Do factors secreted from synovial fibroblasts affect the differentiation of C2C12 cells?

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ABBREVIATIONS

SF=synovial fibroblast

HS= horse serum

FBS = Foetal Bovine Serum

DKK1 = dickkopf-related protein 1

TNF-α = Tumour necrosis factor-alpha

11βHSD1 = 11β-hydroxysteroid dehydrogenase type 1

RA = Rheumatoid arthritis

RASF = Rheumatoid arthritis synovial fibroblasts

Dex = dexamethasone

Fz = frizzled receptor

TCF = transcription factor

LEF = lymphoid enhancing factor

LRP5/6 = lipoprotein-related 5 and 6

dNTP = Deoxyribonucleotide

RNA = Ribonucleic acid

mRNA = messenger ribonucleic acid

ABSTRACT

The Wnt signalling pathway plays a key role within muscle differentiation. Wnt3a, Wnt5a, and DKK1 all have pivotal roles within this pathway. It's hypothesised that due to their role within the Wnt pathway, Wnt3a, Wnt5a, and DKK1 will affect the differentiation of muscle, demonstrated by the murine C2C12 cell line.

Differentiation of the C2C12 cells was induced by adding DMEM differentiation media at day 0. Treatments (Wnt3a, Wnt5a, DKK1, control conditioned media, TNF-α conditioned media, and dexamethasone conditioned media) were added day 0, and mRNA levels of differentiation markers, MyoD, myogenin, α-actinin, and 11βHSD1 were measured using RT-PCR at days 1, 3 and 6. Wnt3a and control conditioned media gave no significant change in differentiation. Wnt5a, DKK1, TNF-α conditioned media and dexamethasone conditioned media gave significant decreases in differentiation. DKK1 inhibitor was tested on cells treated with TNF-α conditioned media, resulting in the decrease in the differentiation no longer being significant. 11βHSD1 enzyme activity assays were carried out to test Wnt3a, Wnt5a, DKK1, and DKK1 inhibitor effects, the results followed the trend of the mRNA data, however were not statistically significant. The results suggest that factors secreted from synovial fibroblasts during inflammation affect muscle differentiation.

CONTENTS

1 INTRODUCTION ... 9

1.1 Rheumatoid arthritis ... 9

1.2 The role of TNF-α in rheumatoid arthritis ... 11

1.3 Glucocorticoids ... 12

1.4 Wnt pathway ... 12

1.5 Muscle differentiation ... 15

1.6 Hypothesis and aims ... 16

2 METHODS ... 17

2.1 C2C12 ... 17

2.2 C2C12 cell culture ... 17

2.3 Preparation of C2C12 cells for treatment ... 18

2.4 C2C12 Cell treatment ... 18

2.5 RNA extraction ... 19

2.6 Assessment of RNA quality and concentration ... 20

2.7 Reverse transcription ... 20

2.8 Real time (RT) PCR ... 20

2.9 Analysis of relative real-time PCR data ... 21

2.10 11β-HSD1 enzyme activity assay ... 21

2.11 Statistics ... 22

3 RESULTS ... 23

3.1 Myocyte differentiation to myotubules ... 23

3.2 Myocyte differentiation marker mRNA levels during differentiation ... 24

3.3 The effects of Wnt3a, Wnt5a, or DKK1 treatment on the morphology of differentiating C2C12 myoblasts 26

3.4 The effects of control SF-conditioned media, TNF-α SF-conditioned media, or dexamethasone SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts 29

3.5 The effects of Wnt3a, Wnt5a, and DKK1 treatment on myocyte differentiation markers in differentiating C2C12 myoblasts 32

3.6 The effects of control SF-conditioned media, TNF-α SF-conditioned media and dexamethasone SF-conditioned media on the differentiation markers in differentiating C2C12 myoblasts 37

3.7 The effect of DKK1 inhibitor treatment on reversing the effects of TNF-α SF-conditioned media treatment on differentiating C2C12 myoblasts 42

3.8 11βHSD1 activity assay testing the effects of Wnt3a, Wnt5a, and DKK1 on treatment on the differentiation of C2C12 myoblasts 47

3.9 11βHSD1 activity assay testing the effect of a DKK1 inhibitor on reversing the effects seen with TNF-α SF-conditioned media treatment of differentiating C2C12 myoblasts 48

4 DISCUSSION 50

4.1 Myocyte differentiation to myotubles 50

4.2 Effect of Wnt3a on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels 51

4.3 Effect of Wnt5a on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels 52

4.4 Effect of DKK1 on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels 53

4.5 Effect of control SF-conditioned media on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels 54

4.6 Effect of TNF-α SF-conditioned media on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels 54

4.7 Effect of Dexamethasone conditioned media on 11βHSD1, MyoD, Myogenin and a-actinin mRNA levels 55

4.8 The effect of DKK1 inhibitor on reversing the effects of TNF-α conditioned media55

4.9 Conclusions and future work 56

5 REFERENCES 58

TABLE OF FIGURES

Figure 1 Schematic of normal and arthritic joints. 9

Figure 2 Schematic of the canonical Wnt pathway. 14

Figure 3 Muscle differentiation 16

Figure 4 Morphological changes during myocyte differentiation. 23

Figure 5 mRNA levels of key markers of myocyte differentiation. 25

Figure 6 The effects of DKK1 treatment on the morphology of differentiating C2C12 myoblasts 27

Figure 7 The effects of Wnt5a treatment on the morphology of differentiating C2C12 myoblasts 28

Figure 8 The effects of TNF-α SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts 30

Figure 9 The effects of dexamethasone SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts 31

Figure 10 mRNA level of α-actinin following treatment. 33

Figure 11 mRNA level of 11βHSD1 following treatment. 34

Figure 12 mRNA level of MyoD following treatment. 35

Figure 13 mRNA level of key myogenin following treatment. 36

Figure 14 mRNA levels of 11βHSD1 following treatment. 38

Figure 15 mRNA levels of α-actinin following treatment 39

Figure 16 mRNA levels of MyoD following treatment. 40

Figure 17 mRNA levels of myogenin following treatment. 41

Figure 18 Effect of DKK1 inhibition on TNF-α conditioned media induced changes in C2C12 differentiation markers. 43

Figure 19 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (MyoD). 44

Figure 20 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (α-actinin). 45

Figure 21 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (Myogenin). 46

Figure 22 11βHSD1 activity assay testing the role of Wnt3a, Wnt5a, and DKK1 effect on C2C12 muscle differentiation..47

Figure 23 11βHSD1 activity assay to test the effect of a DKK1 inhibitor on reversing the effects of TNF-α SF-conditioned media on C2C12 cell differentiation. 49

1 INTRODUCTION

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease, which primarily affects the synovial joints leading to their inflammation of the synovial membrane[1]. The most common joints affected are the hands, feet and wrists[4]. As well as causing inflammation RA can also cause extreme weakness in the muscles and tendons and could also affect other organs[3]. RA is a chronic disease which, if left untreated, could result in destruction of joints from erosion of cartilage and bones leading to deformity. The disease usually progresses from the periphery to more proximal joints, and if treatment is unsuccessful RA could lead to significant long-term locomotor disability within 10 to 20 years.

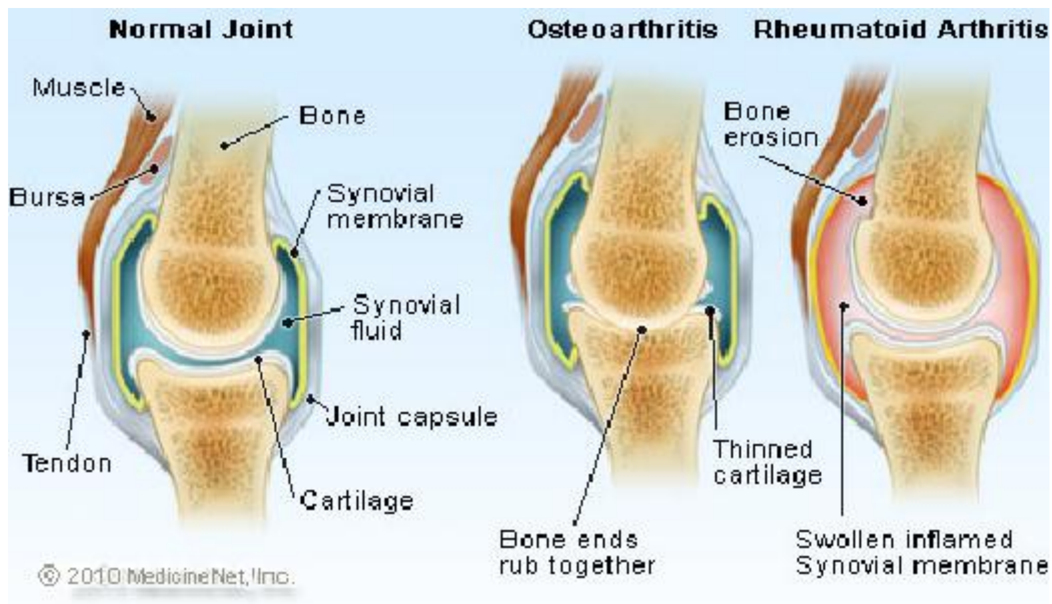

Figure 1 Schematic of normal and arthritic joints.
A comparison between normal joints, and joints suffering from either osteoarthritis or rheumatoid arthritis. The joint which is suffering from rheumatoid arthritis has a swollen and inflamed membrane and also some bone erosion. This will lead to pain during movement. Cited from, www.bestclocosamine.co.uk/rheumatoidartheritis

Around 580,000 people in England are affected by RA which is more common in females. The disease occurs most commonly in people aged between 40 and 60 years, however RA could occur at any time. Overall RA represents one of the most common autoimmune-related diseases, affecting as much as 1% of Western populations[4].

The main symptoms of RA include swelling, soreness and stiffness within the joints. Bone degradation can also occur and this can lead to a lack of movement in the joint, and muscle wasting within the surrounding area. Due to the systemic nature of RA it could affect whole body and internal organs, including the lungs, heart, and eyes.

Muscle weakness and muscular atrophy occur early in the onset of RA[2]. The exact cause of this muscle atrophy is not yet known. However, the loss of muscle is clearly not solely due to the inactivity resulting from reduced mobility of the joints due to pain[2]. Although this may be a contributing factor.

Rheumatoid cachexia, which is a loss of muscle mass and strength and an increase in fat mass is very common in patients with RA[5]. Following successful treatment for RA it has been shown that rheumatoid cachexia persists even after joint inflammation improves. This adds further evidence to the concept that lack of muscle tone is not solely due to inactivity. Rheumatoid cachexia may also be an important risk factor for cardiovascular disease and excess mortality in RA[5].

RA is an autoimmune disease and as a consequence of this there is a strong involvement of immune cells. Due to this macrophages, T-cells and their cytokines have a critical role to play

in RA[4] where they infiltrate the synovial joints[8]. Recently it has been suggested that resident fibroblast-like cells make a major contribution to the perpetuation of the disease, and could also have a role to play in the initiation[4]. The synovial fibroblasts involved in rheumatoid arthritis (RASFs) represent a unique cell type, and themselves set RA apart from other inflammatory conditions of the joints[4]. Many studies have been undertaken on RASFs and these have demonstated that RASFs show alterations in morphology and behaviour. These alterations include molecular changes in signalling cascades, apoptosis responses and in the expression of adhesion molecules as well as matrix-degrading enzymes[4]. As a result of these findings RASFs are believed to play a pivotal role in the symptoms of RA.

1.2 The role of TNF-α in rheumatoid arthritis

TNF-α is found within synovial joints affected by RA[22], it is hypothesised that TNF-α causes the secretion of factors from synovial fibroblasts (SF) which leads to a decrease in muscle differentiation.

TNF-α causes an increase in the inflammatory response of RA[6]. It is a pro-inflammatory cytokine produced by macrophages and T cells, that in RA leads to synovitis and joint destruction[7]. This knowledge of the effect of TNF-α has led to the development of anti-TNF-α therapies which are commonly given to patients who are suffering with RA for example disease-modifying anti-rheumatic drugs (DMARDs). Chronically elevated levels of TNF-α is a catabolic factor that mediates cachectic muscle wasting[9].

1.3 Glucocorticoids

It is hypothesised that dexamethasone causes the secretion of factors from synovial fibroblasts (SF) which leads to a decrease in muscle differentiation.

Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid drugs[11]. It is often given as a treatment for RA due to it acting as an anti-inflammatory and immunosuppressant[11]. Despite these positive effects of Dexamethasone an excess of glucocorticoids (GC) can lead to undesirable side effects such as muscle atrophy, osteoporosis, and diabetes[12].

11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) is an enzyme which is NADPH dependent. It is most highly expressed in the key metabolic tissues, for example the liver, adipose tissue, and the central nervous system. 11βHSD1 functions by converting inactive cortisone into its active form, cortisol, via a reduction reaction. This cortisol then activates glucocorticoid receptors. Within this study 11βHSD1 is primarily used as a muscle differentiation marker.

1.4 Wnt pathway

The Wnt pathway affects may processes within a system due to control of the expression of many target genes. The Wnt pathways effect on muscle differentiation will be studied using the C2C12 cell line.

The Wnts are a family of glycoproteins characterised by several conserved cysteine residues[16]. They act in both autocrine and paracrine ways in order to regulate adult tissue homeostasis and remodelling by initiating a signal transduction cascade[14]. Wnts also control essential developmental processes such as embryonic patterning, cell growth, migration, and differentiation. If there are mutations or errors within the Wnt pathway then the target genes will be unable to be transcribed, which leads to abnormal Wnt signaling.

There are two processes by which Wnt signalling occurs. These are the canonical and the non-canonical pathways[16]. The canonical pathway begins with the ligand binding to the seven-transmembrane Frizzled receptor and the co-receptor lipoprotein-related 5 and 6 (LPR5/6)[13]. Binding triggers signals which lead to the increase of β-catenin levels within the cytoplasm and its dephosphorylation[15]. β-catenin is a co-activator which works in conjunction with the TCF/LEF family of transcription factors. β-catenin translocates to the nucleus where it activates the downstream Wnt-responsive genes[13].

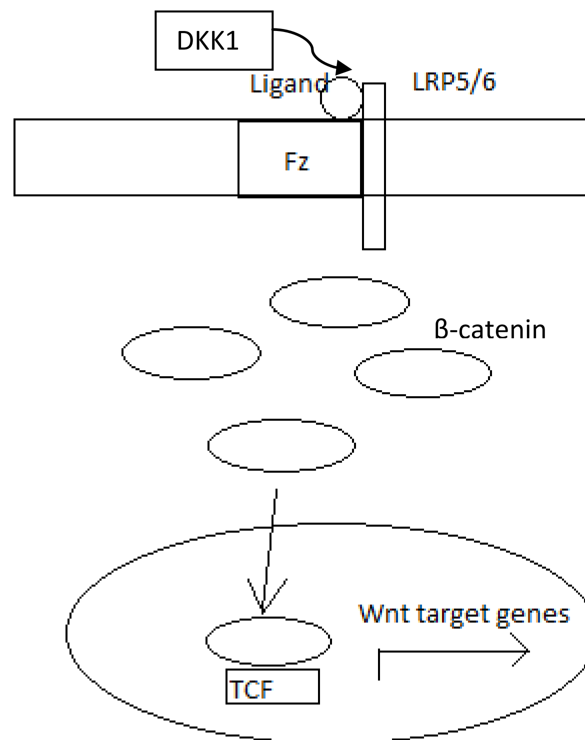

Figure 2 Schematic of the canonical Wnt pathway.
Wnt interacts with the frizzled (Fz) receptor and the co receptor LRP5/6 on the cells surface. This leads to a cascade where β-catenin is increased in expression. The β-catenin is then transported into the nucleus where it interacts with transcription factor (TCF) resulting in the increased expression of Wnt target genes. DKK1 is able to bind to Lipoprotein-related 5 and 6 (LRP5/6) preventing the formation of the LRP5/6, Fz and ligand complex.

The non-canonical pathway is diverse and is mediated through various signalling molecules such as MAP kinase and Protein kinase C. These kinases induce rapid cellular responses such as alteration in cell shape. If neither of these signalling pathways are activated then the un-stimulated β-catenin is phosphorylated by a complex of proteins, and causes the β-catenin to be tagged for degradation by the proteasome.

Dickkopf-related protein 1 (DKK1) and Wnt5a are both inhibitors of the Wnt pathway. Within the study their effect on muscle differentiation will be analysed. DKK1 is a secreted

protein[13] which in humans is encoded by the DKK1 gene, it is the prototype of a family of secreted proteins which are structurally unrelated to Wnt or frizzled[14]. DKK1 contains two cysteine rich regions and is involved in embryonic development. DKK1 interacts with LRP5/6[15] to form a ternary complex with another receptor, kremen. Once this ternary complex is formed there is endocytosis[14] of the complex and therefore the consequential removal of LRP5/6 from the cell surface Another way which has been proposed for the inhibition of the Wnt signalling pathway by DKK1 is that it binds to LRP6 thereby disrupting the Wnt-induced frizzled-LRP6 complex formation.

Wnt5a suppresses β-catenin/TCF-dependent transcriptional activity and also leads to down-regulation of the expression of cyclin D1, which is a downstream target gene of the canonical Wnt signalling pathway. Wnt5a has been classified as a non-canonical Wnt family member[18]. Wnt5a inhibits Wnt3a protein-induced reporter gene expression[18]. Despite being shown to function as a suppressor, Wnt5a can also activate β-catenin signalling in the presence of the appropriate Frizzled receptor, Frizzled 4[18].

Wnt3a activates the Wnt/β-catenin signalling pathway[24], thereby acting as a positive regulator of the canonical Wnt pathway. Wnt3a induces transcription of the LEF-1 promoter through both β-catenin-dependent and LEF-1-independent mechanisms[24].

1.5 Muscle differentiation

Muscle differentiations occurs in three stages, from myoblast to myocyte, to myotuble and finally to myofibre at terminal differentiation. The understanding of muscle-cell commitment and differentiation has been rapidly advanced with the discovery of a group of transcription

factor proteins referred to as the myogenic regulatory factors (MRFs)[25]. The primary MRFs, Myf5 and MyoD, are required for the formation, propagation, and survival of skeletal myoblasts[25]. Both Myf5 and MyoD are required for the development of myoblasts to myocytes. MyoD is also required for the conversion of the myocytes to myotubles. The latter process involves the permanent cessation of DNA synthesis, activation of muscle-specific gene expression, and the fusion of single cells into multinucleated muscle fibres[26].

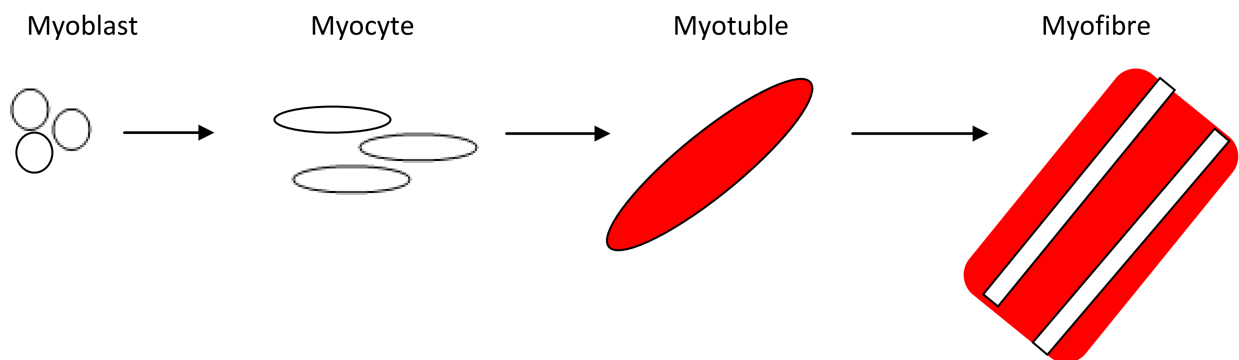

Figure 3 Muscle differentiation
Muscle differentiation occurs in three stages. Firstly the myoblast differentiates into a myocyte utilising Myf5 and MyoD. MyoD then contributes to the development of myocytes to myotubles, and finally myotubles form terminally differentiated myofibres.

1.6 Hypothesis and aims

The hypothesis for the experiment were as follows:

1. Substances present during joint inflammation (DKK1, Wnt5a, Wnt3a) affect muscle differentiation.
2. Substances secreted from synovial fibroblasts treated with either TNF-α or dexamethasone affect muscle differentiation.
3. The effects seen with TNF-α conditioned media can be reversed by the addition of a DKK1 inhibitor.

2 METHODS

2.1 C2C12

C2C12 cells were generated from mouse muscle tissue. They were first generated by Yaffe and Saxel in 1977. This was achieved via selective serial passage of myoblasts cultured from the thigh muscle of C3H mice 70 hours after crush injury[21]. C2C12 are a useful model to study the differentiation of muscle cells due to their capacity for rapid differentiation. Upon differentiation the C2C12 cells form contractile myotubes and express characteristic muscle proteins. The formation of the myotubules is enhanced when the media used to culture cells is supplemented with 10% horse serum instead of foetal bovine serum.

2.2 C2C12 cell culture

C2C12 cells were cultured in DMEM High Glucose [10% Foetal Bovine Serum (FBS), 1% L-Glutamine, Glucose (4.5g/l)]. They were incubated in a humidified atmosphere at 37˚C with 5% CO_2. The reagents used for cell culture were from Sigma Aldrich (Dorset, UK) and the plastic ware was from Corning (Surrey, UK). When the cells had reached around 70% confluence they were split into fresh flasks with fresh media. However when the cells were going to be used for experimentation they were allowed to reach 100% confluence. Any experimentation carried out on the cell line was conducted within a cell culture cabinet.

Cells were passsaged by first removing the old media and adding 1.5ml of trypsin, which itself was removed. 3ml of trypsin was then added and the cells incubated for 5 minutes at 37˚C to dislodge the cells from the flask. Any remaining adherent cells were dislodged by gentle tapping. 13ml of DMEM High Glucose was then added to inactivate the trypsin. An

appropriate amount of this media was added to a fresh 75cm^2 flask depending on the required split. The volume was then made up to 10ml with the appropriate volume of fresh media.

2.3 Preparation of C2C12 cells for treatment

When the cells were to be used in an experiment they were passaged into either 6 or 12 well plates, whichever was most appropriate. The media was removed from the cells within the 75cm^2 flask and 1.5mls of trypsin was added and then removed. 3mls of trypsin was added and the flask incubated at 37˚C for 5 minutes. This removed the adherent cells, and remaining adherent cells were removed by gentle tapping. The cells were then counted using a haemocytometer. An average count was made and the number of cells in the flask was calculated. The cells were then spun down at 12,000rpm for 5 minutes and the excess media removed. The cells were re-suspended in an appropriate volume of DMEM High Glucose to give 100,000 cells per ml. For 6 well plates 1ml of the cells was added to each of the wells and then the wells were made up to 2ml with fresh media. For 12 well plates 0.5ml of the cells was added and 0.5ml of fresh media was added. This allowed the cells to reach around 100% confluence after 24 hours. Once this was achieved the DMEM High Glucose was removed and replaced with DMEM differentiation media [5% Horse Serum (HS), 1% L-Glutamine, Glucose (4.5g/l)] and the appropriate test substances.

2.4 C2C12 Cell treatment

Once the cells had reached 100% confluence within the wells the media was replaced with DMEM differentiation media. The FBS was replaced with HS as the HS causes the C2C12 myocytes to differentiate. Test conditions were Wnt3a, Wnt5a, DKK1, control conditioned media, TNF-α conditioned media, and dexamethasone conditioned media. Wnt3a, Wnt5a, and

DKK1 control SF-conditioned media, TNF-α SF-conditioned media, and dexamethasone SF-conditioned media were added at a concentration of 50ng/ml. The conditioned media was obtained by treating synovial fibroblasts (SF) with either TNF-α or dexamethasone (dex), then removing the TNF-α and dex and collecting the media containing the products from the treated SFs. The treatments were added at day 0 and remained within the media until day 6.

2.5 RNA extraction

After either 1, 3 or 6 days of exposure to the substances or conditioned media the medium was removed from the cells and 1ml of TRI-reagent was added. The cells were removed from the adherent surface by scraping. The solution was then placed into micro-centrifuge tubes and left to stand for 5 minutes at room temperature. 200µl of chloroform was then added and the solution shaken vigorously for 15 seconds. It was then left to stand for 10 minutes. The eppendorfs were then centrifuged at 12,000g for 30 minutes at 4˚C in order to separate the aqueous and the organic phases. The aqueous phase containing the RNA was then removed and placed in a fresh eppendorf and the organic phase was discarded. 1µl of glycoblue was then added along with 500µl of isopropanol. The tubes were inverted a few times and then centrifuged at 12,000g for 30 minutes at 4˚C. Following this the supernatant was removed and discarded leaving the glycoblue stained pellet, and 500µl of 70% ethanol was added. The sample was then vortexed and centrifuged for 5 minutes at 12,000g at 4˚C. The supernatant was then removed and the sample centrifuged for a further minute at 12,000g at 4˚C. The remaining supernatant was then removed and the eppendorfs were left open to air dry for 10 minutes. The pellet was then re-suspended in 20µl of RNase free water, and then placed on ice.

2.6 Assessment of RNA quality and concentration

The quantity of the RNA which had been extracted was determined using a Nanodrop ND-1000 spectrophotometer (Wilmington, Delaware, USA). The value given via the Nanodrop allowed for the calculation of the volume of RNA needed to obtain the 500ng/µl concentration used for reverse transcription. This also allowed for an assessment of the quality of the RNA which had been extracted. This made use of the A260/A280 ratio. This was required to fall between 1.8 and 2.0.

2.7 Reverse transcription

Once the RNA had been extracted it was reverse transcribed to cDNA and used as a template in the real-time PCR. The reverse transcription generated 50ng of cDNA/µl from 20µl of the initial reaction volume. (2.0µl of 10xRT Buffer, 4.4µl 25mM $MgCl_2$, 4.0µl dNTPs (10mM), 1.0µl random hexamers, 0.4µl RNase inhibitor, 1.25 MultiScribe reverse transcriptase, 7µl RNA, and nuclease free water [the amount of RNA used gave a final amount of 1µg, nuclease-free water was added to bring the combined RNA/water volume to 7µl]). One cycle was used (25˚C for 10 minutes, 37˚C for 60 minutes, and 95˚C for 5 minutes). All reagents were supplied by Applied Biosystems (Warrington, UK).

2.8 Real time (RT) PCR

RT-PCR allowed the effects of the treatments on the mRNA levels of key genes (11β-HSD1, MyoD, Myogenin, and α-actinin) to be analysed. This was achieved by comparing the change in the mRNA expression of the genes with the 18S housekeeping gene. The RT-PCR reactions were undertaken using a 96 well microarray reaction plate (MicroAmp; Applied

Biosystems, Warrington, UK). Each of the wells contained 5µl TaqMan gene expression assays (Applied Biosystems) including 1µl of the primers and probes for the gene of interest, 0.5µl of 18S, 0.5µl of Assay on Demand master mix, and 3µl of RNase-free water. This gave each well a total volume of 10µl. The plate was analysed on a 7500 Real Time PCR System (Applied Biosystems) using a 96 minute programme (95˚C for 1 minute, 40 cycles of 95˚C (12 seconds) cooling to 60˚C (60 seconds)).

2.9 Analysis of relative real-time PCR data

The mRNA expression of the gene of interest was measured in comparison to the 18S housekeeping gene. This was done by calculating the ΔCt (change in cycle threshold) values. This was calculated using; (ΔCt = Ct (Gene of interest) – Ct (18S housekeeping gene)). Also the fold change was calculated using $2^{-\Delta Ct}$.

2.10 11β-HSD1 enzyme activity assay

Once the cells had reached terminal differentiation (day 6) the media was removed from the cells and replaced with 1ml of fresh media containing 5µl of hot A (^{3}H 11 dehydrocorticosterone) and cold A (not radio-labelled), which had been previously prepared within the lab. The cells were placed at 37˚C over a period of 2 hours. The media was then removed and placed into a glass thin layer chromatography (TLC) tube. Following this 5ml of dichloromethane was added and the solution was vortexed for 20 seconds, and then centrifuged for 15 minutes at 15,000rpm. This allowed for separation of the aqueous and organic phases. The aqueous phase was then removed and discarded. The sample was then placed at 55˚C to evaporate the remaining organic phase. The remaining sample was then re-suspended in 70µl of dichloromethane which was then spotted on a silica plate. The steroids

were then separated over 1.5 hours by TLC, using chloroform:ethanol (92:8) as a mobile phase (of 200ml total volume). The silica plate was then analysed using a Bioscan Imaging scanner (BioScan, Washington DC, USA). This confirmed the steroid running distance against the migrated distance of the standard. From this information the conversion of inactive to active steroid was calculated.

2.11 Statistics

The experiments were each conducted in triplicate and the three values obtained were averaged for presentation on the graphs. The fold changes for the differentiation were all compared to day 1 for the respective treatment. A t-test was also carried out in order to determine the significance. With the T-test the values of the treated cells were compared to those of the untreated cells for the respective time point, the changes were taken to be significant if the value was less than 0.05.

3 RESULTS

3.1 Myocyte differentiation to myotubules

C2C12 cells were differentiated by replacing DMEM high glucose with DMEM differentiation media once the cells had reached 100% confluence. mRNA was extracted at days 1, 3, 6 following initiation of differentiation on day 0, this allowed for the measurement of mRNA levels for key factors indicative of the change in the differentiation of the cells. Images were also obtained at each time point.

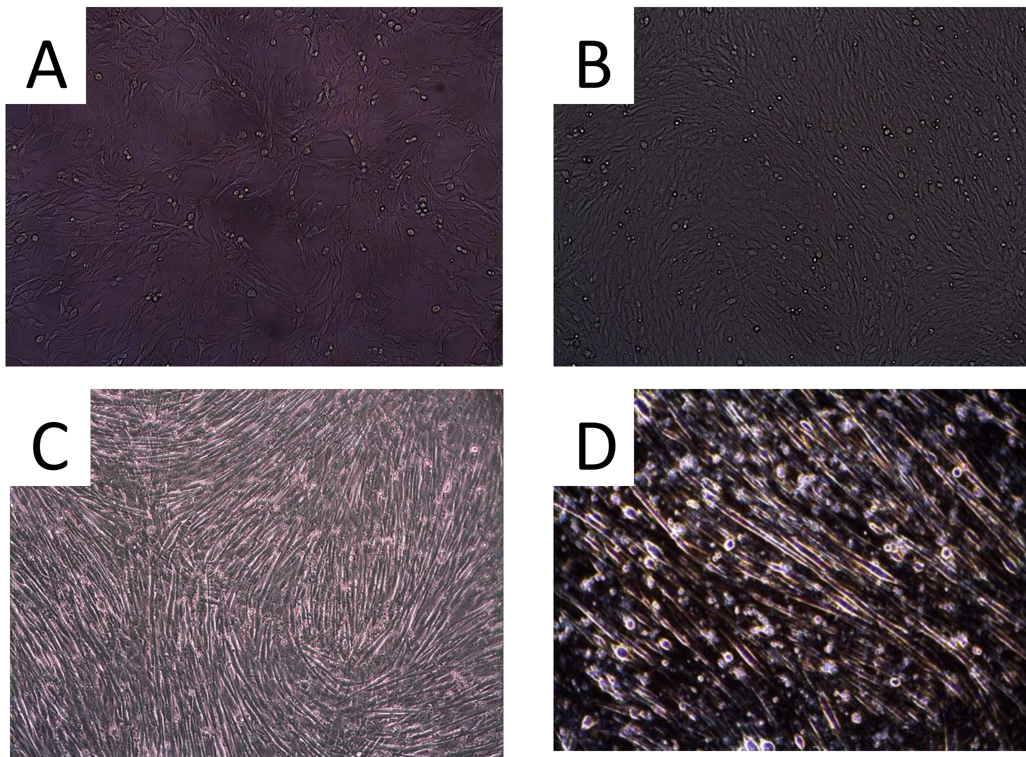

Figure 4 Morphological changes during myocyte differentiation.
The images (A, B, C, and D) show control C2C12 cells after the addition of DMEM differentiation media [5% HS (Horse serum), 1% L-Glutamine, Glucose (4.5mg/ml)] on day 0. The images A, B, C, and D were obtained on day 0, day 1, day 3, and 6 respectively. The images show that the C2C12 cells develop thicker syncytia over the six days of differentiation. It can also be seen that it is not until day 3 (C) that the cells show clear evidence of undergoing differentiation. All photographs were taken at 10x magnification with automatic white light adjustment.

The morphological changes in the C2C12 cells shown in figure 4 show that there is differentiation induced by the change in media, and that there is an increase in the level of differentiation throughout the days. The differentiation is seen to be greatest at day 6 (figure 4-1 D), and this represents the time point where the C2C12 are terminally differentiated.

3.2 Myocyte differentiation marker mRNA levels during differentiation

mRNA levels of the myocyte differentiation markers, MyoD, myogenin, α-actinin, and 11βHSD1 were measured at days 1, 3, and 6 following the initiation of differentiation. This was achieved by collecting the total RNA from the cells at the respective time points. The levels of mRNA of the differentiation markers were measured using RT-PCR, and the values were compared to the control cells from day 1.

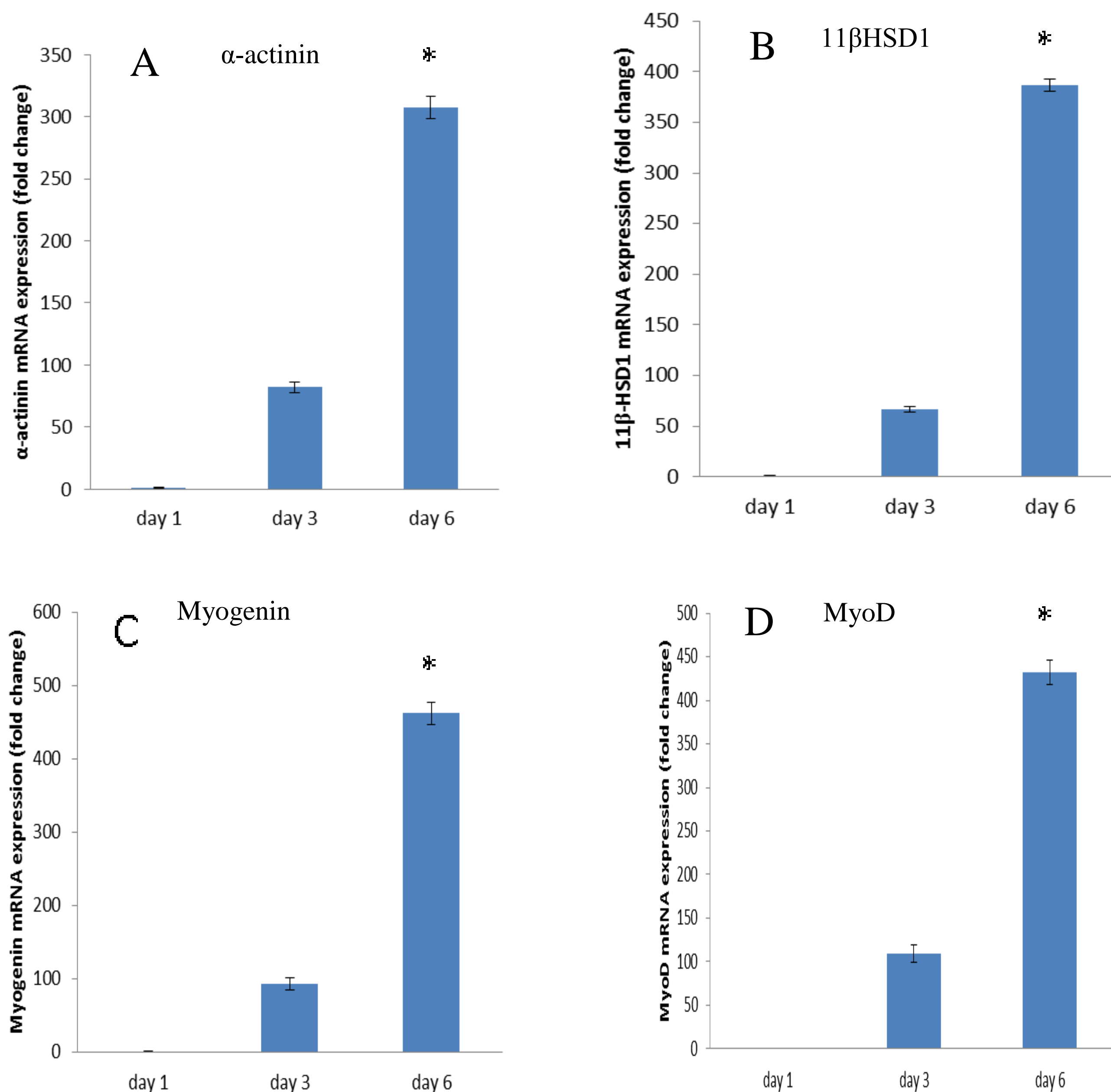

Figure 5 mRNA levels of key markers of myocyte differentiation.
The differentiation of the C2C12 myocytes was induced by replacing the existing media with DMEM differentiation media [5% HS, 1% L-Glutamine, Glucose (4.5g/l)] at day 0. The media was replaced on day 3 of differentiation. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA levels of the myocyte differentiation markers, α-actinin(A), 11βHSD1(B), MyoD(C), and myogenin(D) was measured using relative real-time PCR. The amount of differentiation after day 6 compared to the amount of differentiation after day 1 was seen to be significant in all four markers when assessed using a t-Test.

The level of mRNA of all four markers of differentiation showed a clear increase over the 6 days of differentiation, (figure 5) with all genes showing the largest increase between days 3 and 6. α-actinin mRNA (figure 5 A) showed an increase from day 1 to day 6 of 307-fold during the transformation of myoblasts (day 1) to terminally differentiated myotubles (day 6), whilst MyoD (figure 5 C) mRNA levels increased 432-fold. Myogenin (figure 5 D) mRNA levels increased 462-fold. 11βHSD1 (figure 5 B) mRNA levels increased 386-fold. For all the differentiation markers tested there was a significant increase in mRNA expression on day 6 as compared to day 1. Thus, along with the morphological evidence, there is an increase in differentiation of the cells over the 6 days, and successful differentiation from myoblasts to terminally differentiated myotubules was achieved.

3.3 The effects of Wnt3a, Wnt5a, or DKK1 treatment on the morphology of differentiating C2C12 myoblasts

During the differentiation of the C2C12 myocytes from myoblasts to myotubules over the six days images were obtained to enable changes in the morphology of the cells to be visualised. Control cells were left untreated whereas treated cells had the addition of either Wnt3a, Wnt5 or DKK1. Images were taken with 10x magnification using automatic white light adjustment. Please note the ssame control cell image is used in figures 6 and 7.

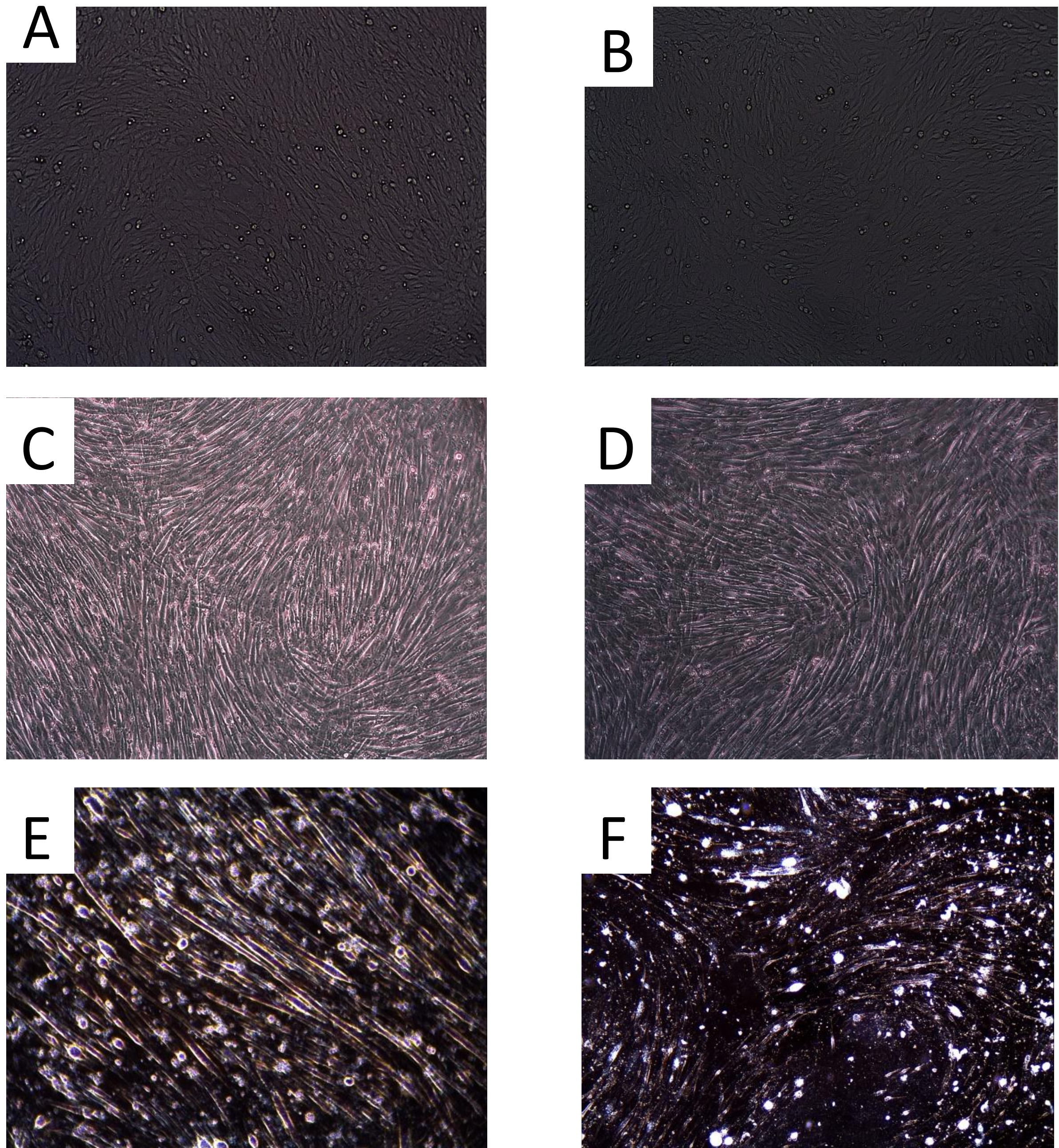

Figure 6 The effects of DKK1 treatment on the morphology of differentiating C2C12 myoblasts

C2C12 myoctes were differentiated from myoblasts to myotubules over a period of 6 days. The images are shown for control cells (A, C, E) and cells treated with DKK1 (B, D, F). After day 1 (A, B) and day 3 (C, D) there is very little morphological difference between the cells, however on day 6 (E, F) the control cells (E) have larger syncytia than the DKK1 (F)-treated cells and also have higher confluence. Photographs were taken at 10x magnification using automatic white light adjustment.

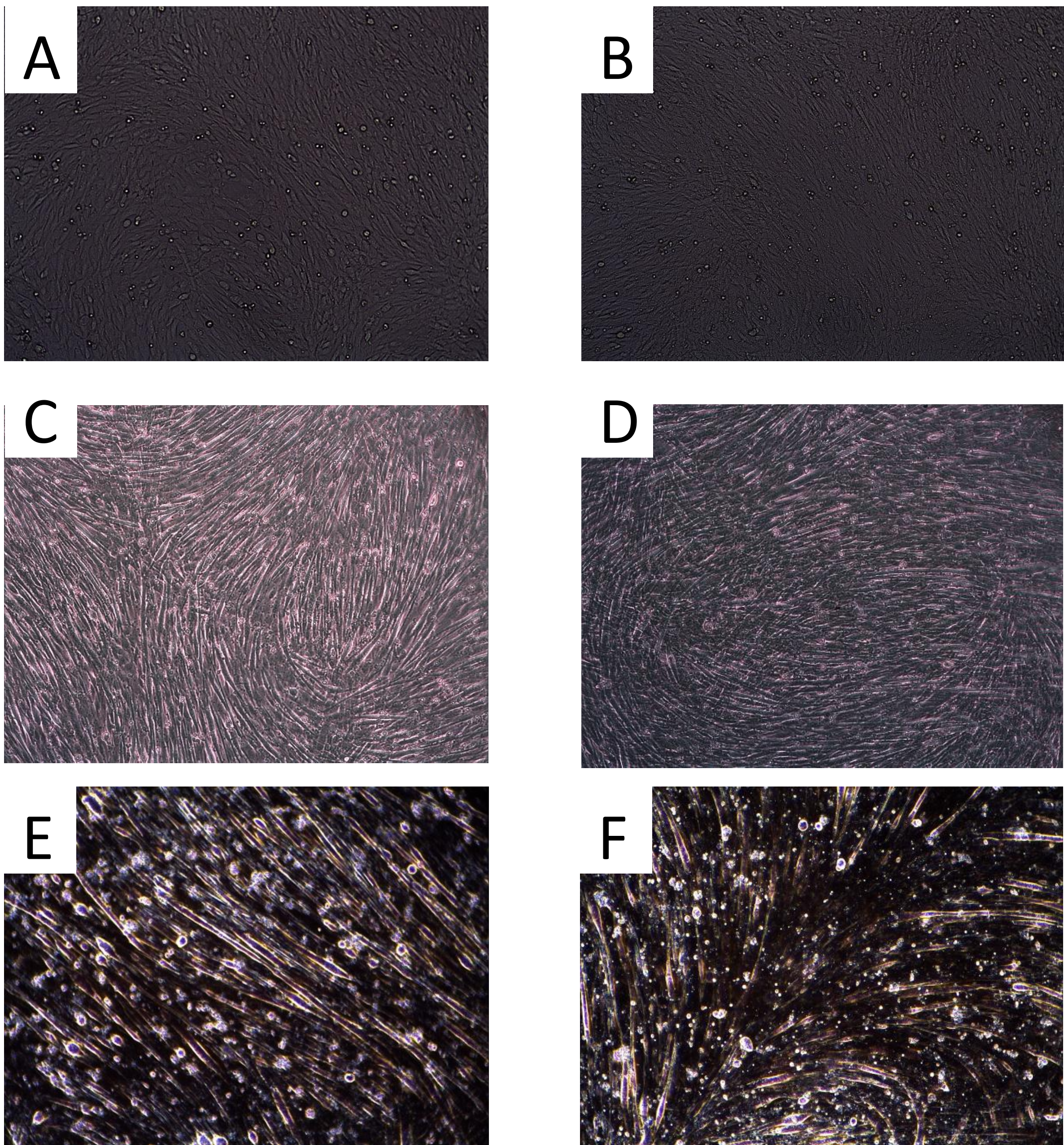

Figure 7 The effects of Wnt5a treatment on the morphology of differentiating C2C12 myoblasts
C2C12 myocytes were differentiated from myoblasts to myotubules over a period of 6 days. The images are shown for control cells (A, C, E) and cells treated with Wnt5a (B, D, F). After day 1 (A, B) and day 3 (C, D) there is very little morphological difference between the cells, however on day 6 (E, F) the control cells (E) have larger syncytia than the Wnt5a (F) treated cells and also have higher confluence. Photographs were taken at 10x magnification using automatic white light adjustment.

Figure 6 and figure 7 show that there is no clear difference between the cells after days 1 and 3, however by day 6 there is a clear morphological difference between untreated cells and those treated with DKK1 or Wnt5a respectively. The changes shown in figure 6 (B, D, and F) for those treated with DKK1 are similar to those which are seen with Wnt5a (figure 7). The control cells appear more confluent and to have differentiated to a greater degree than those which have undergone treatment with Wnt5a or DKK1. The images appear to show that there is possible apoptosis or another form of cell loss which has occurred with the cells that were treated with Wnt5a or DKK1. These differences in the morphology of cells following treatment with Wnt5a and DKK1 is not reflected with the cells treated with Wnt3a (data not shown).

3.4 The effects of control SF-conditioned media, TNF-α SF-conditioned media, or dexamethasone SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts

In order to test the effect of secretory factors obtained from synovial fibroblasts under control conditions, or after TNF-α or dexamethasone stimulation, differentiating C2C12 cells were incubated with DMEM differentiation media and the respective conditioned media. Images were obtained to enable changes in the morphology of the cells to be compared at days 1, 3 and 6. Control cells were left untreated whereas treated cells had the addition of either control conditioned media, TNF-α conditioned media, or dexamethasone conditioned media. Images were taken with 10x magnification using automatic white light adjustment.

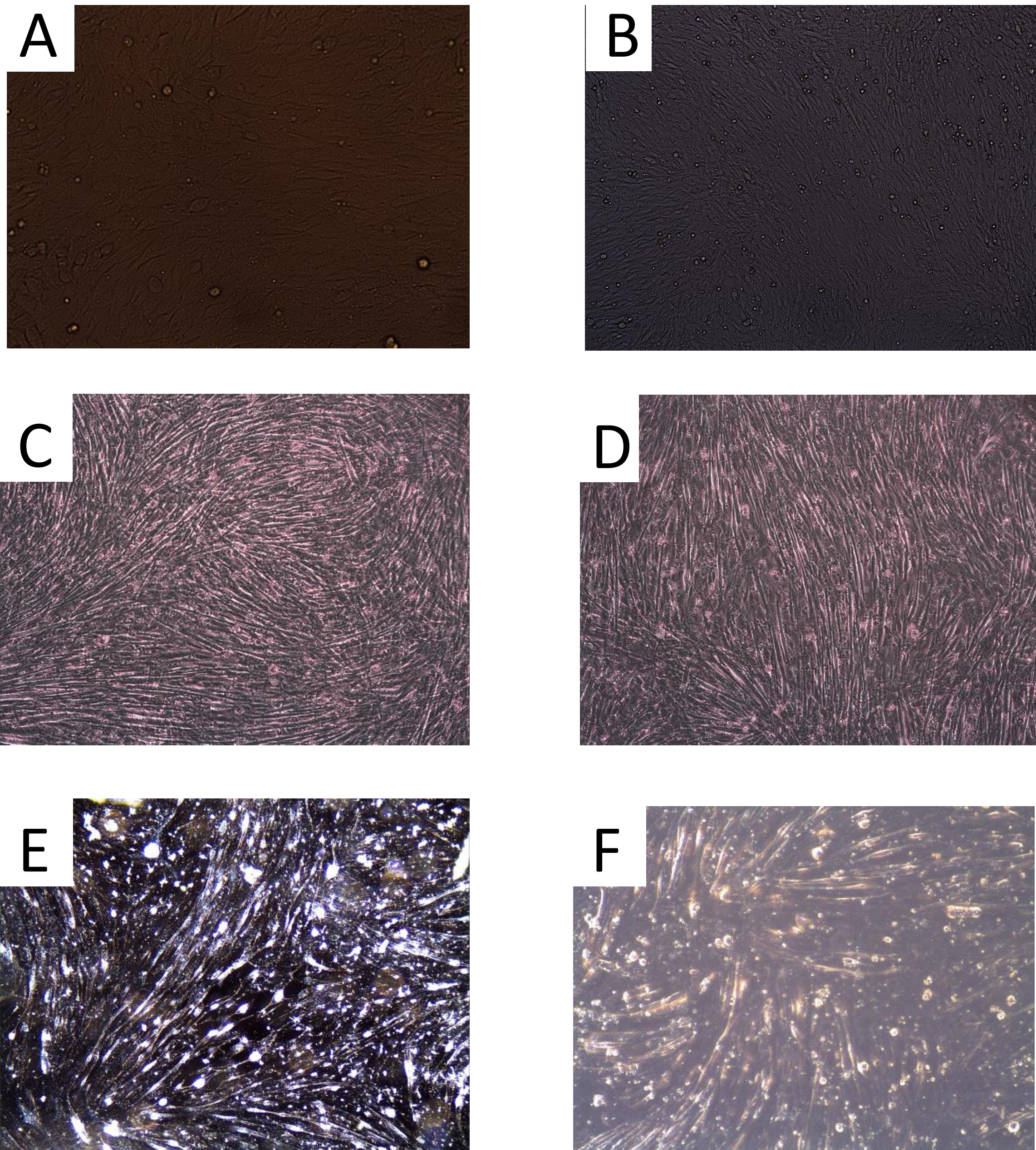

Figure 8 The effects of TNF-α SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts

C2C12 myocytes were differentiated from myoblasts to myotubules over a period of 6 days following the addition of DMEM differentiation media with the media changed at day 3 of differentiation. The images are shown for control cells (A, C, E) and cells treated with TNF-α conditioned media (B, D, F). After day 1 (A, B) and day 3 (C, D) there is very little morphological difference between the cells, however on day 6 (E, F) the control cells (E) have larger syncytia than the TNF-α conditioned media (F) treated cells and also have higher confluence. Photographs were taken at 10x magnification using automatic white light adjustment.

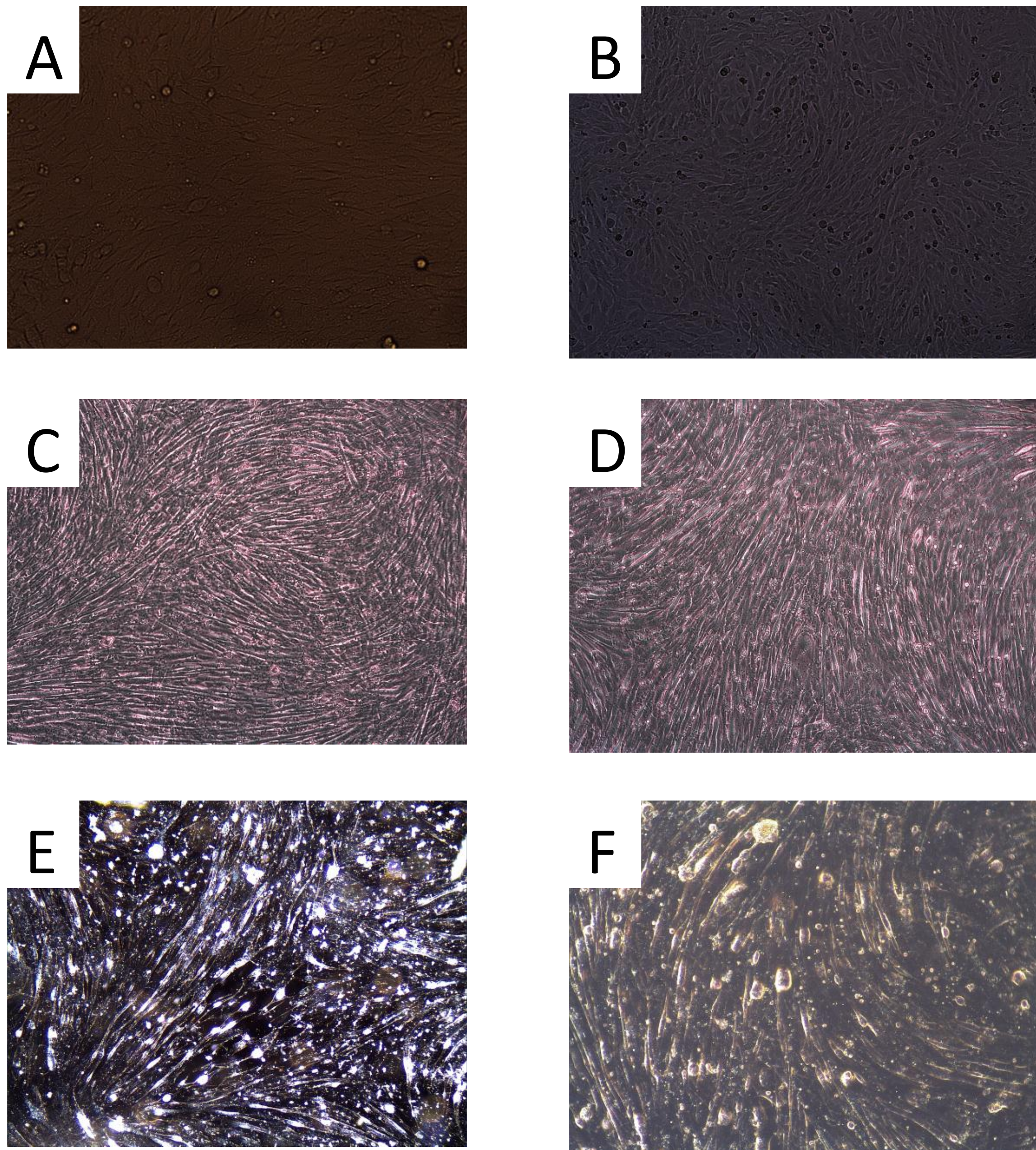

Figure 9 The effects of dexamethasone SF-conditioned media treatment on the morphology of differentiating C2C12 myoblasts

C2C12 myocytes were differentiated from myoblasts to myotubules over a period of 6 days following the addition of DMEM differentiation media with the media changed at day 3 of differentiation. The images are shown for control cells (A, C, E) and cells treated with dexamethasone conditioned media (B, D, F). After day 1 (A, B) and day 3 (C, D) there is very little morphological difference between the cells, however on day 6 (E, F) the control cells (E) have larger syncytia than the dexamethasone conditioned media (F) treated cells and also have higher confluence. Photographs were taken at 10x magnification using automatic white light adjustment.

Figure 8 shows that there is no clear difference between the cells after days 1 (Figure 8 A and B) and 3 (Figure 8 C and D), however by day 6 (Figure 8 E and F) there is a clear morphological difference between untreated cells (Figure 8 E) and those treated with TNF-α conditioned media (Figure 8 F). This is also the case for the images in figure 9 where the cells are treated with dexamethasone conditioned media. The control cells appear more confluent and to have differentiated to a greater degree than those which have undergone treatment with TNF-α conditioned media or dexamethasone conditioned media. The images appear to show that there is possible apoptosis or another form of cell loss which has occurred with the cells that were treated with dexamethasone conditioned media. These differences in the morphology of cells following treatment with TNF-α conditioned media and dexamethasone conditioned media is not reflected with the cells treated with Wnt3a (data not shown).

3.5 The effects of Wnt3a, Wnt5a, and DKK1 treatment on myocyte differentiation markers in differentiating C2C12 myoblasts

Differentiation of the C2C12 cells was initiated by replacing the existing media with differentiation media at day 0. The cells were treated with either Wnt3a, Wnt5a (an inhibitor of the canonical Wnt pathway), or DKK1 (negative regulator of the Wnt signalling pathway) at day 0. The effect that the additions had on the differentiation of the C2C12 cells was assessed by measuring the mRNA concentrations of key differentiation markers, (MyoD, myogenin, α-actinin, and 11βHSD1) at days 1, 3 and 6 following the initiation of differentiation by RT-PCR. These concentrations were compared to the control cells at day 1 and also the control cells at the respective days. The n number for this study is 4.

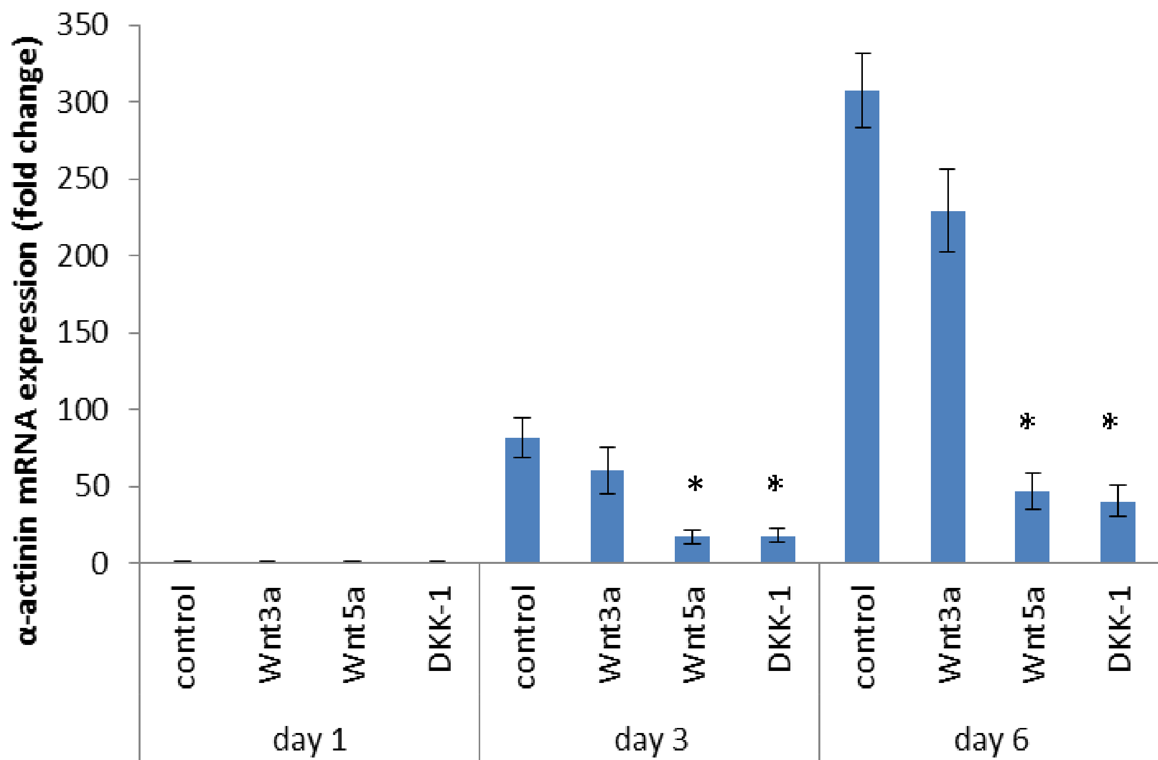

Figure 10 mRNA level of α-actinin following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either Wnt3a, Wnt5a, DKK-1 added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA expression of the myocyte differentiation markers α-actinin was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells from day 1. *p <0.05. Statistical analysis was performed using a t-test.*

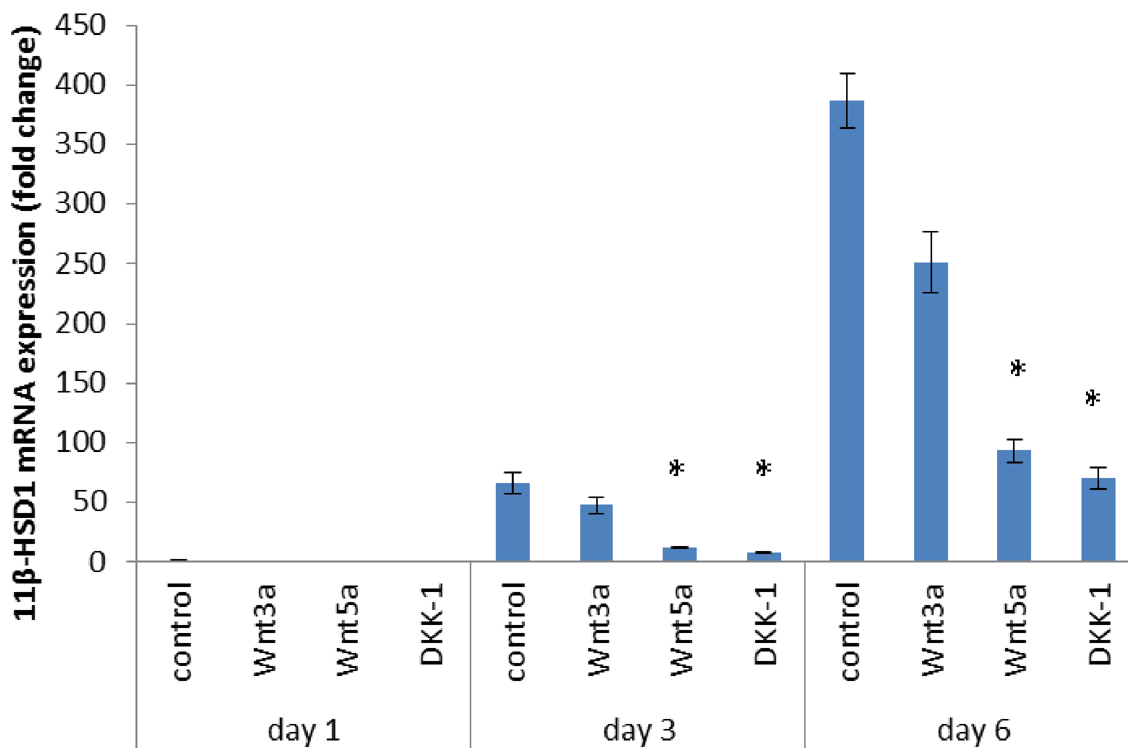

Figure 11 mRNA level of 11βHSD1 following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either Wnt3a, Wnt5a, DKK-1 added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA expression of the myocyte differentiation marker 11βHSD1 was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells from day 1. *p <0.05. Statistical analysis was performed using a t-test.*

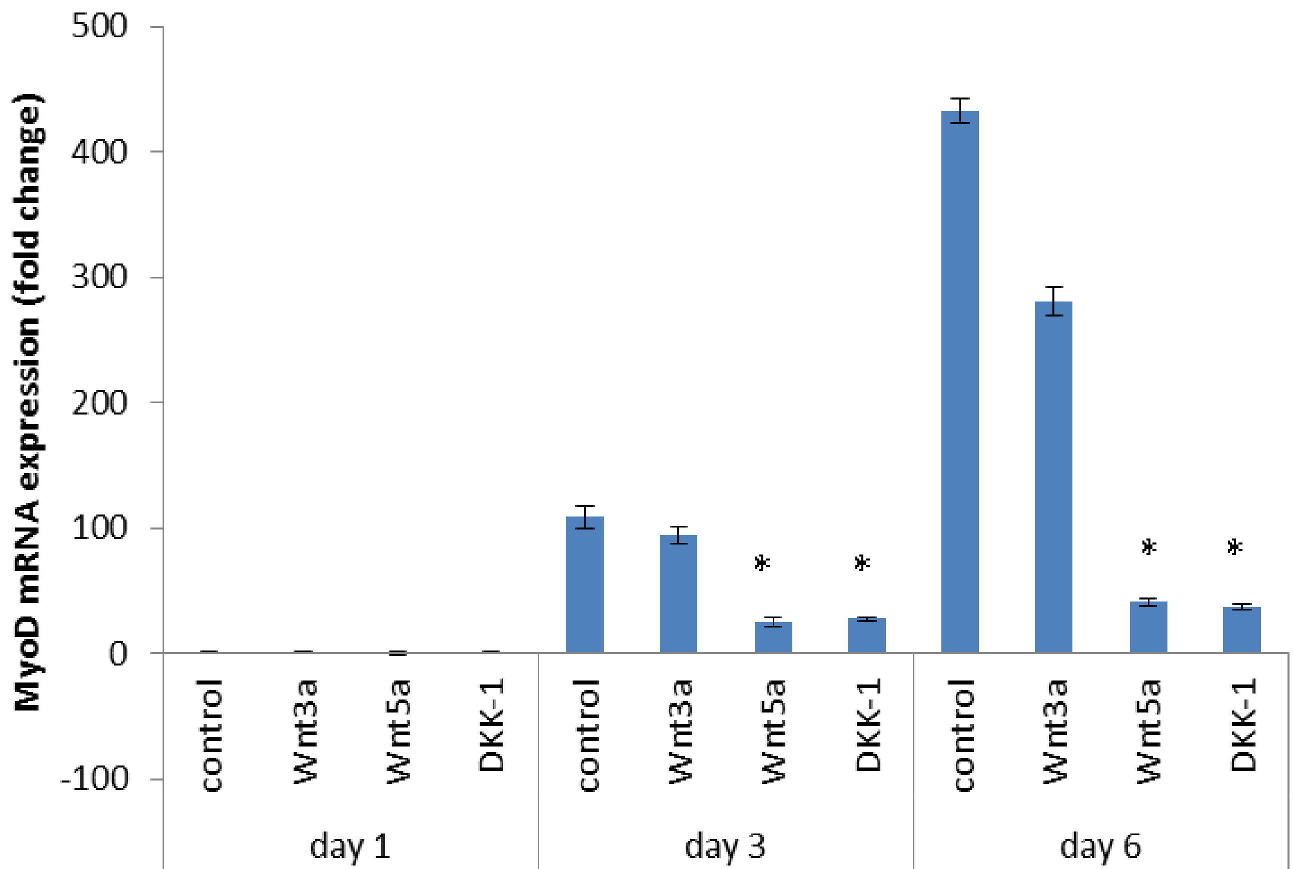

Figure 12 mRNA level of MyoD following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either Wnt3a, Wnt5a, DKK-1 added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA expression of the myocyte differentiation markers MyoD was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells from day 1. *p <0.05. Statistical analysis was performed using a t-test.*

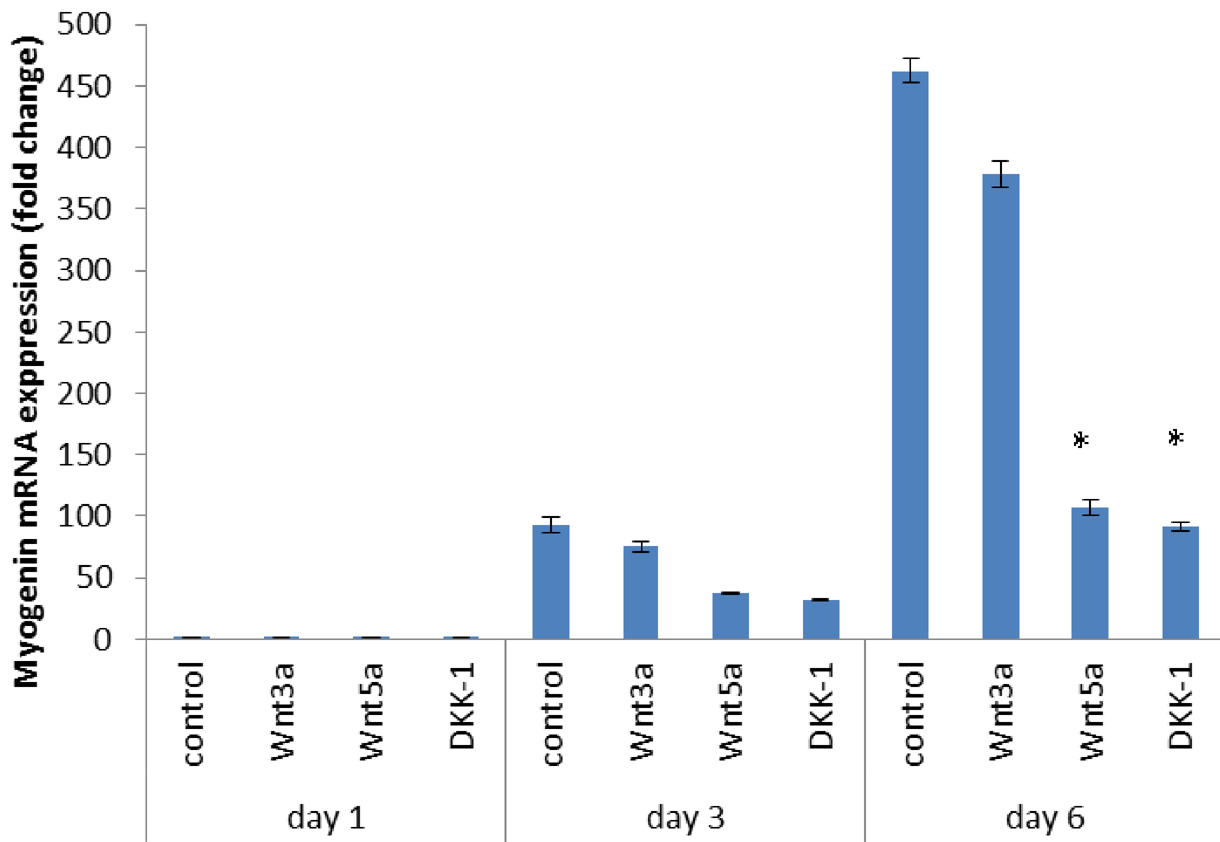

Figure 13 mRNA level of key myogenin following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either Wnt3a, Wnt5a, DKK-1 added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA expression of the myocyte differentiation marker myogenin was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells from day 1. *p <0.05. Statistical analysis was performed using a t-test.*

There is a clear increase in the amount of 11βHSD1 mRNA produced by the C2C12 cells by day as shown in figure 11. This in turn shows that there is an increase in differentiation of the C2C12 cells throughout the 6 days despite the addition of the substances. These increases in mRNA expression are greatest for the control cells closely followed by the cells treated with

Wnt3a which have day 6 fold changes of 386 and 251 respectively when compared to the respective measurements for day 1. The increases seen for the cells with Wnt5a or DKK1 added at day 6 of differentiation are significantly lower than the control cells with values of 93 and 70 respectively. Similar mRNA level changes are observed with MyoD, myogenin and α-actinin (Figure 10-13).

There is a significant decrease in C2C12 differentiation with respect to the control cells on day 6 with all of the differentiation markers when either Wnt5a or DKK1 was added. There was also a significant decrease at day 3 with all of the differentiation markers except α-actinin when either Wnt5a or DKK1 was added. With the addition of Wnt3a there is a consistent trend showing a decrease in differentiation compared to the control cells for the respective time points, however none of the decreases were significant.

3.6 The effects of control SF-conditioned media, TNF-α SF-conditioned media and dexamethasone SF-conditioned media on the differentiation markers in differentiating C2C12 myoblasts

The C2C12 myocytes were also treated with either SF-conditioned media, TNF-α SF-conditioned media or dexamethasone SF-conditioned media at day 0 to determine the role which they had in the differentiation of C2C12 myoblasts to myotubules. Total RNA was extracted from the cells and the levels of the differentiation markers, 11β-HSD1, MyoD, myogenin, and α-actinin were measured as before (3.5). The n number for this study is 3.

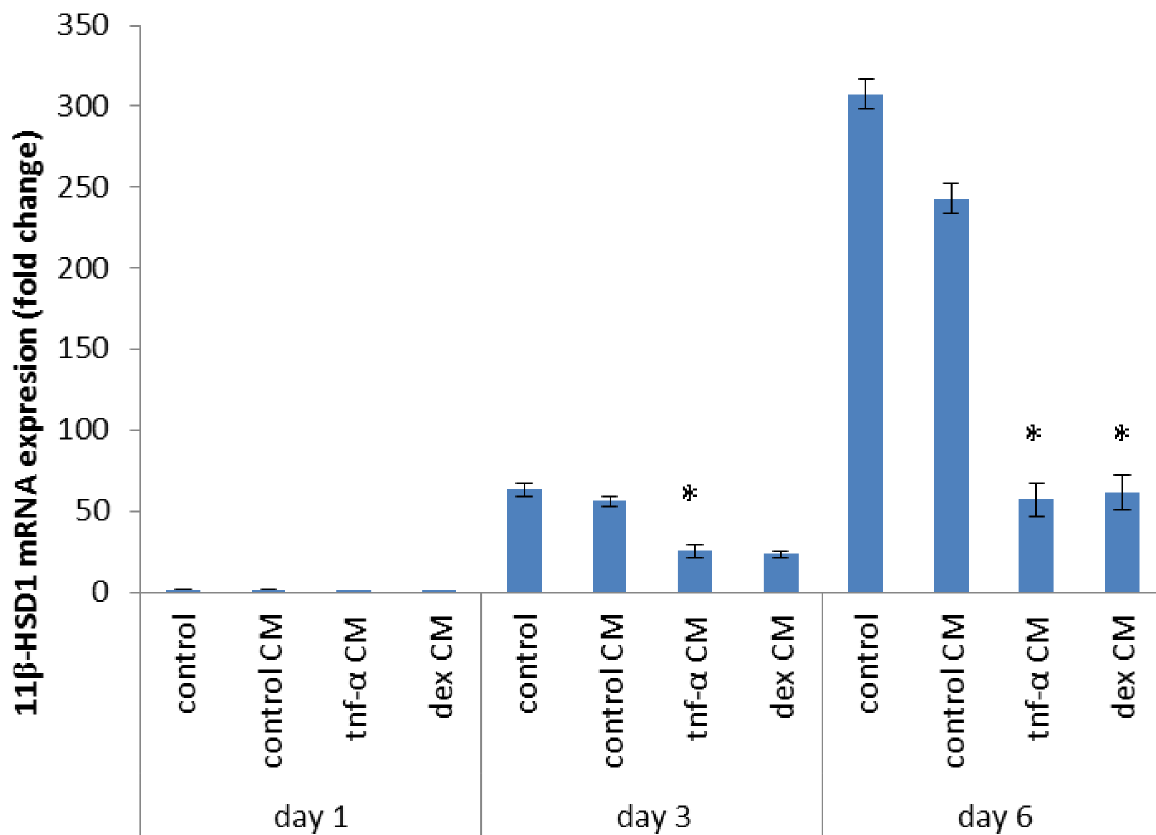

Figure 14 mRNA levels of 11βHSD1 following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either control SF-conditioned media, TNF-a SF-conditioned media, or dexamethasone SF-conditioned media added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA of the myocyte differentiation marker 11BHSD1 was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells for day 1. *p <0.05. Statistical analysis was performed using a t-test.*

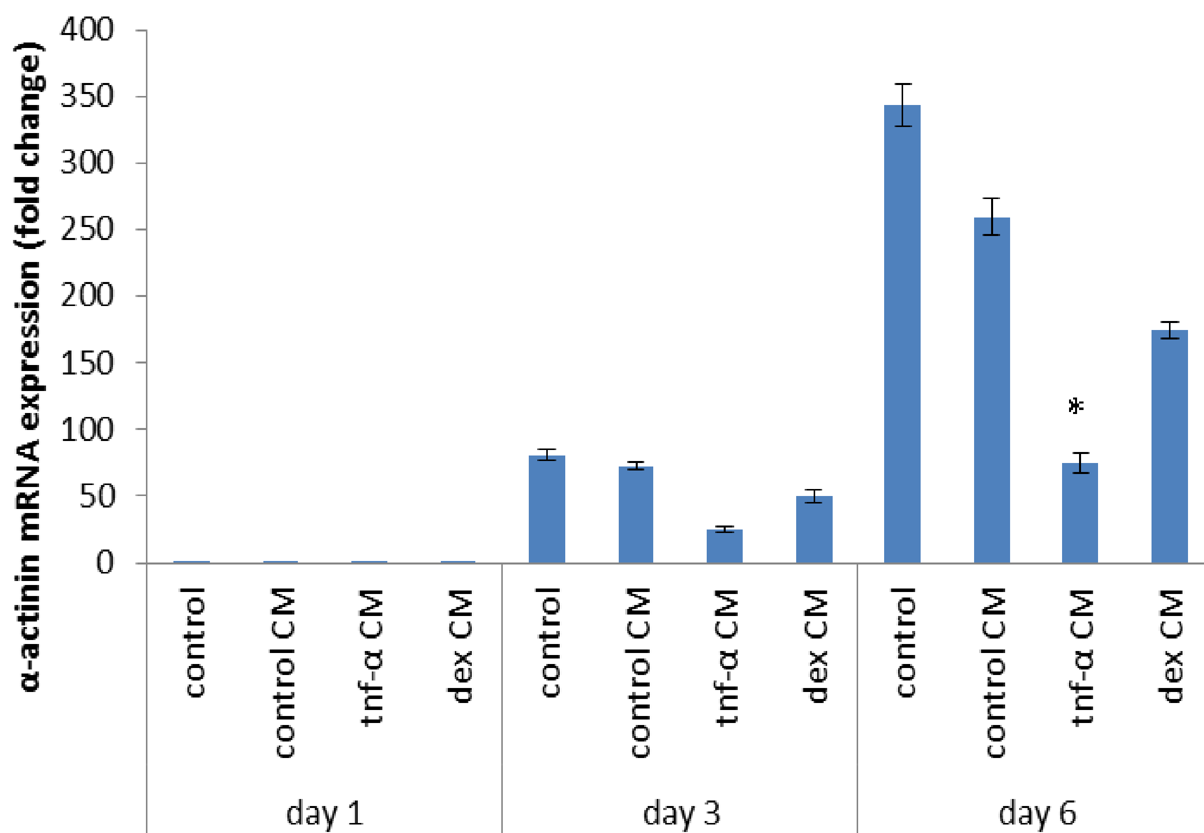

Figure 15 mRNA levels of α-actinin following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either control SF-conditioned media, TNF-a SF-conditioned media, or dexamethasone SF-conditioned media added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA of the myocyte differentiation markers a-actinin was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells for day 1. *p <0.05. Statistical analysis was performed using a t-test.*

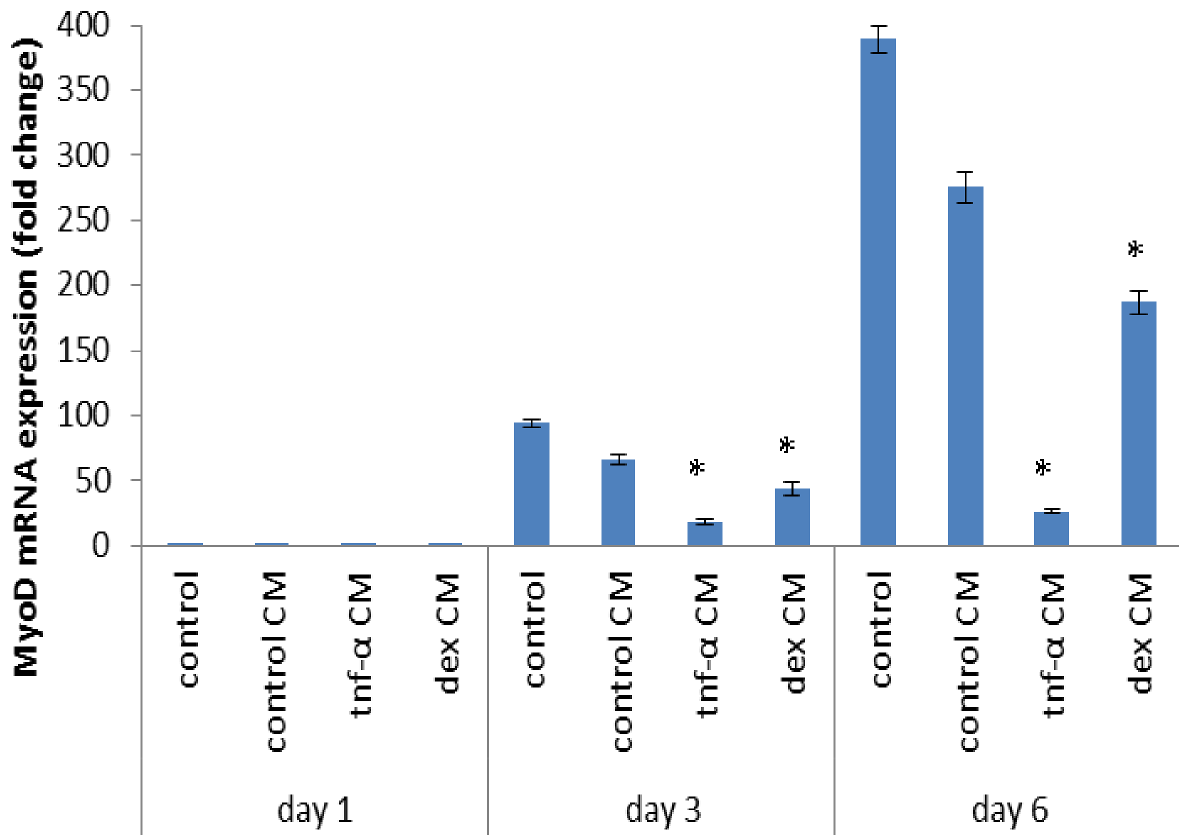

Figure 16 mRNA levels of MyoD following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either control SF-conditioned media, TNF-a SF-conditioned media, or dexamethasone SF-conditioned media added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA of the myocyte differentiation marker MyoD was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells for day 1. *p <0.05. Statistical analysis was performed using a t-test.*

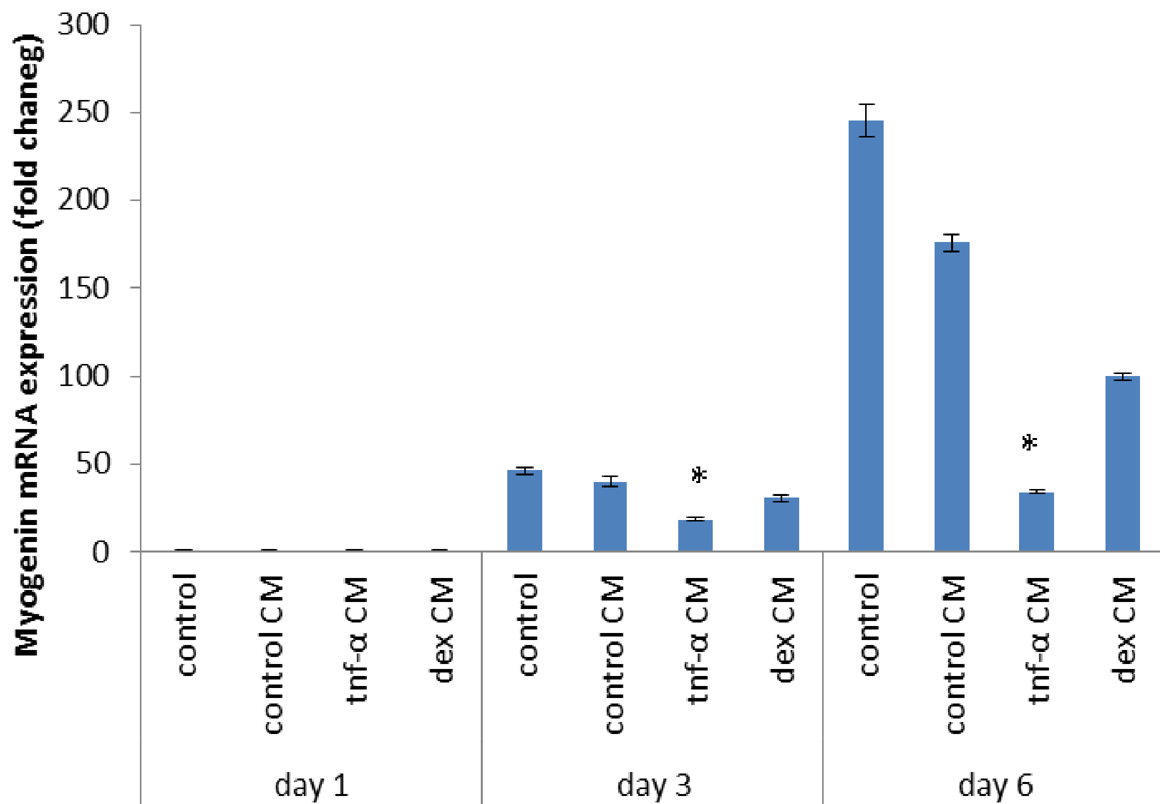

Figure 17 mRNA levels of myogenin following treatment.
*The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either control SF-conditioned media, TNF-a SF-conditioned media, or dexamethasone SF-conditioned media added to them. The RNA was extracted from the cells at day 1, day 3 and day 6. The changes in the level of mRNA of the myocyte differentiation marker myogenin was measured using relative real-time PCR. The levels of mRNA expression are shown as fold change with regard to the control cells for day 1. *p <0.05. Statistical analysis was performed using a t-test.*

There was a clear increase in 11βHSD1 expression in C2C12 cells with differentiation compared to day 1, as shown in figure 14. These increases were largest for the control cells and the cells treated with the control conditioned media, which had fold changes of 307 and

243 respectively. This is reflected with all of the markers of differentiation tested. The size of the increase in mRNA expression on day 6 is lower with the cells treated with TNF-α conditioned media and dexamethasone conditioned media. This trend is also reflected with all of the markers of differentiation tested, shown in figure 15 for α-actinin, figure 16 for MyoD, and figure 17 for myogenin.

There was also a significant decrease at day 3 with all of the differentiation markers except α-actinin when TNF-α conditioned media was added, when the treated cells were compared to the control cells at day 3. With the addition of dexamethasone conditioned media there was a consistent trend showing a decrease in differentiation compared to the control at day 3, however none of the decreases were significant.

At days 3 and 6 with the addition of control conditioned media there is a slight decrease in mRNA expression seen with all the differentiation markers, however this is not significant at any of the time points.

3.7 The effect of DKK1 inhibitor treatment on reversing the effects of TNF-α SF-conditioned media treatment on differentiating C2C12 myoblasts

Following on from the results from the conditioned media and Wnt3a/Wnt5a/DKK1 experiments, we hypothesised that the decrease in differentiation seen with the TNF-α conditioned media could be due to the effect of DKK1 which it would contain. This hypothesis was based on the previous experiments conducted within this study showing that DKK1 is an inhibitor of C2C12 differentiation. To test this we differentiated the C2C12 myocytes at day 0 by adding DMEM differentiation media and also adding either, TNF-α

conditioned media , DKK1 inhibitor, or both TNF-α conditioned media and DKK1 inhibitor. The RNA was extracted at day 6 when terminal differentiation had occurred and when the largest significance was seen with the addition of DKK1 and TNF-α conditioned media. The mRNA levels of the markers of differentiation (MyoD, myogenin, α-actinin, and 11βHSD1) were measured using RT-PCR. The n number for this experiment is 3.

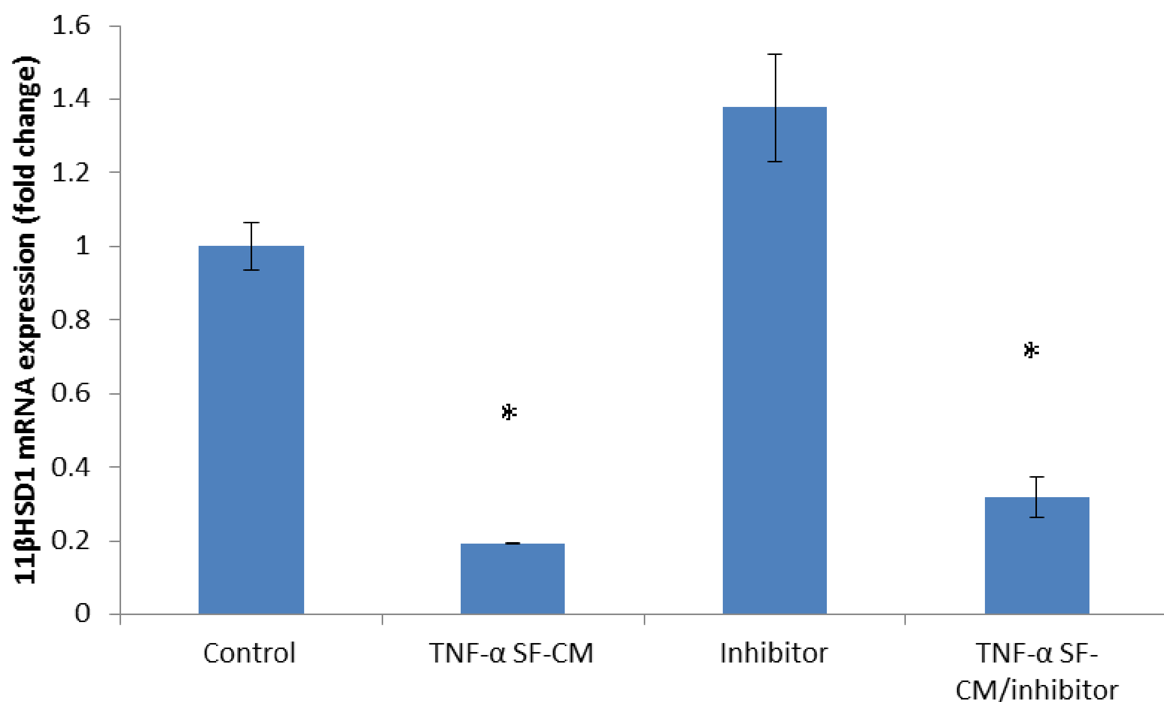

Figure 18 Effect of DKK1 inhibition on TNF-α conditioned media induced changes in C2C12 differentiation markers.
*The addition of the DKK1 inhibitor had no significant effect on TNF-α conditioned media induced decrease in C2C12 differentiation(11βHSD-1). *$p < 0.05$. Statistical analysis was performed using a t-test.*

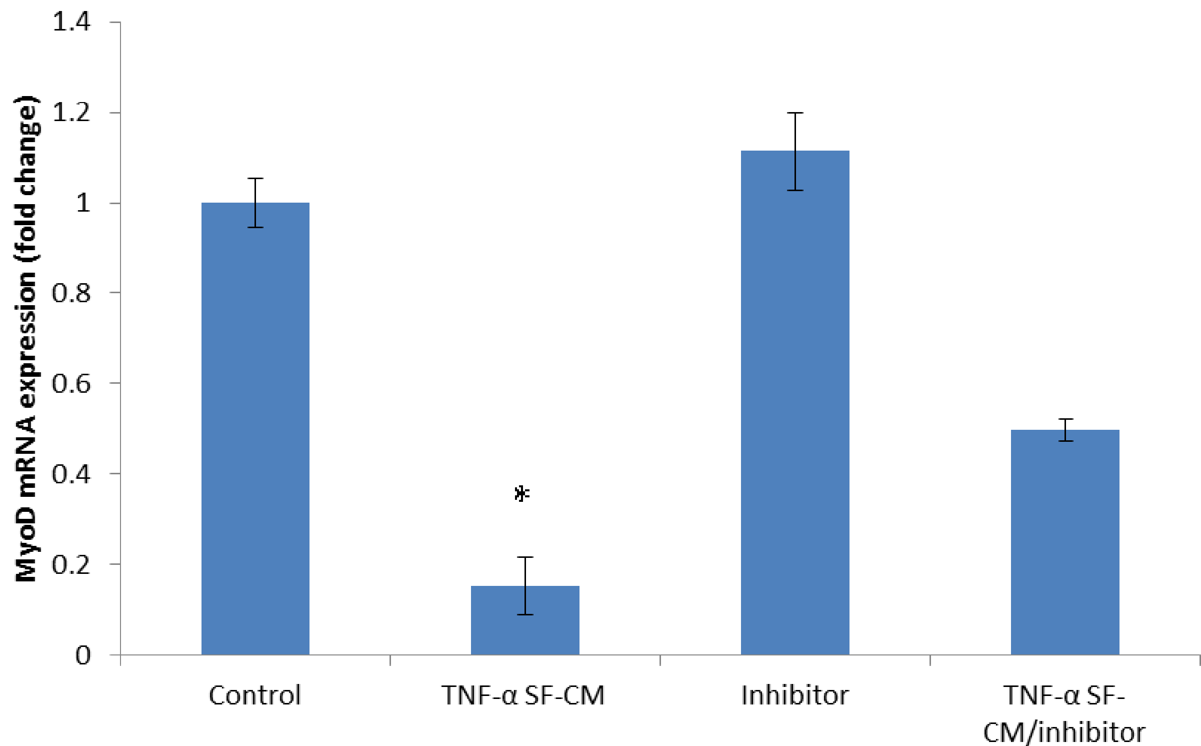

Figure 19 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (MyoD).
*The addition of the DKK1 inhibitor had a significant effect on reversing the effects seen by TNF-α conditioned media on C2C12 cells, which is to significantly decrease the differentation of C2C12 cells. With the addtion of the DKK1 inhibitor there was no longer a significant decrease in muscle differentation caused by the addition of TNF-α conditioned media. *p <0.05. Statistical analysis was performed using a t-test.*

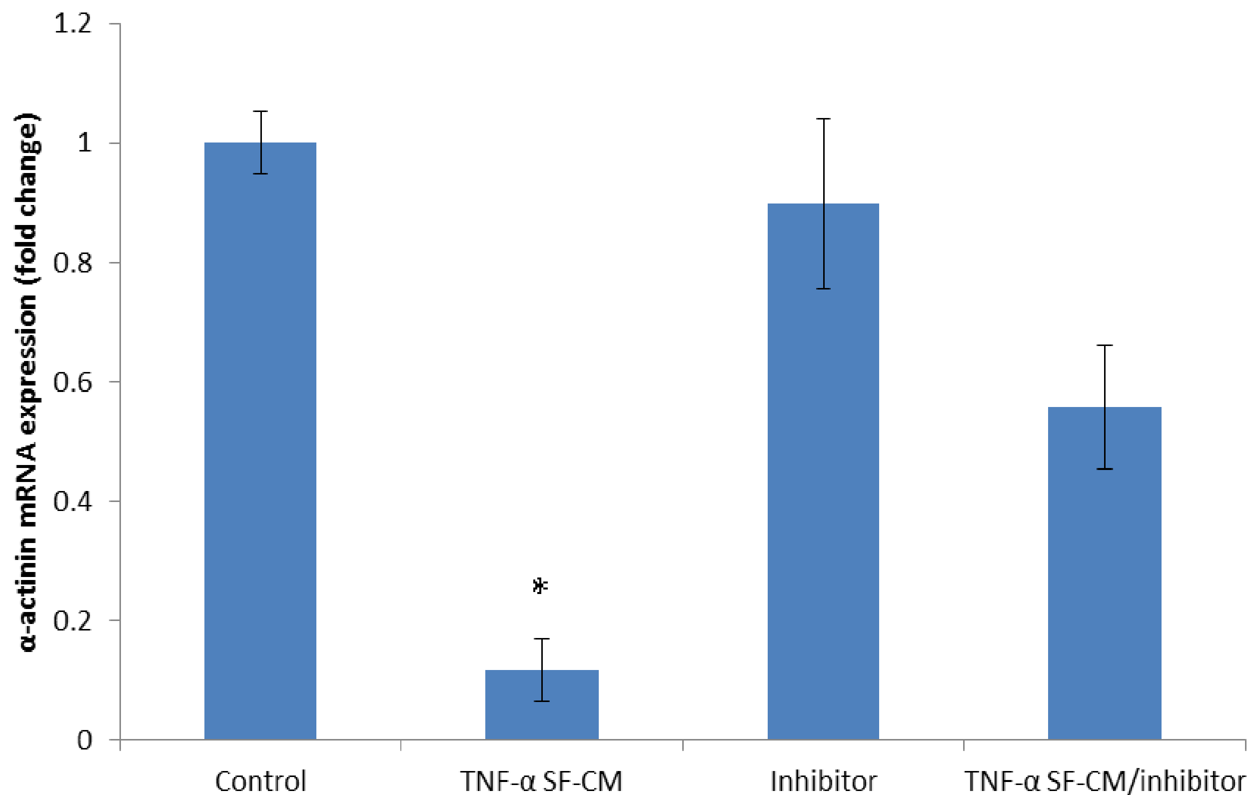

Figure 20 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (α-actinin).
*The addition of the DKK1 inhibitor had a significant effect on reversing the effects seen by TNF-α conditioned media on C2C12 cells, which is to significantly decrease the differentation of C2C12 cells. With the addtion of the DKK1 inhibitor there was no longer a significant decrease in this muscle differentation marker caused by the addition of TNF-α conditioned media. *p <0.05. Statistical analysis was performed using a t-test.*

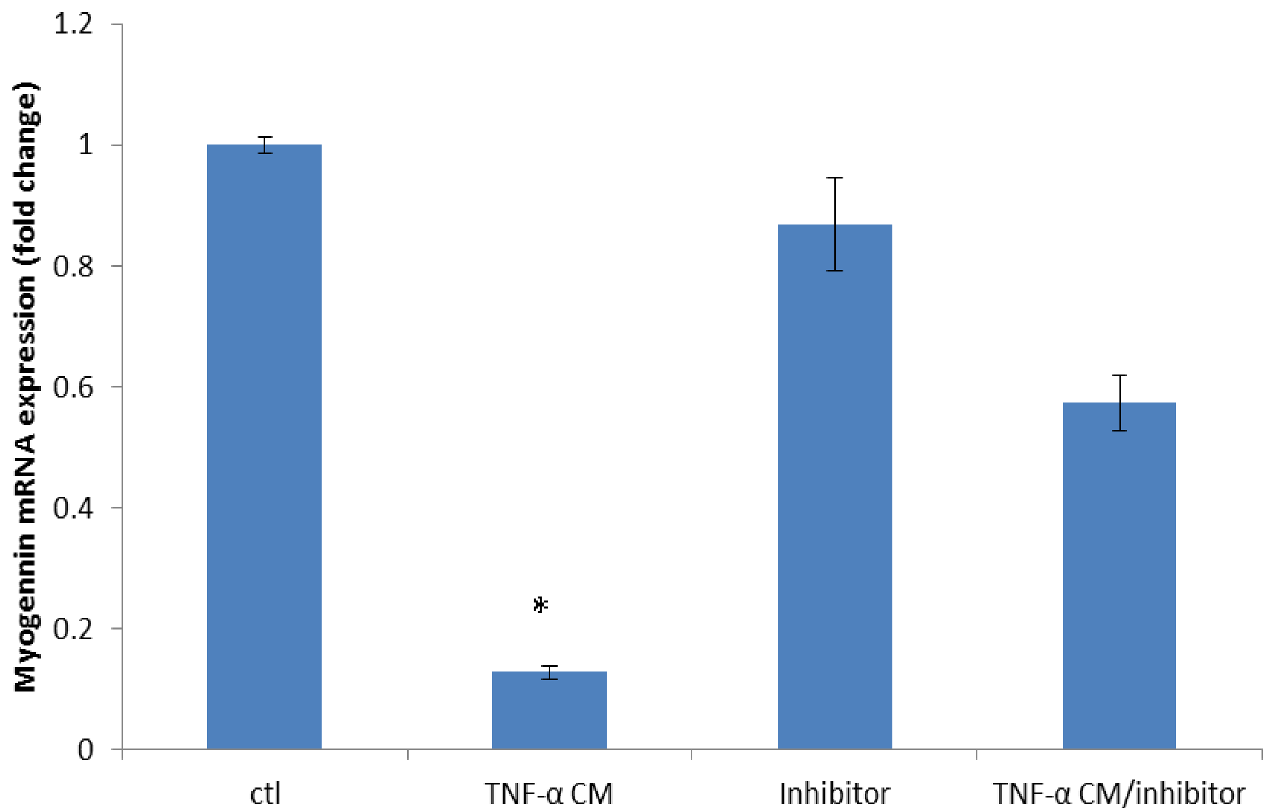

Figure 21 Effect of DKK1 inhibition on TNF-α conditioned media induced C2C12 cell mRNA levels (Myogenin).
*The addition of the DKK1 inhibitor had a significant effect on reversing the effects seen by TNF-α conditioned media on C2C12 cells, which is to significantly decrease the differentation of C2C12 cells. With the addtion of the DKK1 inhibitor there was no longer a significant decrease in this muscle differentation marker caused by the addition of TNF-α conditioned media. There is however still a clear non-significant decrease in C2C12 mucle differentiaiton. *p <0.05. Statistical analysis was performed using a t-test.*

When the mRNA of the genes MyoD, myogenin, and α-actinin were measured at day 6 there was a significant decrease in mRNA expression following the addition of TNF-α conditioned media, when the values were compared to the control cells, as before (3.5). However when the DKK1 inhibitor was added along with the TNF-α SF-conditioned media the significant decrease in mRNA levels was no longer significant (MyoD, myogenin, and α-actinin).

However when the mRNA of 11βHSD1 was measured there was a significant decrease when TNF-α SF-conditioned media was added, with or without the DKK1 inhibitor. The difference between these markers of differentiation could be due to the number of repetitions of the experiment not being high enough or the gene is differently regulated.

3.8 11βHSD1 activity assay testing the effects of Wnt3a, Wnt5a, and DKK1 on treatment on the differentiation of C2C12 myoblasts

Following on from the mRNA analysis and morphological evidence of the effect of Wnt3a, Wnt5a, and DKK1 on C2C12 differentiation, 11βHSD1 activity assays were carried out at day 6 of differentiation. The n number for this experiment is 1.

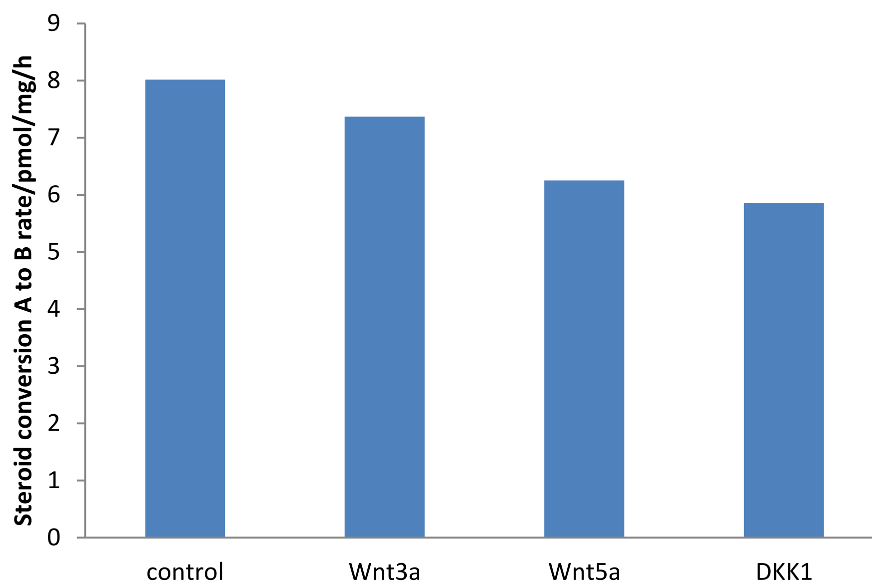

Figure 22 11βHSD1 activity assay testing the role of Wnt3a, Wnt5a, and DKK1 effect on C2C12 muscle differentiation.
The differentiation of the C2C12 myocytes was induced by supplementing the cells with DMEM differentiation media at day 0. The media was replaced on day 3 of differentiation. The treated cells also had either Wnt3a, Wnt5a, or DKK1 added to them. The activity of the cells was measured on day 6 which represents terminal differentiation by performing an 11βHSD1 activity assay on the cells.

The largest amount of activity was seen with the control cells which had a steroid conversion rate of 8 pmol/mg/h closely followed by the cells which were supplemented with Wnt3a which had a steroid conversion rate of 7 pmol/mg/h. There is a larger decrease in activity seen with the cells treated with Wnt5a which had a steroid conversion rate of 6 pmol/mg/h and an even further decrease in the cells which had been supplemented with DKK1 which had a steroid conversion rate of 5 pmol/mg/h. However none of the decreases seen were significant (figure 22).

3.9 11βHSD1 activity assay testing the effect of a DKK1 inhibitor on reversing the effects seen with TNF-α SF-conditioned media treatment of differentiating C2C12 myoblasts

Following on from the experiments testing the role of the DKK1 inhibitor in reversing TNF-α conditioned media effects in C2C12 cells a 11βHSD1 activity assay was carried out. Although there was no significant effect of the DKK1 inhibitor on the TNF-α SF-conditioned media induced decrease in 11βHSD1 mRNA levels (figure 18) an activity assay was performed to rule out any post-transcriptional effect. This was performed on day 6 of differentiation when the cells were terminally differentiated and when the largest and significant changes in gene expression were seen. Differentiation was initiated on day 0 following the addition of DMEM differentiation media and the addition of the respective treatments. The media was changed on day 3 of differentiation. The n number for this experiment is 1.

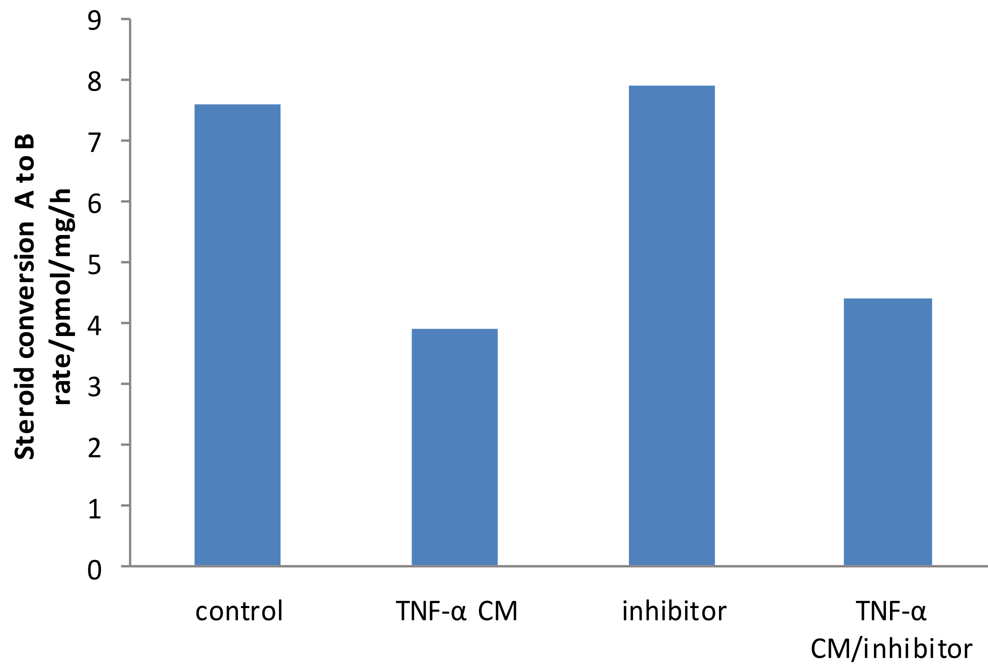

Figure 23 11βHSD1 activity assay to test the effect of a DKK1 inhibitor on reversing the effects of TNF-α SF-conditioned media on C2C12 cell differentiation.
The results show a decrease in activity with the cells treated with TNF-α conditioned media with respect to the control cells, and also a decrease in activity with the cells which were treated with both TNF-α conditioned media and the DKK1 inhibitor. However neither of these changes are significant.

4 DISCUSSION

The study confirmed that substances which are present during joint inflammation affect muscle differentiation, namely Wnt3a, Wnt5a, and DKK1. The most effective of these was shown to be DKK1. It was also confirmed that substances which are secreted from synovial fibroblasts treated with either TNF-α or dexamethasone lead to a decrease in muscle differentiation, as measured morphologically and with differentiation marker mRNA levels. I was able to confirm that effects seen with TNF-α conditioned media could be successfully reversed with the addition of the DKK1 inhibitor, when the mRNA levels of α-actinin, myogeninin and MyoD were measured, however not when the mRNA levels of 11βHSD-1 were measured.

4.1 Myocyte differentiation to myotubles

There was obvious differentiation from the myocytes at day 0 into myotubules at day 6. The morphology show in figure 4 shows clearly that there is development of myotubles which are more elongated than the myocytes, and also the development of multinucleated myotubles. The undifferentiated cells are star-shaped mono-nucleated cells[17]. During differentiation there is a steady change in the morphology of the cells where they become more elongated. The fully differentiated cells are elongated, with thick syncytia and contain more than 20 nuclei[17]. The changes shown in figure 4 for the differentiation of the myotubules over time was seen consistently throughout the experiment whenever the cells were differentiated. This allows confidence in that the changes in mRNA expression seen are due solely to the treatments and not to other influences, for example the passage of the cells.

The mRNA data further supports the successful differentiation of the myocytes at day 0 to myotubles at day 6. It is seen with all the markers of differentiation, that there is an increase in their expression as the cells become more differentiated. MyoD plays a key role in muscle differentiation; it belongs to a family of proteins known as myogenic regulatory factors. Myogenin is a regulatory factor which controls myogenesis. In mice, myogenin is essential for the development of functional skeletal muscle. It is involved in the coordination of skeletal muscle development and repair. α-actinin is located to the z-disc and analogous dense bodies. Its function is to help anchor the myofibrillar actin filaments. So it would be expected that the mRNA levels of these genes would increase as differentiation occurred and this is what the results reflect.

All of this evidence together shows strongly that there was myocyte differentiation induced throughout the experiment once the media was replaced by DMEM differentiation media.

4.2 Effect of Wnt3a on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels

When the cells were treated with Wnt3a there is still a clear increase in the amount of differentiation marker mRNA expression seen with the C2C12 cells throughout the experiment. This shows that there is still clear and obvious differentiation of the cells. The level of mRNA expression seen however is slightly lower than the control cells at all of the time points, however not significantly lower and this trend is seen throughout the differentiation markers. Although not significant, the finding of, if anything, a decrease in Wnt3a was unexpected given the positive role in Wnt signalling and therefore muscle differentiation. Therefore an increase in expression with respect to the control cells would be

expected. The reason this has not been seen could be due to the effect which the addition of Wnt3a had on the C2C12 cells away from the actual direct effect of Wnt3a itself.

When activity assays were carried out to test the role which Wnt3a may have on 11βHSD1 activity it reflected what was seen with both the mRNA data and also the morphology of the cells, in that there was no significant decrease or increase in the differentiation of the C2C12 at day 6.

4.3 Effect of Wnt5a on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels

The mRNA levels of 11βHSD1, MyoD, Myogenin, and α-actinin were measured at day 1, 3 and 6. They were used as they represent markers of differentiation. The mRNA levels of each of these markers increased throughout the time points for differentiation from day 1 to day 6. This shows that despite the addition of Wnt5a there is still some differentiation of the C2C12 cells, which was induced at day 0 after the addition of DMEM differentiation media.

Despite the mRNA levels of the differentiation markers increasing the level of increase is less with Wnt5a than that seen for the control. This curtailed increase in the level of mRNA expression is seen at all of the time points and becomes significant on day 6 for all of the markers. The reason for this is most likely due to the role which Wnt5a plays in the canonical Wnt pathway. Wnt5a promotes beta-catenin degradation through a GSK-3-independent pathway which involves down-regulation of beta-catenin-induced reporter gene expression[18]. Due to Wnt5a causing degradation of beta-catenin it functions as an inhibitor of the Wnt pathway. The enhanced turnover of β-catenin prevents it from being translocated to the nucleus and therefore there is no activation of downstream target genes.

It has already been shown that canonical Wnt signaling plays a critical role in muscle development[20], and is thought to regulate muscle differentiation[20]. Therefore by blocking this pathway with Wnt5a muscle development is interrupted perhaps explaining why Wnt5a decreases C2C12 differentiation.

The inhibition on C2C12 cell differentiation by Wnt5a is also evident morphologically. It is most evident on day 6 where there is less formation of the elongated differentiated C2C12 myotubules.

4.4 Effect of DKK1 on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels

With DKK1 there is however a decrease in the expression of 11βHSD1, MyoD, myogenin, and α-actinin mRNA with respect to the control for all of the time points and this is significant at day 6 upon the terminal differentiation of the myoblasts to myotubles. This is likely due to DKK1 being a negative regulator of the Wnt signalling pathway. Acting through removal of LRP5/6 from the cell surface, or by DKK1 binding to LRP6, thereby disrupting the Wnt-induced frizzled-LRP6 complex formation[15]. Overall DKK1 inhibits β-catenin signaling activated by the Wnt family of secreted signalling proteins. This effect which DKK1 has on the Wnt signalling pathway could explain why there is a decrease in the mRNA expression of the differentiation markers. However the morphological evidence shows that there is possible apoptosis of the cells or necrosis which could have been caused by the addition of the DKK1.

4.5 Effect of control SF-conditioned media on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels

With the addition of control SF-conditioned media to the C2C12 cells, there was a clear increase in the amount of differentiation of the cells throughout the days of differentiation as shown with all the markers of differentiaition in figures 14, 15, 16, and 17. This control SF-conditioned media contains the substances which are released from synovial fibroblasts when nothing has been added to them. The slight decreases in mRNA levels of the markers of differntiation for the cells treated with the control SF-conditioned media as compared to the control cells seen in figures 14, 15, 16, and 17 could show that there is something which is released by the synovial fibroblasts naturally which inhibits the differentiation of the cells. Alternatively due to the results not being significant, the decrease could be due to experimental error and not from a substance released from the synovial fibroblasts. The control conditioned media also provides the required negative control for the later TNF-α and dex SF-conditioned media experiments.

4.6 Effect of TNF-α SF-conditioned media on 11βHSD1, MyoD, Myogenin and α-actinin mRNA levels

With the addition of conditioned media from TNF-α-treated SF cells a decrease with respect to the control cells at day 6, where there was terminal differentiation from myoblasts to myotubules. This could be explained due to the TNF-α conditioned media containing Wnt5a which had previously been shown to decrease the level of 11βHSD1, MyoD, Myogenen, and α-actinin mRNA expression.

4.7 Effect of Dexamethasone conditioned media on 11βHSD1, MyoD, Myogenin and a-actinin mRNA levels

Differentiation of C2C12 cells was suppressed, most markedly at day 6. This could be due to the dexamethasone conditioned media containing Wnt5a and DKK1 which had previously been shown to decrease the level of 11βHSD1 mRNA expression.

The decrease in expression of differentiation markers is less with the dexamethasone conditioned media than with the TNF-α conditioned media. This could be due to dexamethasone conditioned media containing DKK1 as well as Wnt5a.

Having seen that DKK1 causes a decrease in myocyte differentiation (figure 10, 11, 12, and13) it was hypothesised that the decrease in expression seen with dexamethasone SF-conditioned media, and TNF-α SF-conditioned media was at least in part due to DKK1. To test this the experiment was repeated but this time with the addition of a DKK1 inhibitor, to see if this eliminated the significant decrease in mRNA levels of the markers of differentiation which was seen with the addition of TNF-α conditioned media.

4.8 The effect of DKK1 inhibitor on reversing the effects of TNF-α conditioned media

DKK1 inhibitor was added at day 0 along with the DMEM differentiation media and any other treatments to test whether by removing the action of DKK1 from the conditioned media, the effect which conditioned media had on the C2C12 cell differentiation could be reversed. Confirming earlier results TNF-α SF-conditioned media caused a decrease in the

differentiation of C2C12 cells, shown with all the markers of differentiation. When the DKK1 inhibitor was added alone to the C2C12 cells there was no significant decrease in the differentiation of the C2C12 cells, showing that the DKK1 inhibitor alone does not affect the differentiation. When the inhibitor was added along with the TNF-α conditioned media there was no longer a significant decrease in the mRNA expression of the majority of the markers of differentiation (α-actinin, MyoD, and myogenin). It suggests that DKK1 is a factor within TNF-α conditioned media which leads to it causing a significant decrease in C2C12 differentiation. Clearly DKK1 is not the only factor contained within the TNF-α conditioned media to have an affect on the differentiation of the C2C12 cells. There is also the potential that the DKK1 inhibitor concentration or indeed the conditioned media preparation concentration was not optimised and this would need to be investigated further. In contrast to α-actinin, myogenin, and MyoD the levels of 11βHSD1 were still significantly decreased when the DKK1 inhibitor was added to the cells treated with TNF-α SF-conditioned media. This implies either other factors contained within the TNF-α conditioned media which contribute to the decrease in the expression of the genes on top of DKK1, or that these factors differentially regulate 11βHSD1 and other muscle differentiation marker genes.

4.9 Conclusions and future work

The data presented clearly demonstrates that, Wnt5a, DKK1, TNF-α conditioned media and dexamethasone conditioned media lead to a significant decrease in muscle differentiation, and that the effects seen with TNF-α conditioned media could be a result of DKK1. This confirms both parts one and two of the hypothesis, that substances present within inflamed joints (Wnt5a, and DKK1) can lead to a decrease in muscle differentiation, and that TNF-α conditioned media and dexamethasone conditioned media can lead to a decrease in muscle

differentiation. It also goes some way to testing the third part of the hypothesis that by inhibiting DKK1 the effects of TNF-α conditioned media can be reversed. Due to both Wnt5a and DKK1 being present in the joint during inflammation[22], it is possible that these are factors which lead to the decrease in muscle differentiation seen in people with RA[2]. The C2C12 model which was used throughout this project have the benefit of being a clean single cell source that has a good chance of showing a direct effect of any factor that might affect the system.

In future work the inhibition of DKK1 action by the DKK1 inhibitor could be further explored by finding the optimum concentration. Also the same work could be carried out on human muscle cell lines in order to bring the research closer to humans, and also on mice models to see how the substances affect the muscle differentiation in the whole organism, and if the optimised DKK1 inhibitor could reverse any effects seen. Improvements could be made to this experiment by optimising the dosage of the substances used to treat the C2C12 cells and also by using human cell lines. The main limitation of this project was that all the work was carried out on the same C2C12 cell line throughout, and there was no work done on animals which would have given results which included all interactions and substances found within a complete mammal.

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