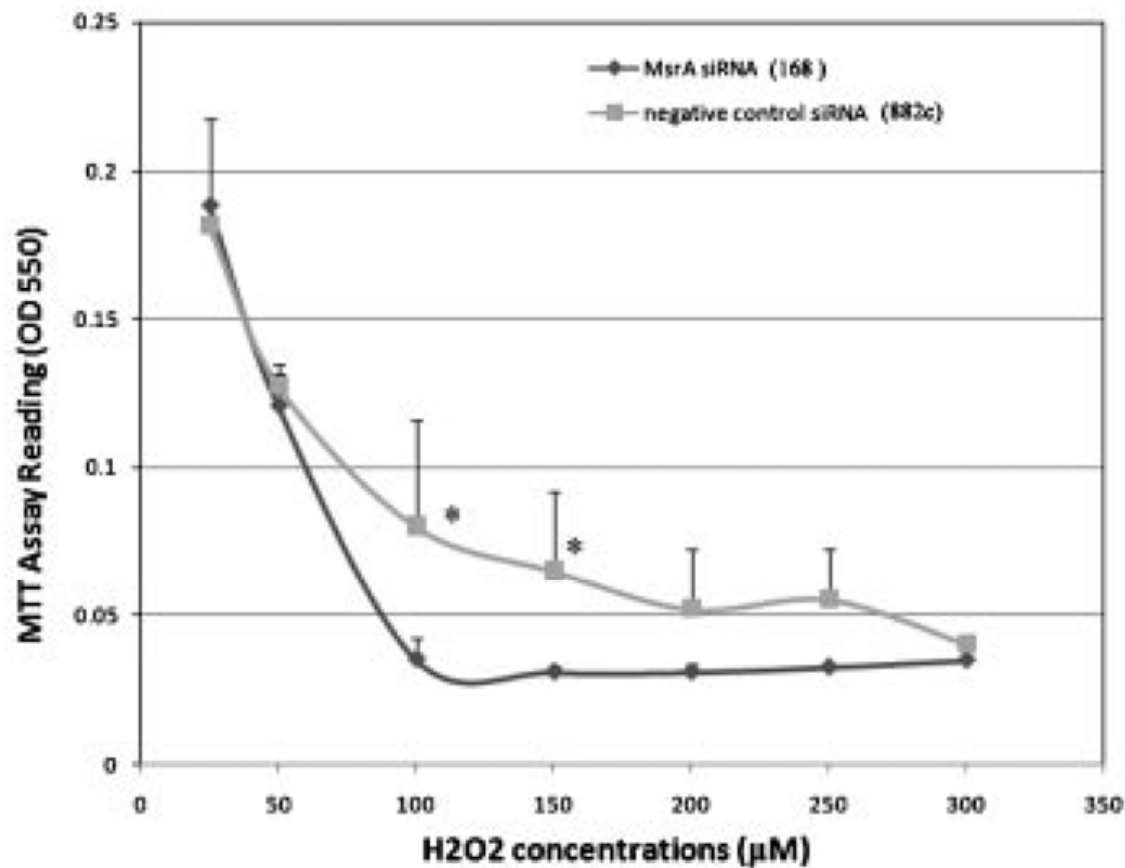


Fig. 6. MTT assays on stem cells treated by MsrA siRNA (168) versus a negative control siRNA (882c). After MsrA-specific siRNA (168) transfection for 24 h, the cells are significantly more prone to oxidative damage mediated by high concentrations of H<sub>2</sub>O<sub>2</sub> treatment compared to a negative control siRNA (882c) transfected cells. The asterisks indicate  $P < 0.05$ . (Reprinted with permission of the authors from Zhang et al., 2010 J. Cell. Biochem. 111(1):94-103).

**5. Confirmation of the maintained potency to differentiate into multiple adult cell types from mouse embryonic stem cells in feeder-layer-free culture conditions**

To avoid interference of feeder layer embryonic fibroblast cells in our earlier stem cell studies, we employed a feeder-layer-free culture system for our mouse embryonic stem cell culture [Narayanan et al., 1993]. Cells were maintained at a low passage number (less than 20) and cultured on gelatin-coated culture dishes with Leukemia Inhibitory Factors (LIF) added to the medium under conditions previously published [Zhang et al., 2011]. Cells using these conditions maintain their typical stem cell morphology, growing into round colonies with smooth edges (Figure 7A).

To confirm that cells used for subsequent studies would carry genuine stem cell characteristics, we routinely stained the cells for embryonic stem cell specific markers and

tested the capability of the cells to differentiate into various adult cell types at the same cell passages we used for the studies of Msr gene expression regulation [Zhang et al., 2011]. The cells under these culture conditions remain undifferentiated and positively stain for Stage-specific Embryonic Antigen-1 (SSEA-1) antibody (Abcam, MA) (Figure 7B). From immunostaining, we have determined that there are 90-95% SSEA-1 stainable cells in the population. After induction of embryoid bodies from the stem cell cultures and subsequent cell differentiation for 2-4 weeks [Muller et al., 2000], we have been able to stain differentiated cells by various cell lineage markers and confirmed multi-potency of the cells using our feeder-layer-free culture conditions [Zhang et al., 2011]. Capability of these cells to

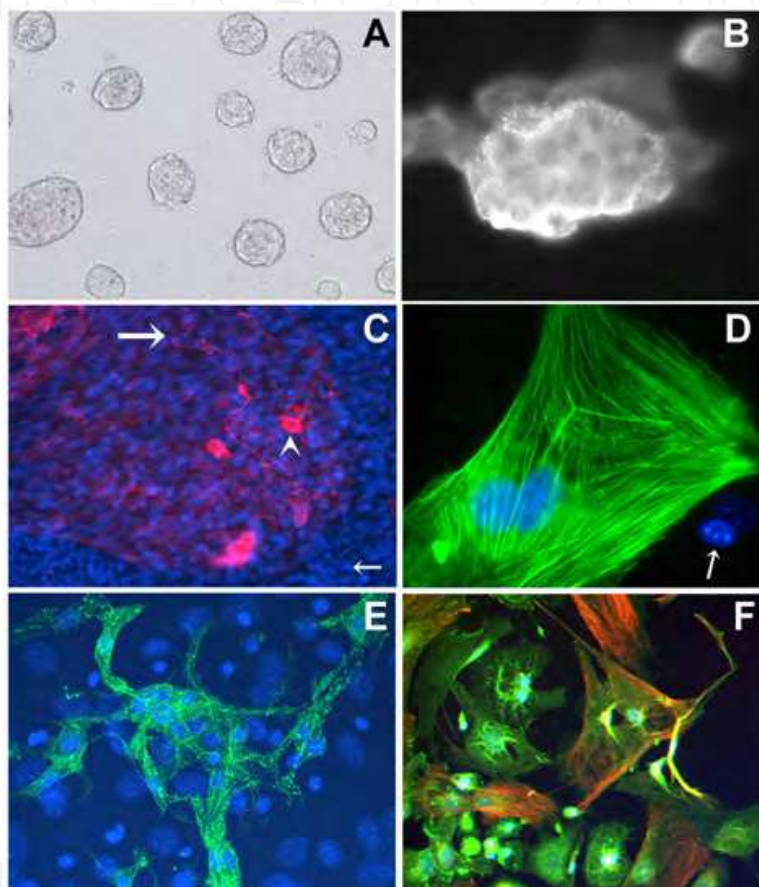


Fig. 7. Confirmation of stem cell identity and multipotent differentiation capability of the cultured mouse embryonic stem cells (MESC) in feeder-layer-free culture system. A: Phase-contrast image of cultured MESC without a feeder layer maintaining their typical round colonies with smooth edges indicating lack of differentiation. B: Positive staining with stem cell specific marker SSEA-1. C: Positive staining for the neuronal cell marker: neurofilament (NF). Antibodies to NF stain both cell bodies (arrowhead) and axons (large arrow). Nuclei are stained blue with DAPI (small arrow). D: Positive staining (green stain) for the cardiac cell marker: cardiac Troponin T (cTnT). Organized myofibrils are seen. E: Positive staining for the skeletal muscle cell marker, fast skeletal Troponin T (fsTnT) (green stain). DAPI, a blue nuclear fluorescent dye shows nuclear staining. F: Positive staining for both  $\alpha$ -actinin (green) and desmin (red), markers for mesoderm-derived cell types. Magnifications for Figure 1: (A) 100x; (B) 250x; (C) 100x; (D) 600x; (E) 250x; (F) 250x. (Reprinted with permission of the authors from Zhang et al., 2011 J. Cell Biochem. 112(1):98-106).

differentiate into neurons and cardiac muscle cells were of particular interest to us since strokes and heart attacks are most likely the two most prominent health problems resulting from ischemia. It is evident that both areas might benefit from advancing stem cell therapy. Differentiated cells stained with neurofilament antibody were used to identify neuronal cells (Figure 7C), while cardiac troponin T is specific for cardiomyocytes (Figure 7D). In addition, fast skeletal muscle troponin T monoclonal antibodies stain only skeletal muscle troponin T (Figure 7E), and desmin and  $\alpha$ -actinin strongly suggest, but are not an absolute conformation of general muscle cell types (Figure 7F).

### 5.1 Responses of Msr gene expression to anoxia/reoxygenation treatments

Using real-time RT-PCR, we have compared the regulation of methionine sulfoxide reductase gene expression at the transcription level under oxidative stress [Zhang et al., 2011]. Different levels of oxygen depletion and oxidative stress were induced by treating the embryonic mouse stem cells with increasing time periods of anoxia (in 90% N<sub>2</sub>, 5% H<sub>2</sub> and 5% CO<sub>2</sub>) and reoxygenation (in air) in combined treatments. Results indicate that MsrA mRNA levels were gradually decreased with prolonged anoxic and reoxygenation treatments. RT-PCR results of MsrB1 and MsrB2 do not show an oxygen-dependent or oxidative stress regulated expression pattern, however, an oxygen-dependent expression pattern for MsrB3 is evident [Zhang et al., 2011]. Sensitivity of the MsrB3 mRNA expression level to oxygen depletion has been unequivocally demonstrated with significantly decreased mRNA levels at four, eight, and twelve hours of anoxic conditions to nearly half of the normal levels of normal oxygen levels. We found that the mRNA levels return to normal values after prolonged reoxygenation (> 4 hours) [Zhang et al., 2011]. The same oxygen-dependent expression pattern has been observed in matrix metalloprotein 2 and matrix metalloprotein 9 (MMP9). All real-time RT-PCR results have been normalized to  $\beta$ -actin levels for comparison.

### 5.2 Knockdown of MsrA expression at the mRNA level has no direct impact on MsrB1 or B2 gene expression but significantly decreases MsrB3 expression at the mRNA level

In view of earlier reports showing the potential for gene expression level interactions between MsrA and MsrB [Moskovitz, 2007], we repeated real-time RT-PCR experiments on all three reported MsrB genes (MsrB1, MsrB2 and MsrB3) using cells with MsrA mRNA downregulated by the siRNA transfection approach [Zhang et al., 2011]. Results confirm that all three MsrBs are expressed in mouse embryonic stem cells. Neither MsrB1 nor MsrB2 expression at the mRNA level were altered in the cells with MsrA mRNA downregulation (Figure 8).

Nonetheless, MsrB3 expression was significantly decreased in MsrA-specific siRNA transfected cells compared to the cells transfected with negative control siRNA. On the basis of our data, we propose that there are no direct interactions between MsrA and MsrB1 or MsrB2 gene expression at the mRNA level in mouse embryonic stem cells. However, MsrB3 mRNA expression is shown to be influenced by the levels of MsrA mRNA concentrations in the cells. A reduction of about 50% MsrB3 mRNA expression was noted as early as day 2 after MsrA gene knockdown. It is noteworthy that MsrA-specific siRNA downregulates MsrA mRNA for several days while its protein levels in the cell start to return towards normal after only 24 hours post-transfection (Figure 8E and 8F) [Zhang et al., 2010, 2011].

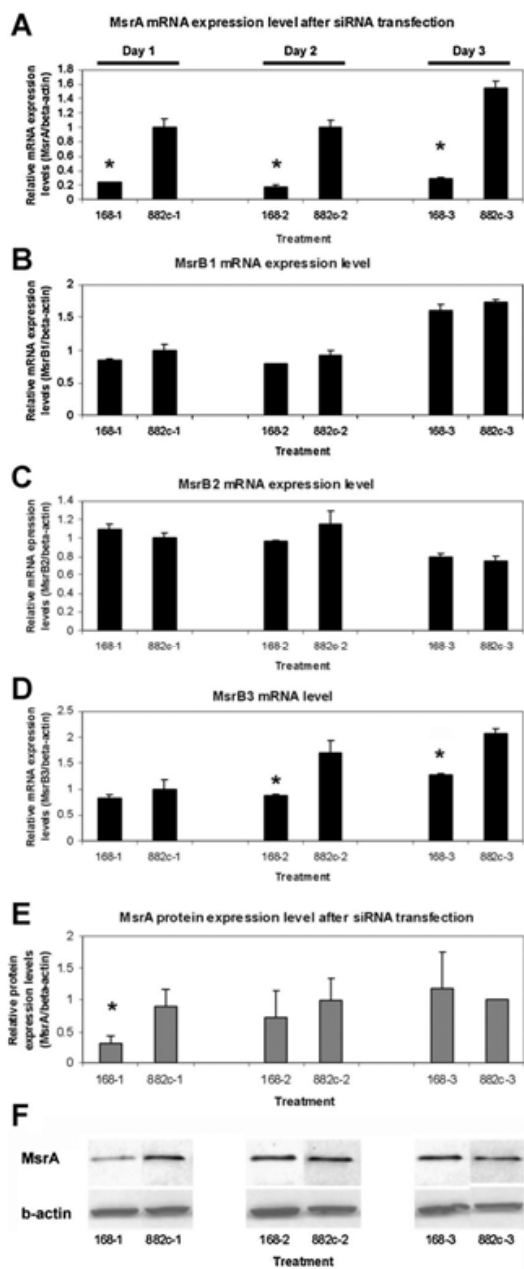


Fig. 8. Real-time RT-PCR studies on MsrB gene expression at the mRNA level after downregulation of MsrA expression by siRNA (168) transfection. A: MsrA mRNA levels; B: MsrB1 mRNA levels; C: MsrB2 mRNA levels. D: MsrB3 RNA levels. MsrB3 is the only one showing significantly increased mRNA expression after MsrA knockdown. E: MsrA protein levels from densitometry of Western blots shown in F. F: Western blotting analysis of MsrA protein and  $\beta$ -actin after treating MESC with MsrA-specific siRNA(168) or negative control siRNA(882) for days 1, 2, and 3. Triplets were used in each experiment. Two independent experiments were performed for real-time RT-PCRs and three Western blotting experiments were done. Results were averaged with standard errors of mean (SEM) presented with error bars. 168-1 represents samples collected on day 1 with siRNA (168) treatment. Results from MsrA-specific siRNA (168) treated samples labeled with asterisks (\*) show statistically significant differences from negative control siRNA (882) treated groups ( $P \leq 0.05$ ). (Reprinted with permission of the authors from Zhang et al., 2011 J. Cell Biochem. 112(1):98-106).



### 5.3 The pH of the culture medium influences Msr gene expression in cultured mouse embryonic stem cells

In our published work on MsrA, it was observed that prolonged culture of mouse embryonic stem cells (MES) in the same medium, without refreshing, promotes MsrA mRNA expression [Zhang et al., 2010, 2011]. To further explore this phenomenon, we cultured the cells in media pre-adjusted to pH 7.5, 6.8 or 6.4 by acetic acid or hydrochloric acid, with either acid showing similar results. Media was changed every 24 hours for three days and cells were collected just before the next culture media change. Total RNA and proteins were extracted for real-time RT-PCR and Western blotting studies. When comparing the different pH conditions on the same days, we found that MsrA, B1 and B3 genes have significantly increased their mRNA expression in cells cultured in the more acidic media on day 3. Significant increases of MsrB3 mRNA were also found in pH 6.8 media on day 2. The most significant change was noticed in MsrB3 which had a 4-6 fold increase of its mRNA in either pH 6.4 or 6.8 culture media compared to pH 7.5 on day 3. However, even with the significant increase in MsrA mRNA expression in pH 6.4 medium on day 3, no increase of protein level in stem cells was observed using MsrA antibody, the only antibody available for Western blotting on mouse Msr family genes (Figure 9E and 9F).

### 6. Evidence of the existence of a truncated form of MsrA in mouse embryonic stem cells

From our previous studies on MsrA [Zhang et al., 2010], we have consistently found that there is an observable protein band with a molecular weight (MW) of about 16 kD from Western blotting experiments using anti-MsrA antibody (Figure 10A, thick arrow), and we examined this in much more detail, recently publishing our study in which we will summarize the results and discussion in the following section of this review chapter [Jia et al., 2011]. The hybridization signal is low, and becomes evident on the X-ray film only after the longer form MsrA bands are overexposed (Figure 10A, thin arrow). There are also protein bands with molecular weights of ~46 and ~48 kD indicating homodimers of MsrA long form proteins (Figure 10A, arrow head), even with protein samples thoroughly treated by reducing agents before SDS-PAGE, as has been reported by other laboratories [Lee et al., 2006]. On our films, there is also a band with a molecular weight of 39 kD, possibly heterodimers formed by a long form protein of MsrA (MW: 23 kD) and a smaller form of MsrA protein (MW 16 kD) (Figure 10A, \*). In addition, a minor band with a molecular weight of ~32 kD, possibly formed by homodimers of two molecules of the smaller form of MsrA proteins (Figure 10A, \*\*). The 32kD and 39kD bands cannot readily be explained by the dimerization from cytosolic isoforms of MsrA which are 19-20kD in size [Kim & Gladyshev, 2006; Lee et al., 2006]. To confirm the existence of this smaller form of protein, we have carried out RT-PCR using total RNA extracted from mouse embryonic stem cells to amplify the full cDNA [Jia et al., 2011]. The forward and reverse primers were designed based on the 5'-UTR and 3'-UTR of the known full length MsrA cDNA respectively (Genebank#: NM 026322.3). Products from RT-PCR were loaded onto an agarose gel for electrophoresis; and two bands are readily visible on the gel, with the smaller one (Figure 10B, thick arrow) showing approximately 1/20<sup>th</sup> of the intensity and being about 100bp smaller than the larger band (Figure 10B, thin arrow).



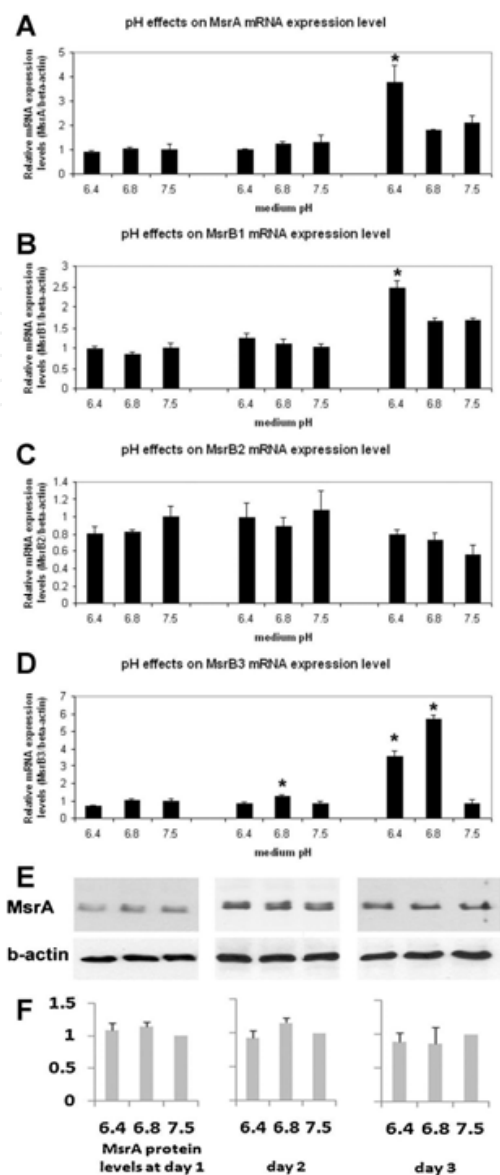


Fig. 9. Real-time RT-PCR studies on Msr gene expression in culture media with different pH conditions (pH 6.4, 6.8, and 7.5) at 1, 2, and 3 days in culture. All Msr genes, except for MsrB2, show increased expression at the mRNA level after 3 days in culture with acidic media with MsrB3 showing the most dramatic responses. A: MsrA mRNA levels; B: MsrB1 mRNA levels; C: MsrB2 mRNA levels; D: MsrB3 mRNA levels. E: Western blotting assays on MsrA protein and  $\beta$ -actin after culturing MESC cells in media with different pH for days 1, 2, and 3. F: Densitometry of MsrA bands normalized by  $\beta$ -actin after Western blotting shows equal levels of MsrA protein expression in cells cultured in media with different pH on the same days. In all comparisons, data from cells cultured at pH 7.5 are arbitrarily set as unit 1. Results with statistically significant differences from control groups (pH 7.5) were labeled with asterisks (\*) ( $P \leq 0.05$ ). Triplets were used in each experiment and at least two independent experiments were performed. Results were averaged with standard errors of mean (SEM) presented as error bars. Results are labeled with asterisks (\*) if there are statistically significant differences ( $P \leq 0.05$ ) while comparing samples treated with acidic culture media and cells cultured at pH 7.5. (Reprinted with permission of the authors from Zhang et al., 2011 J. Cell Biochem. 112(1):98-106).

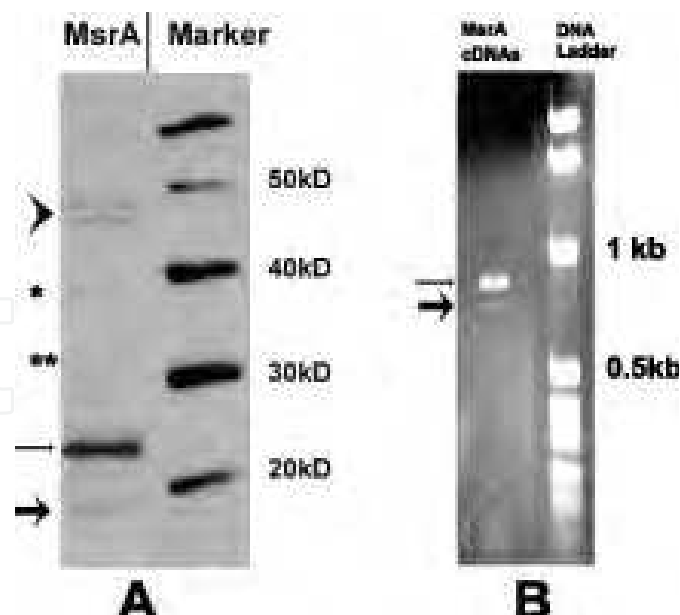


Fig. 10. Western blotting experiment using MsrA antibody reveals a smaller MsrA protein in cultured mouse embryonic stem cells. A. Thick arrow: the smaller form (~16 kD); thin arrow: the large form (~23kD); arrowhead: possible dimers from the long form MsrA proteins; \*: possible heterodimers from a large form and a shorter form protein; \*\*: possible homodimers from two shorter form proteins. B. Agarose gel electrophoresis of the amplified cDNA of MsrA showing a smaller band. PCR bands: long form: 857bp (thin arrow); short form: 744bp (thick arrow). (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

### 6.1 Cloning of the truncated cDNA form of MsrA

RT-PCR products of the smaller band described above were recovered from agarose gels and ligated to the pGEM-T-easy vector (Invitrogen, CA). After determining the DNA sequence, the smaller form sequence was aligned and compared to the full length GenBank cDNA. A deletion of the fifth exon (113bp) was found in the smaller form which ends up with a frame shift in the sixth exon directly attached to the fourth generating a new premature stop codon (Figure 11 A and B). The total length of the truncated protein is 148 amino acids, compared to the full length protein of 233 amino acids in length, both containing a mitochondrial signal peptide at the N-terminus (Figure 2). The truncated form still retains the GCFWG functional motif (catalytic active site) but contains neither of the two cysteines at the c-terminus (Figure 11B). Due to the functional importance of the two c-terminal cysteines in the redox reaction, it is reasonable to believe that the enzyme activity for methionine sulfoxide reduction will decrease dramatically, which requires confirmation by studies on purified proteins translated from this truncated template. It is interesting that we have also identified a virtually identical mouse EST sequence from a kidney cDNA library in GenBank (GenBank ID: BG970953.1) with the same intron splicing pattern as the truncated form of MsrA cloned from embryonic stem cells, indicating that this isoform might not be stem cell specific. The comparison between the EST sequence and truncated MsrA is illustrated in Figure 12. Except for missing ten nucleotides at the end of the third exon (boxes in Figure 12 and Figure 11A), the EST sequence showed 100% identity to the truncated cDNA from the 113<sup>th</sup> nucleotide (at the 5' UTR) to the very end of truncated form of the Msr cDNA.

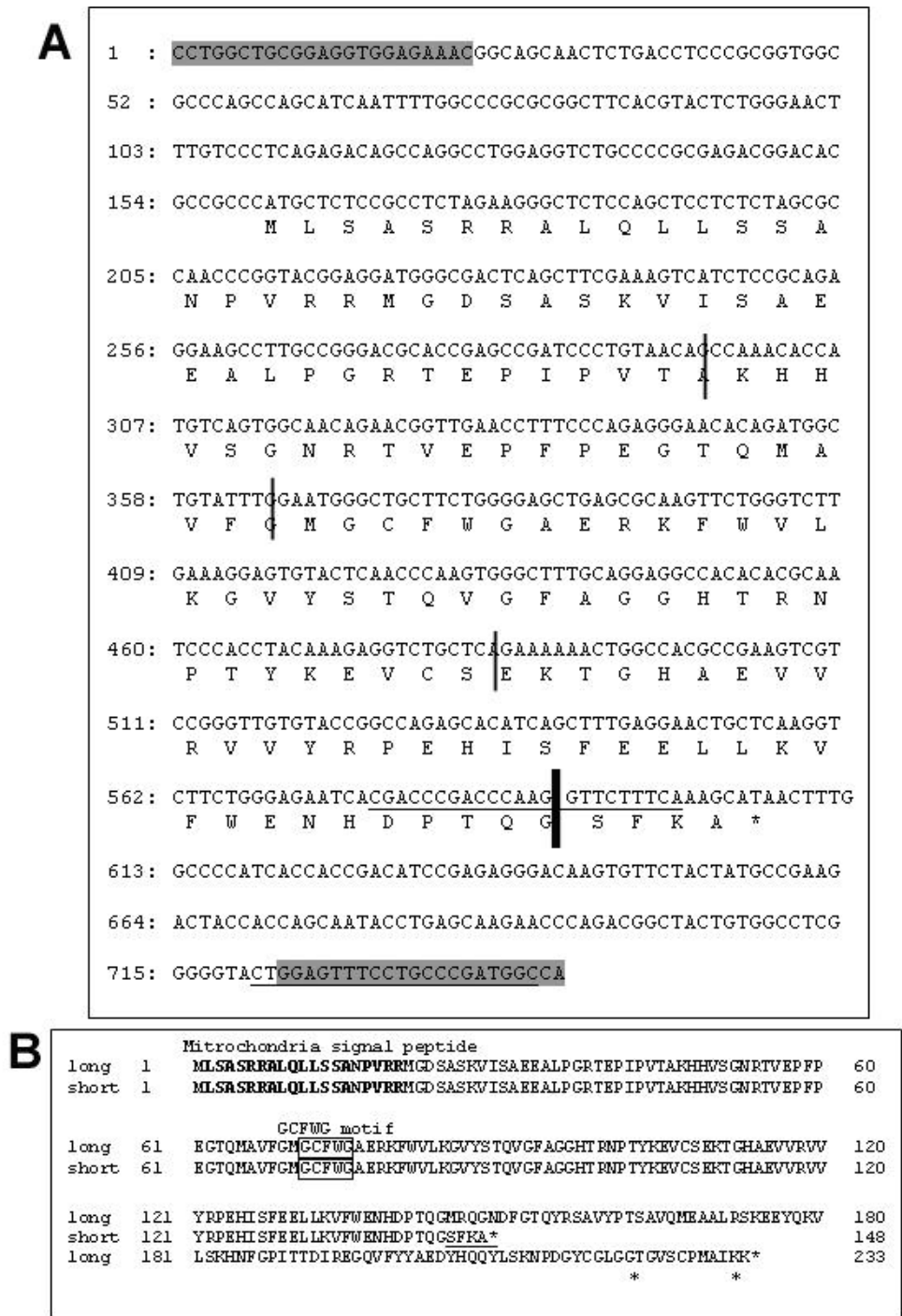


Fig. 11. The cDNA sequence of the truncated form of MsrA with the predicted open reading frame. A. Thin lines indicate exon-exon junctions. The thick line points to the junction of exon 4 and 6 skipping exon 5 in the truncated form but is included in the long form of MsrA. Shaded sequences are the two primers used to amplify the whole cDNA. Sequences underlined show the primer pairs for real time PCR to specifically amplify the truncated form with the forward primer designed on the junction of exon 4 and 6. B. The truncated form contains 148 amino acids with a mitochondrial signal peptide at the N-terminus and a GCFWG motif but no c-terminal cysteines. (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

EST	1	GAGACAGCCAGGCCTGGAGGTCTGCCCCGCGAGACGGACACGCCGCCCATGCTCTCCGCC	60
Trunc	113	GAGACAGCCAGGCCTGGAGGTCTGCCCCGCGAGACGGACACGCCGCCCATGCTCTCCGCC	172
EST	61	TCTAGAAGGGCTCTCCAGCTCCTCTCTAGCGCCAACCCGGTACGGAGGATGGGCGACTCA	120
Trunc	173	TCTAGAAGGGCTCTCCAGCTCCTCTCTAGCGCCAACCCGGTACGGAGGATGGGCGACTCA	232
EST	121	GCTTCGAAAGTCATCTCCGCAGAGGAAGCCTTGCCGGGACGCACCGAGCCGATCCCTGTA	180
Trunc	233	GCTTCGAAAGTCATCTCCGCAGAGGAAGCCTTGCCGGGACGCACCGAGCCGATCCCTGTA	292
EST	181	ACAGCCAAACACCATGTCACTGGCAACAGAACGGTTGAACCTTTCCAGAGGGAACACAG	240
Trunc	293	ACAGCCAAACACCATGTCACTGGCAACAGAACGGTTGAACCTTTCCAGAGGGAACACAG	352
EST	241	ATGGCTGTATTTGGAATGGGCTGCTTCTGGGAGCTGACCGCAAGTTCTGGGTCTTGAAA	300
Trunc	353	ATGGCTGTATTTGGAATGGGCTGCTTCTGGGAGCTGACCGCAAGTTCTGGGTCTTGAAA	412
EST	301	GGAGTGTA	359
Trunc	413	GGAGTGTA	472
EST	360	G-----AGAAAAAACTGGCCACGCCGAAGTCGTCCGGGTTGTGTACCGGCCAGAG	410
Trunc	473	GAGGCTGCTCAGAAAAAACTGGCCACGCCGAAGTCGTCCGGGTTGTGTACCGGCCAGAG	532
EST	411	CACATCAGCTTTGAGGAACTGCTCAAGGTCTTCTGGGAGAATCACGACCCGACCCAAGGT	470
Trunc	533	CACATCAGCTTTGAGGAACTGCTCAAGGTCTTCTGGGAGAATCACGACCCGACCCAAGGT	592
EST	471	TCTTTCAAAGCATAACTTTGGCCCCATCACCACCGACATCCGAGAGGGACAAGTGTTCTA	530
Trunc	593	TCTTTCAAAGCATAACTTTGGCCCCATCACCACCGACATCCGAGAGGGACAAGTGTTCTA	652
EST	531	CTATGCCGAAGACTACCAACAGCAATACCTGAGCAAGAACCAGACGGCTACTGTGGCCT	590
Trunc	653	CTATGCCGAAGACTACCAACAGCAATACCTGAGCAAGAACCAGACGGCTACTGTGGCCT	712
EST	591	CGGGGGTACTGGAGTTTCCTGCCCCGATGGCCA	622
Trunc	713	CGGGGGTACTGGAGTTTCCTGCCCCGATGGCCA	744

Fig. 12. Comparison between truncated form of MsrA cDNA (Trunc) and a mouse EST sequence from a kidney cDNA library in Genbank (EST). The EST sequence lacks ten nucleotides comparing with the truncated cDNA which is shown in the box. The same area is also shown in a box in Figure 2A, which is located at the very end of the third exon. (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

## 6.2 Confocal microscopy reveals different subcellular localizations for the truncated MsrA protein compared to the full length using eGFP fusion constructs

The long form of the MsrA full length protein conjugated with the eGFP tag has been studied previously in our laboratory and was found to be localized predominantly in mitochondria with some staining in the cytosol [Zhang et al., 2010, 2011]. Further studies confirmed this finding with the MsrA long form-eGFP fluorescence signals (green, figure 13B); the mitochondria stained by MitoTracker (red, figure 13A) mostly overlap each other and show an orange color (figure 13G).

After our discovery of the truncated form of the MsrA, we did a detailed study of this form and have published these results [Jia et al., 2011], from which we describe and summarize these recent findings in the following paragraphs. The MsrA truncated form-eGFP fusion protein shows a more nonspecific localization, mostly in the cytosol, although green fluorescent signals in mitochondria are detectable (Figure 13 D, E, F and H). To confirm this observation, we have generated a combination of three single slices of confocal scans top view (A), upper-side view (B) and right-side view (C) on a stem cell colony with the truncated-MsrA-eGFP transfection (Figure 14A, B and C) [Jia et al., 2011]. This provides a clear three dimensional image showing the localization of the truncated protein. On the focal point of the scans (cross-point of the horizontal green line and the vertical pink line), the green fluorescent signal is excluded from nuclei stained by DAPI. Most of the green signals are not overlapping with the red mitochondria in all three view angles (arrowhead, Figure 14C) although there are some detectable co-localizations evident (arrow, Figure 14B) [Jia et al., 2011].

Studies reported by Lee et al. [2006] and Pascual et al. [2009] show the existence of two alternative promoters for the MsrA gene that encode different isoforms of MsrA proteins in mitochondria or cytosol/nuclei due to the presence or absence of a N-terminal mitochondrial signal peptide. Studies from Kim and Gladyshev [2006], using GFP fusion techniques and deletion mutagenesis, reveal functional domains in the MsrA peptide sequence, including sequences close to the c-terminus, that may also direct the specific locations of the protein in subcellular compartments. Localization of the mitochondrial form of MsrA in the cytosol and nuclei were also noted by Kim and Gladyshev [2005] in MsrA overexpression experiments. Although syntheses of different isoforms with or without the N-terminal signal peptide might be the optimal way for cells to direct protein sorting, it should not be overlooked that the same isoform might be able to locate to multiple cellular compartments. While we do not rule out the possibility that the altered localization pattern of the truncated protein, compared to the long form, is due to GFP fusion interference, it is very unlikely considering the fact this same method has been used successfully to reveal subcellular localization of MsrA in all of our cell lines [Zhang et al., 2010, 2011; Jia et al., 2011]. Previous studies from our laboratory on the truncated MsrA-eGFP fusion protein suggest a necessary domain at the c-terminal sequence for permanently docking of the protein on mitochondria. In addition to the mitochondrial signal peptide, there may exist another essential domain at the c-terminal end of the full length protein, without which, the truncated proteins are able to be sorted to the mitochondria but will eventually leak out back into the cytosol. In the deletion mutagenesis studies of Kim and Gladyshev [2005], the deletion is limited only to the very end or very middle of the N-terminus, not totally overlapping the portion omitted in this truncated form which could harbor more functional domain units [Kim & Gladyshev, 2005].



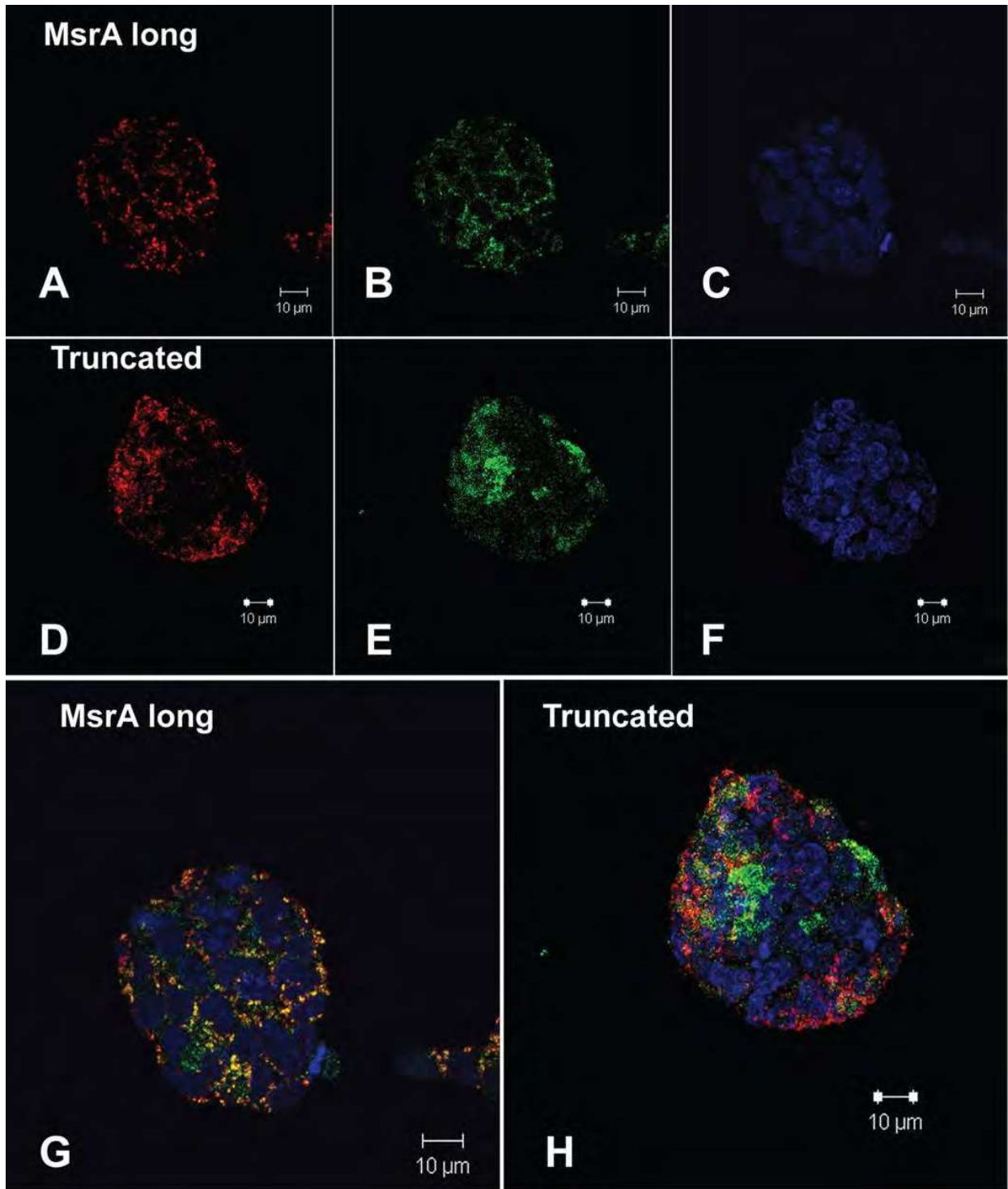


Fig. 13. Confocal microscopy on the long form MsrA-eGFP (A,B, C,G) and truncated MsrA-eGFP (D,E,F,H) transfected mouse embryonic stem cells. A,D: mitochondria stained by propidium iodide; B,E: green fluorescence showing GFP tags; C,F: nuclei stained with DAPI; G: overlapped image from A, B and C, but with higher magnification; H: image overlapped from D, E and F; Bars: 10 µm. (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

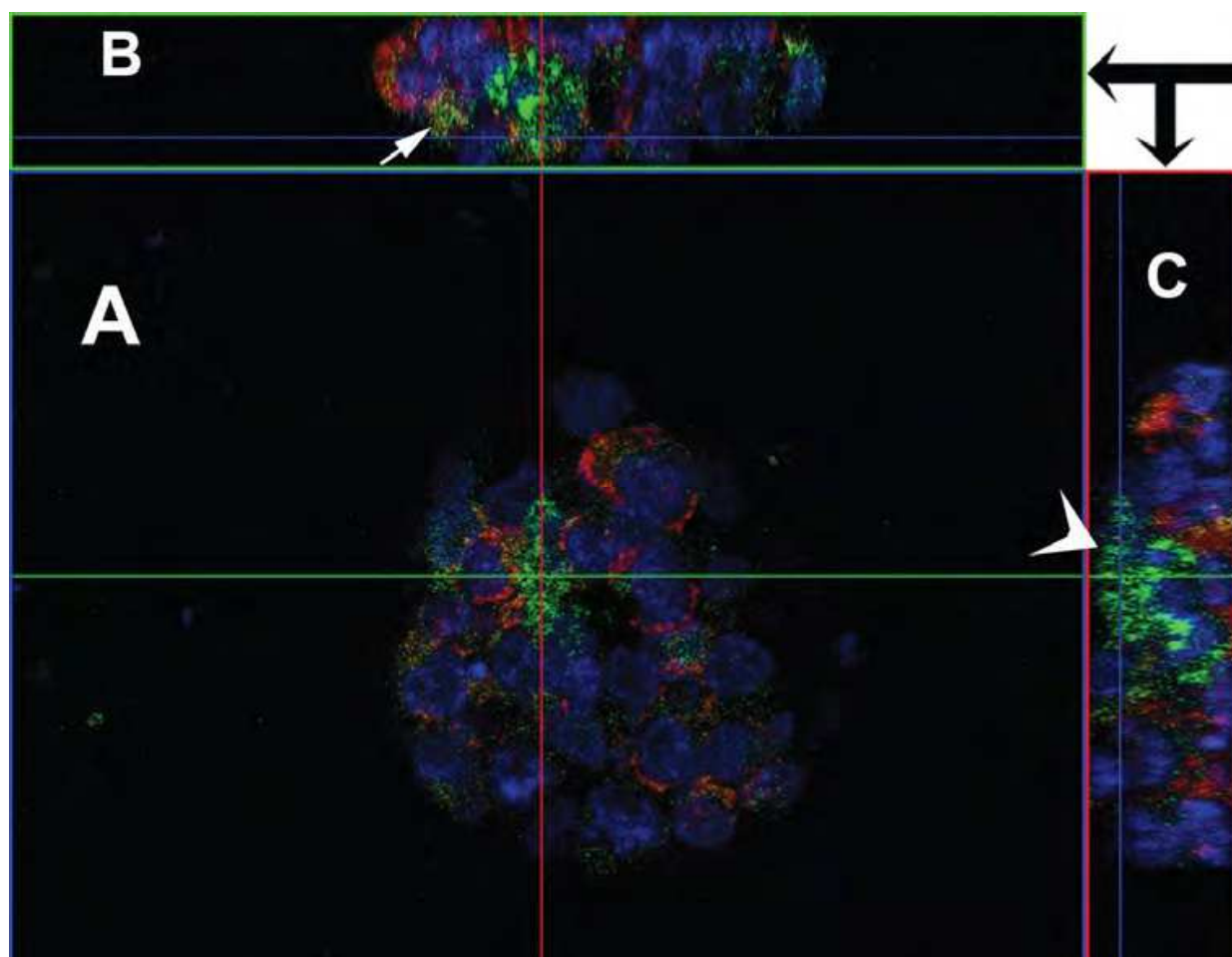


Fig. 14. Confocal microscopy of a single colony of mouse embryonic stem cells. A: top view of a single slice of scanning showing most eGFP signals are not overlapping with mitochondria or nuclei. B: single slice scanning from upper-side view; J-2: single slice scanning from rightside view. The arrow in B indicates some overlapping signal of truncated Msr-eGFP and mitochondria. The arrowhead in C points to the area where the truncated Msr-eGFP signals are not overlapping with mitochondria, but mainly in cytosol. The cross-point of the horizontal green line and vertical red line points to the area we are observing. The blue lines in B and C show the current slice position for this confocal scanning (Reprinted with permission of the authors from Jia et al., 2011 J. Biomed. Sci. 18:46).

### 6.3 Real time RT-PCR shows a different response of mRNA expression levels for the truncated form compared to the full length MsrA

Real time RT-PCR studies done in our laboratory on the long form MsrA expression responses to oxygen deprivation and reoxygenation show that the expression levels decrease along with longer anoxia/reoxygenation treatment combinations [Zhang et al., 2010, 2011]. Similar studies on the truncated MsrA transcripts show different responses compared to the long form except during 4 hours of anoxia treatment in which both truncated and long forms show decreases at the mRNA level, likely an initial response of the stem cells to oxygen deprivation as observed in all Msr genes as well as in other genes

including metalloproteinases (MMP2 and MMP9) [Zhang et al., 2010, 2011; Jia et al., 2011]. mRNA expression of the truncated form decreases dramatically when a short period of reoxygenation (4 hours) was given after a long period (12 hours) of anoxia (12+4 hrs). The level of reactive oxygen species (ROS) in the cells are expected to rise substantially. Expression level partially recovers after 12 hours of reoxygenation following the 12 hours of anoxia treatment (12+12 hrs) to the same level as 12 hours of reoxygenation following 8 hours of anoxia (8+12 hrs), at which point the level of reactive oxygen species might have decreased compared to the point of 12+4 [Jia et al., 2011]. We suggest that the truncated form of the MsrA transcript is responsive to the cellular level of ROS. By comparing expression levels at 12+4 and 12+12 time points for long and truncated forms of MsrA mRNA, it is evident that the truncated form is more sensitive to oxidative stress changes than the long form.

#### **6.4 The c-terminal truncated form of MsrA was cloned from mouse embryonic stem cells by skipping exon 5 and having a frame shift mutation in exon 6, generating a premature stop codon**

The truncated MsrA protein displays a different subcellular localization pattern expression in response to anoxia/reoxygenation treatment of the stem cells. Further studies on the enzymatic activity of this shortened peptide is required to classify it as a functional isoform. One possibility for the evolutionary advantage of retaining such a truncated form might be that producing a smaller size protein while containing an active GCWFG site, may allow this “mini-protein” to readily access the oxidized methionine residues on proteins, while the larger structure hinders access of the long form protein. Since the truncated protein does not contain c-terminal cysteines, perhaps the final relieving of the oxidation step needs the long form MsrA. Interestingly, heterodimers between long form and truncated proteins were observed in our Western blotting experiments [Jia et al., 2011].

### **7. Implications and conclusions on protection of mouse embryonic stem cells from oxidative stress by methionine sulfoxide reductase A (MsrA)**

Previous studies on methionine sulfoxide reductase (Msr) have been performed in various mammalian cell lines [Kantorow et al., 2004; Yermolaieva et al., 2004; Petropoulos et al., 2001] as well as animal models [Ruan et al., 2002; Moskovitz et al., 2003]. Regulation of the Msr system as an antioxidant mechanism for stem cells, however, was totally unknown before our studies [Zhang et al., 2010, 2011; Jia et al., 2011]. It is critical to understand more about stem cell anti-oxidative stress capabilities in order to develop protocols for therapeutic applications of stem cells stressed by high oxidative environments. Our published *in vitro* studies using mouse embryonic stem cell cultures demonstrate the vital importance of the MsrA protein protecting these stem cells from hydrogen peroxide mediated oxidative damage [Zhang et al., 2010]. This same protection may exist *in vivo* and potentially may improve the cell viability of stem cells in patients having ischemia/reperfusion oxidative damage, such as in an infarcted myocardium. Msr genes also could play a major role in shielding adult stem cells or embryonic stem cells stored in adult tissues, from oxidative damage. Such cells apparently are maintained in a quiescent state in adult tissues for long periods that probably extend throughout the entire lifetime of the individual [Ding, 2003, Ryu et al., 2006].

Three MsrBs have been cloned (MsrB1, B2 and B3) from mammals, which are localized in subcellular compartments [Kim and Gladyshev, 2004, 2005, 2006] and show variable expression patterns in different tissues. There is only a single known mammalian MsrA gene and its proteins are localized in both the cytosol and in mitochondria [Vougier et al., 2003]. Our published studies using the eGFP fusion protein to track MsrA expression show that the majority of MsrA proteins are present in the cytosol and the mitochondria components of cells [Zhang et al., 2010]; a similar localization pattern has been shown for rat liver cells [Vougier et al., 2003]. In our experiments, limited fluorescent signals were observed in the cell nuclei, indicating low concentrations of MsrA-eGFP [Zhang et al., 2010]. It is possible that these low eGFP fluorescent levels were due to steric molecular interference being conjugated to MsrA [Hansel et al., 2002]. Studies on human MsrA showed the exclusive mitochondrial protein localization [Hansel et al., 2002]. The mouse MsrA protein, using the eGFP construct, revealed localization of MsrA in mitochondria, cytosol and nuclei [Kim & Gladyshev, 2005]. Kim and Gladyshev [2005] proposed that the N-terminal mitochondrial signal peptide directs the MsrA protein to the mitochondria. It appears there is localization of MsrA variations according to species and type of cell.

In mammalian cells, MsrA is a gene lacking numerous exogenous influences unlike reports for insects suggesting that ecdysone can induce MsrA expression in *Drosophila melanogaster* during development [Cherbas et al., 1986; Roesijadi et al., 2007]. We have demonstrated that combinations of anoxia and reoxygenation to treat mouse embryonic stem cells have shown a unique result in which MsrA mRNA and protein expression decreases with increased periods of anoxia or reoxygenation treatment [Zhang et al., 2010]. Increasing the periods of anoxia or reoxygenation results in elevated levels of reactive oxygen species expression and MsrA mRNA and protein expression in stem cells. These results are consistent with reports [Petropoulos et al., 2001] that rat MsrA gene expression decreases with age and during replicative senescence of human WI-38 fibroblasts. Both scenarios correlate aging with higher levels of oxidative stress. It is relevant to note that all these studies show the MsrA expression levels to decrease, not increase, with prolonged oxidative stress exposure/aging. Increasing MsrA expression would hypothetically provide more protection for cells when exposed to higher levels of oxidative stress [Zhang et al., 2010]. It remains unknown whether this mechanism exists in nature as a protective mechanism to prevent stem cells in the body from turning into cancerous tissue by allowing cells to die by ROS damage once the hypoxic environment is established in a growing tumor [Zhang et al., 2010]. It is also possible that by decreasing MsrA via oxidative stress, embryonic stem cells are allowing higher and longer periods of ROS spikes to signal for differentiation initiation in which case overexpression of MsrA in stem cells will be detrimental to their plasticity. It would be very interesting to further investigate this phenomenon, for future guidance in manipulating MsrA expression in stem cells in response to oxidative damage to increased cell survivability and differentiation [Zhang et al., 2010]. We have found that after siRNA knockdown of MsrA, its mRNA remains at low levels for 72 hours or more. The MsrA protein levels, however, return to normal within 48 hours. Our laboratory was the first to report translational regulation of the MsrA protein in stem cells [Zhang et al., 2010]. The mechanism of regulation remains unknown. It seems evident, however, that the stringent control in mouse ESC to keep the MsrA protein level high, even with low levels of mRNA present, suggests that this protein is essential to cellular function and survivability [Zhang et al., 2010].



We confirmed the successful knockdown of MsrA mRNA levels in the stem cells after 24 hours following MsrA siRNA transfection. There was almost an 80% reduction of mRNA levels and a 70% reduction of protein in the cells at this time point. In addition we added H<sub>2</sub>O<sub>2</sub> to the cell cultures after 24 hours to determine a possible loss of the protective effect after MsrA knockdown at high H<sub>2</sub>O<sub>2</sub> concentrations. We found that the protected loss after MsrA knockdown was not significant if H<sub>2</sub>O<sub>2</sub> concentrations are low (< 50μM), suggesting that the protective effects from other methionine sulfoxide reductases, such as MsrBs, are intact in the stem cells to fight against oxidative damage [Zhang et al., 2010]. In order to test our hypothesis and show that loss of protective effects is from MsrA alone rather than concurrent loss of MsrBs' expression after MsrA siRNA transfection, we performed experiments using real-time RT-PCR on the three MsrB genes (MsrB1, MsrB2 and MsrB3) using samples with MsrA siRNA transfection. These experiments proved that all three MsrBs are present in stem cells after 24 hours of siRNA post-transfection, without any changes in MsrB mRNA expression levels. Our earlier work confirms that there is no direct interaction between MsrA and MsrBs gene expression in mouse embryonic stem cells.

These results reiterate the existence of additional protective effects of MsrA overexpression in mouse embryonic stem cells. We conclude, based on our studies, that overexpression of MsrA in stem cells provides major protection to stem cells in harsh environments such as ischemia/reperfusion during strokes or heart attacks. New approaches to treat diseases, including neurodegenerative diseases and ischemia/reperfusion-induced brain or heart damage using adult or embryonic stem cell transplantation are being developed. A major roadblock of using stem cells is their low survivability after surgery due to the harsh oxidative environments into which these cells are placed. Without preparing and protecting stem cells to resist high oxidative stress prior to transplanting them into damaged tissue areas dictates likely failure and death of the implanted cells. In our recent studies, we believe we have found a very promising approach to solving this problem by enhancing the resistance of stem cells to oxidative damage once they are transplanted into an oxidatively stressing environment [Zhang et al., 2010]. We believe it will be very important to further investigate the differentiation capability of stem cells overexpressing MsrA. Since the Msr genes can directly function to reduce oxidized proteins back to their functional forms by indirectly controlling the ROS levels in these cells, it seems reasonable that overexpressing Msr genes in stem cells might function to reduce ROS damage to the cells without losing the cells' sensitivity to ROS as a differentiation signal [Zhang et al., 2010]. Thus the Msr gene family could potentially serve a key role in engineering stem cells to obtain higher resistance to oxidative damage, while retaining the cells' potential for differentiation into the adult cell types described in a given clinical application.

## 8. Potential regulatory interactions of the Msr genes

In our earlier studies, we demonstrated that knockdown of the MsrA gene in mouse embryonic stem cells causes a loss of protection against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage [Zhang et al., 2010]. Studies from other laboratories have shown a potential intergenic relationship of gene expression between the MsrA and MsrB genes from the MsrA knockout mouse model; MsrA knockout mice showed parallel losses at the levels of the MsrB1 mRNA and the MsrB1 protein [Moskovitz et al., 2003]. Subsequent studies on MsrA knockout mice placed on selenium deficient diets showed a reduction of MsrB activity in a tissue specific manner [Moskovitz, 2007]. In our studies [Zhang et al., 2011], a short term (three days) loss



of MsrB1 or MsrB2 expression was not detected and the long term effect of MsrA knockdown of MsrB1 and MsrB2 expressions is not known. MsrB3, however, shows decreased expression at days 2 and 3. Although the mRNA of MsrA is still down 70% compared to negative control siRNA transfected cells at day 3, the MsrA protein expression has returned to normal levels. It is possible that the signal to decrease MsrB3 expression is at the level of MsrA mRNA, and not its protein. The mechanism by which MsrA regulates MsrB expression remains to be elucidated and will form a most interesting topic for further studies, considering the fact that MsrB3 and MsrA genes are located on different chromosomes in both human and mouse. Earlier studies from our laboratory, together with previous findings from other laboratories, strongly suggest that MsrA plays a significant role in MsrB gene expression [Zhang et al., 2010, 2011].

There have been a few studies on regulatory factors that influence Msr gene expression. In prokaryotes, studies on *H. pylori* demonstrate that certain stress conditions such as treatment with peroxide, peroxyxynitrite, or iron starvation, results in a 3- to 3.5-fold transcriptional up-regulation of the Msr gene [Alamuri & Maier, 2006]. In *H. pylori*, Msr codes for a 42-kDa protein with fused MsrA- and MsrB-like domain [Weissbach et al., 2002]. The only available evidence that Msr gene expression is influenced by pH stems from studies on *Streptococcus gordonii* which when entering the blood stream (pH 7.3) from the oral cavity (pH 6.2) promotes MsrA expression and possibly protects and increases survivability of the bacteria [Vriesema et al., 2000]. In *Drosophila*, ecdysone was found to be effective in promoting MsrA but not MsrB expression [Roesijadi et al., 2007]. In mouse, a selenium deficient diet results in decreased enzymatic activities for both MsrA and MsrB [Uthus & Moskovitz, 2007]. MsrA is also found downregulated in human hepatitis B positive hepatocellular carcinoma (HCC) with metastasis compared to HCCs without metastasis [Lei et al., 2007]. Recent studies on insulin/IGF receptor (IIR)/FOXO pathways indicate that downregulation of signaling in this pathway has been shown to extend lifespan in worms and flies [Minniti et al., 2009]. FOXO-mediated transcription is required for the long lifespan, thus there is great interest in identifying FOXO target genes. Also, it was reported recently that methionine sulfoxide reductase A expression is regulated by the DAF-16/ FOXO pathway in *Caenorhabditis elegans* [Minniti et al., 2009]. Moreover, another study has shown that Spx, a global transcriptional regulator of the disulfide specific oxidative stress response in *B. subtilis* plays a central role in the paraquat-specific induction of MsrA and MsrB expression [You et al., 2008].

Previously, we have demonstrated, for the first time that Msr genes are responsive to environmental culture condition changes that mimic pathological situations [Zhang et al., 2010, 2011; Jia et al., 2011]. Since MsrA is the only enzyme currently known for Met-S-(O) reduction and since MsrB1 has the highest enzymatic activity for Met-R-(O) reduction [Kim & Gladyshev, 2004], and both show only slight responses to oxygen level and media pH changes, MsrA and MsrB1 (and possibly also MsrB2) likely function as housekeeping enzymes for normalizing oxidative status in the cells [Zhang et al., 2011]. However, MsrA and MsrB1 do possess the capability of being regulated in a severely harsh environment. MsrB3, on the other hand, shows the lowest expression level in real time RT-PCR studies compared to MsrB1 and B2, but the most dramatic response to both oxygen level and media pH among all the Msr genes [Zhang et al., 2011]. Thus, MsrB3 could be a major player responding to increased cellular oxidative stress. It seems also reasonable to hypothesize that an acidic pH environment is an important signal for MsrB3 expression induction based

on current results, while oxygen depletion, although also a tissue damaging signal, shuts down MsrB3 transcription [Zhang et al., 2011].

Using real time RT-PCR, we have shown that MsrB3 responds most dramatically, among all Msr genes, to oxygen deprivation, culture media pH changes and MsrA knockdown [Zhang et al., 2010, 2011]. MsrB3 expression decreases significantly under anoxic conditions but increases dramatically after two days of culture in acidic medium, suggesting that MsrB3 is a major player in response to changes of tissue oxidative stress in embryonic stem cells. Knockdown of MsrA by siRNA in these cells also has shown a parallel decrease of MsrB3 transcription (30-50%) compared with the negative controls, but not for MsrB1 and B2, indicating different intergenic interactions between MsrA and members of the MsrB group. In the present studies, we suggest new evidence to examine the expressional regulation of Msr genes. The flexibility of MsrB3 expression levels provides a potential target for future research to improve oxidative stress resistance in therapeutic stem cells or even to reduce this resistance in harmful tissues such as in cancer cells [Zhang et al., 2011].

## 9. Truncated form of methionine sulfoxide reductase A

Methionine sulfoxide reductase A (MsrA), an enzyme in the Msr gene family, is important in the cellular anti-oxidative stress defense mechanism. It acts by reducing the oxidized methionine sulfoxide in proteins back to sulfide and by reducing the cellular level of reactive oxygen species. MsrA, the only enzyme in the Msr gene family that can reduce the S-form epimers of methionine sulfoxide, has been located in different cellular compartments including mitochondria, cytosol and nuclei of various cell lines [Zhang et al., 2010, 2011]. One possibility for the existence of a truncated form of the MsrA transcripts could be that with a smaller protein size, yet retaining a GCWFG action site, this protein might have easier access to oxidize methionine residues on proteins than the longer form of the MsrA protein, thus having an evolutionary selection advantage. This research opens the door for further study on the role and function of the truncated MsrA embryonic mouse stem cells [Jia et al., 2011].

## 10. Conclusions

Studies from our laboratories and others support the concept that the Msr family of enzymes protects cells from oxidative sulfide groups of methionine residues in proteins. As a consequence, normal protein functions are restored and there is a reduction of intracellular reactive oxygen species. Further experiments suggest that the four Msr family genes are under differential regulation by anoxia/reoxygenation, acidic culture conditions and MsrA and MsrB interactions. Results of experiments on mouse embryonic stem cells reveal that the gene family functions in oxidative stress protection with each of the Msr genes responding differently to environmental stimuli at the tissue level. Based upon our experiments using mouse embryonic stem cell cultures (ESC), it is clear that one member of the Msr gene family, methionine sulfoxide reductase A (MsrA) reduces methionine sulfoxide residues in proteins formed by oxidation of methionine by reactive oxygen species (ROS). In these mouse ESC cultures, knockdown of MsrA expression resulted in a significantly lowered resistance of the mouse ESCs to hydrogen peroxide treatment, while overexpression of the MsrA gene resulted in increased survivability of the ESCs. Thus,

MsrA appears to play a significant role in the resistance of ESCs to oxidative stress. As future studies go forward on the use of embryonic stem cells in therapeutic applications, the Msr family of genes may prove to be important in producing stem cells that have the ability to differentiate into the desired adult end tissues, while at the same time possessing a high resistance to the severe oxidative environment that stem cells are subjected to, especially at the early phases of transplantation. Further studies on the methionine sulfoxide reductases (Msr) at the enzyme as well as molecular genetic levels may be a key to future successful stem cell therapeutic applications.

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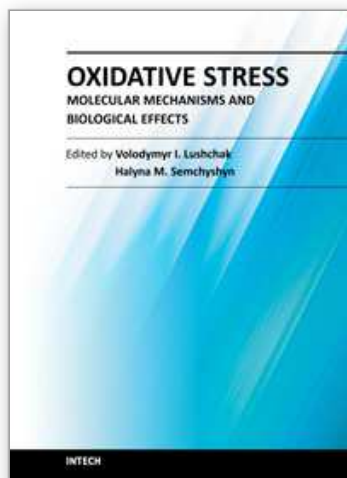
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Since the discovery of free radicals in biological systems researchers have been highly interested in their interaction with biological molecules. Denoted in 1980, and due to fruitful results and ideas, oxidative stress is now appreciated by both basic and applied scientists as an enhanced steady state level of reactive oxygen species with wide range of biological effects. This book covers a wide range of aspects and issues related to the field of oxidative stress. The association between generation and elimination of reactive species and effects of oxidative stress are also addressed, as well as summaries of recent works on the signaling role of reactive species in eukaryotic organisms. The readers will gain an overview of our current understanding of homeostasis of reactive species and cellular processes they are involved in, as well as useful resources for further reading.

### **How to reference**

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