Myostatin Induces DNA Damage in Skeletal Muscle of Streptozotocin-induced Type 1 Diabetic Mice*

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One of the features of uncontrolled type 1 diabetes is 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 culminating in muscle atrophy are not fully known. Here, we show that in Streptozotocin-induced diabetic wild type mice, hypophosphorylation of Akt, resulted in activation of Foxa2 transcription factor in the muscle. Foxa2 transcriptionally up-regulated Myostatin, contributing to exaggerated oxidative stress leading to DNA damage via p63/REDD1 pathway in skeletal muscle of STZ-induced type 1 diabetic mice. Incubation with Foxa2 inhibitor to treated wild type mice. In Myostatin (Mstn) transgenic mice, STZ-induced Mstn levels were higher compared with wild type mice. These results, relatively less DNA damage was observed in Myostatin−/− mutants compared with streptozotocin treatment. Taken together, our results for the first time show the role of Foxa2 in Myostatin regulation of skeletal muscle in diabetic mice. Altogether, these results demonstrate the mechanism by which Myostatin contributes to DNA damage in skeletal muscle of the diabetic mice that would lead to myofiber degeneration.

Background: Uncontrolled type 1 diabetes leads to DNA damage and skeletal muscle atrophy.

Results: STZ-induced Foxa2 up-regulates Mstn leading to DNA damage via p63/REDD1 pathway in skeletal muscle.

Conclusion: Mstn is a target of Foxa2. Blocking Mstn can attenuate DNA damage in the diabetic muscle.

Significance: The findings reveal a mechanism of induction of Mstn and DNA damage during diabetes.

Type 1 diabetes is caused by lack of insulin secretion in the body due to pancreatic β cell death. 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 cellular ROS, especially hydroxyl (-OH) and superoxide (O2•−) can modify DNA by addition to deoxyguanosine (dG) in DNA to form 8-hydroxy-2′-deoxyguanosine (8-OHdG), a mutagenic DNA adduct and a marker for oxidative damage. 8-OHdG is a major base lesion present in type 1 diabetes (1). Additionally, DNA damage can be identified by 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 (2). 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 (3). 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 (4), has been identified to be involved in oxidative stress-induced DNA damage in skeletal muscle during chronic hypoxia (5).

Myostatin (Mstn), a growth and differentiation factor, has been associated closely with skeletal muscle wasting. Our laboratory has shown recently that Mstn can induce ROS and in turn Anti-oxidant enzymes in skeletal muscle through TNF-α and NADPH oxidase (Nox) in a feed-forward manner (6). 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) (7, 8, 9). Furthermore, Mstn expression was attenuated by insulin administration in STZ-induced type 1 diabetic mice (7). 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.

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Here, we present evidence that STZ-induced hyperglycemia up-regulated Mstn via Foxa2 transcription factor, which in turn induced ROS via TNF-α and Nox. The excessive ROS levels caused by high glucose and elevated Mstn levels led to p63/REDD1 regulated DNA damage. Using in vitro techniques, we demonstrate that Mstn induced single-strand and double-strand breaks 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.

**EXPERIMENTAL PROCEDURES**

**Animals**—7-week-old C57Bl/6 male mice (WT) were obtained from National University of Singapore-Centre for Animal Resources. Mstn−/− male mice (7-week-old) were obtained as previously described (6) and maintained at Nanyang Technological University 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, Singapore.

**Reagents and Proteins**—STZ was purchased from Sigma-Aldrich. Mstn protein containing conditioned medium was obtained from the Mstn expressing CHO cell line (10); control conditioned medium is denoted as CCM and conditioned medium containing Mstn is denoted as CMM in this study. To antagonize Mstn, we used Ant1, a C-terminal truncated protein which is a dominant negative (11). Ant1 was produced and purified as previously described (11).

**Induction of Type 1 Diabetes**

**Injection**—To generate an acute type 1 diabetes mouse model, WT and Mstn−/− mice were intraperitoneally injected with a single high-dose (35 mg/kg body weight). Mice were fasted 9–10 h and 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 C2C12 myoblasts were transfected with 1.7 kb promoter and liver tissue, and RT-qPCR was performed exactly as described in Sriram et al. (6). The forward and reverse primers used are available upon request.

**Isolation of Primary Myoblasts**—The hind limb skeletal muscles from WT and Mstn−/− mice were isolated, and primary myoblasts were isolated according to the previously established protocol (14, 15).

**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 WT and Mstn−/− mice for 48 h. The conditioned medium from CHO cells (CMM) was found to have Mstn at a concentration of 3.5 ng/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 h. Ant1 at a concentration of 1 ng/ml was used to pretreat cells 1 h before STZ or CMM treatment.

**RT-qPCR (Reverse Transcriptase Quantitative Polymerase Chain Reaction)**—RNA was isolated from Gastrocnemius muscle and liver tissue, and RT-qPCR was performed exactly as described in Sriram et al. (6). The forward and reverse primers used are available upon request.

**Western Blot Analysis**—Western blotting was performed as previously described (6). The forward and reverse primers used are available upon request.

**Electrophoretic Mobility Shift Assay**—The Foxa2 binding site (18) was identified using the TFSEARCH tool. The oligonucleotides containing the Foxa2 binding site on mouse Mstn promoter (5′-TTTTTTCCCCCTCAATATTTGGTTTTAGAACA-3′) were labeled at the 3′-end with Biotin Tetra-ethylene glycol (Sigma-Aldrich). The nuclear extracts from WT-C and WT-STZ Biceps femoris muscle were used for the assay. The electrophoretic mobility shift assays were performed using the Lightshift Chemiluminescent EMSA kit (Thermo Scientific) as previously described (6).

**Chromatin Immunoprecipitation (ChIP) Assay**—HepG2 cells were transfected with 1.7 kb mouse Mstn promoter construct (1.7P) (18) using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). ChIP assay was performed according to the published protocol (19). The following set of primers was used for PCR: Foxa2 forward primer 5′-GTCAGCTTCTCTAGTGGTTTACTTCTC-3′ and Foxa2 reverse primer 5′-TCCTTAAGACTGTGGATGCGTGT-3′. The resulting PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

**Luciferase Assay**—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), per the manufacturer’s guidelines. Sixteen hours after transfection, the medium was replaced with fresh proliferation medium and the myoblasts were incubated for a further 24 h. Luciferase assays were performed using the Dual Luciferase Assay System.
as per the manufacturer’s protocol (Promega). Relative luciferase activity was measured in triplicate using the Fluoroskan Ascent Microplate Fluorometer and Luminometer (Thermo Fisher Scientific Inc.).

Preparation of Muscle Homogenates for Enzyme Assays—Quadriceps muscle homogenates were made according to a previously published protocol (6), and total protein concentration was measured by Bradford’s assay (17).

Estimation of Lipid Peroxidation Product—The lipid peroxidation product (malonaldehyde) was determined as described previously (20).

Estimation of Superoxide Dismutase and Glutathione Peroxidase—The activity of Superoxide dismutase was estimated as described by Sriram et al. (6). Glutathione peroxidase enzyme assay was performed by the modified method of Rotruck et al. (21) as described previously (6).

Estimation of Reduced Glutathione—Reduced glutathione levels were determined by the method of Moron et al. (22).

Analysis of Intracellular ROS Production—ROS production was assayed in C2C12 myoblasts treated with STZ during proliferation as previously described (6) using the fluorescent dye, CM-H2DCFDA (Molecular Probes).

Myoblast Proliferation Assay—Myoblast proliferation assay was performed as previously described (6) using the methylene blue photometric end point assay (23).

Immunohistochemistry for REDD1 and OGG1—C2C12 primary myoblasts were plated on 4% paraformaldehyde in PBS. The immunostaining for REDD1 or OGG1 primary antibodies was performed according to manufacturer’s instructions (Trevigen Inc.). Images were taken at 10× magnification using a Leica upright microscope.

Immunocytochemistry for REDD1 and OGG1—C2C12 primary myoblasts were seeded on 4% paraformaldehyde in PBS. The next day, proliferation medium containing CCM, CMM, STZ, and/or Ant1 was added and treated with Ant1 for 48 h. The immunostaining for 8-oxo-dG was performed according to manufacturer’s instructions (Trevigen Inc.). Images were obtained at 10× magnification using a Leica upright microscope.

Comet Assay—C2C12 myoblasts were grown and treated as described in the previous section. A single cell suspension was made and comet assay was performed as previously described (24). The alkaline lysis method was used to detect the combination of single strand breaks, double strand breaks, and alkali-labile sites in the DNA, and neutral lysis method was performed to detect only DNA double strand breaks. Using propidium iodide (PI), the comets were visualized and at least 50 comet images/slide and 3 slides/treatment were examined. Comet image analysis software was used to quantify various parameters.

Transient Transfection of shRNA to Knockdown Mstn—C2C12 myoblasts were transfected with 4 μg/well of 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 medium containing CCM, CMM, STZ, and/or Ant1 was added for a further 48 h. The myoblasts’ protein lysates were made as described previously (6).

Statistical Analysis—The p value was calculated using ANOVA, 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 ± S.E. of three independent experiments.

RESULTS

STZ Treatment Induced Muscle Atrophy in Mice—STZ treatment has been shown to induce hyperglycemia in rodents (25), hence we injected mice with STZ to establish a type 1 diabetes model. The results showed maximum induction of ROS, indicated by expression of TNF-α (Fig. 1A) and Nox1 (Fig. 1B) and anti-oxidant enzymes (data not shown) on Day 7 in STZ-treated muscles; hence, further experiments were performed using Day 7 STZ-treated muscles. Furthermore by Day 7, the percentage loss of Gastrocnemius and Quadriceps muscle weights normalized to body weight were significantly reduced in WT-STZ and Mstn−/− mice (Fig. 1C) when compared with their respective controls (data not shown).

Histological analysis of muscle revealed that muscle fiber area (% of WT) in STZ and Mstn−/−—STZ treatment groups (Data not shown). Furthermore, the muscle fiber number was reduced in the WT-STZ and Mstn−/−—STZ groups when compared with their respective controls (Fig. 1D).

P-Smad2/3 (Fig. 1C) were significantly increased in Mstn−/−—STZ compared with WT-C muscles (Fig. 1A, lane 1), while the P-p38 percentage loss of Gastrocnemius and Quadriceps muscles is less in Mstn−/−—STZ compared to WT-C muscles (Fig. 1B, lane 2). These results confirmed that STZ treatment led to extensive skeletal muscle atrophy in WT mice and relatively less muscle atrophy in Mstn−/− mice.

Foxa2 Mediated Up-regulation of Mstn in Response to STZ—The expression and levels of Mstn and its downstream targets, p-Smad2/3 (Fig. 1D, lane 1), and Mstn (Fig. 1D, lane 2) were significantly up-regulated in WT-STZ muscle (lane 2) when compared with WT-C muscle (lane 1). To investigate the mechanism involved in STZ-induced Mstn transcription, an in silico analysis was performed on the 1.7 kb upstream sequence of the mouse Mstn gene to identify various transcription factor binding sites. The sequence analysis identified a putative Foxa2 binding site (5’-CAATATTGGTT-3’) within the 1.7 kb sequence of the mouse Mstn promoter. Foxa2 has been shown to regulate glucose homeostasis and glucose-induced insulin release (26).

RT-qPCR and Western blot analysis of Foxa2 indicated that Foxa2 mRNA expression and protein level (Fig. 2A (i) and (ii)) were significantly up-regulated in WT-STZ muscle (lane 2) and barely detectable in Mstn−/−—STZ 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, electrophoretic mobility shift assay was performed. As shown in Fig. 2B (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 Biceps femoris muscles were incubated with increasing concentrations of competitor oligos, the disappearance of the shifted band was

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observed (Fig. 2B (ii)- lanes 3 and 4). The specificity of Foxa2 binding was confirmed using Foxa2 specific antibody; the results showed a supershift of the Foxa2 specific band (Fig. 2B (ii)- lane 5). ChIP assay further demonstrated enhanced binding of Foxa2 to the Mstn promoter in HepG2 cells transfected with the Mstn promoter (lane 10) when compared with control (lane 9, Fig. 2C).

Myoblasts transfected with 1.7 kb mouse Mstn promoter construct showed an ~8.0-fold increase in Luciferase activity, when compared with myoblasts transfected with the empty vectors (Fig. 2D). 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.
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A (i) Fold change (Foxa2) vs. WT

(ii) Foxa2 (50 KDa) vs. GAPDH (37 KDa)

B (i) Biotinylated probe

(ii) Biotinylated probe vs. WT STZ

C (i) Ladder, H2O, Endo, No Ab, Anti-pG, Anti-FOXa2, Endo, H2O, Ladder

D (i) Fold change (RLU)

E (i) Foxa2 (50 KDa) vs. Mef2c

p-Smad2/3 (Ser423/425) (60 KDa)
Smad2/3 (60 KDa)
p-Akt1/2/3 (Ser473) (56 KDa)
Akt1/2/3 (56 KDa)
GAPDH (37 KDa)

F (i) Normalized protein levels

(i) pFLAG-CMV2 vs. pFLAG-Foxa2

Scrambled -ve control siRNA vs. Foxa2-siRNA
when compared with myoblasts transfected with control vectors (Fig. 2D). These data confirm that Foxa2 regulates transcription of Mstn gene.

In addition, overexpression of Foxa2 in C2C12 cells led to up-regulated levels of Mstn and p-Smad2/3 and reduced levels of p-Akt1/2/3 (Figs. 2E (i) and 2F (ii)-lane 2). Knockdown of Foxa2 using Foxa2-siRNA resulted in reduced p-Smad2/3 levels, increased p-Akt1/2/3 levels and no change in Mstn levels (lane 2) (Fig. 2, E (ii) and F (iii)). Previously, insulin signaling (27) and Akt phosphorylation (28) has been shown to regulate Foxa2 activity. Western blot results showed that the levels of p-Akt1/2/3 and Akt1/2/3 were reduced in WT-STZ muscles (lane 2), when compared with WT-C muscles (lane 1), while the levels were significantly higher in Mstn−/− mice (lanes 3 and 4) (Figs. 3A (i) and (ii)). IRS-1 levels were significantly decreased in Mstn−/−/STZ mice (lane 4). IGF-1 levels were significantly reduced in WT-STZ muscles (lane 2) compared with controls while the levels were higher in Mstn−/− muscles even upon STZ treatment (lane 4) (Figs. 3A (i) and (ii)).

Absence of Mstn Abrogated STZ-induced Changes in p63 and REDD1 Signaling—STZ-induced ROS has been implicated in DNA damage, thus we investigated if STZ-induced Mstn can cause DNA damage in skeletal muscle. STZ treatment in WT mice reduced REDD1 and p63 levels and increased OGG1 levels, significantly (lane 2, Fig. 3B (i) and (ii)). REDD1 and p63 levels were elevated in Mstn−/−/C muscles (lane 3) when compared with WT-C muscles (lane 1). Upon STZ treatment, Mstn−/− mice (lane 4), no change in REDD1 and p63 levels was observed when compared with WT-STZ muscles (lane 4), while REDD1 (lane 5) and p63 levels (lane 6) was observed in p53 knockout (KO) muscles (Fig. 3B (i) and (ii)). In addition, the level of Foxa2 in the muscles of Tibialis anterior muscles was significantly reduced in WT-STZ muscles (lane 5) compared with WT-C sections (Fig. 3C (i)), while Foxa2 expression was higher in both Mstn−/−/C and -STZ sections (Fig. 3C (ii)). Only STZ-treated WT muscle showed increased OGG1 staining as compared with WT-C muscle, Mstn−/− with WT-STZ muscle sections (Fig. 3D (i) and (ii)). These results indicated that STZ-induced changes in p63/REDD1 signaling are rescued in the absence of Mstn.

STZ-induced Mstn Signaling in Vitro via Foxa2 Leads to Oxidative Stress-induced DNA Damage—STZ (STZ1–0.25 mg/ml) treatment on C2C12 cells resulted in increased ROS (Fig. 3E) as previously shown (29) and Foxa2 levels in protein lysates (lane 2) (Fig. 4A (i) and (ii)). As shown in Fig. 4B (i) and (ii), STZ treatment (STZ2 - 1 mg/ml) showed increased levels of Mstn (lane 3), while Ant1 (11) treatment alone significantly inhibited Mstn levels (lane 4). Ant1 treatment along with STZ partially reduced Mstn levels (lanes 5 and 6). Dose-dependent increase in p-Smad2/3 levels were observed upon STZ treatment (lanes 2 and 3), while Ant1 treatment partially rescued the increase (lanes 4 and 5). p-Akt1/2/3 levels were significantly reduced upon STZ treatment (lane 3) (Fig. 4B (i) and (ii)).

Western blot analysis for p63, REDD1, and OGG1 was also performed on protein lysates obtained from STZ-treated C2C12 cells with gain or loss of Foxa2 expression. As shown in Fig. 4C (i) and (ii), overexpression of Foxa2 reduced the levels of REDD1 in the untreated (lane 2) and STZ-treated (lanes 4 and 6) cells; while OGG1 levels were increased upon overexpression of Foxa2 and STZ treatment (lane 6). In agreement with these results, p63 and REDD1 levels were higher in cells transfected with Foxa2-siRNA and treated with STZ1, compared with control (lanes 3, 4-Fig. 4D (i) and (ii)). However, higher concentration of STZ (STZ1) reduced the levels of REDD1 and p63 even in cells transfected with Foxa2-siRNA, and transfected with STZ1-siRNA. No significant differences were observed in muscles transfected with STZ1 or STZ2-siRNA (Fig. 4D).

FIGURE 2. Foxa2 mediated 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 WT-C, WT-STZ, Mstn−/−/C and Mstn−/−/STZ muscle (**, p < 0.01, ***, p < 0.001 when compared with WT-C muscle, n = 7). B, (i) left panel, representative electrophoretic mobility shift assay gel showing increased Foxa2 binding to the DNA upon STZ treatment 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 when nuclear extracts of WT-STZ Biceps femoris muscles were incubated with increasing concentrations of competitor oligos (100× and 500×). Supershift of the Foxa2 specific band when WT-STZ Biceps femoris muscle nuclear extracts were pre-incubated with Foxa2 antibody (lane 1-oligo only, lane 2-WT-STZ, lane 3-100× competitor oligo, lane 4-500× competitor oligo, lane 5-500× Foxa2 antibody) (n = 3). C, representative agarose gel image showing the binding of Foxa2 to 1.7 kb 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, expressed as relative luminescence units (RLU) 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 < 0.001; ****, p < 0.0001; n = 3). E, Western blot and (F) densitometric analysis of Foxa2, Mstn, p-Smad2/3, Smad2/3, p-Akt1/2/3, and Akt1/2/3 in protein lysates obtained from proliferating C2C12 cells treated with either (i) p-FLAG-CMV2 or p-FLAG-Foxa2 or (ii) scrambled -ve control siRNA or Foxa2-siRNA. GAPDH was used as an internal control for equal protein loading on the gel (*, p < 0.05; **, p < 0.01; ***, p < 0.001; n = 3).
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FIGURE 3. Absence of Mstn abrogated STZ-induced changes in p63 and REDD1 signaling. A, Western blot analysis (i) and densitometric analysis (ii) of p-Akt1/2/3, Akt1/2/3, IRS-1 and IGF-1 in WT-C (lane 1), WT-STZ (lane 2), Mstn−/−C (lane 3) and Mstn−/−STZ (lane 4) Gastrocnemius muscle protein lysates. *, p < 0.05; **, p < 0.01 when compared with WT-C; ˆˆ, p < 0.01 when compared with Mstn−/−C. GAPDH was used as an internal control for equal protein loading on the gel (n = 7). B, Western blot analysis (i) and densitometric analysis (ii) of 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) Gastrocnemius muscle. GAPDH was used as an internal control for equal protein loading on the gel (n = 5). *, p < 0.05; ***, p < 0.001 when compared with WT-C. Immunohistochemistry for REDD1 (C) and OGG1 (D) was performed on cryosections of Tibialis anterior muscles 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 5× magnification. Increased or decreased fluorescence (green) indicates changes in expression of REDD1 (C) or OGG1 (D); scale bar represents 100 μm (n = 3). E, ROS production was measured in proliferating C2C12 myoblasts treated with STZ1 for 48 h in Permanox chamber slides using the CM-H2DCFDA fluorescent probe. The fluorescence was viewed under a Leica upright microscope and images were taken at 10× magnification. Increased fluorescence (green) intensity is directly proportional to increased ROS production; scale bar represents 100 μm (n = 2).

height, in C2C12 myoblasts caused by STZ. Pretreatment with Ant1 reduced the comet formation to the level similar to untreated myoblasts (quantitation data not shown).

Similarly, CMM1 and CMM2 induced single strand breaks in DNA, detected by the alkaline lysis method (Fig. 6B (i)). Only higher concentrations of CMM (CMM2) caused double strand breaks in DNA, as detected by neutral lysis of cells (Fig. 6B (ii)). Pretreatment with Ant1 reduced the comets formed by CMM1 or CMM2 treatment. CMM1 and CMM2 treatment significantly increased comet length and comet height (Fig. 6, C (i))
and D (i)), % DNA in tail (Fig. 6C (iii)), tail length (Fig. 6, C (iv) and D (ii)), tail area (Fig. 6C (iv)), tail moment and Olive moment (Fig. 6 (v)) upon alkaline lysis and neutral lysis, while only CMM2 increased % DNA in tail (Fig. 6C (iii)), tail moment, and Olive moment (Fig. 6D (iv)) upon neutral lysis. Ant1 significantly reduced the above mentioned parameters (Fig. 6C (i), (iii), (iv), (v), and D (i), (ii), (iii), (iv)). CCM1- and CCM2-treated myoblasts showed significant decrease in comet mean intensity (Fig. 6C (ii)), head mean intensity (Fig. 6, C (ii) and D (ii)) and % DNA in head (Fig. 6C (iii)) upon alkaline lysis and neutral lysis, while only CMM2 showed a decrease in % DNA in head of comet upon neutral lysis (Fig. 6D (iii)). Ant1 pretreatment

FIGURE 4. STZ-induced Mstn signaling in vitro via Foxa2 leads to oxidative stress-induced DNA damage, which was attenuated by Ant1. A, representative Western blot (i) and densitometric analysis (ii) showing Foxa2 levels in protein lysates obtained from proliferating C2C12 cells treated with STZ1 for 48 h (lane 1 - Untreated; lane 2 - STZ1 treated). GAPDH was used as an internal control for equal protein loading on the gel (*, p < 0.05; n = 3). B, (i) Western blot and (ii) densitometric analysis showing protein levels of Mstn, p-Smad2/3, Smad2/3, p-Akt1/2/3, and Akt1/2/3 in proliferating C2C12 cells untreated (lane 1), treated with STZ1 (lane 2) or STZ2 (lane 3) or Ant1 (lane 4) for 48 h, pretreated with Ant1 for 1 h followed by either STZ1 (lane 5) or STZ2 (lane 6) treatment for 48 h. GAPDH was used as an internal control for equal protein loading on the gel (n = 3) (*, p < 0.05, **, p < 0.01 when compared with untreated cells; †, p < 0.05 when compared with Ant1-treated cells). Western blot analysis (i) and densitometric analysis (ii) of p63, REDD1, and OGG1 in protein lysates obtained from proliferating C2C12 cells transfected with either (C) p-FLAG-CMV2 or p-FLAG-Foxa2 or (D) scrambled -ve control siRNA or Foxa2-siRNA. (Lanes 1 and 2 - untreated; lanes 3 and 4 - STZ1 treated; lanes 5 and 6 - STZ2 treated). GAPDH was used as an internal control for equal protein loading on the gel (*, p < 0.05). (n = 3).
brought the levels of these parameters back to levels similar to CCM-treated myoblasts (Fig. 6, C and D).

**STZ Treatment Altered Genes Involved in DNA Repair in Vivo**—In muscles, Western blot analyses for XRCC1, a protein involved in repair of single strand breaks in DNA, and Histone H2A.X, involved in DNA double strand break repair were performed. 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 with WT-C muscles (lane 1) (Fig. 7A (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 with WT-C muscles (lane 1) (Fig. 7A (i) and (ii)).

**STZ and CMM Alter p63/REDD1 Signaling and DNA Repair Genes in Proliferating Primary Myoblasts**—The effect of STZ and Mstn on p63 and REDD1 levels and DNA repair genes was also analyzed in STZ- (data not shown) or Mstn-treated (Fig. 7B (i) and (ii)) WT and Mstn$^{-/-}$ primary myoblasts. The results show that p63 levels were decreased in CMM1- and CMM2-treated WT myoblasts in a dose-dependent manner (lanes 2 and 3), while only CMM2 treatment reduced p63 level in Mstn$^{-/-}$ myoblasts (lane 9) (Fig. 7B (i) and (ii)). CMM1 and CMM2 treatment increased OGG1 protein levels (lanes 2 and 3), while Ant1 pretreatment rescued this increase (lanes 4–6), when compared with CCM-treated WT primary myoblasts (lane 1). In Mstn$^{-/-}$ myoblasts, the increase in OGG1 levels on CMM treatment (lanes 8 and 9) was lesser than CMM-treated WT myoblasts. CMM2 treatment on WT myoblasts reduced REDD1 and p-Histone H2A.X levels (lane 3), while Ant1 pretreatment rescued the levels (lanes 4–6). CMM2 treatment on
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Mstn−/− myoblasts (lane 9) increased p-Histone H2A.X and total H2A.X levels (Fig. 7B (i) and (ii)).

Knockdown of Mstn by shRNA Partially Attenuates STZ- and CMM-induced DNA Damage—C2C12 myoblasts were transfected with shMstn and treated with STZ (data not shown) or CMM (Fig. 8A (i) and (ii)). Results showed that indeed Mstn levels were significantly reduced in shMstn transfected myoblasts (data not shown). As shown in Fig. 8A (i) and (ii), 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 a decrease in REDD1 levels in shMstn-transfected myoblasts (lane 9) (Fig. 8A (i) and (ii)). 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 total H2A.X in shMstn-transfected myoblasts (lanes 7–9) (Fig. 8A (i) and (ii)).

**DISCUSSION**

Type 1 diabetes is characterized by lack of insulin, hyperglycemia and oxidative stress. In this report, we elucidate the mechanisms by which STZ treatment leads to an increase in Mstn expression through activation of Foxa2 transcription factor. Enhanced Mstn contributes to ROS that 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 (7–9). Indeed, in silico analysis clearly revealed the presence of Foxa2 binding site regulated by insulin, such that in the absence of insulin, Foxa2 activity was enhanced in the liver. Foxa2 activity is implicated in glucose (26) and lipid metabolism (28) in the liver. Our results indicated that transcriptional targets of Foxa2 involved in glucose (26) and insulin signaling (28) were also down regulated in the liver (28).

We show for the first time that significantly higher levels of ROS led to DNA damage and inhibited cell proliferation (29). Regardless of direct or indirect effect, we noticed not only an increase in ROS but also Foxa2 expression in CMM-treated myoblasts thus recapitulating in vivo effects of STZ on muscle.

Previously it has been reported that insulin signaling inhibits Foxa2 activity by phosphorylation mediated by Akt (28, 30). Accordingly, Akt phosphorylation was down regulated upon STZ treatment in WT mice (Fig. 3A (i) and (ii), lanes 1 and 2) leading to higher Foxa2 and Mstn levels. These findings not only indicate that one of the transcriptional targets of Foxa2 is Mstn but also extend the role of Foxa2 in skeletal muscle of diabetic mice. However, despite developing overt diabetes, the levels of Foxa2 were significantly lower in Mstn−/− muscle even after treatment with STZ, which we propose could be due to enhanced Akt signaling in these mice (Fig. 3A (i) and (ii), lanes 3 and 4). Furthermore, Foxa2 has been reported to auto regulate its expression through a positive feedback loop (31). Such an autoregulatory mechanism would help maintain increased levels of Foxa2 in STZ-treated WT mice and lower levels in Mstn−/− mice.

In line with earlier findings (32, 33, 34), increased ROS was observed (and thus Antioxidant enzymes) in skeletal muscles of WT diabetic mice but not Foxa1 (Fig. 1, A and B) and lipid peroxidation (4). However, our results revealed no change in antioxidant levels in Mstn−/−-STZ mice (Fig. 1, A and C). Enhanced Akt signaling in these mice (Fig. 3A (i) and (ii), 4) indicated that in the absence of Mstn these mice resisted the induction of ROS by STZ. ROS are potent molecules involved in programmed cell death. It is evident that STZ-induced DNA damage (35, 36). This could be due to ROS-induced DNA damage (39).

Type 1 diabetes is characterized by lack of insulin, hyperglycemia and oxidative stress. In this report, we elucidate the mechanisms by which STZ treatment leads to an increase in Mstn expression through activation of Foxa2 transcription factor. Enhanced Mstn contributes to ROS that causes DNA damage mediated by p63/REDD1 in the muscle of diabetic mice.

**FIGURE 6.** STZ or CMM treatment leads to single strand breaks and double strand breaks in DNA in proliferating myoblasts. 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 h during proliferation. Representative images showing the head and tail of comets detected by PI stain during various treatments. DNA fragmentation is observed by the formation of tail in the comet as indicated by arrows. Comet images were taken using a Leica upright microscope (n = 3). B, 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 + CMM1, or Ant1 + CMM2 for 48 h during proliferation. Representative images showing the head and tail of comets detected by PI stain during various treatments. DNA fragmentation is observed by the formation of tail in the comet as indicated by arrows. Comet images were taken using a Leica upright microscope. Quantitative analysis of various parameters of comet assay showing alkaline lysis (C) and neutral lysis (D); comet length and comet height (C) and (D), comet mean intensity (C), head mean intensity (D) and (D), % DNA in head and % DNA in tail (C) and (D), tail area (C) and (D), tail moment and olive moment (C) and (D).

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 when compared with CCM-treated myoblasts; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 when compared with CMM1- or CMM2-treated myoblasts (n = 3).
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Redd1 is known to regulate cellular ROS through p63 and hence plays an important role in stress response and modulation of growth factors (4). Here, our results have established Mstn-mediated down-regulation of REDD1 and p63 levels in STZ-treated mice at week 1 of STZ treatment plausibly due to higher dose of STZ (180 mg/kg) and/or the type of muscles used in the study (8). Our results indicated that the basal levels of REDD1 and p63 were higher in Mstn−/− mice probably in part due to higher levels of AMPK because AMPK was shown to stimulate REDD1 in vitro (41). Another reason could be due to the elevated IGF-1 signaling in Mstn−/− mice, since IGF-1 treatment was reported to up-regulate the protein levels of REDD1 in muscle (42). Nevertheless, deficiency of p63 is associated with inefficient DNA repair (43) as well as with reduced transcription of REDD1 (4). Consistently, in vitro experiments confirmed Foxa2-mediated up-regulation of Mstn upon STZ treatment leading to DNA damage via p63/REDD1 signaling (Fig. 7A and D).

At the cellular level, both 8-oxo-dG staining and comet assay showed that both concentrations of CMM were able to induce DNA single strand breaks, while only a higher concentration of CMM (CMM2) was able to induce double strand breaks in DNA. Furthermore, XRCC1, involved in single strand break repair, was decreased in WT-STZ muscle (Fig. 7A (i) and (ii)) indicating that in the diabetic condition due to enhanced ROS, an increase in DNA single strand break would occur and the repair would be impaired due to lower levels of the repair enzyme. XRCC1 is a subunit enzyme involved in various stages of DNA repair via its interactions with other repair proteins. An increase in the levels of Mstn-mediated down-regulation of XRCC1 that results in inefficient single strand break repair and build-up of repair intermediates in Mstn−/− mice, which remained 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 of mouse embryonic and induced pluripotent stem cells that is independent of DNA damage response function (45). Overall, our findings suggest that deletion/inhibition of Mstn abrogates DNA damage in the diabetic muscle/cells.

Our results showed that STZ administration in Mstn−/− mice resulted in high glucose levels and greater decrease in body weights initially when compared with the WT mice (data not shown). 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 (data not shown), which is in agreement with Wang et al. (46). As demonstrated previously, Mstn−/− mice have reduced amount of adipose tissue (47) indicating that muscle would be the predominant source to provide precursors for the enhanced gluconeogenesis following CMM treatment.

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FIGURE 7. STZ treatment altered genes involved in DNA repair in vivo; changes in p63/REDD1 signaling and DNA repair genes occur in proliferating primary myoblasts upon CMM treatment. A, Western blot analysis (i) and densitometric analysis (ii) 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) Gastrocnemius muscle. GAPDH was used as loading control on the gel (n = 5). **, p < 0.01 when compared with WT-C, B, representative Western blot (i) and densitometric analysis (ii) showing protein levels of p63, REDD1, OGG1, p-Histone H2A.X, and H2A.X in 48 h proliferating WT (lanes 1–6) and Mstn−/− (lanes 7–9) primary myoblasts; lanes 1 and 7 CMM-treated, lanes 2 and 8 CMM1-treated, lanes 3 and 9 CMM2-treated, lane 4 Ant1-treated, pretreated for 1 h 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 (*, p < 0.05, **, p < 0.01 when compared with WT-C-treated cells; †, p < 0.05 when compared with Mstn−/−-CCM-treated cells) (n = 3).

FIGURE 8. Knockdown of Mstn reduces DNA damage. A, representative Western blot analysis (i) showing p63, REDD1, H2A.X and GAPDH (37 KDa) expression. B, densitometric analysis (ii) showing p63, REDD1, OGG1, Histone H2A.X and H2A.X protein levels in 48 h proliferating C2C12 myoblasts transfected with empty vector control (lanes 1–3), scrambled shRNA (lanes 4 – 6) and Mstn shRNA (lanes 7–9) primary myoblasts; lanes 1–6 CCM-treated, lanes 7–9 -Ant1-treated, pretreated for 1 h with Ant1 followed by CMM1 (*) or CMM2 (†) treatment. GAPDH was used as internal loading control on the gel (*, p < 0.05, **, p < 0.01 when compared with empty vector control transfected CCM-treated cells; †, p < 0.05 when compared with shMstn-transfected CCM-treated cells) (n = 3).
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STZ treatment, thus accounting for the increased muscle loss observed in Mstn $^{-/-}$ mice. However, even though Mstn $^{-/-}$ mice exhibit higher glucose levels upon STZ treatment, appreciable DNA damage was not observed in these mice. The inactivation/inhibition of Mstn was not able to improve the primary defect of type 1 diabetes but was able to rescue skeletal muscle from oxidative stress-induced DNA damage to a certain extent.

In summary, our results illustrate the mechanism of Mstn regulation in type 1 diabetes and Mstn-mediated DNA damage in skeletal muscle. Our findings also indicate that inhibition of Mstn could be an effective preventive measure to mitigate DNA damage in diabetic muscle.

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