University of Cape Town

Over-expression of NRF-1 in C2C12 myotubes increases GLUT4 content via a transcriptional cascade involving MEF2A

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PUBLICATIONS ASSOCIATED WITH THIS DISSERTATION

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LIST OF ABBREVIATIONS

AMPK - adenosine monophosphate (AMP) activated protein kinase

BSA - bovine serum albumen

C terminus - carboxyl terminus

C2C12 - mouse muscle cell line

C2C12-Tet-On-NRF-1 - double-stable C2C12 cell line

CaMK - Ca^{2+}/calmodulin dependent protein kinase

cDNA - complementary DNA

ChIP - chromatin immunoprecipitation

Co-IP - co-immunoprecipitation

COX - cytochrome oxidase

DMEM - dulbecco‟s modified eagles medium

DNA - deoxyribonucleic acid

dNTP - deoxyribonucleotide triphosphate

Dox- doxycycline

E. coli - Escherichia coli

FBS - fetal bovine serum

FCS- fetal calf serum

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GEF - GLUT4 enhancer factor

GLUT1/4 - glucose transporter-1/4 protein

HDAC - histone deacetylase

HRP - horseradish peroxidase

HS - horse serum

IgG - immunoglobulin G

Input - sample used for ChIP assay prior to immunoprecipitation with antibody

MADS - MCM1 Agamus Deficiens Serum

MEF-2 - myocyte enhancer factor 2

mRNA - messenger ribonucleic acid

N terminus - amino terminus

Na_3VO_4 - sodium orthovanadate

$Na_4P_2O_7$ - sodium pyrophosphate

NaCl - sodium chloride

NaF - sodium flouride

$NaHCO_3$ - sodium bicarbonate

NaOH - sodium hydroxide

NRF- nuclear respiratory factor

PAGE - polyacrilamide gel electrophoresis

PBS - phosphate buffered saline

PCAF - p300/CBP-associated factor

PGC-1α - peroxisome proliferator receptor gamma coactivator -1 alpha

pTet-On - Tet-On regulatory plasmid

pTRE2hyg - Tet-On response plasmid

qRT-PCR - quantitative real time polymerase chain reaction

RNA - ribonucleic acid

SDS - sodium dodecyl sulphate

TBST - Tris buffered saline (TBS) containing 0.1% Tween 20

TE - buffer containing Tris-Cl and EDTA

TFAM - mitochondrial transcription factor

Tet-On- Tetracycline-On gene expression system

ABSTRACT

Previous studies have shown that over-expression of nuclear respiratory factor (NRF)-1 in mice increases glucose transporter (GLUT)-4 and myocyte enhancer factor (MEF-) 2A content, but the mechanisms have not been elucidated. Because NRF-1 has a binding site on the *mef2a* gene, and MEF2A binds the *glut4* gene as a MEF2A-MEF2D heterodimer, the aims of this study were to determine whether NRF-1 over-expression a) enhanced GLUT4 expression indirectly via MEF2A and b) alters MEF2A-MEF2D dimer formation in C2C12 myotubes.

The Tet-on gene expression system was used to over-express NRF-1 in C2C12 myotubes (C2C12-Tet-On-NRF-1) after treatment with 2 µg/ml doxycycline (Dox) for 72 h. Control myotubes were treated with vehicle. To assess if the effects of NRF-1 were mediated via MEF2A, some C2C12-Tet-On-NRF-1 myotubes were transfected with 80 pmol MEF2A-siRNA to degrade MEF2A mRNA. NRF-1, GLUT4, MEF2A, MEF2D, δ-ALAS and α-tubulin protein levels were assessed using immunoblotting. Chromatin immunoprecipitation (ChIP) assays were used to measure NRF-1 and MEF2A binding onto *mef2a* and *glut4* genes, respectively. Co-immunoprecipitation assays using MEF2A or MEF2D antibody were carried out to assess MEF2A-MEF2D dimer formation after NRF-1 over-expression.

Myotubes treated with 2 µg/ml Dox showed ~3-fold higher contents of NRF-1 and δ-ALAS compared to controls. MEF2A and GLUT4 were increased 3- and 2-fold, respectively, in Dox-treated myotubes, but MEF2D was not changed. MEF2A-siRNA transfection prevented MEF2A increase while myotubes transfected with control-siRNA showed ~3 fold increase after Dox treatment. ChIP assays showed a 3-fold increase in NRF-1 binding to *mef2a*, and 2-fold increase in MEF2A binding to *glut4* compared to controls. MEF2A and GLUT4 mRNAs were also increased ($p<0.05$) after NRF-1 over-expression. Co-IP assays showed 2-fold increase in MEF2A-MEF2D dimer formation in Dox-treated myotubes compared to controls.

In conclusion, the study shows that NRF-1 over-expression increases GLUT4 content via MEF2A; and enhances MEF2A-MEF2D dimer content in C2C12 myotubes.

CHAPTER 1

Introduction and Justification

Insulin-mediated disposal of plasma glucose by skeletal muscle plays an important role in glucose homeostasis (8). Some studies have found that up to 90% of blood glucose is taken up by skeletal muscles in this manner (8; 28; 69). By binding to its receptors on muscle plasma membranes, insulin triggers translocation of GLUT4 transporters from intracellular vesicles to muscle cell membranes to facilitate glucose uptake. Inadequate secretion of insulin by pancreatic β-cells and/or disruption of its signalling pathway may result in accumulation of glucose in plasma, leading to the development of diabetes mellitus (28). GLUT4 also translocates to muscle cell membranes to enhance glucose uptake when skeletal muscles are contracting. However, under this condition, insulin secretion is inhibited and GLUT4 translocation occurs by an insulin-independent mechanism (48). In light of this finding, regular physical activity is prescribed as an intervention for improving glucose disposal and controlling plasma glucose levels in insulin-resistant and diabetic patients (89).

Although the cellular mechanisms involved in the exercise-induced up-regulation of glucose disposal into skeletal muscle have not been fully elucidated, there is strong evidence that increased abundance of muscle GLUT4 that results from physical activity is an important contributing factor (36; 40; 41; 48; 62; 73). It is well established that GLUT4 expression in muscle is regulated by many transcription factors including myocyte enhancer factor-2 (MEF-2) and GLUT4-enhancer factor (GEF) (76) (31; 33; 52; 62) which bind to their *cis* elements on the *glut4* gene (52; 68). Some studies further showed that MEF-2 transcription factors bind to the *glut4* gene as a MEF2A-MEF2D heterodimer (38; 58; 59).

A study by Mora & Pessin (58) demonstrated that in skeletal muscle of normal rats MEF2A is dimerized to MEF2D. In this study, western blots of MEF2D immunoprecipitates from nuclear extracts showed co-immunoprecipitation with MEF2A. Co-immunoprecipitation of MEF2D in western blots that were immunoprecipitated with MEF2A was also observed. This study further indicated that the MEF2A-MEF2D dimer is reduced in heart nuclear extracts of streptozotocin (STZ)-induced diabetic rats compared to controls after an electrophoretic mobility shift assay (EMSA). Although this experiment demonstrated increased binding activity of MEF2A-MEF2D dimers to the consensus MEF-2 oligonucleotide of the *glut4* promoter, it did not sufficiently demonstrate MEF2-DNA binding activity for GLUT4 expression. However, this was the first study to show that MEF2A-MEF2D is also associated with GLUT4 expression. The interaction of MEF2A with MEF2D for GLUT4 expression was further demonstrated in a study by Knight et al (38) where COS 7 cells were transfected with the human GLUT4 promoter plasmid, which expressed firefly luciferase (hG4-Luc), and GEF plasmid in conjunction with either MEF2A, MEF2D or both plasmids. This study showed that luciferase expression was slightly elevated after co-transfection with both MEF2A and MEF2D plasmids, however luciferase expression was higher in cells co-transfection with GEF and MEF2A plasmids compared cells co-transfection with GEF, MEF2A and MEF2D. Although this study indicates that MEF2A-MEF2D dimer binds to the MEF-2 domain of the *glut4* promoter, it demonstrates contradictory results from the Mora & Pessin (58) study, and this could be due to the different models that these two studies used.

In addition to increasing GLUT4 expression, studies have shown that muscle contraction/exercise also up-regulates mitochondrial biogenesis and increases muscle oxidative capacity (54) (13; 45; 55). Mitochondrial biogenesis is regulated by many

transcription factors including nuclear respiratory factor (NRF)-1 (13; 16; 92; 98). In a study by Baar et al. (5), it was reported that when NRF-1 is over-expressed in skeletal muscle of mice, both mitochondrial proteins and GLUT4 levels increased. In light of this observation, some researchers have suggested that GLUT4 expression and mitochondrial biogenesis may share a common signalling pathway involving NRF-1 (5; 64). The mechanism by which NRF-1 regulates GLUT4 expression has also not been studied. Because the NRF-1 binding element is absent on the *glut4* gene, it is unlikely that NRF-1 regulates *glut4* gene expression directly; rather, it is more likely that NRF-1 regulates the gene indirectly via some intermediary. Support for this notion comes from studies by Wan & Moreadith (78) and Ramachandran et al. (71): Wan & Moreadith (94), showed that the muscle-specific cytochrome *c* oxidase 6a ($COX6a_H$) which does not contain an NRF-1 *cis* element is nevertheless influenced by NRF-1 over-expression. Subsequently, Ramachandran et al. (71) demonstrated that NRF-1 indirectly regulates $COX6a_H$ through an NRF1→MEF2A→$COX6a_H$ transcriptional cascade; i.e. NRF-1 binds to its element on the *mef2a* gene to increase MEF2A expression and MEF2A in turn regulates $COX6a_H$ expression. The presence of an NRF-1 binding domain on *mef2a* gene, and the fact that GLUT4 expression is regulated by MEF-2 transcription factors support the hypothesis that over-expression of NRF-1 might also increase GLUT4 expression via a transcriptional cascade involving one or more of the MEF-2 isoforms. This hypothesis is supported by the observation that NRF-1 over-expression increased both MEF2A and GLUT4 levels in NRF-1 transgenic mice (5). Having showed that exercise increases NRF-1 and GLUT4 expression via a mediation of coactivators such as peroxisome proliferator receptor gamma coactivator-1 alpha (PGC-1α), they further wanted to know the specific effect of NRF-1 over-expression in skeletal muscle of transgenic mice. The use of transgenic mice in this study was effective in demonstrating the role of NRF-1 over-expression in skeletal muscle by overriding the

activation of other pathways that may have been confounding factors for this study. The results in this study effectively showed that NRF-1 over-expression does not only enhance the expression of mitochondrial proteins, but it also increases MEF2A and GLUT4 expression. The disadvantage of mouse models however, is that does not guarantee replicate results in human studies, and that the formation of transgenic mouse models is rather a long process. The cell culture model would therefore be an ideal model to use as there are mouse and human myoblasts available that can be transfected with plasmids designed to over-express NRF-1 in a short period of time.

The primary purpose of the present study was to characterize the role that NRF-1 plays in GLUT4 expression using a cell culture model. Specifically the study sought to find out if: a) NRF-1 binding to the *mef2a* gene is increased in response to NRF-1 over-expression in C2C12 myotubes, and b) the increase in MEF2A expression that is seen in response to NRF-1 over-expression is essential for GLUT4 up-regulation. Although GLUT4 expression has been shown to require MEF2A-MEF2D dimer formation (58; 59), a study by Baar et al. (5) showed that NRF-1 over-expression leads only to MEF2A and not MEF2D; the secondary purpose of this study was therefore, to assess the effect of NRF-1 over-expression on MEF2A-MEF2D dimer formation globally.

In summary, the specific objectives of this study were to: a) determine whether NRF-1 over-expression increases MEF2A and GLUT4 mRNA and protein levels in C2C12 myotubes, b) demonstrate whether silencing MEF2A expression in NRF-1 over-expressing muscle cells prevents the increase in GLUT4, c) verify if NRF-1 over-expression increases NRF-1 binding to the *mef2a* promoter, d) ascertain whether NRF-1 over-expression altered MEF2A-MEF2D

dimer formation globally and e) find out if NRF-1 over-expression alters MEF2A binding to the *glut4* gene.

CHAPTER 2

Literature Review

2.1. Introduction.

This literature review discusses: a) the mechanism by which glucose is transported into skeletal muscle, with a major focus on the role played by GLUT4; b) how GLUT4 expression is regulated, with the main focus being placed on the role played by MEF2A, the transcription factor that is required for this expression; c) the various MEF-2 isoforms and the dimers they form in order to bind to DNA; and lastly, d) the role that NRF-1 plays in the expression of the various MEF-2 isoforms and GLUT4.

2.2. Skeletal muscle is a major site of glucose disposal.

Skeletal muscle is the major site for glucose disposal and whole body glucose homeostasis (31; 36). Studies have reported that skeletal muscle accounts for up to 75-90% of plasma glucose disposal in the body (8; 46), a process mediated by facilitative proteins known as glucose transporters (GLUTs). To date, 13 distinct GLUTs have been identified in various body tissues (36; 47). The isoforms that are expressed in skeletal muscle include GLUT1 and GLUT4 (47; 84). While GLUT1 is constitutively localized on the plasma membrane of skeletal muscle, GLUT4 traffics between intracellular vesicles and the cell membrane (73; 84). In the basal state, GLUT4 is predominantly found in intracellular vesicles, but in the fed state, in response to insulin, or during muscle contraction, it translocates to the sarcolemma to improve glucose uptake by muscle cells (66; 87).

*2.2.1. Insulin-stimulated glucose disposal***:** Binding of insulin to its receptor causes autophosphorylation and activation of tyrosine kinase on the receptor, resulting in activation of downstream protein kinases, including insulin receptor substrate (IRS)-1 and phosphatidylinositol-3-kinase (PI3K). This signalling cascade results in the translocation and redistribution of GLUT4 proteins on the plasma membrane (21; 85). Studies have indicated that insulin resistance in skeletal muscle significantly reduces GLUT4 activation and translocation for glucose uptake. Obesity and physical inactivity have been shown to be the main causes of insulin resistance which lead to hyperglycaemia and subsequently type 2 diabetes mellitus (8; 19; 88; 89).

*2.2.2. Exercise-induced glucose disposal***:** It is well established that exercise increases glucose transport and GLUT4 expression in an insulin-independent manner (27; 48; 73; 95). Various studies have demonstrated that glucose transport and GLUT4 expression increase at varying bouts of exercise in skeletal muscle (15; 19; 48). Glucose transport has been shown to immediately increase after a single bout of exercise (15; 27; 48; 75). GLUT4 expression has been shown to increase after at least 1 to 10 days of regular exercise training (20; 41; 42; 46) as an adaptation response of skeletal muscle to regular or endurance exercise (6; 41). Although exercise has been demonstrated to increase glucose transport independent of the insulin-activated pathway, a study by Lund et al. (48) showed that muscle contraction and insulin stimulation further increased glucose transport in skeletal muscle. Exercise also increases glucose uptake by increasing GLUT4 protein and its translocation to the sarcolemma. Two main pathways that have been identified in exercise-induced glucose transport involve calcium calmodulin-dependent kinase (CaMK) and AMP-activated protein kinase (AMPK) (41; 62; 63; 97). The CaMK pathway is reported to be activated by an

increase in cytosolic calcium (Ca^{2+}) due to muscle contraction. CaMK was shown to be phosphorylated due to increased Ca^{2+} release in skeletal muscle (97). AMPK, a heterotrimeric enzyme consisting of the catalytic α-subunit and regulatory β- and γ-subunits is said to be activated as a result of intracellular energy changes caused by increases in the AMP-to ATP ratio (44; 53). Similar to CaMK, AMPK increases glucose transport by increasing GLUT4 translocation to the sarcolemma. Both CaMK and AMPK have been shown to activate GLUT4 expression by targeting its transcription factors as described in the section below (51; 53).

2.3. Regulation of GLUT4 expression in skeletal muscle.

2.3.1. Transcription factors for GLUT4 expression: The transcription factors that have been shown to be associated with GLUT4 expression are the GLUT4 enhancer factor (GEF), myocyte enhancer factor (MEF)-2, thyroid receptor α_1 ($TR\alpha_1$) and myogenic determination 1(MyoD) to mention a few (53; 102). Of those, MEF-2 and GEF have been extensively studied (33; 38; 58; 68). Studies have shown that transcriptional activation of GLUT4 is activated when MEF-2 and GEF bind to their respective domains on the *glut4* gene (46; 66; 68; 87) and that binding of MEF-2 is essential for transcription (46; 66; 87).

2.3.2. MEF-2 transcription factor: MEF-2 belongs to the MADS-box family (MCM1 Agamus Deficiens Serum) of transcriptional regulators (3; 11; 67; 99). These transcription factors were previously reported to be specifically expressed in muscle cells, hence the name, but have since been found to be present in non-muscle cell lines such as neuronal cells (67; 99). They have been shown to bind a conserved binding sequence (-CTAAAAATAG-) now

called the MEF-2 DNA-binding domain (46; 87). Four MEF-2 isoforms (MEF2A; 2B; 2C & -2D) that bind as homodimers and/or heterodimers have been identified (67). These MEF-2 isoforms have been shown to be involved in muscle development; MEF2B and MEF2D being expressed during myoblast proliferation whereas MEF2A and MEF2C are expressed during and after muscle differentiation respectively (10; 58).

The MEF-2 isoforms that have been identified to be involved in GLUT4 expression are MEF2A, MEF2C and MEF2D (55; 58; 59; 87). A study by Michael et al. (55) indicated that MEF2C participates in GLUT4 expression when it is co-activated by PGC-1α, while MEF2A is activated by a number of kinases, including CaMK and AMPK (33; 52; 65). At the basal state, MEF2A is bound to class II histone deacetylases (HDACs) that repress its binding and/or transcriptional activity. Activation of CaMK and AMPK, which occurs during exercise, for example, was shown to phosphorylate HDACs which cause them to dissociate from MEF2A, leading to increased binding onto the *glut4* gene for transcriptional activation (38; 58). McGee & Hargreaves (51) earlier showed that a single bout of exercise of reduces HDAC association with MEF-2. They later showed that AMPK phosphorylates HDAC5 which results in its dissociation from MEF2A thus activating GLUT4 transcription (53). Activation of AMPK was not shown to phosphorylate HDACs, but Holmes et al. (33) also demonstrated AMPK phosphorylates GEF which leads to its nuclear localisation and binding to the *glut4* gene. The role of CaMK was further showed in a study where transgenic mice expressing CaMKII indicated that CaMKII phosphorylates particularly HDAC4 in cardiomyocytes and also activates MEF-2 (100). These studies indicate a mechanism by which GLUT4 regulation does not solely depend on the level of transcription factors but that

GLUT4 is also regulated by the HDACs through activation and/or repression of its transcription factors (e.g. MEF2A and GEF).

2.3.3. MEF2A and MEF2D dimers: Protein dimerization is the interaction of proteins to form large complexes that are required for regulation of cellular processes such as gene expression and signal transduction (37). Protein dimers act as switches for activation or inhibition of cellular processes during cell proliferation, apoptosis and transcriptional activation (2; 23; 49; 74).

In the basal state dimerization of transcription factors plays important roles in expression of genes that regulate the cell cycle, development and other biological processes (2). The importance of transcription factor dimerization is that they increase DNA binding affinity on their target genes for transcriptional activation. Transcription factors are known to contain a dimerization domain located at the N-terminus which plays a role in DNA binding. Mutation of the dimerization domain in some transcription factors has been shown to prevent DNA binding (57). Transcription factor families that undergo dimerization include the basic helix-loop-helix (bHLH), the signal transducers and activators of transcription (STAT) and the MADS-box family (81).

The MADS-box family has a conserved 57 amino acid MADS-box domain located at the N-terminus, which plays a role in dimer formation (70). Dimers of the MADS-box family of proteins bind well to promoters and enhancers due to the dyad symmetry or reverse complementarity they form which enables each protein of the dimer complex to interact with the DNA binding site on their target genes (22; 81). One of the most studied MADS-box

transcription factors is the MEF-2 family that is predominantly involved in the transcription of muscle-specific genes (70).

In addition to having the MADS-box for dimerization, the MEF-2 transcription factors have a unique sequence called the MEF-2 domain which together with the MADS-box domain regulates dimer formation and DNA-binding. Some studies have reported that mutations in the MEF-2 domain results in diminished dimerization which decreases DNA binding affinity. MEF-2 isoforms also form homodimers (e.g. MEF2A-MEF2A) and heterodimers (e.g. MEF2A-MEF2D). The MEF2A-MEF2A homodimer has been reported to regulate muscle differentiation (67). The MEF2A-MEF2D heterodimer has been implicated in GLUT4 expression in differentiated muscle cells (47; 52). However, the factors which regulate the formation of the dimer species under different conditions have not been studied.

2.4. The role of NRF-1 in GLUT4 expression.

Nuclear respiratory factors (NRF-1 and NRF-2) are nuclear-localised transcription factors that were first described to be involved in the transcription of nuclear-encoded cytochrome *c*, (13; 17; 78). They have since been found to activate the expression of other nuclear-encoded genes that are involved in mitochondrial transcription and replication machinery, as well as heme biosynthesis (17; 90). NRF-1 has been reported to activate the transcription of most nuclear-encoded mitochondrial genes, and NRF-2 has been demonstrated to be involved in the activation of nuclear genes encoding proteins required for mitochondrial DNA transcription and replication (12; 24).

2.4.1. NRF activation and DNA binding mechanism: NRF-2 consists of five distinct subunits namely α, β_1, β_2, γ_1 and γ_2 subunits that form distinct DNA-complexes on tandem recognition sites to induce high-affinity DNA binding (26; 77). It binds a highly conserved GGAA motif that is common to the recognition sites found on the ETS-domain family of transcriptional activators that either activate or repress transcription according to the number of base pairs NRF-2 binds to on DNA-dinding domain (29; 80; 86; 90; 91). The C-terminus of NRF-2 contains a transactivation domain which consists of hydrophobic residues found on the glutamine-containing clusters (25). NRF-2 is known to regulate the expression of Mitochondrial Transcription factor (TFAM) as well as Translocase of the Outer Membrane (TOM) complex receptors involved in mitochondrial assembly (11).

Apart from being a transcriptional activator for nuclear-encoded genes, NRF-1 has also been shown to play a role in the expression of non-mitochondrial genes such as those involved in cell cycle regulation as well as myogenesis (55; 67). In this regard, NRF-1 is known to stimulate the expression of MEF-2 and has been implicated in GLUT4 expression (5; 65).

The N-terminus has the DNA binding domain and the nuclear localisation signal (NLS) as well as serine residues that are phosphorylated for DNA binding and transcriptional activation (24; 25; 90). NRF-1 consensus region is a GC rich motif (-CGCACATGCGCA-) (10; 76) which binds as a palindrome.

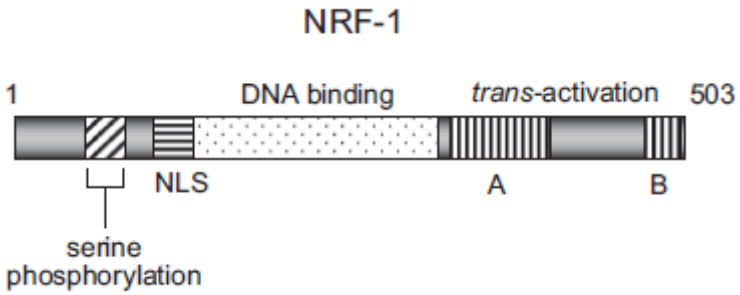

Figure 2.1: NRF-1 structure. The diagram shows NRF-1 domains, including a DNA-binding domain that is adjacent to the nuclear localisation site (NLS). Diagram adapted from R. Scarpulla (79).

2.4.2. NRF-1 regulates the expression of genes that lack NRF-1 binding domain: It is well established that NRF-1 is a transcriptional regulator of a majority of nuclear-encoded mitochondrial genes. However, it has also been reported that the expression of some tissue-specific nuclear-encoded respiratory genes are regulated by NRF-1, although they lack NRF-1 binding sites at their promoter regions (43; 94). It is thought that NRF-1 regulates these genes through intermediary factors that contain the NRF-1 binding site. Some of the genes that lack the NRF-1 binding site are cytochrome *c* oxidase (COX) 6a and COX 8 which are expressed in cardiac and skeletal muscle (71; 94). The intermediary factor for NRF-1-induced COX $6a_H$ expression was demonstrated to be MEF2A by Wan & Moreadith (94). Ramachandran et al. (71) further showed that the *mef2a* gene contains an NRF-1 binding site at its promoter region, and that NRF-1 uses MEF2A as an intermediary for the co-ordinate expression of COX $6a_H$ in cardiac and skeletal muscle. Furthermore, Ramachandran et al. (71) demonstrated that the *mef2a* promoter activity is regulated by NRF-1, and that silencing of NRF-1 by RNA interference significantly reduced *mef2a* promoter activity. This study by Ramachandran was the first to provide evidence of a transcriptional cascade in which NRF-1

uses other transcription factors as intermediaries to co-ordinately regulate the expression of tissue-specific mitochondrial genes.

2.4.3. Regulation of GLUT4 expression by NRF-1: A study performed by Baar et al. (5) revealed that during NRF-1 over-expression in mice, GLUT4 and MEF2A protein levels were increased. The increase in GLUT4 in this study could not be explained at the time since the *glut4* gene does not contain binding sites for NRF-1. However, since it is now known that NRF-1 has a binding site on the *mef2a* gene and regulates MEF2A expression, it is reasonable to hypothesize that NRF-1 up-regulates GLUT4 indirectly via a transcriptional cascade involving MEF2A in the same manner as it regulates COX $6a_H$. The primary purpose of this study was therefore to explore this hypothesis; to provide evidence for or against a transcriptional cascade involving NRF-1→MEF2A→GLUT4, in order to explain the increase in GLUT4 expression when NRF-1 is over-expressed in cells. Given that MEF-2 transcription factors bind the *glut4* gene and regulate its expression as dimers of MEF2A and MEF2D, our secondary purpose was to explore the effect of NRF-1 over-expression on the expression of these MEF-2 subunits and on MEF2A-MEF2D heterodimer formation.

CHAPTER 3

Methodology

3.1. Introduction.

The primary aim of the study was to determine if NRF-1 over-expression in C2C12 myotubes increased MEF2A and GLUT4 protein levels via the NRF-1→MEF2A→GLUT4 cascade. The second aim was to determine whether NRF-1 over-expression in C2C12 myotubes altered MEF2A-MEF2D heterodimer formation globally. In this chapter, we describe the procedures used to a) culture muscle cells, b) over-express NRF-1 in C2C12 myotubes, c) analyse MEF2A, MEF2D and GLUT4 protein levels, d) quantify NRF-1 binding on the *mef2a* gene and MEF2A binding on the *glut4* gene, and e) measure MEF2A & GLUT4 mRNA.

3.2. Source of Materials used in the study.

Mouse myoblasts (C2C12) were obtained from the American Type Culture Collection (Manassas VA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BLR (Auckland, New Zealand). Fetal calf serum/fetal bovine serum (FCS/FBS), Penicilin/streptomycin & Fungizone (PSF), dimethylsulphoxide (DMSO), 0.25% trypsin/EDTA (Mg^{2+} & Ca^{2+} free) and horse serum (HS) were purchased from Highveld Biologicals (Lyndhurst, South Africa). Complete Roche protease inhibitors (RCPIs) were acquired from Roche Diagnostics (Randburg, South Africa). The MEF-2 siRNA and δ-ALAS primary antibody were ordered from Santa Cruz Biotechnology (California USA). The

primary antibodies MEF2A, NRF-1 and MEF2D were procured from Abcam (Cambridge UK). GLUT4 and α-Tubulin were obtained from Cell Signalling Technology, Inc. (USA). The polyclonal horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies were supplied by Dako (Carpinteria, CA). All other reagents that were used for western blots were purchased from Sigma Aldrich (St Louis, MO), while polyvinylidene diflouride (PVDF) membrane was supplied by Amersham (Buckinghamshire, UK). The enhanced chemiluminescent (ECL) solution was from Thermo Scientific (Rockford, IL) and photographic film from Kodak. Chromatin immunoprecipitation assay (ChIP) kit was supplied by Upstate, Millipore (Massachusetts, USA). Primers used for qPCR (Table 3.2) and RT-PCR (Table 3.3) were synthesised by the University of Cape Town, South Africa. SuperTherm Taq DNA polymerase, 10X Buffer and 25 mM Magnesium chloride were purchased from Medox Biotech (Chennai, India).

3.3. Cell culture.

This study used a cell culture model in which mouse myoblasts (C2C12) were the model of choice. All tissue culture was performed under Bioflow II Labotec laminar flow (Midrand, South Africa). Approximately 2 x 10^5 C2C12 myoblasts were seeded on 10-cm collagen-coated Petri-dishes and cultured in a Thermoforma incubator (Labotec, USA) at 37 °C. The myoblasts were cultured in medium (DMEM) supplemented with 10 mM creatine monohydrate, 100 µU/ml each of penicillin and streptomycin & 0.25 µg/ml fungizone (PSF), and 10% heat-inactivated FCS in an atmosphere containing 5% CO_2, until they were 80-90% confluent. When cells were confluent, they were either passaged or differentiated into myotubes by incubation in DMEM containing 2% horse serum instead of 10% FCS for ~ 14

days. The C2C12 myotubes were used for the transfection of Tet-On plasmids to develop a double-stable cell line that over-expressed NRF-1.

3.4. Transformation, amplification and harvesting of Tet-On plasmids.

In our study, NRF-1 was over expressed in C2C12 myotubes using a Tet-On gene expression system (Clontech, Paulo, CA). C2C12 myoblasts were transfected with two plasmids i.e. pTet-On and pTRE2hygNRF-1 (Fig. 3.1). The NRF-1 cDNA was previously cloned into the pTRE2hyg plasmid by a member of our research group. The pTet-On plasmid expresses a regulatory protein called reverse tetracycline transactivator (rtTA), and the response plasmid called pTRE2HygNRF-1 which expresses NRF-1when rtTA binds to its tetracycline response element (TRE) in the presence of the tetracycline derivative-doxycycline (Dox).

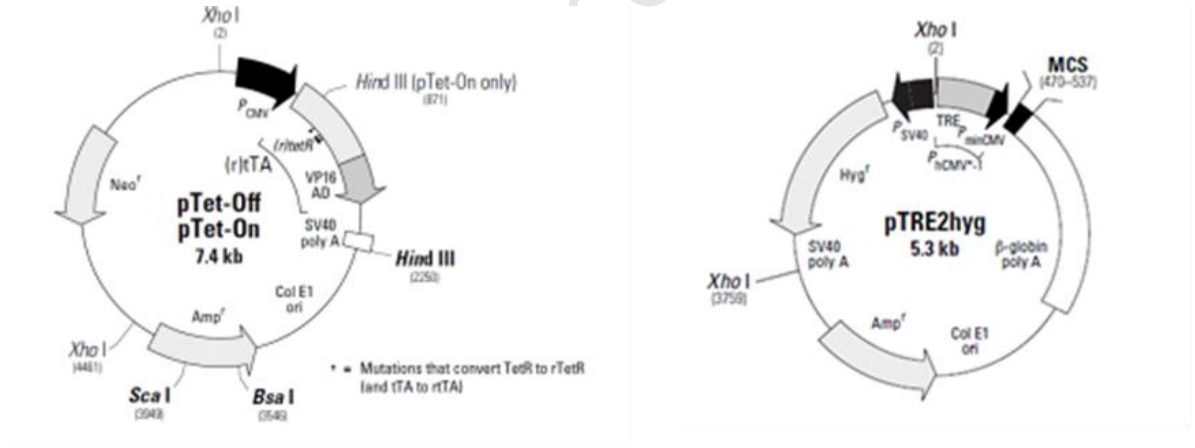

Figure 3.1: Tet-On plasmids that were used for NRF-1 over-expression in C2C12 cells. Clontech Laboratories Inc.

3.4.1. Bacterial transformation and amplification: The Tet-On plasmids (pTet-On; pTRE2hyg; pTRE2hygNRF-1) were respectively amplified by transformation using *E. coli* DH5α competent cells. Fifty nanograms of the plasmids and a control empty vector, pRL-TK, were separately added into 100 µl of competent DH5α cells in 1.5 ml eppendorf tubes and gently mixed and incubated on ice for 30 minutes (min). The tubes were thereafter placed in a water bath at 42 °C for 50 seconds (s) to heat shock the DH5α cells and allow pores on bacterial membrane to open so that plasmids could be taken into bacteria (transformation). To retain the plasmids in bacteria the tubes were again incubated in ice for 2 min. Nine hundred microlitres of Soc medium (2% bacto tryptone; 0.5% yeast extracts; 2.5mM KCl; 10 mM $MgCl_2$ 10 mM $MgSO_4$; 1.5% agar; 20 mM glucose) were added to the cells and incubated at 37 °C for 20 min. The transformed cells were thereafter plated on SOB agar (2% Bacto tryptone; 0.5% yeast extracts; 2.5 mM KCl; 10 mM $MgCl_2$ 10 mM $MgSO_4$; 1.5% agar) containing 100 µg/ml ampicilin using the sterilised glass rod, and incubated overnight at 37 °C. Following overnight incubation it was observed that the cells that contained Tet-On plasmids formed colonies as they were resistant to ampicilin, while the cells that contained the pRL-TK vector which had no ampicilin resistance did not grow.

3.4.2. Bacterial Lysis and harvesting of Tet-On plasmids: The bacterial colonies that had taken up the Tet-On plasmids were transferred into 2 ml SOC medium that contained 100 µg/ml ampicilin and incubated overnight at 37 °C with shaking. After overnight incubation 1.5 ml of the SOC medium was transferred into a microfuge tube and centrifuged at 12 000 x g for 60 s at 4 °C. The supernatant was removed and the pellet re-suspended in 200 µl of ice-cold Solution I [50 mM glucose; 25 mM Tris-HCl (pH8.0); 10 mM EDTA]. Four-hundred microliters of Solution II [0.2 N sodium hydroxide (NaOH); 1% SDS] were then added and

mixed with Solution I by inversion and kept on ice for 5 min. A 300 µl volume of Solution III (5 M potassium acetate; glacial acetic acid) was added to the solution and mixed by inverting the tube for 10 s followed by 5 min incubation on ice. The bacterial lysates were thereafter centrifuged at 12 000 x g for 5 min at 4° C and the supernatants transferred into new tubes. Equal volume of phenol/chloroform was added to each tube and mixed by vortexing, followed by centrifugation at 12 000 x g 4° C for 2 min. The supernatants were thereafter transferred into new tubes and two volumes of 100% ethanol (EtOH) was added to the supernatants, vortexed and allowed to stand at room temperature for 2 min for DNA precipitation. The solution was then centrifuged at 12 000 x g for 5 min at 4° C and the supernatants removed. The DNA pellet was rinsed with 1 ml of 70% EtOH at 4° C and the DNA precipitated by centrifugation at 12 000 x g for 5 min at 4° C. The pellet containing the Tet-On plasmids was thereafter air-dried, dissolved in 50 µl of sterilised distilled water and stored at -20° C.

3.4.3. DNA quantification & verification: The purity of the plasmids was determined by spectrophotometry using the $O.D_{A260}/O.D_{A280}$ ratio. The DNA was considered pure when the $O.D_{A260}/O.D_{A280}$ ratio was 1.8 - 2.0. The concentration of the plasmids was determined using the following formula:

$$[\text{DNA}]\ (\mu l/ml) = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}$$

DNA concentration of 2.0 - 4.0 µg/ml was obtained for the Tet-On plasmids. To verify that the harvested plasmids were indeed Tet-On plasmids, they were digested with the *Xho I* restriction enzyme for 4 h and the DNA fragments were separated using 0.7% agarose gel

electrophoresis (Fig. 3.2). For pTet-On which is 7.4 Kb, there were two fragments sizes of 5.0 kb and 2.4 kb (Fig. 3.2A), which are the expected fragment sizes according to manufacturer's protocol. pTRE2hyg (a plasmid whose size is 5.8 Kb) also produced the expected digestion fragments of sizes 3.75 kb and 1.55 kb (Fig. 3.2B). The pTRE2hygNRF-1 plasmid produced two fragments of sizes 3.75 Kb and 1.55 Kb (Fig.3.2C). Because the NRF-1 cDNA that was cloned into pTRE2hyg was 1.74 kb and has no *Xho 1* restriction site, this digestion profile was also predicted. The Tet-On plasmids were thereafter stored at -20° C and later used for transfection into C2C12 cells.

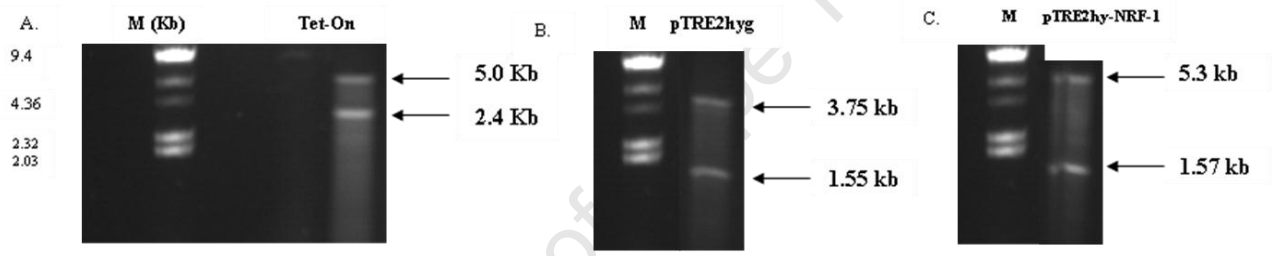

Figure 3.2: Agarose gel for Tet-On plasmids. A 0.7% agarose gel indicating the fragments produced when Tet-On plasmids were digested with the *Xho I* restriction enzyme and separated by agarose gel electrophoresis.

3.5. Production of double-stable C2C12 Tet-On-NRF-1 cells.

An overview of the steps involved in producing double-stable C2C12 Tet-On-NRF-1 cells is illustrated below (Fig. 3.3). A detailed description of the protocol for each step is provided in the sections that follow.

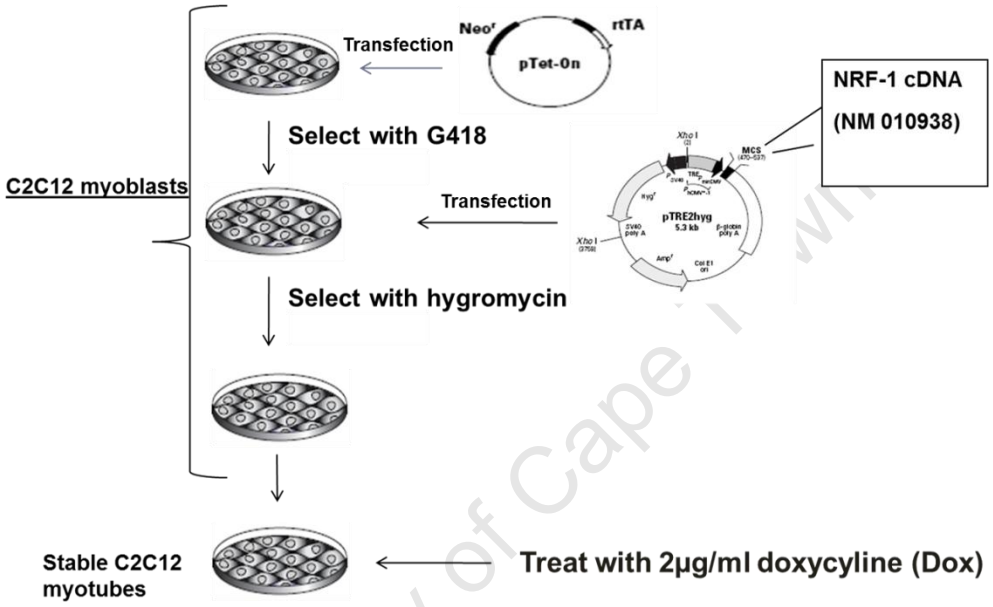

Figure 3.3: Tet-On transfection and selection protocol. A schematic diagram demonstrating the transfection protocol of Tet-On plasmids into C2C12 myoblasts to form the double-stable C2C12 Tet-On-NRF-1 cell line.

3.5.1. Transfection of pTet-On plasmids into C2C12: The C2C12 myoblasts were cultured in 6-well plates containing 2 ml growth medium (DMEM; 10% FBS/FCS; 1% PSF). When the cells were ~80% confluent they were transfected with 10 µg of either pTet-On or pTRE2hyg plasmids using the Fugene HD transfection reagent (Roche Diagnostics). The pTRE2hyg plasmid, which does not contain a neomycin-resistance gene, was used as a control.

Transfections were performed in 6-well plates using 3:2, 4:2 and 8:2 Fugene (µl) to DNA (µg) ratios in order to determine the ratio that induced the highest transfection efficiency. Transfection complexes were made by adding 10 µg of pTet-On or pTRE2hyg (control) plasmids into different microfuge tubes containing 15, 20, or 40 µl of Fugene HD and 0.5 ml of serum-free DMEM at room temperature (20 °C). C2C12 myoblasts were washed twice with 1X PBS at 37 °C, and 1 ml of serum free DMEM was added to the cells. After this initial incubation period, 1 ml of the Fugene: DNA transfection complex was added drop by drop into each plate and incubated overnight at 37 °C. Following overnight incubation the cells were trypsinised and transferred to 10-cm culture plates containing fresh growth medium.

3.5.2. Selection of Neomycin-resistant clones: To select C2C12 myocytes that had taken up the pTet-On, myoblasts were cultured in medium containing 500 µg/ml of G418 (neomycin analogue) for ~14 days. Medium was changed every 2 days. By day 14 the majority of cells that had been plated had died off but small clones of neomycin-resistant cells had begun to form at the bottom of the plate. Individual clones were isolated by trypsinisation inside cloning cylinders using manufacturer's protocol (Corning Inc., Massachusetts USA). Briefly, cells were washed twice with warm 1X PBS and cloning cylinders were gently placed onto the bottom of the culture plate to surround individual clones. Administration of a small layer of petroleum jelly onto the bottom edge of the cylinder allowed it to form a tight seal with the bottom of the culture plate. Trypsin/EDTA (200 µl) was added to the cylinder-isolated clones and incubated for 2 min at 37 °C to lift the cells. Growth medium (300 µl) was added to deactivate trypsin and the medium containing cells was collected and centrifuged at 2000 x g for 5 min at room temperature (20 °C). The supernatant was removed and the cells were re-

suspended in 1 ml growth medium. These cells, called Tet-On C2C12 cells were amplified by continuous culturing in growth medium and passaging, as described earlier. There were neomycin-resistant clones from C2C12 myoblasts that had been transfected using 3:2, 4:2, 6:2 and 8:2 Fugene: plasmid ratios.

3.5.3. Selection of Tet-ON C2C12 clones with high induction and low back ground: It is reasonable to assume that the isolated clones contained pTet-On plasmid because the plasmid confers neomycin resistance. However, it was still necessary to select clones that expressed rtTA. For this reason, we transfected the various Tet-On C2C12 clones with pTREhyg-Luc (Clontech, Inc), a response plasmid which expresses lucifarase when rtTA binds to its tetracycline response element (TRE) in the presence of Dox (7). An assay was conducted to quantify the amount of luciferase that the various clones expressed in the presence or absence (vehicle) of Dox. Briefly, some pTet-On C2C12 myoblasts from the various clones (obtained from various Fugene: plasmid ratios) were cultured in 6-well plates, and upon reaching 80% confluence the pTRE2hyg-Luc plasmid was transfected into Tet-On C2C12 cells according to the Fugene HD protocol for 6-well plates; and the cells incubated at 37 °C and 5% CO_2 for 6 h in serum-free DMEM. Transfection medium was then replaced by growth medium in the presence or absence of 2 µg/ml Dox and clones were maintained in this medium for 48 h. Cells were rinsed twice with 1X cold PBS, and 900 µl of a 1X Cell Culture Lysis Reagent (CCLR) was added onto the cells and the lysates scraped and transferred into microfuge tubes, and centrifuged at 12000 x g for 2 min at room temperature. The supernatants were transferred into new microfuge tubes. Ten µl of the supernatants were mixed with 50 µl of the luciferase reagent (Luciferase assay buffer; Luciferase assay substrate) in a 96-well plate. A luminometer, programmed to perform a 2 s delay and a 10 s read was used to measure

luciferase produced. The pTet-On clones that were transfected with 8:2 Fugene: plasmid transfection ratio indicated a 7-fold high induction with a low background compared to the controls (Fig. 3.4).

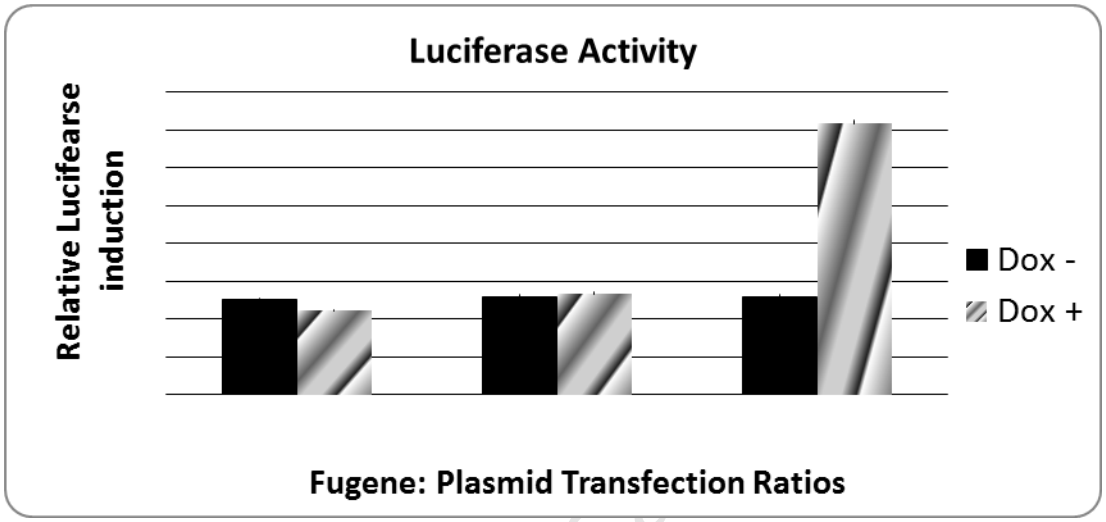

Figure 3.4: Luciferase activity graph for C2C12 cells transfected at various Fugene: plasmid ratios. C2C12-Tet-On clones transfected with pTRE2hyg-Luc plasmid were treated with 2 µg/ml doxycycline (Dox +) or vehicle (Dox -) for 72 h. Graph shows results from luciferase assay to determine clones that had effectively taken up the pTet-On plasmids. Note the clones that had 8:2 Fugene: plasmid transfection ratio had high luciferase activity under Dox and low background. This clone was selected for transfection of the second plasmid (pTRE2hyg-NRF-1).

3.5.4. Production of double-stable C2C12-Tet-On NRF-1 cells: The C2C12 Tet-On clones which had high expression of luciferase and low background i.e. the clones that resulted from a Fugene: plasmid transfection ratio of 8:2 was thereafter cultured in 6-well plates and transfected with either a pTRE2hyg-NRF-1 or a Tbx3-pGL3 plasmid using Fugene: plasmid transfection ratio of 8:2 as described earlier. Tbx3-pGL3 was used as a control vector because

it does not contain hygromycin-resistance genes. After overnight transfection, the cells were treated with 300 µg/ml hygromycin for a period of 10 days to select the double-stable C2C12-Tet-On-NRF-1 cells. The hygromycin-resistant clones were isolated and amplified as described for C2C12 Tet-On clones. Cells that were transfected with the Tbx3-pGL3 plasmid did not survive.

3.5.5. Determination of NRF-1 expression by C2C12-Tet-On NRF-1 clones: The isolated C2C12 Tet-On-NRF-1 clones were cultured in 10-cm culture plates until they were 80% confluent and thereafter differentiated into myotubes by use of 2% horse serum. The clones were maintained in differentiation medium until myotubes were well formed. Medium was changed every 2 days during the differentiation period. To determine the clones that expressed a high level of NRF-1 in response to 2 µg/ml Dox and a low level in the absence of Dox (background), differentiated myotubes were treated with 2 µg/ml Dox or equal volume of vehicle for 72 h and NRF-1 levels measured by western blot (Fig. 3.5) (see section 3.6 below). Clones with high NRF-1 expression in the presence of Dox and low background were amplified and used for this study. This cell line is referred to as a double-stable C2C12 Tet-On-NRF-1 cell line. A transfection efficiency assay was not performed for this study, rather the results of high luciferase and NRF-1 induction with low background after Dox treatment of each transfection step served as an indication and an indirect measurement of transfection efficiency that the majority of cells had taken up the transfected plasmids.

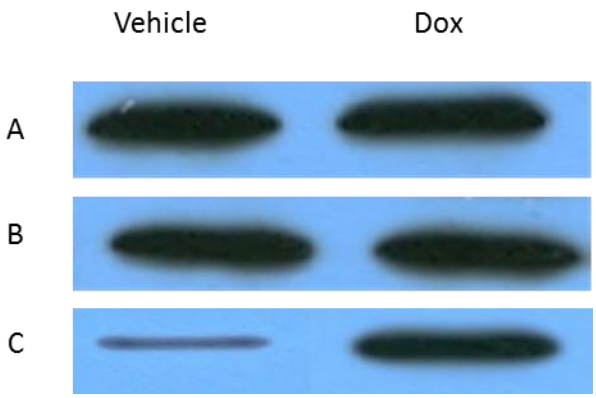

Figure 3.5: Blots showing NRF-1 expression in clones that were rejected or accepted. NRF-1 expression was measured in double-stable Tet-On-NRF-1 clones treated with 2 µg/ml doxycycline (Dox) or vehicle by immunoblotting. Clones A and B indicate rejected clones because NRF-1 induction was high in vehicle-treated cells (high back ground). The clone that was selected had low background and high NRF-1 induction in response to Dox (clone C).

3.6. Measurement of the contents of selected proteins in the C2C12-Tet-On-NRF-1 myotubes.

The purpose of this work was to investigate the mechanisms by which NRF-1 over-expression causes GLUT4 protein to increase in muscle cells. Therefore we assayed GLUT4, MEF2A and MEF2D levels in C2C12-Tet-On-NRF-1 cells that were treated with Dox or vehicle using western blot. Because it is well established that NRF-1 regulates the expression of a number of mitochondrial proteins, we also measured δ-ALAS to validate our experiments (The method is described below). Alpha tubulin (α-tubulin) was used as a loading control for the assays.

3.6.1. Cell harvesting and Protein concentration determination: C2C12-Tet-On-NRF-1 cells were cultured on 10 cm plates and differentiated into myotubes as described earlier. They were then treated with 2 µg/ml Dox for 3 days. The cells were thereafter harvested on ice by adding 300 µl of the radioimmunoprecipitation (RIPA) buffer [56 mM Tris-HCl, pH 7.4; 150 mM sodium chloride (NaCl); 1mM ethylenediaminetetraacetic acid (EDTA); 0.1% sodium dodecyl sulphate (SDS); 10 mM sodium pyrophosphate ($Na_4P_2O_7$); 20 mM sodium fluoride (NaF); 0.15 µM Okadaic acid; 4 mM sodium orthovanadate (Na_3VO_4); 1X Roche complete protease inhibitors (RCPI); 1% Triton-X100], and the lysates were transferred into 1.5 ml tubes on ice. Total protein concentrations for lysates were measured using the Bradford method. Briefly, 5 µl of lysates were added to 0.95 ml of the Bradford reagent [0.5% Coomassie G250 powder; 8.5% phosphoric acid (H_3PO_4); 4.7% Ethanol], mixed by vortexing, and incubated for 5 min. Light absorbance at 595 nm was measured by a spectrophotometer and the protein concentration was deduced from a standard BSA curve. The assay was performed in triplicates. An equal volume of the 2X Laemmli Sample Buffer (250 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 0.01% bromophenol blue) was added to the remaining lysate and frozen at -87 °C.

3.6.2. Western Blots: Frozen lysates were thawed on ice and 5% (v/v) of β-mercaptoethanol was added and boiled at 95 °C for 60 s. For GLUT4 assessment 5% (v/v) β-mercapto-ethanol was added but the sample was not boiled according to manufacturer's protocol. Proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 1X electrophoresis running buffer (0.25M tris base; 1.28M glycine; 1% SDS) at 120 V for 1 h at room temperature, followed by electrotransfer from gels to PVDF membranes in a transfer buffer (25 mM Tris base; 190 mM glycine; 20% methanol) overnight

at 30 V. The PVDF membranes were washed twice with TBS-T (Tris-buffered saline-0.1% Tween 20 pH 7.4) for 10 min and blocked in 5% non-fat milk in TBS-T for 60 min at room temperature. Membranes were incubated with primary antibodies (Table 3.1) in either 1% milk or 5% BSA in TBS-T overnight at 4 °C. Membranes were thereafter washed twice with 1X TBS-T for 10 min and incubated with horseradish peroxidise-conjugated anti-rabbit or anti-mouse secondary antibodies diluted at 1:2500 or 1:5000 respectively in 1% milk in TBS-T (Table 3.1) for 1 h at room temperature. After the secondary antibody incubation the membranes were washed twice for 5 min with TBS-T and treated for 3 min with Enhanced Chemiluminnescent substrate (Supersignal; West Pico), and the protein bands visualised on Kodak film.

Table 3.1: Antibody dilutions used for each target protein in western blots

Target Protein	Primary antibodies	1° Ab Dilution	Secondary antibodies	2° Ab Dilution
NRF-1	Anti-NRF-1	1: 2000	Anti-rabbit/HRP	1:8000
Δ-ALAS	Anti-δ-ALAS	1:500	Anti-mouse/HRP	1:5000
MEF2A	Anti-MEF2A	1:1000	Anti-rabbit/HRP	1:5000
MEF-2D	Anti-MEF-2D	1:1000	Anti-rabbit/HRP	1:5000
GLUT-4	Anti-GLUT4	1:2500	Anti-rabbit/HRP	1:2500

3.7. MEF2A silencing in C2C12-Tet-On-NRF-1 myotubes.

To ascertain whether NRF-1 over-expression increases GLUT4 expression via MEF2A, MEF2A-specific small interfering RNA (MEF2A-siRNA; Santa Cruz Biotechnology, Inc.) was used to block MEF2A expression. The siRNA degrades newly synthesised mRNA at a cytosolic level thus preventing mRNA translation for protein synthesis. C2C12-Tet-On-NRF-1 cells Myotubes were then transfected with MEF2A siRNA using the Fugene X-tremeGene transfection reagent (Roche Diagnostics, South Africa) as follows:

Cells were washed with 1X warm PBS and incubated with 4 ml serum-free DMEM for 20 min before 1 ml of transfection complex was introduced drop-wise. The transfection complex consisted of 10 µl transfection reagent: 1 µg MEF2A siRNA in 0.9 ml of serum-free DMEM. The cells were thereafter maintained at 37 °C in a 5% CO_2 incubator for 6 h. After the incubation period, the myotubes were maintained in differentiation medium containing 2 µg/ml Dox or vehicle for 72 h, harvested and analysed by western blot.

3.8. Chromatin Immunoprecipitation (ChIP) & Co-immunoprecipitation Assays.

The ChIP assays were performed to measure the binding of NRF-1 to its binding site on the *mef2a* gene and for assessing the binding of MEF2A to the *glut4* gene. C2C12-Tet-On-NRF-1 cells were cultured as above, differentiated into myotubes and treated with 2 µg/ml Dox or equivalent volume of vehicle for 72 h. A ChIP assay kit was used for these assays.

3.8.1. Formaldehyde cross-linking and sonication: After treatment with Dox or vehicle cells were incubated in medium containing 1% formaldehyde at 37 °C for 10 min to cross-link protein-DNA and protein-protein interactions. Cross-linking was stopped by adding 0.125 M glycine and incubating at 37 °C for 5 min. Cells were then washed twice with cold 1X PBS and lysed with SDS lysis buffer (1% SDS; 10 mM EDTA; 1X RCPI; 50 mM Tris, pH8.1) on ice. Cell lysates were transferred to microfuge tubes and sonicated on ice using MISONIX-Ultrasonic Liquid Processor (XL-2000 series) sonicator set at 6 watts. We found that 10 x 15 s bursts of sonication produced the desired DNA fragment size of 200-800 bp. Following sonication, lysates were centrifuged at 13 000 x g for 10 min at 4 °C, and the supernatants containing the cross-linked DNA-protein fragments were collected into 1.5 ml tubes. A Bradford assay was then performed to determine total protein concentration for each sample.

3.8.2. Pre-clearing: Aliquots of the supernatant containing 150 µg of total protein were diluted 10-fold with the ChIP dilution buffer (0.01% DSD; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.1; 167 mM NaCl). The final volume was ~1 ml for each sample. Endogenous immunoglobulins in the diluted supernatant were pre-cleared using Protein A agarose/salmon sperm DNA (50% slurry) for 1 h with rotation at 4 °C, followed by centrifugation at 2000 x g for 2 min. The resulting supernatants, called input (IN) samples, were collected into new 1.5 ml tubes. Fifty microliters (50 µl) of IN samples were stored at -80 °C and the remaining samples were used in immunoprecipitation experiments.

3.8.3. Immunoprecipitation of DNA-bound NRF-1, MEF2A and MEF2D proteins: The remaining input sample (~950 µl) was incubated with 5 µl antibodies directed against NRF-1, MEF2A or MEF2D at 4 °C with rotation for 36 h. For a negative control, a no-antibody immunoprecipitation was performed. Thereafter the antibody-bound protein-DNA complexes were immunoprecipitated by adding 50% slurry of Protein A agarose/salmon sperm DNA and incubating at 4 °C with rotation for 4 - 6 h followed by centrifugation at 2000 x g and 4 °C for 2 min. The supernatant was discarded but the pellet containing DNA-bound NRF-1, MEF2A or MEF2D proteins (depending on the antibody used) was washed with rotation for 5 min in 1 ml low salt (0.1% SDS; 1% TritonX-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 150 mM NaCl)) for 5 min and centrifuged for 2 min at 2000 x g. Immunoprecipitates were washed and centrifuged two more times as described above except when high-salt (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl, pH 8.1; 500 mM NaCl) and Lithium chloride [0.25 mM LiCl; 1% IGEPAL-CA630; 1% deoxycholic acid (sodium salt); 1mM EDTA; 10mM Tris-HCl, pH 8.1] buffers were used, respectively. Lastly, the precipitated complexes were washed 2X with T.E buffer (pH 8.0) for 5 min and centrifuged as above. Following centrifugation, 150 µl of the elution buffer [1% SDS; 0.1M sodium carbonate ($NaHCO_3$)] was added to the beads, rotated for 15 min at room temperature and centrifuged. The eluate which contained the protein-DNA complex was saved. The beads were eluted a second time as described above and the eluates from the two elutions were combined to form the immunoprecipitated (IP) sample.

3.8.4. De-crosslinking and protein digestion procedures: The IN and IP samples were reverse cross-linked by adding 0.2M NaCl and heating at 65 °C for 4 h. Following reverse-cross-linking, the proteins were digested by proteinase K by incubation in a buffer containing 10 µl of 0.5M EDTA, 20 µl Tris-HCl (pH 6.5) and 2 µl (10 mg/ml) Proteinase K at 45 °C for 1 h. The resulting solution contained DNA fragments that had been co-immunoprecipitated by the antibodies.

3.8.5. Phenol/chloroform DNA Extraction: To recover DNA from solution, equal volume of phenol was added to IP and IN samples and, rotated for 5 min at room temperature followed by centrifugation at 13 000 x g for 2 min at 4 °C. The supernatants were transferred into new 1.5 ml tubes and an equal volume of phenol/chloroform/isoamyl (25:24:1) was added, vortexed for 20 s, incubated with rotation for 5 min at room temperature and the supernatants collected into new tubes. An equal volume of chloroform/isoamyl (24:1) was then added to the supernatants, vortexed for 20 s and incubated for 5 min with rotation at room temperature, centrifuged (13 000 x g; 4° C) for 2 min. The supernatants were again transferred into new tubes and 2 µl of glycogen (20 mg/ml), 30 µl of 3 M sodium acetate (pH 5.2) as well as 2.5 volume of 100% ethanol (EtOH) were added and the mixture incubated overnight at -20 °C to precipitate DNA. The tubes were centrifuged at 13000 x g (4 °C) for 20 min until a pellet appeared. The supernatants were discarded and the DNA pellet rinsed with 500 µl of 70% EtOH and centrifuged again as above. The 70% EtOH was removed and the pellets air-dried and thereafter dissolved in 25 µl of sterilised distilled water. DNA from IP and IN samples were then amplified by Polymerase Chain Reaction (PCR) and analysed by 2% agarose gel electrophoresis.

3.8.6. Polymerase Chain Reaction (PCR) and Electrophoresis: PCRs were performed to quantify the binding of a) NRF-1 onto the *mef2a* gene and b) the binding of MEF2A onto the *glut4* gene in response to the various treatments. For assessment of MEF2A binding onto the *glut4* gene, a 25 µl mastermix consisting of 0.5 µM of forward and reverse primers (Table 2), 0.5U Taq DNA polymerase, 2 mM magnesium chloride ($MgCl_2$), 1X Thermo pol buffer (2.5 µl), 2 mM deoxynucleotide triphosphates (dTNPs) and 1 µg DNA from IN and IP samples were used. For assessment of NRF-1 binding to the *mef2a* gene the composition of the mastermix was identical as above except that 30 pmol for forward and reverse primers were used instead. Thirty four (34) PCR cycles were performed in an XP cycler PCR machine (Bioer) under the following conditions: one cycle of denaturation at 94 °C for 5 min and 33 cycles at 94 °C for 30 s, annealing at 63.5 °C for 30 s and the extension at 72 °C for 45 s, followed by final extension at 72 °C for 10 min. Separate PCRs with control primers that do not bind the NRF-1 binding domain on the *mef2* gene or MEF-2 *cis* element on the *glut4* gene were also performed as negative controls. Ten microlitres of PCR products and 5 µl of DNA marker were mixed with 2 µl of SYBR gold and loaded into wells of 2% agarose gels and electrophoresed at 100 V for 55 min.

Table 3.2: Primers used for ChIP assays

Primers	Amplicon size (bp)	Forward (F) & Reverse (R) Primer Sequences
NRF-1-*mef-2* (+ve)	315	F: 5' −CCT TCC TGT GCC GGG TGA TCT− 3' R: 5' −CTA TTT TTA GGA GTC AGG CCC GG− 3'
NRF-1-*mef-2* (-ve)	250	F: 5' −AGT TGT GCC ACC TGT CCC A− 3' R: 5' –CAA TGT CAG CTC ACA CTC A− 3'
MEF-2-*glut4* (+ve)	268	F: 5' −CAG GCA TGG TCT CCA CAT ACA− 3' R: 5' –GGT AAC TCC AGC AGG ATG ACA− 3'
MEF-2-*glut4* (-ve)	315	F; 5' – CCA ACA GCT CTC AGG CAT− 3' R: 5' –CCA TTC CAC AGG CAA GCA G− 3'

3.9. mRNA Analysis.

3.9.1. RNA Extraction: Differentiated C2C12-Tet-On-NRF-1 myotubes were incubated in medium containing with 2 µg/ml Dox or vehicle for 6 h. After the treatment, the medium was replaced by Dox-free medium and the myotubes were maintained in this medium for 0 h, 4 h or 12 h. Following this, cells were washed with cold 1X PBS and lysed with 400 µl of the lysis buffer (Roche Applied Sciences, RSA) and the lysates transferred into High Pure Filter tubes (Roche Applied Sciences, RSA) and centrifuged at 8 000 x g for 15 s at room temperature. The flow-through was discarded and 90 µl of the DNase was added to each filter and incubated at room temperature for 15 min. After 15 min, Buffer I was added to the filter and centrifuged for 15 s at 8 000 x g. The flow-through was again discarded and Buffer II was added to the filter and centrifuged as before. Buffer II was again added to the filter tubes

and centrifuged at 13 000 x g for 2 min and again the flow-through discarded. Fifty µl of elution buffer was finally added and the filter tube centrifuged at 8000 x g for 1 min and the flow-through which contained RNA, was then collected into a new microfuge tubes. The RNA was then stored at -80 °C for later use.

3.9.2. Determination of RNA concentration: Analysis of RNA concentration was performed in triplicates using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technology). RNA concentration was measured by placing 1 µl of the RNA onto a nanodrop and the RNA was considered pure when the A260/A280 nm ratio was between 1.9 and 2.1.

3.9.3. cDNA synthesis: One µg of RNA was mixed with 1 µl of oligo dT primer and made up to 5 µl with nuclease-free water. The mixture was then incubated at 70 °C for 5 min and thereafter put on ice for 5 min. The mastermix [1X reaction buffer (Promega); 0.5 mM deoxyribonucleotide triphosphates (dNTPs); 3.0 mM magnesium chloride (MgCl2); 20 U RNase inhibitor (Promega); 1X reverse transcriptase buffer (Promega)] was then added to synthesise cDNA from the RNA using InProm-II TM reverse transcription system (Promega A3800) according to the manufacturer's protocol. The cDNA aliquots were thereafter stored at -20 °C.

3.9.4. Real time quantitative PCR: Real Time quantitative PCR (qRT-PCR) was performed to measure relative GLUT4 and MEF2A mRNA using Sensimix lite kit (Quantace QT 405-05, USA) and a LightCycler Version 4 (Roche, Switzerland) RT-PCR machine. Nine microliters of PCR mastermix which contained 1X Sensimix reagent, 1X enzyme mixture, 1X SYBR

green as well as 0.5 μM of forward and reverse primers (Table 3.3) were used with 1 μl of cDNA. The assay was performed in duplicates using the following conditions: 1 cycle at 95 °C for 15 min, followed by 35 cycles at 95 °C for 5 s, 55 °C for 3 s, and 72 °C for 15 s. A melting cycle was also performed at 65 °C for 15 s followed by a cooling cycle at 40 °C for 30 s. A negative control without cDNA template was also included in every assay to assess background signal. The relative GLUT4 and MEF2A mRNA concentrations were determined based on the 2-ΔΔCt method (change in threshold cycle - Ct). Ct values for GLUT4 and MEF2A mRNA were normalised to values from glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 3.3 cDNA Primers

cDNA Primers	Amplicon size (bp)	Forward (F) & Reverse (R) Primer Sequences
mMEF2A	187	F: 5' –GTGTACTCAGCAATGCCGAC -3' R: 5' –AACCCTGAGATAACTGCCCTC -3'
mGLUT4	243	F: 5' –GCAGCGAGTGACTGGAACA -3' R: 5'-CCAGCCACGTTGCATTGTAG-3'
mGAPDH	100	F: 5'-GCACAGTCAAGGCCGAGAAT-3' R: 5'-GCCTTCTCCATGGTGGTGAA-3'

3.10. Assessment of MEF2A-MEF2D dimers.

To quantify MEF2A-MEF2D dimer in myotubes, C2C12-Tet-On-NRF-1 cells were cultured in 10-cm culture dishes and differentiated as described earlier (section 3. 5. 5). Differentiated myotubes were treated with 2 µg/ml Dox or vehicle for 72 h and harvested. On the day of harvesting, the cells were cross-linked, sonicated and pre-cleared as described in Sections 3.8.1 and 3.8.2. The resulting IN samples were immunoprecipitated with 6 µl of either anti-MEF2A or anti-MEF2D antibodies as described in Section 3.8.3. To check if all MEF2A protein was removed from the supernatant after MEF2A immunoprecipitation, the supernatant (Sup) was air-dried, diluted with 1X LSB and thereafter probed with MEF2A antibody. A similar process was done with regard to MEF2D immunoprecipitation. The immunoblots in figure 3.6 indicate that all of the MEF2A and MEF2D proteins were immunoprecipitated from the supernatants and collected into the pellets.

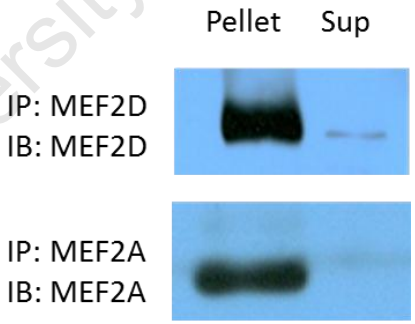

Figure 3.6: Analysis of MEF2A and MEF2D Immunoprecipitates. The western blots indicate MEF2A and MEF2D proteins were effectively immunoprecipitated.

Immunoprecipitated (IP) complexes were washed and reverse cross-linked (Section 3.8.3 and 3.8.4). Equal volume of the 1X Laemmli sample buffer as well as 5% (v/v) β-mercaptoethanol were added to the IP samples and heated at 95° C for 30 s. The samples were loaded in 7.5% polyacrylamide gel, electrophoresed at 120 V for 1 h and electro-transferred at 30 V overnight at 4° C. The membranes were washed with 1X TBS-T and blocked for 90 min with 5% milk in TBS-T at room temperature. This was followed by overnight incubation with MEF2A or MEF2D antibody in 1% TBS-T milk. The membranes were washed with 1X TBS-T for 10 min and incubated with 1:5000 HRP-conjugated anti-rabbit secondary antibody for 90 min at room temperature followed by visualising protein bands using the Kodak film (Amersham, USA).

3.11. Statistical Analysis

STATISTICA 10 software was used for all analyses. A student *t-test* was used to compare statistical difference between variables. The One-Way ANOVA was used to analyse statistical significant difference among different groups that had more than two variables. Where the One-Way ANOVA showed significant difference ($P \leq 0.05$) the Tukey's honestly post hoc test was conducted. All data are presented as means ± S.D.

CHAPTER 4

Results

4.1. Introduction.

The aims of the study were to: a) determine whether NRF-1 over-expression increases MEF2A and GLUT4 levels in skeletal muscle, and b) to examine if NRF-1 over-expression enhances MEF2A-MEF2D dimerization in C2C12 myotubes. Western blots were performed to measure MEF2A, δ-ALAS, GLUT4 and MEF2D expression following NRF-1 over-expression. Binding assays (ChIP) were also performed to measure NRF-1 binding to *mef2a* as well MEF2A binding to the *glut4* binding site. Co-immunoprecipitation (Co-IP) assays where C2C12-Tet-On-NRF-1 lysates were immnuoprecipitated with either MEF2A or MEF2D antibody and thereafter probed with either antibody to measure MEF2A-MEF2D dimerization in C2C12-Tet-On-NRF-1 myotubes were also performed. The schematic diagram below indicates the manner in which the results will be presented in this chapter.

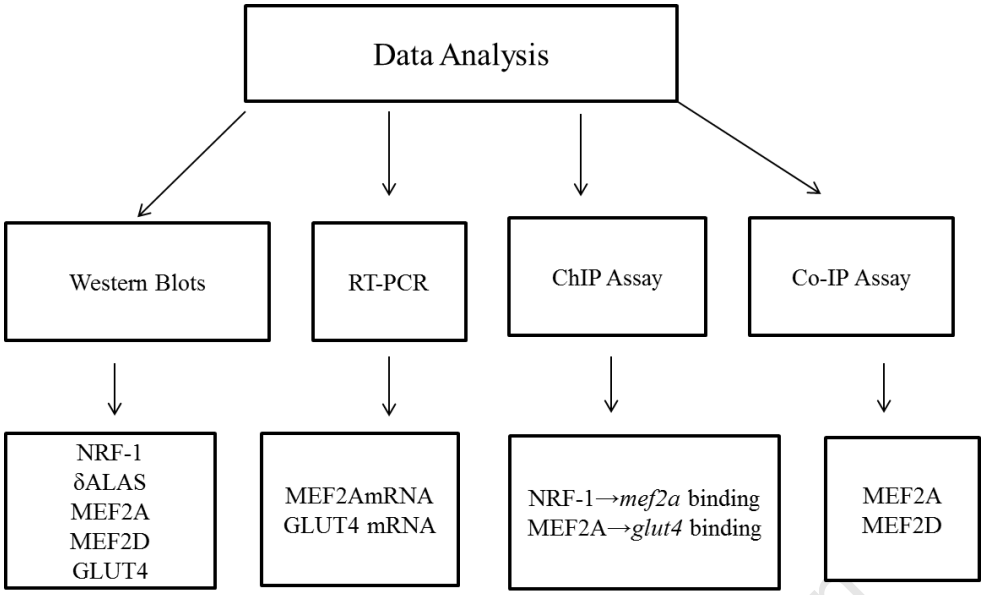

Fig 4.1: Analysis of Results. Western blots were performed to measure the protein levels after NRF-1 over-expression. RT-PCR was performed to analyse mRNA expression of MEF2A and GLUT4 at different time points. The ChIP assay was performed to measure NRF-1 binding to *mef2a* and MEF2A binding to *glut4*. Co-IP assay was done to MEF2A-MEF2D dimer formation in C2C12 myotubes

4.2. NRF-1 is over-expressed in C2C12-Tet-On-NRF-1 myotubes.

To test whether NRF-1 enhances GLUT4 expression via MEF2A, Tet-On plasmids (pTet-On & pTRE2hyg-NRF-1) were transfected into C2C12 myoblasts to form double-stable C2C12-Tet-On-NRF-1 to induce NRF-1 over-expression following treatment with 2 µg/ml of Dox for 72 h. To determine whether the double-stable C2C12-Tet-On-NRF-1 myotubes over-expressed NRF-1, the myotubes were treated for 72 h with 2 µg/ml Dox or vehicle. The cells were harvested and western blots performed to measure NRF-1 expression. The result shown in Figure 2A indicates that NRF-1 was over-expressed ~3-fold compared to the control (vehicle) treatment.

It is well established that NRF-1 over-expression induces δ-ALAS expression (17). Therefore, as a positive control for NRF-1 over-expression, δ-ALAS protein levels were measured in C2C12-Tet-On-NRF-1 myotubes treated with Dox or vehicle. The results (Fig 4.2B) demonstrate that there was a 2.8-fold ($p < 0.05$) increase in δ-ALAS protein levels in C2C12-Tet-On-NRF-1 myotubes following NRF-1 over-expression compared to control myotubes where NRF-1 was not over-expressed.

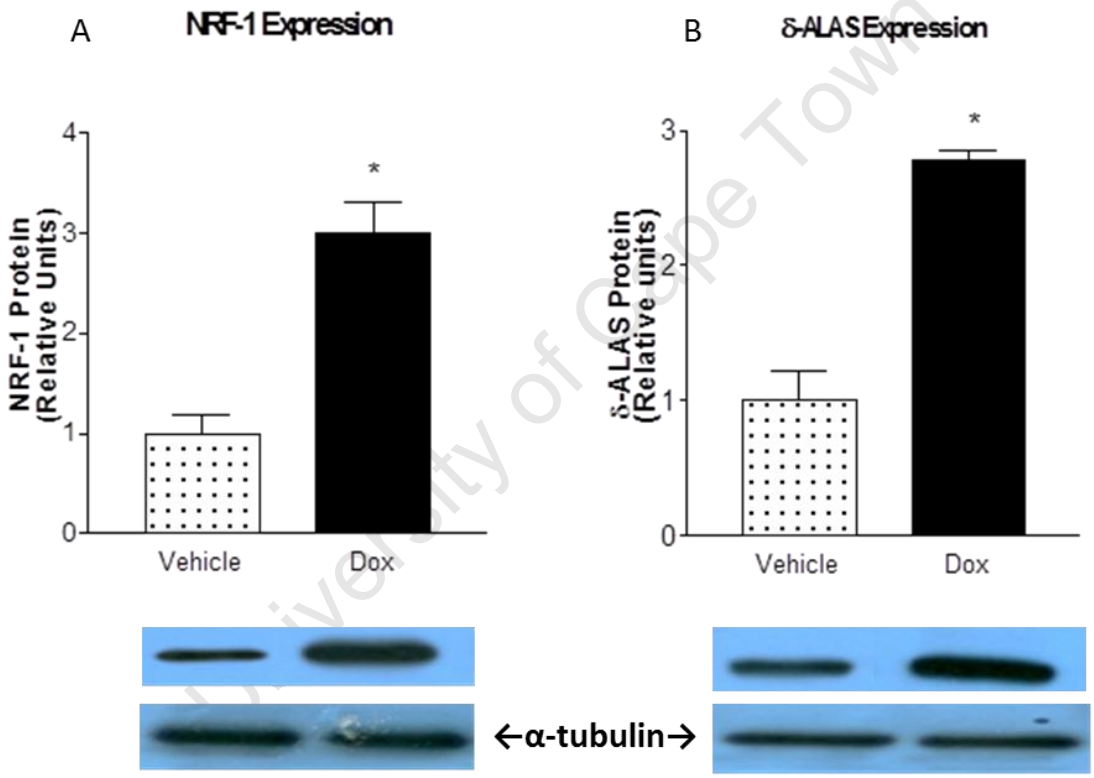

Figure 4.2: NRF-1 and δ-ALAS expression in C2C12-Tet-On-NRF-1 myotubes. C2C12-Tet-On-NRF-1 myotubes were treated for 72 h with 2 µg/ml of Dox or vehicle. Western blots were performed to measure NRF-1 (A) and δ-ALAS expression (B). Data presented as Means ± S.D (n=6). * $P < 0.05$ vs. vehicle.

4.3. MEF2A but not MEF2D is up-regulated following NRF-1 over-expression.

It is known that MEF2A and MEF2D are both involved in GLUT4 expression (38; 58). We therefore tested whether NRF-1 over-expression alters MEF2A and MEF2D in C2C12-Tet-On-NRF-1 myotubes treated for 72 h with 2 µg/ml Dox compared to those treated with vehicle. Western blots were performed to analyse protein expression of MEF2A and MEF2D. The results (Fig 4.3A) show that there was ~3.1-fold ($p < 0.05$) increase in MEF2A compared to the control. There was no change in MEF2D expression following NRF-1 over-expression. GLUT4 levels (Fig. 4.3B) were also increased ~2-fold following NRF-1 over-expression.

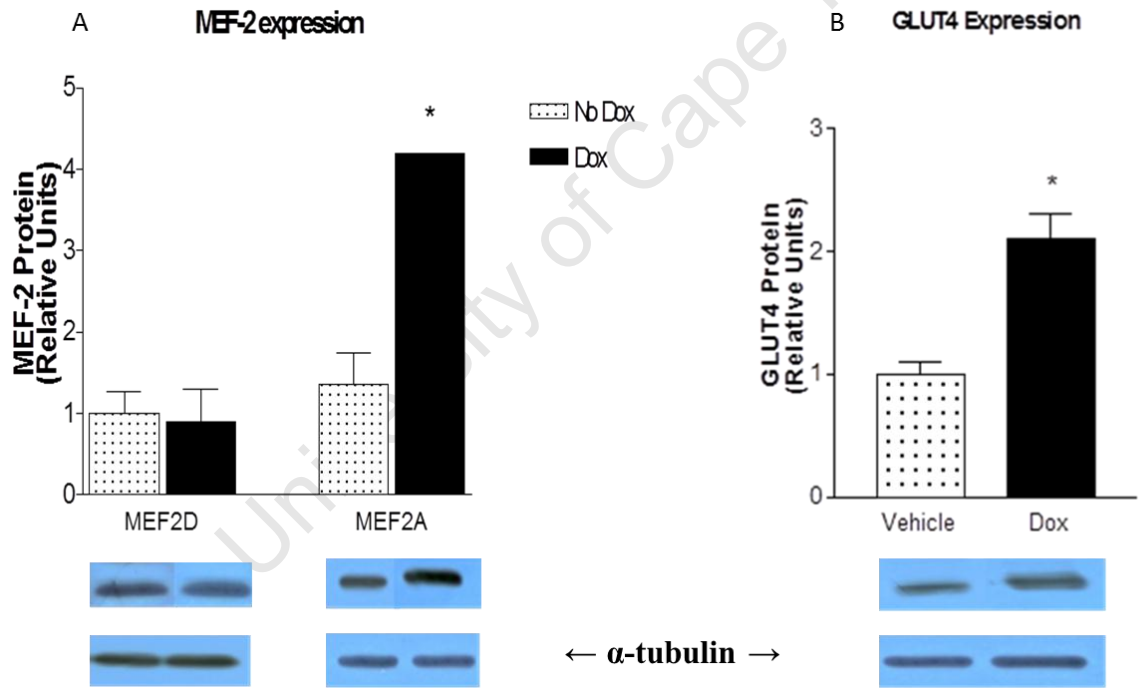

Fig. 4.3: MEF2A, MEF2D and GLUT4 expression in C2C12-Tet-On-NRF-1 myotubes. Protein levels of MEF2A, MEF2D and GLUT4 in C2C12-Tet-On-NRF-1 myotubes were analysed by western blotting after 72 h treatment with 2 µg/ml Dox or vehicle. Data presented as Means ± S.D (n = 4). * $P < 0.05$ Dox vs. vehicle.

4.4. NRF-1 over-expression increases GLUT4 via MEF2A.

To determine whether MEF2A is necessary for GLUT4 increase by NRF-1, C2C12-Tet-On-NRF-1 myotubes were transfected with 80 pmol of MEF2A-siRNA to silence MEF2A expression or control-siRNA which does not target endogenous mRNA. Thereafter transfected myotubes were treated with 2 µg/ml of Dox, or equivalent volumes of vehicle for 72 h. Western blots were performed to determine the expression levels of MEF2A. The graph below shows that Dox-treatment increased MEF2A protein levels ~ 3-fold ($p < 0.05$) in myotubes transfected with control-siRNA compared to MEF2A-siRNA-transfected myotubes (Fig. 4.4A). These results indicate that MEF2A was effectively knocked out by MEF2A-siRNA in Dox treated myotubes. Transfection with MEF2A-siRNA showed no statistical differences in MEF2A levels compared to wild-type C2C12.

It is known that MEF2A is an essential transcription factor for GLUT4 expression (58; 87). Since our results show that NRF-1 over-expression increases MEF2A which is required for GLUT4 expression, we measured GLUT4 levels in C2C12-Tet-On-NRF-1 myotubes that have been transfected with MEF2A-siRNA or control-siRNA, and treated them with 2 µg/ml Dox or vehicle for 72 h. Figure 4.4B shows that GLUT4 levels are increased ~2.1-fold ($p < 0.05$) in myotubes transfected with control-siRNA compared to wild-type C2C12 and myotubes transfected with MEF2A-siRNA. This therefore indicates that MEF2A is required for GLUT4 regulation, and that NRF-1 increases GLUT4 levels using MEF2A as an intermediary.

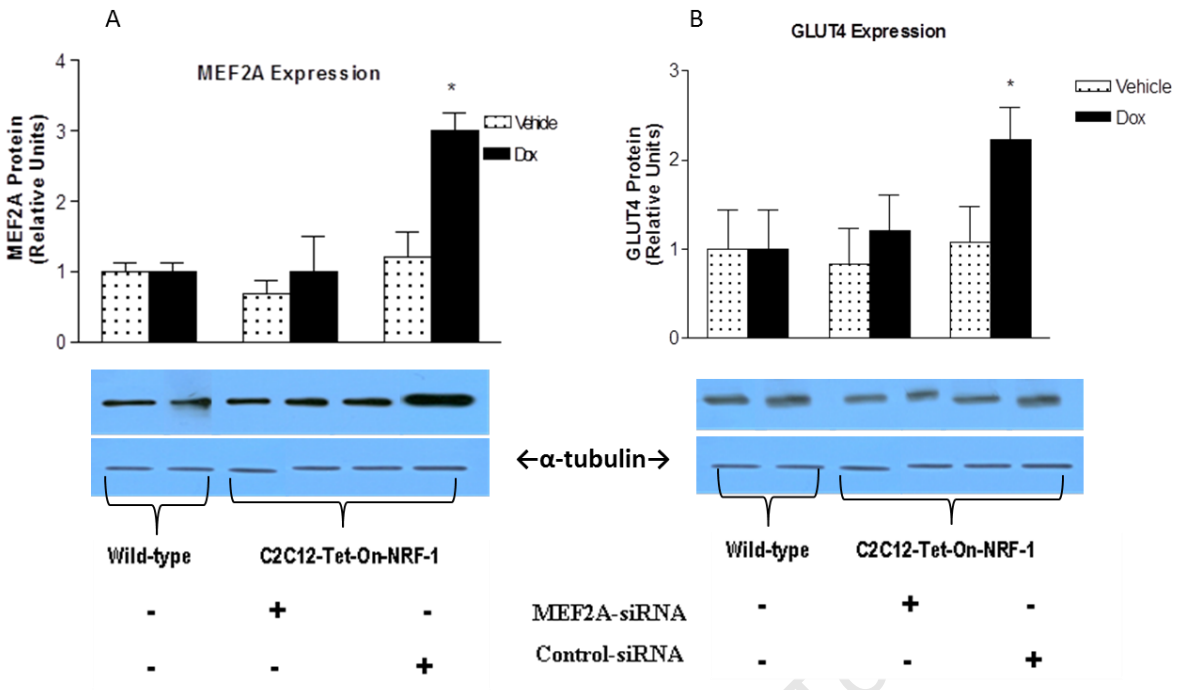

Fig 4.4: MEF2A and GLUT4 expression in C2C12-Tet-On-NRF-1 myotubes. Myotubes were transfected with MEF2A- or control-siRNA, and treated with 2 µg/ml Dox or vehicle for 72 h. Western blot was used to measure MEF2A (A) and GLUT4 (B) expression. Data are presented as Means ± S.D (n = 4). * $P < 0.05$.

4. 5. NRF-1 over-expression increases *mef2a*-bound NRF-1 and *glut4*-bound MEF2A.

To determine whether the increase in MEF2A and GLUT4 levels after Dox-treatment occurred as a result of increased interaction of NRF-1 and MEF2A transcription factors with the *mef2a* and *glut4* genes, respectively, ChIP assays were performed to measure *mef2a* promoter-bound NRF-1 and *glut4* promoter-bound MEF2A. Figure 4.5A demonstrates a ~ 3-fold ($p < 0.05$) increase in NRF-1 binding to the *mef2a* promoter and a 2-fold increase ($p < 0.05$) in MEF2A binding to the *glut4* promoter (Fig. 4.5B) after C2C12-Tet-On NRF-1 myotubes were treated with 2 µg/ml Dox for 72 h compared to vehicle-treated myotubes.

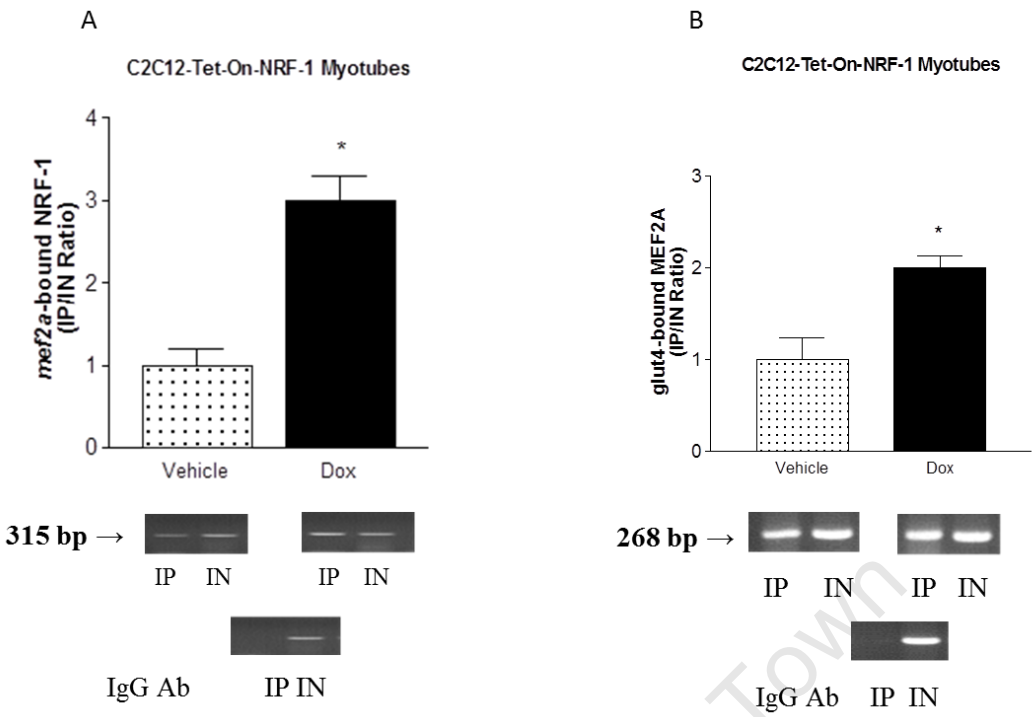

Figure 4.5: NRF-over-expression increases NRF-1 binding to the *mef2a* promoter and MEF2A-binding to the *glut4* promoter. C2C12-Tet-On-NRF-1 myotubes were treated with 2 µg/ml Dox or vehicle for 72 h. The cross-linked lysates were immunoprecipitated with NRF-1 (A) or MEF2A (B) or IgG antibody. The co-immunoprecipitated (IP) and total (IN) DNA were PCR amplified using appropriate promoters and electrophoresed using 2% agarose gel. Data Presented as Means ± S.D (n=4) * $P < 0.05$.

4.6. NRF-1 over-expression increases MEF2A and GLUT4 mRNA.

Quantitative RT-PCR was performed to measure MEF2A and GLUT4 mRNA following NRF-1 over-expression. The vehicle controls for MEF2A mRNA and GLUT4 mRNA represent the controls for each timeline as they all showed similar expression levels. Our results demonstrate that MEF2A mRNA was increased 2.5-fold ($P < 0.05$) 4 h post-Dox treatment and GLUT4 mRNA was increased by 50% ($P < 0.05$) 12 h post-Dox treatment.

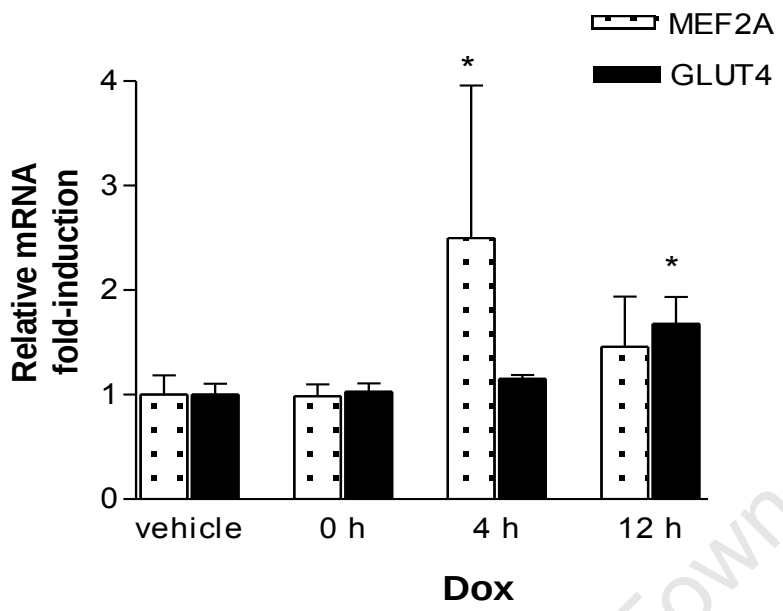

Fig. 4.6: NRF-1 over-expression increases MEF2A and GLUT4 mRNA. The graphs indicate the increase in MEF2A and GLUT4 mRNA at 4 h and 12 h post-Dox treatment respectively. * indicates significance ($P < 0.05$) compared to corresponding control (vehicle).

4.7. NRF-1 over-expression alters MEF2A-MEF2D dimer formation.

It is well established that MEF-2 isoforms bind to the *glut4* gene as MEF2A-MEF2A homodimer or MEF2A-MEF2D heterodimer in skeletal muscle. Since NRF-1 over-expression increased MEF2A but not MEF2D (Fig. 4.3A) in this study, we determined whether this change altered MEF2A-MEF2D dimer formation. C2C12-Tet-On-NRF-1 myotubes were treated with 2 µg/ml Dox or vehicle for 72 h and homogenized. MEF2A and MEF2D from 150 µl of the protein lysates were immunoprecipitated with excess (8 µl) MEF2A or MEF2D antibody for 36 h. The supernatants and immunoprecipitates were then collected and probed with MEF2A or MEF2D antibody.

4.7.1. Dox treatment increases MEF2A-MEF2D dimer formation: After immunoprecipitation with MEF2A, we analysed MEF2A and MEF2D levels in supernatants by western blot.

Panels A and C in figure 4.7 indicate that after immunoprecipitation with MEF2A and MEF2D antibodies respectively, there was minimal to no MEF2A and MEF2D proteins that were found in the supernatants. Panels B shows that there was co-immunoprecipitation of the MEF2D protein in lysates that were immunoprecipitated with the MEF2A antibody after NRF-1 over-expression as MEF2D protein is greatly reduced in the supernatant. Given that MEF2D was not changed by Dox treatment (fig. 4.3A) it is reasonable to conclude this treatment increased MEF2A/D dimer formation compared to vehicle treatment. Panel D shows similar protein levels of MEF2A in treated and untreated lysates that were immunprecipitated with MEF2D. These results are not surprising as NRF-1 over-expression after Dox treatment increases MEF2A (fig. 4.3A). Given that MEF2A was up-regulated by Dox, this conclusion implies that more MEF2A was co-immunoprecipitated by the MEF2D antibody, i.e. it indicated increased MEF2A/D dimer content.

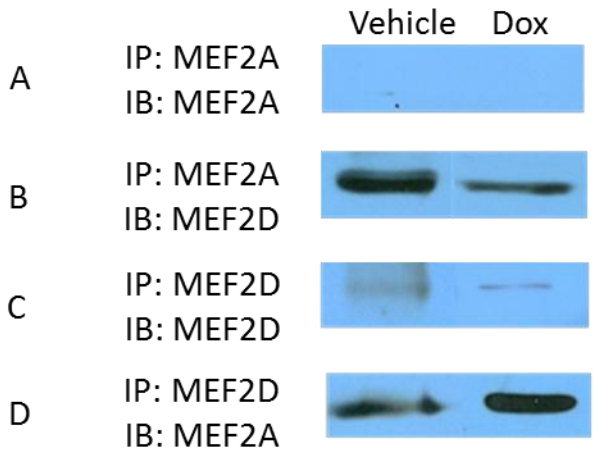

Figure 4.7: Western blots of supernatants after immunoprecipitation with MEF2A and MEF2D antibodies. C2C12-Tet-On-NRF-1 myotubes were treated with 2 µg/ml Dox or Vehicle, homogenized and immunoprecipited (IP) with excess MEF2A or MEF2D antibodies. Supernatants were then immunoblotted (IB) with MEF2A or MEF2D antibodies as indicated. Panel A shows a blot that was IP'd and IB'd with with MEF2Aindicating effective immuoprecipitation of MEF2A protein. Panel B shows a blot that was IP'd with MEFA and IB'd with MEF2D, indicating that some MEF2D protein was co-immunopricitated together with MEF2A after Dox treatment. Panel C shows a blot that was IP'd and IB'd with MEF2D antibody demonstrating MEF2D immunoprecipitation was effective. Panel D shows a blot that was IP'd with MEF2D and IB'd with MEF2A indicating association of MEF2D with MEF2A.

4.7.2. MEF2A and MEF2D co-immunoprecipitation increase MEF2A-MEF2D dimer formation after Dox treatment: When homogenates were immunoprecipitated with MEF2A antibody, the amount of MEF2D that was co-immunoprecipitated was increased by Dox treatment 2-fold relative to vehicle treatment (Figure 4.8A). Similarly, the amount of MEF2A that was co-immunoprecipitated with the MEF2D antibody was increased ~ 2.2-fold (Figure 4.8B). These results show that NRF-1 over-expression in C2C12-Tet-On-NRF-1 myotubes by Dox treatment increased MEF2A-MEF2D dimer formation.

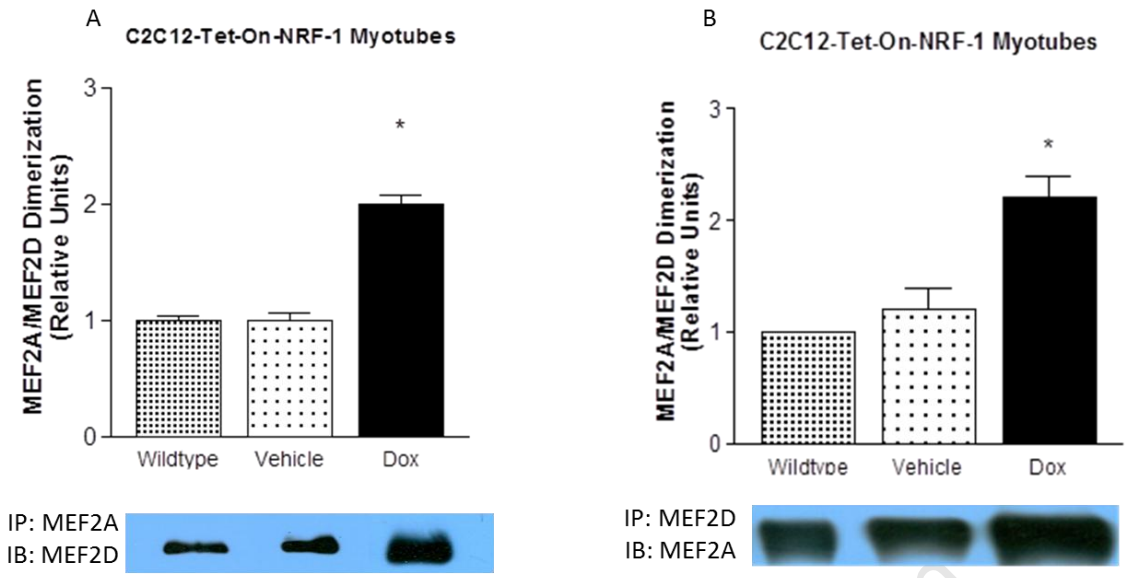

Figure 4.8: Dox treatment increases MEF2A-MEF2D dimer formation in C2C12-Tet-On-NRF-1 myotubes. C2C12-Tet-On-NRF-1 myotubes were treated with 2 µg/ml Dox or vehicle for 72 h. The lysates were immunoprecipitated with MEF2A (A) or MEF2D (B) antibody and immunoblotted with MEF2D or MEF2A antibody. Data are presented as Mean ± S.D (n=3) * $P < 0.05$.

CHAPTER 5

Discussion and Conclusion

5. 1. Summary of findings

The present study investigated the mechanism by which NRF-1 over-expression enhances GLUT4 expression by testing the hypothesis that it acts indirectly via MEF2A. Our results show that NRF-1 over-expression increases mRNA and protein levels of MEF2A & GLUT4, and protein contents of δ-ALAS but not MEF2D. Silencing of MEF2A using siRNA prevented the increase in GLUT4 expression following NRF-1 over-expression. ChIP assays indicated that NRF-1 over-expression increased NRF-1 binding to the *mef2a* gene, which was followed by increases in MEF2A mRNA and protein levels. Increased MEF2A binding onto the *glut4* gene also resulted in augmented GLUT4 mRNA and protein. Results of the present study are generally consistent with those from a previous study by Baar et al. (5), who over-expressed NRF-1 mice. However, we were only able to achieve a 3-fold induction of NRF-1 with our NRF-1 expression system relative to controls whereas Baar et al. (5) achieved ~10-fold increase. The relatively low induction of NRF-1 in our model, compared to Baar et al. (3), is possibly due to the different model systems used. Nevertheless, it is apparent that NRF-1 is a potent factor in both MEF2A and GLUT4 expression because our modest over-expression of NRF-1 still led to significant increases in both proteins.

In studies where GLUT4 mRNA and protein levels were measured at different time points, an increase was shown to occur between 6 h and 18 h after exercise or AMPK and/or CaMK activation treatments in muscle biopsies and cell culture models (40; 41; 60; 60; 101). Some of these studies further indicated that GLUT4 levels were unchanged after these time

periods. The time points that can be added to measure GLUT4 mRNA in this study can be up to 18 h, with 4 h intervals to determine if NRF-1 over-expression can further increase GLUT4 expression.

5. 2. MEF2A is a necessary intermediate in NRF-1-induced GLUT4 expression.

A study by Ramachandran et al. (71) showed that the *mef2a* gene has an NRF-1 binding site in its promoter region. They demonstrated that over-expression of NRF-1 significantly increased *mef2a* promoter activity in C2C12 myotubes whereas silencing NRF-1 expression had the opposite effect. These findings have provided strong evidence that NRF-1 is a potent regulator of MEF2A expression. The same authors also demonstrated that NRF-1 over-expression increases the expression of *cytochrome c oxidase (COX) 6* and *8* genes which have MEF2A but not NRF-1 binding domains (71). Based on these results, they concluded that the increases in COX 6 and COX 8 expression occurred as result of a transcriptional cascade involving NRF-1→MEF2A→COX V6/8 because the *mef2a* gene has an NRF-1 binding site at its promoter region, and silencing of NRF-1 significantly reduces MEF2A promoter activity. Further support for this conclusion came from Wan & Moreadith (94) who showed that mutations at the MEF-2 site on the *cox 6* gene diminished transcription of COX 6a in myotubes, demonstrating that the MEF-2 site is required for COX 6.

The results of the present study provide evidence that NRF-1 over-expression also regulates GLUT4 expression via a transcriptional cascade involving NRF-1 →MEF2A→GLUT4. Lines of evidence for the cascade include:: a) increased content of *mef2a* bound NRF-1 (Figure 4.2A), b) increased MEF2A mRNA and protein (Fig. 4.6 & Fig. 4.3A), c) increased *glut4*-bound MEF2A (Fig 4.5 B), d) increased GLUT4 mRNA and

protein (Fig. 4.6 & Figure 4.3B), and e) the fact that MEF2A silencing prevented GLUT4 up-regulation following NRF-1 over-expression (Figure 4.4B), and f) a time-line of increases in MEF2A and GLUT4 mRNA which are consistent with a transcriptional cascade (Fig. 4.6). Further evidence for a transcriptional cascade derives from a previous study by Mukwevho et al. (under review) who, using a Tet-On system to over-express NRF-1 in C2C12 myotubes, observed that NRF-1 over-expression precedes increases in MEF2A and GLUT4 proteins by ~6 h and 12 h, respectively.

The *mef2a* gene is also known to contain the MEF-2 binding site at its promoter region, and had been shown to undergo transcriptional auto-regulation (14; 72). MEF-2 auto-regulation has been shown to occur in differentiated muscle (14). With this in mind, it is therefore reasonable to assume that the increase in MEF2A mRNA and protein content in this study may have also resulted from this transcriptional auto-regulation.

5.3. Does NRF-1 co-ordinate the simultaneous expression of mitochondrial biogenesis and GLUT4 expression in response to exercise?

Studies have reported that exercise induces the expression of mitochondrial proteins and GLUT4 expression simultaneously (6; 89). However, the signalling mechanism responsible for synchronizing these events has not been defined. The finding from this study, showing that NRF-1 increases the expression of both GLUT4 and some mitochondrial proteins, provides evidence that NRF-1 may be part of the signalling pathway.

Although a number of pathways (e.g. AMPK, CaMK, calcineurin) have been shown to regulate GLUT4 expression via activation of GLUT4 transcription factors (e.g. MEF2A and GEF) (32; 33; 53; 65; 82), there are very few studies that have indirectly implicated other

mechanisms by which NRF-1 regulates GLUT4 expression. Exercise has been shown to also increase NRF-1 protein levels but the mechanism has not been elucidated (6; 93). One of the exercise-induced signalling pathways that has been implicated in increased NRF-1 levels and transcriptional activation for mitochondrial and GLUT4 biogenesis is the AMPK pathway (6; 9; 30; 61); however, the transcription factors that lead to increased NRF-1 in this pathway have not been identified. Other studies have shown that PGC-1α is a potent co-activator of NRF-1 and to a certain extent its expression during exercise or muscle contraction (18; 96; 98). Co-activation of NRF-1 by PGC-1α in these studies also indicated increased expression of NRF-1 target genes. These studies therefore demonstrate pathways by which NRF-1 can be activated and further expressed; therefore it is rational to suggest that the NRF-1→MEF2A→GLUT4 cascade can also be activated by PGC-1α. It should however, be taken into consideration that though PGC-1α is a potent NRF-1 co-activator, it has also been implicated in GLUT4 expression via MEF2C (51; 55). The signalling pathways can therefore be summarised in Figure 5.1, demonstrating the pathways that may lead to synchronised mitochondrial biogenesis and GLUT4 expression by NRF-1.

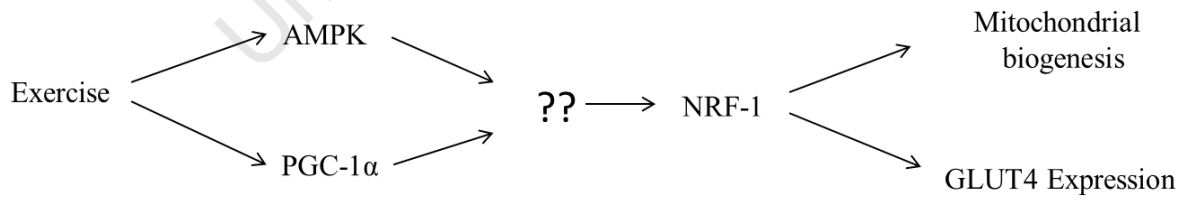

Figure 5.1: Possible pathways for simultaneous induction of mitochondrial biogenesis and GLUT4 expression.

5.4. NRF-1 as a potential drug target.

From the results obtained in this study, that NRF-1 over-expression increases GLUT4 in a cascade manner involving MEF2A, it can be suggested that NRF-1 becomes a target for drug intervention studies. Drug intervention studies for the treatment of metabolic diseases such as type 2 diabetes can also target to increase NRF-1 expression to activate the aforementioned transcriptional cascade for regulation of GLUT4 expression. Metmorfin and thiazolidinediones (e.g. rosiglitazone) are well known drug treatments for insulin resistance and hyperglycaemia by increasing insulin sensitivity, glucose transport and to some extent GLUT4 expression in insulin-responsive tissue (e.g adipose tissue and skeletal muscle) (1; 4; 35; 39). Recent cancer studies have shown that Tamoxifen and estradiol treatment in cancer cells increase NRF-1 expression (34; 50). A study by Ivanova et al. (34) has indicated that treatment of MCF-7 cells (breast cancer cells) with 4-Hydroxytamoxifen increases NRF-1 expression by activating and recruiting oncogenes such as cJun and cFos as well as estrogen receptor β (ERβ). Estradiol (E_2) was shown to target and increase NRF-1 at a transcriptional level in MC-7 cells (50). It would therefore be of interest to determine whether Tamoxifen does increase NRF-1 and the possibly GLUT4 in muscle cell lines, and if so, by which mechanism does this increase occur.

5. 5. What regulated MEF2A-MEF2D dimer formation?

It has been reported that MEF-2 factors bind to the GLUT4 promoter as a MEF2A-MEF2D heterodimer (58; 59). Since MEF2A but not MEF2D content was increased by NRF-1 over-expression, we were curious to know how MEF2A-MEF2D dimer formation was affected globally. Co-immunoprecipitation studies revealed that despite the absence of MEF2D

increase following NRF-1 over-expression, the MEF2A-MEF2D dimer content was nonetheless increased. The western blots performed to measure MEF2A and MEF2D protein levels after co-immunoprecipitation with MEF2A or MEF2D antibody revealed that MEF2D is expressed in relatively higher levels compared to MEF2A at basal conditions in differentiated C2C12 myotubes. This therefore made us speculate that MEF2A-MEF2D dimer formation for GLUT4 expression is dependent on the amount of MEF2A protein available. And since MEF2A appears less abundant in C2C12 myotubes compared to MEF2D, it is reasonable to assume that MEF2A is a limiting factor in MEF2A-MEF2D dimer formation. Another study that suggested that MEF2A may be a limiting factor for GLUT4 expression and MEF2A-MEF2D dimer formation is a study by Mora et al. (59), who demonstrated that MEF2A levels were lower while MEF2D content remained unchanged in cardiac muscle of mice with SZT-induced diabetes. Further studies need to be performed to demonstrate that MEF2A is indeed a limiting factor in MEF2A-MEF2D dimer formation.

5.6. Contribution of other MEF2 isoforms in GLUT4 expression.

Many authors have reported that GLUT4 expression is regulated by the MEF2A-MEF2D heterodimer (38; 52; 58; 59). Some authors have reported that MEF2C protein also binds to the *glut4* gene as a homodimer (38). The role of MEF2C in regulating GLUT4 expression is, however, controversial: Knight et al., (38) indicated that co-activation of MEF2C by PGC-1α increases GLUT4 content, but Miura et al. (56) have shown otherwise. In our study we did not measure the content of MEF2C or investigate its associated dimers because the *mef2c* gene does not have the NRF-1 binding site, and therefore is unlikely to be changed by NRF-1 over-expression. MEF2A-MEF2A homodimer was not measured in this study, as literature has mainly reported on MEF2A-MEF2D dimer formation for GLUT4 expression (52; 58; 59;

87). However, it would be of interest, to perform an experiment to determine the binding affinity of MEF2A-MEF2A and MEF2A-MEF2D dimer on the glut4 gene. The assay that can perhaps demonstrate the binding affinity of these dimers would be EMSA where the MEF2A antibody or both MEF2A and MEF2D antibodies are separately incubated with a radiolabeled MEF-2 consensus double stranded oligonucleotide that matches the MEF-2 binding site on the glut4 gene. The MEF-2 dimers that display a supershift in EMSA will indicate the MEF-2 dimer that has a higher binding affinity on the MEF-2 site found on the glut4 gene.

5. 7. Summary and future experiments.

Firstly, we have shown that NRF-1 over-expression increases GLUT4 levels via a transcriptional cascade involving NRF-1→MEF2A→GLUT4. Secondly, we have provided evidence suggesting that MEF2A content is one of the factors that limit MEF2A-MEF2D formation in C2C12 myotubes.

This study has demonstrated a crucial mechanism by which NRF-1 increases GLUT4 expression. And though NRF-1 acts via MEF2A for GLUT4 upregulation, MEF2A does not singularly increase GLUT4 expression, but rather requires GEF for activation of GLUT4 expression (33; 38; 87). A study by Knight et al. (38) reported that GEF and MEF2A form a protein-protein complex for GLUT4 expression. Another study by Sparling et al. (83) demonstrated that MEF2A physically interacts with GEF to increase its binding affinity to Domain I on the *glut4* gene. It is therefore suggested that future work for the current study include immuprecipitation assays where C2C12-Tet-On-NRF-1 lysates be immunoprecipitated with the MEF2A antibody and co-immunoprecipitated with the GEF

antibody to determine if NRF-1 over-expression also increases MEF2A-GEF protein interaction.

Because artificial methods of increasing NRF-1 in muscle have indicated that NRF-1 increases GLUT4 content and improves glucose clearance capacity (5) (and current study), further work needs to be conducted to investigate natural methods such as exercise or phytochemicals of increasing NRF-1 in skeletal muscle in vivo as mentioned ealier in the discussion. These methods might find value in the treatment of insulin resistance and type II diabetes mellitus.

Future work should also investigate the effects of NRF-1 over-expression on MEF2A-MEF2D binding to the *glut4* gene. We were unable to perform this assay because we did not have the necessary technology. The limitation of the ChIP assay in this study is that it primarily showed a global increase in MEF2A-MEF2D dimerization following NRF-1 over-expression. The assays used for this study were however, unable to demonstrate increased MEF2A-MEF2D binding on the MEF-2 binding site of the *glut4* promoter. As previously mentioned, EMSA will be a preferable assay that can be performed for future work to demonstrate increased MEF2A-MEF2D dimer formation on the *glut4* gene after NRF-1 over-expression. The assay will not only explicitly demonstrate MEF2A-MEF2D dimer formation on the *glut4* promoter which the ChIP assay could not achieve, but it will also measure the migration pattern of MEF2A-MEF2D complex on the *glut4* gene between Dox treated and control groups thus indicating the binding affinity of the dimer complex on the *glut4* promoter. Measuring glucose uptake after NRF-1 over-expression will also be further evidence that NRF-1-induced GLUT4 expression also results in increased glucose uptake. The factors that influence dimer formation between the various MEF-2 proteins also merit further investigation.

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