Role of Myostatin in oxidative stress in skeletal muscle

Sandhya Sriram

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Thesis Erratum

ROLE OF MYOSTATIN IN OXIDATIVE STRESS IN SKELETAL MUSCLE
-SANDHYA SRIRAM

This Thesis Erratum is prepared and submitted by Sandhya Sriram ONLY.

A handwritten signature in black ink, appearing to read 'Sandhya', with a long horizontal line extending from the end of the signature.

11th November 2015.

Sandhya Sriram.

The edits/insertions have been highlighted in yellow.

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Figure 3.7 *Mstn induces ROS through TNF- α via NF- κ B signaling.* Effect of Mstn on NF- κ B and I κ B- α in proliferating C2C12 myoblasts. Western blotting analysis was performed on whole cell lysates, nuclear and cytoplasmic extracts obtained from C2C12 cells treated with Mstn (3.5 ng/ml) for indicated time points. (i) Left panel, representative immunoblot showing protein levels of NF- κ B (p65), p-NF- κ B (p65), I κ B- α , p-I κ B- α and IKK α in negative control (Lanes 1 and 3) and Mstn (Lanes 2 and 4) treated whole protein lysates, nuclear and cytoplasmic extracts at indicated time points. α -tubulin was used as an internal control for equal protein loading on the gel. Other lanes from the representative whole cell lysate NF- κ B (p65) blot in (i) were excluded as not being relevant to the comparison illustrated here so that only pertinent lanes from the original blots are shown. Right panel, corresponding densitometry analysis of (ii) p-NF- κ B (p65) and (iii) p-I κ B- α showing significant increase or decrease in protein content upon Mstn treatment (* p <0.05, ** p <0.01) (n=2).

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Figure 3.8 *Mstn-mediated enhancement of NF- κ B binding activity on mouse TNF- α promoter.* EMSA was performed using nuclear extracts from proliferating C2C12 cells treated with Mstn (3.5 ng/ml) for 48 hours. (i) Left panel, representative gel showing the increased activity of NF- κ B upon Mstn treatment. Lane 1-oligo only, Lane 2-untreated, Lane 3-Mstn treated. EMSA was also performed with nuclear extracts preincubated with the specific antibody. Right panel, representative gel showing NF- κ B complexes containing p65 or p50 subunits. Lane 1-no antibody added, Lane 2-p65 specific antibody added, Lane 3-p50 specific antibody added (n=2). All lanes are from one representative gel. Lane 3 in panel (i) is also shown in Lane 1 panel (ii) to allow for direct comparison.

This image (i) is the scan of the original blot for Figure 3.8

This image (ii) shows the labeling of lanes 1-5 in the original blot shown above (in (i))

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Figure 3.9 5-ASA and ascorbic acid inhibit Mstn induced ROS via TNF- α and NF- κ B signaling. mRNA expression analysis was performed on C2C12 cells treated with 5-ASA (10 μ M/ml) and ascorbic acid (100 μ M/ml) along with Mstn (3 μ g/ml). Representative graphs show fold change in the mRNA expression of TNF- α (A) and *Nox1* (B). The values are mean $\pm$ S.E of 2 independent experiments. ** $p < 0.01$ and *** $p < 0.001$ denotes significant increase when compared to untreated cells; ^^ $p < 0.01$ and ^^ $p < 0.001$ denotes significant decrease when compared to Mstn treated cells ($n=2$). (C) Western blotting analysis showing the level of NF- κ B-p65 at the indicated time point in whole cell lysates, nuclear and cytoplasmic extracts of proliferating C2C12 cells, untreated (Lane 1), treated with Mstn (Lane 2), 5-ASA (Lane 3), 5-ASA and Mstn (Lane 4), ascorbic acid (Lane 5), and ascorbic acid and Mstn (Lane 6). α -tubulin was used as an internal control for equal protein loading on the gel. Lanes 1 and 2 of the NF- κ B (p65) blot for whole cell lysate in Figure 3.9 (C) are the same as lanes 1 and 2 in the NF- κ B (p65) blot for whole cell lysate in Figure 3.7 (i). Other lanes from the representative whole cell lysate NF- κ B (p65) blot in (C) were excluded as not being relevant to the comparison being made and only pertinent lanes from original blots are shown.

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Figure 3.10 Mstn induces the muscle specific E3 ligase expression via ROS dependent pathway. Western blotting analysis showing the levels of p-Akt and Akt (A), Atrogin-1 and MuRF-1 (B) at indicated time points in the C2C12 cells, untreated (Lane 1), treated with Mstn (3 μ g/ml) (Lane 2), ascorbic acid (100 μ M/ml) (Lane 3), and ascorbic acid and Mstn (Lane 4) ($n=2$). α -tubulin was used as an internal control for equal protein loading on the gels. (C) Representative gel showing p-Smad2/3 protein levels in C2C12 cells, untreated (Lane 1), treated with Mstn (3 μ g/ml) (Lane 2), ascorbic acid (100 μ M/ml) (Lane 3), and

ascorbic acid and Mstn (Lane 4) for 48 hours during proliferation. GAPDH was used as an internal control for equal protein loading on the gel. Samples for Figure 3.10 (C) and Figure 3.11 (D) were run on the same gel. Other lanes from the representative p-Smad2/3 blot and the corresponding GAPDH blot in (C) were excluded as not being relevant and only pertinent bands are shown in respective figures. Lanes 1 and 2 of Figure 3.10 (C) are the same as lanes 1 and 2 of Figure 3.11 (D).

Lane 1- -ve control
 Lane 2- Mstn
 Lane 3- TNF- α
 Lane 4- 5-ASA
 Lane 5- 5-ASA+Mstn
 Lane 6- 5-ASA+TNF- α
 Lane 7- Ascorbic acid
 Lane 8- Ascorbic acid+Mstn

This image (iii) shows the original blot for p-smad2/3 used in Figure 3.10 (C) and 3.11 (D)

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Figure 3.11 *TNF- α induces Mstn expression, secretion and signaling in a feed forward mechanism.* (A) Representative graph showing mRNA expression of *Mstn* and (B) representative gel showing protein levels of Mstn (~56 KDa-full length) in C2C12 cells, untreated (Lane 1), treated with TNF- α (10ng/ml) (Lane 2), 5-ASA (10 μ M/ml) (Lane 3), and 5-ASA and TNF- α (Lane 4) for 72 hours in proliferation media. The values are mean $\pm$ S.E of 2 independent experiments. Level of significance was compared to the untreated control (****p<0.0001) (n=2). GAPDH was used as an internal control for equal protein loading on the gel. (C) Level of secreted Mstn in the cell culture media from C2C12 cells treated with TNF- α (10 ng/ml) and 5-ASA (10 μ M/ml) was determined by EIA. The values are mean $\pm$ S.E of 2 independent experiments. The concentration of Mstn is given as ng/ml and the level of significance was determined with respect to the negative control (**p<0.01) (n=2). (D) Representative gel showing p-Smad2/3 protein levels in C2C12 cells, untreated (Lane 1), treated with Mstn (3 μ g/ml) (Lane 2), TNF- α (10 ng/ml) (Lane 3), 5-ASA (10 μ M/ml) (Lane 4), 5-ASA and Mstn (Lane 5), and 5-ASA and TNF- α (Lane 6) for 72 hours during proliferation. GAPDH was used as an internal control for equal protein loading on the gel. Samples for Figure 3.11 (D) and Figure 3.10 (C) were run on the same gel. Other lanes from the representative p-Smad2/3 blot and the corresponding GAPDH blot in (D) were excluded and only pertinent bands are shown in respective figures. Lanes 1 and 2 of Figure 3.10 (C) are the same as lanes 1 and 2 of Figure 3.11 (D).

Figure 3.13 H_2O_2 induces *Mstn* expression, secretion and signaling. (A) Representative graph showing mRNA expression of *Mstn* and (B) representative gel showing protein levels of *Mstn* in C2C12 cells, untreated (Lane 1), treated with H_2O_2 (0.05 mM) (Lane 2) and ascorbic acid (100 μ M/ml) (Lane 3) for 72 hours in proliferation media. The values are mean $\pm$ S.E of 2 independent experiments. Significant increase (** $p < 0.01$) or decrease (***) $p < 0.001$) in mRNA expression was compared to the untreated control (n=2). GAPDH was used as an internal control for equal protein loading on the gel. Other lanes from the representative *Mstn* blot in (B) were excluded as not being relevant and only pertinent bands from the blots are shown. (C) Level of secreted *Mstn* in the cell culture media from C2C12 cells treated with H_2O_2 (0.05 mM) and ascorbic acid (100 μ M/ml) was determined by EIA. The values are mean $\pm$ S.E of 2 independent experiments. The concentration of *Mstn* is given as ng/ml and the level of significance was determined with respect to the negative control (** $p < 0.01$) (n=2). **H_2O_2 inhibits proliferation and differentiation of C2C12 myoblasts.** (D) C2C12 cells were treated with two different concentrations of H_2O_2 (0.05 mM and 0.1 mM), the cells were fixed at the indicated time and proliferation assay was performed (**** $p < 0.0001$, ^^^ $p < 0.0001$) (n=4). (E) C2C12 cells were cultured in differentiation media in the presence of 0.05 mM H_2O_2 for 48 and 72 hours. The cells were fixed and stained with H & E and images were taken using a Leica upright bright field microscope at 10X magnification (n=2).

The internal control for Figure 3.14 (A) has been inadvertently shown as Tubulin in the thesis, which has now been corrected with the appropriate internal control (GAPDH). The corrected figure and legend are shown below. Original blot of GAPDH shown in (iv) below.

Figure 3.14 Absence of *Mstn* during aging decreases NF- κ B levels, TNF- α and Nox1 expression and increases AOE gene expression in skeletal muscle. (A) Western blotting analysis showing the level of NF- κ B-p65 in protein lysates from Gas muscle of young (6 week old) and aged (2 year old) WT and *Mstn*^{-/-} mice; young WT (Lane 1), young *Mstn*^{-/-} (Lane 2), aged WT (Lane 3) and aged *Mstn*^{-/-} (Lane 4). GAPDH was used as an internal control for equal protein loading on the gel (n=3). mRNA expression analysis in Gas muscle

from young and aged WT and *Mstn*^{-/-} mice was performed. Representative graphs show fold change in the mRNA expression of *TNF-α* and *Nox1* in Young (B) and Aged (C) WT and *Mstn*^{-/-} mice. The values are mean ± SEM of 3 independent experiments. ***p<0.001 denote significant decrease when comparing WT and *Mstn*^{-/-} mice (n=3). Representative graphs show fold change in the mRNA expression of *Sod1* (D) and *Cat* (E). The values are mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001, ****p<0.0001 denote significant increase when comparing young and aged mice and ^^^p<0.0001 denotes significant increase when comparing WT and *Mstn*^{-/-} mice (n=3).

The above is the original GAPDH blot for the corresponding NF-κB (p65) represented in Figure 3.14

- Lane 1- Young WT mouse 1
- Lane 2- Young *Mstn*^{-/-} mouse 1
- Lane 3- Aged WT mouse 1
- Lane 4- Aged *Mstn*^{-/-} mouse 1
- Lane 5- Young WT mouse 2
- Lane 6- Young *Mstn*^{-/-} mouse 2
- Lane 7- Aged WT mouse 2
- Lane 8- Aged *Mstn*^{-/-} mouse 2

This image (iv) shows the original GAPH blot used in corrected Figure 3.14 above. The bands shown are for mouse 2 (lanes 5-8) as the corresponding NF-κB (p65) blot is of mouse 2 as well.

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Figure 4.3 Loss of *Mstn* relieves oxidative stress in *Smad3*^{-/-} muscle.

(A) (i). Representative gel showing protein carbonylation in protein lysates of Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice as detected using the Oxyblot assay kit. (A) (ii). Corresponding densitometric analysis of the gel showing the percentage increase or decrease in carbonylation over and above WT muscle (*p<0.05, **p<0.01); ^^p<0.01: double-KO muscle as compared to *Smad3*^{-/-} muscle. α-tubulin was used as an internal control for equal protein loading on the gel (n=3). The same protein extracts were used in blots shown in Figure 4.3 (A) (i) and Figure 4.4 (A) (i), with one representative α-tubulin shown for both figures. Representative graph showing mRNA expression of *TNF-α* (B) and *Nox1*

(C) in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. **p*<0.05, ***p*<0.01: when compared to the WT muscle; ^*p*<0.01, ^^*p*<0.001: when compared to *Smad3*^{-/-} muscle (n=3). Representative graph showing enzyme assays for XO (D), SOD (E) and GSR (F) performed on whole muscle lysates of Quadriceps muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. **p*<0.05, ***p*<0.01: when compared to the WT muscle; ^*p*<0.01: when compared to *Smad3*^{-/-} muscle (n=3).

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Figure 4.4 *Smad3* is needed for IKKα-mediated activation of NF-κB (p65) by *Mstn*.

(A) (i). Representative Western blot analysis of NF-κB (p65), p-NF-κB (p65), p-IκB-α, IκB-α and IKKα protein levels in whole muscle lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from Biceps femoris muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. Corresponding densitometric analysis of p-NF-κB (p65) (ii), p-IκB-α (iii) and IKKα (iv) protein levels. α-tubulin was used as an internal control for equal protein loading on the gel (n=3). The same protein extracts were used in blots shown in Figure 4.3 (A) (i) and Figure 4.4 (A) (i), with one representative α-tubulin shown for both figures. (B) (i). Western blot analysis of NF-κB (p65), p-NF-κB (p65), p-IκB-α, IκB-α and IKKα protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from C2C12 cells treated with CCM (Lane 1), CMM (Lane 2), SIS3 and CCM (Lane 3) and SIS3 and CMM (Lane 4). Corresponding densitometric analysis of p-IκB-α (ii) and IKKα (iii) protein levels. α-tubulin was used as an internal control for equal protein loading on the gel (n=2).

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Figure 4.6 ROS is generated through p38 and ERK MAPK pathways in *Smad3*^{-/-} muscle.

Western blot analysis of p-p38 MAPK (A), p38 α/β (A), p-TAK1 (A), TAK1 (A), p-MKK3/6 (A), p-JNK (B), JNK (B), p-MKK4 (B), p-ERK1/2 (C), ERK1/2 (C) and p-MEK1/2 (C) protein levels in protein lysates obtained from Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α-tubulin was used as an internal control for equal protein loading on the gels (n=3). The same protein extracts were used in blots shown in Figure 4.6 and Figure 4.9, with one representative α-tubulin shown for Figure 4.6 (A), (B) and between Figure 4.6 (C) and Figure 4.9 (A) (ii).

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Figure 4.9 The JAK/STAT pathway participates in XO-mediated ROS production in *Smad3*^{-/-} mice.

(A) (i). Representative graph showing mRNA expression of IL-6 in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. **p*<0.05, ***p*<0.01, *****p*<0.0001: when compared to the WT muscle; ^*p*<0.05: when compared to *Smad3*^{-/-} muscle (n=3). (A) (ii) Western blot analysis of IL-6 protein level in Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α-tubulin was used as an internal control for equal protein loading on the gel (n=3). The same protein extracts were used in blots shown in Figure 4.6 and Figure 4.9, with one representative α-tubulin shown for Figure 4.6 (A), (B) and between Figure 4.6 (C) and Figure 4.9 (A) (ii). (B) Western blot analysis of JAK1, JAK2, JAK3, p-STAT3, STAT3 and p-STAT5 protein levels in protein lysates obtained from Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α-tubulin was used as an internal control for equal protein loading on the gel (n=3). (C) Densitometric analysis of

Western blots to detect the protein levels of p-STAT3 (Tyr705) (i) and p-STAT5 (ii) in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05: when compared to the WT muscle; ^p<0.05: when compared to *Smad3*^{-/-} muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3).

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Figure 4.13 Enhanced binding of CHOP mediates MuRF1 up-regulation in *Smad3*^{-/-} mice.

(A) EMSA was performed using nuclear extracts from WT and *Smad3*^{-/-} Biceps femoris muscle. (i) Left panel, representative gel showing increased CHOP binding in *Smad3*^{-/-} muscle as indicated by the shifted band in Lane 3 (Lane 1-oligo only, Lane 2-WT, Lane 3-*Smad3*^{-/-}). EMSA was also performed with nuclear extracts from WT and *Smad3*^{-/-} Biceps femoris muscle pre-incubated with CHOP specific antibody. (ii) Right panel, representative gel showing the diminished band intensity of CHOP specific band. Also, the disappearance of the shifted band in the nuclear extracts incubated with 500X concentration of competitor oligos is observed. (Lane 1-oligo only, Lane 2-WT, Lane 3-*Smad3*^{-/-}, Lane 4-WT with CHOP antibody, Lane 5-*Smad3*^{-/-} with CHOP antibody, Lane 6-WT with 500X competitor oligos, Lane 7-*Smad3*^{-/-} with 500X competitor oligos). All lanes are from one representative gel. Lanes 1, 2 and 3 in panel (i) are also shown in Lanes 1, 2 and 3 in panel (ii) to allow for direct comparison. (B) Representative agarose gel image showing the binding of CHOP to MuRF1 promoter (Lanes 7 and 8), as assessed by ChIP, in C2C12 myoblasts treated with SIS3 for 48 hours during differentiation. The relative amounts of the input DNA in both untreated (Lane 1) and SIS3 treated (Lane 2) myoblasts were also assessed. Both no antibody (No Ab) (Lanes 3 and 4) and isotype specific IgG (Lanes 5 and 6) controls are shown. (C) (i) Representative graph showing mRNA expression of *Chop* in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05, **p<0.01: when compared to the WT muscle; ^^^p<0.0001: when compared to *Smad3*^{-/-} muscle (n=3). (C) (ii) Western blot analysis of CHOP and MuRF1 protein levels in Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (D) Western blot analysis of CHOP protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) and MuRF1 levels in whole cell lysates obtained from untreated C2C12 myoblasts (Control) (Lane 1) and SIS3 treated (for 48 hours during differentiation) myoblasts (Lane 2). α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

This image (v) shows the original blot from the EMSA performed in Figure 4.13

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Figure 5.4 STZ treatment up-regulates *Mstn* and affects Akt phosphorylation. (A) Representative graph (i) showing mRNA expression of *Mstn* and representative Western blot (ii) showing protein levels of *Mstn*, p-Smad2/3 and Smad2/3 in WT-C and WT-STZ Gas muscle (** $p < 0.001$; Lane 1-WT-C, Lane 2- WT-STZ). GAPDH was used as an internal control for equal protein loading on the gel ($n=7$). (B) Representative Western blot showing p-Akt1/2/3 and Akt1/2/3 protein levels in WT-C (Lane 1), WT-STZ (Lane 2), *Mstn*^{-/-}-C (Lane 3) and *Mstn*^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as an internal control for equal protein loading on the gel ($n=7$). The same protein extracts were used in blots shown in Figure 5.4 (B), Figure 5.8 (A) and Figure 5.15 (i), with one representative GAPDH shown.

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Figure 5.5 FoxA2 mediates up-regulation of *Mstn* in response to STZ. (A) Representative graph (i) showing mRNA expression of *FoxA2* and representative Western blot (ii) showing protein levels of *FoxA2* in protein lysates from Gas muscle of WT-C, WT-STZ, *Mstn*^{-/-}-C and *Mstn*^{-/-}-STZ mice (** $p < 0.01$, *** $p < 0.001$ when compared to WT-C muscle; Lane 1-WT-C, Lane 2-WT-STZ, Lane 3- *Mstn*^{-/-}-C, Lane 4- *Mstn*^{-/-}-STZ). GAPDH was used as an internal control for equal protein loading on the gel ($n=7$). (B) EMSA was performed using nuclear extracts from WT-C and WT-STZ BF muscles. (i) Left panel, representative gel

showing increased FoxA2 binding upon STZ treatment in WT mice as indicated by the shifted band in Lane 3 (Lane 1-oligo only, Lane 2-WT-C, Lane 3-WT-STZ). (ii) Right panel, representative gel showing the disappearance of the shifted band in nuclear extracts of WT-STZ BF muscles incubated with increasing concentrations of competitor oligos (100 X and 500 X). Supershift assays were performed with nuclear extracts from WT-STZ BF muscles pre-incubated with FoxA2 specific antibody. Supershift of the FoxA2 specific band is observed. (Lane 1-oligo only, Lane 2-WT-STZ, Lane 3-with 100 X competitor oligo, Lane 4-with 500 X competitor oligo, Lane 5-with FoxA2 antibody) (n=3). All lanes are from one representative gel. Lanes 1 and 3 in panel B (i) are also shown in Lanes 1 and 2 of panel B (ii) to allow for direct comparison. Other lanes from the representative EMSA blot were excluded as not being relevant to the comparison illustrated here so that only pertinent lanes from the original blots are shown. (C) Representative agarose gel image showing the binding of FoxA2 to 1.7kb murine *Mstn* promoter (1.7P) (Lanes 9 and 10), as assessed by ChIP. The relative amounts of both the control and *Mstn* promoter in the input were also assessed (Lanes 3 and 4). Both no antibody (No Ab) (Lanes 5 and 6) and isotype specific IgG (Lanes 7 and 8) controls are shown. (D) Assessment of promoter-luciferase reporter activity in C2C12 myoblasts transfected with either pGL3-basic and pFLAG-CMV2 empty vectors or 1.7 kb mouse *Mstn* promoter construct (1.7P) and pFLAG-CMV2 or 1.7P and FoxA2 expression vector (pFLAG-FoxA2), together with the control Renilla luciferase vector pRL-TK. (****p<0.0001, ^^^p<0.0001; n=3).

This image (vi) shows the original blot of the EMSA represented in Figure 5.5 (604 is the mouse number for WT-C and 601 is mouse number for WT-STZ)

Figure 5.8 Absence of *Mstn* abrogates STZ-induced changes in p63 and REDD1 signaling. (A) Representative Western blot showing REDD1, OGG1, p53 and p63 protein levels in WT-C (Lane 1), WT-STZ (Lane 2), *Mstn*^{-/-}-C (Lane 3) and *Mstn*^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as an internal control for equal protein loading on the gel (n=5). The same

protein extracts were used in blots shown in Figure 5.4 (B), Figure 5.8 (A) and Figure 5.15 (i), with one representative GAPDH shown. IHC for REDD1 (B) and OGG1 (C) was performed on cryosections of TA muscle isolated from WT-C and WT-STZ (i) and *Mstn*^{-/-}-C and *Mstn*^{-/-}-STZ (ii) mice. The fluorescence was viewed under a Leica upright microscope and images were taken at 5X magnification. Increased fluorescence (green) indicates increase in expression of REDD1 (B) or OGG1 (C); scale bar represents 100 μ m (n=3).

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Due to inadvertent human error the original image used in the WT-CMM1 (8-oxo-dG) panel for Figure 5.12 was incorrect. The corrected figure is shown below.

This is the original image (vii-correct) for WT-CMM1 (8-oxo-dG) panel in Figure 5.12

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Figure 5.13 STZ treatment leads to SSB and DSB in DNA in proliferating myoblasts as detected by Comet assay. (A) Comet assay was performed by alkaline lysis method (i) and neutral lysis method (ii) on C2C12 myoblasts treated with STZ1, Ant1 or Ant1+STZ1 for 48 hours during proliferation. Representative images showing the head and tail of comets formed during various treatments, stained using PI. DNA fragmentation is observed by the formation of tail in the comet as indicated by the arrows. Comet images were taken using a Leica upright microscope (n=3), and have been enlarged to show the comets clearly. Quantitative analysis of various parameters of comet assay (analysis was performed on original images) following alkaline lysis (B) and neutral lysis (C); comet length and comet height B (i) and C (i), comet mean intensity B (ii), head mean intensity B (ii) and C (ii), % DNA in head and % DNA in tail B (iii) and C (iii), tail length B (iv) and C (ii), tail area B (iv), tail moment and olive moment B (v) and C (iv). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to untreated myoblasts; ^ $p < 0.01$, ^^ $p < 0.001$, ^^^ $p < 0.0001$ when compared to STZ1-treated myoblasts (n=3).

The following images (viii) are the original images (no enlarging) for Figure 5.13 A (i) and (ii)

Alkaline-Media

Alkaline-STZ1

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Figure 5.14 *CMM treatment leads to SSB and DSB in DNA in proliferating myoblasts as detected by Comet assay.* (A) Comet assay was performed by alkaline lysis method (i) and neutral lysis method (ii) on C2C12 myoblasts treated with CCM, CMM1, CMM2, Ant1+CCM, Ant1+CMM or Ant1+CMM2 for 48 hours during proliferation. Representative images showing the head and tail of comets formed during various treatments, stained using PI. DNA fragmentation is observed by the formation of tail in the comet as indicated by the arrows. Comet images were taken using a Leica upright microscope (n=3), and have been enlarged to show the comets clearly. Quantitative analysis of various parameters of comet assay (analysis was performed on original images) following alkaline lysis (B) and neutral lysis (C); comet length and comet height B (i) and C (i), comet mean intensity B (ii), head mean intensity B (ii) and C (ii), % DNA in head and % DNA in tail B (iii) and C (iii), tail length B (iv) and C (ii), tail area B (iv), tail moment and olive moment B (v) and C (iv). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to CCM-treated myoblasts; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$, ^^^^ $p < 0.0001$ when compared to CMM1- or CMM2-treated myoblasts (n=3).

Due to inadvertent human error, the original images used in Figure 5.14 A (i) Alkaline Ant1+CCM2, A (ii) Neutral (Ant1+CCM), and A (ii) Neutral Ant1+CMM1 were incorrect. The correct images have been inserted now and the revised figure is shown below.

The following images (ix) are the original images (no enlarging) used in the corrected Figure 5.14 A (i) and (ii)

Alkaline

CCM

CMM1

CMM2

Ant1+CCM

Ant1+CMM1

Ant1+CMM2

Neutral

CCM

CMM1

CMM2

Ant1+CCM

Ant1+CMM1

Ant1+CMM2

Figure 5.15 *STZ treatment alters genes involved in DNA repair in vivo.* (i) Representative gel showing protein levels and (ii) (iii) densitometric analysis of XRCC1, p-Histone H2A.X and H2A.X in WT-C (Lane 1), WT-STZ (Lane 2), Mstn^{-/-}-C (Lane 3) and Mstn^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as loading control on the gel (n=5). The same protein extracts were used in blots shown in Figure 5.4 (B), Figure 5.8 (A) and Figure 5.15 (i), with one representative GAPDH shown.

NANYANG
TECHNOLOGICAL
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**ROLE OF MYOSTATIN IN OXIDATIVE STRESS IN
SKELETAL MUSCLE**

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SCHOOL OF BIOLOGICAL SCIENCES

2013

**ROLE OF MYOSTATIN IN OXIDATIVE STRESS IN
SKELETAL MUSCLE**

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School of Biological Sciences

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ABSTRACT

Skeletal muscle is a metabolically active tissue that is highly susceptible to wasting via oxidative stress. Oxidative stress, caused by excessive generation of reactive oxygen species, leads to skeletal muscle wasting during various diseases including cancer, diabetes and sarcopenia via various signaling molecules including TNF- α , NADPH oxidase, NF- κ B, MAPKs and IL-6. However, the exact mechanism involved in oxidative stress-induced muscle wasting is not known. In the recent years, Myostatin, a member of the TGF- β superfamily and a negative regulator of skeletal muscle growth and development, has been identified as a potent inducer of muscle wasting. Here, in this thesis using various *in vitro* and *in vivo* approaches, I present that Myostatin acts as a pro-oxidant leading to skeletal muscle wasting.

Firstly, this thesis demonstrates that Myostatin induces reactive oxygen species in skeletal muscle cells through TNF- α signaling via NF- κ B and NADPH oxidase. In addition, Myostatin and TNF- α are components of a 'feed forward' loop in which Myostatin triggers reactive oxygen species generation via TNF- α and the elevated TNF- α in turn stimulates Myostatin expression. The sustained reactive oxygen species production leads to increased proteasomal-mediated degradation of proteins resulting in skeletal muscle wasting. Genetic inactivation of Myostatin in young and aged mice increased the basal Antioxidant Enzyme levels and lowered NF- κ B levels, thus offering resistance to oxidative stress. Since aged Myostatin^{-/-} mice also have lower levels of NF- κ B in the muscle, I propose that Myostatin would be a good candidate to target during sarcopenia.

To further understand Myostatin signaling of NF- κ B and reactive oxygen species, Smad3^{-/-} mice was used as a model since Smads are crucial downstream targets of Myostatin. Recently, our laboratory showed that Smad3^{-/-} mice have pronounced muscle

atrophy which was attributed to the increased Myostatin levels in these mice. Hence, I investigated if Smad3^{-/-} mice have increased reactive oxygen species and our results indicated that indeed these mice have elevated oxidative stress in skeletal muscle. Inactivation of Myostatin in the absence of Smad3 partially alleviates the oxidative stress. Even though, increased Myostatin in Smad3^{-/-} mice induced excessive reactive oxygen species, NF-κB signaling was not activated in these mice. This led us to propose that Smad3 is necessary for Myostatin-induced oxidative stress via NF-κB. Reactive oxygen species generation by Myostatin in mice lacking Smad3 was by activation of complex signaling pathways that include p38, ERK MAPK and crosstalk with the JAKs and STATs. Activation of these signaling cascades increased TNF-α, NADPH oxidase and Xanthine oxidase levels resulting in exaggerated reactive oxygen species production in Smad3^{-/-} muscles. The excessive reactive oxygen species generated led to increased binding of CHOP transcription factor to MuRF1 promoter resulting in enhanced muscle atrophy.

To advance our understanding of the effect of Myostatin-induced reactive oxygen species in skeletal muscle, a type 1 diabetes mouse model via Streptozotocin administration was used. During type 1 diabetes, there is pronounced muscle wasting due to excessive oxidative stress also leading to DNA damage. In the recent years, Myostatin has been shown to be up-regulated during Streptozotocin-induced type 1 diabetes in rodents. Since I showed that Myostatin can cause oxidative stress in muscle cells leading to protein degradation, I wanted to investigate if Myostatin can induce DNA damage also. Using an integrative approach from cell cultures to mice, I show that Myostatin can induce DNA damage via p63/REDD1 pathway in skeletal muscle. Moreover, inhibition/inactivation of Myostatin partially rescued DNA damage by

regulating DNA damage/repair mechanisms. Additionally, our results also demonstrate that hypoinsulinemia during type 1 diabetes increased Myostatin levels via FoxA2.

In conclusion, the data presented in this thesis indicates that Myostatin is a pro-oxidant that can induce oxidative stress leading to skeletal muscle wasting, by not only increasing protein degradation but by also causing DNA damage.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
3'	3 prime
5'	5 prime
AA	Amino acid
A band	Anisotropic band
ActRIIB	Activin type II receptor
ADP	Adenosine diphosphate
AF	Alexa Fluor
AIDS	Acquired Immuno Deficiency Syndrome
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
5-ASA	5-Aminosalicylic acid
AS160	Akt substrate 160
AOE	Antioxidant enzyme
ATCC	American Type Culture Collection
ATF-2	Activating transcription factor-2
ATP	Adenosine triphosphate
β-gal	β-galactosidase
BF	Biceps femoris
BMI	Body Mass Index
BSA	Bovine Serum Albumin

Ca ²⁺	Calcium
CAT	Catalase
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CCM	Control conditioned media
Cdk	Cyclin dependant kinase
cDNA	Complementary DNA
CE	Cytoplasmic extract
CEE	Chick embryo extract
ChIP	Chromatin Immunoprecipitation assay
CHO	Chinese hamster ovary
CHOP	C/EBP homology protein
cm	Centimeter
CM-H2DCFDA	5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester
CMM	Conditioned media containing Myostatin
CO ₂	Carbon dioxide
COOH	Carboxylic acid
COPD	Chronic obstructive pulmonary disease
CRP	C-Reactive Protein
CuZnSOD	Copper Zinc superoxide dismutase
Cyt C	Cytochrome C
DAPI	4', 6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DIDS	4-4'-Diisothiocyanatostilbene-2-2'-disulphide

dL	Deciliter
DMD	Duchenne Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
DPI	Diphenylene iodonium
DSB	Double strand breaks
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example
EIA	Enzyme immunoassay
ELISA	Enzyme linked immune sorbent assay
EMSA	Electrophoretic mobility shift assay
eNOS	Endothelial Nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ETC	Electron Transport Chain
EtOH	Ethanol
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FoxA2	Forkhead box protein 2

FoxO1	Forkhead box O1
FoxO3	Forkhead box O3
FRTA	Free-radical theory of aging
g	Gram
G6P	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gas	Gastrocnemius
Gasp-1	Growth and differentiation factor-associated serum protein-1
GDF-8	Growth and Differentiation Factor-8
GFP	Green fluorescence protein
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
GR	Glucocorticoid
GSH	Reduced glutathione
GSR	Glutathione reductase
GSK3	Glycogen synthase kinase 3
GSSG	Oxidized glutathione
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
h	Hour
H & E	Hematoxylin and eosin
Hb	Hemoglobin
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGF	Hepatocyte growth factor
HIV	Human Immunodeficiency Virus
HNF-3 β	Hepatocyte nuclear factor-3 β
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HOCl ⁻	Hypochlorous acid
HRP	Horse radish peroxidase
HS	Horse Serum
HSP	Heat Shock Protein
I band	Isotropic band
ICC	Immunocytochemistry
IFN- γ	Interferon- γ
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
I κ B	Inhibitor κ B
I κ B- α C	I κ B- α Control
I κ B- α SR	I κ B- α super-repressor
IKK	I κ B kinase
IL	Interleukin
IMM	Inner mitochondrial membrane
IPTG	Isopropyl thio- β -galactoside
IRS-1	Insulin receptor substrate-1
IUCAC	Institutional Animal Care and Use Committee
JAK-STAT	Janus kinase-signal transducers and activators of transcription

JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
kDa	Kilo Dalton
L	Liter
LAP	Latency-associated protein
LB	Luria Bertani
Lbx1	Ladybird homeobox 1
L-NAME	<i>N</i> ω-nitro-L-arginine methyl ester
μg	Microgram
μl	Microliter
μM	Micromolar
M	Molar
Mad	Mothers against decapentaplegic
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MB/Mb	Myoblast
MDA	Malonaldehyde
Mef2	Myocyte enhancer factor-2
MEK	MAP kinase/ERK kinase
MEKK1	MAP/ERK kinase kinase 1
mETC	Mitochondrial Electron Transport Chain
mg	Milligram
MgSO ₄	Magnesium sulphate
MHC	Major histocompatibility complex

MIT	Mitochondria
MKK	MAPK kinase
MKP	MAPK phosphatase
ml	Milliliter
mM	Millimolar
MnSOD	Manganese superoxide dismutase
MOPS	3-(N-morpholino)propanesulfonic acid
Mrf4	Myogenic regulatory factor 4
mRNA	Messenger ribonucleic acid
Mstn	Myostatin
Mstn ^{-/-}	Myostatin null
Mstn ^{-/-} -C	Myostatin null Control group
Mstn ^{-/-} -STZ	Myostatin null Streptozotocin group
MT/Mt	Myotube
mtDNA	Mitochondrial Deoxyribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	mTOR Complex 1
MuRF-1	Muscle ring finger-1
Myf5	Myogenic factor 5
MYH	Myosin heavy chain
MyoD	Myogenic differentiation factor
NaCl	Sodium chloride
NAD ⁺	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide dehydrogenase

NADH-Q	Nicotinamide Adenine Dinucleotide dehydrogenase coenzymeQ
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate dehydrogenase
Na ₂ -EDTA	Disodium ethylenediaminetetraacetic acid
NaF	Sodium fluoride
NaOH	Sodium hydroxide
N.C.	Notochord
NE	Nuclear extract
NF-κB	Nuclear factor-kappa B
ng	nanogram
NGS	Normal goat serum
Ni	Nickel
nm	Nanometre
nmoles	Nanomoles
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NAD(P)H oxidase
NP-40	Nonidet P-40
N.T.	Neural tube
NTU	Nanyang Technological University
NUS	National University of Singapore
NUS-CARE	National University of Singapore-Centre for Animal Resources

O ₂	Oxygen
O ₂ ⁻	Superoxide radical
OCT	Optimal cutting temperature
OD	Optical density
OH ⁻	Hydroxyl ion
OGG1	8-Oxoguanine glycosylase
ONOO	Peroxynitrite
8-oxo-dG	8-Oxo-2'-deoxyguanosine
PAGE	Polyacrylamide gel electrophoresis
Pax	Paired box gene
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1 α	Peroxisome proliferator-activated receptor γ coactivator-1 α
PI	Propidium iodide
PIF	Proteolysis inducing factor
PI(3)K	Phosphoinositide 3-kinase
PMSF	Phenylmethanesulfonylfluoride
PPAR β/δ	Peroxisome proliferator-activated receptor β/δ
PPase	Protein phosphatase
P/S	Penicillin/Streptomycin
PVP	Polyvinylpyrrolidone
Quad	Quadriceps

qPCR	Quantitative/Real time Polymerase Chain Reaction
Rb	Retinoblastoma
REDD1	Regulated in development and DNA damage response
RFP	Red fluorescence protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
ROS	Reactive oxygen species
RT	Reverse Transcriptase
RT-qPCR	Reverse Transcriptase-Quantitative/Polymerase Chain Reaction
SAPK	Stress-activated protein kinases
SC	Satellite cell
SDS	Sodium dodecyl sulphate
S.E.	Surface ectoderm
SEM	Standard Error of Mean
shRNA	Short hairpin RNA
Smad	Small mothers against decapentaplegic
SOD	Superoxide dismutase
SP	Signal peptide
SSB	Single strand breaks
STZ	Streptozotocin
TA	Tibialis anterior
TAE	Tris-Acetate EDTA
TAK1	Transforming growth factor- β -activated kinase 1
TBARS	Thiobarbituric acid reactive substrate

TBE	Tris-Borate-EDTA
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween 20
TCA	Trichloro acetic acid
TE	Tris EDTA
TF	Transcription factor
TFSEARCH	Transcription factor search
TGF- β	Transforming growth factor- β
TNF- α	Tumor necrosis factor- α
TNF-R	Tumor necrosis factor-Receptor
TRAF2	TNF receptor associated factor 2
TTFA	Thenoyltrifluoro acetone
U	Units
UQ	Ubiquinone
USA	United States of America
UV	Ultraviolet
V	Volt
VO ₂ max	Maximal oxygen consumption
WT	Wild type
WT-C	Wilt type Control group
WT-STZ	Wilt type Streptozotocin group
XD	Xanthine dehydrogenase
XO	Xanthine oxidase
XRCC1	X-ray repair cross-complementing protein 1
ZnSO ₄	Zinc sulphate

*This thesis is dedicated to my dad, my hero and idol,
whom I miss so much
and to
my mom and husband,
the two most important people in my life*

Ω

CHAPTER 1

LITERATURE REVIEW

1.1 Skeletal muscle

Muscle is a contractile tissue and derived from the mesodermal layer of embryonic germ cells. There are three types of muscle: skeletal, cardiac and smooth muscle. Skeletal muscle makes up 40-50 % of the body weight and there are 640 individually named skeletal muscles, making it one of the largest organs of the human body. Skeletal muscles are voluntary muscles that help in locomotion and movement and play a major role in carbohydrate and lipid metabolism. This section reviews literature on the structure and function of skeletal muscle.

1.1.1 Skeletal muscle structure and function

Skeletal muscle is a highly structured, striated, well-organized body tissue consisting of several distinct compartments. Skeletal muscles articulate into bones via tendons and this aids in locomotion. Skeletal muscle is enclosed by a connective tissue sheath termed the epimysium. A layer of connective tissue, termed the perimysium, further divides the muscle into a number of compartments called fascicles. The fascicles are bound together by a type of connective tissue called fascia. Each fascicle contains a bundle of skeletal muscle fibers, which are themselves enclosed within a third layer of connective tissue, the endomysium (Figure 1.1A) (Seeley *et al.* 2006).

Muscle fibers are made up of smaller units called myofibrils (Figure 1.1B) (Seeley *et al.* 2006). The myofibrils move as the skeletal muscle contracts; and this gives skeletal muscle its functional ability. Myofibrils are composed of repeating sections of sarcomeres, which consists of thick filaments, myosin and thin filaments, actin (Figure 1.1C) (Seeley *et al.* 2006). Sarcomeres are the basic units of muscle and each muscle

cell is surrounded by a sarcolemma, which consists of a true plasma membrane. Myofibrils are connected to the sarcolemma which in turn is connected to collagen in the tendon; thus when a muscle fiber contracts, the strength is transferred to the bone via tendons facilitating movement.

Each sarcomere has two different banding patterns, A (anisotropic) band and I (isotropic) band, based on the pattern they exhibit when exposed to polarized light. The A band is located in the center of the sarcomere and extends to the entire length of thick (myosin) filaments. It consists of M line in the central portion where the thick filament is connected to its neighbor and helps in stability. H zone is the lighter region on either side of the M line. The I band contains thin filaments made up of actin polymers. A Z disc made up of actinin connects two thin filaments (Figure 1.2) (Marieb 2006).

Figure 1.1 Skeletal muscle structure

(A) Skeletal muscle is made up of fascicles, which are enclosed in the perimysium. Fascicles are made up of muscle fibers, which are enclosed in the endomysium. The entire skeletal muscle is enclosed in the epimysium. (B) Muscle fibers are made up of myofibrils, which are in turn made up of sarcomeres. (C) Sarcomeres are composed of actin and myosin filaments. Figure modified from Seeley *et al.* 2006

Figure 1.2 Sarcomere structure

Each sarcomere has two different banding patterns, A band and I band. M line is in the central portion and H zone is the lighter region on either side of the M line. The I band contains thin filaments made up of actin polymers. A Z disc connects two thin filaments. Figure modified from Marieb 2006.

1.1.2 Myogenesis

Myogenesis is the process of muscle development which involves various stages including delamination and migration of muscle progenitor cells, proliferation and differentiation of muscle cells, specific muscle formation and satellite cell development. This section reviews on embryonic myogenesis, post-natal myogenesis and muscle regeneration.

1.1.2.1 Embryonic myogenesis

Skeletal muscle originates from the paraxial mesoderm during embryogenesis. The formation of somites is the first step in skeletal myogenesis. The presomitic mesoderm is segmented into somites, which are found on either side of the neural tube. The dorsal part of the somites gives rise to dermomyotome, which forms the dermatome (dermis) and myotome (skeletal muscle). The epaxial dermomyotome gives rise to the deep back muscles, whereas the hypaxial dermomyotome gives rise to the rest of body musculature and the limb muscles (Figure 1.3A) (Buckingham *et al.* 2003).

As shown in Figure 1.3B, muscle progenitor cells delaminate from the epithelium of the hypaxial dermomyotome and migrate into the limb field with the help of Hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-met (Dietrich *et al.* 1999). Pax3, a transcription factor regulates c-met, however, it is unknown as to how it functions in the embryo (Epstein *et al.* 1996). Mice lacking either HGF (Schmidt *et al.* 1995) or c-met (Bladt *et al.* 1995) or Pax3 (Tajbakhsh *et al.* 1997) have no limb muscles and cells do not delaminate from the hypaxial dermomyotome. Lbx1 is another transcription factor which is involved in migration of cells from the somites (Schafer and Braun, 1999). Once the muscle precursor cells have migrated, they proliferate extensively and they begin to express *MyoD* and *Myf5*, which are the myogenic determination genes

(Tajbaksh and Buckingham 1994). In the absence of these two factors, cells that would normally form muscle would form other cell types (Kablar *et al.* 1999). Mutation of the *MyoD* gene in mice leads to delayed myogenesis, however the musculature is normal, while mutation in *Myf5* leads to slightly reduced muscle fiber size and muscle mass with normal musculature (Kablar *et al.* 1997). However, mice with mutation of both *MyoD* and *Myf5* lack skeletal muscle completely (Rudnicki *et al.* 1993).

The presence of *Myogenin*, *Mrf4*, *Mef2* and *Six* is necessary for activation of the differentiation programme of the muscle cells (Figure 1.3B). The formation of specific muscles depends on *Lbx1* (Schafer and Braun 1999; Gross *et al.* 2000), *Mox2* (Mankoo *et al.* 1999) and *c-met* (Maina *et al.* 2001) as mutation of either of these genes in mice shows varied effect on specific muscles.

Figure 1.3 Somitogenesis and embryonic myogenesis

(A) Schematic representation of somitogenesis. (B) Formation of skeletal muscles in the limb. Different stages and the various genes involved are indicated. N.T. Neural tube; N.C. Notochord; S.E. Surface ectoderm. The dermomyotome gives rise to the epaxial myotome, which in turn gives rise to back musculature, and hypaxial myotome, which gives rise to limbs and body musculature. Figure modified from Buckingham *et al.* 2003.

1.1.2.2 Post-natal myogenesis

The number of muscle cells is pre-determined at birth and will not increase or decrease during the lifespan of an individual. The increase in muscle weight and size is attributed to increased length and diameter (hypertrophy), respectively, and not increase in muscle cell number (hyperplasia). Post-natal muscle growth is very rapid; the total skeletal muscle mass increases several folds in the first 3 weeks in mice (Gokhin *et al.* 2008). Muscle hypertrophy occurs by the addition of myonuclei to the existing muscle fibers. These new myonuclei are formed from a population of muscle cells known as satellite cells (SCs). The SCs are found in between the basal lamina and the sarcolemma of individual muscle fibers. SCs are often referred to as muscle stem cells, and are located throughout the skeletal muscle tissue. SCs were first discovered in 1961 by Katz and Mauro while studying the skeletal muscles of the frog (Katz 1961; Mauro and Adams 1961). At birth, SCs account for 30 % of the sub-laminar nuclei in mice, however, later in adult mice, the number rapidly decreases to about 5 % (Bischoff and Heintz 1994). The origin of SCs is still under debate and many articles are published on the specific origin of SCs. However, generally SCs are a progenitor population of cells that express Pax3 and Pax7 that arise from the central portion of the dermomyotome and maintained throughout embryogenesis within the developing skeletal muscle (Kassar-Duchossoy *et al.* 2005; Relaix *et al.* 2005). Later in fetal development, the resident progenitor population generates cells in a SC position around myofibers, which are marked by the expression of Pax7 (Kassar-Duchossoy *et al.* 2005; Relaix *et al.* 2005).

SCs are generally quiescent and reside in the niche between basal lamina and sarcolemma of their associated muscle fibers. Quiescent SCs are activated in response to stress, exercise, myotrauma or injury (Rosenblatt *et al.* 1994; Grounds 1998, Hawke *et al.* 2003; Yan *et al.* 2003); they enter the cell cycle to undergo further proliferation.

Quiescent SCs express a range of proteins including CD34 (Beauchamp *et al.* 2000), c-met (Cornelison and Wold 1997) and M-cadherin (Irintchev *et al.* 1994) (Figure 1.4A). Pax7 is a marker for both quiescent and activated proliferating SCs (Cornelison *et al.* 2001; Zammit *et al.* 2004). The proliferating SCs start expressing *Myf5* and/or *MyoD*, depending on their fate, as SCs have the capacity to form differentiated myotubes as well as the ability to self-renew their population (Weissman *et al.* 2001). The SCs that are committed to differentiation lose Pax7 expression and begin to express *MyoD* and the proliferating myoblasts fuse to form myotubes and further mature into myofibers by expressing Myogenin; these cells are the fusion-competent cells. The fusion-incompetent cells or the self-renewing population of cells are a small portion of cells which continue expressing Pax7 while down-regulating *MyoD* expression (Figure 1.4B) (Olguin and Olwin 2004).

The fusion-competent cells proliferate and fuse into myotubes, and further mature into myofibers, thus leading to hypertrophy. The most common type of muscle hypertrophy occurs due to physical training and exercise, by the induction of SC activation and increase in muscle protein synthesis (Marini and Veicsteinas 2010). The signals which regulate SC activation and number are not fully understood. Various articles have shown that factors such as HGFs (Tatsumi *et al.* 1998), Nitric oxide synthase (NOS) (Anderson 2000; Tatsumi *et al.* 2002; Tatsumi *et al.* 2006), Insulin-like growth factor (IGF-1), Fibroblast growth factor (FGF) (Johnson and Allen 1995), and various other factors are involved in activation and proliferation of SCs in skeletal muscle (Brzoska *et al.* 2011). IGF-1 has been shown to stimulate proliferation of primary SCs isolated from transgenic mice overexpressing IGF-1. The increase in SC number is shown to be associated with increased G1/S cell cycle progression and the activation of the phosphoinositide 3-kinase (PI(3)K)/Akt signaling pathway (Chakravarthy *et al.* 2000). IGF-1 has also been

shown to induce skeletal muscle hypertrophy via the PI(3)K/Akt/mammalian target of rapamycin (mTOR) and PI(3)K/Akt/ glycogen synthase kinase 3 (GSK3) pathways (Rommel *et al.* 2001). Binding of IGF-1 to its receptor leads to auto-phosphorylation and activation of its intrinsic tyrosine kinase, leading to phosphorylation of insulin receptor substrate (IRS). Phosphorylated IRS then activates PI(3)K and eventually activation of Akt. Akt inhibits protein degradation by phosphorylating thus repressing the transcription factors of the FoxO family, and stimulates protein synthesis via mTOR and GSK3 β .

Post-natal myogenesis also includes the adult skeletal muscle's powerful ability to regenerate and repair itself, especially in response to injury. The repair process has been shown to be associated with the SC population present within the skeletal muscle. The process of muscle regeneration will be reviewed in the next section.

Figure 1.4 Post-natal myogenesis: Satellite cell (SC) activation, differentiation and self-renewal

(A) SCs normally exist in a quiescent state and are activated upon injury or myotrauma. Quiescent SCs express CD34, Pax7, Myf5 and β -gal. Upon activation, *MyoD* expression is up-regulated along with the expression of Pax7. The SCs committed to differentiation express Myogenin and fuse into myotubes and further mature into myofibers. Figure modified from Zammit *et al.* 2006 (B) SCs expressing *MyoD* with a loss of Pax7 are committed towards differentiation and are fusion-competent cells. The fusion-incompetent cells or the self-renewed population of SCs express Pax7 with down-regulation of *MyoD*. Figure modified from Zammit *et al.* 2004.

1.1.2.3 Muscle regeneration

As mentioned earlier, skeletal muscle has the remarkable property to regenerate itself upon injury or trauma. The repair process is multi-step and includes three steps: degeneration, regeneration and remodeling. During the degenerative phase, chemotactic factors are released by the necrotic muscle, which promotes phagocytosis of fiber debris by recruitment of inflammatory cells in the damaged muscle area (Figure 1.5). The activation of SCs also occurs at this stage (Tidball 1995; Tidball 2005). During the regeneration phase, the activated SCs give rise to myoblasts, which then proliferate and differentiate to repair the damaged myofiber or fusing together to form nascent myofibers (Figure 1.5). During the remodeling phase, the nascent myofibers undergo maturation by increasing in size and the infiltrated fibroblasts form the extra-cellular matrix (ECM). Excessive accumulation of ECM leads to scar tissue formation during muscle regeneration (Sato *et al.* 2003).

Figure 1.5 Skeletal muscle regeneration

Myotrauma activates SCs and the inflammatory response begins. Macrophages and SCs migrate to the site of injury; the activated SCs proliferate at the injury site and the resulting myoblasts (MB) fuse with the damaged myofiber or form a new myotube. Figure modified from McCroskery *et al.* 2005.

1.1.3 Muscle metabolism

The main function of skeletal muscle is to provide movement by contraction. In order for muscles to contract, energy in the form of adenosine triphosphate (ATP) must be available in the muscle fiber. However, the intracellular ATP depletes very quickly and various other metabolic pathways are activated to supply the ATP required by catabolizing different molecules including glucose, lipids, glycogen and phosphocreatine (Sahlin *et al.* 1998). Glucose is an essential energy substrate and is stored as glycogen in skeletal muscle (Graham *et al.* 2010). Through the metabolic process of glycogenolysis, glycogen is broken down to release glucose. ATP is then generated from glucose by cellular respiration. Glucose can be broken down by either aerobic (oxidative phosphorylation) or anaerobic (substrate level phosphorylation) pathways. Glucose released from glycogen is further broken down to pyruvic acid, and two ATP molecules are generated without the use of oxygen. During this anaerobic process, pyruvic acid is then converted to lactic acid. Lactic acid can be converted to pyruvic acid with the help of liver enzymes and the presence of oxygen. This pyruvic acid can enter the mitochondria and aid in production of ATP. Anaerobic pathway is relatively rapid, and does not require oxygen, however, the ATP production is much lesser when compared to the aerobic pathway. The lactic acid produced by anaerobic pathway diffuses out of the muscle cell into the bloodstream and is subsequently absorbed by the liver, but some of the lactic acid remains in the muscle fibers, where it contributes to muscle fatigue. During strenuous exercise, a lot of ATP is needed and the body tries to produce a large amount of ATP via the anaerobic pathway. ATP is also generated using oxygen, but the ATP production via aerobic respiration is slower. Hence, after exercise, the liver and muscles need to convert the excessive lactic acid produced back to pyruvic acid.

Based on the metabolic activities, muscle fibers are divided into 3 types: Type I-slow oxidative fibers, Type IIa-fast oxidative/glycolytic fibers and Type IIb-fast glycolytic fibers. Type I fibers are also known as slow twitch fibers. They are red in color due to the presence of large volumes of myoglobin, more oxygen, and have more number of mitochondria, hence these are oxidative fibers. They are resistant to fatigue and are capable of producing repeated low level contractions by producing large amounts of ATP through aerobic metabolic cycle. Type IIa fibers are also sometimes known as fast oxidative fibers and are a hybrid of type I and II fibers. These fibers contain a large number of mitochondria and myoglobin, hence their red colour. They manufacture and split ATP at a fast rate by utilising both aerobic and anaerobic metabolism and so produce fast, strong muscle contractions, although they are more prone to fatigue than type I fibers. Often known as fast glycolytic fibers, Type IIb fibers are white in colour due to a low level of myoglobin and they contain less number of mitochondria. They produce ATP at a slow rate by anaerobic metabolism and break it down very quickly. This results in short, fast bursts of power and rapid fatigue. Type IIb fibers can be converted into type IIa fibers by resistance training (Kramer *et al.* 1998).

1.2 Reactive oxygen species (ROS) and skeletal muscle

Since skeletal muscle is a metabolically active tissue. Reactive oxygen species (ROS) are produced as a by-product in the muscle. ROS have both physiological and non-physiological roles in muscle. ROS play a double-edged sword; low-dose of ROS is necessary for cell signaling while high dose causes toxicity. Excessive generation of ROS leads to oxidative stress, eventually causing skeletal muscle wasting. This section reviews on ROS, roles of ROS in skeletal muscle and various mechanisms involved in induction of ROS.

1.2.1 ROS, Antioxidant enzymes (AOEs) and their generation

1.2.1.1 ROS generation

ROS are present in all tissues, especially produced in the mitochondrial respiratory chain. ROS are molecules or ions formed by the incomplete one-electron reduction of oxygen. These reactive oxygen intermediates include singlet oxygen, superoxides, peroxides, hydroxyl radical, and hypochlorous acid. ROS are generated through the TNF- α pathway, NAD(P)H oxidase (Nox) pathway, eNOS (endothelial Nitric Oxide Synthase) pathway, Xanthine oxidase (XO) pathway and ETC (Electron Transport Chain) in mitochondria (mETC) (Figure 1.6) (Cai and Harrison 2000).

1.2.1.2 Physiological roles of ROS

Excessive production of ROS results in pathology of many diseases, but in recent years it has become apparent that low levels of hydrogen peroxide (H_2O_2) may be required for normal cellular functioning and intracellular signaling. This physiological ROS is generated mainly at the plasma membrane by Nox (Veal *et al.* 2007). Recently, contrary to the ‘bad boy’ reputation of ROS, it has been found to be essential for the development of immune cells in the fly (Theopold 2009).

Figure 1.6 Major pathways of ROS generation and metabolism

Incomplete reduction of oxygen (O_2) leads to formation of superoxide radical ($O_2^{\cdot-}$). As a result of the ROS generated, AOE production is triggered in the cells. $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase, which is further broken down into water (H_2O) and O_2 by catalase and glutathione peroxidase and the antioxidant, glutathione reductase. Figure modified from Aitken and Roman 2008.

ROS generation is a ubiquitous phenomenon and at relatively low cellular levels, ROS influences metabolism in physiological conditions. ‘Redox homeostasis’ is maintained by ROS; coupled oxidation-reduction reactions are required for normal cell survival. Along with growth factors and chemokines, ROS participate in a cascade of events including neutrophil and macrophage invasion during inflammation leading to muscle regeneration and repair (Tidball 2005). ROS generated during exercise promote mitochondrial biogenesis, important for muscle differentiation, through peroxisome proliferator-activated-receptor- γ -coactivator-1 α -(PGC-1 α) (Adhihetty *et al.* 2003). The differentiation of myoblasts is also capable of redirecting the cell through different signaling pathways including Ca²⁺ signaling, MAPK pathways, AMPK signaling and ROS. ROS can trigger and/or affect Ca²⁺ signaling, FoxO and NF- κ B transcription factors which are relevant to skeletal muscle cells' homeostasis and adaptation (Figure 1.7) (Barbieri and Sestili 2012). ROS may be also involved in the activation of ‘insulin-like’ metabolic effects by activating other non-insulin-initiated signaling pathways like the stimulation of glucose transport in skeletal muscle during exercise (Coderre *et al.* 1995; Lund *et al.* 1995).

1.2.1.3 Generation of Anti-oxidant enzymes (AOEs)

Under normal physiological conditions, the intracellular Antioxidant Enzymes (AOEs) are capable of detoxifying the ROS produced due to cellular metabolism (Figure 1.6). The superoxide radicals produced are immediately acted upon by superoxide dismutase (SOD) and converted to hydrogen peroxide (H₂O₂), which is further broken down into water and oxygen by catalase (CAT) and glutathione peroxidase (GPx) and the antioxidant, glutathione reductase (GSR). It has been shown that muscle cells do regulate antioxidant defences in response to oxidative stress during the process of differentiation (Franco *et al.* 1999).

Figure 1.7 Physiological and non-physiological roles of ROS in skeletal muscle

Low levels of physiological ROS activate PGC-1 α , AMP-activated protein kinase (AMPK), and mitogen-activated protein kinase (MAPK), which control oxidative metabolism, mitochondrial biogenesis, and generation of antioxidant enzymes. Slight ROS accumulation also promotes the phosphorylation of many proteins involved in the muscle signaling responses. Low ROS levels induce up-regulation of IGF-1, which has beneficial effects in muscle protein balance and contributes to the development of an oxidant-resistant phenotype, therefore preventing oxidative damage and chronic diseases. Thus, low levels of ROS elicit positive effects on physiological muscle responses. In contrast, high levels of ROS cause functional oxidative damages of proteins, lipids, nucleic acids and cell components, induce a significant rise of intracellular [Ca²⁺], and promote signaling cascades for apoptosis or autophagy via NF- κ B or FoxO. Hence ROS induces inflammation, muscle atrophy, sarcopenia, muscle wasting and myopathies. Figure modified from Barbieri and Sestili 2012.

1.2.1.4 Pathophysiological roles of ROS

Skeletal muscle cells produce ROS transiently in response to a variety of diverse stimuli, such as intense contractile activity (Reid *et al.* 1992; Diaz *et al.* 1993), heat stress (Zuo *et al.* 2000), acute osmotic stress (Martins *et al.* 2008), stretch (Cheng *et al.* 1995), cytokines, hormones, growth factors (Goossens *et al.* 1995; Thannickal *et al.* 2000; Thannickal and Fanburg 2000; Espinosa *et al.* 2009). Considering the numerous stimuli involved in ROS production and also their lack of chemical specificity, it is hard to explain the exact role of ROS in skeletal muscle physiology even though many studies have reported its importance in normal skeletal muscle growth and differentiation, as explained in Section 1.2.1.2. Moderate muscle contraction does not result in elevated levels of ROS, implying that the intracellular redox balance was maintained. Intense muscle contractions have been shown to elevate levels of H₂O₂ and NO (Silveira *et al.* 2003). H₂O₂ is a potent pro-oxidant that causes excessive damage to cells at increasing concentration by generating hydroxyl groups. Different muscles have specific responses to ROS. The oxidative injury by H₂O₂ to differentiated myotubes is greater than to undifferentiated myoblasts (Franco *et al.* 1999). The increased susceptibility of myotubes to H₂O₂ correlated with the decreased AOE levels as the muscle cells differentiated, both at the transcript and activity level. C2C12 myoblasts when exposed to H₂O₂, showed dose-dependent cell death and concomitant loss of total cellular protein (Escobedo *et al.*, 2004).

An imbalance between the systemic manifestation of ROS and the body's ability to readily detoxify the reactive intermediates or to repair the resulting damage leads to a pathological condition. Oxidative stress has been shown to be involved in the development or exacerbation of a number of diseases/pathological conditions including cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, cardiovascular

diseases, muscular dystrophy and sarcopenia (Halliwell 2007; Valko *et al.* 2007; Ramond *et al.* 2011). Persistence of ROS sustained by infiltrated neutrophils may cause oxidative damage to differentiating myoblasts and myotubes (Barbieri and Sestili 2012). ROS also target mitochondria and mitochondrial DNA (mtDNA) thus blocking myogenic differentiation (Rochard *et al.* 2000). Extremely high ROS levels can cause oxidative damage to proteins, lipids, nucleic acids and cell components, increase calcium signaling and promote signaling cascades for apoptosis or autophagy via NF- κ B or FoxO. Hence ROS induces inflammation, muscle atrophy, sarcopenia, muscle wasting, and myopathies (Figure 1.7) (Barbieri and Sestili 2012).

1.2.1.5 Role of ROS in DNA damage

As stated earlier, exaggerated ROS levels can cause damage to nucleic acids, especially DNA. ROS can induce oxidative DNA damage, including single and double strand breaks (SSB and DSB, respectively) and base and nucleotide modifications (Burney *et al.* 1999a; Burney *et al.* 1999b). A robust repair mechanism is induced due to oxidative DNA damage which involves excision of modified bases and nucleotides and also activation of DNA repair enzymes. Excessive oxidative stress is known to be detrimental for nuclear DNA as well as mtDNA, which is a critical target for oxidative damage (Guidi *et al.* 2008). Increased ROS can damage DNA by superoxide addition to double bonds of DNA bases resulting in increased 8-Oxo-2'-deoxyguanosine (8-oxo-dG) levels (Figure 1.8A), which is used as a marker for oxidative stress. 8-oxo-dG is a mutagenic base by-product that is formed as a result of ROS. DNA fragmentation, increase in levels of apoptosis-inducing factor (AIF), mitochondrial release of cytochrome c and up-regulation of p53 were observed in differentiated C2C12 myotubes following H₂O₂ exposure (Siu *et al.* 2009).

DNA repair mechanisms are affected by ROS resulting in DNA fragmentation and eventually necrosis of cells. Down-regulation of DNA repair enzymes like 8-Oxoguanine glycosylase (OGG1) has been shown to be involved in oxidative DNA damage during type 1 diabetes (Figure 1.8A) (Simone *et al.* 2008). OGG1 is a DNA glycosylase enzyme involved in base excision repair, especially aids in the excision of 8-oxo-dG. In recent years, regulated in development and DNA damage response (REDD1), a component of stress response and a developmentally regulated transcriptional target of p63 and p53, has been identified to be involved in oxidative stress-induced DNA damage in skeletal muscle during chronic hypoxia (Favier *et al.* 2010) and type 1 diabetes (Hulmi *et al.* 2012). These reports also show that p63 can regulate REDD1 which further alters the Akt/mTOR/MAPK pathway in skeletal muscle (Figure 1.8B). p63 is a tumor protein and is a member of the p53 family of transcription factors involved in apoptosis and premature aging. Antioxidants like ascorbic acid, trolox and probucol protect mice against type 1 diabetes-induced DNA damage (Imaeda *et al.* 2001a; Imaeda *et al.* 2001b); confirming further the role of ROS in DNA damage. All tissues of aged organisms contain phospholipids, DNA and proteins damaged by oxidative stress (Nohl 1993; Schoneich 1999). Aged mitochondria damaged by oxidative stress are associated with a marked increase in the number of rearrangements of mitochondrial DNA (Melov *et al.* 1995). Hiona *et al.* reported that mitochondrial DNA mutations induce sarcopenia in skeletal muscle of mice (Hiona *et al.* 2010). Angiotensin-II (a hormone that causes vasoconstriction and increase in blood pressure)-mediated oxidative DNA damage has been shown to accelerate aging in muscle cells via telomere-dependent and independent pathways, both mediated via p53 (Herbert *et al.* 2008).

Figure 1.8 Oxidative DNA damage during diabetes

(A) Increased glucose due to diabetes induces ROS production which causes DNA damage by increasing 8-oxo-dG levels. ROS also affects the PI3K/Akt pathway thus regulating DNA repair enzymes including OGG1. Figure modified from Simone *et al.* 2008. (B) Signal transduction and DNA damage mechanisms in skeletal muscle during type 1 diabetes. DNA damage is mediated through p63/REDD1/mTOR/Akt pathway during hyperglycemia. Figure modified from Hulmi *et al.* 2012.

1.3 Skeletal muscle wasting and ROS

As mentioned in the previous section, one of the main pathophysiological roles of ROS is skeletal muscle wasting. Muscle wasting is associated with high morbidity and mortality of many diseases including cancer, acquired immunodeficiency syndrome (AIDS) and diabetes. Hence, skeletal muscle wasting and involvement of oxidative stress will be reviewed in the following sections with emphasis on cachexia, sarcopenia, diabetes and molecular mechanisms responsible for progression of muscle wasting.

1.3.1 Skeletal muscle wasting

Muscle wasting is characterized by the loss of muscle mass and progressive change in post-natal musculature leading to decreased quality of life. Skeletal muscle wasting, or atrophy, is observed during various conditions including diseases like cachexia, muscular dystrophy, diabetes (Type 1 and Type 2), during starvation, disuse atrophy observed due to immobilization, denervation and muscle unloading and sarcopenia (aging). Muscle wasting occurs due to decreased protein synthesis and increased protein degradation (Figure 1.9) (Pasini *et al.* 2008; Sandri 2008). Muscle wasting is characterized by a decrease in muscle fiber cross sectional area, a loss in muscle nuclei and loss in muscle strength (Mitchell and Pavlath 2001; Edgerton *et al.* 2002; Hudson and Franklin 2002; Sandri *et al.* 2004). However, Gundersen and Bruusgaard have shown that muscle atrophy is not related to loss of myonuclei but due to apoptosis confined to stromal and satellite cells (Gundersen and Bruusgaard 2008). Increased catabolic stimuli by cytokines, cortisol and other growth factors and decreased anabolic stimuli including IGF signaling and insulin signaling, reduces protein synthesis and increases protein degradation leading to excessive amino acid pool in circulation, hence inducing muscle wasting.

Figure 1.9 *Muscle wasting is caused due to increased catabolic stimuli*

Increased catabolic stimuli including cytokines, cortisone and glucagon can lead to an imbalance in the system, causing increased protein degradation and decreased protein synthesis. Decreased anabolic stimuli including insulin and IGF-1 signaling pathways lead to excessive amino acid (AA) pool in circulation, leading to skeletal muscle atrophy. Figure modified from Pasini *et al.* 2008.

1.3.1.1 Cachexia

The word cachexia is derived from the Greek word “kakos” (bad) and “hexis” (condition) and is associated with many chronic diseases including cancer, AIDS, sepsis, chronic obstructive pulmonary disease (COPD), diabetes and renal failure (Mitch and Goldberg 1996; Jackman and Kandarín 2004; Lecker *et al.* 2004; Boonyarom and Inui 2006). Cachexia is characterized by loss of weight, muscle atrophy, fatigue, weakness and significant loss of appetite (Figure 1.10). The exact mechanism in which various diseases lead to cachexia is not well understood, but is mostly due to both tumoral and humoral factors including inflammatory cytokines such as tumor necrosis factor- α (TNF- α) (also known as cachectin), interferon- γ (IFN- γ), interleukin (IL-6) and proteolysis inducing factor (PIF) (Argiles *et al.* 2005; Argiles *et al.* 2006). Cancer cachexia describes a syndrome of progressive weight loss in response to malignant growth. The increased protein degradation is mediated by three proteolytic systems: the calcium-dependent calpain pathways, the lysosomal protease system and the ubiquitin-proteasome pathway. Increased ROS are regarded as crucial players for increasing protein degradation by stimulating the ubiquitin-proteasome system. This increase in ROS is attributed to significantly lower activities of AOE (Mantovani *et al.* 2003). Due to metabolic derangements seen in cancer cachexia, effective nutritional treatment regimens which employ food rich in antioxidants have been shown to be one of the treatments for maintaining body weight. In addition, insulin therapy, corticosteroids, and exercise are also other treatments for cachexia which increase appetite, may reverse weight loss but have no evidence of reversing muscle loss (Ardies 2002; Lundholm *et al.* 2007).

Figure 1.10 Cachexia

Chronic illnesses like COPD, renal failure, chronic heart failure and cancer lead to cachexia. Cachexia is characterized by weight loss, decreased muscle strength, fatigue and increased inflammatory markers. Figure modified from Evans *et al.* 2008.

1.3.1.2 Sarcopenia

Sarcopenia is the loss of skeletal muscle mass, function and strength during aging. It is the degenerative loss of skeletal muscle and its efficiency. The muscle fibers are replaced by fibroblasts and fat (fibrosis), thus reducing the myogenic capacity of a muscle. Increased fibrosis occurs in regenerating muscles of aged animals (Carlson and Faulkner 1989; Ullman *et al.* 1990). Loss of muscle strength and muscle wasting are characteristic of aging and are caused by atrophy of fibers (Porter *et al.* 1995). With aging and inactivity, the most atrophy is seen in the fast twitch fibers and fiber type switch from fast to slow occurs (Kugelberg 1976; Alnaqeeb and Goldspink 1987; Musaro *et al.* 1995). In addition, aging has been associated with a relative increase in muscle fibres co-expressing two or more major histocompatibility complex (MHC) isoforms, signifying that there is less distinction between the slow and fast fibre types with age (Andersen *et al.* 1999).

Aged muscle has impaired SC function, however whether SC numbers change during aging remains controversial (Gibson and Schultz 1983; Nnodim 2000; Conboy *et al.* 2003; Schafer *et al.* 2005; Shefer *et al.* 2006). The ability of SC to become activated and their proliferative capacity decreases with age (Schultz and Lipton 1982; Dodson and Allen 1987; Johnson and Allen 1993; Barani *et al.* 2003; Conboy *et al.* 2003; Machida and Booth 2004). This loss has been shown to be associated with loss of Notch signaling (Conboy *et al.* 2003; Luo *et al.* 2005), but this is still under debate. Furthermore, parabiosis studies, in which young and old mice were surgically joined to establish a shared circulatory system, have shown that as yet unknown circulating factors in the young mice restored both Notch signaling and the muscle regeneration capacity in the aged mice (Conboy *et al.* 2005).

Many factors, including physical inactivity, motor-unit remodelling, decreased hormone levels, oxidative stress and decreased protein synthesis, may all contribute to muscle wasting during aging, sarcopenia (Vella and Kravitz 2002). The free-radical theory of aging (FRTA) proposed by Denham Harman in the 1950s states that organisms age because cells accumulate free radical damage over time. Based on studies to date, oxidative stress as a lifespan determinant is still debatable. However, extensive studies based on FRTA show that human average life expectancy at birth and maximum life span can be extended when living conditions are improved (Harman 2006). The involvement of oxidative stress-induced skeletal muscle wasting during aging has been proven by numerous studies. Bejma & Ji reported that homogenates of skeletal muscle from aged animals showed increased oxidative stress (Bejma & Ji 1999). An increase in mitochondrial superoxide and H₂O₂ production with increasing age has also been reported (Sohal *et al.* 1994). The chronic activation of NF- κ B in muscles of aged mice is associated with chronic increase in the content and activities of AOE, such as the SOD and CAT (Broome *et al.* 2006), increased content of heat-shock proteins (HSPs) (Broome *et al.* 2006; Vasilaki *et al.* 2006; Kayani *et al.* 2008) and increased production of pro- and anti-inflammatory cytokines (Pedersen and Febbraio 2005). It has been shown that aging-associated oxidative stress in aged muscle exacerbated the inflammatory response (Ghaly and Marsh 2010), but evidence as to whether the mRNA expression of pro-inflammatory cytokines like IL-1 β and TNF- α in skeletal muscle increases with aging is still inconclusive (Hamada *et al.* 2005; Raue *et al.* 2007). Reduced IGF-1 signaling due to increased TNF- α has been shown to contribute to sarcopenia (Grounds 2002). Marzani *et al.* reported that ROS-mediated oxidative damage showed variation in different muscles (based on fiber types) from humans

during aging (Marzani *et al.* 2005). This implies that a relationship between aging and ROS exist but is dependent on muscle fiber type.

Fortunately, sarcopenia is partly reversible with appropriate exercise interventions, testosterone replacement and over expression of IGF-1 (Klitgaard *et al.* 1990; Snyder *et al.*, 1999; Musaro *et al.* 2004; Mourkioti and Rosenthal 2005). Resistance training programs, such as weightlifting is a more powerful stimulus of muscle hypertrophy than endurance-type exercise regimes such as swimming (Klitgaard *et al.* 1990). Inhibition of ROS by antioxidants reduced oxidative stress and improved skeletal muscle function during sarcopenia (Kim *et al.* 2010; Ryan *et al.* 2011).

1.3.1.3 Type 1 diabetes

Type 1 diabetes is caused due to insulin-deficiency, thus leading to increased glucose in circulation (hyperglycemia). One of the characteristic features of uncontrolled type 1 diabetes is skeletal muscle wasting. Studies have shown the involvement of oxidative stress in the pathogenesis of skeletal muscle wasting during hyperglycemia in streptozotocin (STZ)-induced type 1 diabetic rats (De Angelis *et al.* 2000; Mastrocola *et al.* 2008; Chen *et al.* 2011). Also, skeletal muscle repair is impaired due to increased oxidative stress in STZ-induced type 1 diabetic rats (Aragno *et al.* 2004). STZ is an antibiotic that is toxic to pancreatic β cells, hence used widely to induce type 1 diabetes in rodents as it selectively destroys the insulin-producing β cells of the pancreas. STZ is transported into the β cell by the GLUT2 transporter, hence STZ has enhanced transport and cytotoxicity in cells expressing the GLUT2 transporter (Schnedl *et al.* 1994). STZ causes DNA alkylation and DNA damage by inducing ROS (NO and XO), depleting intracellular NAD^+ and ATP generation (Figure 1.11), resulting in irreversible damage to β cells and hence no synthesis of insulin. Down-regulation of DNA repair enzymes like 8-Oxoguanine glycosylase (OGG1) has been shown to be involved in oxidative DNA damage during STZ-induced type 1 diabetes (Simone *et al.* 2008). STZ has also been shown to inhibit C2C12 myoblast growth by arresting the G2/M cell cycle transition (Johnston *et al.* 2007). However, studies have shown that control of diabetes by insulin administration and/or reduction in levels of ROS by AOE supplementation reduces muscle wasting to a certain extent. An oral mixture of amino acid supplements was able to improve the AOE status in the skeletal muscle of diabetic mice (Brocca *et al.* 2008). Recently, Bravard *et al.* showed that inhibition of XO in skeletal muscle of STZ-induced diabetic mice is able to reduce the oxidative stress caused due to hyperglycemia (Bravard *et al.* 2011).

Figure 1.11 Mode of action of STZ in pancreatic β cells

STZ is transported into the β cells via GLUT2. DNA alkylation and DNA damage is induced by STZ via ROS (NO and XO) resulting in increased generation of superoxides (O_2^-) and hydrogen peroxide (H_2O_2). This depletes intracellular NAD^+ and ATP generation leading to irreversible damage; MIT: mitochondria. Figure modified from Szkudelski 2001.

1.3.2 Skeletal muscle wasting systems

Skeletal muscle wasting is predominantly due to increased protein degradation and decreased protein synthesis. The degradation of skeletal muscle proteins is controlled mainly by three proteolytic systems: the calcium-dependent calpain pathways, the lysosomal protease system and the ATP-dependent ubiquitin-proteasome pathway (Figure 1.12) (Jackman and Kandarín 2004).

Calcium-dependent proteolysis is mediated through calcium-activated cysteine proteases called calpains (Costelli *et al.* 2005). Among the calpains (calpain 1, calpain 2, calpain 3), calpain3 has been reported to be expressed in skeletal muscle specifically (Bartoli and Richard 2005). Calpains help release actin and myosin from the muscle fibers and these are acted upon by the ubiquitin-proteolytic system to further degrade them. Calpains have been shown to play an important role in muscle wasting during sepsis (Hasselgren and Fischer 2001; Fareed *et al.* 2006). The calpain pathway is predominant in the severe pathology associated with muscular dystrophy, a muscle disease caused due to the lack of a large cytoskeletal protein, dystrophin. The absence of dystrophin weakens the sarcolemma, thus aiding influx of extracellular calcium into the myofibril and increasing calpain expression and eventually protein degradation (Costelli *et al.* 2005).

Lysosomes cause degradation of membrane lipids and proteins including receptors and ion channels (Tisdale 2005). Lysosomes are membrane-bound vesicles which contain acid hydrolases and proteases, mainly cathepsins, L, B, H and D (Bechet *et al.* 2005). Studies have shown the up-regulation of cathepsin B and cathepsin L during skeletal muscle wasting (Tsujinaka *et al.* 1997).

Ubiquitin-mediated protein degradation is a step-wise process which eventually results in poly-ubiquitination of substrate proteins and degradation by proteasome. The

ubiquitin-proteasome pathway primarily degrades damaged or defective myofibrillar proteins; it is unable to degrade the proteins while the myofibrils are intact (Solomon and Goldberg 1996), suggesting that more than one proteolytic pathway is involved in the process of skeletal muscle wasting. The muscle-specific E3 ubiquitin ligases Atrogin-1 and MuRF-1 are two of the most important markers of skeletal muscle atrophy (Bodine *et al.* 2001; Gomes *et al.* 2001). The expression of both these enzymes is significantly up-regulated during muscle wasting; during denervation (Bodine *et al.* 2001), hind limb unloading, glucocorticoid (GR)-dexamethasone (Dex)-induced muscle wasting (Bodine *et al.* 2001; Krawiec *et al.* 2005), starvation, diabetes, renal failure and cancer cachexia (Gomes *et al.* 2001; Lecker *et al.* 2004; Costelli *et al.* 2006). Thus Atrogin-1 and MuRF-1 are useful molecular markers for detecting skeletal muscle atrophy.

Figure 1.12 Muscle wasting systems

The degradation of skeletal muscle proteins is mainly through three proteolytic systems: the calcium-dependent calpain pathways, the lysosomal protease system and the ATP-dependent ubiquitin-proteasome pathway. Figure modified from Jackman and Kandarin 2004.

1.3.3 Signaling molecules involved in oxidative stress-induced skeletal muscle wasting

As said earlier, ROS plays an important role in muscle differentiation and muscle wasting and various diseases associated with it. They are crucial players in the onset of protein hyper catabolism leading to muscle wasting during cancer and diabetes, mainly through the ubiquitin-proteasome pathway (Li and Reid 2000; Mantovani *et al.* 2003; Tisdale 2005; Moylan and Reid 2007). ROS in skeletal muscle can be induced by a number of mechanisms including enzymes involved in mETC, TNF- α , Nox, XO, NOS, hypoxia and signaling mechanisms including MAPK and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways. Various signaling molecules and pathways involved in oxidative-stress induced skeletal muscle wasting are discussed below.

1.3.3.1 Mitochondrial electron transport chain (mETC)

The main function of the mitochondria is to produce ATP in the electron transport chain (ETC) (Figure 1.13A); it also produces superoxides when leakage of electrons occurs in the chain (Figure 1.13B). Mitochondria themselves are susceptible to damage by ROS, although they produce it, leading to a vicious circle involving mitochondrial DNA (mtDNA) damage and fragmentation, decrease in oxidative phosphorylation potential, further increase in mitochondrial superoxide production and decline in efficiency of mitochondrial scavenging enzymes including MnSOD (Figure 1.14) (Madamanchi and Runge 2007; Sharon *et al.* 2008). The four complexes involved in the mETC are NADH-Q reductase/NADH dehydrogenase (complex I), Succinate-Q reductase/Succinate dehydrogenase (complex II), Cytochrome C reductase (complex III) and Cytochrome oxidase (complex IV). Diphenylene iodonium (DPI), 4-4'-

Diisothiocyanatostilbene-2-2'-disulphide (DIDS) and Thenoyltrifluoro acetone (TTFA) are inhibitors of complex I of mETC, superoxide release from mitochondria and complex II of mETC, respectively. Recently, it has been shown that cancer cachexia is associated with decreased mitochondrial oxidative capacity and increased *Atrogin-1* and *MuRF-1* gene expression (Julienne *et al.* 2012). ROS generation via hypoxia has been shown to decrease mitochondrial membrane stability and cause dysregulation of mETC (Chen *et al.* 2012).

Figure 1.13 Mitochondrial electron transport chain and superoxide production

(A) mETC couples electron transfer between an electron donor (such as NADH) and an electron acceptor (such as O_2) with the transfer of H^+ ions (protons) across a membrane. The resulting electrochemical proton gradient is used to generate chemical energy in the form of ATP. This entire process is known as oxidative phosphorylation. (B) A small percentage of electrons do not complete the ETC and instead leak, resulting in the formation of free-radical superoxides ($O_2^{\bullet -}$), which are detoxified by the SOD system, which includes CuZnSOD and MnSOD. Figure modified from Szeto 2006a.

Figure 1.14 Mitochondrial damage caused by ROS

Mitochondrial damage caused by free radicals. Free radicals generated by the ETC can result in oxidative damage to mitochondrial DNA (mtDNA) and proteins, lipid peroxidation, and opening of the mitochondrial permeability transition pore; $O_2^{\bullet -}$: superoxides; $OH^{\bullet}$: hydroxyl radicals, H_2O_2 : hydrogen peroxide; H_2O : water; SOD: superoxide dismutase. Figure modified from Szeto 2006b.

1.3.3.2 NAD(P)H oxidase (Nox)

Reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Nox) is a complex enzyme that catalyzes the production of superoxides from NAD(P)H and oxygen, consisting of two membrane-bound elements (gp91^{PHOX} and p22^{PHOX}), three cytosolic components (p67^{PHOX}, p47^{PHOX} and p40^{PHOX}), and a low molecular weight G-protein (rac 1 or rac 2) (Figure 1.15). The activation of Nox is caused by the migration of the cytosolic components to the cell membrane so the complete enzyme is assembled (Ago *et al.* 1999; Babior 1999; Babior 2004). NAD(P)H oxidase can be inhibited by apocynin, quinacrine and DPI. Apocynin prevents the assembly of its subunits (Palmen *et al.* 1995) and DPI binds strongly to flavoproteins and inhibits platelet aggregation and several important enzymes like NADH oxidase/reductase and NO synthase (Cross and Jones 1986). Superoxide production induced by palmitate has been shown to be via mETC and Nox activity in muscle cells (Lambertucci *et al.* 2008). Nox is up-regulated in dystrophic skeletal muscle which results in oxidative stress (Shkryl *et al.* 2009). Cabello-Verrugio *et al.* showed that fibrotic response induced by angiotensin-II in skeletal muscle cells requires Nox-induced ROS (Cabello-Verrugio *et al.* 2011).

Figure 1.15 *NAD(P)H oxidase enzyme complex*

Schematic representation of the NAD(P)H oxidase enzyme complex. The membrane complex has two subunits: p22phox and gp91phox, There are two cytosolic subunits: p67phox and p47phox; a p40phox accessory protein and a Rac-GTP binding protein that translocates to the cell membrane upon cell activation to form the NADPH oxidase complex which generates a respiratory burst. Superoxide (O₂⁻) can react to form hydrogen peroxide (H₂O₂) and hypochlorus acid (HOCl⁻). Figure modified from Assari 2006.

1.3.3.3 Tumor necrosis factor- α (TNF- α)

TNF- α is a pro-inflammatory cytokine, also known as cachexin or cachectin, that regulates immune cells and is involved in inflammation, apoptotic cell death. TNF- α is also involved in development of skeletal muscle wasting. Circulating TNF- α level is increased during various inflammatory diseases, cancer, AIDS, cachexia, after strenuous exercise and during necrotic cell death, all involving oxidative stress. TNF- α binds to two receptors, TNF-R1 and TNF-R2; TNF-R1 is found in most tissues and activated by membrane-bound and soluble trimeric forms whereas TNF-R2 is found in immune system only and is activated by membrane-bound homotrimer of TNF- α . Following binding of TNF- α to the receptors, three signaling pathways can be initiated; activation of NF- κ B, activation of MAPK pathways and induction of death signaling.

TNF- α induces free radical production mainly through Nox and mETC. TNF- α induces necrotic-like cell death due to ROS via the activation of Nox (Morgan *et al.* 2008). Pharmacological inhibition of TNF- α has been shown to reduce necrosis of dystrophic myofibers in mdx mouse model (Grounds *et al.* 2008). In a murine model of muscle cachexia, TNF- α induces oxidative stress in skeletal muscles (Buck and Chojkier 1996). Studies show that serum pro-inflammatory cytokines and oxidant-antioxidant parameters, especially TNF- α , ROS, glutathione and vitamin E were significantly increased during cancer cachexia (Fortunati *et al.* 2007). TNF- α has been shown to independently induce protein degradation in murine myotubes, leading to muscle atrophy and myofiber necrosis (Eley *et al.* 2008). NF- κ B is one of the central players in both these processes (Figure 1.16) (Grounds *et al.* 2008). It has been observed that the basal TNF- α expression level in differentiating C2C12 myoblasts is up-regulated and the NF- κ B activity is also increased by serum restriction (Li and Schwartz 2001). At the post-transcriptional level, in C2C12 differentiating myoblasts, TNF- α induces NF- κ B to

inhibit skeletal muscle differentiation by suppressing *MyoD* mRNA, whereas in differentiated myotubes, both TNF- α and IFN- γ are needed for down-regulation of *MyoD* and dysfunction of muscle fibers by NF- κ B (Guttridge *et al.* 2000). The damaging effects of TNF- α in skeletal muscle cells are partially mediated by crosstalk with IGF-1 receptor signaling (Figure 1.16) (Grounds *et al.* 2008). C-Jun N-terminal kinase 1 (JNK1) has been identified as one of the signaling molecules that mediates such crosstalk (Figure 1.16). JNK1 can associate with the IGF-1 signaling and inhibit its activity, which would lead to down-regulation of biological processes of IGF-1 like stimulation of protein synthesis, inhibition of protein degradation and cell survival. However, TNF- α has been also identified to be a key regulator of myogenesis and muscle regeneration. TNF- α regulates early differentiation of C2C12 myoblasts in an autocrine fashion (Li and Schwartz 2001). TNF- α regulates myogenesis and regeneration by activating MAPK pathways. Differentiating myoblasts and injured muscles have increased TNF- α level which activates p38 to induce myogenesis. If this pathway is blocked, this leads to impaired muscle development and increased degeneration in adult muscle (Chen *et al.* 2007). In C2C12 and primary myotubes, TNF- α increases the protein content through TNF-R1, PI(3)K and myocyte enhancer factor pathways (Plaisance *et al.* 2008).

Figure 1.16 *Crosstalk between TNF- α and IGF-1 signaling in skeletal muscle*

TNF- α -mediated protein degradation and necrosis in skeletal muscle cells is partially mediated by crosstalk with IGF-1 receptor signaling. JNK1, a mediator molecule, can associate with the IRS-1 in IGF-1 signaling and inhibit its activity, which would lead to decreased protein synthesis and cell survival, by down-regulating PI3K and Akt. TNF- α via NF- κ B can up-regulate protein degradation, leading to muscle atrophy and myofiber necrosis. Figure modified from Grounds, *et al.* 2008.

1.3.3.4 Nuclear factor-kappa B (NF- κ B)

One of the most important regulators of gene transcription and metabolism in response to oxidative stress is NF- κ B. NF- κ B is a transcription factor involved in a broad range of biological functions, including immune responses, stress responses and cell survival. Hence NF- κ B is an important pro-survival factor. Two NF- κ B signaling pathways have been described: the canonical pathway that mediates inflammatory response and the non-canonical/alternate pathway involved in lymphoid organogenesis, B-cell survival and dendritic cell activation. Generally, the NF- κ B proteins are sequestered in the cytoplasm bound to inhibitor κ B- α (I κ B- α) in an inactive complex. Once activated by a stimulus including ROS and TNF- α , I κ B gets phosphorylated by I κ B kinase (IKK), thus releasing active NF- κ B subunits which translocate from the cytoplasm into the nucleus and regulate the expression of its target genes (Figure 1.17). Canonical NF- κ B pathway involves activation of RelA/p65 and p50 subunits of NF- κ B, while the non-canonical pathway involves RelB and p52 NF- κ B subunits (Figure 1.17). The target genes of NF- κ B are extensive and include ILs, TNF- α , Nox, immunoreceptors, cell adhesion molecules, stress response genes like genes coding for AOE, regulators of apoptosis, various growth factors which include activin A, angiopoietin, androgen receptor and enzymes involved in calcium signaling.

The ubiquitin-proteasome pathway is induced by activation of the transcription factor NF- κ B in response to PIF (Wyke and Tisdale 2005). NF- κ B plays an important role in PIF- and angiotensin-II-induced ROS which leads to protein degradation in murine myotubes (Russell *et al.* 2007). Activation of NF- κ B in mice caused profound muscle wasting that resembled clinical cachexia. Recently, Activin A has been shown to be associated with transforming growth factor- β -activated kinase 1 (TAK1), p38 MAPK

and NF- κ B pathway during human skeletal muscle cells differentiation (Trendelenburg *et al.* 2012).

Figure 1.17 Canonical and non-canonical NF-κB signaling

Schematic of NF-κB signaling; inflammatory cytokines like TNF-α on binding to their receptors (TNFR), activate the NF-κB signaling pathway. Canonical signaling: IκB gets degraded after getting phosphorylated by IKK and this activates NF-κB p50 and p65 subunits to translocate into the nucleus. Non-canonical/alternate signaling: p100 subunit is broken down to p52 and RelB by IKK-α, leading to regulation of its target genes by NF-κB. Figure modified from Annunziata *et al.* 2007.

1.3.3.5 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are important regulators of metabolism in response to oxidative stress. MAPKs are a family of protein kinases which regulate extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events. The MAPK super-family is composed of three major sets of kinases: the extracellular-receptor kinases (ERKs) and two types of MAPK-related kinases that respond to cellular stress and inflammatory signals: the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 MAPKs. The various signaling molecules and components of MAPK pathways include MEK, MAPK kinase (MKK) and TAK and crosstalk between molecules is very common (Figure 1.18). p38 MAPKs, JNKs and ERKs were found to be activated by H₂O₂ in myoblasts and ERKs and p38 MAPKs, in particular, were found to be involved in NF-κB transactivation by oxidative stress (Kefaloyianni *et al.* 2006). ROS production via mETC and Nox has been shown to involve JNK and ERK signaling pathways in mice (Carnesecchi *et al.* 2009).

Chronic activation of MAPK and NF-κB signaling pathways has been implicated in the development and perpetuation of skeletal muscle wasting during cachexia and diabetes. Contrarily, MAPK signaling pathways are also activated during exercise, which is often employed to mitigate muscle atrophy during aging or chronic diseases. However, this increase in MAPK activities during diseased and healthy states is actually a necessary response to chronic and intermittent stress, respectively. Activation of p38 MAPK and increased oxidative stress in skeletal muscle occur after Dex (glucocorticoid) treatment in mice, concurrent with up-regulation of autophagy-related genes (McClung *et al.* 2010).

Cytokines (like TNF- α) and ROS can activate MAPKs via various signaling mechanisms including oxidative modifications and inactivation of MAPK phosphatases (MKPs) (Son *et al.* 2011). TNF- α acts via p38 MAPK to stimulate expression of Atrogin-1 and MuRF-1 in skeletal muscle (Li *et al.* 2005). TNF- α increases phosphorylation of ERK1/2 and JNK, concomitant with increased serine and reduced tyrosine phosphorylation of IRS-1. These effects are associated with impaired PI3K activation and phosphorylation of Akt substrate 160 (AS160), thus causing skeletal muscle insulin resistance (Plomgaard *et al.* 2005).

Figure 1.18 MAPK signaling pathways

MAPK signaling pathways are organized in modular cascades in which activation of upstream kinases by cell surface receptors lead to sequential activation of a MAPK pathway (MAPKKK → MAPKK → MAPK). The major MAPK pathway components and examples of the MAPK pathway target proteins are shown here. Figure modified from Junttila *et al.* 2008.

1.3.3.6 Janus kinase and signal transducers and activators of transcription (JAKs and STATs)

Besides these signaling molecules and pathways involved in ROS production, IL-6, an important cytokine, has been shown to regulate the Janus kinases-signal transducers and activators of transcription (JAK-STAT) pathway in cells having increased oxidative stress. Along with IL-6, cytokines such as IFN- α/β and IFN- γ exert their effect by binding to specific receptors in the cell membrane that subsequently activate intracellular signaling through JAK-STAT signaling. Activated STAT translocates into nucleus and activates transcription of cytokine-responsive genes (Figure 1.19), leading to increased ROS generation (Darnell 1997). p38 MAPK and ERK are involved critically in ROS-mediated induction of IL-6 (Sano *et al.* 2001). IL-6 and soluble IL-6 receptor have been shown to regulate JAK-STAT and ERK MAPK signaling pathways (Aida *et al.* 2012). A report shows that stimulation of the JAK-STAT cascade by angiotensin II is via Nox and IL-6 (Schieffer *et al.* 2000). Furthermore, activation of one of the STAT components, STAT3 is observed during skeletal muscle wasting and cancer cachexia (Bonetto *et al.* 2011).

Figure 1.19 *IL-6-induced JAK-STAT signaling*

Binding of cytokines like IL-6 to their receptors induces JAK activation. The activated JAKs phosphorylate the cytokine receptors, leading to the recruitment and subsequent activation of STAT family proteins. The activated STAT proteins translocate into the nucleus and activate transcription of a range of cytokine responsive genes. Figure modified from Yajima and Knowlton 2009.

1.3.3.7 Xanthine oxidase (XO)

XO, a form of xanthine oxidoreductase, is another important enzyme that generates ROS by catalyzing the oxidation of hypoxanthine to xanthine and further catalyzes oxidation of xanthine to uric acid (Figure 1.20). It is a molybdoenzyme that plays a major role in catabolism of purines and the enzyme can be converted to xanthine dehydrogenase by reversible sulfhydryl oxidation. Xanthine dehydrogenase reduces NAD^+ , whereas XO reduces molecular oxygen, resulting in production of O_2^- and H_2O_2 (Cai and Harrison 2000). Inhibitors of XO are allopurinol, oxypurinol and phytic acid. Allopurinol is a structural isomer of hypoxanthine and is a prodrug that is converted to oxypurinol by XO (Grootveld *et al.* 1987; Spector 1988; Muraoka and Miura 2004; Pacher *et al.* 2006). But it has been demonstrated that at lower XO concentrations, or at higher temperatures, less conversion of allopurinol to oxypurinol occurs, making allopurinol a conventional substrate that generates superoxide radicals during its oxidation. Oxypurinol as such does not produce any free radicals (Galbusera *et al.* 2006).

Superoxides are known to be generated by XO in the cytosol of contracting rat skeletal muscles cells (Gomez-Cabrera *et al.* 2005). However, human skeletal muscles contain lower levels of XO than rat muscle cells, and the question whether XO plays an important role in ROS generation in human skeletal muscle is still open (Gomez-Cabrera *et al.* 2005). Several ROS-generating mechanisms may be simultaneously operative, as in the case of prolonged muscle ischemia where ROS production via XO has been observed (Baudry *et al.* 2008). ROS generation is linked with sequential activation of the JAK-STAT pathway leading to induction of XO (Wang *et al.* 2008). Inhibition of XO by allopurinol reduced oxidative stress and improved skeletal muscle function in aged mice (Ryan *et al.* 2011).

Figure 1.20 XO-induced ROS

XO generates superoxides by catalyzing the oxidation of hypoxanthine to xanthine and further catalyzes oxidation of xanthine to uric acid; NO: nitric oxide; O₂⁻: superoxide anion; OONO⁻: peroxynitrite; XD: xanthine dehydrogenase; XO: xanthine oxidase. Figure modified from Puddu *et al.* 2012.

1.3.3.8 Transforming growth factor- β (TGF- β) superfamily members

Transforming Growth Factor- β (TGF- β) superfamily is a large group of extracellular proteins involved in autocrine, paracrine and endocrine regulation of various processes including proliferation, differentiation of cells, immune and inflammatory response as well as in diseases such as cancer and diabetes. TGF- β superfamily members are secreted proteins that activate the TGF- β receptors through ligand binding. TGF- β has been shown to suppress epithelial cell growth by inducing mitochondrial ROS production via Nox (Yoon *et al.* 2005). The ROS generated via Nox in turn mediates TGF- β -induced alterations in cells (Hu *et al.* 2005). TGF- β has been shown to also regulate AOE expression in smooth muscle cells (Michaeloudes *et al.* 2011).

TGF- β superfamily members signal through a cell surface receptor complex and a group of intracellular signal transducers, called Smad proteins (Figure 1.21) (Kollias and McDermott 2008). Smad (small mothers against decapentaplegic) proteins are a family of proteins identified to be homologous to the *Drosophila* proteins, encoded by gene, mothers against decapentaplegic (Mad) (Riggins *et al.* 1996). Upon TGF- β ligand binding to the cell surface receptor complex, Smad2/3 proteins are recruited and phosphorylated. A heteromeric complex of Smad2/3 and co-Smad, Smad4 is formed, which translocates into the nucleus to regulate TGF- β superfamily target gene expression. Black *et al.* showed that TGF- β mediates apoptosis of cells through Smad3-mediated generation of ROS (Black *et al.* 2007).

Additionally, Smad-independent pathways are also found to be involved in TGF- β family signaling, such as the ERK, JNK and p38 MAPK pathways. TGF- β also activates PI(3)K and Rho-like GTPases, which have been shown to further regulate Smad activation (Derynck and Zhang 2003; Kane *et al.* 2010). ROS play an important role in

TGF- β -induced activation of MAPK- and ERK-mediated Smad pathway in epithelial cells (Rhyu *et al.* 2005).

Figure 1.21 *TGF-β signaling pathway*

Upon TGF-β ligand binding, the cell surface receptor complex recruits and phosphorylates Smad2/3 proteins. After phosphorylation, Smad2/3 forms a heteromeric complex with the co-Smad, Smad4 and the complex translocates to nucleus to regulate expression of TGF-β superfamily target genes. Figure modified from Dijke and Hill 2004.

1.3.3.9 Myostatin (Mstn)

The secreted growth factor, Myostatin (Mstn), is a member of the TGF- β superfamily and is a negative regulator of muscle growth and development. Numerous studies have implicated Mstn in the regulation of skeletal muscle wasting by reducing muscle mass via ubiquitin-mediated protein degradation. However, the role of Mstn in ROS signaling and whether Mstn can induce oxidative stress has not been studied yet. A detailed review of Mstn biology and physiological effects is discussed in the following section.

1.4 Myostatin

As mentioned in the previous section, an important regulator of skeletal muscle growth is Myostatin (Mstn). Mstn has been shown to be a negative regulator of muscle growth and is involved in skeletal muscle atrophy. A detailed review of Mstn and its role in muscle wasting is discussed in the following sections.

1.4.1 Myostatin and its functions in myogenesis

Mstn (also known as Growth and Differentiation Factor-8; GDF-8) is a protein secreted in the blood stream and is a member of the TGF- β family. The gene encoding Mstn was discovered in 1997 by generating a strain of mutant mice that lacked the Mstn gene and it found that these mice have twice as much muscle as normal mice (Figure 1.22A) (McPherron and Lee 1997). The enhanced muscle mass in Mstn-null mice (Mstn^{-/-}) was due to a combination of both muscle hyperplasia and hypertrophy (McPherron and Lee 1997). Also there are double muscled cattle-Belgian blue and Piedmontese (Figure 1.22C) (Kambadur *et al.* 1997), whippet dogs and humans (Figure 1.22B), lacking the functional Mstn gene. Mstn is mainly produced by skeletal muscle cells, however, low levels of Mstn are also seen in adipose tissue and cardiac muscle (Sharma *et al.* 1999). The secreted Mstn circulates in the blood stream and affects skeletal muscle predominantly, but also has its effects on other organs including liver, kidney, cardiac and adipose tissue. Over-expression of Mstn in mice leads to muscle atrophy and muscle wasting (Zimmers *et al.* 2002), reduced skeletal muscle mass, decreased muscle fiber size and myonuclei number (Reisz-Porszasz *et al.* 2003).

Figure 1.22 Double muscling caused by mutations in *Mstn*

(A) Double muscling due to mutations in *Mstn* gene is observed in mice in the absence of *Mstn* (*Mstn*^{-/-} mice). Figure modified from McPherron and Lee 1997. (B) Photograph of natural mutations in *Mstn* gene leading to increased muscle growth in a 7-month old human child. Figure modified from Schuelke *et al.* 2004. (C) Photograph showing the heavy muscling observed in the Belgian Blue cattle (Kambadur *et al.* 1997). Figure modified from Haliba '96 Catalogue.

Similar to other TGF- β superfamily members, Mstn is synthesized as an inactive full length protein (pre-proprotein) and a homodimer of the precursor proteins are linked together by disulfide bonds. The precursor protein undergoes two proteolytical cleavage events to generate mature Mstn. As a result of the two cleavages, an N-terminal signal peptide (SP) (removed to target the protein for secretion), an N-terminal latency-associated protein (LAP or propeptide) and a C-terminal mature protein are formed. The disulfide-linked C-terminal mature Mstn is the active ligand (Figure 1.23).

Figure 1.23 *Schematic representation of structure of Mstn*

Mstn protein has a signal peptide (SP) for secretion and a RSRR proteolytic processing site. Proteolytic processing of Mstn gives rise to LAP and mature Mstn. Figure modified from Dominique and Gerard 2006.

Mstn inhibits proliferation (Thomas *et al.* 2000; Taylor *et al.* 2001; McFarlane *et al.* 2011), differentiation (Rios *et al.* 2001; Langley *et al.* 2002; McFarlane *et al.* 2011), and negatively regulates satellite cell (SC) activation and self-renewal (McCroskery *et al.* 2003; McFarlane *et al.* 2008). Mstn has also been shown to increase protein degradation and reduce muscle protein synthesis (Taylor *et al.* 2001; McFarlane *et al.* 2006; Welle *et al.* 2009).

Different mechanisms are involved in inhibition of myoblast proliferation by Mstn. Mstn up-regulates p21 expression, a Cdk2 (cyclin-dependant kinase 2) inhibitor involved in cell cycle progression (Langley *et al.* 2000; McFarlane *et al.* 2011). The cyclin-Cdk complex induces phosphorylation of retinoblastoma (Rb) family (tumor suppressor proteins) and abrogates their inhibitory activity on cell cycle progression (Langley *et al.* 2000; McFarlane *et al.* 2011). Mstn inhibits Cdk2 activity and degrades cyclin D1 which leads to cell cycle arrest in G1 phase resulting in inhibition of myoblast proliferation (Langley *et al.* 2000; McFarlane *et al.* 2011).

Langley *et al.* showed that Mstn inhibits myoblast differentiation by down-regulating *MyoD* expression (Langley *et al.* 2002). Mstn inhibits activation of the Akt/mTOR/p70S6 protein synthesis pathway thus inhibiting differentiation of myoblasts (Trendelenburg *et al.* 2009). Myogenin is also inhibited by Mstn; recently, it has been shown that Mstn negatively regulates human myoblast differentiation by down-regulating *MyoD* and *Myogenin* expression via Notch signaling (McFarlane *et al.* 2011). Recently, our laboratory demonstrated that peroxisome proliferator-activated receptor β/δ (PPAR β/δ) positively regulates myogenesis, by negatively modulating Mstn activity via growth and differentiation factor-associated serum protein-1 (Gasp-1) (Bonala *et al.* 2012).

However, a controversial study by Manceau *et al.* showed that Mstn promotes terminal differentiation of embryonic muscle progenitors (Manceau *et al.* 2008). Over expression of Mstn in embryos resulted in muscle hypotrophy, due to a reduction in the muscle progenitor population during embryogenesis. However, loss of Mstn in embryos delayed the onset of the terminal differentiation program and stimulated continuous proliferation of progenitors. The different environment context between *in vivo* muscle progenitors and *in vitro* myoblasts may explain this different role of Mstn in the regulation of myogenic differentiation (Manceau *et al.* 2008).

In addition to regulating proliferation and differentiation of myoblasts, Mstn also regulates SC activation and self-renewal. Mstn not only inhibits SC activation, through negatively regulating cell cycle progression from the G1 phase to S phase (McCroskery *et al.* 2003), but also inhibits SC self-renewal by signaling through Pax7 (McFarlane *et al.* 2008). Mstn negatively regulates muscle regeneration also by controlling SC activation, migration of myoblasts and macrophages to injury site (Figure 1.24) (McCroskery *et al.* 2005).

Mstn^{-/-} mice (Figure 1.22A) have been used as a model for studying skeletal muscle differentiation and regeneration, extensively. The reason for muscle overgrowth (hypertrophy) due to Mstn deficiency is by increased activation of Akt/mTOR signaling pathway, causing global RNA content, polysome formation and protein synthesis to be increased in Mstn^{-/-} myotubes (Rodriguez *et al.* 2011). Mstn regulates fiber-type composition via myocyte enhancer factor 2 (MEF2) and MyoD, thus Mstn^{-/-} muscles have increased type IIB fibers with concomitant decrease in type I and type IIA fibers (Hennebry *et al.* 2009). Mstn^{-/-} muscle have been found to have enhanced muscle regeneration, reduced fibrosis and improved healing when compared to injured WT muscle (McCroskery *et al.* 2005). Mstn plays a role in diet-induced obesity as well as in

glucose metabolism and insulin signaling (Zhao *et al.* 2005; Chen *et al.* 2010; Dilger *et al.* 2010; Hittel *et al.* 2010). Studies from our laboratory suggest that Mstn^{-/-} mice have reduced fat accumulation and increased insulin sensitivity (Zhang *et al.* 2011).

Figure 1.24 Role of Mstn in skeletal muscle healing and regeneration

SCs and inflammatory response are activated by myotrauma. The SCs and macrophages migrate to the site of injury and help either myoblast (MB) fusion to form new myotubes or MB fusion with existing damaged myofibers. Mstn inhibits SC activation and proliferation, SC and macrophage migration. Figure modified from McCroskery *et al.* 2005.

1.4.2 Myostatin signaling and regulation

1.4.2.1 Canonical signaling

Like other TGF- β superfamily members, Mstn signal transduction also begins with ligand binding to the activin type II receptor (ActRIIB). The type II receptor associates with its corresponding type I receptor (ALK4 or ALK5), forming an activated heterotetrameric receptor complex, which then phosphorylates Smad2/3 protein that oligomerizes with a co-Smad (Smad4). Specifically, Smad3 is required by Mstn to inhibit differentiation of myoblasts (Langley *et al.*, 2002) and to activate proliferation of fibroblasts (Li *et al.*, 2008). The Smad2/3 and Smad4 oligomer translocates into the nucleus where it regulates its target genes (Figure 1.25) (Zhu *et al.* 2004) including *MyoD*. Recently, Smad3 signaling has been shown to be required for SC function and myoblast differentiation; lack of Smad3 (in Smad3^{-/-} mice) leads to decreased muscle mass, loss of SC functionality, impaired proliferation and differentiation of myoblasts (Ge *et al.* 2011). Absence of Smad3 also resulted in increased *Mstn* expression in skeletal muscle and inactivation of Mstn in Smad3^{-/-} mice was able to rescue the impaired myogenesis (Ge *et al.* 2011). Lack of Smad3 signaling also leads to impaired skeletal muscle regeneration, maybe due to increased *Mstn* expression (Ge *et al.* 2012). Smad7 or inhibitory Smad serves to auto-regulate Mstn signaling via an inhibitory feedback loop (Forbes *et al.* 2006). The Smad7 inhibition is enhanced by Smurf1 co-expression (Zhu *et al.* 2004).

1.4.2.2 Non-canonical signaling

As mentioned earlier, Smad-independent pathways are also involved in TGF- β family signaling which include JNK (Huang *et al.* 2007), ERK (Yang *et al.* 2006), p38 MAPK (Philip *et al.* 2005) and/or Akt/mTOR (Yang *et al.* 2007; Ji and Liu 2008; Amirouche *et al.* 2009) pathways (Figure 1.25).

Mstn was found to activate p38 MAPK through TGF- β activated kinase 1 (TAK1) to up-regulate p21 and inhibit myoblast proliferation (Philip *et al.* 2005). Mstn can also activate the ERK1/2 pathway to inhibit muscle cell differentiation and growth (Yang *et al.* 2006). Likewise, Mstn can regulate pre-adipocyte differentiation and adipocyte lipid metabolism via ERK1/2 signaling pathway (Li *et al.* 2011). Mstn activates JNK pathway to inhibit proliferation and differentiation of C2C12 myoblasts (Huang *et al.* 2007). Moreover, Mstn induces fibroblast proliferation by sequentially stimulating Smad2/3, p38 MAPK and Akt pathways (Li *et al.* 2008).

Akt/mTOR signaling plays an important role in skeletal muscle growth by regulating both protein synthesis and protein degradation (Bodine *et al.* 2001). Muscle size is regulated via a complex interplay of Mstn with the IGF-1/PI(3)K/Akt pathway, which is responsible for increase in protein synthesis in muscle. Decreased phosphorylation of the Akt/mTOR pathway leading to increased protein ubiquitination results in skeletal muscle protein degradation. Mstn inhibits Akt phosphorylation thus increasing FoxO1, allowing increased protein degradation (McFarlane *et al.* 2006). Also, Mstn overexpression leads to down-regulation of Akt/mTOR signaling leading to muscle atrophy (Amirouche *et al.* 2009). Thus, it is well established that Mstn signals through multiple signaling pathways to elicit biological function, and regulation of Mstn is mediated by cross-talk between these signaling pathways.

Figure 1.25 Mstn signaling

Canonical signaling: Mstn on binding to ActRIIB receptor, associates with ALK4 or ALK5 receptor, which leads to phosphorylation of Smad2/3 and oligomerization with Smad4 (co-Smad). The oligomer then translocates into the nucleus to regulate its target genes. Non-canonical signaling: Mstn can also signal via JNK, ERK, p38 MAPK and/or Akt. Figure modified from Tsuchida *et al.* 2009.

1.4.3 Myostatin and skeletal muscle wasting

Apart from the role of Mstn in regulating myogenesis, numerous evidences implicate Mstn in the induction of skeletal muscle wasting. Mstn has been associated with muscle wasting observed during disease conditions like cancer, AIDS, COPD, diabetes and aging. Mstn has also been found to be an important gene target in Duchenne muscular dystrophy (DMD). *Mstn* expression was up-regulated in human immunodeficiency virus- (HIV-) infected individuals with muscle wasting (Gonzalez-Cadavid *et al.* 1998) and in chronic human disuse muscle atrophy (Reardon *et al.* 2001). Over-expression of Mstn in mice induced cachexia and muscle wasting (Zimmers *et al.* 2002). Fasting and food deprivation also increased *Mstn* expression (Allen *et al.* 2010; Carneiro *et al.* 2012). Elevated Mstn levels are also seen in patients with liver disease and liver cirrhosis (Dasarathy *et al.* 2004; Garcia *et al.* 2010), chronic kidney disease (Verzola *et al.* 2011; Zhang *et al.* 2011) and in response to hypoxic stimuli (Hayot *et al.* 2011).

Our laboratory demonstrated that Mstn is a pro-cachectic growth factor that induces cachexia by activating the Ubiquitin-proteolytic system through a FoxO1-dependent mechanism (McFarlane *et al.* 2006). The two well-known mechanisms of muscle wasting are increased protein degradation by up-regulating Atrogin-1 and other atrophy related genes and decreased myogenesis and protein synthesis by blocking *MyoD* expression. Mstn promotes the wasting of human myoblasts by inducing Ubiquitin-proteasome pathway which mediates the loss of sarcomeric proteins including myosin light chain and myosin heavy chain. This process is mediated via E3 ligase MuRF-1, Smad3-dependent regulation of FOXO1 and another E3 ligase, Atrogin-1 (Figure 1.26) (Lokireddy *et al.* 2011a; Lokireddy *et al.* 2011b). Recently, our laboratory reported that Mstn is a novel tumoral factor that induces cancer cachexia due to the activation of Atrogin-1, MuRF-1, Smad and NF- κ B signaling and reduced activation of IGF-

1/PI(3)K/Akt pathway (Lokireddy *et al.* 2012a). Mstn is also involved in inducing autophagy in skeletal muscle *in vitro* by up-regulating autophagy-related genes and also by increasing auto phagosome formation (Lee *et al.* 2011).

Figure 1.26 *Mstn-induced muscle wasting*

Mstn enhances FOXO1 expression via Smad3 signaling. Through inhibition of Akt phosphorylation, Mstn treatment results in the accumulation of active FOXO transcription factors, which activates Atrogenin-1 and MuRF-1, leading to the ubiquitination of sarcomeric proteins, increased protein degradation and reduced protein synthesis. Figure modified from Lokireddy *et al.* 2011a.

A number of studies including from our laboratory have shown the involvement of Mstn in muscle wasting during sarcopenia (Seibert *et al.* 2001; Kawada *et al.* 2001; Corsi *et al.* 2002; Yarasheski *et al.* 2002; Baumann *et al.* 2003; Raue *et al.* 2006; Siriatt *et al.* 2006; Siriatt *et al.* 2007; Leger *et al.* 2008). The levels of Mstn during aging is still under debate as some studies revealed no changes in *Mstn* mRNA expression (Welle *et al.* 2002) and serum Mstn levels (Ratkevicius *et al.* 2011) upon aging. However, aged *Mstn*^{-/-} mice have lesser muscle wasting and reduced loss of regenerative capacity (Siriatt *et al.* 2006) and also antagonism of Mstn enhanced muscle regeneration upon injury and during sarcopenia in aged mice (Siriatt *et al.* 2007). The Mstn antagonist increased SC activation, Pax7 and MyoD protein levels, myoblast and macrophage migration following injury in aged mice. Resistance training and acute exercise have shown to reduce *Mstn* expression in skeletal muscle even upon aging (Kim *et al.* 2005; Louis *et al.* 2007; Dalbo *et al.* 2011).

The role of Mstn in diabetes is also extensively investigated during type 1 and type 2 diabetes. Mstn expression and level is up-regulated in skeletal muscle during type 1 diabetes in rodents (Chen *et al.*, 2009; Hulmi *et al.*, 2012; Dutra *et al.*, 2012). However, the mechanism behind Mstn induction due to hyperglycemia during type 1 diabetes is not understood. Mstn induces insulin resistance during type 2 diabetes. *Mstn*^{-/-} mice have been shown to have reduced fat accumulation and increased insulin sensitivity (Zhang *et al.* 2011) and also inhibition of Mstn protects against diet-induced obesity by enhancing fatty acid oxidation (Zhang *et al.* 2012). Mstn inhibition prevents reduced blood glucose level and hyperphagia in mice with lipodystrophy (Guo *et al.* 2012).

Thus, Mstn can be considered as a target for treatment of muscle wasting during various conditions including cancer, diabetes and aging, keeping in mind that Mstn can have cross-talk with various signaling molecules involved in normal muscle development.

1.5 Aims and objectives

Skeletal muscle wasting is a complication associated commonly with cancer, AIDS, diabetes, COPD, disuse, muscular dystrophy, aging and other inflammatory conditions. Oxidative stress is an important factor involved in all these conditions and has been shown to be induced by various inflammatory cytokines and signaling pathways. In the recent years, Myostatin (Mstn), a TGF- β super-family member has been well established as an important growth and differentiation factor that causes skeletal muscle wasting mainly by negatively regulating myogenesis and muscle growth. However, relationship between Mstn and ROS and whether Mstn can induce oxidative stress has not been studied yet. Therefore, the overall aim of this thesis was to investigate if Mstn is a pro-oxidant that can induce ROS in skeletal muscle causing muscle wasting in various conditions and whether absence of Mstn can rescue these effects. Therefore, the objectives of this thesis are as follows:

1. To establish the role of Mstn in inducing oxidative stress *in vitro* and *in vivo*.
2. To delineate the mechanism by which Mstn induces the generation of ROS using various mouse models.
3. To determine whether lack of Mstn reduces ROS-mediated muscle wasting.
4. To determine the effect of aging and ROS during sarcopenia in skeletal muscle of wild type and Mstn^{-/-} mice.
5. To investigate if Mstn can induce DNA damage via ROS in type 1 diabetic mice and to study whether inactivation of Mstn rescues the phenotype observed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

This section outlines general materials used in this thesis, including reagents, oligonucleotides, antibodies, chemicals and solutions. Details of other specific materials used during this study are included in Chapters 3-5.

2.1.1 Oligonucleotides

All oligonucleotides used in this study were synthesized by Sigma-Aldrich, Singapore. The stock solutions (100 μ M) were stored in -20°C and working concentrations of primers used were 2.5 μ M for Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) and 10 μ M for genotyping PCR. The oligonucleotides used are listed in Table 2.1 and 2.2.

Table 2.1 RT-qPCR primers

Gene	Forward 5' to 3'	Reverse 5' to 3'
<i>Cat</i>	CAGATGAAGCAGTGGAAGGA G	AGGAATCCGCTCTCTGTCAA
<i>Chop</i>	CTGCCTTTCACCTTGGAGAC	GGACGCAGGGTCAAGAGTAG
<i>FoxA2</i>	TAAGCGAGCTAAAGGGAGCA	GTGGTTGAAGGCGTAATGGT
<i>GAPDH</i>	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
<i>G6P</i>	TCTGTCCCGGATCTACCTTG	GTAGAATCCAAGCGCGAAAC
<i>IL-6</i>	GGCCTTCCCTACTTCACAAGTC CG	TGCACAACCTCTTTTCTCATTTCC CACGA
<i>Mstn</i>	AGTGGATCTAAATGAGGGCAG T	GTTTCCAGGCGCAGCTTAC
<i>Nox1</i>	ACAGCACTACCAATGCCCAG G	GCTTGTTGTCGGCAGCTGG
<i>PEPCK</i>	GCAGAACACAAGGGCAAGAT	CTTCCGGAACCAGTTGACAT

<i>Sod1</i>	CCAGTGCAGGACCTCATTTT	TTGTTTCTCATGGACCACCA
TNF- α	ATGAGCACAGAAAGCATGATC CGC	CCAAAGTAGACCTGCCCCGGAC TC

Table 2.2 PCR primers for genotyping

Primer	Sequence 5' to 3'
Smad3-p1	CCACTTCATTGCCATATGCCCTG
Smad3-p2	CCCGAACAGTTGGATTACACA
Smad3-p3	CCAGACTGCCTTGGGAAAAGC
Myostatin-P1	GATGTGCTCTCACTTCCTTG
Myostatin-P2	CAGCCATGGTAGTAGACCG
Myostatin-P3	TCTATCGCCTTCTTGACGAG

2.1.2 Antibodies

The primary and secondary antibodies used in this study are listed in Table 2.3, 2.4, 2.5.

Table 2.3 Primary antibodies used for Western blot, EMSA and ChIP

Antibody	Source	Catalogue #	Dilution used
Akt1/2/3	Santa Cruz	sc-8312	1:10,000
p-Akt1/2/3 (Ser473)	Santa Cruz	sc-7985-R	1:1,000
Atrogin-1	Dr. Esther Latres*	-	1:4,000
CHOP ^{###}	Cell Signaling	5554	1:500
ERK1/2	Santa Cruz	sc-94	1:500
p-ERK1/2 (Thr202/Tyr204)	Santa Cruz	sc-16982	1:200
FoxA2 ^{###}	Cell Signaling	3143	1:1,000

GAPDH	Santa Cruz	sc-32233	1:10,000
GDF-8 (Mstn)**	Sigma-Aldrich	HPA021681	1:3,000
H2A.X	Cell Signaling	7631	1:1,000
p-Histone H2A.X (Ser139)	Cell Signaling	9718	1:500
I κ B- α	Santa Cruz	sc-203	1:1,000
p-I κ B- α (Ser32)	Cell Signaling	2859	1:500
IKK α	Santa Cruz	sc-166231	1:1,000
IL-6	Abcam	ab6672	1:500
JAK1	Cell Signaling	3344	1:1,000
JAK2	Cell Signaling	3230	1:1,000
JAK3	Cell Signaling	3775	1:1,000
p-MEK1/2 (Ser217/221)	Cell Signaling	9154	1:1,000
p-MKK3 (Ser189)/MKK6 (Ser207)	Cell Signaling	9231	1:1,000
Mstn***	R&D Systems	MAB788	1:3,000
MuRF-1	Dr. Esther Latres*	-	1:1,000
MuRF-1****	Santa Cruz	SC-32920	1:500
NF- κ B (p50) [#]	Santa Cruz	SC-7178	-
NF- κ B (p65) ^{##}	Santa Cruz	sc-372	1:1,000
p-NF- κ B (p65) (Ser536)	Santa Cruz	sc-33020	1:500
OGG1	Proteintech	15125-1-AP	1:1,000
p38 α/β	Santa Cruz	sc-7972	1:500
p-p38 MAPK (Thr180/Tyr182)	Cell Signaling	4511	1:1,000
p53	Santa Cruz	sc-71818	1:500
p63	Santa Cruz	sc-8343	1:500
REDD1	Proteintech	10638-1-AP	1:200

SAPK/JNK	Cell Signaling	9252	1:1,000
p-SAPK/JNK (Thr183/Tyr185)	Cell Signaling	9251	1:1,000
p-SEK1/MKK4 (Thr261)	Cell Signaling	9151	1:1,000
Smad2	Cell Signaling	5339	1:1,000
p-Smad2 (Ser465/467)	Cell Signaling	3101	1:500
Smad2/3	Santa Cruz	sc-8332	1:1,000
p-Smad2/3 (Ser423/425)	Santa Cruz	sc-11769-R	1:1,000
STAT3	Cell Signaling	4904	1:2,000
p-STAT3 (Tyr705)	Cell Signaling	9145	1:1,000
p-STAT5 (Tyr694)	Cell Signaling	9359	1:1,000
TAK1	Cell Signaling	4505	1:1,000
p-TAK1 (Thr184/187)	Cell Signaling	4508	1:1,000
α -tubulin	Sigma-Aldrich	T6199	1:10,000
XRCC1	Cell Signaling	2735	1:1,000

*Atrogin-1 and MuRF-1 antibodies were a kind gift from Dr. Esther Latres (Regeneron Pharmaceuticals, USA)-Used in Chapter 3

**Used in Chapter 3

*** Used in Chapters 4 and 5

****Used in Chapter 4

Used for Electrophoretic mobility shift assay (EMSA)

Used for EMSA and Western blot

Used for EMSA, Western Blot and ChIP

Table 2.4 Primary antibodies used for Immunocytochemistry (ICC) and Immunohistochemistry (IHC) and

Antibody	Source	Catalogue #	Dilution used
8-oxo-dG (ICC)	Trevigen	4354-MC-050	1:250
OGG1 (IHC)	Proteintech	15125-1-AP	1:50
REDD1 (IHC)	Proteintech	10638-1-AP	1:50

Table 2.5 Secondary antibodies

Antibody	Source	Catalogue #	Dilution used	Usage
Alexa Fluor 488 (AF 488) goat anti-mouse IgG	Invitrogen	A-11029	1:400	ICC
Alexa Fluor 488 (AF 488) goat anti-rabbit IgG	Invitrogen	A-11034	1:1,000	IHC
Goat anti-mouse IgG HRP conjugate	Bio-Rad	170-6516	1:5,000	Western blot
Goat anti-rabbit IgG HRP conjugate	Bio-Rad	170-6515	1:5,000	Western blot
Goat anti-rat IgG HRP conjugate	Santa Cruz	sc-2006	1:2,000	Western blot

2.1.3 Reagents and chemicals

Sigma-Aldrich, St. Louis, MO, USA: H₂O₂, Methylene blue, Collagenase type 1A, Ascorbic acid, SIS3, BAY 11-7085, Aprotinin, Bovine Serum Albumin (BSA), Epinephrine, reduced glutathione, sodium azide, Trichloroacetic acid (TCA), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), thiobarbituric acid, 1,1,3,3-tetramethoxy propane, Indomethacin, L-NAME, Oxypurinol, Quinacrine, DPI, DIDS, CCCP, TTFA, Xanthine, Sodium citrate, Streptozotocin (STZ), paraformaldehyde, low-gelling temperature agarose, sodium lauryl sarcosinate, sarkosyl, PI, PVP, PEG and other reagents.

PAA Laboratories: DMEM.

HyClone Laboratories, Inc., Logan, Utah, USA: FBS and HEPES pH 7.8.

Gibco: P/S, Trypsin-EDTA and HS.

US Biologicals, Swampscott, MA, USA: Chick embryo extract (CEE) and Dithiothreitol (DTT).

Bio-Rad Laboratories: Bradford assay reagent, Ssofast Evagreen and nitrocellulose membrane.

Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA: 5-Aminosalicylic acid (5-ASA) and phenylmethanesulfonylfluoride (PMSF).

1st Base Pte. Ltd.: EDTA.

GE Healthcare Life Sciences: Nucleic acid blotting nylon membrane (Hybond-N+).

Fluka, St. Louis, MO, USA: Igepal (NP-40) and Xanthine oxidase (XO).

Merck & Co., Inc., NJ, USA: Ethanol, Formaldehyde, HCl, methanol, acetone and AG-490.

Enzo Life Sciences, Inc., PA, USA: SB203580, SP600125 and U0126.

Invitrogen: Normal goat serum and DAPI.

2.1.4 Enzymes

All restriction enzymes were acquired from New England Biolabs (NEB, USA) or Fermentas (Maryland, USA). All restriction enzyme buffers were supplied by the respective companies. The remaining enzymes used in this study are listed in Table 2.6.

Table 2.6 *Enzymes*

Enzymes	Source
Proteinase K	Invitrogen
RNase A	Fermentas
iScript Reverse Transcriptase	Biorad
Taq DNA polymerase	Fermentas

2.1.5 Solutions

The common solutions used in this study are listed in Table 2.7.

Table 2.7 *Composition of common solutions used*

Solution	Working concentration	Composition
Formal saline fixative	10 % Formaldehyde 0.9 % Saline	37 % Formaldehyde = 250 ml NaCl = 8.7 g MilliQ water = 750 ml
Borate buffer	0.01 M (pH 8.5)	Sodium tetraborate = 7.63 g MilliQ water = 2 L Adjust pH to 8.5
Methylene blue stain	1 % methylene blue	Methylene blue = 1 g 0.01 M borate buffer (pH 8.5) = 100 ml
1 M HCl	1 M	37 % fuming HCl = 8.33 ml MilliQ water = 100 ml
HCl:Ethanol	1:1 0.1 M HCl:Ethanol	Absolute ethanol = 500 ml 1 M HCl = 50 ml MilliQ water = 450 ml
Ethanol:formaldehyde:acetic acid	20:2:1	Absolute ethanol = 87 ml 37 % Formaldehyde = 8.7 ml Glacial acetic acid = 4.3 ml
0.1 % HCl	0.1 %	37 % fuming HCl = 0.1 ml MilliQ water = 99.9 ml
Scott's tap water	-	Sodium bicarbonate = 3.5 g MgSO ₄ . 7 H ₂ O = 20 g MilliQ water = 1 L

Collagenase 1A solution	0.2 %	Collagenase 1A (490 U) = 40 mg DMEM = 20 ml Freshly prepared. Filter sterilized.
Matrigel	10 %	Matrigel = 0.4 ml Cold DMEM = 3.6 ml
Protein lysis buffer	50 mM Tris (pH 7.6) 250 mM NaCl 5 mM Na ₂ -EDTA 0.1% Igepal-NP-40 1X Protease inhibitor cocktail 2 mM NaF 2 mM PMSF 1.5 mM Sodium vandate	1 M Tris (pH 7.6) = 2.5 ml 5 M NaCl = 2.5 ml 0.5 M Na ₂ -EDTA = 0.5 ml Igepal-NP-40 = 0.5 ml 100X Protease inhibitor cocktail = 0.5 ml 100 mM NaF = 1 ml 100 mM PMSF = 1 ml 700 mM Sodium vandate = 107 µl Make up to 50 ml with MilliQ water. Store at 4°C.
<u>Solutions for cytoplasmic and nuclear extract isolation (freshly prepared):</u>		
Lysis buffer	50 mM KCl 0.5 % Igepal-NP-40 25 mM HEPES (pH 7.8) - 125 µM DTT 1 mM PMSF	1 M KCl = 250 µl Igepal-NP-40 = 25 µl 1 M HEPES (pH 7.8) = 125 µl 1.7 mg/ml Aprotinin = 58.8 µl 250 mM DTT = 2.5 µl 100 mM PMSF = 50 µl MilliQ water = 4.48 ml
Wash buffer	50 mM KCl 25 mM HEPES (pH 7.8) - 125 µM DTT 1 mM PMSF	1 M KCl = 250 µl 1 M HEPES (pH 7.8) = 125 µl 1.7 mg/ml Aprotinin = 58.8 µl 250 mM DTT = 2.5 µl 100 mM PMSF = 50 µl MilliQ water = 4.51 ml
Extraction buffer	0.5 M KCl 25 mM HEPES (pH 7.8) - - 125 µM DTT 1 mM PMSF	1 M KCl = 2.5 ml 1 M HEPES (pH 7.8) = 125 µl 50 % glycerol = 1 ml 1.7 mg/ml Aprotinin = 58.8 µl 250 mM DTT = 2.5 µl 100 mM PMSF = 50 µl MilliQ water = 1.26 ml

Cell lysis buffer for AOE assays	0.02 M Tris-HCl 150 mM NaCl 1 mM Na ₂ -EDTA pH 7.0	Trizma (Tris-HCl) = 157.6 mg 5 M NaCl = 1.5 ml 0.5 M Na ₂ -EDTA = 0.1 ml Adjust pH to 7.0 Make up to 50 ml with MilliQ water Store at 4°C
Lysis buffer for muscle homogenates for AOE assays	0.02 M Tris-HCl (pH 7.0)	Trizma (Tris-HCl) = 157.6 mg Adjust pH to 7.0 Make up to 50 ml with MilliQ water. Store at 4°C
Carbonate buffer with EDTA	0.1 M (pH 10.2)	A) Sodium carbonate = 1.06 g in 50 ml MilliQ water B) Sodium bicarbonate = 0.336 g in 20 ml MilliQ water For pH 10.2, 33 ml of A + 17 ml of B 28.5 mg Na ₂ -EDTA
Epinephrine	3 mM epinephrine 0.01 M HCl	Epinephrine = 11 mg 37 % fuming HCl = 17 µl Make up to 20 ml with MilliQ water Freshly prepared
0.2 M Phosphate buffer	0.2 M (pH 8.0)	A) Sodium dihydrogen phosphate = 552 mg in 20 ml MilliQ water B) Disodium hydrogen Phosphate = 7.12 g in 200 ml MilliQ water For pH 8.0, 10.6 ml of A + 189.4 ml of B
0.4 M Phosphate buffer	0.4 M (pH 7.0)	A) Sodium dihydrogen phosphate = 1.36 g in 50 ml MilliQ water B) Disodium hydrogen Phosphate = 2.5 mg in 70 ml MilliQ water For pH 7.0, 39 ml of A + 61 ml of B

0.5 M Phosphate buffer	0.5 M (pH 7.0)	A) Sodium dihydrogen phosphate = 345 mg in 50 ml MilliQ water B) Disodium hydrogen Phosphate = 623 mg in 70 ml MilliQ water For pH 7.0, 39 ml of A + 61 ml of B
0.00667 M Phosphate buffer	0.00667 M (pH 7.0)	A) Sodium dihydrogen phosphate = 46 mg in 50 ml MilliQ water B) Disodium hydrogen Phosphate = 83 mg in 70 ml MilliQ water For pH 7.0, 39 ml of A + 61 ml of B
DTNB	0.6 mM in 0.2 M Phosphate buffer (pH 8.0)	DTNB = 23.8 mg 0.2 M Phosphate buffer (pH 8.0) = 100 ml
	0.6 mM in 1 % Sodium citrate	DTNB = 23.8 mg Sodium citrate = 1 g MilliQ water = 100 ml Freshly prepared
Thiobarbituric acid	0.12 M	Thiobarbituric acid = 1.73 g MilliQ water = 100 ml Freshly prepared
Sodium azide	10 mM	Sodium azide = 6.5 mg MilliQ water = 10 ml
Reduced glutathione	4 mM	Reduced glutathione = 12 mg MilliQ water = 10 ml
2.5 mM H ₂ O ₂	2.5 mM	H ₂ O ₂ = 290 µl Make up to 10 ml with MilliQ water

0.20 mg/ml H ₂ O ₂	0.20 mg/ml in 0.00667 M Phosphate buffer (pH 7.0)	H ₂ O ₂ = 4 ml 0.00667 M Phosphate buffer (pH 7.0) = 46 ml
Disodium hydrogen phosphate	0.3 M	Disodium hydrogen phosphate = 2.67 g MilliQ water = 50 ml
MES-SDS running buffer	1 X	20 X MES-SDS = 50 ml MilliQ water = 950 ml
Western blot transfer buffer	-	Tris = 3.03 g Glycine = 14.4 g Methanol = 200 ml Make up to 1000 ml with MilliQ water. Store at 4°C.
TBS (Tris buffered saline)	0.05 M Tris-HCl (pH 7.5) 150 mM NaCl 1 X	1 M Tris-HCl (pH 7.5) = 50 ml 5 M NaCl = 30 ml Make up to 1000 ml with MilliQ water
TBS-T	0.05 M Tris-HCl (pH 7.5) 150 mM NaCl 0.1 % Tween-20 1 X	1 M Tris-HCl (pH 7.5) = 50 ml 5 M NaCl = 30 ml Tween-20 = 1 ml Make up to 1000 ml with MilliQ water
Western blot milk blocker	5 % non-fat milk in 1X TBS-T	Non-fat milk powder = 5 g 1X TBS-T = 100 ml
Western blot PVP blocker	1 % Polyvinylpyrrolidone (PVP), 1 % Polyethylene glycol (PEG) and 0.3 % Bovine serum albumin (BSA) in 1 X TBS-T	PVP = 5 g PEG = 5 g BSA = 1.5 g 1 X TBS-T = 500 ml Filter sterilized

Western blot BSA blocker	5 % BSA in 1X TBS-T	BSA = 5 g 1X TBS-T = 100 ml
<u>Solutions and reagents for EMSA:</u>		
Tris-borate EDTA (TBE) buffer	0.5 X	10 X TBE = 100 ml Make up to 2000 ml with MilliQ Water. Freshly prepared.
5 % TBE gel	-	30 % Acrylamide = 1.66 ml 10 X TBE = 0.5 ml 10 % Ammonium persulfate (freshly prepared) = 0.1 ml TEMED = 10 µl Make up to 10 ml with MilliQ water
Binding buffer	100 mM Tris 500 mM KCl 10 mM DTT pH 7.5	Tris = 60.57 mg KCl = 186.32 mg DTT = 7.71 mg MilliQ water = 5 ml Adjust pH to 7.5. Store at -20°C.
Loading dye	5 X	Bromophenol blue = 25 mg Xylene cyanol – 25 mg 0.2 M Na ₂ -EDTA = 5 ml 30 % glycerol = 3 ml Make up to 10 ml with Milli Q water. Store at -20°C.
Potassium phosphate buffer	50 mM(pH 7.5)	Potassium phosphate, monobasic anhydrous = 1.36 g MilliQ water = 100 ml Adjust pH to 7.5 using 1 M potassium hydroxide
Xanthine solution	0.15 mM (pH 7.5)	Xanthine = 2.28 mg dissolved in NaOH first Make up to 100 ml with MilliQ water. Freshly prepared. Adjust pH to 7.5
Xanthine oxidase solution	0.1-0.2 U/ml	Xanthine oxidase (0.054 U/mg) = 18.5 mg Potassium phosphate buffer (pH 7.5) (Cold) = 10 ml

Tris-acetate EDTA (TAE) buffer	1 X	10 X TAE = 100 ml Make up to 1000 ml with MilliQ water
Sodium citrate buffer	0.1 M (pH 4.5)	Sodium citrate = 294 mg MilliQ water = 10 ml Adjust pH to 4.5
Paraformaldehyde	4 % in 1 X PBS	Paraformaldehyde = 4 g 1 X PBS = 100 ml Stir slowly at 60°C inside fume hood. 1N NaOH added to make clear solution.
H ₂ O ₂ in methanol	3 % H ₂ O ₂ in methanol	H ₂ O ₂ = 3 ml Methanol = 97 ml
Normal goat serum (NGS)	5 % NGS in 1 X TBS	NGS = 0.5 ml 1 X TBS = 9.5 ml
Methanol:Acetone	1:1	Methanol = 50 ml Acetone = 50 ml
0.05 N HCl	0.05 N	1 N HCl = 0.5 ml MilliQ water = 9.5 ml
RNase	100 µg/ml RNase in 150 mM NaCl and 15 mM sodium citrate	10 mg/ml RNase = 100 µl NaCl = 87.6 mg Sodium citrate = 44.1 mg
0.15 N NaOH	0.15 N NaOH in 70 % ethanol	NaOH = 60 mg 100 % ethanol = 7 ml MilliQ water = 3 ml
DAPI	0.2 µg/ml DAPI in 1 X PBS	1 mg/ml DAPI = 2 µl Make up to 10 ml with 1X PBS

Ethanol with formaldehyde	70 % ethanol with 4 % formaldehyde	100 % ethanol = 7 ml Formaldehyde = 0.4 ml Make up to 10 ml with MilliQ water
Proteinase K	5 µg/ml Proteinase K in 20 mM Tris and 1 mM Na ₂ -EDTA pH 7.5	19.2 mg/ml Proteinase K = 2.6 µl Tris = 24.2 mg Na ₂ -EDTA = 3.7 mg Make up to 10 ml with MilliQ water. Adjust pH to 7.5
PBS containing BSA and Tween-20	1 X PBS 1 % BSA 0.01 % Tween-20	1 X PBS = 2 ml BSA = 20 mg Tween-20 = 0.2 µl
PBS-T	1 X PBS 0.05 % Tween-20	1 X PBS = 10 ml Tween-20 = 5 µl
Na ₂ -EDTA	0.5 M	Na ₂ -EDTA = 18.6 g MilliQ water = 100 ml
5 M NaOH	5 M	NaOH = 200 g MilliQ water = 1000 ml
Propidium iodide (PI)	10 µg/ml	1 mg/ml PI = 10 µl MilliQ water = 990 µl
<u>Solutions for Comet assay:</u>		
A1, alkaline lysis solution	1.2 M NaCl 100 mM Na ₂ -EDTA 0.1 % sodium lauryl sarcosinate 0.26 M NaOH pH>13	NaCl = 14.02 g Na ₂ -EDTA = 7.44 g 20 % sodium lauryl sarcosinate = 1ml NaOH = 2.08 g Dissolve in 200 ml MilliQ water Adjust pH>13. Freshly prepared Equilibrate at 4°C
A2, alkaline rinse and electrophoresis solution	0.03 M NaOH 2 mM Na ₂ -EDTA pH~12.3	NaOH = 1.2 g Na ₂ -EDTA = 744 mg MilliQ water = 1000 ml Adjust pH to 12.3

N1, neutral lysis solution	2 % sarkosyl 0.5 M Na ₂ -EDTA 0.5 mg/ml Proteinase K pH 8.0	Sarkosyl = 3 g Na ₂ -EDTA = 27.9 g Proteinase K = 75 mg Dissolve in 150 ml of MilliQ water. Adjust pH to 8.0 Equilibrate at 4°C
N2, neutral rinse and electrophoresis solution	90 mM Tris 90 mM boric acid 2 mM Na ₂ -EDTA pH 8.5	Tris = 43.6 g Boric acid = 22.2 g Na ₂ -EDTA = 2.97 g Make up to 4 L with MilliQ water. Adjust pH to 8.5
DNA loading dye	15 % ficoll 0.25 % bromophenol blue 0.25 % xylene cyanol 1 % SDS 1 mM Na ₂ -EDTA pH 8.0	Ficoll = 1.5 g Bromophenol blue = 25 mg Xylene cyanol = 25 mg SDS = 100 mg Na ₂ -EDTA = 3.7 mg MilliQ water = 10 ml Adjust pH to 8.0
LB broth	-	LB broth = 20 g MilliQ water = 1 L Autoclave. When cool, appropriate antibody was added
LB agar	-	LB agar = 35 g MilliQ water = 1 L Autoclave. When cool, appropriate antibody was added and poured on to petridish
MOPS (10 X)	41.8 % MOPS 50 mM sodium acetate 10 mM Na ₂ -EDTA	MOPS = 418 g Sodium acetate = 4.1 g Na ₂ -EDTA = 3.7 g
MOPS (1 X)	10 X MOPS DEPC treated water	10 X MOPS = 100 ml DEPC treated water = 900 ml

RNA loading dye	10 % 10 X MOPS	10 X MOPS = 1 ml
	20 % deionized	20 % deionized
	formaldehyde	formaldehyde = 2 ml
	50 % deionized	20 % deionized
	formamide	formamide = 5 ml
	0.02 % bromophenol	0.02 % bromophenol blue
	blue	= 2 mg
	5 % glycerol	Glycerol = 0.5 ml
	1 mM Na ₂ -EDTA	Na ₂ -EDTA = 3.7 mg
	40 µg/ml ethidium	10 mg/ml ethidium bromide =
	bromide	40 µl
		Make up to 10 ml with MilliQ
		water

2.1.6 ROS inhibitors

The ROS inhibitors used, the respective enzymes/pathways inhibited and the concentrations used (Iuchi *et al.* 2003) are in Table 2.8.

Table 2.8 *The ROS inhibitors, the respective enzymes/pathways inhibited and the concentration used*

Inhibitor	Enzymes/pathways inhibited	Concentration used (μmol/l)	Duration of treatment (minutes)
Indomethacin	Cyclooxygenase	100	15
L-NAME (Nω-nitro-L-arginine methyl ester)	NOS	1	15
Oxypurinol	Xanthine oxidase	100	15
Quinacrine	NADH oxidase	6	5
DPI (Di Phenylene Iodonium chloride)	NAD(P)H oxidase, NOS and Complex I of mETC	50	5
DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt)	Superoxide release from mitochondria	100	10
CCCP (Carbonyl cyanide 3-chlorophenylhydrazone)	Mitochondrial membrane proton gradient	0.5	Along with Mstn in differentiation media
TTFA (2-Thenoyltrifluoroacetone)	Complex II of mETC	100	Along with Mstn in differentiation media

2.1.7 Bacterial strains

The bacterial strains used in this thesis are outlined in Table 2.9. The bacterial strains were kept at -80°C in a 50% glycerol, 50% LB solution.

Table 2.9 Bacterial strains

Species	Strain	Source
<i>E.coli</i>	DH5 α	Invitrogen
<i>E.coli</i>	JM109	Promega

2.1.8 Cell lines

The cell lines used in this thesis are listed in Table 2.10.

Table 2.10 Cell lines

Cell line	Species	Cell type	Source
C2C12	Mouse	Myoblast	ATCC (Yaffe and Saxel 1977)
CHO	Hamster	Ovary	ATCC
CHO-GDF8	Hamster	Ovary	Prof S J Lee, USA (Zimmers <i>et al.</i> 2002)
I κ B SR C2C12	Mouse	Myoblast	Dr. Denis Guttridge, USA (Guttridge <i>et al.</i> 1999)
shSmad3 C2C12	Mouse	Myoblast	Ge <i>et al.</i> 2011
HepG2	Human	Hepatocyte	ATCC

2.1.9 shRNA

The shRNAs used in this study are listed in Table 2.11.

Table 2.11 *shRNAs used*

shRNA	Vector	Selection marker in <i>E.coli</i>	Source
shSmad2	pRFP-C-RS	Chloramphenicol	Origene
shMstn	pGFP-V-RS	Kanamycin	Origene

2.1.10 Recombinant Mstn protein

Recombinant human Mstn protein was produced and purified in the laboratory according to established protocol (Sharma *et al.* 1999). Briefly, an overnight BL21 *E. coli* culture transformed with the recombinant *myostatin* expression vector (pET 16-B) was diluted and grown to an OD of 0.7 (540 nm) in 1 L of Luria Broth (LB) containing ampicillin (50 mg/L). Expression of the Mstn fusion protein was then induced by isopropyl thio- β -galactoside (IPTG) (800 mg/L) for 3 hours. The bacteria were then harvested by centrifugation and resuspended in 100 mL of lysis buffer (6 M guanidine HCl; 20 mM Tris (pH 8.0); 5 mM β -mercaptoethanol), sonicated and centrifuged at 5,000 rpm for 30 min. Mstn was purified from the supernatant by Ni-Sepharose affinity chromatography (GE Healthcare), according to the manufacturer's protocol. Soluble fractions containing Mstn were pooled and dialyzed at 4°C against two changes of 100 mM Tris-HCl (pH 8.0) containing 500 mM NaCl for 3 hours. The protein concentration was determined by Bradford's assay (Bradford 1976) (Bio-Rad Laboratories Inc.) and the protein was separated on SDS-PAGE gel and stained with Coomassie Blue.

2.1.11 Conditioned media containing Mstn

The CHO cell line engineered to express Mstn from the metallothionein promoter (CHO-myostatin) (Lee and McPherron 2001) was used to obtain Conditioned media containing Mstn (CMM) (Zimmers *et al.* 2002). A clonal cell line transfected with the metallothionein expression vector and similarly selected but shown not to express recombinant protein was used as a control (CHO-control); Control conditioned media (CCM). The CHO cells were seeded at a density of 30,000 cells per cm² and allowed to settle down overnight. The next day, the cells were induced with ZnSO₄ and after 24 hours, the CCM and CMM was collected and filter sterilized. The concentration of Mstn in the conditioned media was determined by EIA (Immundiagnostik AG, Bensheim, Germany). Western blot was performed to detect the presence of Mstn in the conditioned media.

2.1.12 Mstn antagonist Ant1 protein

To antagonize Mstn, Ant1, a C-terminal truncation of Mstn protein (350 amino acid) which is a dominant negative (Mstn mimetic) protein was used. Ant1 was produced and purified as previously published (Siriatt *et al.*, 2007). The protein was purified utilizing a Ni-Sepharose affinity chromatography column (GE Healthcare) (Sharma *et al.* 1999). Soluble fractions containing Ant1 were pooled and dialyzed at 4°C against two changes of 100 mM Tris-HCl (pH 8.0) containing 500 mM NaCl for 3 hours. The protein concentration was determined by Bradford's assay (Bradford 1976) (Bio-Rad Laboratories Inc.). In order to test the purity of the antagonist, the protein was separated on a SDS-PAGE and stained with Coomassie blue.

2.2 Methods

This section describes the experimental procedures used in this thesis, which are not described in detail in the individual chapters. Details of the specific experimental methods used in this thesis are included in Chapters 3-5.

2.2.1 Genotype analysis

Genotype analysis of various transgenic mice lines including $Smad3^{-/-}$, $Mstn^{-/-}$ and double-KO ($Smad3^{-/-}/Mstn^{-/-}$) mice was performed according to Ge *et al.* 2011. A 0.2 x 0.2 cm² section of mouse ear tissue was digested in 300 µl of tissue lysis buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5 % SDS and 100 µg/ml Proteinase K for 2 hours at 56°C and centrifuged at 13,000 rpm at room temperature for 10 minutes. The supernatant was transferred to a new tube and 500 µl isopropanol and 1 ml ice-cold 70 % ethanol was added. The mixture was centrifuged at 13,000 rpm at room temperature for 10 minutes. The supernatant was decanted and DNA pellet was washed with 2 ml of 70 % ethanol. After air drying, the pellet was re-suspended in 100 µl of 10 mM Tris-HCl (pH 8.0) for 5 minutes at 65°C. PCR was performed to identify WT, heterozygous, $Smad3^{-/-}$ and double-KO ($Smad3^{-/-}/Mstn^{-/-}$) mice using the oligonucleotides as listed in Table 2.2. Reaction conditions were as follows: 3 minutes at 94°C and 40 amplification cycles (20 seconds at 94°C, 20 seconds at 60°C, and 20 seconds at 72°C). PCR products were separated on 1.5% agarose gel in 1 X TAE running buffer.

2.2.2 DNA gel electrophoresis

Agarose gels ranged from 0.8-2 % agarose, depending on the size of the fragments to be separated. The gels were made up in 1 X TAE, with ethidium bromide added for

visualization of the DNA bands. The DNA gels were cast and run in 1 X TAE as the running buffer. DNA samples were mixed with DNA loading dye before loading. DNA molecular markers were run beside the DNA samples. Electrophoresis was carried out between 30-100 V until the desired separation of bands was obtained. The separated DNA was then visualized under ultraviolet (UV) light using the Gel Doc system and photos were printed.

2.2.3 Transformation of competent cells and isolation of single bacterial colonies

Transformation of DH5 α or JM109 competent cells was achieved by addition of 5-10 ng of either plasmid DNA to 50 μ L of the competent cells. The DNA-competent cell mixture was incubated on ice for 20 minutes, followed by a heat shock at 42°C for 48 seconds, then further 2 minutes incubation on ice. Following this, 950 μ L of room temperature LB broth (without antibiotic) was added to the transformed cells and incubated at 37°C for 90 minutes with gentle shaking (~150 rpm). The culture was spread over LB agar plates, which contained the appropriate antibiotic, and grown overnight for 16-24 hr at 37°C. Single colonies, from LB agar plates grown overnight were seeded in 4 ml (miniprep) or 100 ml (maxiprep) of LB broth with appropriate antibiotic. The cultures were grown at 37°C with shaking (~250 rpm) for 12-18 hr.

2.2.4 Miniprep plasmid isolation

Plasmid DNA from miniprep cultures was extracted and purified using the QIAprep spin miniprep system (Qiagen), according to the manufacturer's protocol. Bacteria were harvested by centrifugation at 3500 rpm for 5 minutes in a 1.5 ml eppendorf tube. The bacterial pellet was resuspended in 250 μ L of Buffer P1 followed by 250 μ L of Buffer P2, the sample was then mixed by inverting four-six times and allowed to incubate at room

temperature for no more than 5 minutes. After incubation 350 μ l of Buffer P3 was added. After the addition of Buffer P3, the tube was again inverted four-six times to mix. The extract was centrifuged (20,000 $\times$ g for 10 minutes) and the supernatant transferred to a QIAprep spin column. The spin column was centrifuged at 20,000 $\times$ g for 1 minute, washed with 0.75 ml Buffer PE and centrifuged twice to remove any traces of Buffer PE. Plasmid DNA was eluted in 50 μ l Buffer EB by centrifugation (20,000 $\times$ g for 1 minute).

2.2.5 Maxiprep plasmid isolation

Large scale purification of plasmid DNA was performed using the Qiagen Plasmid Maxi kit (Qiagen) as per the protocol. Briefly, 250 ml of overnight culture was harvested by centrifugation at 6000 $\times$ g for 15 minutes. The pellet was then resuspended in 10 ml of buffer P1, mixed with 10 ml of Buffer P2 and incubated on ice for 20 minutes on addition of Buffer P3. The mix was then centrifuged for 30 minutes at 20,000 $\times$ g followed by a second spin at 20,000 $\times$ g for 15 minutes to remove any contaminants. The plasmid DNA containing supernatant was passed through a pre-equilibrated Qiagen-column by gravity flow. Plasmid DNA bound to the column was washed twice with Qiagen Buffer QC and eluted with Qiagen elution buffer. The eluted DNA was precipitated with 0.7 volumes of isopropanol and pelleted by centrifugation at 20,000 $\times$ g for 10 minutes. The pellet was washed twice with 70% ethanol, dried and resuspended in an appropriate volume of TE buffer.

2.2.6 RNA extraction

Total RNA was isolated from cultured cells and Gas muscle using TRIZOL as per the manufacturer's protocol (Invitrogen). For RNA isolation from cells, the culture medium

was removed and the cells were washed twice with 1 X PBS to remove any cell debris. Cells were lysed by the addition of TRIZOL reagent (1 ml per 10 cm plate). For RNA isolation from muscles, 50 mg of muscle tissue was homogenized in 1 ml TRIZOL. Following 5 minutes incubation at room temperature, 200 µl of Chloroform was added and the tubes were shaken vigorously for 15 seconds. After 3 minutes incubation at room temperature, the samples were centrifuged at 12,000 rpm for 15 minutes. The upper clear aqueous phase was then transferred to a new tube and the RNA was precipitated with 500 µl of isopropyl alcohol. Following 10 minutes incubation at room temperature, the samples were centrifuged at 12,000 rpm for 10 minutes. The total RNA was then washed with 1 ml of 75 % ethanol (made in DEPC water) and finally pelleted at 5,000 rpm for 5 minutes and resuspended in an appropriate volume of DEPC-treated MilliQ water. RNA isolated from cells and muscle was quantified using the NanoDrop spectrophotometer to measure concentration and purity. In addition, RNA was subjected to electrophoresis to check RNA quality. All RNA samples were stored at -80°C prior to use.

2.2.7 RNA gel electrophoresis

Formaldehyde-Agarose gels contained between 1-2 % agarose, 1 X MOPS and 0.66 M Formaldehyde. Total RNA was quantified using the NanoDrop spectrophotometer to ensure accurate amounts for RT-qPCR reactions. Total RNA (5 µg) was mixed with an equal volume of RNA loading dye and incubated at 65°C for 5 min prior to loading. Electrophoresis of RNA was done between 40-80 V until the desired separation was achieved. The RNA loading dye contained ethidium bromide and allowed the visualization of the RNA by UV illumination. A Gel Doc system was used to

photograph the gels. The integrity of the RNA was monitored by observing the 28S and 18S ribosomal RNA bands.

2.2.8 cDNA synthesis (Reverse Transcription)

Following RNA isolation and electrophoresis, cDNA was synthesized using Superscript First Strand Synthesis System for RT PCR (Biorad) according to the manufacturer's (Bio-Rad Laboratories, Inc., Hercules, CA, USA) instructions. Each cDNA reaction (20 µl) contained 1 µg of RNA, 1 µl of iScript Reverse transcriptase, 4 µl of 5 X iScript reaction mix and 10 µl of nuclease-free water. All reactions were performed using the following thermal cycler conditions: 25°C for 5 minutes, followed by 42°C for 30 minutes and finally 85°C for 5 minutes.

2.2.9 Myoblast proliferation assay

Proliferating cells in 96-well plates were fixed at 24 hour intervals with 100 µl of formal saline fixative. The proliferation was assessed by methylene blue photometric end point assay (Oliver *et al.* 1989). After removal of fixative, 100 µl of methylene blue stain was added to each well and incubated for 30 minutes at room temperature. The stain solution was then removed and washed 4 times with 200 µl borate buffer. Following this, 200 µl of 0.1 M HCl: Ethanol (1:1) was added to each well. Absorbance was read at 655 nm in a microplate reader (absorbance at 655nm is directly proportional to final cell number).

2.2.10 Myoblast differentiation assay

The differentiating myoblasts and myotubes which were grown on Thermanox cover slips (Nalge Nunc International) in 24-well plates were fixed at 24 hour intervals with 200 µl of fixative (20:2:1-ethanol:formaldehyde:acetic acid). The myotubes were stained

with Hematoxylin and Eosin. 0.5 ml of Hematoxylin was added to the cover slips and incubated for 3 minutes, followed by 1 ml of 0.1 % HCl for 2 seconds. The cells were washed 5 times with 1 ml of Scott's tap water. Then, 0.5 ml of Eosin was added and incubated for 15 seconds to 1 minute. The cells were washed 3 times with 100 % ethanol for 5 minutes and the cover slips were then soaked in xylene for 10 minutes. The stained cover slips were mounted on glass slides with DPX mounting medium. Images of non-overlapping areas were taken in a bright field microscope at 10 X magnification.

2.2.11 Estimation of antioxidant enzyme - Catalase (CAT)

The activity of catalase was determined by the method of Beer and Seizer (Beer and Seizer 1952). 0.05 ml of cell or muscle homogenate was mixed with 0.95 ml of 0.20 mg/ml H₂O₂ in 0.00667 M phosphate buffer. The change in absorbance per 30 seconds was measured at 240 nm for 4 minutes. The activity was expressed as Optical Density of H₂O₂ consumed/min/mg protein.

2.2.12 Estimation of antioxidant-Reduced Glutathione (GSH)

The reduced glutathione was determined by the method of Moron *et al.* (Moron *et al.* 1979). 1 ml of cell or muscle homogenate was precipitated with 2 ml of 5 % TCA by centrifugation at 8,000 x g for 5 minutes at 4°C. 0.5 ml of the supernatant was mixed with 0.5 ml of phosphate buffer (pH 8.0) and 2 ml of DTNB. Absorbance was read at 412 nm and the amount of glutathione was expressed as µg/mg protein.

2.2.13 Estimation of lipid peroxidation product (MDA)

The lipid peroxidation product (MDA) was determined by thiobarbituric acid reaction as described by Ohkawa *et al.* (Ohkawa *et al.* 1979). 1 ml of cell or muscle homogenate

was mixed with 2 ml of 20 % TCA and centrifuged at 4,000 rpm for 20 minutes. 2 ml of the supernatant was mixed with 2 ml of thiobarbituric acid and heated in a boiling water bath. After 20 minutes of heating, the samples were cooled at room temperature and absorbance was read at 532 nm. The lipid peroxide content was expressed as nmoles MDA per 100 mg protein.

2.2.14 Cytoplasmic and nuclear extract isolation

C2C12 cells following various treatments were trypsinized and the cells were centrifuged at 1,500 rpm for 5 minutes at 37°C following 1 X PBS wash twice. The cell pellets (as dry as possible) were stored at -80°C to be processed later or were processed immediately for isolation of cytoplasmic and nuclear extracts as previously described (Ye *et al.* 1996). Muscle samples were homogenized in ice-cold 1 X PBS and processed immediately for isolation of cytoplasmic and nuclear extracts as previously described (Ye *et al.* 1996). To the cell pellet or muscle homogenate, 300 µl of lysis buffer (Table 2.7) was added and the cells were resuspended/muscle homogenate was mixed well and incubated on ice for 4 minutes. The tubes were then centrifuged at 10,000 rpm for 1 minute at 4°C. The supernatant was collected as cytoplasmic extract (CE). The pellet in the tubes were washed with 300 µl of wash buffer (Table 2.7) followed by centrifugation at 10,000 rpm for 1 minute at 4°C. The supernatant was discarded and the pellet was resuspended in appropriate volume (100 µl for cells; 250 µl for muscles) of extraction buffer (Table 2.7). The pellet was pipetted up and down several times in the extraction buffer. The mixture was centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was collected as nuclear extract (NE). The protein concentrations of the nuclear and cytoplasmic extracts were measured by Bradford's assay (Bradford 1976) (Bio-Rad).

2.2.15 Statistical analysis

Statistical analysis was performed using ANOVA and Student's t-test. The prerequisites for ANOVA including sample size, independent samples and normally distributed data were met in all experiments. Results were considered significant at $p < 0.05$. The F-values were calculated and the degrees of freedom were also considered for data to be significant (Bodo Winter 2011). The F-values are represented in one of the figures (Figure 4.2). Results are presented as mean $\pm$ SE of 2 or 3 independent experiments.

CHAPTER 3

MODULATION OF REACTIVE OXYGEN SPECIES IN SKELETAL MUSCLE BY MYOSTATIN IS MEDIATED THROUGH NF- κ B

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Modulation of reactive oxygen species in skeletal muscle by myostatin is mediated through NF- κ B

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Abstract

Abnormal levels of Reactive oxygen species (ROS) and inflammatory cytokines have been observed in the skeletal muscle during muscle wasting including sarcopenia. However the mechanisms that signal ROS production and prolonged maintenance of ROS levels during muscle wasting are not fully understood. Here, it is shown that Myostatin (Mstn) is a pro-oxidant and signals the generation of ROS in muscle cells. Mstn, a Transforming Growth Factor- β (TGF- β) family member, has been shown to play an important role in skeletal muscle wasting by increasing protein degradation. The results here show that Mstn induces oxidative stress by producing ROS in skeletal muscle cells through Tumor Necrosis Factor- α (TNF- α) signaling via NF- κ B and

NADPH oxidase. Aged Mstn-null ($Mstn^{-/-}$) muscles, which display reduced sarcopenia, also show an increased basal Antioxidant Enzyme (AOE) levels and lower NF- κ B levels indicating efficient scavenging of excess ROS. Additionally, the results indicate that both TNF- α and Hydrogen peroxide (H_2O_2) are potent inducers of Mstn and require NF- κ B signaling for Mstn induction. These results demonstrate that Mstn and TNF- α are components of a feed forward loop in which Mstn triggers the generation of second messenger ROS, mediated by TNF- α and NADPH oxidase and the elevated TNF- α in turn stimulates Mstn expression. Higher levels of Mstn in turn induce muscle wasting by activating proteasomal mediated catabolism of intracellular proteins. Thus I propose that inhibition of ROS induced by Mstn could lead to reduced muscle wasting during sarcopenia.

3.1 Introduction

Skeletal muscle is a metabolically active tissue that is highly susceptible to wasting during various disease conditions like cancer, chronic obstructive pulmonary disease (COPD), sepsis and also with aging (sarcopenia). Several studies have reported an increase in ROS in muscle in response to infection, inflammatory stimulus and/or aging (Sohal and Weindruch 1996; Powers *et al.* 2005). ROS are molecules formed by incomplete reduction of oxygen and under normal physiological conditions, ROS get detoxified in the cell with the help of the intracellular AOE's and non-enzymatic antioxidants. Physiological levels of ROS play an important role in myoblast proliferation by regulating G1 to S phase transition (Jahnke *et al.* 2009). Reduced mitochondrial biogenesis, reduced H_2O_2 in the mitochondria and decreased release of Ca^{2+} are shown to be associated with the initiation of differentiation of myoblasts (Jahnke *et al.* 2009). Furthermore, in a recent study, Handayaningsih *et al.* have

demonstrated that IGF-I induced myotubes hypertrophy by signaling via ROS *in vitro* (Handayaningsih *et al.*, 2011). However, excessive generation of ROS cause extensive oxidative damage to nucleic acids and proteins. Two main sources of ROS in the skeletal muscle are mitochondria and peroxisomes. During aging, mitochondrial dysfunction occurs owing to several causes including dysregulated expression of mitochondrial genes and mutations in mitochondrial DNA leading to oxidative stress (Aiken *et al.* 2002; Zahn *et al.* 2006; Herbst *et al.* 2007). In addition, lipid peroxidation is significantly higher in aged human skeletal muscle which is indicative of oxidative stress (Mecocci *et al.* 1999; Pansarasa *et al.* 1999; Fano *et al.* 2001).

In the aging muscle, higher levels of ROS have been linked to the occurrence of chronic inflammation (Peake *et al.* 2010). The combination of increased oxidative stress and inflammation during muscle wasting seems to be causal to the induction of cytokines such as TNF- α and its downstream target NF- κ B and NAD(P)H oxidase (Kim *et al.* 2007). NF- κ B, a transcription factor is generally sequestered in the cytoplasm bound to I κ B in an inactive complex. Once activated by a stimulus including ROS, I κ B gets phosphorylated by IKK and NF- κ B p65 subunit translocates from the cytoplasm into the nucleus and regulates the expression of its target genes. Some of the known downstream targets which NF- κ B up-regulates include muscle specific E3 ligases such as MuRF-1 involved in muscle wasting (Cai *et al.* 2004). NF- κ B is also implicated in the loss of myogenic regulatory factor *myoD* mRNA in skeletal muscle thus enhancing the muscle degeneration (Guttridge *et al.* 2000).

In recent years, a growth and differentiation factor, Mstn has emerged as an inducer of muscle wasting. Mstn is an inhibitor of myogenesis regulating both the proliferation and differentiation of myoblasts (Thomas *et al.* 2000; Langley *et al.* 2002) and over expression of Mstn *in vivo* results in cachexia in mice (Zimmers *et al.* 2002; Reisz-

Porszasz *et al.* 2003). One of the mechanisms by which Mstn has been shown to induce cachexia is by activating the Ubiquitin proteolytic system through FoxO1 (McFarlane *et al.* 2006). In recent past, several studies have reported an increase in *Mstn* mRNA and/or protein levels during aging in humans and rodents (Yarasheski *et al.* 2002; Baumann *et al.* 2003; Raue *et al.* 2006; Leger *et al.* 2008); while, Welle *et al.* reported that *Mstn* mRNA levels were unchanged during aging (Welle *et al.* 2002). Using *Mstn*^{-/-} mice, it was found that lack of Mstn helped muscle regenerate better in the old mice and recently it was reported that Mstn inactivation enhanced bone density, insulin sensitivity and heart function in aged mice (Siriett *et al.* 2007; Morissette *et al.* 2009). Overall, Mstn seems to be playing a role in muscle function during aging also.

It is well established now that skeletal muscle is prone to oxidative stress during aging which augments chronic low level inflammation in the muscle by regulating factors like TNF- α , IL6, IL-1 and NF- κ B which in turn can cause oxidative stress. The combined impact of the oxidative stress and inflammation is thought to be responsible for perturbation of the anabolic signaling proteins such as Insulin-like growth factor 1 (IGF-1) and activating catabolic signals such as proteasomal mediated degradation of proteins. However, what has not been very well understood so far are the mechanisms that initiate TNF- α induction and thereby activate ROS production. Furthermore, the molecular mechanisms that maintain the chronic elevated levels of ROS during aging have also not been thoroughly understood.

Here, in this chapter I present evidence that Mstn is a pro-oxidant capable of signaling ROS production via NF- κ B and TNF- α in muscle cells. Furthermore, this chapter shows that Mstn feeds forward the expression of redox sensitive transcription factor, NF- κ B through induction of pro-inflammatory cytokine TNF- α . Lack of functional Mstn offers resistance to oxidative stress in the aging muscle by enhancing the antioxidant response

and reducing NF- κ B levels. Based on the results presented, it can be speculated that Mstn plays a critical role in integrating redox signaling, inflammatory and catabolic signaling pathways during ROS mediated muscle wasting.

3.2 Materials and methods

3.2.1 Animals

Young 5-8 week old C57Bl/6J male mice (WT) were obtained from National University of Singapore-Centre for Animal Resources (NUS-CARE), Singapore, Republic of Singapore. Young 5-8 week old $Mstn^{-/-}$ were obtained from $Mstn^{-/-}$ heterozygous mice breeding (gifted by Prof S.J Lee (Johns Hopkins University, Baltimore, MD, USA)) at Nanyang Technological University (NTU) Animal house, Singapore, Republic of Singapore. Wild type and $Mstn^{-/-}$ mice (2 year old) were aged at AgResearch, Hamilton, NZ. All animals had free access to chow diet and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Singapore.

3.2.2 Reagents and Proteins

Recombinant human Mstn protein used in the experiments was produced and purified in the laboratory according to established protocol (Sharma *et al.* 1999). Conditioned media containing Mstn was obtained from CHO cell line that expresses Mstn (Zimmers *et al.* 2002). The concentration of Mstn in the conditioned media was determined by EIA (Immundiagnostik AG, Bensheim, Germany). Recombinant human TNF- α was purchased from Miltenyi Biotec., Bergisch Gladbach, Germany.

3.2.3 Cell culture

The established ATCC mammalian (murine) cell line C2C12 myoblasts were used (Yaffe and Saxel 1977). The cell stocks were stored in C2C12 proliferation media: Dulbecco's Modified Eagle Medium (DMEM) (PAA Laboratories, Inc., Pasching,

Austria), 10 % Fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah, USA), 1 % Penicillin/Streptomycin (P/S) (Gibco, Carlsbad, CA, USA) media with 5 % Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) in liquid nitrogen. The cells were passaged in proliferation media and differentiated in C2C12 differentiation media: DMEM, 2 % Horse serum (HS) (Gibco, Carlsbad, CA, USA), 1 % P/S was used to differentiate the myoblasts to myotubes.

The NF- κ B inhibitor cell line which is the I κ B- α super-repressor (I κ B- α SR) C2C12 cells was gifted by Guttridge *et al.* (1999). The I κ B- α control and I κ B- α SR expressing C2C12 cells were cultured in proliferation media containing 0.5 mg/ml Geneticin (Invitrogen, Carlsbad, CA, USA).

3.2.4 Myoblast proliferation assay

C2C12 cells were seeded in 96-well plates in proliferation media at a cell density of 1000 cells per well and incubated at 37°C and 5 % CO₂. After overnight attachment, the cells were treated with H₂O₂ (0.05 mM and 0.1 mM) and the negative control was treated with autoclaved Milli Q water in fresh proliferation media. Proliferating cells were fixed at 24 hour intervals with 100 μ l of formal saline fixative (10 % formaldehyde (Merck & Co., Inc., NJ, USA) and 0.9% saline). The proliferation was assessed by methylene blue photometric end point assay (Oliver *et al.* 1989) (as described in Section 2.2.9).

3.2.5 Myoblast differentiation assay

C2C12 cells were seeded on Thermanox plastic cover slips (Nalge Nunc International, Rochester, NY, USA) in 24-well plates in proliferation media at a cell density of 25,000 cells per cm². After overnight incubation of cells at 37°C and 5 % CO₂, the cells were

treated with various concentrations of H₂O₂ (0.05 mM) and the negative control was treated with autoclaved Milli Q water in differentiation media. The differentiating cells and myotubes were fixed at 24 hour intervals. The myotubes were stained with Hematoxylin and Eosin (as described in Section 2.2.10). Images of non-overlapping areas from two cover slips were taken in a bright field microscope at 10X magnification.

3.2.6 Isolation of primary myoblasts (satellite cells)

Primary myoblasts were isolated according to the established protocol (Partridge 1997; McCroskery *et al.* 2003). Briefly, the hind limb skeletal muscles from mice were dissected and minced in PBS. After the PBS was discarded by centrifugation, the minced muscle was digested in 0.2 % collagenase type 1A in warmed for 90 minutes. The digested muscle after several washes was gently triturated and filtered. The filtered suspension was centrifuged and the pellet was resuspended in Satellite cell (SC) medium: DMEM, 20 % FBS, 10 % HS, 1 % P/S and 1 % Chick embryo extract (CEE). Fibroblasts were removed by pre-plating on uncoated plate for 3 hours. Myoblasts were cultured on 10 % matrigel coated plates and were maintained in SC medium. Isolated satellite cells were trypsinized and seeded for various experiments.

3.2.7 Treatment of C2C12 cells and primary myoblasts

Based on the results obtained from the proliferation and differentiation assays on C2C12 cells treated with H₂O₂, 0.05 mM was chosen as concentration of H₂O₂ to cause mild oxidative stress and hereby this concentration was used for the treatment of cells. Recombinant Mstn, 3 µg/ml concentrations, used in this study inhibited 30-40 % proliferation of myoblasts. Thus, this concentration of Mstn was used to cause mild oxidative stress in the cells. The conditioned media from CHO cells was found to have

Mstn at a concentration of 3.5 ng/ml. Based on earlier studies (Yamashita *et al.* 2008; Grzelkowska-Kowalczyk and Wieteska-Skrzeczyńska 2010), 10 ng/ml TNF- α was used to treat C2C12 cells. Also, 10 μ M/ml of 5-ASA (Yan and Polk 1999) and 100 μ M/ml ascorbic acid (Ciafre *et al.* 2007) was used to treat C2C12 cells during proliferation and differentiation. BAY 11-7085 at the concentration of 20 μ M/ml (Ladner *et al.* 2003) was used to inhibit NF- κ B activity in proliferating C2C12 cells. C2C12 cells and primary myoblasts following various treatments for time indicated were collected in TRIZOL (Invitrogen, Carlsbad, CA, USA) for RNA isolation.

3.2.8 Determination of ROS production

3.2.8.1 Real time intracellular measurement of ROS

ROS was determined using a fluorescent probe, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) (Molecular Probes, Carlsbad, CA, USA). C2C12 cells were seeded in 96-well plates in proliferation media at a cell density of 6000 cells per well and incubated at 37°C and 5 % CO₂. After overnight attachment of cells, they were treated with Mstn in fresh differentiation media. Subsequently, at 24 hour time point intervals, the media was removed and the cells were incubated with 5 μ M of CM-H2DCFDA (Molecular Probes, Carlsbad, CA, USA) in phosphate buffered saline (PBS) for 30 minutes at 37°C and 5 % CO₂. Upon incubation, the dye was removed and the 200 μ l of differentiation media was added in each well. Fluorescence was determined at various time points using a fluorescent multi label plate reader at excitation/emission wavelengths of 495 nm/525 nm respectively.

3.2.8.2 Visualization of Mstn induced ROS

C2C12 cells were seeded onto Permax chamber slides (Nalge Nunc International, Rochester, NY, USA) in proliferation media at a cell density of 25,000 cells per cm² and incubated at 37°C and 5 % CO₂. After overnight attachment, the cells were treated with Mstn in fresh differentiation media. Subsequently, at 24 hour time intervals, the media was removed and the cells were incubated with 5 µM of CM-H2DCFDA (Molecular Probes, Carlsbad, CA, USA) in PBS for 30 minutes at 37°C and 5 % CO₂. Upon incubation, the dye was removed and 0.5 ml of differentiation media was added in each well. The fluorescence was viewed under the Leica upright microscope and images were taken at 10 X magnification.

3.2.9 Protein isolation

C2C12 cells and primary myoblasts following various treatments for the indicated time were collected in Protein lysis buffer [50 mM Tris pH 7.6, 250 mM NaCl, 5 mM EDTA, 0.1% Igepal-Nonidet P-40 (NP-40) (Fluka, St. Louis, MO, USA), Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana, USA)] and protein lysates were made as previously described (McFarlane *et al.* 2006). Gas muscle from young and aged WT and Mstn^{-/-} mice were homogenized in the protein lysis buffer and the clear supernatants were collected. These protein lysates were used for Oxyblot assay (Millipore Corp., Billerica, MA, USA) and Western blotting analysis. Cytoplasmic and nuclear fractions from proliferating C2C12 cells following Mstn treatment were isolated as previously described (Ye *et al.* 1996) (as described in Section 2.2.14).

3.2.10 Oxyblot assay

To detect carbonyl groups introduced into proteins by oxidative reactions due to ROS, Oxyblot assay using Oxyblot Protein Oxidation Detection Kit (Millipore Corp., Billerica, MA, USA) was performed. Briefly, 15 µg of the protein lysate collected from C2C12 cells upon various treatments was derivatized with 1X 2, 4-Dinitrophenylhydrazine (DNPH) solution. The derivatized samples were loaded onto a 4-12 % Bis-Tris polyacrylamide gel (Invitrogen) and electro blotted onto a nitrocellulose membrane. After blocking with 1X PBS-T, BSA, pH 7.2, the membrane was incubated with 1:200 diluted Rabbit anti-DNP primary antibody followed by incubation with 1:300 diluted Goat anti-rabbit IgG (HRP conjugated) secondary antibody. After subsequent washes, the membrane was developed using chemiluminescent reagents (Western Lightning Plus ECL, PerkinElmer, Waltham, MA, USA) and was exposed to x-ray film and the signal intensity was calculated using a densitometer to determine the extent of carbonylation of the proteins.

3.2.11 Cell and muscle homogenates for AOE assays

To obtain cell homogenates for antioxidant enzyme assays, the C2C12 cells and primary myoblasts were seeded in 6-well plates at a cell density of 25,000 cells per cm². After overnight incubation at 37°C and 5 % CO₂, the cells were treated with the above mentioned concentration of recombinant Mstn or conditioned media Mstn; the negative control being the buffer or control conditioned CHO media respectively in differentiation media. The 10 % cell homogenate (1 ml of cell lysis buffer added to the cells and scraped off the plate, and resuspended in 9 ml of cell lysis buffer) was collected at 24 hour time points in cell lysis buffer (0.02 M Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA). Similarly, for AOE assays in muscle samples (Quadriceps), the

muscles were weighed and a 10 % homogenate was made in lysis buffer (0.02M Tris-HCl pH 7.0) using a Teflon homogenizer in ice-cold condition. The homogenates were centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatants were used for determination of various non-enzymatic and enzymatic antioxidants.

3.2.12 Estimation of protein concentrations using Bradford's (Biorad) assay

The protein concentrations of the cell and muscle homogenates, cell protein lysates, nuclear and cytoplasmic extracts were determined using Bradford's assay (Bradford 1976) (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3.2.13 Estimation of antioxidant enzymes

3.2.13.1 Super Oxide Dismutase (SOD)

0.1 ml of cell homogenate was diluted by adding 0.9 ml of distilled water and 1.5 ml of 0.1 M carbonate buffer, pH 10.2, (containing 57 mg EDTA/100ml) was added to it. The reaction was initiated by the addition of 0.4 ml of 3mM epinephrine. The change in absorbance per minute was measured at 480 nm for 3 minutes. Activity is expressed as the Optical density (at 480 nm) of the enzyme required to exhibit 50% inhibition of epinephrine auto oxidation.

3.2.13.2 Catalase (CAT)

The activity of catalase was determined by the method of Beer and Seizer (1952) (as described in Section 2.2.11). The activity was expressed as Optical Density of H₂O₂ consumed/min/mg protein.

3.2.13.3 Glutathione Peroxidase (GPx)

Activity of glutathione peroxidase was determined using the method of Rotruck *et al.* (1973) with some modifications. Briefly, 0.4 M sodium phosphate buffer pH 7.0, 10

mM sodium azide, 4mM reduced glutathione, the cell lysate or muscle homogenate and 2.5 mM hydrogen peroxide were mixed and incubated at 37°C for 3 minutes. After terminating the reaction with 10% TCA, the residual glutathione content was determined by addition of 0.3 M disodium hydrogen phosphate and 0.6 mM DTNB reagent. The activity of GPx was expressed as μg of glutathione consumed/min/mg protein.

3.2.14 Estimation of antioxidant-Reduced Glutathione (GSH)

The reduced glutathione was determined by the method of Moron *et al.* (1979) (as described in Section 2.2.12). The amount of glutathione was expressed as $\mu\text{g}/\text{mg}$ protein.

3.2.15 Estimation of lipid peroxidation product

The lipid peroxidation product (MDA) was determined by thiobarbituric acid reaction as described by Ohkawa *et al.* (1979) (as described in Section 2.2.13). The lipid peroxide content was expressed as nmoles MDA per 100 mg protein.

3.2.16 Identification of signaling pathway and enzymes involved through which Mstn induces ROS

To determine the enzymes involved and the cell signaling pathway involved in Mstn induced ROS, various cell signaling inhibitors were used (Iuchi *et al.* 2003). The inhibitors used are as mentioned in Table 2.8. C2C12 cells were seeded in 96-well plates in proliferation media at a cell density of 6000 cells per well and incubated at 37°C and 5 % CO_2 . After overnight attachment, the cells were treated with Mstn in fresh differentiation media for wells assigned for inhibitors 1-6. For inhibitor 7, the cells in

the assigned wells were treated with Mstn and 0.5 $\mu\text{mol/l}$ CCCP and for inhibitor 8, Mstn and 100 $\mu\text{mol/l}$ TTFA in fresh differentiation media (Table 2.8). After 48 hours of Mstn treatment, inhibitors 1-6 (Table 2.8) in differentiation media were added to the wells. After the stipulated time (Table 2.8), the cells were incubated with 5 μM of CM-H2DCFDA (Molecular Probes, Carlsbad, CA, USA) in PBS for 30 minutes at 37°C and 5 % CO_2 . Upon incubation, the dye was removed and 200 μl of differentiation media per well was added. Fluorescence was determined using a fluorescent multi label plate reader at excitation/emission wavelengths of 495 nm/525 nm respectively.

3.2.17 RT-qPCR (Reverse Transcriptase-quantitative Polymerase Chain Reaction)

RNA was isolated from C2C12 cells that were subjected to various treatments during proliferation and differentiation using TRIZOL protocol (Invitrogen, Carlsbad, CA, USA) and also from muscles (Gas) and cDNA was synthesized using Superscript First Strand Synthesis System for RT PCR (Biorad) according to the manufacturer's (Bio-Rad Laboratories, Inc., Hercules, CA, USA) instructions. Quantitative analysis of the gene expression by real-time PCR was carried out using CFX96 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) system. Each real-time PCR reaction (10 μl) contained 2 μl of cDNA, 5 μl of SsoFast Evagreen (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and forward and reverse primers (Table 2.1) at a final concentration of 2.5 μM . All reactions were performed using the following thermal cycler conditions: 98°C for 3 minutes, followed by 45 cycles of a three-step reaction, denaturation at 98°C for 3 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 10 seconds, and final extension at 95°C for 10 seconds. The reaction was followed by a melt curve from 65°C to 95°C in 5 second increments of 0.5°C to ensure amplification specificity. Transcript

levels were normalized to GAPDH transcript levels. Relative fold change in expression was calculated using the $\Delta\Delta CT$ method.

3.2.18 Western Blot analysis

Total protein (15-40 μ g) was separated on 4-12 % Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane by electro blotting. The membranes for probing with primary antibodies NF- κ B-p65, Mstn, p-Akt1/2/3 (Ser473), Akt1/2/3, Atrogin-1 and MuRF-1 were blocked for 1 hour at room temperature in 0.3 % BSA, 1 % Polyethylene glycol (PEG) (Sigma-Aldrich, St. Louis, MO, USA) and 1 % Polyvinylpyrrolidone (PVP) (Sigma-Aldrich, St. Louis, MO, USA) in 1X TBS-T, then incubated overnight at 4°C with the above mentioned primary antibodies. The membranes for probing with p-Smad2/3, IKK α , α -Tubulin and GAPDH were blocked in 5 % non-fat milk in 1X TBS-T overnight at 4°C and then incubated for 3 hours at room temperature with the above mentioned primary antibodies. The membranes for detecting p-NF- κ B-p65 (Ser536), IKB- α and p-IKB- α (Ser32/36) were blocked for 1 hour at room temperature in 5 % BSA TBS-T, then incubated overnight at 4°C with the specific primary antibodies. The primary antibody dilutions and other information on them are as stated in Table 2.3. After subsequent washes and incubation with respective secondary antibodies (Table 2.5) for 1 hour at room temperature, the HRP activity was detected using chemiluminescent reagents (Western Lightning Plus ECL, PerkinElmer, Waltham, MA, USA).

3.2.19 Electrophoretic mobility shift assay (EMSA)

The oligonucleotides containing the NF- κ B binding site on mouse Mstn promoter (5'-CTCAAACAGGGGGCTTTCCCTCCTCAATATC-3') and mouse TNF- α promoter

(5'-GATCCAGCTCAGGGGGAGGCCCAAGGCCTGAC-3') were hybridized to their respective complementary strands and labeled at the 3' end with Biotin Tetra-ethyleneglycol (TEG) (Sigma-Aldrich, St. Louis, MO, USA). The EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific Pierce Biotechnology, Rockford, IL, USA). Nuclear extracts obtained from Mstn treated proliferating C2C12 cells were incubated with the biotin labeled probe (final concentration 20 fmol) in a final volume of 20 μ l containing Binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), glycerol, MgCl₂, poly(dI-dC), and NP-40. After 20 minutes of incubation at room temperature, samples were subjected to electrophoresis on a 5 % acrylamide gel containing Tris-Borate-EDTA (TBE) (Promega Corporation, Madison, WI, USA) at 80V at 4°C. The samples were then transferred to a nylon membrane at 200 mA for 2 hours at 4°C and UV-crosslinked after transfer. The membrane was probed with stabilized Streptavidin-Horseradish Peroxidase conjugate and developed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific Pierce Biotechnology, Rockford, IL, USA).

To confirm the presence of NF- κ B p65 and NF- κ B p50 in the retarded complex, nuclear extracts from C2C12 cells were preincubated for 20 minutes at room temperature with 1 μ l of 1:1 diluted (in PBS) NF- κ B p65 (sc-372) and NF- κ B p50 (sc-7178) antibody (Table 2.3) before incubation with the labeled probe for another 20 minutes at room temperature. The samples were then subjected to EMSA as mentioned above.

3.2.20 Mstn Enzyme immunoassay (EIA)

To estimate the Mstn secreted into the media, Mstn EIA kit (Immundiagnostik AG, Bensheim, Germany) was used and the competitive immunoassay was performed according to manufacturer's instructions. Briefly, standards with defined Mstn

concentrations and media which were to be assayed for Mstn were incubated with polyclonal antibodies against human recombinant Mstn in glass vials. During the incubation, free Mstn would be bound by the Mstn antibodies. The pre-incubates were then transferred into the microtiter wells coated with recombinant Mstn. The unbound antibodies in the pre-incubates then bind to the immobilized antigen and are detected using a peroxidase-conjugated secondary antibody and TMB as a substrate. The absorbance of the samples is read using an ELISA microtiter plate reader at 450 nm. The concentration of Mstn secreted into the media is expressed as ng/ml.

3.2.21 Statistical analysis

Statistical analysis was performed using ANOVA and results were considered significant at $p < 0.05$. Results were expressed as SEM (Standard Error of Mean).

3.3 Results

3.3.1 Mstn induces ROS in differentiating C2C12 myoblasts

Increased levels of Mstn are associated with cachexia, sarcopenia and disuse atrophy (Langley *et al.* 2002; McFarlane *et al.* 2006). Similarly, ROS or oxidative stress plays an important role in inducing muscle atrophy (Powers *et al.* 2005), sarcopenia and cachexia (Li and Reid 2000; Gomes-Marcondes and Tisdale 2002; Mantovani *et al.* 2004; Moylan and Reid 2007). Hence, whether Mstn could induce ROS production was determined by treating differentiating C2C12 cells with Mstn and estimating the levels of ROS. The results show that ROS production was significantly increased upon Mstn treatment (Figure 3.1A and 3.1B).

3.3.2 Mstn increases protein carbonylation in C2C12 myoblasts

ROS are molecules that cause extensive oxidative damage to nucleic acids, proteins and various components of the cell. The protein oxidation by ROS leads to various protein modification including protein carbonylation (Stadtman 2001). To find out if ROS induced by Mstn results in oxidation and subsequent carbonylation, protein lysates of C2C12 cells treated with H₂O₂ (potent pro-oxidant) or Mstn were subjected to immunoblotting with anti-DNP antibody. Densitometry analysis of the oxyblot clearly indicates an increased protein carbonylation in response to H₂O₂ and Mstn treatment of C2C12 cells (Figure 3.1C). Hence, the results indicate that ROS produced by Mstn causes protein carbonylation in C2C12 myoblasts.

Figure 3.1 *Mstn* induces ROS in C2C12 myoblasts. (A) ROS production was measured using a fluorescent multi label plate reader in differentiating C2C12 cells treated with Mstn (3.5 ng/ml) using a fluorescent probe, CM-H2DCFDA (Molecular Probes, Carlsbad, CA, USA). The graph shows relative intensities expressed as arbitrary fluorescent units. ROS production is significantly increased (** $p < 0.001$) upon Mstn treatment during differentiation when compared to the untreated cells ($n = 4$). (B) ROS production was also measured in Mstn treated C2C12 cells in Permanox chamber slides. The fluorescence was viewed under the Leica upright microscope and images were taken at 10X magnification. Increased fluorescence (green) intensity is directly proportional to increased ROS production in cells ($n = 4$). (C) Effect of Mstn and H₂O₂ on protein carbonylation in C2C12 myoblasts. Left panel, representative gel showing the increased protein carbonylation in H₂O₂ (0.05 mM) and Mstn (3.5 ng/ml) treated C2C12 cells as detected by Oxyblot assay kit (Millipore Corp., Billerica, MA, USA). Right panel, corresponding densitometry analysis of the blot showing the percentage increase in carbonylation as compared to the untreated cells (* $p < 0.05$) ($n = 2$). α -tubulin was used as an internal control for equal protein loading on the gel.

3.3.3 Mstn up-regulates the activity of AOE and their gene expression in differentiating C2C12 myoblasts

Under normal physiological conditions, cells respond to the changes in ROS levels by the production of non-enzymatic antioxidants and AOE. To investigate if ROS induced by Mstn also elicit changes in antioxidant enzyme levels, enzyme activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were determined in proteins extracts from C2C12 cells treated with Mstn. Increase in activity of SOD (Figure 3.2A), CAT (Figure 3.2B) and GPx (Figure 3.2C) was observed in differentiating C2C12 cells treated with Mstn. To determine if Mstn increases the transcription of the genes encoding AOE, RT qPCR analysis was performed. The results show that mRNA expression of *Sod1* (Figure 3.2E) and *Cat* (Figure 3.2F) was up-regulated upon Mstn treatment during C2C12 myoblast differentiation. To determine if Mstn treatment also regulates the levels of non-enzymatic antioxidant reduced glutathione reductase (GSR), the activity of GSR produced in differentiating C2C12 cells was assayed. Results show that GSR levels are significantly elevated on treatment with Mstn (Figure 3.2D). Together these results indicate that Mstn increases the levels of AOE and GSR in differentiating C2C12 myotubes.

Figure 3.2 *Mstn* up-regulates the activity and mRNA expression of AOEs in differentiating C2C12 myoblasts. C2C12 cells were treated with Mstn (3 µg/ml) in the differentiation media and total cell lysates were made. The enzyme assays were performed for SOD (A), CAT (B), GPx (C) and GSR (D). The values are mean ± S.E of 4 independent experiments; **p<0.01 and ***p<0.001 when compared to the negative control (n=4). C2C12 cells were treated with Mstn (3.5 ng/ml) and gene expression analysis was performed for *Sod1* and *Cat*. The values are mean ± S.E of 4 independent experiments. An increase in gene expression of *Sod1* (E, ***p<0.001) and *Cat* (F, ****p<0.0001) was observed upon Mstn treatment during differentiation (n=4).

3.3.4 Basal AOE activity in Mstn^{-/-} mice is higher than the WT mice

To further understand the regulation of antioxidant enzyme gene expression by Mstn, the basal levels of the AOE activity during differentiation were compared between the WT and Mstn^{-/-} primary myoblasts. The results show that Mstn^{-/-} myoblasts have higher basal levels of the antioxidant enzymes (SOD, CAT and GPx) when compared to the WT myoblasts (Figures 3.3A, 3.3B and 3.3C respectively).

Figure 3.3 *Mstn^{-/-} primary myoblasts have elevated basal AOE levels.* Enzyme activities of SOD (A), CAT (B) and GPx (C) were analyzed in the total cell lysates of differentiating primary myoblasts from WT and Mstn^{-/-} mice (8 week old). The values are given as mean ± S.E of 4 independent experiments; **p<0.05, ***p<0.001 and ****p<0.0001 denote significant increase compared to the WT primary myoblasts.

3.3.5 NADH/NAD(P)H oxidase, enzymes of Mitochondrial ETC (mETC) and TNF- α are involved in ROS production by Mstn

To investigate the mechanism involved in Mstn induced ROS generation, cell signaling inhibitors which include Indomethacin, L-NAME, Oxypurinol, Quinacrine, DPI, DIDS, CCCP and TTFA (Table 2.8) that inhibit various pathways and enzymes involved in ROS production were used (Iuchi *et al.* 2003). ROS production by Mstn was significantly inhibited in cells treated with Quinacrine, DIDS, CCCP and TTFA inhibitors (Figure 3.4A). These inhibitors block free radical generation by NADH/NAD(P)H oxidase, the various enzymes and complexes of mitochondria and mETC. Thus, the inhibitor studies show that NADH/NAD(P)H oxidase and mETC are involved in production of ROS by Mstn in muscle cells. To further establish that NADPH oxidase is involved in Mstn signaling, the mRNA expression of NADPH oxidase 1 (*Nox1*) in differentiating C2C12 myoblasts treated with Mstn was analyzed. The results show an up-regulation of *Nox1* (Figure 3.4B) up on treatment with Mstn.

Figure 3.4 Effect of the inhibitors of NADH/NAD(P)H oxidase and enzymes of Mitochondrial ETC (mETC) on Mstn induced ROS generation. (A) C2C12 cells were treated for 48 hours with Mstn (3.5 ng/ml) in the presence of ROS cell signaling inhibitors and ROS content was analyzed using the fluorescent probe, CM-H2DCFDA (Molecular Probes, Carlsbad, CA, USA). ROS production was significantly decreased (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) in cells treated with Quinacrine, DIDS, CCCP and TTFA when compared to the cells treated with Mstn alone (no inhibitor). The data are expressed as percentage increase or decrease in ROS production ($n = 4$). mRNA expression for *Nox1* (B) was determined in C2C12 cells treated with Mstn (3.5 ng/ml) in differentiation media at indicated time points. The values are mean $\pm$ S.E of 4 independent experiments. *** $p < 0.001$ denotes significant fold increase in *Nox1* mRNA expression relative to the negative control.

Lipid peroxidation is also known to give rise to ROS, therefore WT myoblasts were treated with Mstn and analyzed the lipid peroxidation by measuring ThioBarbituric Acid Reactive Substances (TBARS) expressed in terms of malonaldehyde (MDA). TBARS estimation has been commonly used previously but its drawbacks are overestimation of the product and inconsistency (Garcia *et al.* 2005). Nevertheless, Mstn treatment of WT myoblasts decreased TBARS levels (Figure 3.5A) suggesting that Mstn induced the generation of ROS independent of lipid peroxidation. An increase in lipid peroxidation during differentiation in Mstn^{-/-} myotubes resulting in increased production of TBARS was observed (Figure 3.5B). This increase in lipid peroxidation could be attributed to the increased number of mitochondria in the Mstn^{-/-} muscles (Unpublished results from our laboratory).

Figure 3.5 *Mstn* down regulates lipid peroxidation in differentiating WT primary myoblasts. (A) WT primary myoblasts were treated with Mstn (3 μ g/ml) in the differentiation media and total cell lysates were made at indicated time points. The lipid peroxidation was determined and the values are given as nmoles of MDA produced and are mean $\pm$ SEM of 3 independent experiments; ** $p < 0.01$ denotes significant decrease relative to the negative control ($n=3$). ***Mstn^{-/-} primary myoblasts have increased lipid peroxidation.*** (B) The lipid peroxidation was determined in the differentiating primary myoblasts at the indicated time point ($n=4$). ** $p < 0.01$ denotes significant increase relative to the WT primary myoblasts.

3.3.6 Mstn induces ROS through TNF- α and NF- κ B signaling

Since TNF- α is known to regulate NADPH oxidase (Kim *et al.* 2007), next it was investigated if Mstn induces TNF- α gene expression which in turn up-regulates *Nox1* gene. The RT qPCR results confirmed that Mstn treatment potently induces TNF- α expression in differentiating C2C12 cells (Figure 3.6A). To ascertain if Mstn requires NF- κ B activity to induce TNF- α and *Nox1*, stable C2C12 cell line that expresses the I κ B- α SR protein was used. The I κ B- α SR protein acts as a potent and specific inhibitor of NF- κ B activity (Guttridge *et al.* 1999). As shown in Figure 3.6B, treatment of control cells with Mstn leads to an increase in TNF- α (Figure 3.6B (i)) and *Nox1* (Figure 3.6B (ii)) expression. However, in I κ B- α SR expressing C2C12 cells, Mstn fails to induce the expression of TNF- α (Figure 3.6B (i)) and *Nox1* (Figure 3.6B (ii)). In another experiment, NF- κ B activity was inhibited by the compound BAY 11-7085, which prevents NF- κ B activation by blocking I κ B- α phosphorylation (Ladner *et al.* 2003). When C2C12 cells were first treated with BAY 11-7085 compound, Mstn failed to up-regulate TNF- α and *Nox1* mRNA expression (Figure 3.6C; (i) and (ii) respectively). Hence, these results confirm that Mstn requires NF- κ B activity to induce TNF- α and *Nox1* expression.

Figure 3.6 *Mstn induces ROS through TNF- α via NF- κ B signaling.* mRNA expression for TNF- α (A) was determined in C2C12 cells treated with Mstn (3.5 ng/ml) in differentiation media at indicated time points. The values are mean $\pm$ S.E of 4 independent experiments. *** p <0.001 and **** p <0.0001 denote significant fold increase in TNF- α mRNA expression relative to the negative control. (B) Representative graph showing mRNA expression of TNF- α (i) and *Nox1* (ii) in I κ B- α Control and I κ B- α SR expressing C2C12 cells which lack NF- κ B activity treated for 48 hours with Mstn (3.5 ng/ml) in proliferation media. The values are mean $\pm$ S.E of 2 independent experiments. ** p <0.01 denotes significant increase when compared to untreated cells (n=2). (C) Representative graph showing mRNA expression of TNF- α (i) and *Nox1* (ii) in C2C12 cells treated for 1 hour with BAY11-7085 (20 μ M/ml) and for 48 hours with Mstn (3.5 ng/ml) in proliferation media. The values are mean $\pm$ S.E of 2 independent experiments. ** p <0.01 and *** p <0.001 denotes significant increase when compared to untreated cells; ^^ p <0.01 denotes significant decrease when compared to Mstn treated cells (n=2).

Normally, NF- κ B exists in the cytoplasm in an inactive form bound to I κ B- α . Various stimuli, for example, TNF- α are known to transduce the signal starting at proximal kinase IKK, which is known to phosphorylate I κ B- α on Serine32/36. The phosphorylation of I κ B- α is necessary for its degradation and release of NF- κ B from the inactive complex. Concomitantly, the phosphorylation of NF- κ B and its translocation from the cytoplasm to the nucleus renders NF- κ B to bind to the regulatory sequences of the target genes. Therefore, the signaling events leading to the activation of NF- κ B upon Mstn treatment was studied next. Whole cell lysates, nuclear and cytoplasmic extracts of C2C12 cells treated with Mstn during proliferation were analyzed by Western blotting using NF- κ B p65, p-NF- κ B p65, I κ B- α , p-I κ B- α and IKK α antibodies (Figure 3.7 (i)). Whole cell protein lysates did not show a significant difference in NF- κ B p65 level on Mstn treatment (Lanes 2 and 4) however nuclear extracts showed increased NF- κ B p65 level up on 48 and 72 hours of Mstn treatment (Lanes 2 and 4) compared to the untreated cells (Lanes 1 and 3). A decrease in NF- κ B p65 content was observed in the cytoplasmic extract at 48 hours on Mstn treatment (Lane 2), at the same time a decrease in p-NF- κ B p65 level (Figure 3.7 (ii)) was observed in the nuclear extract (Lane 2). An increase in NF- κ B p65 level in cytoplasmic extract and p-NF- κ B p65 level in nuclear extract was observed on 72 hours of Mstn treatment (Lane 4). After 48 hours of treatment with Mstn, I κ B- α content was decreased (Lane 2) and p-I κ B- α level (Figure 3.7 (iii)) remained unchanged (Lane 2), whereas 72 hours of Mstn treatment increased I κ B- α level (Lane 4) and p-I κ B- α level (Lane 4). IKK α level is increased in C2C12 cells treated for 72 hours with Mstn (Lane 4). Thus, these results indicate that Mstn is able to stimulate degradation of I κ B- α by increasing IKK α and activate NF- κ B transcription factor.

Figure 3.7 *Mstn* induces ROS through *TNF-α* via *NF-κB* signaling. Effect of Mstn on NF-κB and IκB-α in proliferating C2C12 myoblasts. Western blotting analysis was performed on whole cell lysates, nuclear and cytoplasmic extracts obtained from C2C12 cells treated with Mstn (3.5 ng/ml) for indicated time points. (i) Left panel, representative immunoblot showing protein levels of NF-κB (p65), p-NF-κB (p65), IκB-α, p-IκB-α and IKKα in negative control (Lanes 1 and 3) and Mstn (Lanes 2 and 4) treated whole protein lysates, nuclear and cytoplasmic extracts at indicated time points. α-tubulin was used as an internal control for equal protein loading on the gel. Right panel, corresponding densitometry analysis of (ii) p-NF-κB (p65) and (iii) p-IκB-α showing significant increase or decrease in protein content upon Mstn treatment (* $p < 0.05$, ** $p < 0.01$) (n=2).

Since Mstn treatment results in increased translocation of NF- κ B into the nucleus, experiments to investigate if Mstn treatment results in enhanced binding of NF- κ B to the NF- κ B binding sites on TNF- α promoter was investigated. EMSA was performed using nuclear extracts made from C2C12 cells treated with Mstn and the oligonucleotides bearing NF- κ B binding site of the mouse TNF- α promoter. Four shifted bands (complex I, II, III and IV) were observed on EMSA and addition of Mstn to C2C12 cells led to increased NF- κ B DNA binding activity as shown by an increased level of complex III (Figure 3.8 (i); Lanes 2 and 3). To prove that complex III and IV contains p65 and p50 respectively, super shift analysis with specific antibodies was performed. The results revealed that complex III could be super shifted (and hence diminished band intensity) with p65 specific antibodies whereas and complex IV was super shifted with p50 subunit specific antibodies (Figure 3.8 (ii); Lanes 2 and 3). These observations further indicate that increased availability of NF- κ B in nucleus due to Mstn signaling results in enhanced binding to the NF- κ B binding sites in TNF- α promoter/enhancer sequences.

Figure 3.8 *Mstn-mediated enhancement of NF- κ B binding activity on mouse TNF- α promoter.* EMSA was performed using nuclear extracts from proliferating C2C12 cells treated with Mstn (3.5 ng/ml) for 48 hours. (i) Left panel, representative gel showing the increased activity of NF- κ B upon Mstn treatment. Lane 1-oligo only, Lane 2-untreated, Lane 3-Mstn treated. EMSA was also performed with nuclear extracts preincubated with the specific antibody. Right panel, representative gel showing NF- κ B complexes containing p65 or p50 subunits. Lane 1-no antibody added, Lane 2-p65 specific antibody added, Lane 3-p50 specific antibody added (n=2).

5-Aminosalicylic acid (5-ASA) inhibits NF- κ B activation and I κ B degradation, therefore 5-ASA was used to further confirm that NF- κ B activity is required for Mstn mediated up-regulation of TNF- α and *Nox1*. The results show that TNF- α (Figure 3.9A) and *Nox1* (Figure 3.9B) expression was significantly down regulated with 5-ASA treatment, along with Mstn in comparison to cells treated with Mstn only. Western blotting analysis for NF- κ B p65 was performed on whole cell lysates, nuclear and cytoplasmic extracts of proliferating C2C12 cells treated for 48 hours with Mstn along with 5-ASA. Whole cell protein lysates showed significant decrease in NF- κ B p65 level in cells treated with 5-ASA (Figure 3.9C; Lanes 3 and 4) when compared to untreated (Lane 1) and Mstn treated cells (Lane 2); nuclear extracts show significant decrease in NF- κ B p65 levels (Figure 3.9C) on treatment with 5-ASA (Lane 3) along with Mstn (Lane 4) when compared to cells treated with Mstn only (Lane 2); cytoplasmic extracts show corresponding increase in NF- κ B p65 levels (Figure 3.9C; Lanes 1-4). These results indicate that Mstn mediated activation and translocation of NF- κ B is required for TNF- α and *Nox1* expression.

Antioxidants are known to detoxify free radicals and protect against ROS in skeletal muscles. Hence proliferating C2C12 cells were treated with ascorbic acid, an antioxidant, along with Mstn to investigate if antioxidants are able to inhibit Mstn induced ROS signaling. The results demonstrate that ascorbic acid significantly down regulated TNF- α (Figure 3.9A) and *Nox1* (Figure 3.9B) mRNA expression mediated by Mstn. Western blotting analysis for NF- κ B p65 on whole cell protein lysates showed significant decrease in NF- κ B p65 level in cells treated with ascorbic acid (Figure 3.9C; Lanes 5 and 6) when compared to untreated (Lane 1) and Mstn treated cells (Lane 2); nuclear extracts show significant decrease in NF- κ B p65 levels (Figure 3.9C) on treatment with ascorbic acid (Lane 5) along with Mstn (Lane 6) when compared to cells

treated with Mstn only (Lane 2); cytoplasmic extracts show corresponding increase in NF- κ B p65 levels (Figure 3.9C; Lanes 5 and 6).

Figure 3.9 5-ASA and ascorbic acid inhibit Mstn induced ROS via TNF-α and NF-κB signaling. mRNA expression analysis was performed on C2C12 cells treated with 5-ASA (10 μM/ml) and ascorbic acid (100 μM/ml) along with Mstn (3 μg/ml). Representative graphs show fold change in the mRNA expression of TNF-α (A) and *Nox1* (B). The values are mean ± S.E of 2 independent experiments. **p<0.01 and ***p<0.001 denotes significant increase when compared to untreated cells; ^^p<0.01 and ^^p<0.001 denotes significant decrease when compared to Mstn treated cells (n=2). (C) Western blotting analysis showing the level of NF-κB-p65 at the indicated time point in whole cell lysates, nuclear and cytoplasmic extracts of proliferating C2C12 cells, untreated (Lane 1), treated with Mstn (Lane 2), 5-ASA (Lane 3), 5-ASA and Mstn (Lane 4), ascorbic acid (Lane 5), and ascorbic acid and Mstn (Lane 6). α-tubulin was used as an internal control for equal protein loading on the gel.

3.3.7 Mstn induces the muscle specific E3 ligase expression via ROS dependent pathway

The results previously showed that Mstn inhibits Akt phosphorylation and is a potent inducer of muscle specific E3 ligases such as Atrogin-1 and MuRF-1 (McFarlane *et al.* 2006). Given that these two E3 ligases can be activated through increased ROS levels (Li *et al.* 2003; Cai *et al.* 2004), it was further investigated if Mstn induces E3 ligases in a ROS-dependent pathway. Western blotting analysis confirmed that treatment with Mstn does indeed inhibit Akt phosphorylation (Figure 3.10A; Lane 2), and up-regulate Atrogin-1 and MuRF-1 level (Figure 3.10B; Lane 2) in C2C12 cells in the absence of ascorbic acid, a potent anti-oxidant. However, when used along with ascorbic acid, Mstn failed to induce the expression of Atrogin-1 and MuRF-1 (Figure 3.10B; Lane 3). Furthermore, ascorbic acid was also able to rescue Mstn mediated inhibition of Akt phosphorylation (Figure 3.10A; Lanes 3 and 4). It is known that Mstn signals by activating receptor Smads, Smad2 and Smad3 (Zhu *et al.* 2004). Western blotting analysis for p-Smad2/3 (Figure 3.10C) revealed that ascorbic acid (Lane 3) treatment along with Mstn (Lane 4) reduced p-Smad2/3 level when compared to cells treated with Mstn only (Lane 2). These results thus indicate that Mstn signals the up-regulation of E3 ligases through ROS.

Figure 3.10 *Mstn* induces the muscle specific E3 ligase expression via ROS dependent pathway. Western blotting analysis showing the levels of p-Akt and Akt (A), Atrogin-1 and MuRF-1 (B) at indicated time points in the C2C12 cells, untreated (Lane 1), treated with Mstn (3 μ g/ml) (Lane 2), ascorbic acid (100 μ M/ml) (Lane 3), and ascorbic acid and Mstn (Lane 4) (n=2). α -tubulin was used as an internal control for equal protein loading on the gels. (C) Representative gel showing p-Smad2/3 protein levels in C2C12 cells, untreated (Lane 1), treated with Mstn (3 μ g/ml) (Lane 2), ascorbic acid (100 μ M/ml) (Lane 3), and ascorbic acid and Mstn (Lane 4) for 48 hours during proliferation. GAPDH was used as an internal control for equal protein loading on the gel.

3.3.8 TNF- α in turn induces Mstn expression, secretion and signaling in a feed forward manner

The above mentioned results clearly indicate that Mstn activates TNF- α expression and thereby induces ROS production. In order to see if elevated TNF- α in turn increases Mstn for a sustained “feed forward” mechanism, *Mstn* mRNA levels in C2C12 cells treated with TNF- α was checked. The results show that *Mstn* mRNA expression was indeed significantly elevated (Figure 3.11A). Consistent with mRNA expression, Mstn protein content was up-regulated with TNF- α (Figure 3.11B; Lane 2) treatment. Consistent with an increase in intracellular Mstn levels, TNF- α treatment also resulted in elevated Mstn secretion (Figure 3.11C). These results confirm that TNF- α induces Mstn at the transcriptional, translation and secretion level. To further investigate if elevated Mstn levels as a result of TNF- α treatment, result in increased Smad2/3 phosphorylation, C2C12 cells were treated with TNF- α and protein extract was analyzed for p-Smad2/3 protein content. Western blotting analysis revealed that TNF- α (Figure 3.11D; Lane 3) significantly increased p-Smad2/3 level when compared to the untreated cells (Figure 3.11D; Lane 1).

3.3.9 TNF- α induces Mstn expression via NF- κ B signaling

TNF- α has been shown to induce gene expression via NF- κ B pathway. Therefore, using 5-ASA it was ascertained if NF- κ B is involved in TNF- α mediated Mstn induction. TNF- α signaling was inhibited with 5-ASA treatment, hence there was no significant increase in *Mstn* mRNA (Figure 3.11A). It was also observed that 5-ASA treatment reduced basal levels and TNF- α mediated up-regulation of *Mstn* protein (Figure 3.11B; Lanes 3 and 4) and Mstn secretion (Figure 3.11C). Consistent with reduced levels of

Mstn, 5-ASA treatment interfered with phosphorylation of Smad2/3 by Mstn (Figure 3.11D; Lanes 4, 5 and 6).

Mstn regulatory region contains more than one NF- κ B binding sites. Since TNF- α induces Mstn expression, secretion and signaling, I wanted to investigate if this induction was by NF- κ B binding to the Mstn enhancer region to regulate its gene expression. EMSA was performed using nuclear extracts of C2C12 cells treated with TNF- α and the oligonucleotides containing NF- κ B binding site from mouse Mstn enhancer region. As shown in Figure 3.12A, treatment of C2C12 cells with TNF- α led to significantly increased NF- κ B DNA binding activity. To further confirm that TNF- α regulates Mstn via NF- κ B, the ability of TNF- α to up-regulate *Mstn* mRNA expression and protein was tested in the C2C12 cell line expressing the I κ B- α SR protein which inhibits NF- κ B activity. As shown in Figure 3.12B (i), TNF- α is unable to increase the mRNA expression of *Mstn* in the absence of NF- κ B activity (I κ B- α SR) when compared to the control cells (I κ B- α Control). Western blotting analysis also revealed that TNF- α does not up-regulate Mstn level when NF- κ B was inhibited by the I κ B- α SR protein (Figure 3.12B (ii); Lanes 3 and 4). In an independent study, the blocker of NF- κ B, BAY 11-7085, was used and the results show that up on inhibiting NF- κ B activity, TNF- α does not up-regulate *Mstn* mRNA expression (Figure 3.12C (i)) and protein level (Figure 3.12C (ii); Lanes 3 and 4). Hence, these results confirm that TNF- α regulates Mstn via NF- κ B in C2C12 myoblasts.

Figure 3.11 *TNF- α induces *Mstn* expression, secretion and signaling in a feed forward mechanism.* (A) Representative graph showing mRNA expression of *Mstn* and (B) representative gel showing protein levels of *Mstn* (~56 KDa-full length) in C2C12 cells, untreated (Lane 1), treated with TNF- α (10ng/ml) (Lane 2), 5-ASA (10 μ M/ml) (Lane 3), and 5-ASA and TNF- α (Lane 4) for 72 hours in proliferation media. The values are mean $\pm$ S.E of 2 independent experiments. Level of significance was compared to the untreated control (**** $p < 0.0001$) (n=2). GAPDH was used as an internal control for equal protein loading on the gel. (C) Level of secreted *Mstn* in the cell culture media from C2C12 cells treated with TNF- α (10 ng/ml) and 5-ASA (10 μ M/ml) was determined by EIA. The values are mean $\pm$ S.E of 2 independent experiments. The concentration of *Mstn* is given as ng/ml and the level of significance was determined with respect to the negative control (** $p < 0.01$) (n=2). (D) Representative gel showing p-Smad2/3 protein levels in C2C12 cells, untreated (Lane 1), treated with *Mstn* (3 μ g/ml) (Lane 2), TNF- α (10 ng/ml) (Lane 3), 5-ASA (10 μ M/ml) (Lane 4), 5-ASA and *Mstn* (Lane 5), and 5-ASA and TNF- α (Lane 6) for 72 hours during proliferation. GAPDH was used as an internal control for equal protein loading on the gel.

Figure 3.12 *TNF- α induces Mstn expression via NF- κ B signaling.* (A) TNF- α -mediated enhancement of NF- κ B binding on mouse Mstn promoter. EMSA was performed using nuclear extracts from proliferating C2C12 cells treated with TNF- α (10 ng/ml) for 72 hours. Representative gel showing the significant increase in the binding activity of NF- κ B upon TNF- α treatment. Lane 1-oligo only, Lane 2-untreated, Lane 3-TNF- α treated. (B) Representative graph showing mRNA expression of *Mstn* (i) and representative gel showing protein levels of Mstn (ii) in IkB- α SR expressing C2C12 cells treated for 72 hours with TNF- α (10 ng/ml) in proliferation media; IkB- α C (control cells) untreated (Lane 1), TNF- α treated (Lane 2), IkB- α SR cells untreated (Lane 3) and TNF- α treated (Lane 4). The values are mean $\pm$ S.E of 2 independent experiments. *** p <0.001 denotes significant increase in mRNA expression when compared to untreated cells ($n=2$). (C) Representative graph showing mRNA expression of *Mstn* (i) and representative gel showing protein levels of Mstn (ii) in C2C12 cells treated for 1 hour with BAY11-7085 (20 μ M/ml) and for 72 hours with TNF- α (10 ng/ml) in proliferation media; Lane 1-untreated, Lane 2-TNF- α treated, Lane 3-BAY11-7085 treated and Lane 4-BAY11-7085 and TNF- α treated cells. The values are mean $\pm$ S.E of 2 independent experiments. **** p <0.0001 denotes significant increase in gene expression when compared to untreated cells; ^^ p <0.01 and ^^ p <0.001 denote significant decrease in mRNA expression when compared to Mstn treated cells ($n=2$). GAPDH was used as an internal control for equal protein loading on the gels.

3.3.10 H₂O₂ also induces Mstn expression, secretion and signaling

In addition to TNF- α , I next tested if pro-oxidants like H₂O₂ could also induce Mstn levels in C2C12 cells. When C2C12 cells were treated with H₂O₂, *Mstn* mRNA expression was significantly elevated (Figure 3.13A); whereas a significant down regulation of *Mstn* expression was observed when ROS was inhibited by ascorbic acid treatment (Figure 3.13A). Consistent with mRNA levels, Mstn protein content was also up-regulated with H₂O₂ (Figure 3.13B; Lane 2) treatment which was down regulated with ascorbic acid (Figure 3.13B; Lane 3) treatment when compared to untreated cells (Figure 3.13B; Lane 1). In addition EIA was also performed and the results showed that Mstn secretion into media was significantly up-regulated on treatment with H₂O₂ while ascorbic acid treatment did not result in any change (Figure 3.13C). Furthermore, treatment of C2C12 with H₂O₂ led to inhibition of proliferation (Figure 3.13D) and differentiation (Figure 3.13E) of myoblasts similar to that seen in case of Mstn. These results confirm that pro-oxidants increase Mstn expression, secretion and signaling.

Figure 3.13 *H₂O₂ induces Mstn expression, secretion and signaling.* (A) Representative graph showing mRNA expression of *Mstn* and (B) representative gel showing protein levels of Mstn in C2C12 cells, untreated (Lane 1), treated with H₂O₂ (0.05 mM) (Lane 2) and ascorbic acid (100 μM/ml) (Lane 3) for 72 hours in proliferation media. The values are mean ± S.E of 2 independent experiments. Significant increase (**p<0.01) or decrease (***p<0.001) in mRNA expression was compared to the untreated control (n=2). GAPDH was used as an internal control for equal protein loading on the gel. (C) Level of secreted Mstn in the cell culture media from C2C12 cells treated with H₂O₂ (0.05 mM) and ascorbic acid (100 μM/ml) was determined by EIA. The values are mean ± S.E of 2 independent experiments. The concentration of Mstn is given as ng/ml and the level of significance was determined with respect to the negative control (**p<0.01) (n=2). ***H₂O₂ inhibits proliferation and differentiation of C2C12 myoblasts.*** (D) C2C12 cells were treated with two different concentrations of H₂O₂ (0.05 mM and 0.1 mM), the cells were fixed at the indicated time and proliferation assay was performed (****p<0.0001, ^^^p<0.0001) (n=4). (E) C2C12 cells were cultured in differentiation media in the presence of 0.05 mM H₂O₂ for 48 and 72 hours. The cells were fixed and stained with H & E and images were taken using a Leica upright bright field microscope at 10X magnification (n=2).

3.3.11 Absence of Mstn helps to maintain AOE levels in skeletal muscle during aging

Elevated ROS levels in skeletal muscle have been shown to be one of the hall marks of sarcopenia. Given that Mstn protein levels have been shown to be increased during aging (Baumann *et al.* 2003), and that Mstn^{-/-} mice have reduced sarcopenia, it was decided to study the role of Mstn induced ROS during aging. Western blotting analysis for NF-κB p65 was performed on protein lysates from gastrocnemius (Gas) muscle from young (6 week old) and aged (2 years) WT and Mstn^{-/-} mice (Figure 3.14A). When compared to the young WT mice (Lane 1), the aged WT mice showed significant increase in NF-κB p65 level (Lane 3). However, both young (Lane 2) and aged (Lane 4) mice of Mstn^{-/-} genotype expressed significantly lower levels of NF-κB p65. Similarly, TNF-α and *Nox1* mRNA expression was also down regulated in Mstn^{-/-} young and aged mice (Figures 3.14B and 3.14C).

To determine the antioxidant status of aging mice, basal AOE levels were measured in Gas muscle isolated from young and aged WT and Mstn^{-/-} mice. A significant increase in the mRNA expression of AOE, *Sod1* (Figure 3.14D) and *Cat* (Figure 3.14E) was noted in young and aged Mstn^{-/-} mice when compared to WT mice of similar age. In fact, AOE expression was up-regulated in aged Mstn^{-/-} mice in comparison to young Mstn^{-/-} mice. With aging, *Sod1* expression was not increased in WT mice, whereas in the absence of Mstn, there was an increase in *Sod1* expression. These results support the notion that the absence of Mstn helps in maintaining the antioxidant status in skeletal muscle during aging.

Figure 3.14 Absence of *Mstn* during aging decreases NF-κB levels, TNF-α and *Nox1* expression and increases AOE gene expression in skeletal muscle. (A) Western blotting analysis showing the level of NF-κB-p65 in protein lysates from Gas muscle of young (6 week old) and aged (2 year old) WT and Mstn^{-/-} mice; young WT (Lane 1), young Mstn^{-/-} (Lane 2), aged WT (Lane 3) and aged Mstn^{-/-} (Lane 4). α-tubulin was used as an internal control for equal protein loading on the gel (n=3). mRNA expression analysis in Gas muscle from young and aged WT and Mstn^{-/-} mice was performed. Representative graphs show fold change in the mRNA expression of TNF-α and *Nox1* in Young (B) and Aged (C) WT and Mstn^{-/-} mice. The values are mean ± SEM of 3 independent experiments. ***p<0.001 denote significant decrease when comparing WT and Mstn^{-/-} mice (n=3). Representative graphs show fold change in the mRNA expression of *Sod1* (D) and *Cat* (E). The values are mean ± SEM of 3 independent experiments. **p<0.01, ***p<0.001, ****p<0.0001 denote significant increase when comparing young and aged mice and ****p<0.0001 denotes significant increase when comparing WT and Mstn^{-/-} mice (n=3).

3.4 Discussion

A major cause of aging and several diseases is thought to be the cumulative oxidative stress, resulting from the production of ROS due to mitochondrial dysfunction (Aiken *et al.* 2002; Zahn *et al.* 2006; Herbst *et al.* 2007). However, the underlying signaling mechanisms that induce ROS during aging are not well understood. Here, it is shown that Mstn is a potent inducer of ROS in myotubes and that lack of Mstn enhances the antioxidant response. Furthermore, it is also shown that the increased ROS levels in turn induce Mstn. Therefore, it is identified that a novel “feed forward” mechanism exists for a sustained ROS production in Mstn-elevated situations such as aging and other muscle wasting conditions.

Mstn, a member of the TGF- β super-family is now widely accepted as an inhibitor of skeletal muscle growth. Over expression of Mstn in mice has been shown to cause cachexia (Zimmers *et al.* 2002; Reisz-Porszasz *et al.* 2003) and increased levels of Mstn are also observed during muscle wasting seen in Human Immunodeficiency Virus (HIV) infection, cancer and chronic kidney diseases. Elevated Mstn in these conditions is believed to increase protein degradation, decrease myogenesis and reduce protein synthesis. Muscle wasting in the above mentioned conditions also involves ROS production, which has been found to be associated with protein catabolism and ubiquitin-proteasome pathway (Li and Reid 2000; Gomes-Marcondes and Tisdale 2002). In addition, oxidative stress during aging is considered to be a major contributor of muscle atrophy (Sohal and Weindruch 1996; Powers *et al.* 2005).

Several lines of evidence presented in this study suggest that Mstn is an inducer of ROS in the muscle cells. The results demonstrate that Mstn induces ROS production in myoblasts during proliferation as well as during differentiation. Increase in ROS levels was evident from the incorporation of the fluorescent dye in the Mstn treated C2C12

cells coinciding with increased carbonylation of the cellular proteins. Even though carbonylation of proteins is considered to be a standard marker for oxidative stress there are limitations of Oxyblot assay for determination of carbonylated proteins in terms of sensitivity (Nystrom 2005). In addition to carbonylation of proteins, previous studies have also reported an increase in AOE levels in C2C12 cells when exposed to pro-oxidants like paraquat (Franco *et al.* 1999). Indeed in Mstn treated muscle cells an increase not only in carbonylation of proteins but also an increase in the levels of AOE was detected. These results thus confirm that increased Mstn levels lead to the induction of oxidative stress in skeletal muscle cells. Considering that Mstn is a pro-oxidant, it is argued that in the absence of Mstn, ROS levels and thus AOE levels should be lower in Mstn^{-/-} mice. However, what was observed was that Mstn^{-/-} mice have higher basal levels of AOE compared to WT mice. This observation could be accounted for by increased muscle mass in Mstn^{-/-} mice as compared to the WT mice. Nevertheless, the higher basal levels of AOE appear to confer resistance to oxidative stress in Mstn^{-/-} mice. Similarly, in the 2 year old Mstn^{-/-} mice the AOE activity remains up-regulated as compared to the old wild type mice. In line with these observations, it has been suggested that AOE and non-enzyme scavengers of ROS like GSR are extremely important to maintain the balance of redox system (Valko *et al.* 2007), thus accentuated AOE activity in Mstn^{-/-} muscle would make it less susceptible to oxidative damage. The results show that with aging, there are changes in the basal activity of different AOE. Aged Mstn^{-/-} mice are found to have higher basal levels of AOE compared to aged wild type mice; this might be one of the reasons for aged Mstn^{-/-} mice to maintain the muscle mass and function. To delineate the mechanism(s) through which ROS may be induced by Mstn, cell signaling inhibitors were used to block the pathways involved in the generation of ROS. The results show that NADH/NAD(P)H oxidase and Mitochondrial ETC are involved in

ROS induction by Mstn since blocking Nox and Mitochondrial ETC reduces ROS production by Mstn. Based on AOE analysis in the Mstn treated myoblasts, NADPH oxidase derived ROS are most likely to be superoxide radicals, hydroxyl radicals and H₂O₂. In addition, the contribution of lipid peroxidation towards ROS was discounted by the observation that TBARS levels were down regulated by Mstn. Previously it has been reported that TNF- α , induces oxidative stress in skeletal muscles (Buck and Chojkier 1996) and its expression is regulated through NF- κ B transcription factor. It is also clearly shown by molecular analysis that treatment of myoblasts with Mstn increases NF- κ B translocation into nucleus which induced the transcription of TNF- α (Figure 3.6A and 3.6B (i)). Induction of ROS by Mstn via TNF- α was further confirmed by the fact that in the absence of Mstn there is a reduction in TNF- α and *Nox1* mRNA levels in young and aged Mstn^{-/-} mice when compared to WT mice (Figures 3.14B and 3.14C). This data is also supported by an earlier report where low circulatory levels of TNF- α was reported in Mstn^{-/-} mice (Wilkes *et al.* 2009). Previously it has been reported that TNF- α , induces oxidative stress by activating Nox1 expression (Kim *et al.* 2007). Indeed in this study it is also clearly shown that the increased TNF- α is responsible for the increased expression of NAD(P)H oxidase. Hence, I propose that Mstn increases the levels of ROS by Nox1 pathway through NF- κ B. Several lines of evidence from the data presented here support this hypothesis. Firstly, the oxidative stress induced by Mstn was alleviated upon treatment with NF- κ B inhibitors (5-ASA and BAY 11-7085). Furthermore, loss of Mstn leads to decrease in NF- κ B levels in Gas muscles of Mstn^{-/-} mice (Figure 3.14A). It is also noteworthy that aging leads to an increase in the levels of NF- κ B in the Gas muscles of 2 year old WT mice. However, such an increase was not observed in the NF- κ B levels in the aged Mstn^{-/-} muscle. Taken together these results confirm a hypothesis that Mstn signals via NF- κ B, TNF- α and Nox1 to induce ROS and

are also exemplified *in vivo*. Furthermore, the oxidative stress induced by Mstn, was alleviated upon treatment with the antioxidant, ascorbic acid which supports evidence indicating that the administration of antioxidants is effective to protect against ROS in chronically loaded muscles of aged rats (Ryan, *et al.* 2008).

A higher level of ROS has previously been shown to inhibit Akt by activating protein phosphatase (PP2A) which dephosphorylates Ser473 on Akt. Mstn has been shown to inhibit Akt phosphorylation (McFarlane *et al.* 2006) and the molecular mechanism for the dephosphorylation has not been identified so far. Current results suggest that induced ROS by Mstn could be one of the possible molecular mechanisms behind Mstn induced inhibition of Akt phosphorylation. Hypophosphorylation of Akt by either blocking IGF or by increasing Mstn levels leads to skeletal muscle wasting by inducing muscle specific E3 ligases, Atrogin-1 and MuRF-1. In the present study, ascorbic acid and 5-ASA treatment of Mstn treated myoblasts was able to rescue p-Akt level and down regulate the protein content of E3 ligases Atrogin-1 and MuRF-1 by Smad2/3 dependent mechanism. These results therefore further suggest that, increased ROS produced by Mstn could potentially induce muscle wasting by activating ubiquitin proteolytic pathway.

Another important discovery that is described here is that ROS itself is a potent inducer of Mstn. Furthermore, it is shown that TNF- α and NF- κ B are required for this “feed forward” induction of Mstn by ROS (Figure 3.15). Inhibition of either TNF- α induction by ROS or NF- κ B activity and binding to Mstn enhancer, results in reduced Mstn levels in myoblasts and myotubes which further confirms that ROS induces Mstn via TNF- α and NF- κ B dependent pathway. It is noteworthy that in a related context of muscle wasting associated with renal failure, recently, Zhang *et al.* reported that Mstn is

responsible for this muscle wasting and that TNF- α stimulates *Mstn* mRNA and protein by a NF- κ B dependent pathway in C2C12 myotubes (Zhang *et al.* 2011).

Figure 3.15 *Proposed mechanism by which Mstn induces ROS in skeletal muscle.* Increased Mstn induces TNF- α production via NF- κ B signaling, increasing the production of ROS by NADPH oxidase. The induced ROS results in a feed forward loop further increasing Mstn levels via NF- κ B signaling of TNF- α . The increased ROS levels result in a decrease in muscle protein synthesis and an increase in protein degradation leading to skeletal muscle wasting. Antioxidants like 5-ASA and ascorbic acid block these pathways to reduce ROS, Mstn and TNF- α .

Therefore, in the present study, using an integrative approach from cell cultures to mice, it is demonstrated that Mstn and TNF- α are components of a feed forward loop in which Mstn triggers the generation of second messenger ROS, mediated by TNF- α and NADPH oxidase. I speculate that higher levels of ROS induce muscle wasting via proteasomal mediated degradation of proteins. Furthermore I also propose that increased ROS in turn induces TNF- α which stimulates Mstn expression via pro-inflammatory transcription factor NF- κ B thus enabling a “feed forward” and sustained induction of ROS during muscle wasting. Mstn protein levels in skeletal muscle and circulation is reported to increase during aging and cachexia (Yarasheski *et al.* 2002; Baumann *et al.* 2003; Raue *et al.* 2006; Leger *et al.* 2008) which would overlap with the increase in pro-inflammatory and redox sensitive NF- κ B. As stated earlier, Mstn^{-/-} aged mice have lower levels of NF- κ B in the muscle, which makes Mstn a perfect candidate to integrate inflammatory and ROS signaling during aging related muscle wasting.

Sandhya Sriram performed majority of the experiments and analyzed the data. Subha Subramanian, Durga Sathiakumar and Rithika Venkatesh helped in performing some of the experiments (isolation of primary myoblasts and EMSA). Monica Senna Salerno and Craig Desmond McFarlane helped in planning experiments on aging animals.

CHAPTER 4

SMAD3 IS NECESSARY FOR MYOSTATIN-MEDIATED NF- κ B SIGNALING BUT DISPENSABLE FOR REACTIVE OXYGEN SPECIES GENERATION

This study has been communicated as a manuscript to Biochemical Journal.

Abstract

Smad2/3 are downstream signaling molecules for TGF- β and Myostatin. Mice lacking Smad3 display skeletal muscle atrophy due to increased Myostatin levels. Recently, Myostatin was shown to induce ROS in skeletal muscle via Smad3, NF- κ B and TNF- α . Hence, the aims were to investigate if Myostatin induced muscle atrophy in Smad3^{-/-} mice by increasing ROS and delineate Smad3-independent signaling mechanism for Myostatin. Herein the results show that Smad3^{-/-} mice have increased ROS levels in skeletal muscle and inactivation of Myostatin in these mice partially ablates the oxidative stress. Furthermore, ROS induction by Myostatin in Smad3^{-/-} muscle was not via NF- κ B (p65) signaling but by activating complex signaling pathways that include p38, ERK MAPK and crosstalk with the JAK-STAT pathway. Consequently, TNF- α , Nox and XO levels were up-regulated that led to an increase in ROS production in Smad3^{-/-} skeletal muscle. The exaggerated ROS in the Smad3^{-/-} muscle also increased binding of CHOP transcription factor to MuRF1 promoter resulting in enhanced MuRF1 levels leading to muscle atrophy. Finally, the results presented in this Chapter identify Smad3 as the crucial transducer for NF- κ B activation by Myostatin and novel adaptive signaling mechanisms (JAK-STAT pathway) engaged by Myostatin in the absence of Smad3 to generate ROS eventually resulting in muscle atrophy.

4.1 Introduction

Smads are a family of intracellular proteins that transduce extracellular signals from TGF- β superfamily members (Derynck and Zhang 2003; Shi and Massagué 2003). Upon TGF- β ligand binding and subsequent receptor activation, Smad2 and Smad3 get phosphorylated to form a complex with the common mediator Smad4. The Smad2/3-Smad4 complex then translocates into the nucleus to activate or repress the transcription of TGF- β target genes. Several reports have indicated that disruption of TGF- β and/or the Smad signaling pathway is causal to the pathogenesis of several disorders (Fiocchi 2001; Huang *et al.* 2010) and fibrogenesis (Bonniaud *et al.* 2005).

Myostatin (Mstn), a member of the TGF- β superfamily signals predominantly through the Smad2/3 pathway to inhibit myogenesis (Liu *et al.* 2001; Liu *et al.* 2004). Specifically, Smad3 is required by Mstn to inhibit differentiation of myoblasts (Langley *et al.* 2002) and to activate proliferation of fibroblasts (Li *et al.* 2008). Recently it has been demonstrated that in the absence of Smad3, pronounced skeletal muscle atrophy, impaired myogenesis, increased protein degradation via muscle-specific E3 ligase, MuRF1, and impaired skeletal muscle regeneration is observed (Ge *et al.* 2011; Ge *et al.* 2012). Elevated levels of Mstn in the absence of Smad3 are reported to contribute to the muscle atrophy and regeneration defects observed in Smad3^{-/-} mice (Ge *et al.* 2011; Ge *et al.* 2012). As shown in Chapter 3, Mstn can induce reactive oxygen species (ROS) via NF- κ B signaling and its downstream targets, including TNF- α and NADPH oxidase 1 (*Nox1*), in skeletal muscle (Sriram *et al.* 2011). Even though the results indicate that Mstn activates NF- κ B in muscle cells, the signaling mechanism(s) through which Mstn promotes the induction of NF- κ B are not known.

In addition to Smad-dependent signaling, Mstn has been reported to signal via Smad-independent mechanisms. For example, Mstn was found to activate p38 mitogen-activated protein kinase (MAPK) through TGF- β activated kinase 1 (TAK1) to up-

regulate p21 and inhibit myoblast proliferation (Philip *et al.* 2005). Likewise, Mstn can activate the extracellular signal-related kinase 1/2 (ERK1/2) pathway to inhibit muscle cell differentiation and growth (Yang *et al.* 2006). Excessive production of ROS via mitochondrial electron transport chain (mETC) and NADPH Oxidase (Nox) has also been shown to involve c-Jun N-terminal kinase (JNK) and ERK signaling pathways during oxidative stress-induced lung injury (Carnesecchi *et al.* 2009). Besides these pathways, ROS generation is also linked with sequential activation of the JAK-STAT pathway leading to induction of Xanthine oxidase (XO), an important enzyme that produces ROS in cells (Wang *et al.* 2008). Furthermore, activation of STAT3 is observed during skeletal muscle wasting and cancer cachexia (Bonetto *et al.* 2011). Also, p38 MAPK and ERK are involved critically in ROS-mediated induction of IL-6 in cardiac fibroblasts (Sano *et al.* 2001). IL-6 has also been shown to signal through JAK-STAT and ERK MAPK signaling pathways to cause ROS production (Aida *et al.* 2012). A report by Black *et al.* underscores the role of Smad3 in the generation of ROS by TGF- β in the hepatocytes (Black *et al.* 2007). Given the fact that Mstn levels are elevated in Smad3^{-/-} mice and that Mstn also acts as a pro-oxidant, I wanted to investigate if Mstn can induce ROS via NF- κ B signaling in the absence of Smad3. Using *in vitro* and *in vivo* approaches herein the results show that Smad3 is required for Mstn-mediated induction of ROS via NF- κ B (p65) signaling. Furthermore, the results demonstrate that Mstn is able to induce ROS through MAPK pathways, including p38 and ERK, in Smad3^{-/-} mice, which could be mitigated through inhibiting Mstn. The results also reveal that Mstn signals through the JAK-STAT pathway to increase XO, another inducer of ROS. In addition, crosstalk was observed between ERK MAPK and the JAK-STAT pathways in Smad3^{-/-} myoblasts, indicating the involvement of a potential nexus of signaling mechanisms in the generation of ROS in Smad3^{-/-} mice. The excessive oxidative stress led to enhanced binding of CHOP, a transcription factor

induced by cellular oxidative stress, to MuRF1, a muscle-specific E3 ligase, promoter. This in turn would result in increased protein degradation and hence muscle atrophy via ubiquitination of muscle-specific proteins by MuRF1.

4.2 Materials and methods

4.2.1 Animals

Smad3^{-/-} mice and Mstn^{-/-} mice were obtained as previously described (Ge *et al.* 2011; Yang *et al.*, 1999; Sections 2.2.1 and 3.2.1). The double-KO mice (Smad3^{-/-}/ Mstn^{-/-}) were generated and genotyped according to previously published protocol (Ge *et al.* 2011) (as described in Section 2.2.1). C57Bl/6J mice (WT) were obtained from National University of Singapore-Centre for Animal Resources (NUS-CARE), Singapore. All mice used in this study were 6-wk-old females and maintained at Nanyang Technological University (NTU) Animal house, Singapore. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Singapore.

4.2.2 Reagents and Proteins

The Mstn expressing CHO cell line was used to obtain Mstn protein containing conditioned medium (Zimmers *et al.* 2002); denoted as CMM in this chapter. Enzyme Immuno Assay (EIA) (Immundiagnostik AG) was used to determine the concentration of Mstn in the conditioned medium. The list of various reagents and chemicals used in the current study are in Section 2.1.3.

4.2.3 Isolation of primary myoblasts (satellite cells)

The hind limb skeletal muscles from WT and Smad3^{-/-} mice were dissected and used for isolation of primary myoblasts. Primary myoblasts were isolated according to the previously described protocol (Partridge 1997; McCroskery *et al.* 2003) (as described in Section 3.2.6).

4.2.4 Cell culture

Established ATCC murine C2C12 myoblast cell line was used (Yaffe and Saxel 1977). Stable shSmad3 and shControl C2C12 cells were established as previously described (Ge *et al.* 2011). The myoblasts were passaged in proliferation media (DMEM, 10 % FBS, 1 % Penicillin/Streptomycin (P/S)) and differentiated in differentiation media (DMEM, 2 % Horse serum (HS), 1 % P/S). The stable cell lines were passaged in proliferation media containing puromycin and differentiated in puromycin containing differentiation media.

4.2.5 Treatment of C2C12, shSmad3 and shControl C2C12 cells

The concentration of Mstn in conditioned medium from CHO cells (CMM) used in all the experiments was 3.5 ng/ml. This concentration was used to treat C2C12 cells, shSmad3 and shControl C2C12 cells during differentiation. Equal volume of control conditioned medium from CHO cells (CCM) was used which served as the control in all experiments. Based on earlier studies, 10 μ M/ml SIS3 (Jinnin *et al.*, 2006), 10 μ M/ml SB203580 (Baeza-Raja *et al.*, 2004), 10 μ M/ml SP600125 (Strle *et al.*, 2006), 10 μ M/ml U0126 (Yu *et al.*, 2007) and 10 μ M/ml AG490 (Spangenburg *et al.*, 2002) were used to treat C2C12, shSmad3 and shControl C2C12 cells during differentiation.

4.2.6 Analysis of ROS production

4.2.6.1 Intracellular visualization of ROS

ROS production was assayed in differentiating WT and Smad3^{-/-} primary myoblasts using the fluorescent dye, CM-H2DCFDA (Molecular Probes), as described previously (Section 3.2.8.2).

4.2.6.2 Analysis of components of signaling pathways and the enzymes involved in ROS generation in Smad3^{-/-} myoblasts

The cell signaling inhibitors used in this study were according to Iuchi *et al.* (Iuchi *et al.* 2003). WT and Smad3^{-/-} primary myoblasts were treated with the inhibitors as described previously (Section 3.2.16), however the myoblasts were not treated with Mstn. ROS production was assayed using the fluorescent dye, CM-H2DCFDA (Molecular Probes), as described previously (Sections 3.2.8.2 and 3.2.16).

4.2.7 RT-qPCR (Reverse Transcriptase-quantitative Polymerase Chain Reaction)

WT and Smad3^{-/-} primary myoblasts as well as shSmad3 and shControl C2C12 cells treated with inhibitors and CCM or CMM were harvested and suspended in TRIZOL (Invitrogen) for RNA isolation. RNA was also isolated from Gastrocnemius muscle of WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice using TRIZOL, as per the manufacturer's protocol (Invitrogen). The RT-qPCR was performed exactly as explained in Sections 2.2.8 and 3.2.17. The forward and reverse primers used are given Table 2.1.

4.2.8 Protein isolation

WT and Smad3^{-/-} primary myoblasts, shSmad3 and shControl C2C12 cells subjected to various treatments with inhibitors and CMM during differentiation were collected at indicated time points in protein lysis buffer. Protein lysates were subsequently made as previously described (Section 3.2.9). Gastrocnemius muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice were homogenized in protein lysis buffer and the clear supernatants were collected. Cytoplasmic and nuclear fractions from Biceps femoris muscle and cells following various treatments were isolated as previously described (Ye *et al.* 1996) (as described in Section 2.2.14).

4.2.9 Cell and muscle homogenates for enzyme assays

The cell homogenates from WT and Smad3^{-/-} primary myoblasts and tissue homogenates of Quadriceps muscles were made according to Section 3.2.11 except that the cells were not treated with Mstn.

The protein concentrations of the cell lysates and muscle homogenates were measured by Bradford's assay (Bradford 1976) (Bio-Rad Laboratories, Inc.).

4.2.10 Xanthine oxidase (XO) assay

XO activity was measured in primary myoblast lysates and in muscle homogenates using a continuous spectrophotometric rate determination assay as described by Bergmeyer *et al.* (Bergmeyer *et al.* 1974). Briefly, 0.1 ml of cell or muscle homogenate was mixed with 1.9 ml of 50 mM of potassium phosphate buffer (pH 7.5) and 1 ml of 0.15 mM xanthine solution. 0.1 ml of XO solution was added and mixed by inversion. Immediately, the increase in absorbance was read at 290 nm for 5 minutes. The activity was expressed as XO units per ml enzyme.

4.2.11 Antioxidant enzyme activity-Super Oxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx)

The activity of SOD was estimated as previously described (Section 3.2.13.1). The method of Beers and Sizer (Beers and Sizer 1952) was used to assay catalase activity. Glutathione peroxidase enzyme assay was performed by the modified method of Rotruck *et al.* (Rotruck *et al.* 1973) as described previously (Section 3.2.13.3).

4.2.12 Antioxidant-Reduced Glutathione (GSH) estimation

The total reduced glutathione was measured by the method of Moron *et al.* (Moron *et al.* 1979) (as described in Section 2.2.12).

4.2.13 Oxyblot assay

To estimate carbonyl groups incorporated into proteins by oxidation due to ROS, Oxyblot assay was performed according to the manufacturers' instructions using the Oxyblot Protein Oxidation Detection Kit (Millipore Corp.) (Section 3.2.10).

4.2.14 Transient transfection of shRNA to knockdown Smad2

C2C12 cells were plated at a density of 10,000 cells per cm² in 6-well plates. Following overnight attachment, the C2C12 cells were transfected with 4 µg per well of either empty vector control (pRFP-C-RS), scrambled negative control non-effective shRNA (scrambled shRNA) or the Smad2-specific shRNA expression vector (shSmad2) (OriGene Technologies, Inc.) using Lipofectamine 2000 (Invitrogen), as per the manufacturer's guidelines. Next day, fresh differentiation media containing either CCM or CMM was added to the cells for a further 48 hours. At the indicated time, the cells were harvested in protein lysis buffer and protein lysates were made as described previously (Section 3.2.9).

4.2.15 Western Blot analysis

Total protein, nuclear and cytoplasmic extracts (20-30 µg) were separated on 4-12 % Bis-Tris polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membrane by electro blotting (as described in Section 3.2.18). The membranes were blocked and probed with specific primary antibodies (Table 2.3), followed by incubation with respective secondary antibodies (Table 2.5). HRP activity was detected using Western Lightning Plus ECL (PerkinElmer) (as described in Section 3.2.18).

4.2.16 Electrophoretic mobility shift assay (EMSA)

The oligonucleotides containing the CHOP binding site on mouse MuRF1 promoter (5'-AAAGTCTCAGTGCAATGCGCAGCCCATAA-3') were hybridized to their respective complementary strands and labeled at the 3' end with Biotin Tetra-ethyleneglycol (TEG) (Sigma-Aldrich). The EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific). Nuclear extracts (NE) (4 µg) obtained from Biceps femoris muscles were incubated with the biotin labeled probe (final concentration 1 nM (1 X)) and EMSA was performed as previously described (Section 3.2.19). Unlabeled probes were used as competitors at concentrations 500 nM (500 X). To confirm specificity of CHOP binding, 4 µl of CHOP antibody (Table 2.3) was added to the NE and pre-incubated for 20 minutes at room temperature before incubation with the labeled probe.

4.2.17 Chromatin Immunoprecipitation (ChIP) assay

C2C12 myoblasts were treated with SIS3 for 48 hours during differentiation. ChIP assay was performed according to the published protocol (Bonala *et al.* 2012). C2C12 myoblasts were treated with SIS3 for 48 hours during differentiation. Following 48 hours of treatment, the C2C12 cells were fixed with 1 % formaldehyde, sonicated (15 cycles with 30 seconds pulse and 30 seconds pause) and immunoprecipitation was conducted overnight at 4°C with 5 µg of anti-CHOP antibody (Table 2.3) or anti-IgG antibody (Dako). Formaldehyde cross-linking was reversed and DNA was isolated using the QIAquick PCR Purification kit (Qiagen). DNA was PCR amplified using HotStart Taq polymerase according to manufacturer's instructions (Kapa Biosystems, Woburn, MA). The following set of primers was used for PCR: *Chop* forward primer 5'-AAGCAGGTGCCACTCTCTGT-3' and *Chop* reverse primer 5'-

GTAGCTGATTTGGCCCATGT-3'. The resulting PCR products were electrophoresed on 1 % agarose gel and stained with ethidium bromide.

4.2.18 Statistical analysis

The p value was calculated using ANOVA and Student's t-test with $p < 0.05$ being considered as significant. Three mice per genotype were used for various experiments.

Results are presented as mean $\pm$ SE of 2 or 3 independent experiments.

4.3 Results

4.3.1 Smad3^{-/-} myoblasts have increased ROS production

Smad3^{-/-} mice have been shown to have impaired myoblast proliferation and differentiation resulting in pronounced skeletal muscle atrophy (Ge *et al.* 2011). The mRNA expression of *Mstn*, a candidate gene that regulates muscle atrophy, was up-regulated in skeletal muscle of Smad3^{-/-} mice (Ge *et al.* 2011). As shown in Chapter 3, *Mstn* is a pro-oxidant and induces the production of ROS in myoblasts (Sriram *et al.* 2011). Hence, to investigate whether the elevated *Mstn* expression in Smad3^{-/-} mice is associated with increased ROS production, ROS levels were measured in differentiating myoblasts of WT and Smad3^{-/-} mice using the fluorescent dye, CM-H2DCFDA. The results show that ROS production was increased in Smad3^{-/-} myoblasts when compared to the WT myoblasts, as shown by the increased fluorescence in the Smad3^{-/-} myoblasts (Figure 4.1A).

4.3.2 ROS in Smad3^{-/-} myoblasts is produced by Mitochondrial ETC (mETC) and XO

In order to understand the mechanism behind ROS production in Smad3^{-/-} myoblasts, cell signaling inhibitors that inhibit various enzymes and pathways involved in ROS production were used (Iuchi *et al.* 2003). DPI, DIDS, CCCP and TTFA block ROS production by Nox and mETC, which have already been shown to be sources of ROS induced by *Mstn* (Chapter 3; Sriram *et al.* 2011). Similarly in Smad3^{-/-} mice, inhibitor treatments revealed that ROS was predominantly produced by Nox and various enzymes of the mETC (Figures 4.1B (i) and 4.1B (ii)). In addition, Oxypurinol inhibited ROS production in Smad3^{-/-} myoblasts (Figures 4.1B (i) and 4.1B (ii)) indicating that XO, another important enzyme which contributes to the generation of ROS is involved in the induction of oxidative stress in Smad3^{-/-} mice.

Figure 4.1 *Smad3^{-/-} myoblasts have increased ROS production.* (A) Representative images showing ROS production at 48 hours during differentiation in WT and Smad3^{-/-} primary myoblasts, probed with the CM-H2DCFDA fluorescent probe. The images were taken using a Leica upright microscope at 10X magnification. Increased fluorescence (green) intensity is directly proportional to increased ROS production in myoblasts. Scale bar represents 100 μ m (n=3). (B) (i). Smad3^{-/-} primary myoblasts were treated for 48 hours during differentiation in the presence of ROS cell signaling inhibitors and ROS content was measured using the CM-H2DCFDA probe using a fluorescent multi-label plate reader. The data is expressed as percentage increase or decrease in ROS production; **p<0.01, ***p<0.001, ****p<0.0001 (n=3). (B) (ii). ROS production in Smad3^{-/-} primary myoblasts treated for 48 hours during differentiation in the presence of ROS cell signaling inhibitors using the CM-H2DCFDA probe. The fluorescence was viewed as mentioned in (A). Scale bar represents 100 μ m (n=3).

To further show that induction of ROS in Smad3^{-/-} myoblasts is via TNF- α regulated Nox, RT-qPCR for TNF- α and *Nox1* was performed in differentiating WT and Smad3^{-/-} primary myoblasts. The results confirmed that TNF- α (Figure 4.2A) and *Nox1* (Figure 4.2B) expression was increased in Smad3^{-/-} primary myoblasts during differentiation. Since the results indicate that XO also produces ROS in Smad3^{-/-} myoblasts, an XO assay was performed in protein lysates from differentiating WT and Smad3^{-/-} primary myoblasts. The results showed significantly increased XO activity in Smad3^{-/-} myoblasts during differentiation (Figure 4.2C). These results further indicate that ROS is produced in the Smad3^{-/-} muscle cells by TNF- α regulation of Nox and also by XO.

4.3.3 Smad3^{-/-} myoblasts have increased Anti-oxidant enzyme (AOE) activity

An important marker for oxidative stress is AOE production, which is enhanced in cells in response to elevated ROS. As it has already been shown in the previous chapter that Mstn induces AOE activity (Chapter 3; Sriram *et al.* 2011), it was decided to find out the antioxidant status of Smad3^{-/-} myoblasts. Thus, the activities of the enzymatic antioxidants, CAT and GPx, and level of non-enzymatic antioxidant, GSR, were measured in differentiating WT and Smad3^{-/-} primary myoblasts. Increased activity of CAT (Figure 4.2D) and GPx (Figure 4.2E) was observed in Smad3^{-/-} myoblasts, together with increased GSR levels (Figure 4.2F). These data indicate that the increased ROS in Smad3^{-/-} myoblasts, potentially induced by Mstn, results in the production of AOE and GSR.

Figure 4.2 *Smad3^{-/-} myoblasts have increased TNF-α, Nox expression and XO activity and also increased AOE production.* mRNA expression of TNF-α (A) and *Nox1* (B) in WT and *Smad3^{-/-}* primary myoblasts in differentiation media for 48 hours. **p*<0.05, ****p*<0.001 (*n*=3). XO production assay (C) and enzyme assays for CAT (D), GPx (E) and GSR (F) were performed on protein lysates from differentiating WT and *Smad3^{-/-}* primary myoblasts at indicated time points. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001 (*n*=3). F-values are represented on the graphs.

4.3.4 Loss of Mstn relieves oxidative stress in Smad3^{-/-} muscle

It has been recently shown that genetic inactivation of Mstn in Smad3^{-/-} mice (double-KO mice), partially alleviates muscle atrophy (Ge *et al.* 2011). Since Smad3^{-/-} myoblasts demonstrated increased ROS production and elevated Mstn levels, double-KO mice were used to test whether the loss of Mstn is able to reduce the oxidative stress in Smad3^{-/-} mice. Firstly, the protein carbonylation, a standard marker for oxidative stress, was analyzed in Gastrocnemius muscle of WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice by immunoblotting, using an anti-DNP antibody (Figure 4.3A (i)). Densitometric analysis (Figure 4.3A (ii)) of the oxyblot revealed that while Smad3^{-/-} muscle had significantly higher levels of carbonylated proteins, Mstn^{-/-} muscle had significantly lower levels of carbonylated proteins, when compared to WT muscle. Furthermore, the level of protein carbonylation in the double-KO muscle was similar to that observed in WT muscle, suggesting that inactivation of Mstn ameliorates the effect of the enhanced ROS production observed in Smad3^{-/-} muscle.

Next, the mRNA expression of TNF- α and *Nox1* was analyzed in Gastrocnemius muscle of WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. The results indicate that Smad3^{-/-} muscle had significantly up-regulated expression of TNF- α (Figure 4.3B) and *Nox1* (Figure 4.3C) when compared to WT muscle; while in double-KO muscle, the expression of TNF- α (Figure 4.3B) and *Nox1* (Figure 4.3C) was similar to that observed in WT muscle and significantly down-regulated when compared to Smad3^{-/-} muscle. The enzyme activity of XO was also assayed in the muscle tissue and results indicated that Smad3^{-/-} muscle has higher XO activity when compared to WT, Mstn^{-/-} and double-KO muscle tissues (Figure 4.3D).

Further, the effect of inactivation of Mstn on the basal activities of AOE and non-enzymatic antioxidants in double-KO mice was determined. The results revealed that Smad3^{-/-} muscle had significantly increased SOD activity (Figure 4.3E) and GSR

(Figure 4.3F) level when compared to the WT muscle, while the double-KO muscle had the lowest SOD activity when compared to WT, $\text{Smad3}^{-/-}$ and $\text{Mstn}^{-/-}$ muscle (Figure 4.3E). The level of GSR in double-KO muscle was similar to that of WT muscle and significantly lower when compared to $\text{Smad3}^{-/-}$ muscle (Figure 4.3F). These findings confirm that inactivation of Mstn in $\text{Smad3}^{-/-}$ mice helps to maintain basal AOE activity in skeletal muscle.

Figure 4.3 Loss of *Mstn* relieves oxidative stress in *Smad3*^{-/-} muscle.

(A) (i). Representative gel showing protein carbonylation in protein lysates of Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice as detected using the Oxyblot assay kit. (A) (ii). Corresponding densitometric analysis of the gel showing the percentage increase or decrease in carbonylation over and above WT muscle (*p<0.05, **p<0.01); ^p<0.01: double-KO muscle as compared to *Smad3*^{-/-} muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). Representative graph showing mRNA expression of TNF- α (B) and *Nox1* (C) in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05, **p<0.01: when compared to the WT muscle; ^p<0.01, ^^p<0.001: when compared to *Smad3*^{-/-} muscle (n=3). Representative graph showing enzyme assays for XO (D), SOD (E) and GSR (F) performed on whole muscle lysates of Quadriceps muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05, **p<0.01: when compared to the WT muscle; ^p<0.01: when compared to *Smad3*^{-/-} muscle (n=3).

4.3.5 Smad3 is required for IKK α -mediated activation of NF- κ B (p65) by Mstn

Results from the previous chapter show that Mstn signals through NF- κ B to induce ROS in skeletal muscle cells (Sriram *et al.* 2011). Hence, whole muscle lysates, nuclear and cytoplasmic extracts of Biceps femoris muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice were analyzed by Western blot to assess the levels of proteins involved in NF- κ B signaling (Figures 4.4A (i), (ii), (iii) and (iv)). No change in the level of NF- κ B (p65) protein was observed in whole muscle lysates collected from Smad3^{-/-} (Lane 2) and double-KO mice (Lane 4), when compared to WT mice (Lane 1); however, a decrease in NF- κ B (p65) level was observed in Mstn^{-/-} mice (Lane 3), a feature consistent with results presented in Chapter 3/previously published work (Sriram *et al.* 2011). A significant decrease in NF- κ B (p65) levels was detected in both cytoplasmic and nuclear extracts of Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) muscle when compared to WT muscle (Lane 1) (Figure 4.4A (i)). The level of p-NF- κ B (p65) in the nuclear extract of Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) muscle was significantly lower than that detected in WT muscle (Lane 1) (Figures 4.4A (i) and (ii)). The level of p-I κ B- α (Figures 4.4A (i) and (iii)) was reduced in Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) cytoplasmic extracts, while reduced I κ B- α levels were detected in the cytoplasmic extract from Smad3^{-/-} muscle (Lane 2), when compared to WT muscle (Lane 1). IKK α levels were also significantly decreased in Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) cytoplasmic extracts (Figures 4.4A (i) and (iv)). Taken together, these results indicate that loss of Smad3 does not result in aberrant activation of NF- κ B signaling and moreover, as Mstn levels are enhanced in Smad3^{-/-} mice, suggesting that Smad3 may be required for Mstn-mediated activation of NF- κ B.

To confirm this hypothesis, C2C12 cells were treated with SIS3, a specific inhibitor of Smad3, in the absence or presence of CMM during differentiation. Western blot analysis

was performed to assess the protein levels of NF- κ B (p65), p-NF- κ B (p65), p-I κ B- α , I κ B- α and IKK α in whole cell lysates, nuclear and cytoplasmic extracts obtained from treated C2C12 cells. As expected, treatment with CMM resulted in activation of NF- κ B (p65) signaling (Figures 4.4B (i)-Lanes 1 and 2, (ii) and (iii)). However, SIS3-mediated blockade of Smad3 prevented NF- κ B (p65) activation even upon addition of CMM (Figures 4.4B (i)-Lanes 3 and 4, (ii) and (iii)). Similarly, Smad3 inhibition by shRNA also resulted in down-regulation of NF- κ B (p65) signaling in shSmad3 C2C12 cells (Figure 4.5). These data confirm that Smad3 is indispensable for Mstn-induced NF- κ B-mediated ROS induction.

Figure 4.4 *Smad3* is needed for *IKK α* -mediated activation of *NF- κ B* (p65) by *Mstn*.

(A) (i). Representative Western blot analysis of NF- κ B (p65), p-NF- κ B (p65), p-I κ B- α , I κ B- α and IKK α protein levels in whole muscle lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from Biceps femoris muscle from WT (Lane 1), Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) mice. Corresponding densitometric analysis of p-NF- κ B (p65) (ii), p-I κ B- α (iii) and IKK α (iv) protein levels. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (B) (i). Western blot analysis of NF- κ B (p65), p-NF- κ B (p65), p-I κ B- α , I κ B- α and IKK α protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from C2C12 cells treated with CCM (Lane 1), CMM (Lane 2), SIS3 and CCM (Lane 3) and SIS3 and CMM (Lane 4). Corresponding densitometric analysis of p-I κ B- α (ii) and IKK α (iii) protein levels. α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

Figure 4.5 *Smad3* is needed for activation of *NF-κB* (p65) by *Mstn*. Western blotting analysis of NF-κB (p65), p-NF-κB (p65), p-IκB-α, IκB-α and IKKα protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from shControl C2C12 cells, treated with either CCM or CMM (Lanes 1 and 2, respectively) or shSmad3 C2C12 cells, treated with either CCM or CMM (Lanes 3 and 4, respectively). α-tubulin was used as an internal control for equal protein loading on the gel (n=2).

4.3.6 ROS is generated through MAPK pathways in Smad3^{-/-} muscle

The results presented so far indicated that Mstn-mediated induction of ROS in Smad3^{-/-} muscle occurs independent of NF-κB signaling. Thus, the aim of the subsequent experiments was to identify potential alternative signaling pathways through which Mstn may induce ROS production in the absence of Smad3. Previous studies have shown that Mstn can activate p38 MAPK through TAK1 (Philip *et al.* 2005), JNK (Huang *et al.* 2007) and/or ERK pathways (Yang *et al.* 2006). Hence, to determine whether p38 MAPK, JNK and/or ERK pathways are involved in Mstn-mediated ROS production in the absence of Smad3, various downstream signaling molecules involved in these pathways were probed for in Gastrocnemius muscle isolated from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. As shown in Figures 4.6A and 4.7A (i), Smad3^{-/-} muscle has higher levels of p-p38 MAPK (Lane 2) when compared to WT muscle (Lane 1), while Mstn^{-/-} muscle (Lane 3) and double-KO muscle (Lane 4) have significantly lower levels of p-p38 MAPK. Total levels of p38 (Figure 4.6A) were significantly lower in WT and Smad3^{-/-} muscle (Lanes 1 and 2, respectively) and significantly higher in Mstn^{-/-} and double-KO muscle (Lanes 3 and 4, respectively). To determine if TAK1 plays a role in mediating Mstn-induced activation of p38, Western blot and densitometric analysis for p-TAK1 and total TAK1 was performed and results (Figures 4.6A and 4.7A (ii), respectively) indicated that p-TAK1 level was significantly increased in Smad3^{-/-} and double-KO muscle (Lanes 2 and 4, respectively) and significantly reduced in Mstn^{-/-} muscle (Lane 3), when compared to WT muscle (Lane 1). An increase in total TAK1 level (Figures 4.6A and 4.7A (iii)) was observed in Mstn^{-/-} (Lane 3) and double-KO muscle (Lane 4). Immunoblotting results for p-MKK3/6, which is a substrate of TAK1 and activator of p38, revealed that there was no significant change in the levels p-MKK3/6 in Smad3^{-/-} muscle (Lane 2) and double-KO muscle

(Lane 4) when compared to the WT muscle (Lane 1); however, lower levels of p-MKK3/6 were detected in the *Mstn*^{-/-} muscle (Lane 3). These results indicate that *Mstn* activates p38 MAPK via TAK1, and not p-MKK3/6, in the absence of *Smad3* and that inactivation of *Mstn* down-regulates the p38 MAPK activity in skeletal muscle.

Next the contribution of the JNK pathway towards *Mstn*-mediated signaling in *Smad3*^{-/-} mice was examined. Western blot and subsequent densitometric analysis for p-JNK (Figures 4.6B and 4.7B (i), respectively) revealed that the levels of p-JNK were reduced in *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) muscle, when compared to WT muscle (Lane 1). The level of p-MKK4, the kinase activator of JNK, was reduced in *Smad3*^{-/-} (Lane 2) and *Mstn*^{-/-} (Lane 3) muscle, while no significant difference was observed in double-KO muscle (Lane 4), when compared to WT muscle (Lane 1) (Figures 4.6B and 4.7B (ii)). These data revealed that there is reduced activity of the JNK pathway in the absence of *Smad3* and *Mstn* in skeletal muscle.

Lastly, it was asked whether the ERK pathway was involved in *Mstn* signaling in *Smad3*^{-/-} mice. Subsequent Western blot and densitometric analysis indicated that the levels of p-ERK1/2 and ERK1/2 (Figures 4.6C and 4.7C (i) and (ii), respectively) were higher in *Smad3*^{-/-} muscle (Lane 2) and in double-KO muscle (Lane 4) when compared to WT muscle (Lane 1). The p-ERK1/2 levels in double-KO muscle (Lane 4) were slightly reduced when compared to *Smad3*^{-/-} muscle (Lane 2). In contrast, no significant difference was observed in the levels of p-ERK1/2 and total ERK1/2 in *Mstn*^{-/-} muscle (Lane 3). The level of p-MEK1/2 was increased in double-KO muscle (Lane 4), relatively low in *Mstn*^{-/-} muscle (Lane 3) and showed no change in *Smad3*^{-/-} muscle (Lane 2) when compared to WT muscle (Lane 1) (Figure 4.6C). These results indicate that ERK pathway is activated in *Smad3*^{-/-} mice and that inactivation of *Mstn* partially rescues the activity of the ERK pathway.

To further confirm that MAPK pathways are involved in Mstn-mediated ROS production in the absence of Smad3, shControl and shSmad3 C2C12 were subjected to treatment with specific inhibitors of p38 MAPK (SB203580), JNK (SP600125) and ERK (U0126) signaling, in the absence or presence of CMM during differentiation. The expression of TNF- α and *Nox1* was determined and the results indicated that treatment with either SB203580 or U0126 significantly suppressed Mstn-mediated induction of TNF- α expression (Figure 4.8A) in shSmad3 C2C12 cells, while treatment with SP600125 only partially inhibited the Mstn-induced increase in TNF- α . Furthermore, all three inhibitors significantly suppressed the Mstn-induced increase in *Nox1* expression in shSmad3 C2C12 cells (Figure 4.8B). Similarly, pre-treatment with p38 MAPK and ERK inhibitors blocked the activation of p-p38 MAPK and p-ERK1/2 respectively, in shSmad3 C2C12 cells treated with CMM (Figures 4.8C and 4.8D, respectively). These results confirm that p38 MAPK and ERK pathways are involved in Mstn-mediated ROS production in the Smad3^{-/-} mice.

Figure 4.6 *ROS is generated through p38 and ERK MAPK pathways in Smad3^{-/-} muscle.* Western blot analysis of p-p38 MAPK (A), p38 α/β (A), p-TAK1 (A), TAK1 (A), p-MKK3/6 (A), p-JNK (B), JNK (B), p-MKK4 (B), p-ERK1/2 (C), ERK1/2 (C) and p-MEK1/2 (C) protein levels in protein lysates obtained from Gastrocnemius muscle from WT (Lane 1), Smad3^{-/-} (Lane 2), Mstn^{-/-} (Lane 3) and double-KO (Lane 4) mice. α -tubulin was used as an internal control for equal protein loading on the gels (n=3).

Figure 4.7 Densitometric analysis. (A) Densitometric analysis of Western blots to detect the protein levels of p-p38 MAPK/p38 α/β (i), p-TAK1 (Thr184/187) (ii) and TAK1 (iii) in Gastrocnemius muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. *p<0.05, **p<0.01: when compared to the WT muscle; ^p<0.05: when compared to Smad3^{-/-} muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (B) Densitometric analysis of Western blots to detect the protein levels of p-SAPK/JNK (Thr183/Tyr185) (i) and p-SEK1/MKK4 (Thr261) (ii) in Gastrocnemius muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. *p<0.05: when compared to the WT muscle; ^p<0.05: when compared to Smad3^{-/-} muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (C) Densitometric analysis of Western blots to detect the protein levels of p-ERK1/2 (Thr202/Tyr204) (i) and ERK1/2 (ii) in Gastrocnemius muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. *p<0.05, **p<0.01: when compared to the WT muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3).

Figure 4.8 MAPK pathways are involved in Mstn-mediated up regulation of TNF- α and Nox1 expression. mRNA expression of TNF- α (A) and Nox1 (B) in shSmad3 C2C12 cells pre-treated for 2 hours with SB203580 or for 1 hour with SP600125 or U0126 followed by 48 hours treatment with CCM or CMM in differentiation medium. The values are expressed as fold change when compared to respective shControl C2C12 cells. ##p<0.01: when compared to CCM treated shControl C2C12 cells; *p<0.05, **p<0.01, ***p<0.001: when compared to CCM treated shSmad3 C2C12 cells; ^p<0.05, ^^p<0.01, ^^^p<0.001: when compared to CMM treated shSmad3 C2C12 cells (n=2). ***p38 and ERK inhibitors inhibit activation of p38 and ERK MAPK pathways, respectively, in shSmad3 C2C12 cells.*** (C) Western blot and (E) densitometric analysis of p-p38 MAPK and p38 α/β protein levels in shControl and shSmad3 C2C12 cells treated with CCM (Lanes 1 and 5) and CMM (Lanes 2 and 6) for 48 hours in differentiation medium. Cells were also treated with CCM (Lanes 3 and 7) or with CMM (Lanes 4 and 8) for 48 hours following 2 hours pre-treatment with SB203580 in differentiation medium. Lanes 1-4: shControl C2C12 cells; Lanes 5-8: shSmad3 C2C12 cells. α -tubulin was used as an internal control for equal protein loading on the gel (n=2). (D) Western blot and (F) densitometric analysis of p-ERK1/2 and ERK1/2 protein levels in shControl and shSmad3 C2C12 cells treated with CCM (Lanes 1 and 5) and CMM (Lanes 2 and 6) for 48 hours in differentiation medium. Cells were also treated with CCM (Lanes 3 and 7) or with CMM (Lanes 4 and 8) for 48 hours following 1 hour pre-treatment with U0126 in differentiation medium. Lanes 1-4: shControl C2C12 cells; Lanes 5-8: shSmad3 C2C12 cells. α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

4.3.7 JAK-STAT pathway participates in XO-mediated ROS production in

Smad3^{-/-} mice

IL-6 has been shown to activate the JAK-STAT pathway and XO in endothelial cells (Wang *et al.* 2008), resulting in the generation of ROS. Hence, the expression and protein level of IL-6 in the absence of Smad3 and/or Mstn was analyzed in Gastrocnemius muscle from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. The results show that the mRNA expression (Figure 4.9A (i)) and protein level (Figure 4.9A (ii)) of IL-6 was significantly up-regulated in Smad3^{-/-} muscle (Lane 2), significantly down-regulated in Mstn^{-/-} muscle (Lane 3) and partially rescued in double-KO muscle (Lane 4), when compared to WT muscle (Lane 1). These data suggest that inactivation of Mstn can partially attenuate increased IL-6 expression in the absence of Smad3. To determine if the increase in IL-6 expression leads to the activation of the JAK-STAT pathway in Smad3^{-/-} mice, Western blot analysis was performed on muscle lysates collected from WT, Smad3^{-/-}, Mstn^{-/-} and double-KO mice. The results revealed that protein levels of JAK1, JAK2 and STAT3 were elevated in Smad3^{-/-} (Figure 4.9B-Lane 2) and double-KO (Lane 4) muscle while significantly reduced in Mstn^{-/-} muscle (Lane 3), when compared to WT muscle (Lane 1). The levels of p-STAT3 and p-STAT5 (Figures 4.9B and 4.8C (i) and (ii)) in Smad3^{-/-} muscle (Lane 2) were lower when compared to the level in WT muscle (Lane 1). However, the level of p-STAT3 and p-STAT5 were increased in double-KO muscle (Lane 4) when compared to the Smad3^{-/-} muscle (Lane 2). Interestingly, JAK3 levels were significantly elevated in Mstn^{-/-} muscle (Lane 3) and barely detectable in WT (Lane 1), Smad3^{-/-} (Lane 2) and double-KO (Lane 4) muscle. These results indicate that the JAK-STAT pathway is dysregulated in the absence of Smad3. Also, inactivation of Mstn in the Smad3^{-/-} genetic background did not have a significant effect on this pathway.

To further verify that JAK-STAT pathway is involved in ROS production in Smad3^{-/-} muscle, AG490, an inhibitor for JAK-STAT signaling, was used in shControl and shSmad3 C2C12 cells treated with or without CMM. The results show that inhibition of JAK-STAT signaling significantly down-regulated IL-6 expression (Figure 4.10A) as well as reduced the level of XO (Figure 4.10B) in shSmad3 C2C12 cells treated with CMM. Furthermore pre-treatment with AG490 inhibited IL-6, JAK1, JAK2, p-STAT3 and p-STAT5 in the shSmad3 C2C12 cells treated with CMM, while STAT3 levels remained unchanged (Figure 4.10C). These results suggest that the induction of ROS by XO in Smad3^{-/-} mice occurs via the JAK-STAT pathway.

Figure 4.9 The JAK/STAT pathway participates in XO-mediated ROS production in *Smad3*^{-/-} mice. (A) (i). Representative graph showing mRNA expression of IL-6 in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05, **p<0.01, ***p<0.0001: when compared to the WT muscle; ^p<0.05: when compared to *Smad3*^{-/-} muscle (n=3). (A) (ii) Western blot analysis of IL-6 protein level in Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (B) Western blot analysis of JAK1, JAK2, JAK3, p-STAT3, STAT3 and p-STAT5 protein levels in protein lysates obtained from Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (C) Densitometric analysis of Western blots to detect the protein levels of p-STAT3 (Tyr705) (i) and p-STAT5 (ii) in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. *p<0.05: when compared to the WT muscle; ^p<0.05: when compared to *Smad3*^{-/-} muscle. α -tubulin was used as an internal control for equal protein loading on the gel (n=3).

Figure 4.10 *The JAK/STAT pathway participates in XO-mediated ROS production in $Smad3^{-/-}$ mice.* Representative graph showing mRNA expression of IL-6 (A) and XO production (B) in shSmad3 C2C12 cells pre-treated for 1 hour with or without AG490 and grown for a further 48 hours with CCM or CMM in differentiation medium. The values are compared to respective shControl C2C12 cells. # $p < 0.05$: when compared to CCM treated shControl C2C12 cells; * $p < 0.05$, ** $p < 0.01$: when compared to CCM treated shSmad3 C2C12 cells; ^ $p < 0.01$: when compared to CMM treated shSmad3 C2C12 cells (n=2). (C) Western blot analysis of IL-6, JAK1, JAK2, p-STAT3, STAT3 and p-STAT5 protein levels in shControl and shSmad3 C2C12 cells treated with CCM (Lanes 1 and 5) or with CMM (Lanes 2 and 6), pre-treated for 1 hour with AG490 and CCM (Lanes 3 and 7) or with AG490 and CMM (Lanes 4 and 8) for 48 hours in differentiation medium. Lanes 1-4: shControl C2C12 cells; Lanes 5-8: shSmad3 C2C12 cells. α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

4.3.8 Crosstalk between ERK and JAK-STAT signaling is observed in the absence of Smad3

The data presented above indicated that ROS production in Smad3^{-/-} mice might occur through p38 MAPK, ERK MAPK and JAK-STAT signaling pathways. Therefore, to investigate whether or not there was crosstalk between the MAPK and JAK-STAT signaling pathways in the absence of Smad3, Western blot analysis was performed for IL-6, JAK1, JAK2, p-STAT3 and STAT3 in shControl and shSmad3 C2C12 cells treated with or without CMM in the absence or presence of the p38 inhibitor (SB203580) or ERK inhibitor (U0126). The results indicate that there was no change in the protein levels of IL-6, JAK1, p-STAT3 and STAT3 in the shSmad3 cells treated with SB203580 and CMM (Figures 4.11A (Lanes 6 and 8) and 4.11B (i)) when compared to the CCM treated shSmad3 cells (Figures 4.11A (Lanes 5 and 7) and 4.11B (i)); however, slight up-regulation was seen in JAK2 levels of shSmad3 cells treated with SB203580 and CMM (Figures 4.11A (Lanes 5-8) and 4.11B (ii)) when compared to the shControl cells treated likewise (Lanes 1-4). Inhibition of ERK activation in shSmad3 cells by U0126 treatment resulted in a significant decrease in JAK2 and p-STAT3 levels (Figures 4.12A (Lanes 5-8) and 4.12B (i) and (ii)) upon treatment with CMM. These results, which reveal decreased abundance of JAK-STAT signaling molecules upon inhibition of ERK, suggest the possibility of crosstalk between ERK activation and JAK-STAT signaling in the absence of Smad3.

Figure 4.11 No crosstalk between p38 MAPK and JAK-STAT signaling is observed in the absence of Smad3. (A) Western blot analysis of IL-6, JAK1, JAK2, p-STAT3 and STAT3 protein levels in shControl and shSmad3 C2C12 cells treated with CCM (Lanes 1 and 5) or CMM (Lanes 2 and 6), pre-treated for 2 hours with SB203580 and further with CCM (Lanes 3 and 7) or CMM (Lanes 4 and 8) for 48 hours in differentiation medium. Lanes 1-4: shControl C2C12 cells; Lanes 5-8: shSmad3 C2C12 cells. α -tubulin was used as an internal control for equal protein loading on the gel (n=2). (B) Densitometric analysis of Western blots to detect the protein levels of p-STAT3 (Tyr705) (i) and JAK2 (ii) in shControl and shSmad3 C2C12 cells treated with CCM, CMM, SB203580 and CCM or SB203580 and CMM. α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

Figure 4.12 Crosstalk between *ERK* and *JAK-STAT* signaling is observed in the absence of *Smad3*. (A) Western blot analysis of IL-6, JAK1, JAK2, p-STAT3 and STAT3 protein levels in shControl and shSmad3 C2C12 cells treated with CCM (Lanes 1 and 5) or CMM (Lanes 2 and 6), pre-treated for 1 hour with U0126 and further with CCM (Lanes 3 and 7) or CMM (Lanes 4 and 8) for 48 hours in differentiation medium. Lanes 1-4: shControl C2C12 cells; Lanes 5-8: shSmad3 C2C12 cells. α-tubulin was used as an internal control for equal protein loading on the gel (n=2). (B) Densitometric analysis of Western blots to detect the protein levels of JAK2 (i) and p-STAT3 (Tyr705) (ii) in shControl and shSmad3 C2C12 cells treated with CCM, CMM, U0126 and CCM or U0126 and CMM. α-tubulin was used as an internal control for equal protein loading on the gel (n=2).

4.3.9 CHOP-mediated MuRF1 up-regulation in Smad3^{-/-} mice

Excess ROS have been demonstrated to induce Atrogin1 and MuRF1, two important muscle-specific E3 ligases and likewise these enzymes are up-regulated by Mstn (Lokireddy *et al.* 2011a). However, in the absence of Smad3, MuRF1 but not Atrogin1 was up-regulated in the skeletal muscle (Quadriceps) (Ge *et al.* 2011). To further understand the molecular basis of differential expression of MuRF1 in Smad3^{-/-} muscle, an *in silico* analysis was performed on the upstream sequences of the mouse MuRF1 and Atrogin1 promoter sequences to identify unique transcription factor binding sites using the TFSEARCH tool (<http://www.cbrc.jp/research/db/TFSEARCH.html>). This analysis identified a putative CHOP binding site (5'-GCAATGC-3') within the MuRF1 promoter which was absent in Atrogin1 promoter. CHOP is a stress-induced transcription factor which induces oxidative stress and apoptosis in different cell types. Hence, to ascertain CHOP binding to MuRF1 promoter sequence, EMSA was performed using nuclear extracts from WT and Smad3^{-/-} Biceps femoris muscle and the double-stranded oligonucleotides, bearing the CHOP binding site on the mouse MuRF1 promoter (5'-AAAGTCTCAGTGCAATGCGCAGCCCATAA-3'). Smad3^{-/-} muscles showed increased CHOP binding activity indicated by the shifted band (Lane 3) when compared to the WT muscle (Lane 2) (Figure 4.13A (i)). To confirm the specificity of CHOP binding, nuclear extracts from WT and Smad3^{-/-} Biceps femoris muscle were pre-incubated with CHOP specific antibody. As shown in Figure 4.13A (ii), the results showed diminished band intensity in nuclear extracts pre-incubated with CHOP antibody (Lane 4 and 5). Furthermore, when nuclear extracts were incubated with 500X concentration of competitor oligos, the disappearance of the shifted band was observed (Figure 4.13A (ii)-Lanes 6 and 7). To confirm the binding of CHOP on the MuRF1 promoter *in vivo*, C2C12 cells were treated with SIS3 during differentiation and ChIP

assay was performed. As shown in Figure 4.13B, enhanced binding of CHOP to the MuRF1 promoter was observed in C2C12 cells upon SIS3 treatment (Lane 8) when compared to the untreated cells (Lane 7).

To investigate whether CHOP levels are altered in skeletal muscle of *Smad3*^{-/-} mice, RT-qPCR and Western blot analysis for CHOP was performed in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. The results indicated that *Chop* mRNA expression (Figure 4.13C (i)) and protein level (Figure 4.13C (ii)) was up-regulated in *Smad3*^{-/-} muscles (Lane 2). mRNA expression of *Chop* was up-regulated in *Mstn*^{-/-} mice, however, no significant change was observed in CHOP protein levels in these mice (Lane 3). *Chop* expression and level in double-KO muscle were similar to that of WT muscle (Lane 4). Western blot analysis was also performed for MuRF1 and the results showed that MuRF1 levels were up-regulated in *Smad3*^{-/-} muscle (Lane 2) and reduced in *Mstn*^{-/-} muscle (Lane 3) and double-KO muscle (Lane 4) (Figure 4.13C (ii)). *Smad3* inhibition by SIS3 also showed significantly higher levels of CHOP in whole cell lysates, nuclear and cytoplasmic extracts obtained from C2C12 myoblasts treated with SIS3 for 48 hours during differentiation (Figure 4.13D-Lane 2). As expected, MuRF1 levels were increased upon SIS3 treatment (Lane 2) when compared to the untreated myoblasts (Control) (Lane 1) (Figure 4.13D). Collectively, these data confirm that oxidative stress induced CHOP levels in the absence of *Smad3* resulting in increased binding of CHOP to MuRF1 promoter.

Figure 4.13 Enhanced binding of CHOP mediates MuRF1 up-regulation in *Smad3*^{-/-} mice. (A) EMSA was performed using nuclear extracts from WT and *Smad3*^{-/-} Biceps femoris muscle. (i) Left panel, representative gel showing increased CHOP binding in *Smad3*^{-/-} muscle as indicated by the shifted band in Lane 3 (Lane 1-oligo only, Lane 2-WT, Lane 3-*Smad3*^{-/-}). EMSA was also performed with nuclear extracts from WT and *Smad3*^{-/-} Biceps femoris muscle pre-incubated with CHOP specific antibody. (ii) Right panel, representative gel showing the diminished band intensity of CHOP specific band. Also, the disappearance of the shifted band in the nuclear extracts incubated with 500X concentration of competitor oligos is observed. (Lane 1-oligo only, Lane 2-WT, Lane 3-*Smad3*^{-/-}, Lane 4-WT with CHOP antibody, Lane 5-*Smad3*^{-/-} with CHOP antibody, Lane 6-WT with 500X competitor oligos, Lane 7-*Smad3*^{-/-} with 500X competitor oligos). (B) Representative agarose gel image showing the binding of CHOP to MuRF1 promoter (Lanes 7 and 8), as assessed by ChIP, in C2C12 myoblasts treated with SIS3 for 48 hours during differentiation. The relative amounts of the input DNA in both untreated (Lane 1) and SIS3 treated (Lane 2) myoblasts were also assessed. Both no antibody (No Ab) (Lanes 3 and 4) and isotype specific IgG (Lanes 5 and 6) controls are shown. (C) (i) Representative graph showing mRNA expression of *Chop* in Gastrocnemius muscle from WT, *Smad3*^{-/-}, *Mstn*^{-/-} and double-KO mice. **p*<0.05, ***p*<0.01: when compared to the WT muscle; ^^^^*p*<0.0001: when compared to *Smad3*^{-/-} muscle (n=3). (C) (ii) Western blot analysis of CHOP and MuRF1 protein levels in Gastrocnemius muscle from WT (Lane 1), *Smad3*^{-/-} (Lane 2), *Mstn*^{-/-} (Lane 3) and double-KO (Lane 4) mice. α -tubulin was used as an internal control for equal protein loading on the gel (n=3). (D) Western blot analysis of CHOP protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) and MuRF1 levels in whole cell lysates obtained from untreated C2C12 myoblasts (Control) (Lane 1) and SIS3 treated (for 48 hours during differentiation) myoblasts (Lane 2). α -tubulin was used as an internal control for equal protein loading on the gel (n=2).

A (i)

(ii)

B

C (i)

(ii)

D

4.4 Discussion

Smad3 is required for normal skeletal muscle growth and development as lack of Smad3 leads to impaired myogenesis and skeletal muscle atrophy (Ge *et al.* 2011; Ge *et al.* 2012). The pronounced muscle atrophy observed in Smad3^{-/-} mice is in part due to the increased Mstn levels, as the inactivation of Mstn in Smad3^{-/-} mice partially rescues the muscle atrophy seen in these mice (Ge *et al.* 2011). As shown in Chapter 3, it has been demonstrated that Mstn induces ROS production in skeletal muscle (Sriram *et al.* 2011), therefore, it was hypothesized that increased levels of Mstn in Smad3^{-/-} muscle would lead to increased ROS. Indeed, the results presented here show that Smad3^{-/-} muscles display increased ROS levels and that Mstn is one of the key signaling factors behind the increased oxidative stress observed in these mice. In concurrence, inactivation of Mstn in Smad3^{-/-} mice partially alleviates the oxidative stress.

The results in Chapter 3 have revealed that NF-κB is one of the downstream targets of Mstn (Sriram *et al.* 2011) and Nox, mETC and XO are well known downstream targets of NF-κB. However, the results in this Chapter indicated that ROS production was independent of IKKα-mediated NF-κB (p65) signaling in Smad3^{-/-} muscle. Mstn has also been shown to signal through Smad2 therefore the phosphorylation of Smad2 was also analyzed. As expected Smad2 phosphorylation was enhanced in Smad3^{-/-} muscle (Figure 4.14A) as well as in myoblasts with down-regulated Smad3 (Figure 4.14B). A point to note here is that despite increased phosphorylation of Smad2 and stimulation by Mstn, NF-κB (p65) signaling remained subdued in these muscles and myoblasts suggesting the requirement of Smad3 for Mstn-mediated NF-κB (p65) signaling (Figure 4.14C). However, inhibition of Smad2 alone in C2C12 cells resulted in repression of basal levels of IKKα and NF-κB (p65) (Figure 4.14C) suggesting distinct roles of Smad2 and Smad3 in NF-κB (p65) signaling.

Figure 4.14 *Smad2 is phosphorylated in the absence of Smad3.* (A) Western blot analysis of p-Smad2 and Smad2 protein levels in protein lysates obtained from Gastrocnemius muscle from WT (Lane 1) and Smad3^{-/-} (Lane 2) mice. GAPDH was used as an internal control for equal protein loading on the gel (n=3). (B) Western blot analysis of p-Smad2 and Smad2 in protein lysates obtained from C2C12 cells treated with CCM (Lane 1), CMM (Lane 2), SIS3 and CCM (Lane 3) and SIS3 and CMM (Lane 4). GAPDH was used as an internal control for equal protein loading on the gel (n=2). (C) Western blotting analysis of NF-κB (p65), p-NF-κB (p65), p-IκB-α, IκB-α and IKKα protein levels in whole cell lysates, nuclear (NE) and cytoplasmic extracts (CE) obtained from C2C12 cells transfected with empty vector control (pRFP-C-RS) (Lanes 1 and 2), scrambled shRNA (Lanes 3 and 4) and shSmad2 (Lanes 5 and 6). Lanes 1, 3 and 5: CCM treated; Lanes 2, 4 and 6: CMM treated. α-tubulin was used as an internal control for equal protein loading on the gel (n=2).

The IKK α levels were rescued in double-KO mice, which may be due to increased p-TAK1 (and TAK1) in these mice, because phosphorylation of TAK1 is postulated to result in increased IKK activation (Kanayama *et al.* 2004; Chen 2012).

Considering the repression of IKK α and NF- κ B (p65) signaling in Smad3^{-/-} muscle, it was speculated that Mstn might be inducing ROS through transducers other than Smad3. Previously, the activation of p38 MAPK pathway by Mstn was shown to be independent of Smad3 signaling and resulted in inhibition of myoblast proliferation (Philip *et al.* 2005). Likewise, the data analysis presented here revealed that in the absence of Smad3, p38 and ERK MAPK pathways were up-regulated and in double-KO mice there was a rescue of p38 and partial rescue of ERK pathway components. An earlier study has demonstrated that Mstn was able to repress myoblast proliferation and differentiation via JNK signaling (Huang *et al.* 2007). However, results presented here indicate that in spite of high levels of Mstn, the phosphorylation of JNK was down-regulated in Smad3^{-/-} muscle (Figure 4.6B). Previously, p38 α MAPK has been shown to activate MAPK phosphatase dual specificity protein phosphatase 1 (DUSP1), which in turn was observed to down-regulate JNK in mouse embryonic fibroblasts (Staples *et al.* 2010). These results support my observations of reduced JNK in Smad3^{-/-} muscle together with increased p38 α MAPK. Thus, it is possible that DUSP1 is responsible for the down-regulation of JNK in Smad3^{-/-} mice.

XO is a known source of ROS in tissues and reports have implicated IL-6 and ROS in inducing XO activity (Kosmidou *et al.* 2002). An increase in IL-6 and IL-17 levels was observed in Th17 cells lacking Smad3 in an earlier report (Bommireddy *et al.* 2011). As the results presented here show that Smad3^{-/-} mice have increased ROS, it is plausible that ROS induces IL-6 directly, leading to increased XO expression in Smad3^{-/-} mice. Furthermore, IL-6 expression has also been shown to be induced by ROS via p38

MAPK and ERK1/2 pathways in cardiac fibroblasts (Sano *et al.* 2001). Thus, increased IL-6 could be due in part to an increase in MAPK signaling. Thus, in Smad3^{-/-} mice there are several active signaling cascades that could up-regulate IL-6 and XO to promote generation of ROS. The reduced levels of IL-6 in Mstn^{-/-} and double-KO mice can be accounted for by reduced MAPK signaling as well as reduced levels of ROS, as indicated by reduced protein carbonylation (Figures 4.3A (i) and (ii)) and expression of TNF- α (Figure 4.3B) and *Nox1* (Figure 4.3C), found in these mice.

Previously, increased IL-6 levels have been associated with STAT3 activation and skeletal muscle wasting during cancer (Bonetto *et al.* 2011). Furthermore, hypoxia-induced production of IL-6 leads to the sequential activation of the JAK-STAT pathway and XO in endothelial cells (Wang *et al.* 2008). Dysregulation or disruption of various components of the JAK-STAT signaling pathway can result in ROS generation leading to immune deficiency syndromes and cancer (Costa-Pereira *et al.* 2011). In the present study, significant dysregulation of JAK1, JAK2, STAT3 and STAT5 in Smad3^{-/-} muscle was observed indicative of involvement of JAK-STAT signaling in ROS production in these mice.

Use of p38 and ERK inhibitors in shSmad3 cells revealed crosstalk between ERK MAPK and JAK-STAT signaling pathways (Figures 4.12A and 4.12B), which may further exacerbate ROS production. Mstn and ROS induced by Mstn up-regulate ubiquitination of proteins by E3 ligases like Atrogin1 and MuRF1 in skeletal muscle (Sriram *et al.* 2011; Lokireddy *et al.* 2011a). Since MuRF1 levels were up-regulated in Smad3^{-/-} muscle, it was speculated that expression/activity of various transcription factors which may be induced by oxidative stress or absence of Smad3 would be altered in these mice. Interestingly, an *in silico* analysis indicated a CHOP binding site only on MuRF1 promoter sequence and not on Atrogin1 promoter. CHOP is a stress-induced

nuclear protein which is known to play a role in apoptosis, proliferation, differentiation and oxidative stress (Cao *et al.* 1991; Maytin *et al.* 2001; Song *et al.* 2008; Alter and Bengal 2011). Further experiments confirmed that CHOP expression/level (Figures 4.13C (i) and (ii)) and binding to MuRF1 promoter (Figures 4.13A (i) and (ii)) is indeed up-regulated in Smad3^{-/-} muscle. SIS3-mediated inhibition of Smad3 in C2C12 myoblasts also increased binding of CHOP to MuRF1 promoter as shown by ChIP assay (Figure 4.13B). Mstn inactivation in Smad3^{-/-} mice resulted in rescue of CHOP protein to WT levels. However, *Chop* expression was up-regulated in Mstn^{-/-} mice (Figures 4.13A (i) and (ii)) suggesting that in Mstn^{-/-} mice, CHOP could be regulated post-transcriptionally. p38 MAPK was shown to mediate increase in activity of CHOP (Maytin *et al.* 2001) supporting my results of enhanced p38 MAPK signaling and CHOP activity in Smad3^{-/-} mice. Previously, CHOP has also been implicated to up-regulate IL-6 transcription (Hattori *et al.* 2003) indicating the possibility of a role of CHOP in IL-6 regulation in Smad3^{-/-} as well.

Various signaling cascades and cross-talk are likely to be involved in Mstn-mediated induction of ROS production. While Smad3 is the canonical/preferred pathway, I propose that in the absence of Smad3, Mstn induces ROS through an alternative mechanism. The activation of p38 and ERK MAPK pathways and dysregulation of JAK-STAT pathways lead to increased ROS production in Smad3^{-/-} muscle in response to increased Mstn (Figure 4.15).

Sandhya Sriram performed majority of the experiments and analyzed the data. Subha Subramanian helped in performing some of the experiments (animal work, isolation of primary myoblasts, EMSA). Prasanna Kumar Juvvuna helped in performing the ChIP assay. Sudarsanareddy Lokkireddy generated the stable shSmad3 C2C12 cell line.

Figure 4.15 Proposed *Smad3*-dependent and –independent mechanisms behind ROS production by *Mstn* in *Smad3*^{-/-} mice. In the absence of *Smad3*, *Mstn* induces *TNF-α* to activate *p38* and *ERK* MAPK to promote *Nox*-mediated induction of ROS. *Smad3*^{-/-} muscles also have increased *IL-6* production which can lead to activation of *JAK-STAT* pathway and increased *XO* production resulting in induction of ROS. Crosstalk between *ERK* MAPK and *JAK-STAT* pathways is also present in the absence of *Smad3* and leads to elevated ROS production through *XO*. The excessive ROS leads to increased *CHOP* levels and up-regulation of *MuRF1* transcription. The increased *CHOP* protein levels also induce ROS production and the enhanced ROS would further lead to increased *Mstn* production.

CHAPTER 5

MYOSTATIN IS INVOLVED IN ROS-MEDIATED DNA DAMAGE IN SKELETAL MUSCLE DURING STREPTOZOTOCIN-INDUCED TYPE 1 DIABETES

This study will be communicated as a manuscript to Journal of Biological Chemistry.

Abstract

One of the main features of uncontrolled type 1 diabetes is hyperglycaemia-induced oxidative stress that induces DNA damage and cell death. Skeletal muscle atrophy is also considerable in type 1 diabetes, however, the signaling mechanisms that induce oxidative stress which culminate in muscle atrophy are not fully known. Here, it is shown that Streptozotocin-mediated reduced insulin signaling led to hypo-phosphorylated Akt, resulting in activation of FoxA2 transcription-factor in the muscle. FoxA2 transcriptionally up-regulated Myostatin that contributed to exaggerated oxidative stress leading to DNA damage in skeletal muscle of Streptozotocin-treated wild type mice. In Myostatin^{-/-} mice however, Streptozotocin-treatment did not alter Akt phosphorylation, and therefore, FoxA2 levels were unaltered. Consistent with this result, appreciable DNA damage was not observed in Myostatin^{-/-} mice. Furthermore, Myostatin induced DNA damage in muscle of Streptozotocin-treated mice via p63/REDD1 pathway and genetic deletion of Myostatin attenuated DNA damage by regulating DNA damage/repair mechanisms.

5.1 Introduction

Type 1 diabetes is caused by lack of insulin secretion in the body due to pancreatic β cell damage. The increased levels of glucose in circulation dysregulate the prooxidant-antioxidant balance, thus enhancing oxidative stress and reducing antioxidant levels. The exaggerated level of reactive oxygen species (ROS) increases protein degradation and reduces protein synthesis eventually leading to skeletal muscle wasting. In addition to proteins, DNA is also susceptible to damage by ROS during type 1 diabetes. Increased ROS, especially hydroxyl ($^{\bullet}\text{OH}$) and superoxide ($\text{O}_2^{\bullet-}$) radicals can damage DNA by addition to double bonds of DNA bases resulting in increased 8-oxo-dG levels, a mutagenic base by-product used as a marker for oxidative stress. A recent study conducted on type 1 diabetic patients has shown that 8-oxo-dG levels exhibit a robust correlation with carbonylated proteins (Su *et al.* 2012). Additionally, DNA damage consists of single and double strand breaks and AP (apurinic/apyrimidinic) sites. The cellular DNA repair machinery comprising base excision repair and nucleotide excision repair is able to repair the above mentioned lesions (Clancy 2008). However, DNA repair mechanisms are also affected by ROS resulting in DNA fragmentation and eventually necrosis of cells. Indeed alterations in the DNA repair capacity and index have been observed in diabetic patients (Pacal *et al.* 2011). In recent years, *regulated in development and DNA damage responses* (REDD1), a component of stress response and a developmentally regulated transcriptional target of p63 and p53 (Ellisen *et al.* 2002), has been identified to be involved in oxidative stress-induced DNA damage in skeletal muscle during chronic hypoxia (Favier *et al.* 2010).

Myostatin (Mstn), a growth and differentiation factor, has been associated closely with skeletal muscle wasting. Results as shown in Chapter 3 indicate that Mstn can induce ROS and in turn Anti-oxidant enzymes (AOEs) in skeletal muscle through $\text{TNF-}\alpha$ and

NADPH oxidase (Nox) in a feed-forward manner (Sriram *et al.* 2011). Previously it was reported that Mstn expression and level was up-regulated in skeletal muscle of rodents with type 1 diabetes induced by Streptozotocin (STZ) (Chen *et al.* 2009; Hulmi *et al.* 2011; Dutra *et al.* 2012). Furthermore, Mstn expression was attenuated by insulin administration in STZ-induced type 1 diabetic mice (Chen *et al.* 2009). However, the signaling mechanisms by which high glucose levels induce Mstn expression are still unknown.

Since, Mstn levels are increased in skeletal muscle during STZ-induced type 1 diabetes and Mstn also acts as a pro-oxidant, the aim of this study was to investigate how Mstn levels are up-regulated during STZ-induced diabetes and whether Mstn can cause DNA damage under hyperglycemic conditions. Here, evidence is presented that Mstn can cause DNA damage and inhibit DNA repair mechanisms via ROS in a STZ-induced type 1 diabetes model. Furthermore, the results show that STZ-induced hypoinsulinemia up-regulated Mstn via FoxA2, which in turn induced ROS via TNF- α and Nox. The excessive ROS levels due to high glucose and elevated Mstn levels lead to p63/REDD1 regulated DNA damage. Using *in vitro* techniques, it is demonstrated here that Mstn induces single-strand (SSB) and double-strand breaks (DSB) in DNA, eventually leading to DNA fragmentation in muscle cells via p63/REDD1 pathway. Furthermore, inactivation or inhibition of Mstn in skeletal muscle or cells attenuated DNA damage by regulating the DNA damage/repair mechanisms.

5.2 Materials and methods

5.2.1 Animals

Seven week-old C57Bl/6J male mice (WT) were obtained from National University of Singapore-Centre for Animal Resources (NUS-CARE). *Mstn*^{-/-} male mice (7 week-old) were obtained as described in Section 3.2.1 and maintained at Nanyang Technological University (NTU) Animal house. All animals had free access to chow diet and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Singapore.

5.2.2 Reagents and Proteins

STZ was purchased from Sigma-Aldrich. *Mstn* protein containing conditioned medium was obtained from the *Mstn* expressing CHO cell line (Zimmers *et al.* 2002); control conditioned medium is denoted as CCM and conditioned medium containing *Mstn* is denoted as CMM in this Chapter. To antagonize *Mstn*, Ant1, a C-terminal truncation of *Mstn* protein which is a dominant negative (*Mstn* mimetic) protein, was used. Ant1 was produced and purified as previously published (Siriett *et al.* 2007). The list of various reagents and chemicals used in the current study are in Section 2.1.3.

5.2.3 Induction of type 1 diabetes (hyperglycemia) by STZ injection

To generate an acute type 1 diabetes mouse model, WT and *Mstn*^{-/-} mice were intraperitoneally injected with a single high-dose (concentration 22.5 mg/ml) of STZ (150 mg/kg body weight) dissolved in 0.1M sodium citrate buffer (pH 4.5). Mice injected with an equal volume of sodium citrate buffer were maintained as control groups. Non-fasting body weight and random blood glucose levels were determined on

the day of injection and subsequently on Day 1, 2, 4, 5, 7, 8, 9, 11, 12, 13, 14, 15, 16 and 18. Mice were fasted for 9-10 hours and body weight and fasting blood glucose was measured on Day 3, 6, 10 and 17. WT-Control and -STZ injected groups are denoted as WT-C and WT-STZ respectively, while $Mstn^{-/-}$ groups are denoted as $Mstn^{-/-}$ -C and $Mstn^{-/-}$ -STZ in this chapter. Mice were sacrificed on Day 4, 7, 11 and 18; Tibialis anterior (TA) muscles were embedded in OCT and frozen while the rest of the hind limb muscles (Gastrocnemius (Gas), Quadriceps (Quad) and Biceps femoris (BF)) were dissected, weighed and snap frozen in liquid nitrogen.

5.2.4 RT-qPCR (Reverse Transcriptase-quantitative Polymerase Chain Reaction)

RNA was isolated from Gas muscle and liver tissue and RT-qPCR was performed exactly as explained in Sections 2.2.8 and 3.2.17. The forward and reverse primers used are given in Table 2.1.

5.2.5 Cell culture

The established ATCC murine C2C12 cell line was used (Yaffe and Saxel 1977) in various experiments. The myoblasts were passaged and grown in C2C12 proliferation medium: DMEM, 10 % FBS, 1 % Penicillin/Streptomycin (P/S), as previously described in Section 3.2.3. The hepatocellular carcinoma cell line, HepG2 was used for ChIP assays. Cells were grown in medium containing DMEM (with high glucose, L-Glutamine and sodium pyruvate), 10 % FBS, 1 % P/S.

5.2.6 Isolation of primary myoblasts

The hind limb skeletal muscles from WT and Mstn^{-/-} mice were dissected and primary myoblasts were isolated according to the previously established protocol (Partridge 1997; McCroskery *et al.* 2003) (Section 3.2.6).

5.2.7 Treatment of C2C12 and primary myoblasts

STZ at two different concentrations (STZ1=0.25 mg/ml; STZ2=1 mg/ml) was used to treat proliferating C2C12 myoblasts and primary myoblasts isolated from the WT and Mstn^{-/-} mice for 48 hours. The conditioned media from CHO cells (CMM) was found to have Mstn at a concentration of 3.5 mg/ml (as determined by Enzyme Immuno Assay (EIA) (Immundiagnostik AG)). Two different concentrations of CMM (CMM1-7 ng; CMM2-17.5 ng) were used to treat C2C12 and primary myoblasts in proliferation medium for 48 hours. Ant1 at a concentration of 1 ng/ml was used to pre-treat cells 1 hour before STZ or CMM treatment.

5.2.8 Protein isolation

Gas muscle from WT and Mstn^{-/-} mice treated with STZ were homogenized in protein lysis buffer (as described in Section 3.2.9) and the clear supernatants were collected. WT and Mstn^{-/-} primary myoblasts and C2C12 myoblasts subjected to various treatments during proliferation were collected in protein lysis buffer. Protein lysates were subsequently made as previously described (McFarlane *et al.* 2006; Section 3.2.9). Cytoplasmic and nuclear fractions from BF muscles were isolated as previously described (Ye *et al.* 1996) (as described in Section 2.2.14). The protein concentrations were measured by Bradford's assay (Bradford 1976).

5.2.9 Western Blot analysis

Western blotting was performed as described previously (Section 3.2.18). List of primary and secondary antibodies are given Tables 2.3 and 2.5.

5.2.10 Electrophoretic mobility shift assay (EMSA)

The oligonucleotides containing the FoxA2 binding site on mouse Mstn promoter (5'-TTTTTCCCTCAAATATTTGTTTGTAGTAACAA-3') were hybridized to their respective complementary strands and labeled at the 3' end with Biotin Tetra-ethyleneglycol (TEG) (Sigma-Aldrich). The EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific). Nuclear extracts (NE) (4 µg) obtained from BF muscles were incubated with the biotin labeled probe (final concentration 1 nM (1 X)) and EMSA was performed as previously described (Section 3.2.19). Unlabeled probes were used as competitors at concentrations of either 100 nM (100X) or 500 nM (500 X). In supershift assays, 2 µl of FoxA2 antibody (Table 2.3) was added to the NE and pre-incubated for 20 minutes at room temperature before incubation with the labeled probe.

5.2.11 Chromatin Immunoprecipitation (ChIP) assay

HepG2 cells were transfected with 1.7 kb mouse Mstn promoter construct (1.7P) (Salerno *et al.* 2004) using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). ChIP assay was performed according to the published protocol (Wu *et al.* 2012). Briefly, following 48 hours of incubation, the cells were fixed by adding formaldehyde to a final concentration of 1 % for 10 minutes at room temperature. The formaldehyde fixation was stopped by adding glycine to a final concentration of 125 mM for 5 minutes at room temperature. The cells were washed twice and collected in

ice-cold PBS. Following centrifugation at 2,000 rpm for 5 minutes, the pellet was resuspended in lysis buffer (20 mM Tris-HCl (pH 8), 85 mM KCl, 0.5 % NP-40 and 1 X Protease Inhibitor Cocktail) by mixing gently at 4°C for 5 minutes. The cell suspension was centrifuged at 3,000 rpm for 5 minutes at 4°C. The resulting crude nuclear extract pellet was resuspended in high-salt lysis buffer (50 mM HEPES-KOH (pH 7.3), 140 mM NaCl, 1 mM EDTA (pH 8), 1 % Triton X-100, 0.1 % Sodium Deoxycholate, 0.1 % SDS and 1 X Protease Inhibitor Cocktail) and incubated on ice for 10 minutes. The nuclear extracts were sonicated on ice using QSONICA sonicator (Model Q55) with a pulse at 40 % amplitude for 30 seconds followed by 1 minute break for 10 times to yield DNA fragments of 200 to 500 base pairs in size. 50 µl of the sonicated sample was removed as input DNA. The protein concentration of the lysates was determined by Bradford's assay (Bradford 1976). The nuclear extracts (300 µg) were initially pre-cleared with pre-blocked Protein A agarose beads (Millipore) for 1 hour at 4°C. The beads were then discarded by centrifugation at 10,000 rpm for 5 minutes at 4°C. Immunoprecipitation was conducted overnight at 4°C with 5 µg of anti-FoxA2 antibody (Table 2.3) or anti-IgG antibody (Dako). Following incubation with antibody, 100 µl of protein A agarose beads (pre-blocked) was added to the samples and incubated for 2 hours with rotation at 4°C. The beads were washed with wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA (pH 8), 500 mM NaCl and 20 mM Tris-HCl (pH 8) and resuspended in elution buffer (1 % SDS and 0.1 M sodium bicarbonate) containing 2 µl of RNase A (0.5 mg/ml). The samples were then incubated for 2 hours at 65°C. Subsequently, the beads were removed by centrifugation for 3 minutes at 10,000 rpm and the supernatant was incubated overnight at 65°C, to reverse formaldehyde cross-linking. DNA was isolated using phenol/chloroform/isoamyl alcohol (25:24:1) extraction and the commercially available QIAquick PCR Purification kit (Qiagen).

DNA was PCR amplified using HotStart Taq polymerase according to manufacturer's instructions (Kapa Biosystems). The following set of primers was used for PCR: *FoxA2* forward primer 5'-GTCAGCTCTTCCTAGTTTTTACTTCTC-3' and *FoxA2* reverse primer 5'-TCCTTTAAGACTTGGAGTGCTGT-3'. The resulting PCR products were electrophoresed on 1.5 % agarose gel and stained with ethidium bromide.

5.2.12 Luciferase assay

C2C12 myoblasts were plated at a density of 10,000 cells/cm² in 6-well plates. Following overnight attachment, the C2C12 myoblasts were transfected with either pGL3-basic and pFLAG-CMV2 empty vectors or 1.7 kb mouse *Mstn* promoter construct (1.7P) and pFLAG-CMV2 or 1.7P and pFLAG-*FoxA2*, together with the control Renilla luciferase vector pRL-TK using Lipofectamine 2000 (Invitrogen), as per the manufacturer's guidelines. Following overnight transfection, the medium was replaced with fresh proliferation medium and the myoblasts were incubated for a further 24h. Luciferase assays were performed using the Dual Luciferase Assay System, as per the manufacturer's protocol (Promega). Relative luciferase activity in each of the extracted protein samples was measured in triplicate using the Fluoroskan Ascent Microplate Fluorometer and Luminometer (Thermo Fisher Scientific Inc.).

5.2.13 Preparation of muscle homogenates for enzyme assays

Quad muscle homogenates were made according to previously published protocol (Section 3.2.11) and total protein concentration was measured by Bradford's assay (Bradford 1976).

5.2.14 Estimation of Superoxide dismutase (SOD) and Glutathione peroxidase (GPx)

The activity of SOD was estimated as described in Section 3.2.13.1. GPx enzyme assay was performed by the modified method of Rotruck *et al.* (Rotruck *et al.* 1973) as described previously (Section 3.2.13.3).

5.2.15 Estimation of lipid peroxidation product

The lipid peroxidation product (malonaldehyde (MDA)) was determined as described previously (Ohkawa *et al.* 1979) (as described in Section 2.2.13).

5.2.16 Estimation of Reduced Glutathione (GSH)

GSH/GSR levels were determined by the method of Moron *et al.* (Moron *et al.* 1979) (as described in Section 2.2.12).

5.2.17 Immunohistochemistry (IHC) for REDD1 and OGG1

OCT embedded TA muscles were sectioned and fixed with 4% paraformaldehyde (dissolved in 1 X PBS). The IHC protocol for REDD1 or OGG1 primary antibodies was followed as per manufacturer's instructions (Proteintech). Briefly, endogenous peroxidase was blocked by incubation with 3 % H₂O₂ in methanol for 30 minutes at room temperature. Following wash with TBS twice, the sections were blocked for 30 minutes at room temperature with 5 % normal goat serum. Subsequently, the sections were incubated with REDD1 or OGG1 primary antibodies (1:50 dilution in TBS) (Proteintech) for 1 hour at room temperature. After two washes with TBS containing Tween-20, the sections were incubated for 1 hour at room temperature with Alexa Fluor 488 (AF 488) goat anti-rabbit secondary antibodies (1:1,000 dilution in TBS)

(Invitrogen). Following washes, the sections were mounted with anti-fade mounting medium and images were taken at 5X magnification using a Leica upright microscope.

5.2.18 Myoblast proliferation assay

C2C12 cells were seeded in 96-well plates in proliferation media at a cell density of 1,000 cells/well. After overnight attachment, the cells were treated with either STZ1, Ant1 or Ant1+STZ1 in fresh proliferation media. After 48 hours, the proliferating cells were fixed and the proliferation was assessed by methylene blue photometric end point assay (Oliver *et al.* 1989) (as described in Section 2.2.9).

5.2.19 Analysis of intracellular ROS production

ROS production was assayed in C2C12 myoblasts treated with STZ during proliferation as previously described (Section 3.2.8.2) using the fluorescent dye, CM-H2DCFDA (Molecular Probes).

5.2.20 Immunocytochemistry (ICC) for 8-oxo-dG

C2C12 and primary myoblasts were seeded at a density of 15,000 cells/cm². The next day, proliferation medium with or without STZ1, STZ2, CCM, CMM1, CMM2 and with or without pre-treatment with Ant1 was added to the myoblasts and incubated for 48 hours. The immunostaining for 8-oxo-dG was performed according to manufacturer's instructions (Trevigen Inc.). At the indicated time point, the cells were fixed for 20 minutes at -20°C in 1:1 methanol:acetone. 0.05 N HCl was added for 5 minutes followed by 3 times wash with 1 X PBS for 5 minutes. The fixed cells were incubated with 250 µl of 100 µg/ml RNase in 150 mM NaCl, 15 mM sodium citrate for 1 hour at 37°C. Sequentially, the cells were washed with 1 X PBS, 35 %, 50 % and 75

% EtOH, for 3 minutes each. DNA was denatured *in situ* with 250 µl 0.15 N NaOH in 70 % EtOH for 4 minutes. The nuclei were then stained with DAPI and washed sequentially in 70 % EtOH containing 4 % v/v formaldehyde, 50 % and 35 % EtOH, and 1 X PBS for 2 minutes each. 250 µl of 5 µg/ml proteinase K in 20 mM Tris, 1 mM EDTA, pH 7.5 (TE) was added and the cells were incubated for 10 minutes at 37°C. Following several washes with 1 X PBS, non-specific binding was blocked with 5 % normal goat serum in 1 X PBS for 1 hour at room temperature. Then the cells were incubated with 250 µl of anti-8-oxo-dG antibody at a concentration of 1:250 diluted in 1 X PBS containing 1 % BSA, 0.01 % Tween-20 at 4°C overnight in a humidified chamber. Next day, the cells were washed several times with 1 X PBS containing 0.05 % Tween-20 for 5 minutes each. Then, 250 µl of secondary antibody AF-488 goat anti-mouse (1:400 dilution) (Invitrogen) diluted in 1X PBS containing 1 % BSA was added and incubated for 1 hour in the dark, at room temperature. After several washes with 1 X PBS containing 0.05 % Tween-20, the cells were mounted with anti-fade mounting medium. The fluorescence images (DAPI-blue; 8-oxo-dG-green) were taken at 10X magnification using a Leica upright microscope.

5.2.21 Comet assay

C2C12 myoblasts were seeded at a density of 15,000 cells/cm² and following overnight attachment of cells, proliferation media with or without STZ1, CCM, CMM1, CMM2 and with or without pre-treatment with Ant1 was added to the myoblasts and incubated for 48 hours. A single cell suspension was made and comet assay was performed as previously described (Olive and Banath 2006). At the indicated time point, a single-cell suspension using 0.005 % trypsin-EDTA was prepared from the myoblasts following various treatments. The cells were kept in ice-cold PBS to minimize cell aggregation

and inhibit DNA repair. Using a hemocytometer, the cell density was adjusted to about 20,000 cells/ml in PBS containing EDTA. 0.4 ml of cells was mixed with 1.2 ml of 1 % low-gelling-temperature agarose at 40°C. 1.2 ml of the cell suspension was rapidly pipetted onto a agarose-pre-coated slide and was allowed to gel for about 2 minutes. The alkaline lysis method was done to detect the combination of SSB, DSB and alkali-labile sites in the DNA and neutral lysis method was performed to detect only DNA DSB. In the alkaline lysis method, the slides were submerged in a covered dish containing A1 lysis solution. The samples were lysed overnight at 4°C in the dark. After overnight lysis, the slides were submerged in A2 rinse solution at room temperature for 20 minutes thrice. The slides were then submerged in fresh A2 solution in an electrophoresis chamber and electrophoresis was performed for 25 minutes at a voltage of 0.6 V/cm. In the neutral lysis method, the slides were submerged in a covered dish containing N1 lysis solution and incubated at 37°C overnight in the dark. The next day, the slides were submerged in room temperature N2 rinse buffer for 30 minutes thrice. Then, the slides were submerged in fresh N2 solution in an electrophoresis chamber and electrophoresis was performed for 25 minutes at a voltage of 0.6 V/cm. Following electrophoresis, the slides were rinsed in 400 ml of MilliQ water and stained with 100 µl of a 10 µg/ml stock solution of propidium iodide (PI) for 20 minutes. The slides were again rinsed in 400 ml of MilliQ water and the comets were visualized using a fluorescent microscope. At least 50 comet images per slide and 3 slides per treatment/time point were examined. Comet image analysis software was used to quantify several parameters including comet height, comet length, comet mean intensity, head mean intensity, % DNA in head, % DNA in tail, tail length, tail area, tail moment and Olive moment. Olive moment is the product of the tail length and the fraction of total DNA in the tail.

5.2.22 Transient transfection of shRNA to knockdown Mstn

C2C12 myoblasts were plated at a density of 10,000 cells/cm² in 6-well plates. Following overnight attachment, the C2C12 myoblasts were transfected with 4 µg/well of either empty vector control (pGFP-V-RS), scrambled shRNA or Mstn-specific shRNA expression vector (shMstn) (OriGene Technologies, Inc.) using Lipofectamine 2000 (Invitrogen), as per the manufacturer's guidelines. Next day, fresh proliferation media containing either CCM, CMM, STZ and/or Ant1 was added for a further 48 hours. The myoblasts were then harvested in protein lysis buffer and protein lysates were made as described previously (Section 3.2.9).

5.2.23 Statistical analysis

The p value was calculated using ANOVA and Student's t-test and $p < 0.05$ was considered as significant. Five or seven mice for each treatment were used for various experiments. The results are presented as mean $\pm$ SE of 2 or 3 independent experiments.

5.3 Results

5.3.1 STZ treatment induced hyperglycemia and reduced body weight and muscle weights in mice

To establish a type 1 diabetes model, both WT and $Mstn^{-/-}$ mice were injected with a high-dose of STZ once. The body weight and blood glucose was analyzed in these mice for a period of 18 days. As shown in Figures 5.1A and 5.1C, body weights of WT-STZ and $Mstn^{-/-}$ -STZ groups decreased for the first 11 days when compared to their respective control groups, but reached a steady state in the next one week. Interestingly, the loss in body weight was more in $Mstn^{-/-}$ -STZ group when compared to the loss observed in WT-STZ group. The random and fasting blood glucose levels in WT-STZ and $Mstn^{-/-}$ -STZ groups were elevated throughout the 18 days. Corresponding to the increased loss in body weight, $Mstn^{-/-}$ -STZ group had much higher blood glucose level when compared to WT-STZ group (Figures 5.1B and 5.1D).

Figure 5.1 *STZ treatment reduced body weight and induced hyperglycemia in mice.* Representative graph showing non-fasting (A) and fasting (C) body weight of WT and Mstn^{-/-} mice on indicated days after a single high-dose of STZ (150 mg/kg body weight) injection (n=7). Random (B) and fasting (D) blood glucose (mmol/L) was measured on indicated days after STZ injection (*p<0.05, **p<0.01, ***p<0.001 when compared to WT-C; ^p<0.05, ^^p<0.01, ^^^p<0.001 when compared to Mstn^{-/-}-C) (n=7).

One of the effects of hyperglycemia is induction of ROS in skeletal muscle. Therefore, hind limb muscles were dissected on Day 4, 7, 11 and 18 after STZ injection and expression of genes involved in ROS production (TNF- α and *Nox1*) and activity of AOE (GSR and GPx) was determined. The results showed maximum induction of ROS and AOE on Day 7 in STZ-treated muscles (Figures 5.2A, 5.2B, 5.2C and 5.2D); hence, further experiments were performed using Day 7 STZ-treated muscles. Furthermore by Day 7, the Gas and Quad muscle weights were significantly reduced in WT-STZ and *Mstn*^{-/-}-STZ groups when compared to their respective control groups (Figure 5.2E). Also, the percentage loss of muscle weight when normalized to body weight was significantly decreased in both STZ groups (Figure 5.2F). These results confirmed that STZ is able to induce hyperglycemia and reduce muscle weights in both WT and *Mstn*^{-/-} mice.

Figure 5.2 STZ induces TNF- α and Nox1 expression and increases AOE production. Representative graph showing mRNA expression of TNF- α (A) and Nox1 (B) in Gas muscle of WT and Mstn^{-/-} mice on indicated days after STZ injection. Enzyme assays were performed using protein lysates from Quad muscle for GSR (C) and GPx (D). *p<0.05, **p<0.01, ***p<0.001 when compared to WT-C muscle; ^p<0.05, ^^p<0.01 when compared to Mstn^{-/-}-C muscle (n=7). **STZ treatment reduced muscle weights in mice.** Skeletal muscles were dissected on Day 7 after STZ injection and the muscles were weighed. Representative graph showing decrease in Gas and Quad muscle weights (E) and decrease in muscle weights when normalized to body weight (F) upon STZ treatment in WT and Mstn^{-/-} mice. (*p<0.05, **p<0.01, ***p<0.001 when compared to WT-C; ^p<0.05, ^^p<0.01, ^^p<0.001 when compared to Mstn^{-/-}-C) (n=7).

5.3.2 $Mstn^{-/-}$ mice have increased expression of genes involved in gluconeogenesis

The results show that the initially high levels of glucose and decrease in body and muscle weight were greater in $Mstn^{-/-}$ -STZ mice when compared to the WT mice. One of the reasons for this could be due to increased gluconeogenesis in $Mstn^{-/-}$ mice as suggested by Wang *et al.*, 2012. Deficiency of insulin leads to increased conversion of non-carbohydrate substrates to glucose via gluconeogenesis; hence RT-qPCR was performed for *PEPCK* and *G6P*, genes involved in gluconeogenesis, on RNA isolated from liver of WT and $Mstn^{-/-}$ mice treated with STZ. The results showed that mRNA expression of *PEPCK* (Figure 5.3A) and *G6P* (Figure 5.3B) were significantly up-regulated upon STZ treatment in WT mice. Interestingly, *PEPCK* (Figure 5.3A) and *G6P* (Figure 5.3B) expression was significantly up-regulated in $Mstn^{-/-}$ -C mice as well, when compared to the WT-C mice and *PEPCK* expression was further up-regulated in $Mstn^{-/-}$ -STZ mice (Figure 5.3A). These results indicated that there is increased glucose production in the $Mstn^{-/-}$ mice via gluconeogenesis and that might be the reason for high levels of glucose in $Mstn^{-/-}$ mice upon STZ administration. The high levels of glucose could be the reason for a greater decrease in body and muscle weights in the $Mstn^{-/-}$ -STZ mice when compared to that of WT mice.

Figure 5.3 *Genes involved in gluconeogenesis are up-regulated in Mstn^{-/-} mice.* Representative graph showing mRNA expression of *PEPCK* (A) and *G6P* (B) in RNA isolated from liver tissue of WT-C, WT-STZ, Mstn^{-/-}-C and Mstn^{-/-}-STZ mice. *p<0.05 and **p<0.01 when compared to WT-C muscle; ^p<0.05 when compared to Mstn^{-/-}-C muscle (n=7).

5.3.3 FoxA2 mediates up-regulation of Mstn in response to STZ

Similar to previously published data (Chen *et al.* 2009; Hulmi *et al.* 2012; Dutra *et al.* 2012), results presented here show a significant increase in Mstn expression (Figure 5.4A (i)) and protein level (Figure 5.4A (ii)) in Gas muscle of WT-STZ group (Lane 2) when compared to WT-C group (Lane 1). Concomitantly, downstream targets of Mstn signaling, p-Smad2/3 levels were significantly up-regulated in WT-STZ muscle (Lane 2) when compared to WT-C muscle (Lane 1) (Figure 5.4A (ii)). Western blot analysis was also performed for p-Akt1/2/3 and Akt1/2/3 and results showed that the levels were reduced in WT-STZ muscles (Lane 2), when compared to WT-C muscles (Lane 1), while the levels were significantly higher in Mstn^{-/-} muscles (Lanes 3 and 4) (Figure 5.4B). These results indicated that STZ treatment is able to induce Mstn and affect its downstream signaling molecules such as Smad2/3 and Akt in WT mice.

To investigate the mechanism involved in STZ-induced Mstn, an *in silico* analysis was performed on the 1.7 kb upstream sequence of the mouse Mstn promoter to identify various transcription factor binding sites using the TFSEARCH tool (<http://www.cbrc.jp/research/db/TFSEARCH.html>). Subsequent sequence analysis identified a putative FoxA2 binding site (5'-CAAATATTTGTT-3') within the 1.7 kb upstream sequence of the mouse Mstn promoter. FoxA2 has been shown to be involved in glucose homeostasis and implicated to control glucose-induced insulin release (Wang *et al.* 2002). Therefore, firstly RT-qPCR and Western blot analysis for FoxA2 was performed in Gas muscle of STZ-treated WT and Mstn^{-/-} mice. The results indicated that FoxA2 mRNA expression (Figure 5.5A (i)) and protein level (Figure 5.5A (ii)) was significantly up-regulated in WT-STZ muscles (Lane 2) and barely detectable in Mstn^{-/-} muscles even upon STZ treatment (Lanes 3 and 4). Next, to determine whether high glucose levels induced by STZ can enhance FoxA2 binding to Mstn promoter, EMSA

was performed using nuclear extracts from WT-C and WT-STZ BF muscles and the double-stranded oligonucleotides bearing the FoxA2 binding site on the mouse *Mstn* promoter (5'-TTTTTCCCTCAAATATTTGTTTGTAGTAACAA-3'). As shown in Figure 5.5B (i), STZ treatment in WT mice led to increased FoxA2 binding as indicated by the shifted band (Lane 3). Furthermore, when nuclear extracts from WT-STZ BF muscles were incubated with increasing concentrations of competitor oligos, the disappearance of the shifted band was observed (Figure 5.5B (ii)-Lanes 3 and 4). In addition, to confirm the specificity of FoxA2 binding, when supershift assays were performed with nuclear extracts from WT-STZ BF muscles pre-incubated with FoxA2 specific antibody, the results showed a supershift of the FoxA2 specific band (Figure 5.5B (ii)-Lane 5). These observations confirm that STZ-induced hypoinsulinemia would be able to up-regulate *Mstn* levels through enhanced FoxA2 binding on the *Mstn* promoter.

To confirm the binding of FoxA2 on the *Mstn* promoter, HepG2 cells, which have high levels of endogenous FoxA2, were transfected with the 1.7kb murine *Mstn* promoter and ChIP assay was performed. As shown in Figure 5.5C, enhanced binding of FoxA2 to the *Mstn* promoter was observed in HepG2 cells transfected with the *Mstn* promoter (Lane 10) when compared to cells transfected with the control vector (Lane 9).

To establish whether FoxA2 can activate *Mstn* transcriptionally by binding to the *Mstn* promoter as shown in EMSA and ChIP, C2C12 myoblasts were transfected either pGL3-basic and pFLAG-CMV2 empty vectors or 1.7 kb mouse *Mstn* promoter construct (1.7P) and pFLAG-CMV2 or 1.7P and pFLAG-FoxA2. Myoblasts transfected with 1.7 kb mouse *Mstn* promoter construct resulted in a ~8.0-fold increase in Luciferase activity, when compared to the myoblasts transfected with the empty vectors (Figure 5.5D). A further significant increase in Luciferase activity was observed in

myoblasts transfected with both FoxA2 expression vector and 1.7 kb mouse Mstn promoter construct when compared to myoblasts transfected with pFLAG-CMV2 and 1.7P (Figure 5.5D). These data confirm that FoxA2 regulates *Mstn* expression by binding to Mstn promoter and activating its expression.

Figure 5.4 STZ treatment up-regulates *Mstn* and affects *Akt* phosphorylation. (A) Representative graph (i) showing mRNA expression of *Mstn* and representative Western blot (ii) showing protein levels of *Mstn*, p-Smad2/3 and Smad2/3 in WT-C and WT-STZ Gas muscle (***) $p < 0.001$; Lane 1-WT-C, Lane 2- WT-STZ). GAPDH was used as an internal control for equal protein loading on the gel ($n=7$). (B) Representative Western blot showing p-Akt1/2/3 and Akt1/2/3 protein levels in WT-C (Lane 1), WT-STZ (Lane 2), *Mstn*^{-/-}-C (Lane 3) and *Mstn*^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as an internal control for equal protein loading on the gel ($n=7$).

Figure 5.5 *FoxA2* mediates up-regulation of *Mstn* in response to STZ. (A) Representative graph (i) showing mRNA expression of *FoxA2* and representative Western blot (ii) showing protein levels of *FoxA2* in protein lysates from Gas muscle of WT-C, WT-STZ, *Mstn*^{-/-}-C and *Mstn*^{-/-}-STZ mice (**p<0.01, ***p<0.001 when compared to WT-C muscle; Lane 1-WT-C, Lane 2-WT-STZ, Lane 3- *Mstn*^{-/-}-C, Lane 4- *Mstn*^{-/-}-STZ). GAPDH was used as an internal control for equal protein loading on the gel (n=7). (B) EMSA was performed using nuclear extracts from WT-C and WT-STZ BF muscles. (i) Left panel, representative gel showing increased *FoxA2* binding upon STZ treatment in WT mice as indicated by the shifted band in Lane 3 (Lane 1-oligo only, Lane 2-WT-C, Lane 3-WT-STZ). (ii) Right panel, representative gel showing the disappearance of the shifted band in nuclear extracts of WT-STZ BF muscles incubated with increasing concentrations of competitor oligos (100 X and 500 X). Supershift assays were performed with nuclear extracts from WT-STZ BF muscles pre-incubated with *FoxA2* specific antibody. Supershift of the *FoxA2* specific band is observed. (Lane 1-oligo only, Lane 2-WT-STZ, Lane 3-with 100 X competitor oligo, Lane 4-with 500 X competitor oligo, Lane 5-with *FoxA2* antibody) (n=3). (C) Representative agarose gel image showing the binding of *FoxA2* to 1.7kb murine *Mstn* promoter (1.7P) (Lanes 9 and 10), as assessed by ChIP. The relative amounts of both the control and *Mstn* promoter in the input were also assessed (Lanes 3 and 4). Both no antibody (No Ab) (Lanes 5 and 6) and isotype specific IgG (Lanes 7 and 8) controls are shown. (D) Assessment of promoter-luciferase reporter activity in C2C12 myoblasts transfected with either pGL3-basic and pFLAG-CMV2 empty vectors or 1.7 kb mouse *Mstn* promoter construct (1.7P) and pFLAG-CMV2 or 1.7P and *FoxA2* expression vector (pFLAG-*FoxA2*), together with the control Renilla luciferase vector pRL-TK. (****p<0.0001, ^^^p<0.0001; n=3).

5.3.4 Absence of Mstn attenuates STZ-induced ROS and AOE production in skeletal muscle

STZ induces ROS (and thus AOE) in skeletal muscle via TNF- α (Aragno *et al.* 2004; Mastrocola *et al.* 2008). Similarly, RT-qPCR analysis indicated that there was significant up-regulation in TNF- α (Figure 5.6A) and *Nox1* (Figure 5.6B) expression in WT-STZ muscles when compared to WT-C muscles, while *Mstn*^{-/-}-STZ muscles showed no change when compared to *Mstn*^{-/-}-C muscles. Enzyme assays indicated that there was significantly increased lipid peroxidation in WT-STZ muscle and the levels were significantly reduced in *Mstn*^{-/-}-STZ muscle (Figure 5.6C). *Sod1* (Figure 5.6D) and *Cat* (Figure 5.6E) expression was significantly up-regulated in WT-STZ muscles, while *Mstn*^{-/-}-STZ muscles showed significant down-regulation of the genes. Results of SOD enzyme activity assay coincided with the expression of *Sod1* (Figure 5.6F). The amount of GSR was increased upon STZ treatment in WT mice, while no change was observed in *Mstn*^{-/-} mice (Figure 5.6G). These data revealed that STZ induces TNF- α , Nox and AOE, while absence of Mstn attenuates STZ-induced ROS production by up-regulating AOE and down-regulating ROS-inducing genes.

Figure 5.6 Absence of *Mstn* attenuates STZ-induced ROS and AOE production. Representative graph showing mRNA expression of TNF-α (A) and *Nox1* (B) in Gas muscle from C and STZ groups of WT and Mstn^{-/-} mice. (C) Lipid peroxidation was determined in Quad muscle of WT-C, WT-STZ, Mstn^{-/-}-C and Mstn^{-/-}-STZ mice. The values are given as nmol of MDA/100 mg of protein. Representative graph showing mRNA expression of *Sod1* (D) and *Cat* (E) in Gas muscle. Enzyme assays were performed on protein lysates from Quad muscle for SOD (F) and GSR (G); *p<0.05, **p<0.01 when compared to WT-C muscle; ^p<0.05, ^^p<0.01 when compared to Mstn^{-/-}-C muscle (n=7).

5.3.5 Absence of Mstn rescued STZ-induced decrease in JNK and ERK MAPK signaling

The results establish that STZ-induced Mstn is able to increase ROS production in skeletal muscle via TNF- α and Nox. Next, I wanted to investigate if Mstn inactivation helps to rescue the decreased MAPK signaling involved in ROS production upon STZ treatment (as shown by Hulmi *et al.* 2012). Western blot analysis for various molecules involved in MAPK pathways was performed and as shown in Figure 5.7, results revealed that p-p38 MAPK, p-JNK and p-ERK1/2 levels were decreased in WT-STZ muscles (Lane 2) when compared to WT-C muscles (Lane 1). p-p38 MAPK was significantly decreased in Mstn^{-/-}-STZ muscle (Lane 4), however, p-JNK, JNK, p-ERK1/2 and ERK1/2 levels were significantly higher in both Mstn^{-/-} groups (Lanes 3 and 4) when compared to WT groups (Lane 1 and 2) (Figure 5.7). These results suggest that absence of Mstn can rescue STZ-induced decrease in JNK and ERK MAPK signaling.

Figure 5.7 Absence of *Mstn* rescued STZ-induced changes in MAPK signaling. Western blot analysis showing protein levels of p-p38 MAPK, p38 α/β, p-JNK, JNK, p-ERK1/2 and ERK1/2 in Gas muscle from WT-C (Lane 1), WT-STZ (Lane 2), Mstn^{-/-}-C (Lane 3) and Mstn^{-/-}-STZ (Lane 4) mice. GAPDH was used as an internal control for equal protein loading on the gel (n=5).

5.3.6 Absence of Mstn abrogates STZ-induced changes in p63 and REDD1 signaling

STZ-induced ROS has been implicated in DNA damage, thus it was investigated if STZ-induced Mstn can cause DNA damage in skeletal muscle. Based on previous reports (Simone *et al.* 2008; Hulmi *et al.* 2012), Western blot analysis (Figure 5.8A) for various genes involved in DNA damage and repair was performed and results revealed that STZ treatment in WT mice (Lane 2) reduced REDD1 and p63 levels and increased OGG1 levels, significantly. REDD1 and p63 levels were elevated and OGG1 levels were reduced in Mstn^{-/-}-C muscles (Lane 3) when compared to WT-C muscles (Lane 1). Upon STZ treatment in Mstn^{-/-} mice (Lane 4), a slight increase in REDD1 and no change in OGG1 levels were observed when compared to Mstn^{-/-}-C group (Lane 3). A decrease in p63 levels were observed in Mstn^{-/-}-STZ muscles (Lane 4), which were comparable to WT-C muscles (Lane 1) (Figure 5.8A). No change was observed in p53 levels in WT muscles (Lanes 1 and 2); slightly higher levels of p53 were observed in Mstn^{-/-}-C muscles (Lane 3) (Figure 5.8A). To further confirm that DNA damage was induced by STZ and reduced in the absence of Mstn, IHC for REDD1 and OGG1 was performed on cryosections of TA muscle. Subsequent analysis revealed that REDD1 staining was slightly reduced in WT-STZ muscle sections when compared to WT-C sections (Figure 5.8B (i)), while the staining was higher in both Mstn^{-/-}-C and -STZ sections (Figure 5.8B (ii)). STZ-treated WT muscle showed increased OGG1 staining, while it was similar to WT-C muscle (Figure 5.8C (i)) in Mstn^{-/-}-C and STZ muscle sections (Figure 5.8C (ii)). These results indicated that STZ-induced changes in p63/REDD1 signaling are partially rescued in the absence of Mstn.

Figure 5.8 Absence of *Mstn* abrogates STZ-induced changes in p63 and REDD1 signaling. (A) Representative Western blot showing REDD1, OGG1, p53 and p63 protein levels in WT-C (Lane 1), WT-STZ (Lane 2), Mstn^{-/-}-C (Lane 3) and Mstn^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as an internal control for equal protein loading on the gel (n=5). IHC for REDD1 (B) and OGG1 (C) was performed on cryosections of TA muscle isolated from WT-C and WT-STZ (i) and Mstn^{-/-}-C and Mstn^{-/-}-STZ (ii) mice. The fluorescence was viewed under a Leica upright microscope and images were taken at 5X magnification. Increased fluorescence (green) indicates increase in expression of REDD1 (B) or OGG1 (C); scale bar represents 100 μ m (n=3).

5.3.7 STZ-induced DNA damage in proliferating myoblasts is attenuated by antagonizing Mstn

Next, the effect of STZ and/or Mstn on DNA damage *in vitro* was investigated and also Mstn was antagonized to determine whether inhibition of Mstn can rescue the muscle cells from DNA damage. In order to block the effect of Mstn *in vitro*, Ant1, an antagonist of Mstn, was used (Siriett *et al.* 2007). Firstly, to confirm that STZ treatment causes ROS production in C2C12 myoblasts (as shown by Johnston *et al.* 2007), ROS levels were analyzed in proliferating C2C12 myoblasts treated with STZ1 using the fluorescent probe, CM-H2DCFDA. The results showed that ROS production was increased upon STZ1 treatment, as shown by increased fluorescence (Figure 5.9A). To determine if Ant1 can block Mstn upon STZ treatment, C2C12 myoblasts were pre-treated with Ant1 followed by treatment with STZ1 and a proliferation assay was performed. As shown in Figure 5.9B, STZ1 significantly decreased proliferation of C2C12 myoblasts which was rescued by Ant1 treatment. Hence, Ant1 was used in this study to antagonize Mstn in myoblasts during proliferation.

To determine the effect of STZ and/or Mstn on DNA damage in myoblast cell culture system, ICC for 8-oxo-dG, a sensitive marker of ROS-induced DNA damage, was performed on proliferating C2C12 myoblasts treated with STZ1 or CMM1 with or without Ant1 pre-treatment. The intensity of 8-oxo-dG staining was increased upon STZ1 (Figure 5.10A) and CMM1 (Figure 5.10B) treatment, while Ant1 pre-treatment reduced the staining intensity. In addition, primary myoblasts isolated from WT and Mstn^{-/-} mice were treated with two different concentrations of STZ or CMM during proliferation and 8-oxo-dG ICC was performed. WT primary myoblasts were also pre-treated with Ant1 before STZ or CMM treatment. The results revealed that STZ (Figure 5.11) and CMM (Figure 5.12) increased DNA damage in a dose-dependent manner and

Ant1 pre-treatment was able to protect WT myoblasts from DNA damage, even upon treatment with higher concentrations of STZ (STZ2) or CMM (CMM2). In Mstn^{-/-} myoblasts, there was no change in the intensity of 8-oxo-dG staining upon treatment with two different concentrations of STZ (Figure 5.11) or CMM (Figure 5.12). These results indicated that STZ and Mstn can induce DNA damage in myoblasts which can be attenuated by inhibition of Mstn.

Figure 5.9 STZ induces ROS in proliferating C2C12 myoblasts. (A) ROS production was measured in proliferating C2C12 myoblasts treated with STZ1 in Permaox chamber slides using the CM-H2DCFDA fluorescent probe. Representative images showing ROS production at 48 hours during proliferation in untreated and STZ1-treated C2C12 myoblasts. The fluorescence was viewed under a Leica upright microscope and images were taken at 10X magnification. Increased fluorescence (green) intensity is directly proportional to increased ROS production in myoblasts; scale bar represents 100 μ m (n=2). (B) C2C12 myoblasts were allowed to proliferate for 48 hours with or without STZ1 treatment and with or without pre-treatment of Ant1 for one hour followed by STZ1 treatment. The proliferation rate was determined using methylene blue assay. Representative graph showing increase or decrease in proliferation following various treatments (* $p < 0.05$, ** $p < 0.01$ when compared to untreated myoblasts; ^ $p < 0.05$ when compared to STZ1-treated myoblasts) (n=2).

Figure 5.10 *STZ-induced increase in 8-oxo-dG levels in proliferating C2C12 myoblasts is attenuated by antagonizing Mstn.* (A) ICC was performed for 8-oxo-dG on C2C12 myoblasts treated for 48 hours during proliferation with or without STZ1 and with or without pre-treatment of Ant1 for one hour followed by STZ1 treatment. The fluorescence for 8-oxo-dG (green) and DAPI (blue) was viewed under a Leica upright microscope and images were taken at 10X magnification. Increased fluorescence is directly proportional to increased DNA damage; scale bar represents 100 μ m (n=3). (B) 8-oxo-dG immunostaining showed increased DNA damage shown by increased fluorescence in C2C12 myoblasts treated for 48 hours during proliferation with CCM, CMM1, Ant1+CCM or Ant1+CMM1. Images were taken as mentioned above in (A); scale bar represents 100 μ m (n=3).

Figure 5.11 *STZ-induced increase in 8-oxo-dG levels in proliferating primary myoblasts is attenuated by inhibition/inactivation of Mstn.* Immunostaining for 8-oxo-dG was performed on WT and Mstn^{-/-} primary myoblasts treated for 48 hours with STZ1, STZ2, Ant1 alone, Ant1+STZ1 or Ant1+STZ2. Representative images showing the increase or decrease in 8-oxo-dG staining (8-oxo-dG-green; DAPI-blue). Images were taken as mentioned above in Figure 5.10A; scale bar represents 100 μ m (n=2).

Figure 5.12 *CMM increased 8-oxo-dG levels in proliferating primary myoblasts.* Immunostaining for 8-oxo-dG was performed on WT and Mstn^{-/-} primary myoblasts treated for 48 hours with CCM, CMM1, CMM2, Ant1+CCM, Ant1+CMM1 or Ant1+CMM2. Representative images showing the increase or decrease in 8-oxo-dG staining (8-oxo-dG-green; DAPI-blue). Images were taken as mentioned above in Figure 5.10A; scale bar represents 100 μ m (n=2).

5.3.8 STZ and CMM cause SSB and DSB in DNA in proliferating myoblasts

Since the results in this Chapter showed that STZ and Mstn can induce DNA damage in myoblasts, comet assay was performed on C2C12 myoblasts treated with STZ1 during proliferation to detect SSB and DSB in DNA. The arrows in Figure 5.13A (i) and (ii) indicate DNA fragmentation and damage, as detected by the increased comet tail length, in C2C12 myoblasts caused by STZ. Pre-treatment with Ant1 reduced the comet formation to the level similar to untreated myoblasts. STZ1 treatment on C2C12 myoblasts significantly increased comet length and comet height (Figure 5.13B (i) and 5.13C (i)), % DNA in tail (Figure 5.13B (iii) and 5.13C (iii)), tail length (Figure 5.13B (iv) and 5.13C (ii)), tail area (Figure 5.13B (iv)), tail moment and Olive moment (Figure 5.13B (v) and 5.13C (iv)). Ant1 pre-treatment significantly reduced the above mentioned parameters to the level similar to untreated myoblasts (Figure 5.13B (i), (iii), (iv), (v) and 5.13C (i), (ii), (iii), (iv)). STZ1-treated C2C12 myoblasts showed significant decrease in comet mean intensity (Figure 5.13B (ii)), head mean intensity (Figure 5.13B (ii) and 5.13C (ii)) and % DNA in head of the comets (Figure 5.13B (iii) and 5.13C (iii)), while Ant1 pre-treatment brought these levels back to the levels of untreated myoblasts.

Comet assay was also performed on C2C12 myoblasts treated with CMM1 and CMM2 during proliferation. CMM1 and CMM2 induced SSB in DNA, shown by the arrows indicated on Figure 5.14A (i), as detected by the alkaline lysis method, while only higher concentration of CMM i.e. CMM2 (indicated by arrows in Figure 5.14A (ii)) caused DSB in DNA, as detected by neutral lysis of cells. Pre-treatment with Ant1 reduced the comets formed by CMM1 or CMM2 treatment, similar to CCM-treated myoblasts. CMM1 and CMM2 treatment significantly increased comet length and comet height (Figure 5.14B (i) and 5.14C (i)), % DNA in tail (Figure 5.14B (iii)), tail

length (Figure 5.14B (iv) and 5.14C (ii)), tail area (Figure 5.14B (iv)), tail moment and Olive moment (Figure 5.14B (v)) upon alkaline lysis, while only CMM2 increased % DNA in tail (Figure 5.14C (iii)), tail moment and Olive moment (Figure 5.14C (iv)) upon neutral lysis. Ant1 significantly reduced the above mentioned parameters (Figure 5.14B (i), (iii), (iv), (v) and 5.14C (i), (ii), (iii), (iv)). CCM1- and CCM2-treated myoblasts showed significant decrease in comet mean intensity (Figure 5.14B (ii)), head mean intensity (Figure 5.14B (ii) and 5.14C (ii)) and % DNA in head (Figure 5.14B (iii)) upon alkaline lysis, while only CMM2 showed a decrease in % DNA in head of comet upon neutral lysis (Figure 5.14C (iii)). Ant1 pre-treatment brought the levels of these parameters back to levels similar to CCM-treated myoblasts. These data showed that lower concentration of Mstn can induce SSB and alkali labile sites in DNA, while higher concentration can induce DNA DSB and this phenomenon can be attenuated by antagonism of Mstn.

Figure 5.13 STZ treatment leads to SSB and DSB in DNA in proliferating myoblasts as detected by Comet assay. (A) Comet assay was performed by alkaline lysis method (i) and neutral lysis method (ii) on C2C12 myoblasts treated with STZ1, Ant1 or Ant1+STZ1 for 48 hours during proliferation. Representative images showing the head and tail of comets formed during various treatments, stained using PI. DNA fragmentation is observed by the formation of tail in the comet as indicated by the arrows. Comet images were taken using a Leica upright microscope (n=3). Quantitative analysis of various parameters of comet assay following alkaline lysis (B) and neutral lysis (C); comet length and comet height B (i) and C (i), comet mean intensity B (ii), head mean intensity B (ii) and C (ii), % DNA in head and % DNA in tail B (iii) and C (iii), tail length B (iv) and C (ii), tail area B (iv), tail moment and olive moment B (v) and C (iv). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to untreated myoblasts; ^^ $p < 0.01$, ^^^ $p < 0.001$, ^^^^ $p < 0.0001$ when compared to STZ1-treated myoblasts (n=3).

Figure 5.14 CMM treatment leads to SSB and DSB in DNA in proliferating myoblasts as detected by Comet assay. (A) Comet assay was performed by alkaline lysis method (i) and neutral lysis method (ii) on C2C12 myoblasts treated with CCM, CMM1, CMM2, Ant1+CCM, Ant1+CMM or Ant1+CMM2 for 48 hours during proliferation. Representative images showing the head and tail of comets formed during various treatments, stained using PI. DNA fragmentation is observed by the formation of tail in the comet as indicated by the arrows. Comet images were taken using a Leica upright microscope (n=3). Quantitative analysis of various parameters of comet assay following alkaline lysis (B) and neutral lysis (C); comet length and comet height B (i) and C (i), comet mean intensity B (ii), head mean intensity B (ii) and C (ii), % DNA in head and % DNA in tail B (iii) and C (iii), tail length B (iv) and C (ii), tail area B (iv), tail moment and olive moment B (v) and C (iv). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 when compared to CCM-treated myoblasts; ^p<0.05, ^^p<0.01, ^^^p<0.001, ^^^^p<0.0001 when compared to CMM1- or CMM2-treated myoblasts (n=3).

5.3.9 STZ treatment alters genes involved in DNA repair *in vivo*

In cells, DNA damage is followed by DNA repair, thus DNA repair system was analyzed in STZ-treated muscles. Western blot analysis for XRCC1, a protein involved in repair of SSB in DNA, and Histone H2A.X, involved in DNA DSB repair were performed on protein lysates from STZ-treated WT and *Mstn*^{-/-} muscles. The results indicated that XRCC1 protein levels were significantly reduced in WT-STZ muscles (Lane 2), while there was no change observed in *Mstn*^{-/-} muscles (Lanes 3 and 4), when compared to WT-C muscles (Lane 1) (Figures 5.15 (i) and (ii)). The level of phosphorylated Histone H2A.X was increased and total Histone H2A.X was correspondingly decreased in WT-STZ muscles (Lane 2) when compared to WT-C muscles (Lane 1) (Figures 5.15 (i) and (iii)). *Mstn*^{-/-}-C and *Mstn*^{-/-}-STZ muscles (Lanes 3 and 4, respectively) have higher levels of phosphorylated and total Histone H2A.X when compared to WT-C muscles (Lane 1) (Figures 5.15 (i) and (iii)). These results suggest that STZ treatment *in vivo* causes changes in DNA repair genes, while genetic inactivation of *Mstn* may offer protection of the muscle from DNA damage.

Figure 5.15 STZ treatment alters genes involved in DNA repair in vivo. (i) Representative gel showing protein levels and (ii) (iii) densitometric analysis of XRCC1, p-Histone H2A.X and H2A.X in WT-C (Lane 1), WT-STZ (Lane 2), Mstn^{-/-}-C (Lane 3) and Mstn^{-/-}-STZ (Lane 4) Gas muscle. GAPDH was used as loading control on the gel (n=5).

5.3.10 STZ and CMM alter p63/REDD1 signaling and DNA repair genes in proliferating primary myoblasts

To further confirm effect of STZ on p63 and REDD1 levels and to study the DNA repair genes, Western blot was performed on protein lysates from STZ-treated WT and *Mstn*^{-/-} primary myoblasts. Subsequent analysis (Figure 5.16A) revealed that STZ2 significantly decreased p63 levels in WT myoblasts while Ant1 pre-treatment partially rescued this decrease. No change was observed in p63 levels in *Mstn*^{-/-} myoblasts upon STZ treatment. Both concentrations of STZ (Lanes 2 and 3) caused decrease in REDD1 levels in WT primary myoblasts when compared to untreated myoblasts (Lane 1), while Ant1 pre-treatment (Lanes 4-6) rescued the decrease in REDD1 levels. In *Mstn*^{-/-} myoblasts, the decrease in REDD1 levels was lesser than STZ-treated WT myoblasts. p-Histone H2A.X levels were increased in Ant1 (Lane 4) and Ant1+STZ2 (Lane 6) treated WT myoblasts, however, total H2A.X was also increased in these myoblasts (Figure 5.16A). STZ treatment (Lanes 8 and 9) increased p-Histone H2A.X and total H2A.X levels in *Mstn*^{-/-} myoblasts when compared to untreated myoblasts (Lane 7). These data showed that STZ alters p63/REDD1 signaling and DNA repair genes in proliferating primary myoblasts.

To study changes in p63/REDD1 signaling and DNA repair genes upon *Mstn* treatment, Western blot analysis was performed and the results showed that p63 levels were decreased in CMM1 and CMM2 treated WT myoblasts in a dose-dependent manner (Figure 5.16B-Lanes 2 and 3), while Ant1 pre-treatment inhibited the decrease in p63 levels (Figure 5.16B-Lanes 4-6). Also, the decrease in p63 levels in *Mstn*^{-/-} myoblasts (Lane 9) was lesser than the decrease observed in WT myoblasts (Lanes 2 and 3). CMM2 treatment on WT myoblasts reduced REDD1 and p-Histone H2A.X levels (Lanes 2 and 3), while Ant1 pre-treatment rescued the levels (Lanes 4-6). CMM2

treatment on $Mstn^{-/-}$ myoblasts (Lane 9) increased p-Histone H2A.X and total H2A.X levels (Figure 5.16B). These results confirm that Mstn causes changes to p63/REDD1 signaling and DNA repair genes in primary myoblasts during proliferation.

Figure 5.16 *Changes in p63/REDD1 signaling and DNA repair genes occur in proliferating primary myoblasts upon STZ or CMM treatment.* (A) Western blot analysis of p63, REDD1, p-Histone H2A.X and H2A.X protein levels in 48 hour proliferating WT (Lanes 1-6) and Mstn^{-/-} (Lanes 7-9) primary myoblasts; Lanes 1 and 7-untreated, Lanes 2 and 8-STZ1-treated, Lanes 3 and 9-STZ2-treated, Lane 4-Ant1-treated, pre-treated for 1 hour with Ant1 followed by STZ1 (Lane 5) or STZ2 (Lane 6) treatment. GAPDH was used as an internal control for equal protein loading on the gel (n=2). (B) Representative Western blot showing protein levels of p63, REDD1, p-Histone H2A.X and H2A.X in 48 hour proliferating WT (Lanes 1-6) and Mstn^{-/-} (Lanes 7-9) primary myoblasts; Lanes 1 and 7-CCM-treated, Lanes 2 and 8-CMM1-treated, Lanes 3 and 9-CMM2-treated, Lane 4-Ant1-treated, pre-treated for 1 hour with Ant1 followed by CMM1 (Lane 5) or CMM2 (Lane 6) treatment. GAPDH was used as an internal control for equal protein loading on the gel (n=2).

5.3.11 Knockdown of Mstn by shRNA partially attenuates STZ- and CMM-induced DNA damage

To further prove that inhibition of Mstn is able to reduce STZ- and CMM-induced DNA damage, C2C12 myoblasts were transfected with shMstn and treated with STZ or CMM during proliferation. First, to confirm that shMstn is able to knockdown the gene efficiently, Western blot for Mstn was performed on C2C12 myoblasts transfected with shMstn. Results showed that indeed Mstn levels were significantly reduced in shMstn transfected myoblasts (Lane 3) (Figure 5.17A). Subsequently, Western blot analysis for p63, REDD1, p-Histone H2A.X and H2A.X (Figure 5.17B) showed that STZ (STZ1 and STZ2) treatment reduced p63 and REDD1 levels significantly in empty vector (Lanes 1-3) and scrambled shRNA (Lanes 4-6) transfected myoblasts, while only STZ2 treatment caused decrease in REDD1 levels in shMstn transfected myoblasts (Lanes 9). STZ treatment elevated p-Histone H2A.X levels in empty vector (Lanes 2 and 3), scrambled shRNA (Lanes 5 and 6) and shMstn (Lanes 8 and 9) transfected myoblasts in a dose dependent manner, however, the increase was significantly higher in shMstn (Lanes 8 and 9) transfected myoblasts (Figure 5.17B)

As shown in Figure 5.17C, p63 levels were reduced upon CMM treatment in a dose-dependent manner (Lanes 1-9). REDD1 levels were reduced in CMM-treated empty vector (Lanes 2 and 3) and scrambled shRNA (Lanes 5 and 6) transfected myoblasts while only CMM2 showed decrease in REDD1 levels in shMstn transfected myoblasts (Lane 9) (Figure 5.17C). CMM1 treatment decreased the levels of p-Histone H2A.X (Lanes 1-9), while CMM2 treatment increased the levels of p-Histone H2A.X and H2A.X in shMstn transfected myoblasts (Lanes 7-9) (Figure 5.17C). These data confirmed that knockdown of Mstn by shRNA is able to partially attenuate STZ- and CMM-induced DNA damage.

Figure 5.17 Knockdown of Mstn by shRNA partially attenuates STZ- and CMM-induced DNA damage. (A) Western blot analysis showing protein level of Mstn in 48 hours proliferating C2C12 myoblasts transfected with either empty vector control (pGFP-V-RS) (Lane 1), scrambled shRNA (Lane 2) or shMstn vector (Lane 3). GAPDH was used as internal control on the gel (n=2). (B) Representative gel showing protein levels of p63, REDD1, p-Histone H2A.X and H2A.X in 48 hours proliferating C2C12 myoblasts transfected with empty vector control (pGFP-V-RS) (Lanes 1-3), scrambled shRNA (Lanes 4-6) or shMstn vector (Lanes 7-9) and treated with two different concentrations of STZ; Lanes 1, 4, 7-untreated, Lanes 2, 5, 8-STZ1-treated, Lanes 3, 6, 9-STZ2-treated. GAPDH was used as loading control on the gel (n=2). (C) Western blot showing p63, REDD1, p-Histone H2A.X and H2A.X protein levels in 48 hours proliferating C2C12 myoblasts transfected with empty vector control (pGFP-V-RS) (Lanes 1-3), scrambled shRNA (Lanes 4-6) or shMstn vector (Lanes 7-9) and treated with two different concentrations of CMM; Lanes 1, 4, 7-CCM-treated, Lanes 2, 5, 8-CMM1-treated, Lanes 3, 6, 9-CMM2-treated. GAPDH was used as internal loading control on the gel (n=2).

5.4 Discussion

Type 1 diabetes is characterized with lack of insulin, hyperglycemia and oxidative stress. In this Chapter, the mechanisms by which hypoinsulinemia leads to an increase in Mstn expression through activation of FoxA2 transcription factor is elucidated. Enhanced Mstn contributes to ROS which causes DNA damage mediated by p63/REDD1 in the muscle of diabetic mice.

Over the recent years, various studies have shown that Mstn expression is increased in STZ treated mice (Chen *et al.* 2009; Hulmi *et al.* 2012; Dutra *et al.* 2012), however the mechanism behind the increase in Mstn levels was not identified. Since the results presented here showed an increase in *Mstn* RNA and protein levels, it was surmised that Mstn could be regulated at the transcriptional level during hyperglycemic conditions. Indeed, *in silico* analysis of Mstn 5'-upregulatory sequences revealed the presence of a binding site for FoxA2, a transcription factor regulated by insulin. Previous studies by Wolfrum *et al.* (2004) demonstrated that in the absence of insulin, FoxA2 activity and level was enhanced. The results also indicate that in the muscle from STZ treated WT mice which have become insulin-deficient, the FoxA2 expression and protein was up-regulated (Figure 5.5A (i) and (ii), respectively). Further confirmation of increased FoxA2 levels and activity came from the bandshift experiments (EMSA) and Mstn promoter reporter assays. The ChIP results clearly indicated the binding of endogenous FoxA2 on the Mstn promoter transfected in HepG2 cells. These results for the first time demonstrate that activation of FoxA2 due to hypoinsulinemia induces Mstn expression.

Insulin is known to signal via IRS-1 and Akt. Previously it has been reported that insulin inhibits FoxA2 activity by phosphorylation mediated by Akt (Wolfrum *et al.* 2004; Puigserver and Rodgers 2006). Activation of Akt is affected by high glucose levels as shown in Figure 5.4B (Lanes 1 and 2). However, in spite of developing overt diabetes,

Akt signaling was not inhibited in *Mstn*^{-/-} mice (Figure 5.4B-Lanes 3 and 4) possibly due to elevated IGF-1 signaling in *Mstn*^{-/-} mice (Williams *et al.* 2011). Furthermore, the levels of FoxA2 were significantly lower in *Mstn*^{-/-} muscle even after treatment with STZ which could be again due to enhanced IGF-1 signaling in these mice.

In congruence with earlier findings (Aragno *et al.* 2004; Mastrocola *et al.* 2008), STZ induced ROS (and thus AOE) in skeletal muscles of WT mice via TNF- α , *Nox1* and lipid peroxidation (Figures 5.6A to 5.6G). The results in this Chapter also revealed no change in these parameters in *Mstn*^{-/-}-STZ mice indicating that in the absence of *Mstn* these mice resisted the induction of ROS by STZ. Interestingly, even though TNF- α and *Nox1* are up-regulated, STZ-induced skeletal muscle atrophy is not found to be associated with NF- κ B canonical cascade activation (Kelleher *et al.* 2010). Hence, ROS generation during type 1 diabetes may be through other mechanisms including dysregulation of MAPK pathways as shown in Figure 5.7. However, genetic inactivation of *Mstn* is able to rescue the STZ-induced JNK and ERK MAPK inhibition in muscle. MAPK signaling is involved in normal muscle growth and dysregulation of its components will lead to impaired muscle differentiation and development. JNK and ERK MAPK signaling are involved in insulin-mediated metabolism and are required for a diverse array of extracellular stimuli and signal transduction. Hence appropriate regulation of JNK and ERK MAPK signaling in the absence of *Mstn* even upon STZ treatment will help the muscle cells to respond to intermittent stress and inflammatory cytokines.

ROS are potent molecules that are toxic to the cells; if uncontrolled, lead to protein degradation as well as DNA damage. It is evident that STZ-induced ROS can lead to DNA damage (Imaeda *et al.* 2002; Blasiak *et al.* 2004). In this Chapter, it is shown for the first time that significantly higher levels of *Mstn* induce ROS which inflict DNA

damage and inhibit DNA repair system. Previously, oxidative DNA damage during STZ-induced type 1 diabetes was observed with a concomitant increase in oxidized DNA bases like 8-oxo-dG in the liver of STZ treated diabetic rats (Andican and Burcak 2005). Furthermore, 8-oxo-dG content was found to be higher in the skeletal muscle of diabetic patients (Suzuki *et al.* 1999). Similarly, occurrence of higher levels of OGG1 has been reported in pancreas of diabetic patients which was correlated with DNA damage (Tyrberg *et al.* 2002). Consistently, the results demonstrate that both STZ and Mstn can cause an increase in 8-oxo-dG and OGG1 indicative of DNA damage and repair respectively and inhibition of Mstn partially reversed these changes.

REDD1 is known to regulate cellular ROS through p63 and hence plays an important role in stress response and modulation of growth factors (Ellisen *et al.* 2002). Here, the results have established Mstn-mediated down-regulation of REDD1 and p63 levels via ROS production. However, recently, Hulmi *et al.* reported an increase in p63 and REDD1 levels in STZ treated mice at week 1 of the STZ treatment which could be due to higher dose of STZ (180 mg/kg) and/or the type of muscles used in the study (Hulmi *et al.* 2012). The basal levels of REDD1 and p63 were higher in Mstn^{-/-} mice probably in part due to higher levels of AMP activated protein kinase (AMPK) in these mice (Zhang *et al.* 2011) because AMPK was shown to stimulate REDD1 *in vitro* (Sofer *et al.* 2005). Another factor could be the elevated IGF-1 signaling in Mstn^{-/-} mice since IGF-1 treatment was reported to up-regulate the protein levels of REDD1 in Gas muscle and in C2C12 myotubes (Frost *et al.* 2009). Nevertheless, deficiency of p63 is associated with inefficient DNA repair (Lin *et al.* 2009) as well as with reduced transcription of REDD1 (Ellisen *et al.* 2002). Taken together, the results presented here suggest that inactivation/inhibition of Mstn can protect the cells against DNA damage by regulating DNA damage/repair mechanisms.

At the cellular level, both 8-oxo-dG staining and comet assay showed that STZ and CMM treatments increased DNA damage in the myoblasts while Ant1 pre-treatment prevented the DNA damage in the myoblasts. Interestingly, both concentrations of CMM were able to induce DNA SSB, while only a higher concentration of CMM (CMM2) was able to induce DSB in DNA. Pre-treatment with Ant1 reduced the DNA fragmentation even upon treatment with higher concentration of CMM suggesting inhibition of Mstn resisted DNA damage.

Furthermore, XRCC1, involved in SSB repair, was decreased in WT-STZ muscle (Figures 5.15 (i) and (ii)) which indicates that in the diabetic condition due to enhanced ROS, an increase in DNA SSB would occur and the repair would be impaired due to lower levels of the repair enzyme. SSB arise frequently from direct attack by intracellular metabolites and from spontaneous DNA decay. When only one of the two strands of a double helix has a defect, the other strand can act as a template to correct the damaged strand. In order to repair damage to one of the two strands of DNA, a number of excision repair mechanisms including base excision repair and nucleotide excision repair exist. If SSB are not repaired rapidly or appropriately, they can cause a serious threat to cell survival. XRCC1 is a critical enzyme involved in various stages of DNA repair via its interactions with other repair proteins and an increase in ROS is known to down-regulate XRCC1 which results in inefficient SSB repair and build-up of repair intermediate products (Narciso *et al.* 2007). In the *Mstn*^{-/-} mice, the levels of XRCC1 remain unchanged even after STZ treatment suggesting that SSB repair mechanism was not compromised in these muscles.

DSB, in which both strands of the double helix are damaged, pose a greater hazard to the cell because they can lead to genome rearrangements. DSB can be repaired by either non-homologous end-joining or homologous recombination. Defects in the repair of

DNA damage are implicated in increased genetic instability and a variety of diseases. Errors in DSB repair may cause cancer-associated translocations. Genes involved in DSB repair are H2A.X, XRCC2, and XRCC3. Histone H2A.X, involved in DSB repair, was activated upon STZ treatment in WT mice thus indicating the response of the cell survival system against the DNA damage due to increased oxidative stress in these mice. The basal levels of both H2A.X and p-H2A.X in *Mstn*^{-/-} muscles are higher than WT muscles, which remain unaltered even after STZ treatment. It is difficult to predict the significance of this result however a recent report by Turinetto *et al.* suggests a role of p-H2A.X in self-renewal that is independent of DNA damage response function (Turinetto *et al.* 2012). Overall, the findings suggest that deletion/inhibition of *Mstn* abrogates DNA damage in the diabetic muscle/cells.

Our results also showed that STZ administration in *Mstn*^{-/-} mice resulted in high glucose levels and greater decrease in body and muscle weights initially when compared to the WT mice. This anomaly could be due to higher gluconeogenesis in these mice. In fact, the expression of genes involved in gluconeogenesis like *PEPCK* and *G6P* was elevated in the *Mstn*^{-/-} mice relative to the WT mice in agreement with Wang *et al.* (2012). As demonstrated previously, *Mstn*^{-/-} mice have reduced amount of adipose tissue (Zhang *et al.* 2012) indicating that muscle would be the predominant source to provide precursors for the enhanced gluconeogenesis following STZ treatment thus accounting for more muscle loss.

Finally, I propose a model, whereby it is hypothesize that hypoinsulinemia during type 1 diabetes induces *Mstn* via FoxA2 and excess *Mstn* in turn induces ROS via TNF- α and Nox in skeletal muscle (Figure 5.18). The ROS levels can be further induced directly by hyperglycemia which in turn induces *Mstn*. The exaggerated ROS levels induce DNA damage and inhibit DNA repair pathways via p63/REDD1 signaling (Figure 5.18).

Sandhya Sriram performed majority of the experiments and analyzed the data. Subha Subramanian helped in performing some of the experiments (animal work-injection of STZ, monitoring of body weights and blood glucose, isolation of primary myoblasts, EMSA, comet assay). Prasanna Kumar Juvvuna helped in performing the ChIP assay.

Figure 5.18 *Proposed mechanism for Mstn-induced DNA damage in skeletal muscle in a STZ-induced type 1 diabetes model.* Single high-dose injection of STZ induces hypoinsulinemia and hence hyperglycemia leading to type 1 diabetes (T1D). Hypoinsulinemia induces Mstn via FoxA2 and the excess Mstn in turn induces ROS via TNF- α and Nox. The high levels of glucose also can directly induce ROS production via facilitated diffusion/active transport. The exaggerated ROS production induces DNA fragmentation by inhibiting p63 and REDD1 signaling, resulting in DNA damage during type 1 diabetes leading to skeletal muscle wasting eventually.

CHAPTER 6

FINAL DISCUSSION

Oxidative stress due to excessive reactive oxygen species (ROS) levels causes extensive protein and DNA damage and if not controlled, leads to cell death and eventually death of the organism during various disease conditions like cancer, AIDS, and diabetes. One of the most metabolically active tissues in the body is skeletal muscle. Skeletal muscle is extremely susceptible to oxidative stress-induced muscle wasting especially due to contractions. During aging, skeletal muscle is one of the most affected tissues. There is a gradual decrease in the ability to maintain skeletal muscle mass and function during aging. However, the exact signaling mechanism behind muscle wasting due to excessive ROS is not known. Over the recent years, Myostatin (Mstn), a potent negative regulator of muscle growth and development, has been identified as an important inducer of muscle wasting. However, it is not known if a relationship exists between Mstn and ROS and whether Mstn can induce ROS in skeletal muscle leading to wasting. Thus the overall aim of this thesis was to investigate if Mstn can induce ROS in skeletal muscle under various muscle wasting conditions *in vitro* and *in vivo*.

Under physiological conditions, ROS are crucial mediators of intracellular signaling cascades and are most important for maintaining the innate immunity system. ROS are generated as by-products of cellular metabolism and are involved in almost all the cellular processes. ROS are diverse in nature and abundantly produced in all the cells. Mammalian cells have the amazing capacity to modulate redox levels by regulating oxidant-antioxidant balance. When excessive cellular production of ROS overwhelms its antioxidant capacity, damage to macromolecules such as proteins, lipids and DNA may occur. Thus ROS are a crucial parameter in determining normal cellular processes and a disease condition.

Skeletal muscle is a highly metabolic tissue and as a consequence, it can be affected by excessive oxidative stress. Numerous signaling molecules are involved in oxidative stress-induced muscle wasting including TNF- α (Buck and Chojkier 1996; Fortunati *et al.* 2007; Eley *et al.* 2008), NADPH oxidase (Lambertucci *et al.* 2008; Shkryl *et al.* 2009), NF- κ B (Russell *et al.* 2007; Trendelenburg *et al.* 2012), MAPKs (McClung *et al.* 2010; Li *et al.* 2005), IL-6, JAKs, STATs (Bonetto *et al.* 2011) and xanthine oxidase (Baudry *et al.* 2008; Ryan *et al.* 2011). Treatment of muscle cells with antioxidants like ascorbic acid attenuates the effects of oxidative stress hence, as various studies suggest, administration of antioxidants would definitely reduce the hazards caused by excessive ROS levels. However, it should be kept in mind that excess of antioxidants can also cause damage to the cells as minimal ROS levels are required for normal cell survival and metabolism.

An important growth and differentiation factor of skeletal muscle, Mstn, was discovered in 1997. Since its discovery, our laboratory has performed extensive research to identify various molecular mechanisms behind Mstn-induced muscle wasting (Langley *et al.* 2002; McFarlane *et al.* 2006; Ge *et al.* 2011; McFarlane *et al.* 2011; Lokireddy *et al.* 2011a; Lokireddy *et al.* 2011b; Ge *et al.* 2012; Lokireddy *et al.* 2012a; Lokireddy *et al.* 2012b). Initially, a significant amount of research showed that lack of Mstn or mutation in the *Myostatin* gene leads to increased musculature. In the recent past, the role of Mstn in muscle wasting has been studied especially during cancer cachexia, AIDS, COPD, diabetes and aging as over expression of Mstn or increased level of Mstn results in skeletal muscle wasting.

In this thesis I have characterized a mechanism through which Mstn induces muscle wasting via induction of oxidative stress and proved that Mstn is a potent pro-oxidant. I have also shown that if Mstn is inactivation or inhibited, resistance to oxidative stress is

observed due to high basal levels of AOE_s. However, the increase in AOE_s can be attributed to the ‘double muscling’ seen in the Mstn^{-/-} mice in which Mstn is genetically inactivated. But the lower TNF- α , *Nox1* and NF- κ B levels, decreased ubiquitination and increased protein synthesis would indicate that oxidative stress is indeed reduced in these mice.

In this thesis, I have shown that Mstn induces oxidative stress via TNF- α and NF- κ B in a ‘feed forward’ loop to sustain the increased ROS production. NF- κ B, a very important transcription factor, involved in almost all cellular processes, has been shown to regulate skeletal myogenesis independent of Mstn (Bakkar *et al.* 2005). But the results presented in this thesis prove otherwise. It must be kept in mind that NF- κ B signaling is very transient and has to be studied at the appropriate time point. Mstn signaling of NF- κ B differs during proliferation and differentiation of myoblasts. As shown by Bakkar *et al.*, transient activation of NF- κ B is indeed observed on Mstn treatment (Bakkar *et al.* 2005). I have also shown that Mstn induces NF- κ B translocation and activation only at certain time points during proliferation of myoblasts leading to oxidative stress via TNF- α and Nox.

Genetic inactivation of Mstn reduced expression of TNF- α and *Nox1* and basal NF- κ B levels in skeletal muscle and this phenomenon was observed even on aging. Aberrant activation of NF- κ B leads to muscle wasting, but decreased levels of NF- κ B can also affect various processes including inflammation. These results indicate that even though Mstn is a negative regulator of muscle growth, it is present through evolution for a reason. Only excessive Mstn in the system leads to negative regulation. Basal Mstn levels are required for normal proliferation and differentiation of myoblasts. I speculate that basal Mstn levels are also needed to maintain the oxidant-antioxidant balance in the

system. This makes Mstn a very interesting molecule to be studied further especially with relationship to inflammation, immunity and wound healing.

Therefore, further studies were carried out to find out the exact signaling of NF- κ B by Mstn. For this, mice lacking Smad3 were used since Smad3 is a crucial downstream target of Mstn signaling. Results from our laboratory showed that Smad3^{-/-} mice have defective myogenesis, pronounced muscular atrophy (Ge *et al.* 2011) and impaired regeneration (Ge *et al.* 2012). Interestingly, these mice showed increased *Mstn* expression despite the absence of Smad3 and the excessive muscle wasting seen in these mice is attributed to increased Mstn. This confirmed that Mstn can signal via other transcription factors like Smad2 and molecules involved in various signaling pathways. The increased Mstn levels in the Smad3^{-/-} mice led to the hypothesis that these mice have excessive ROS leading to muscle atrophy. The results presented in this thesis confirm this hypothesis and also show that Mstn can induce ROS via MAPK and JAK-STAT signaling pathway through TNF- α , Nox and XO. The dysregulation of JAK-STAT pathway in the absence of Smad3 and Mstn is very interesting as my results for the first time show a relationship between Mstn and JAKs and STATs. Further studies need to be performed to understand the biological significance and the role of Mstn in this pathway by looking into various other upstream and downstream signaling molecules involved in the IL-6/JAK-STAT pathway.

An interesting result obtained from this study was that Mstn is unable to induce NF- κ B activation in the absence of Smad3 and basal NF- κ B levels are reduced in the absence of Smad2. However, further studies have to be performed on the role of Smad2 in NF- κ B signaling. Together these results show that Mstn signaling is essential for maintaining muscle mass and muscle growth. However, if the threshold is exceeded, multiple signaling pathways can cause severe muscle wasting. It would be interesting and at the

same time extremely difficult to study the exact threshold levels of Mstn, but if found, it would open doors to effective therapies for cancer, diabetes and sarcopenia.

The role of Mstn as a pro-oxidant which induces muscle wasting via a cascade of signaling pathways is now well established. Further, I wanted to study if inhibition/inactivation of Mstn can really help overcome or rather improve muscle wasting during a severe and widely prevalent disease condition like type 1 diabetes. Also, results from our laboratory and my studies show that Mstn causes increased protein degradation via carbonylation and ubiquitination. This led me to investigate if Mstn can induce DNA damage as well in skeletal muscle. Type 1 diabetes model was especially used for this study as it has been shown that there is extensive DNA damage induced by hyperglycemia (Simone *et al.* 2008; Hulmi *et al.* 2012) and also Mstn levels are increased in STZ-induced type 1 diabetic mice (Chen *et al.* 2009; Hulmi *et al.* 2012; Dutra *et al.* 2012). Hence, this model was used to investigate if Mstn is capable of inducing DNA damage and whether in the absence of Mstn, skeletal muscle is protected against such damage. As presented in this thesis, Mstn is indeed able to induce SSB and DSB in DNA while its absence/inhibition is partially able to rescue the muscle cells from DNA damage majorly by up-regulating DNA repair genes. The results demonstrate that both STZ and Mstn can cause an increase in DNA damage and that inhibition of Mstn partially reversed these changes. However, one report also shows that STZ can directly cause DNA damage to C2C12 myoblasts thus causing G2/M cell cycle arrest (Johnston *et al.* 2007). Hence, further experiments have to be carried out to distinguish the DNA damage induced due to high glucose/low insulin levels during type 1 diabetes and those caused by STZ directly. A genetically modified non-obese diabetic mouse model can be used to establish our proposed mechanism.

Type 1 diabetes model was also used to investigate the mechanism by which high glucose levels induce Mstn. The results show that Mstn is induced by high glucose levels via FoxA2-mediated activation of the Mstn promoter, which is insulin-independent. FoxA2, a very interesting transcription factor, is involved in insulin signaling and maintaining glucose homeostasis. This is one of the first evidences of glucose-mediated Mstn regulation. The decrease of FoxA2 levels in the Mstn^{-/-} mice is justified by the increased Akt signaling and insulin sensitivity in these mice. To expand on this research, a set of experiments using FoxA2 over-expression vectors and knockdown of FoxA2 will have to be performed. Also, regulation of Mstn and FoxA2 can be studied in relation to insulin sensitivity and insulin resistance, as Mstn has been shown to be involved in insulin signaling.

In conclusion, Mstn is a pro-oxidant that is capable of inducing muscle wasting through a complex cascade of signaling pathways which regulate protein synthesis, degradation and DNA damage (Figure 6.1). Keeping this in mind, Mstn can be targeted and inactivated, especially in patients suffering from severe and chronic diseases like cancer and diabetes. Mstn can be inactivated/inhibited by using soluble ActRIIB, antibody against Mstn or Mstn antagonist, Ant1. Clinical trials involving ACVR2B, a monoclonal antibody that neutralizes Mstn, was conducted by Merck and Co. However, since ACVR2B can block various growth factors other than Mstn, it is not known as to how many other muscle-limiting proteins may be affected due to this potent agent (Samson 2007). Thus, methods to inhibit Mstn signaling with least side/after effects may help improve quality of life during cancer and aging.

Figure 6.1 *Mstn is a pro-oxidant that induces skeletal muscle wasting through multiple signaling pathways.* Mstn through TNF- α induces the activation of NF- κ B signaling to generate ROS via NADPH oxidase. The activation of NF- κ B by Mstn requires Smad3. In the absence of Smad3, Mstn causes increased ROS production via p38 and ERK MAPK pathways through TNF- α induced Nox. A crosstalk between ERK MAPK and IL-6 induced JAK-STAT signaling pathway also generates ROS via Xanthine oxidase. In the absence of Smad3, the excessive ROS leads to increased MuRF1 levels via CHOP. Hence exaggerated ROS levels induced by Mstn can lead to increased protein degradation and decreased protein synthesis via multiple mechanisms. Mstn-induced ROS can also induce DNA damage via p63/REDD1 pathway. Increased ROS can in turn induce Mstn in a ‘feed forward’ manner via TNF- α and NF- κ B (as shown in Figure 3.15). Hypoinsulinemia can also induce Mstn levels via FoxA2 leading to ROS-mediated DNA damage (as shown in Figure 5.18). The sustained ROS production due to the ‘feed forward’ loop leads to skeletal muscle wasting.

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