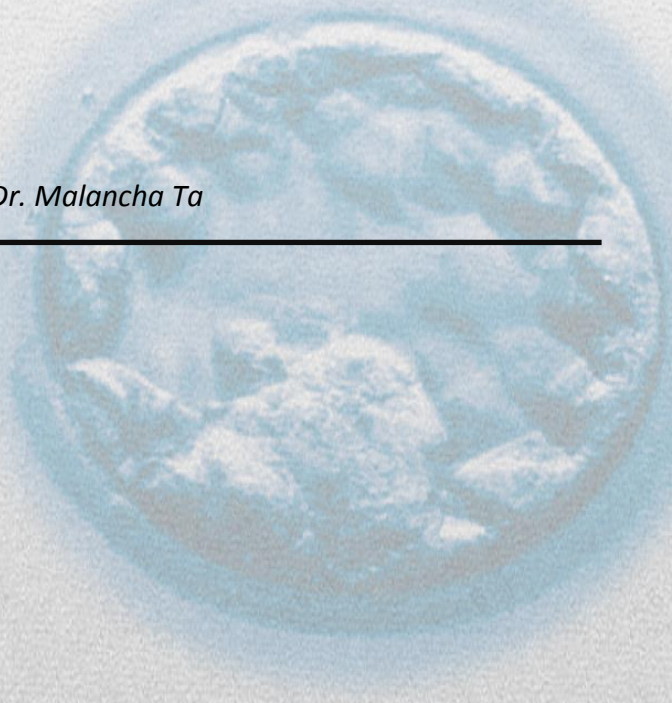
IISER-Kolkata MS Thesis Dissertation

Characterizing the role of Non Muscle Myosin II in proliferation and the effect of serum deprivation on maintenance of pluripotency of human Wharton's Jelly Mesenchymal Stem Cells

by Tulika Sharma, M.S.

Under the guidance of Dr. Malancha Ta



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Indian Institute of Science Education and Research- Kolkata (IISER-K)

Characterizing the role of Non Muscle Myosin II in proliferation and the effect of serum deprivation on maintenance of pluripotency of human Wharton's Jelly Mesenchymal Stem Cells

A *Thesis Submitted*

In Partial Fulfillment of the Requirements for the BS-MS Dual Degree

by

Tulika Sharma

(08MS45)

to the

Department of Biological Sciences

Indian Institute of Science Education and Research, Kolkata

Declaration

I hereby declare that the thesis titled — "Characterizing the role of Non Muscle Myosin II in proliferation and the effect of serum deprivation on maintenance of pluripotency of human Wharton's Jelly Mesenchymal Stem Cells" is an original work of mine carried out for the partial fulfillment of the requirements for the BS-MS dual degree. I declare that this report submitted is the result of investigations carried out by me under the supervision of Dr. Malancha Ta at the Department of Biological Sciences, Indian Institute of Science Education and Research, Kolkata.

Date:

Tulika Sharma

Indian Institute of Science Education and Research, Kolkata Department of Biological Sciences

Certificate

It is certified that the work contained in the thesis titled — "Characterizing the role of Non Muscle Myosin II in proliferation and the effect of serum deprivation on maintenance of pluripotency of human Wharton's Jelly Mesenchymal Stem Cells" by Tulika Sharma has been carried out under my supervision to be submitted to partially fulfill the requirements for awarding the BS-MS dual degree by Indian Institute of Science Education and Research, Kolkata and that this work has not been submitted elsewhere for a degree.

Date:

Dr. Malancha Ta

Department of Biological Sciences

Indian Institute of Science Education and Research

Kolkata

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-Tulika Sharma

Contents

Abstract

Wharton's Jelly Mesenchymal Stem Cells (WJMSC) holds the potency of becoming a tool for cell-based therapy. Their multipotent, immunomodulatory, homing capacity, non-tumorogenic character and easy availability give these cells an edge over other forms of stem cells i.e. embryonic stem cells and adult stem cells.

Stem cells, in general, are present in low number in their niche. To be able to use WJMSCs for clinical purpose, we need to expand the culture in vitro. Non muscle myosin, being a motor protein involved in cell division (cytokinesis), cell migration, and cell-cell and cell-matrix adhesion, becomes a contending candidate for investigating its role in proliferation capability of WJMSCs. blebbistatin, an inhibitor of NMII activity, inhibits the ATPase activity by interfering with the phosphate release process which reduce the affinity of NMII to bind with actin filament. In this study, we have isolated the WJMSCs from human umbilical cord and characterized them through immunophenotyping and differentiating the cells to adipocyte and osteocyte lineages. Through this study, it has been shown that disruption of actomyosin cytoskeleton leads to reduced proliferation of WJMSCs. WJMSCs in presence of blebbistatin exhibit a profound change in cell morphology i.e. a loss in spindle shape, flattened out cell shape, formation of stress fibre. Flow cytometry data and senescence data confirm that the WJMSCs grown in presence of blebbistatin show a G0/G1 growth arrest with cells going to premature senescence.

Serum deprivation at the site of infection/inflammation pose a major issue towards the success of cell based therapies. *in vitro* studies in bone marrow MSC (BMMSC) has shown that when BMMSC were subjected to serum deprived condition, it led to the selection of a population of cell with higher expression of pluripotent markers. Hence we wanted to study whether WJMSC are able to survive in serum deprived condition and if yes then how do they behave in terms of stem cell marker expression. Our study has shown that when WJMSC were subjected to serum deprived condition they also led to a selection of population that expressed higher levels of pluripotent markers.

1 INTRODUCTION

1.1 Stem Cells

Every human starts with a single cell – a single cell that gives rise to all kinds of cells present in our body. This has always been a topic of fascination for mankind. It is this property of a cell to divide and form cells of different lineages (**differentiation**) and the potential to divide and renew their population for long periods (**self-renewal**) which justify the term stem cells.[1, 2]

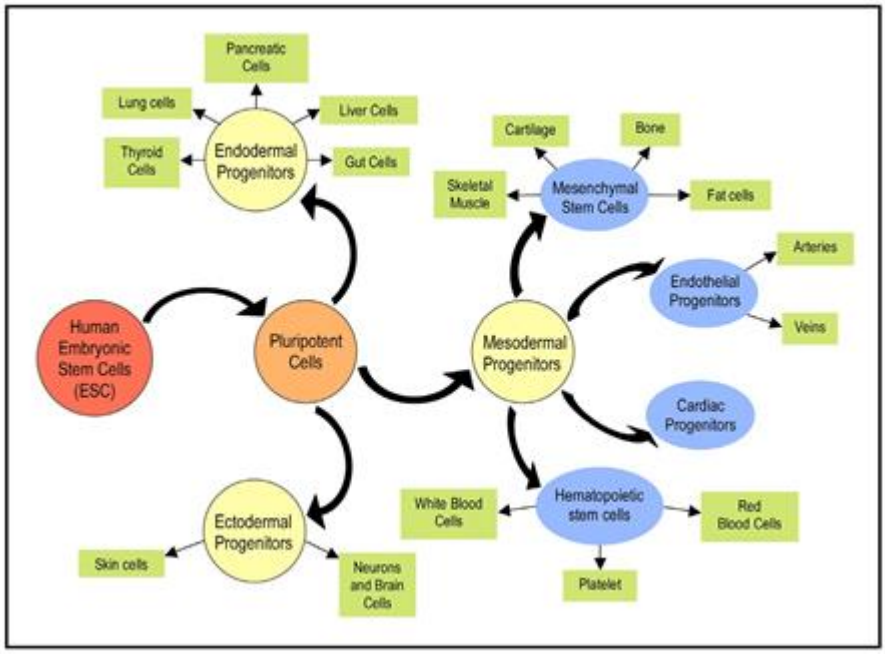


Fig 1: Various lineages of stem cell
Source: http://www.bakerinstitute.org/publications/stemcell-intro-0208.pdf

Stem cells exist in almost all tissues of an adult body and mostly stay in a dormant or non-dividing state within the tissues. They act as an internal repair system during tissue damage replacing dead/non-functional cells in the body and hence maintaining homeostasis in terms of cell number. Whenever a cell becomes non-functional and is required to be replaced, the appropriate stem cell is invoked. Stem cells then divide and give rise to the required cell type and also replenish their own population for future requirement. Hence, stem cells form an essential part in the day to day basics of our life- from blood formation to the replacement of non-functional specialized cells in the gut lining.[3]

Stem cells have far more vital and potential application in today's world than their daily roles in our lives. A few stem cell therapies have been designed and successfully implemented in humans. For example- bone marrow transplant, treatment of myocardial infarction with mesenchymal stem cells.[4] Stem cells hold the key to improving if not curing diseases which are incurable presently - Alzheimer's, diabetes to name a few. Hence it becomes our responsibility to expand our knowledge base about these cells, thereby enabling us to design therapies through which these cells can be used efficiently with minimal risks.

1.2 Types of Stem Cells

On the basis of the ability of stem cells to differentiate into various lineages, they are classified into the following categories:

Differentiation Potential	Number of cell types	Example of stem cell	Cell types resulting from differentiation
Totipotential	All	Zygote (fertilized egg), blastomere	All cell types
Pluripotential	All except cells of the embryonic membranes	Cultured human ES cells	Cells from all three germ layers
Multipotential	Many	Hematopoietic cells	skeletal muscle,cardiac muscle, liver cells, all blood cells
Oligopotential	Few	Myeloid precursor	5 types of blood cells (Monocytes, macrophages, eosinophils, neutrophils, erythrocytes)
Quadripotential	4	Mesenchymal progenitor cell	Cartilage cells, fat cells, stromal cells, boneforming cells
Tripotential	3	Glial-restricted precursor	2 types of astrocytes, oligodendrocytes
Bipotential	2	Bipotential precursor from murine fetal liver	B cells, macrophages
Unipotential	1	Mast cell precursor	Mast cells
Nullipotential	None	Terminally differentiated cell e.g. Red blood cell	No cell division

Table 1: Classification of stem cells on the basis of differentiation potential
Source: http://www.csa.com/discoveryguides/stemcell/overview.php

Based on the source, stem cells are broadly categorized into three categories i.e. Embryonic Stem Cells (ESCs), Adult Stem Cells (ASCs) and induced Pluripotent Stem Cells (iPSCs).

1.2.1 *Embryonic Stem Cells (ESCs)*

ESCs are isolated exclusively from the inner cell mass of 5-8 days old blastocysts in the preimplantation-stage. ESCs can differentiate into all the specialized cells in the adult body. However, they fail to form the tissues that are formed from the trophectoderm layer e.g. amnion, hence are categorized as pluripotent and not totipotent cells. ESCs can be induced to produce a number of different specific cells which are important from clinical perspective for e.g. liver, bone, muscle or blood cells.

1.2.2 *Adult Stem Cells (ASCs)*

Adult stem cells are undifferentiated and unspecialized cells found among specialized cells in an adult tissue or organ where they multiply to replenish dying cells and regenerate damaged tissues. ASCs, in general, are multipotent with their differentiation potential restricted to the types of cells found in the tissue that they reside in. The environments in which ASCs are present have crucial effect in determining the fate and the ability of an adult stem cell to differentiate into other cell types. However, till date we do not have a clear understanding on how it happens.

Adult stem cells are further classified on the basis of their tissue of origin and lineage into which they can differentiate, for example hematopoietic stem cells, epithelial stem cells, Bone Marrow Mesenchymal Stem/Stromal Cells (BMMSC), Wharton's Jelly Mesenchymal Stem/Stromal Cells (WJMSC), endothelial stem cells, neural stem cells etc.

1.2.3 *induced Pluripotent Stem Cells (iPSCs)*

Induced pluripotent stem cells, generally abbreviated as iPS cells or iPSCs are produced in the lab by reprogramming somatic cells to express ESC type characteristics. iPSCs have been shown to possess similar properties as natural pluripotent stem cells. Some similarities are the expression of certain stem cell genes, pluripotent markers and proteins, doubling time, teratoma formation, embryoid body formation, potency and differentiability. iPSCs have been successfully generated from adult stomach, skin, liver and blood cells.

1.3 Mesenchymal stem cells

Due to the variety of sources and methods by which Mesenchymal stem cells (MSCs) can be isolated and expanded, it becomes imperative to define certain basal requirements for a culture to qualify as a MSC culture. Addressing this issue, in 2006, the Mesenchymal and Tissue Stem Cells Committee of the International Society for Cellular Therapy (ISCT) proposed three criteria that are essential for defining MSCs: [5]

1. MSC must be able to demonstrate plastic adherence when grown under standard culture conditions.
2. It should be positive for the surface antigen expression of CD105, CD73, and CD90 and minimal/negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II proteins.
3. Finally, the property that most specifically defines MSC is their capabiliity for classical tri-lineage mesenchymal differentiation i.e. cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* tissue culture-differentiating conditions.

    a In the case of osteogenic differentiation, an osteogenic cocktail which consists of dexamethasone, ascorbic acid-2phosphate and beta-glycerophosphate is used and the culture is maintained for three weeks. The mineral deposition, can be viewed by staining with Alizarin Red or von Kossa staining. Furthermore, osteogenic differentiation is accompanied by the expression of genes such as osterex, cbfa1, osteopontin, osteocalcin, bone sialoprotein, which can be monitored at the RNA and protein level.

    b In the case of Adipocyte differentiation, a medium supplemented with dexamethasone, isobutylmethylxanthine (IBMX), insulin, indomethosine and a PPARgamma agonist such as BRL 49653 is used. The appearance of adipocytes containing lipid-filled droplets can be demonstrated by staining with oil red O and the expression of genes adipsin, aP2 and PPARgamma can be monitored.

    c In the case of Chondroblast differentiation, presence of dexamethasone, ascorbic acid phosphate and ITS+ supplement, which consists of bovine insulin, transferrin, selenous acid, linoleic acid and bovine serum albumin is required and is demonstrated by staining with Alcian blue.

As mentioned earlier that MSCs can be extracted from various sources, one such rich source for extracting MSCs is Wharton's Jelly present in the umbilical cord.

The focus of this report will be on Wharton's Jelly MSCs (WJMSCs).

1.4 Wharton's Jelly

The mucoid connective tissue of the umbilical cord present between the amniotic epithelium and the umbilical vessels is called Wharton's Jelly. Thomas Wharton in 1656 first noticed that this gelatinous substance is primarily composed of proteoglycans and various isoforms of collagen. The primary purpose of the Wharton's Jelly is to prevent the blood vessels present in the umbilical cord from bending, compression and torsion. During early phase of embryogenesis, a primitive MSC population migrates towards the AGM (aorta-gonad-mesonephros) region through the developing cord. It is believed that a sub-population of this primitive MSC population gets trapped in the connective tissue matrix i.e. Wharton' Jelly[6,7,8,9]

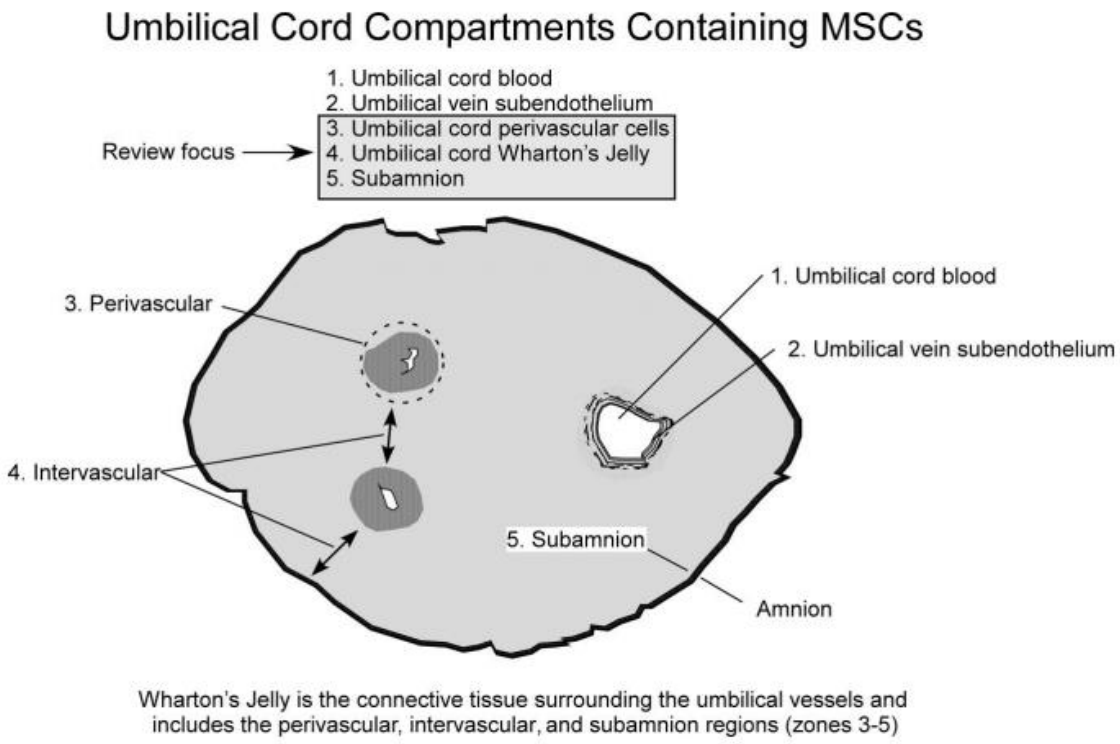


Fig 2: Cross Section view of Umbilical Cord and WJMSC's are isolated from the perivascular, intercascular and subamnion regions. Source: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3311226/

To be more precise, WJMSCs should be categorized as fetal stem cells as they represent a bridge between embryonic and adult stem cells in terms of potency (plasticity) and pluripotent marker expression. They can differentiate into wider lineages eg: osteogenic, chondrogenic, adipogenic, cardiomyocytes, neurogenic and glial cells, and dopaminergic neurons. Interestingly, WJMSCs not only possess MSC properties but they exhibit properties similar to those attributed to ESCs. Specifically, WJMSCs express human ESC markers Tra-1-60, Tra-1-81, SSEA-1 (stage-specific embryonic antigen-1), SSEA-4, alkaline phosphatase. Additionally, WJMSCs express the pluripotency markers Oct-4, Sox-2, and Nanog, at relatively lower levels than ESCs.[10,11]

1.5 Advantages of Using Wharton's Jelly Mesenchymal Stem Cells

Both adult and embryonic stem cells have major drawbacks in spite of their inherent stem cell characteristics. These limitations can be overcome with the utilization of WJMSCs.

- Unlike Embryonic stem cells, WJMSCs **do not form teratomas** when injected subcutaneously in severe combined immunodeficient mice.[12,13]
- WJMSCs are **immuno-suppressive** in nature; possess anti-inflammatory and immuno-modulatory effects. Several studies currently suggest, WJMSCs, like BM-MSCs, do not require tissue matching, thus, allowing for an allogeneic cell therapy source, as any donor can give cells to any other person without rejection or need of immuno-suppressant drugs.
- *In vitro* differentiation to adipogenic, osteogenic or chondrogenic lineages, does not lead to the loss of expression of key immunomodulatory molecules in WJMSCs.[13,14,15,16]
- They possess **homing property** i.e., have a tendency to move towards the site of infection/ inflammation and is able to heal inflammation mediated tissue damage.
- WJMSCs are procured from the umbilical cord that would otherwise be discarded as medical waste. The **ease of collection** of these cells (which is non-invasive and non-controversial) has obvious advantage over collection of ASCs or ESCs. ASCs involve invasive surgical procedures whereas ESCs are a controversial source as they are acquired by destruction of the embryo.
- WJMSCs have **better proliferative potential** than adult stem cells and are easier to expand ex vivo.[17]

- WJ is a much richer source of stem cells than umbilical cord blood.[18]

Due to these advantages over other sources of stem cells, WJMSC form an ideal source of MSCs for designing cell-based therapies. Large number of cells is required to carry out cell based therapies and stem cells are generally present in very few numbers in their respective niches. Hence, a better understanding of parameters affecting the proliferative potential of stem cells may help us to harvest large number of cells. Thus it becomes important to thoroughly study their proliferative properties. Hence we wanted to investigate the role of cytoskeletal elements such as non muscle myosin II (NMII) in proliferation and cell cycle progression of WJMSCs. NMII has been earlier shown to take part in cell division, cell migration and cell adhesion.

1.6 Myosin

Myosin is a superfamily that comprises ATP-dependent actin based motor proteins. It performs primary functions in muscle contraction and a huge number of eukaryotic motility processes. Various types of myosin have been discovered that carry out several specific processes in cells e.g. Myosin I aids in vesicle transport, Myosin II in muscle contraction. There are lot of inter-class variations in the myosin superfamily in terms of morphology and the purpose they fulfill. However, ATP hydrolysis, actin binding and force transduction are common to all myosins.[19]

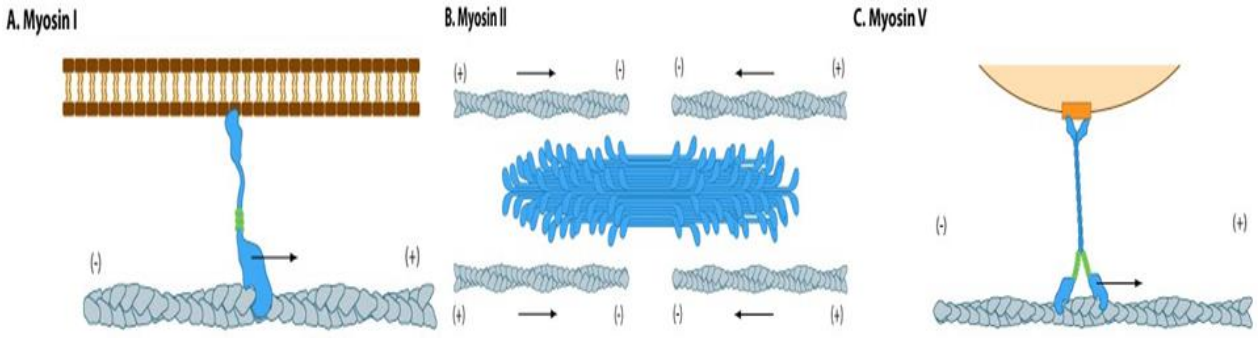


Fig 3: Various types of Myosins.
Source: http://www.mechanobio.info/Home/topics/Mechanobiology/What-are-Motor-Proteins/myosin-isoforms

1.7 Non muscle myosin II (NMII)

Non-muscle eukaryotic cells contain an isoform of myosin II called non-muscle myosin II, which resembles myosin II in terms of structure and function. NMII proteins form an integral part of the actomyosin cytoskeleton. These proteins are known to play an important role in many basic cellular processes such as cytokinesis, cell-cell adhesion, cell-matrix adhesion and cell migration.

Three different types of NM II has been discovered in mammals namely, NM II-A, II-B, II-C. MYH9 (II-A), MYH10 (II-B) and MYH14 (II-C) are the three genes which are responsible for the expression of these isoforms in humans present. These genes are present on three different chromosomes. Although all three isoforms share a number of biochemical and structural properties, there are also important differences among them that are being investigated at the cellular level. [20, 21]

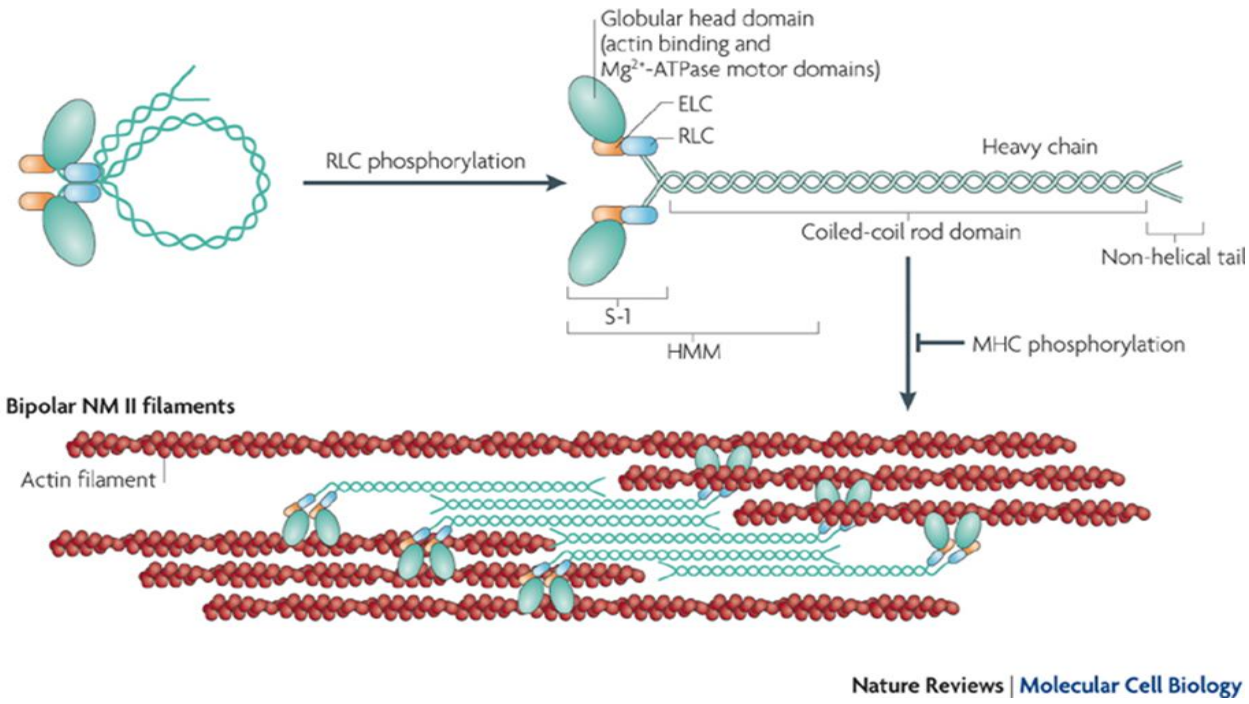


Fig 4: Structure of Non muscle Myosin II and the formation of bipolar filaments
Source: http://www.nature.com/nrm/journal/v10/n11/fig_tab/nrm2786_F1.html

1.7.1 Structure, Function and Regulation

Structure:

All NMII molecules are hexamers composed of Myosin Heavy Chain (MHC) dimers and two pairs of myosin light chains (MLCs-essential light chain (ELC) and regulatory light chain (RLC)). NMII contains an ATP binding cleft and an actin binding site in each of the two globular heads. The globular head is followed by the neck region which is responsible for providing lever action to amplify head rotation when ATP is hydrolyzed. Binding sites for the MLCs are present in the neck domain. The neck region is trailed by a long α-helical coiled coil, which gives rise to an extended rod-shaped domain. This domain terminates in a relatively short non-helical tail and helps in the dimerization of heavier two chains. The rod domain can associate to other rod domains and hence, helps in bipolar filament formation which is required for contractile activity. Although this contractile activity is mostly required in the sarcomeres of the differentiated muscle tissues, such as the beating heart, but it is also evident in nonmuscle cells in varied cellular processes such as motility, cell division (cytokinesis) and adhesion.[21]

Function:

NMII form bipolar filaments, similar to that formed by myosin II in sarcomeres. This type of assembly helps NMII to provide strong force transduction in non-muscle cells. Strong contractile motions are required in a non-muscle cell at the time of cell migration, cytokinesis, cell-cell adhesion and cell-matrix adhesion.[21,22,23,24]

NMII heads interact with actin and are responsible for the translocation of the NMII filament on the actin filament. In a reaction cycle, there are interactions between myosin heads and actin filaments:

ATP Hydrolysis Cycle:

- Myosin head binds firmly to the actin filament in an ATP unbound state.
- When ATP binds to the catalytic domain, it leads to a conformational change resulting in release of the myosin head from actin.

- Further conformation change occurs due to ATP hydrolysis which results in the myosin head moving forward towards the next docking site on the actin filament.
- Release of inorganic phosphate causes stronger binding.
- Finally ADP dissociates thereby leaving the myosin head tightly attached to actin.

Regulation:

The MLCs play a very important role in stabilizing and regulating the NMII. MLCs bind non-covalently but tightly to the neck region of MHCs and aid in the stabilization and regulation of the myosin structure and activity. Few of the kinases, such as Ca^{+2}-calmodulin-dependent MLC kinase (MLCK) or Rho kinase, phosphorylate the MLC which leads to an increase in actin-activated-ATPase activity, filament formation and contractile activity. On the other hand myosin phosphatase (MYPT) dephosphorylates leading to a decrease in contractile activity.

The regulation of vertebrate non muscle myosin II is primarily through phosphorylation of the 20 kDa Myosin Light Chain. This allows non muscle cells to respond to numerous intra and extra cellular signals.[21]

1.7.2 (-) Blebbistatin

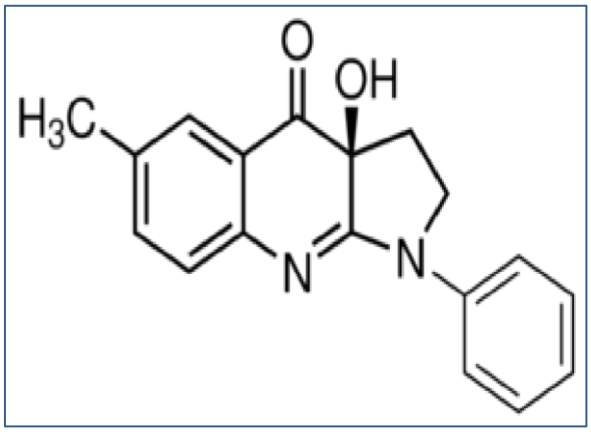


Fig 5a : Structure of (-) blebbistatin

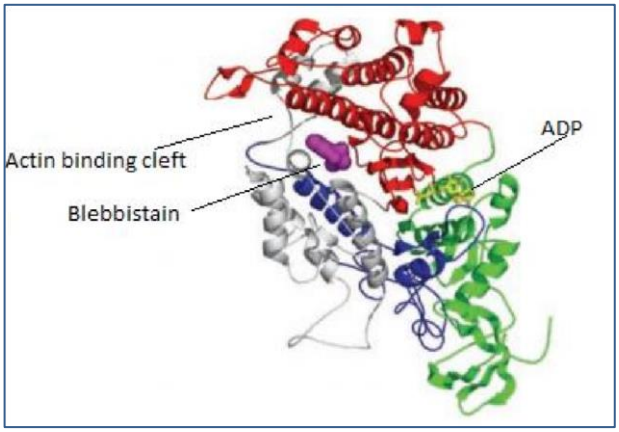


Fig 5b : Structure of NMII with binding site for blebbistatin[31]

The (-) enantiomer of blebbistatin ((-))-1-phenyl-1,2,3,4-tetrahydro-4-hydroxypyrrolo[2,3-b]-7-methylquin-olin-4-one) inhibits the motor function of NMII by specifically and reversibly blocking the ATPase activity.

This inhibitory effect is brought into action by blebbistatin binding tightly to the myosin-ADP-Pi complex and interfering with the phosphate release process. Thus, blebbistatin suppresses motor function of myosin in an actin-detached state, and prevents rigid actomyosin cross-linking.[25]

Due to the specificity and efficiency in blocking NMII activity, blebbistatin has become a very important tool for the study of the specific role of NMII in non-muscle cells and hence is widely used for the same.

Through literature review, it becomes clear that NMII plays a central role in few vital life processes, for example cytokinesis, adhesion and cell motility.[21] However, not much is known about the role of NMII in other processes like proliferation (cell cycle). Through, this study we aim to answer questions regarding the role of NMII in the cell cycle in WJMSCs.

1.8 Serum Deprivation

Hypoxia and serum deprivation are two main features of ischemic conditions. Ischemia is generally found in those parts of our body where there is a wound or poor blood circulation, infection, tumor or myocardial infarction. Almost all regions having tissue damage suffer from ischemia. Hence, it becomes important that before designing any cell based therapy, we should know how the cells are going to behave in an ischemic environment.[26]

WJMSCs are developing fast in the therapeutic world and have proven themselves competent or even better than other sources of stem cells, on some occasions to be used for designing cell based therapies. A study on how they proliferate under ischemic conditions is going to help us to develop our foresight into crafting better cell based therapies with these cells.

2 *OBJECTIVES*

2.1 Role of NMII in the cell cycle progression of WJMSCs

A stem cell can either chose to be in an undifferentiated state (self-renewing state) or differentiated state (non self-renewing state). Hence, differentiation and self-renewal are complementary process for a stem cell at any given time. The pathways that help the cell to move towards a differentiated state inhibit pathways that support self-renewal of the stem cell (ex. pluripotent markers, Oct4, Sox2, RexI) and vice versa.

If a protein associated with a differentiated state is inhibited in a stem cell, then it is expected that the cells would tend to be in the self-renewing state by default. One such pathway, the Rho/Rock pathway, is associated with differentiated state of ESCs. Non-muscle myosin II is a molecule that acts downstream of the Rho/Rock Pathway.

It has been shown previously that by inhibiting NMII activity, the self-renewal property, proliferation and pluripotent markers of human pluripotent stem cells (hPSC) increase.[27] NMII is not expressed significantly in hPSCs and knock-out mutants of hPSCs of NMIIA has indicated intricate role of NMII in regulating the cell death of hPSCs. Inhibiting its activity helped to maintain the self-renewing population off hPSCs. These cells could be carried till a higher passage number under *in vitro* conditions in a healthy self-renewing state.[27]

This report has lot of significance as stem cells are generally present in low number in their niches. Transplantation or any other therapeutic application requires harvesting a large number of cells. Hence, there exists a need for extensive expansion of MSCs to meet the clinical demand. Having a better understanding of the parameters and identification of the factors and pathways that control MSC self-renewal and proliferation hence become important. Non muscle myosin is involved in cell division (cytokinesis), cell migration, and cell-cell and cell-matrix adhesion and

maintenance of self-renewal property in hPSCs.[21,27] These properties make NMII, a contending candidate for investigating its role in the proliferation capability of WJMSCs.

NMII is expressed in WJMSCs, so will inhibiting NMII activity help in maintaining the self-renewal state of WJMSCs for a longer term? So WJMSCs were exposed to blebbistatin and various parameters were studied for e.g. growth kinetics, cell size estimation, cell cycle analysis, senescence assay to account for the proliferation rate of WJMSC when cultured in presence of blebbistatin.

2.2 Effect of Serum Deprivation on WJMSCs

Many reports suggest successful use of MSCs (bone marrow MSCs) in the treatment of ischemia caused due to myocardial infarction or stroke. As WJMSCs are a new upcoming source of MSCs, it is possible that they would soon be used for therapeutic purposes. However 90% of the MSCs are known to get removed from the system as early as 48 hours after the transplantation.[28] As the MSCs grown *in vitro* are not conditioned to stand harsh conditions like ischemia, they tend to disappear after releasing important factors onsite. It is not clearly understood why these transplanted MSC are removed through circulation and fail to give a prolonged effect. Ischemia at the site of infection/inflammation can be one of the possible causes. Hence, it becomes important to know the dynamics of MSCs in presence of serum deprived or hypoxic conditions.

As mentioned earlier, before designing therapeutic cell based therapies through WJMSC cells, we need to check whether they will be able to cope up with the onsite conditions. Hence, knowing the proliferation and pluripotent marker expression of these cells when they are subjected to serum deprivation (one of the features of ischemia) will help to design better therapies in future.

Earlier studies on BMMSCs have shown that these cells were able to survive under serum deprived conditions *in vitro*. In fact they show an upregulation in pluripotent markers like OCT4. When subjected to serum deprivation for longer duration, it led to the isolation of a sub-population of a group of highly proliferative cells that retained the MSC characters as well as having higher expression levels of the pluripotent markers, showing longer telomere length.[29]

Wang et al. in 2008 reported that third week of human embryogenesis, there is first wave of migration of hematopoietic cells and MSCs from the yolk sac and AGM through the umbilical cord towards the placenta. During later development, these cells migrate back from the placenta via the umbilical cord to the fetal liver and then finally homed in the fetal bone marrow. These migrating colonies contain primitive HSCs and MSCs. During these migrations, some of the MSCs likely get trapped in the umbilical cord matrix and then persisted there throughout gestation.[9,30] Hence WJMSCs get deposited in the umbilical cord around that period from which they survive till birth, under low nutrient and hypoxic conditions. Therefore, here we wanted to study the dynamics of WJMSCs when exposed to serum deprived condition, more specifically the expression level of pluripotent markers. The level of expression of pluripotent markers was checked at the RNA level along with immunofluorescence studies to determine the amount of stress imposed by serum deprivation.

3 MATERIALS AND METHODS

3.1 Buffers and Media

Standard medium used to culture WJMSCs:

- Dulbesso's Modified Eagle Medium-Knock Out medium (optimized for Embryonic Stem cells) (DMEM-KO)
- Foetal Bovine Serum (FBS) 10%
- Glutamine 1%
- Penstrep 0.5% (1% for growth of primary culture)

STEMPRO Adipocyte/Osteocyte/Chondrocyte culture – Basal medium + differentiation supplement (10%)

Serum Deprived Culture – Standard MSC culture medium without FBS.

D-PBS (Dulbecco's Phosphate Buffered Saline) - facilitates a buffer system which helps to maintain cell culture media in the 7.2-7.6 physiological range. For a fixed period of time, the cell tonicity and viability of cells in vitro is maintained.

STEMPRO Antibiotic-Antimycotic – 100X Pencillin + Streptomycin (Penstrep)

10X TAE buffer (500mL)

- Tris-Cl 24.2 gm
- Acetic acid 5.72 ml
- 0.5M EDTA (pH 8) 10 ml
- [18.612 gm EDTA dissolved in 100 ml dH_2O and pH adjusted to pH 8 with NaOH pellets]

3.2 Isolation of mesenchymal stem cells from wharton's jelly of human umbilical cord tissue

3.2.1 Materials Required

1. Normal saline (0.9% w/v sodium chloride).
2. Normal saline containing Antibiotic-Antimycotic in a 50 ml falcon [200ul Anti-Anti (Gibco) in 20ml saline].
3. D-PBS
4. 70% Isopropyl alcohol-Which acts as a disinfectant.
5. 10% Bleach.
6. Complete DMEM-KO with 1X Penstrep
7. Sterilized surgical instruments.
8. Laminar Air Flow hood.

9. 50 ml Falcon Tubes.
10. Beaker.
11. Serological pipettes.
12. Marker.
13. 10cm Dishes.
14. 35mm culture dishes--takes 2 ml of medium. It has an area of 8.55 square cm.
15. CO_2 Incubator.
16. Personal protective equipment (sterile gloves, laboratory coat)

3.2.2 Protocol

1. Human umbilical cord sample was collected after normal or caesarian delivery, the placenta portion was cut off using a sterile blade and then rinsed thoroughly in saline to remove the blood or any visible clots (place the cord in a falcon in ice if it needs to be carried).
2. The cleaned cord was then cut into 2-3 cm long pieces using a sterile scalpel, the pieces placed in a 50 ml falcon containing 200 µl antibiotic (Gibco) in 20 ml saline (1% antibiotic solution) and stored at 4°C for ~2 hrs.
3. The falcon was then taken to a laminar hood and the saline carefully poured off in a discard beaker containing 10% bleach. The cord pieces were then washed with DPBS thrice.
4. This was followed by a 30 sec wash in 70% isopropyl alcohol (IPA) for disinfection and another 4 washes with DPBS to remove all traces of IPA. (All waste was discarded in beaker with bleach)
5. A piece of cord was then taken in a 10 cm petri dish and slit longitudinally to expose the Wharton's jelly using a sterile scalpel. The piece was straightened as much as possible by holding opposite ends with two forceps prior to slitting.
6. The cord blood vessels i.e. the 2 small arteries and the bigger vein (optional) were then cleared off using scissors, scalpel and forceps.
7. The epithelial perivascular region (more translucent than the rest of the tissue) was then separated with scalpel and forceps and cut into small pieces.
8. The pieces were then plated on a 35mm petri dish and allowed to attach for 7-8 mins with the lid open inside the hood after which 1X DMEM-KO medium was added drop wise, ensuring the pieces were not dislodged.
9. The plates were then incubated at 37°C, 5% CO_2 with a medium change on every 3^{rd} day till cells started to come out by the 7^{th}-9^{th} day. The tissue pieces were then removed and the cell patches were allowed to become confluent.
10. Around the 9^{th}-day the cells were trypsinised and replated/frozen depending on cell number obtained and experiment to be started.
11. Trypsinisation, plating and freezing were done according to protocol.

3.3 Passaging cells and Medium changes

Passaging is the process of transferring a small number of cells into a new culture flask. It is also known as subculture or splitting of cells. Cells can go to senescence if cultured at high density for long durations, hence regular passaging of cells is required. Ideally the cells should always remain in exponential growth. When the cells are approximately 70-80% confluent (80% of surface of flask covered by cell monolayer) they require sub-culturing. (If the cells become over confluent, they will begin to die off and recovery may not be possible).

As these WJMSCs are adherent cells, which attach themselves to surface of tissue culture flasks or dishes using proteins, secreted by the cells to form a tight bridge between the cell and the surface,these bridges must be broken to detach cells from the flask surface. Trypsin, a proteolytic enzyme, is used to achieve this. EDTA (ethylene diamine tetra acetate) is often used with trypsin to chelate metal ions that might inhibit trypsin's activity. Cells should not be exposed to trypsin for long duration, as it might affect the cells' health. Appropriate exposure time is about 2-3 min.

3.3.1 Materials Required

All the reagents should be at room temperature before use:

1. Trypsin-EDTA (0.025%)
2. DPBS
3. Standard WJMSC medium
4. Inverted Microscope
5. Hemocytometer
6. Centrifuge
7. Laminar Air Flow hood
8. Aspirator pump
9. 15 ml Falcon Tubes
10. Beaker
11. Serological pipettes (1ml, 2ml)
12. Pipetteman, Pipette aid
13. Tips (1ml, 200ul)
14. Eppendorfs
15. Parafilm
16. Marker

17. 10cm Dishes
18. 35mm culture dishes/ T25 Flask
19. CO_2 Incubator maintained at 37˚C, 5% CO_2
20. Personal protective equipment (sterile gloves, laboratory coat).

3.3.2 Protocol

1. The culture dish was taken to LAF hood from the incubator and sealed with parafilm. The cells were then checked under inverted microscope for the confluency.
2. The culture dish was taken back to LAF hood and then media was aspirated off.
3. The cells were rinsed with 1ml of DPBS.
4. Added trypsin (0.2ml for 35mm dish and 0.5ml for T25 culture flask) and kept it in incubator for 23 mins.
5. Observed the dish under light for the small particulates floating in the solution.
6. Added media (1.5ml for 35mm dish and 3ml for T25 culture flask), transfered them to a prelabeled 15ml falcon tube and centrifuged at 1800rpm for 2 mins at room temperature.
7. Discarded the supernatant and then resuspended the pellet in 1ml of media.
8. Took 5ul of Trypan blue and added it to 5ul of resuspend media with cells .Mixed them properly and carefully dropped the suspension in to a hemocytometer covered with a cover slip.
9. Placed the chamber on the microscope stage and performed cell counting (Count the cells present in the corner four squares in the chamber. Cells that touch the top and right lines of a square should not be counted, cells on the bottom and left side should be counted.).
10. Calculated the number of cells present in 1ml of suspension with help of following formula:

    No. Of cells counted/$4*2*1.7*10^4$--------For 35mm culture Dish

    No. Of cells counted/$4*2*3.5*10^4$--------For T25 culture Flask

11. Plated the cells with seeding plate density ranging from 1000-5000 cells per cm^2[usually $5*10^4$ cells in 35mm culture dish(area-10cm^2) and $1.25*10^5$ cells in T25 culture flask(25cm^2)—If the seeding density is 5000 cells per cm^2]
12. Calculated the volume of resuspended cells required to give a final density of$5*10^4$ cells/ml and transfer it to 35mm culture dish.
13. Added media (2ml for 35mm culture dish and 4ml for T25 culture Flask).
14. Made sure that the culture dishes are properly labeled with passage number, which is the number of sub-cultures the cells have gone through.
15. Placed the culture dishes in incubator (at 37 °C and 5% CO_2).

Medium Changes:

Metabolic processes in a cell, result in acid produced, thereby a reduction in pH. Nutrient depletion can be measured by a pH indicator is added to the medium. When the medium color changes from pink to yellow, indicates a need for medium change. Usually, for this culture medium change is required every 2 days.

1. Medium change was given on 3rd day in case of WJMSCs primary culture at P0 passage and on 4th day in case regular WJMSCs culture at other passages.
2. The culture dishes were carefully taken out from the incubator and transferred to LAF hood.
3. Medium from the dish is discarded with the help of aspirator or pipette.
4. 2ml of fresh medium was then added to the culture dishes and placed them back in the incubator (at 37 °C and 5% CO2.)

3.4 Cryopreservation

Cryopreservation is a process of cell preservation by cooling to attain low sub-zero temperatures, (typically) 77 K or −196 ˚C (the boiling point of liquid nitrogen). Such low temperatures, lead to the stoppage of any biological activity. This includes the cell death biochemical reactions. Cryoprotectants are used to save the cells from damages caused due to freezing during the approach to low temperatures or warming to room temperature. So commonly a cryoprotectant like DMSO is used.

3.4.1 Materials Required

1. Freezing media consisting of 10%DMSO and 90% FBS [4.5ml of FBS+0.5ml of DMSO].
2. Isopropanol freezing container----drops the temperature 1˚C per min.
3. Freezing Vials
4. -80 ˚C Freezer
5. Liquid Nitrogen Tank

3.4.2 Protocol

1. Follow the above passaging protocol up to the cell counting step and calculate the volume of cell suspension that consists of desired no. of cells (must be 10^5- 10^6cells/ml) ,next the cells were spun down and upernatant was discarded and then suspended the cells in

freezing medium at a density of $1x10^6$ cells/ml and transfer them in to a pre-labeled freezing vial.

2. Placed the vials into an isopropanol freezing container (which lowers the temperature in a regulated manner (1˚C per min)) and keep it in -80 ˚C overnight.
3. Next day, transferred the vials to Liquid Nitrogen (-196 ˚C).

3.5 Revival of cryopreserved cells

3.5.1 Materials Required:

1. Water in a beaker at 37˚C.
2. Laboratory Thermometer
3. Standard WJMSC medium
4. Centrifuge
5. Laminar Air Flow hood
6. Aspirator pump
7. 15 ml Falcon Tubes
8. Beaker
9. Serological pipettes (1ml, 2ml)
10. Pipette aid
11. Marker
12. 10cm Dishes
13. 35mm culture dishes/ T25 Flask
14. CO_2 Incubator maintained at 37˚C, 5% CO_2
15. Personal protective equipment (sterile gloves, laboratory coat).

3.5.2 Protocol

1. Cell vials were taken out from liquid nitrogen (-196°C) and immersed in water at 37°C till the ice melted.
2. The cells were then immediately resuspended in about 5 ml medium (removes DMSO) and centrifuged (1800rpm, 2 mins, room temperature).
3. Supernatant was then removed and the cells were again resuspended in 2 ml medium and plated in a 35mm dish and incubated at 37°C, 5% CO_2 overnight.

3.6 Differentiation potential of WJMSCs

One of the hallmark characteristics of MSCs (WJMSCs) is their ability to differentiate into adipocytes, chondrocytes and osteocytes in culture.

3.6.1 Adipogenesis growth medium

Standard DMEM KO Complete culture media was used for controls.

Adipogenesis Differentiation Medium	Conc.	For 10mL
STEMPRO® Adipocyte Differentiation Basal Medium	1X	9 ml
STEMPRO ® Adipogenesis Supplement	1X	1 ml

Adipogenic media in general contains 0.5mM isobutylmethylxanthine (IBMX), 200µM Indomethosin, 10µM Insulin, 1µM Dexamethasone

1. First the cells were cultured in normal complete media following the above mentioned protocol at 1000 cells/cm^2 for control and 3000cells/cm^2 for adipocytes.
2. After attaining 60%-70% of cell confluency (will take about 3 days, depends upon seeding cell no.), media was aspirated and then the Adipogenesis Differentiation Medium was added.
3. Media change has to be given on every 4th day for about 18-19 days and should be monitored under phase-contrast microscope before giving media change.
4. After 18 days cells were stained using Oil Red O stain to observe oil droplets and can compare with control dish in which only normal complete media was added but not differentiation medium.

<u>Oil Red O staining:</u>

1. Aspirate media carefully from the dish
2. Fix with 4% PFA for 30 minutes at room temperature
3. Wash with PBS for 3 times 22 min each
4. Aspirate and Rinse twice with water
5. Aspirate water, Add 2ml of OIL RED O stain
6. Incubate at room temperature for 1hr
7. Wash 3 times with water, observe under microscope
8. Add water to dish to prevent cells from drying

3.6.2 Osteogenesis growth medium

DMEM KO medium+10% FBS+1% Glutamine----Complete culture media

STEMPRO® Osteogenesis Differentiation	Conc.	For 10mL
STEMPRO® Osteocyte/Chondrocyte Differentiation Basal Medium	1X	9 ml
STEMPRO ® Osteogenesis Supplement	1X	1 ml

Osteogenesis media in general contains 10mM β-Glycero phosphate, 0.1µM Dexamethasone, 0.2µM Ascorbic acid

1. First the cells were cultured in normal complete media following the above mentioned protocol at 1000 cells/cm^2 for control and 5000cells/cm^2 for osteoocytes.
2. After attaining 60%-70% of cell confluency (will take about 3 days, depends upon seeding cell no.), media was aspirated and the Osteogenesis Differentiation Medium was added.
3. Media change has to be given for every 3-4 days for about 21 days and should monitor under phase-contrast microscope before giving media change.
4. After 21 days cells were stained using Vankossa stain to observe calcium deposits and can compare with control dish in which only normal complete media was added but not differentiation medium.

<u>Vankossa staining:</u>

1. Remove the media completely
2. Wash twice with PBS
3. Fix with 4% PFA for 15 min
4. Wash with water twice
5. Incubate with1% Silver nitrate(dissolved in water) under UV light for 60 mins
6. Remove solution, Wash with water once
7. Observe under microscope(still in water, don’t allow the cells to dry)

3.7 Immunophenotyping (cell surface marker analysis)

Different types of cells express different cell surface proteins, this is the principal on which immunophenotyping is based. In Immunophenotyping, cells are first labeled with a fluorescein tagged specific antibody and then run through a flow cytometer. Number of cells showing positive for the surface marker is noted. Sample labeled with fluorescein tagged non-specific antibody acts as control. If there is a significant difference between the number of cells positive in control and experiment, that sample is considered to have positive expression of that marker.

3.7.1 Materials Required

1. Trypsin-EDTA 0.025%
2. 1X PBS (Phosphate Buffered Saline)
3. Complete DMEM-KO with 0.5X Penstrep
4. Antibodies - IgG isotype control, anti-CD34, anti-CD73, anti-CD90 - all tagged with Phycoerythrin (PE) fluorophore.
5. FACS Tubes

3.7.2 Protocol

1. Cells were trypsinised, neutralized and counted followed by centrifugation at 1000rpm for 2 mins to pellet the cells.
2. The pellet was then washed once with PBS (same centrifugation conditions) and then resuspended in fresh 1X PBS such that around 1*10^5 cells are present in 50 µl of PBS.
3. 50 µl aliquots were made in appropriately labeled flow tubes and required volume of respective antibodies (labeled with Phycoerythrin) were added.
4. The tubes were then incubated in the dark at 4˚C for an hour.
5. The Flow cytometer was switched on 20 mins before use, log scale measurements for 10000 events were made. Voltage settings used were: Forward scatter – 30.3V; Side scatter – 246.7V; Phycoerythrin (PE) – 335V.

Antibody concentration used for 1*10^5 cells –

Antibody	Volume added	Stock concentration	Isotype control (Iso-PE)*
CD90	1 µl	0.2 mg/ml	1 µl of neat
CD73	1 µl	12.5 µg/ml	1 µl of 1:16 dilution
CD34	1 µl	12.5 µg/ml	1 µl of 1:16 dilution
CD166	1 µl	12.5 µg/ml	1 µl of 1:16 dilution

* Iso-PE stock concentration – 0.2 mg/ml

3.8 Treatment of WJMSCs with blebbistatin

3.8.1 Materials Required

All the reagents should be at room temperature before use:

1. Trypsin-EDTA (0.025%)
2. DPBS
3. Standard WJMSC medium
4. 2.5 mM blebbistatin stock
5. DMSO stock
6. Ice Bucket filled with ice
7. Inverted Microscope
8. Hemocytometer
9. Centrifuge
10. Laminar Air Flow hood
11. Aspirator pump
12. 15 ml Falcon Tubes
13. Beaker
14. Serological pipettes (1ml, 2ml)
15. Pipetteman, Pipette aid
16. Tips (1ml, 200ul)
17. Eppendorfs
18. Parafilm
19. Marker
20. 10cm Dishes
21. 35mm culture dishes/ T25 Flask
22. CO_2 Incubator maintained at 37˚C, 5% CO_2
23. Personal protective equipment (sterile gloves, laboratory coat).

3.8.2 Protocol

1. Followed the steps of passaging protocol mentioned above until up step-9 (till the counting of cells).
2. Labeled two culture dishes/T25 flasks, one with CT+DMSO (which acts as a mock control as blebbistatin is dissolved in DMSO) and other with 5B.
3. Added 2ml of fresh media to culture dishes(4 ml for T25 flask) and then added 4µl of DMSO to CT+DMSO labeled dish(8µl for T25 flask) and 4µl of 2.5mM blebbistatin to 5B labeled dish (8µl for T25 flask). Note: Care should be taken not to expose blebbistatin to light as it is light sensitive and repeated thawing should also be avoided.

4. Then added the cell suspension according to desired seeding density (generally 3000cells/cm^2,so that mostly cells will reach 70%-80% confluence in 3 days)
5. The culture dishes were placed inside the incubator maintained at 37 ˚C and 5% CO_2.

Cells were exposed to two rounds/passages of blebbistatin in order to further process for experiments like flow cytometry, immunofluorescence and three rounds of exposure for senescence experiments.

3.9 Cell cycle analysis by Flow cytometry using propidium iodide

The cell cycle is typically divided into three phases: G0/G1, S and G2/M phase. In the G0 phase cells are mostly quiescent and do not take part in cell division. In the G1 phase the cells prepare to divide through cell division. Both these phases have 2n DNA content and cannot be distinguished solely with the help of a DNA probe like Propidium Iodide. In the S phase DNA is synthesized, which leads to a gradual increase in DNA content through S phase (between 2n and 4n DNA). In the G2 and M phases the DNA content is 4n (G2: just prior to mitosis; M: mitosis). Again, these phases cannot be distinguished solely with the help of a DNA probe and flow cytometry.

Propidium Iodide (PI) was the DNA probe in the flow experiments. PI is a DNA fluorochrome which intercalates to DNA base pairs. Blue light is required as the excitation source (e.g., 488 nm argon ion laser). Because PI also ends up staining double-stranded RNA; RNase A is added to the staining solution to remove the latter. More than 10,000 events should be acquired for the data to be considered authentic.

3.9.1 Materials Required

1. Trypsin-EDTA 0.025%
2. 1X PBS (Phosphate Buffered Saline) ice cold
3. 70% ethanol/tablet PBS in a clean falcon (ice cold) (4.5 mL/sample)
4. Complete DMEM-KO with 0.5X Penstrep
5. Propidium Iodide Stock (1mg/mL)
6. RNase A (10mg/mL)
7. FACS Tubes
8. 37 ˚C Water Bath

9. Inverted Microscope
10. Hemocytometer
11. Centrifuge
12. Laminar Air Flow hood
13. Aspirator pump
14. 15 ml Falcon Tubes
15. Beaker
16. Serological pipettes (1ml, 2ml)
17. Pipetteman, Pipette aid
18. Tips (1ml, 200ul)
19. Eppendorfs
20. Parafilm
21. Marker
22. 10cm Dishes
23. 35mm culture dishes/ T25 Flask
24. CO_2 Incubator maintained at 37˚C, 5% CO_2
25. Personal protective equipment (sterile gloves, laboratory coat).

3.9.2 Protocol

Day 1:

1. The 50-70% confluent cell culture is trypsinized and taken in a falcon tube. The cell number should be at least 5 X 10^5 or more.
2. Centrifuged (1800 rpm, 2 min, Room Temperature).
3. Aspirated the medium and added 2-3 mL tablet PBS (ice-cold) for washing. Mixed well with a 1 mL pipetteman.
4. Centrifuged (1800 rpm, 2 min, Room Temperature).
5. Aspirated the tablet PBS and add 0.5 mL tablet PBS. Mix well with a 1 mL pipetteman.
6. Added the cell suspension drop wise to ice cold 4.5 mL 70% ethanol/PBS with continuous vortexing.
7. Incubated at 4 °C overnight. The cells can also be stored at -20 °C for 2 – 3 weeks.

Day 2:

Small crystals can be seen in the cell suspension.

Switch on a 37 °C water bath.

1. Centrifuged (2000 rpm, 10 min, Room Temperature)

2. During centrifugation, prepared PI Solution. In a fresh 1.5 mL eppendorf: 475µL D PBS + 28µL Stock PI (1mg/mL) + 5µL RnaseA (10mg/mL) {Cover the eppendorf properly with aluminum foil.}
3. Decanted the supernatant carefully in a fresh falcon tube (Don't use aspirator or pipetteman).
4. Centrifuged the supernatant (2000 rpm, 10 min, Room Temperature).
5. Meanwhile, added PI solution to the cell pellet and mixed well with a pipetteman. Cover the falcon properly with aluminum foil.
6. Took 5 µL cell suspensions on a hemocytometer for counting the number of cells and also for checking if the cells are present or not.
7. If a pellet is seen in the falcon (step 4) then discard the supernatant completely. Pool the cells in PI Solution.
8. Incubated the cells in 37 °C water bath for 30 min.
9. Took the cells for flow cytometry.

Please notice that the FACS has been calibrated just before running the sample.

3.10 Senescence assay

Senescent cells are known to express biochemical markers such as acidic senescence-associated ß-galactosidase (SA-ß-Gal). This is the principle on which the senescence detection assay is based. The senescent cells have active pH dependent SA-ß-Gal. SA-ß-Gal, at acidic pH of 5.9-6.1, become active and digest X-Gal to give indole which gives a blue coloration that is easily detectable through eyes. A senescent cell is large in size. Also the pattern of gene expression is changed.

Cell signalling Senescence β-Galactosidase kit was used for senescence detection.

3.10.1 Materials Required

1. 1X PBS (Phosphate Buffered Saline):17 mM KH2PO4, 5 mM Na2HPO4, 150 mM NaCl (pH 7.4)
2. N-N-dimethylformamide (DMF)
3. Polypropylene tubes
4. 37°C dry incubator (no CO2)
5. Phase contrast or light microscope
6. 70% glycerol (optional)
7. PBS: Prepare at least 6 ml 1X PBS per 35 mm culture dish.
8. FACS Tubes

Solutions stated below should be freshly prepared. (copied)

1. Staining Solution: Re-dissolve the 10X Staining Solution by heating to 37°C with agitation. Dilute the 10X Staining Solution to a 1X solution with distilled water. Need about 930 µl of the 1X Staining Solution per 35 mm culture dish.
2. Fixative Solution: Dilute the 10X Fixative Solution to a 1X solution with distilled water. Need about 1 ml of the 1X solution per 35 culture dish.
3. X-gal: Dissolve 20 mg of X-gal in 1 mL DMF to prepare a 20X stock solution. Excess X-gal solution can be stored at -20°C in a light resistant container for one month. Always use polypropylene plastic or glass to make and store X-gal solutions. Do not use polystyrene.
4. β-Galactosidase Staining Solution: For each 35 mm culture dish to be stained, combine the following in a polypropylene container:

    a. 930 µl 1X Staining Solution (see step 8)

    b. 10 µl Staining Supplement A

    c. 10 µl Staining Supplement B

    d. 50 µl 20 mg/ml X-gal in DMF (see step 4)

Due to variations in water pH, please be sure that the solution has a final pH of 5.9-6.1. HCl can be used to lower the pH if necessary.

3.10.2 Protocol

Senescence β-Galactosidase Cell Staining Protocol:

1. Removed the growth media from the cells.
2. Rinsed the plate one time with 1X PBS.
3. Added 1 ml of 1X Fixative Solution to each 35 mm culture dishes. Allowed cells to fix for 15minutes at room temperature.
4. Rinsed the plate two times with 1X PBS.
5. Added 1 ml of the β-Galactosidase Staining Solution to each 35 mm well (see Setup, Step 4) Incubate the plate at 37°C overnight in a dry incubator (no CO2).
6. While the β-Galactosidase Staining Solution is still on the plate, checked the cell under a microscope (200X total magnification) for the development of blue colour.
7. For long-term storage of the plates, removed the β-Galactosidase Staining Solution and overlayed the cells with 70% glycerol. Store at 4°C.

3.11 Immunoflourescence

Immunofluorescence is a characteristic antigen-antibody reaction. The fluorescent dye tagged antibodies bind with the antigen thereby forming the antigen-antibody complex which is visualized using ultra-violet (fluorescent) microscopy. Direct and indirect are two primary methods of immunofluorescent labeling. The direct method is less frequently used. In the direct method, a fluorescent dye is chemically conjugated to the antibody against the molecule of interest. In indirect immunofluorescence, there is a secondar antibody involved. The unlabeled primary antibody attaches itself to the molecule of interest. A second anti-immunoglobulin antibody, tagged with the fluorescent dye then attaches to the constant part of the primary antibody (called the secondary antibody). Fluorescein isothiocyanate (green) and tetra methyl rhodamine isothiocyanate (red) are the fluorochromes commonly used in immunofluorescence. Fluorochromes are ultra-violet ray absorbing dyes. They then emit visible light thereby having characteristic colours. This process is called fluorescence. Acridine Orange, Rhodamine, Lissamine and Calcofluor white are typically used fluorochromes for general fluorescence. When fluorescein (FITC) is irradiated with a blue (wavelength 488nm) light, green (520nm) colour is emitted. Phycoerythrin (PE) gives out an orange (570nm) colour.

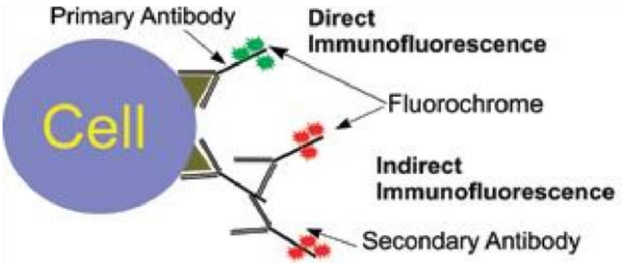


Fig 6: Principle of Immunofluorescence

3.11.1 Materials Required

1. 4% Para-Formaldehyde
2. 1X Phosphate Buffered Saline (PBS)
3. 0.1% Bovine Serum Albumin (BSA) in PBS
4. Blocking solution – (0.3% Triton X + 10% Goat serum) in 0.1% BSA in PBS
5. Primary antibody dilutions – anti-Oct4 – 1:400

(Made in 10% Goat serum	anti-Vimentin – 1:100
in 0.1% BSA in PBS)	anti-NMHCIIa – 1:50
	anti-NMHCIIb – 1:200

6. Secondary antibody [goat Anti rabbit-IgG Fab2 Alexa flour(R) 488] dilution – 1:1000 (made in 2% goat serum in 0.1% BSA in PBS)
7. ProLong Gold Antifade reagent with DAPI – 5 µl
8. 8 well Chamber slides.
9. 60mm cover slip

3.11.2 Protocol

1. Cells were seeded (500 cells/cm^2) in the wells of chamber slide.
2. After reaching 70%-80% confluency, removed the media completely
3. Washed once with PBS
4. Fixed with 4% PFA for 20 min @RT
5. Washed with 0.1% BSA in PBS three times for 2 min each
6. Removed 0.1% BSA-PBS
7. Added 0.3% triton X and 10% blocking serum in 0.1% BSA-PBS and incubated for 1hr @RT(blocking serum should be from the animal in which secondary antibody has been raised)
8. Removed the above solution(no washes)
9. Added primary antibody(vimentin--1:100 and oct4-1:400--dilutions prepared in 10% blocking serum in 0.1% BSA-PBS)
10. Incubated at 4°C overnight, seal the slide with parafilm and put it in a moistened dish
11. Washed with 0.1% BSA-PBS for three times 2 min each
12. Added secondary antibody(1:1000 dilution in 2% blocking serum in 0.1% BSA-PBS) and kept in dark @RT for 1hr
13. Washed again with 0.1% BSA-PBS 3times for 2 min each
14. Added DAPI(1 µg/ml) and incubate for 5 min and then wash with PBS
15. Mounted with DABCO (mounting media)
16. Observed under microscope
17. If anti fade mounting media with DAPI is used, then leave the slide @RT in dark for 24hrs for curing and then observe under microscope
18. Sealed the slide with transparent nail paint and store at 4°C in dark.

3.12 Serum deprivation of WJMSCs

3.12.1 Material Required

1. Standard WJMSC medium
2. Serum deprived medium
3. Inverted microscope
4. Laminar Air Flow hood
5. Aspirator pump
6. Beaker
7. Serological pipettes (1ml, 2ml)
8. Pipetteman, Pipette aid
9. Tips (1ml, 200ul)
10. Parafilm
11. Marker
12. 10cm Dishes
13. 35mm culture dishes/ T25 Flask
14. CO_2 Incubator maintained at 37˚C, 5% CO_2
15. Personal protective equipment (sterile gloves, laboratory coat).

3.12.2 Protocol

Cells are plated with standard media at a density of 1000cells/cm^2 in 35mm dish for control and serum deprived dish.

Allowed the cells to attach and grow for two days.

On third day media was aspirated from both dishes and serum deprived dish was washed with D-PBS to remove traces of FBS.

Complete DMEM KO media added in case of control and Complete DMEM KO media without FBS is added to serum deprived dish.

The cells were monitored daily under phase contrast microscope.

Media change given every 3^{rd} day.

Culture stopped on the 11^{th} day and cells taken for RNA isolation by trizol method.

3.13 RNA Isolation – Trizol method

3.13.1 Materials Required

1. Trizol
2. Chloroform
3. Isopropanol
4. 75% ethanol
5. Nuclease free water
6. Dry heat bath
7. 1.5mL eppendorf
8. Centrifuge
9. Pipetteman,
10. Tips (1ml, 200ul)
11. Eppendorfs
12. Marker

3.13.2 Protocol

1. Cells were trypsinised and collected after centrifugation or directly detached from the plate surface in Trizol, pH 4 (1 ml for 5 *10^5 cells) in an eppendorf.
2. Cells were then lysed by repeated pipetting and the sample was allowed to stand for 5 mins at room temperature.
3. 200 µl of chloroform was added (for 1 ml Trizol) sample was shaken vigorously for 15 seconds and allowed to stand for 10 mins at room temperature.
4. The eppendorf was then centrifuged at 12000g for 15 mins at 4°C.
5. The upper aqueous phase containing RNA was transferred to a fresh tube and mixed with 500 µl isopropanol (for 1 ml Trizol) and allowed to stand at room temperature for 8 mins, followed by centrifugation at 12000g for 10 mins at 4°C.
6. The supernatant was removed and the RNA pellet was then washed in 75% ethanol (made in nuclease free water) by vortexing followed by a 5 min centrifugation at 7500g, 4°C.
7. Pellet was briefly air-dried and then dissolved in appropriate amount of water by incubating for ~5 mins at 55-60°C and then kept at room temperature for 5 mins.
8. Final storage was done at -80°C.

3.14 DNase Treatment

3.14.1 Material Required

1. Ambion DNA free kit
2. Nuclease free water
3. 1.5mL eppendorf
4. Dry heat bath maintained at 37˚C
5. Centrifuge
6. Pipetteman,
7. Tips (1ml, 200ul)
8. Eppendorfs
9. Marker

3.14.2 Protocol

To 21.5 µl of RNA sample, 0.1 volume of 10X DNase I buffer (2.5 µl) and 1 µl rDNase I were added and mixed gently followed by a 30 min incubation at 37°C.

Following this 0.1 volume of DNase inactivating resin A (~2 µl) was added and the mix was incubated for 2 mins with gentle shaking and then centrifuged at 10000 rpm for 1.5 mins to collect the RNA in the supernatant.

RNA quantification was then done using Nanodrop 1000 (Thermo scientific) and equal amount of RNA was used for cDNA synthesis for each experimental set.

3.15 cDNA Synthesis- Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

3.15.1 Materials Required

1. Applied Biosystems' High capacity cDNA reverse transcription kit
2. Nuclease free water
3. Thermocycler
4. 1.5mL eppendorf
5. Centrifuge
6. Pipetteman,
7. Tips (1ml, 200ul)
8. Eppendorfs
9. Marker

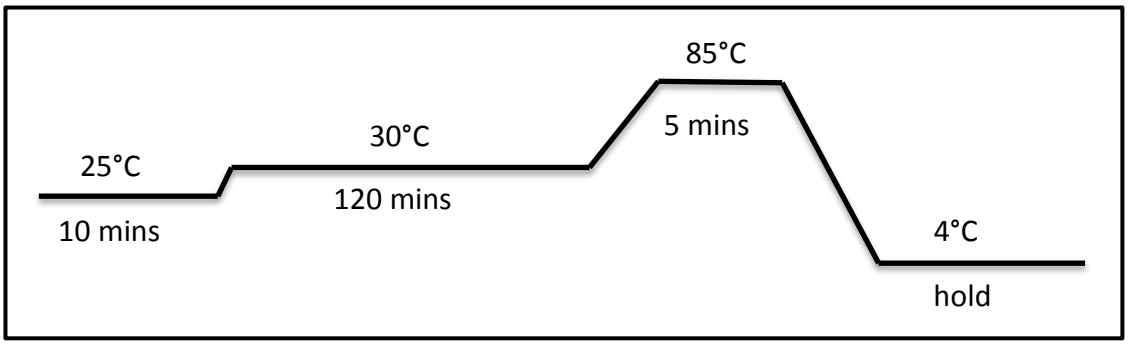
3.15.2 Protocol

RT-PCR was done in order to synthesize the cDNA of the RNA sample. Amount of RNA was calculated so as to be between 200ng – 1 µg (such that it's same for the control and experimental set)

Reaction mix for 10 µl volume –

Ingredient	Stock conc.	Final conc.	Volume used in 5 µl
RT Buffer	10X	1X	1 µL
dNTP MIX	25X	1X	0.40 µl
RT Random primers	10X	1X	1 µl
Reverse Transcriptase	Units	Units	0.5 µl
Nuclease free water			2.1 µl
RNase inhibitor			1 µl
RNA sample			5 µl

Reaction cycle for RT-PCR (cDNA synthesis)



3.16 Semi-Quantitative Polymerase Chain Reaction (PCR)

cDNA synthesized above was used to amplify the desired genes using gene specific primers. The primer sequences were obtained from scientific papers (used in validation, differentiation markers).

3.16.1 Materials Required

1. Taq DNA Polymerase and buffer (Sigma)
2. $MgCl_2$ (25mM)
3. Respective forward and reverse primers (5µM)
4. Nucleotide mix (dNTP 10mM)
5. Nuclease free water

6. Thermocycler
7. 1.5mL eppendorf
8. Centrifuge
9. Pipetteman,
10. Tips (1ml, 200ul)
11. Eppendorfs
12. Marker

3.16.2 Protocol

The reaction mix (for total reaction volume of 12.5 µl) was set up as given below –

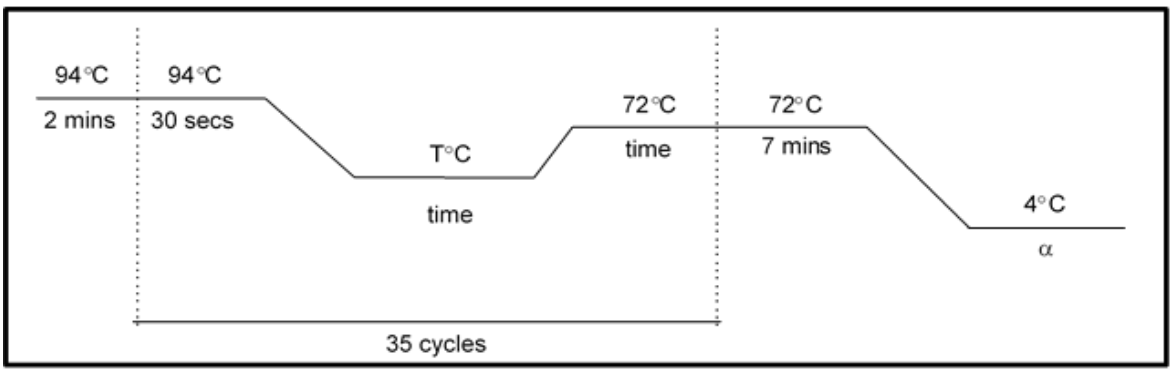
Ingredient	Volume used in 12.5 µl
Nuclease free water	8.9 µl
Buffer	1.25 µl
$MgCl_2$ (25mM)	0.75 µl
Forward Primer(5µM)	0.5 µl
Reverse Primer (5µM)	0.5 µl
dNTP (10mM)	0.25 µl
Taq polymerase	0.125 µl
cDNA*	0.5 µl

* Volume of cDNA + Nuclease free water varied according to that optimized.

Annealing temperature of the primers was calculated according to the given formula –
T = 62.3 + (0.41 * %GC) – 500/N (where N is number of nucleotides in primer)

The lower temperature was used for standardization (in case forward and reverse primers have slightly different melting temperatures) and conditions made more stringent (increasing temperature; decreasing $MgCl_2$ conc.) in order to eliminate non-specific bands.

PCR programme –



T°C = annealing temperature of primers used.
time = 30 secs for product length <500bp and 1 min for product length ≥ 500 bp.

Gene expression was then checked by electrophoresis of the PCR reaction mix after the reaction on an agarose gel (concentration of gel used varied according to size of product). Bands were viewed by irradiating gel with UV wavelength using Gel Doc XR software.

3.17 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA isolation and cDNA synthesis was done as written above. 5 µl of reaction mix was set up in duplicates for the genes whose expression was to be analysed (Oct4, ABCG2) with 18S rRNA used as control. The tubes were then set in the platform, the cDNA amplified according to the given temperature conditions and the amplification was measured at every cycle. The run was performed on an ABI Prism 7500HT (Applied Biosystems, Foster City, CA, USA), and SDS v1.4 software was used to analyze the results. Samples with a cycle threshold of 35 or less were considered for calculating the fold change in expression.

3.17.1 Materials Required

1. Taqman Real Time PCR Master Mix
2. Taqman Real Time PCR Assay Mix
3. Nuclease free water
4. Thermocycler
5. 1.5mL eppendorf
6. Centrifuge
7. Pipetteman,
8. Tips (1ml, 200ul)
9. Eppendorfs
10. Marker

3.17.2 Protocol

Composition of 5 µl reaction mix:

Ingredient	Stock conc.	Final conc.	Volume used in 5 µl
Buffer	2X	1X	2.5 µL
Assay mix	20X	1X	0.25 µl
cDNA			0.5 µl
Nuclease free water			1.75 µl

Buffer: polymerase buffer, dNTPs, $MgCl_2$, Polymerase

Assay mix: primers, Taqman probe (mix separate for each gene)

The results were analysed using the $\Delta\Delta C_T$ method (Fold increase in expression = $2^{-\Delta C_T}$). A C_T value greater than 35 was discarded and the average value for each gene was normalized with that of 18S. The fold increase/decrease in expression was calculated.

4 RESULTS

4.1 Characterization of Wharton's Jelly Mesenchymal Stem cells

In 2006, the Mesenchymal and Tissue Stem Cells Committee of the International Society for Cellular Therapy (ISCT) proposed three criteria that are essential for defining MSCs:

1. MSCs must be able to demonstrate plastic adherence when grown under standard culture conditions.
2. It should be positive for the surface antigen expression of CD105, CD73, and CD90 and minimal/negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II proteins.
3. Finally, the property that most specifically defines MSCs is their capabiliity for classical tri-lineage mesenchymal differentiation i.e. cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts using standard *in vitro* tissue culture-differentiating conditions.

4.1.1 Plastic adherence

Human umbilical cords were collected and WJMSCs were isolated by processing and seeding the tissue pieces in DMEM-KO medium according to the protocol. Cells were observed emerging from tissue pieces (Fig 7a) under phase contrast microscope after 7-8 days of culture. The morphology of cells were observed to be fusiform (spindle shaped).

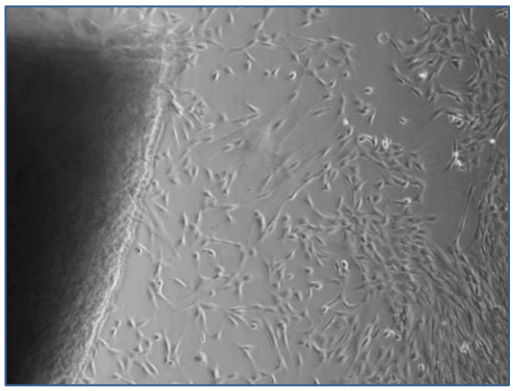


Fig 7a: WJMSCs coming out of umbilical cord tissue

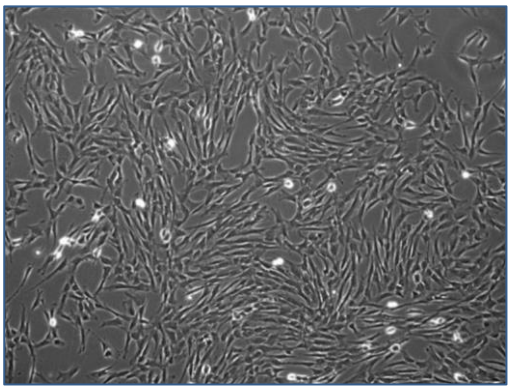


Fig 7b: WJMSCs growing in culture at P_o

4.1.2 *Immunophenotyping*

It is important to characterize a MSC culture through cell surface marker analysis. A typical MSC culture is expected to test positive for the surface antigen expression of CD105, CD73, and CD90 and minimal/negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II proteins.

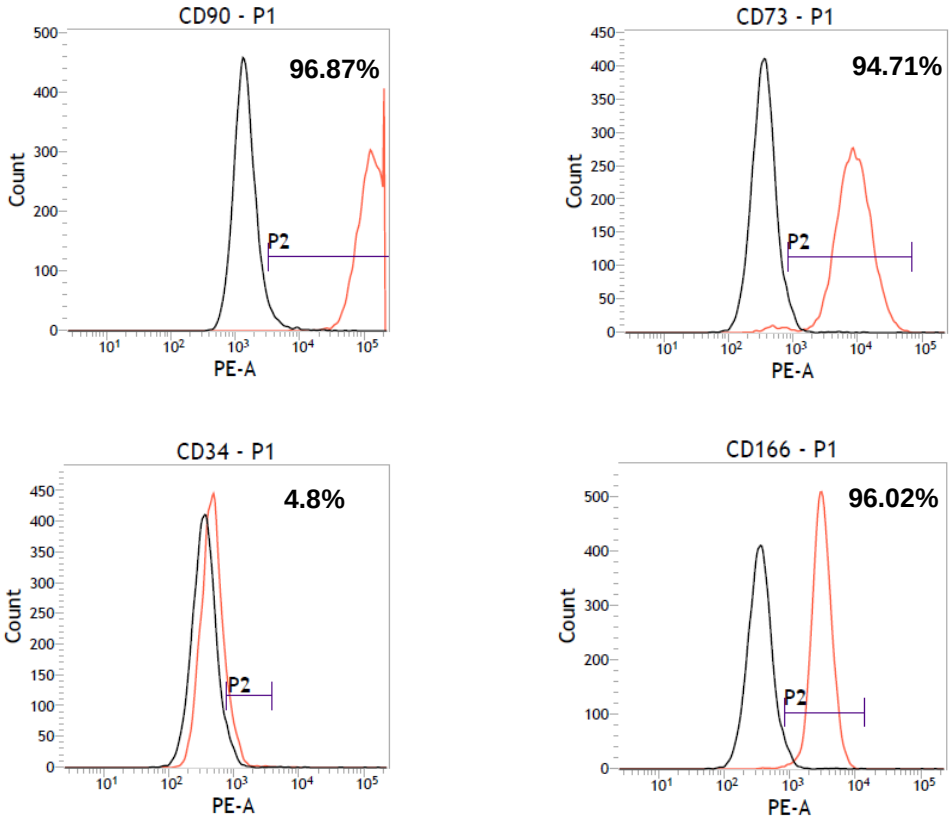


Fig 12: Immunophenotyping for various cell surface markers for MSCs. The black peak represents negative control in each case with the cells stained with non-specific antibody. The red peak represents the cells that were stained with specific antibody. P2 population is gated such that in the experiment 5% of the population represents non-specific binding.

- Positive expression of CD90, 73, 166 and negative expression of CD34 is shown with the help of Flow cytometry.
- Positive expression of CD105 and negative expression of CD45, CD14 or CD11b, CD79a or CD19 and HLA-DR needs to be demonstrated.

4.1.3 Differentiation potential of WJMSCs

In order to confirm the isolated cells as mesenchymal stem cells, the cells were induced to Osteogenic and Adipogenic lineages. Along with the control cells (non-induced, which were cultured in normal DMEM-KO media), cells were cultured in Osteogenic differentiation media and Adipogenic differentiation media respectively. In the case of Adipogenic differentiation, cells were cultured for about 18 days and then stained with Oil Red O staining in which the formation of oil droplets were observed (Fig 8 & 9).In the case of Osteogenic differentiation, cells were cultured for about 21 days and then stained with Van Kossa staining in which the mineralization of calcium deposits were observed (Fig 10 & 11). Stronger staining was observed in the case of induced cells compared to non-induced control cells.

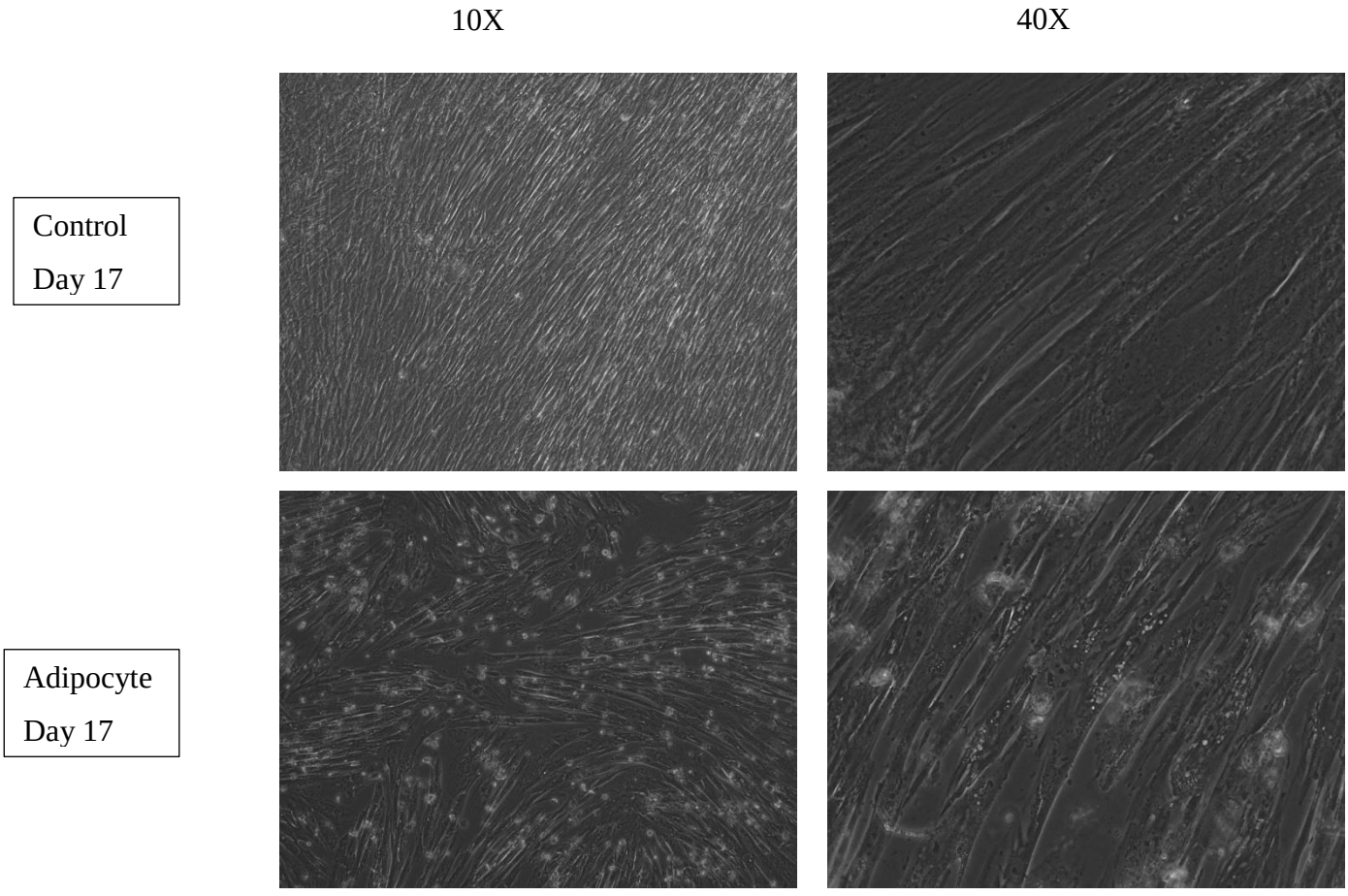


Fig 8: Unstained Adipocyte Differentiation Culture after 17 days

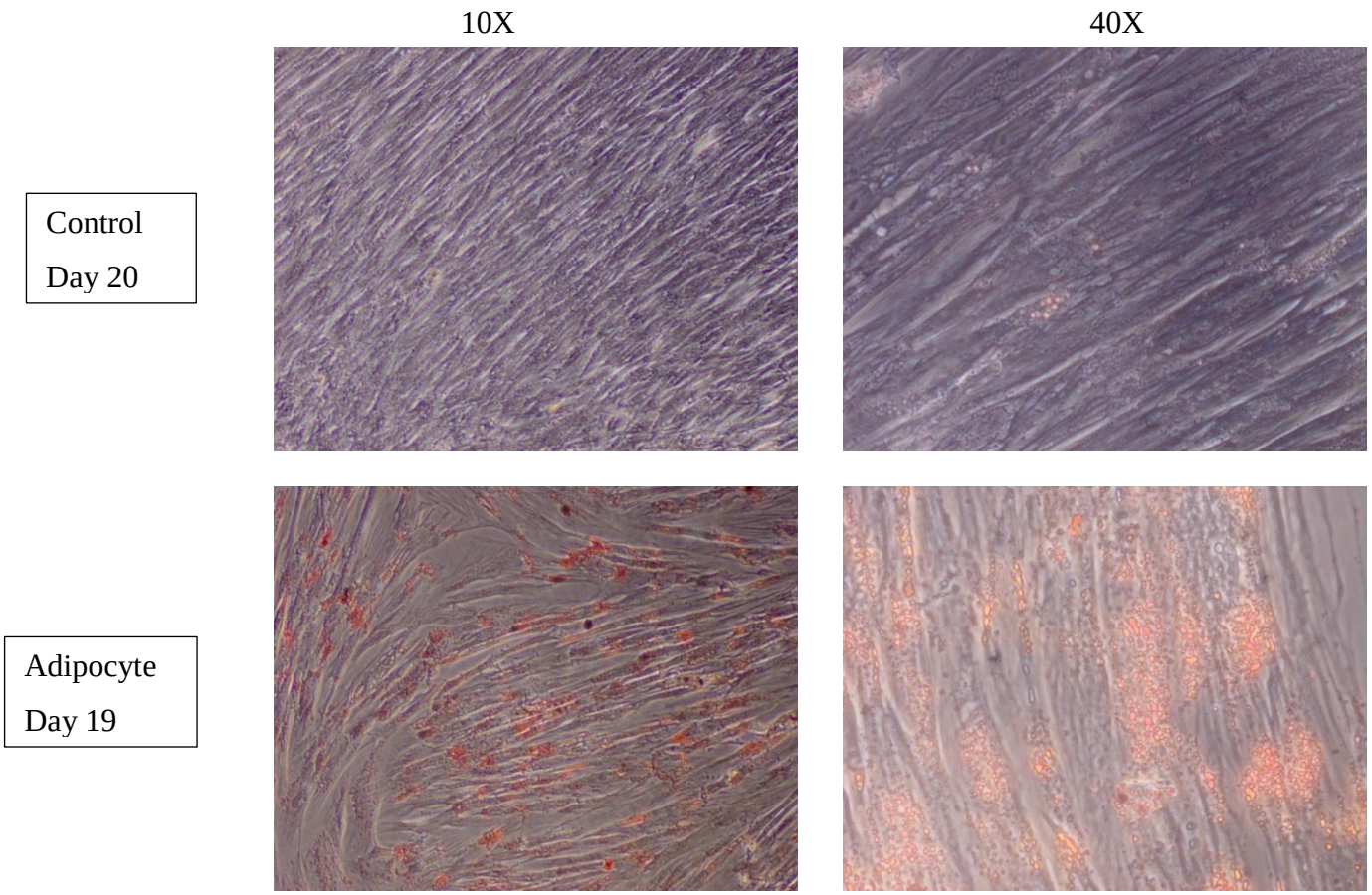


Fig 9: Oil Red O Stained Adipocyte Differentiation Culture after 19 days

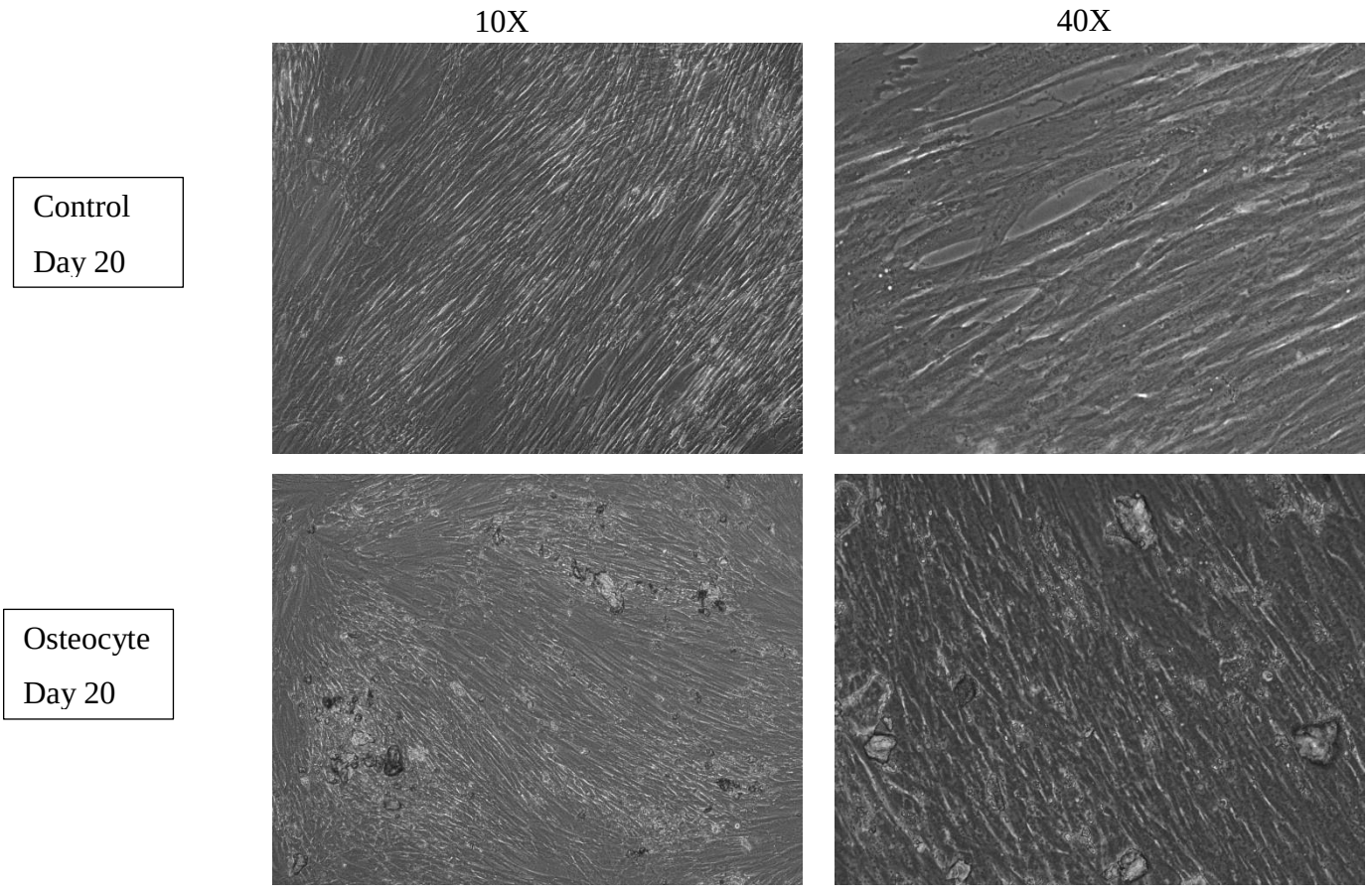


Fig 10: Unstained Osteoocyte Differentiation Culture after 20 days

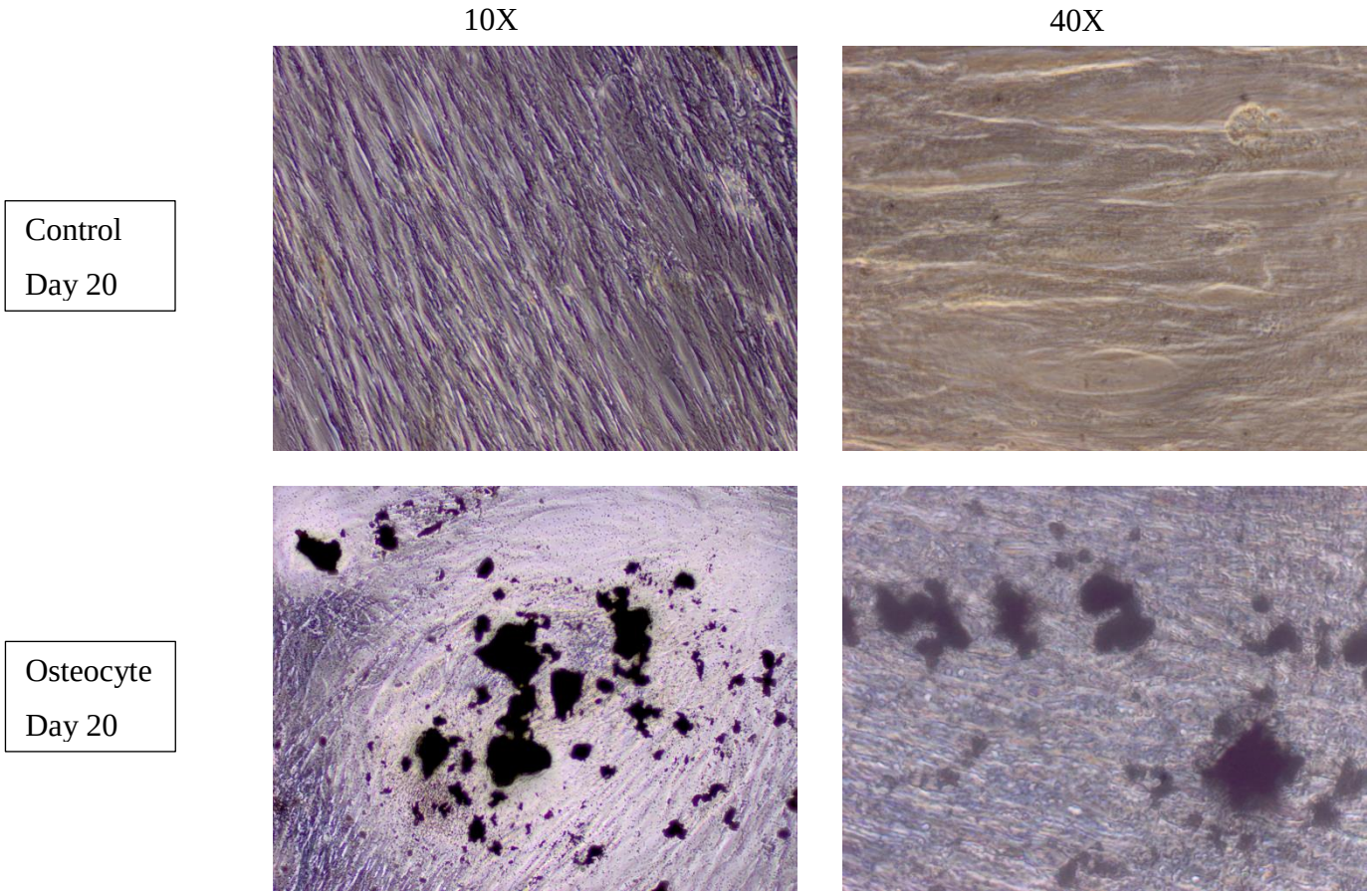


Fig 11: Von Kossa Stained Osteoocyte Differentiation Culture after 20 days

4.2 Inhibition of Non muscle Myosin II activity, hinders cell cycle progression in Wharton's Jelly Mesenchymal Stem Cells

4.2.1 Differences in Morphology and cell proliferation (Growth kinetics)

WJMSCs isolated from the umbilical cord were cultured in the DMEM-KO media, plated at a density of 1000-5000 cells/cm^2 mostly. The experiments carried out on three different human umbilical cord samples and the collective data were shown here. In the case of every experiment, two conditions were considered. One is the control in which cells were cultured in DMEM-KO media with 4uL DMSO (as blebbistatin is dissolved in DMSO-it acts as a mock control) and the other one is 5B in which cells were cultured in DMEM-KO media with 5µm blebbistatin (4uL of 2.5mM stock). The drug exposure to the cells was carried out for four passages and the data were collected. In the case of morphology, observed a profound difference in blebbistatin treated cells which were flattened out with large cell size and have shown neuronal like projections (Fig 13 right) when compared to the control cells which were in normal fusiform (spindle) shape (Fig 13 left).In addition to these, there were some bi-nucleated cells observed in blebbistatin treated cells when compared to the control cells.

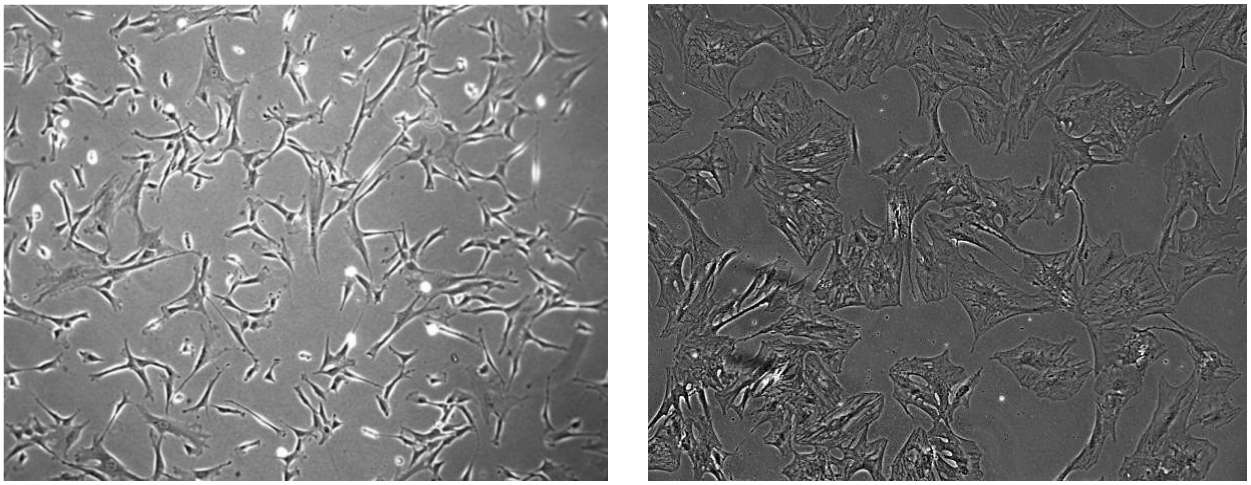


Fig 13: Effect of blebbistatin on the morphology of WJMSCs. Untreated control cells (left) which are in spindle shape. Treated cells with 5µm blebbistain (right) which are flattened out and large with some neuron like projections

In the case of cell proliferation, when plated at the same cell density (inoculum cell no-NI) in the both control and 5B cultures, more cell number (harvest cell no-NH) was observed in the case of control when compared to 5B (approximately half of cell no. of control cells were observed in 5B) indicating that blebbistatin at 5µm concentration, has inhibitory role in cell proliferation

activity of WJMSCs. Formula for population doubling (PD) and population doubling time (PDT):
PD = ($\log_{10}$ (NH) - $\log_{10}$ (NI)) / $\log_{10}$ (2)
PDT = Time of the culture (72 hrs) * $\log_{10}$ (2) / **(**$\log_{10}$ (NH) - $\log_{10}$ (NI)) or
PDT=Time of the culture (72 hrs)/PD

PD and PDT were calculated for 3rd round of exposure to blebbistatin. The following graph was plotted by the data collected from three different samples. (Fig 14)

	CT	**2.5B**	**5B**
PD (numbers)	2.96	1.69	0.66
PDT (hrs)	24.33	42.57	108.64

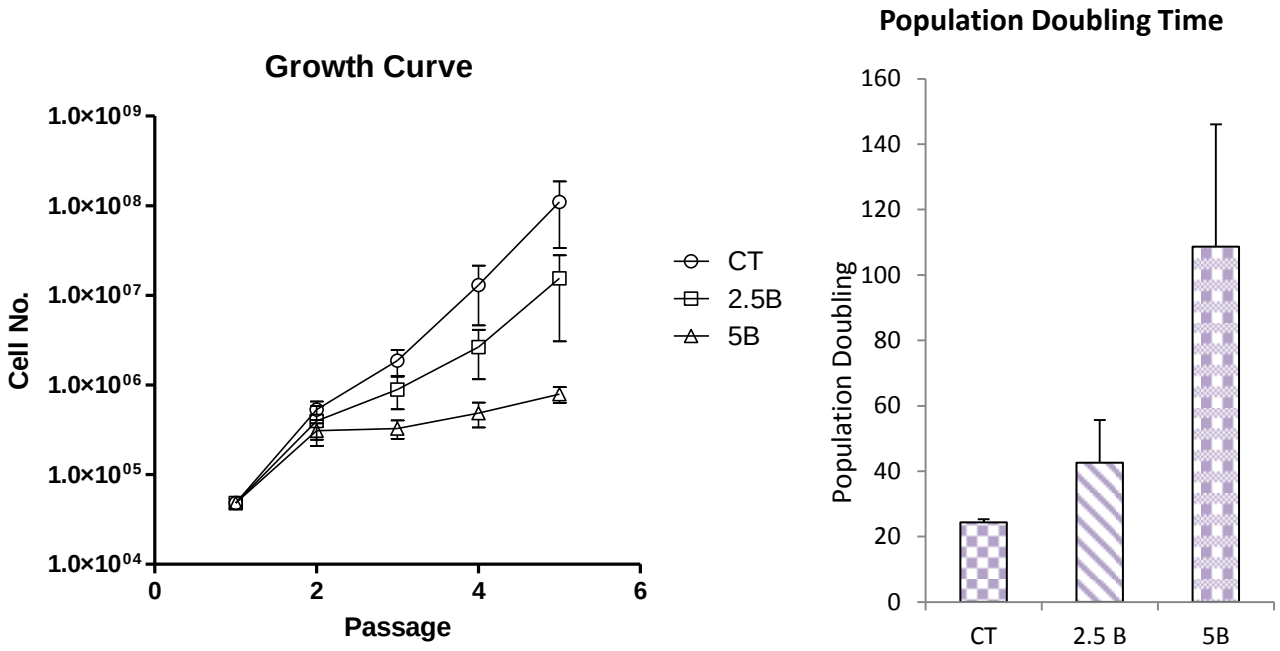


Fig 14: Effect of blebbistatin on the proliferation rate of WJMSCs. Untreated mock control has the highest proliferation rate which reduced with increasing concentrations of blebbistatin i.e. 2.5µm and 5µm. Right Panel: Population Doubling Time of Control, 2.5µm treated cells and 5µm treated cells.

4.2.2 *Cell Size estimation*

With respect to the morphology, treated cells looked much more flattened out and larger than the untreated cells. To quantify this, images were taken after exposing the cells to 5µM blebbistatin for two passages. Cell^P software was used to measure the cell size. The software was first standardized for the objectives used such that the area measured of the enclosed area will be read in µm2. Cell area was measured for 20 cells for each sample selected from two different fields. The experiment was repeated for three samples. (Fig 15)

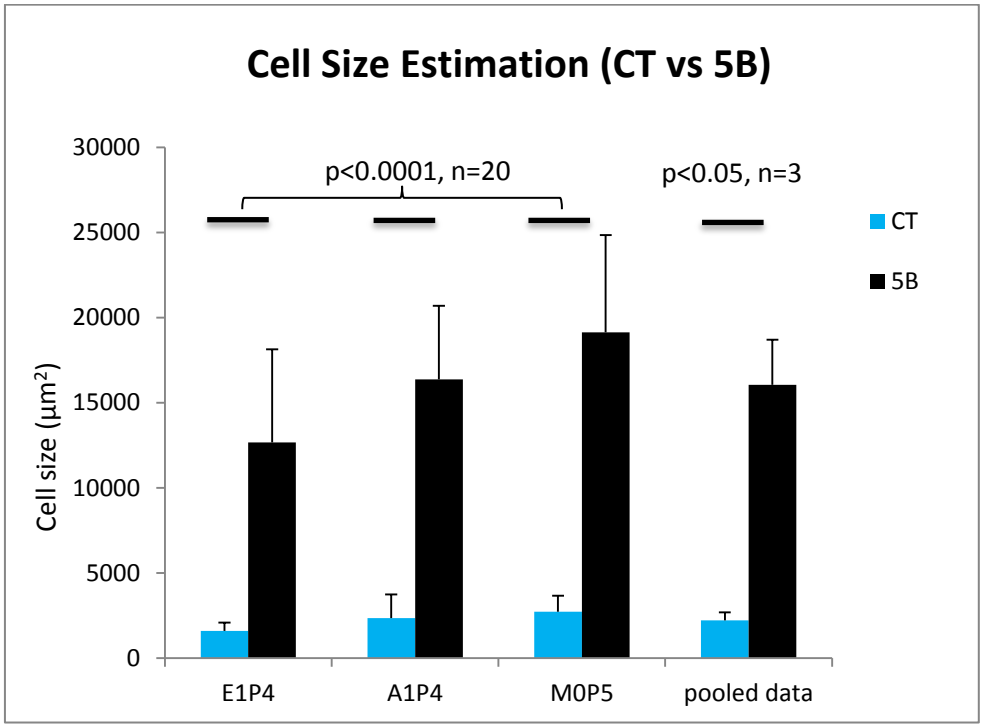


Fig 15: Effect of blebbistatin on the morphology of WJMSCs. Untreated mock control has the smaller size whereas the size of treated cells is significantly larger. (student's t-test p-value<0.0001 for individual samples, n=20 fields counted; p-value<0.05 for pooled data, n=3 times repeated with 20 fields counted for each)

4.2.3 *Immunofluorescence*

The cultured WJMSCs were exposed to blebbistatin (5µm conc.) for three passages and immunofluorescence was carried out by using primary antibody anti-vimentin (which is an intermediate filament and a mesenchymal marker) and counter staining with DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride-stains nucleus) following the protocol mentioned in the materials and method section. In the case of blebbistatin treated cells, a clear morphological

difference was observed in which cells were flattened out with more vigorous staining of vimentin compared to control (Fig 16).

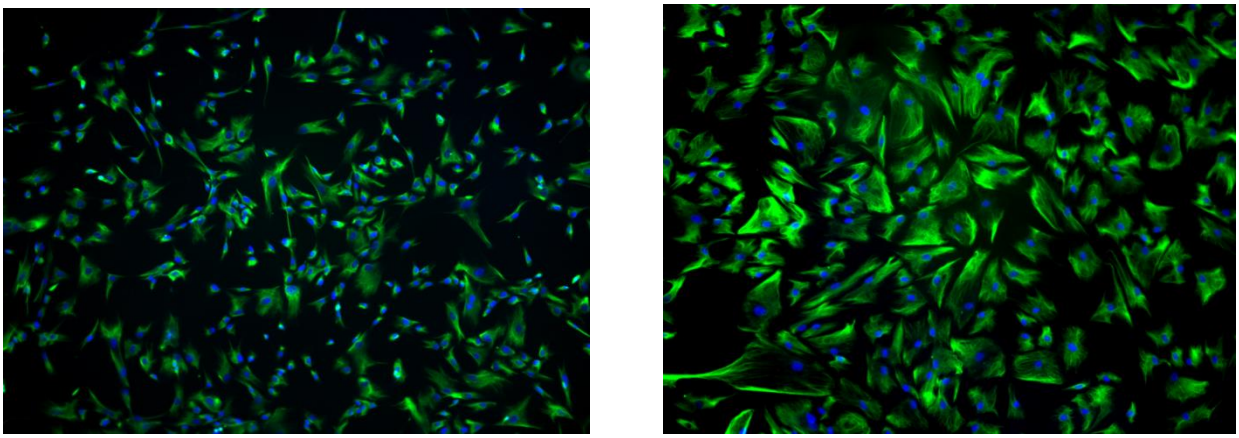


Fig 16: Immunofluorescence staining of mesenchymal marker vimentin in treated and untreated WJMSCs. Blebbistatin treated cells (right) show a flattened out morphology when compared to control cells (left) which are in spindle shape. Green colour-vimentin; Blue colour-nucleus (DAPI counter stain). Images taken at 10X.

4.2.4 Cell Senescence Assay

In vitro WJMSCs cells proliferate for a limited number of passages (app maxim up to P20) prior to which undergoes a stable cell cycle arrest and altered phenotype, which are the characteristics of cell senescence. In the case of senescent cells, the expression and activity (at pH6) of β-Galactosidase was increased which is the principle behind the senescence assay. In the experiment, WJMSCs cells were cultured and the cells were exposed to the blebbistatin (5µm conc.) for three passages and senescence assay was performed by cell signaling β-Galactosidase Staining kit according to the protocol mentioned in the materials and methods. Cells were observed for the development of blue color under microscope .In the case of blebbistatin treated cells, a profound increased (intense) development of blue color (Fig 17) was observed compared to the control cells (Fig 17) which indicate that the blebbistatin treated cells were undergoing early senescence depicting a role of blebbistatin in activating the senescence pathway by targeting the molecules that are involved in the cell senescence. The cells stained blue were counted along with the total number of cells in each field. The experiment is repeated for two samples.

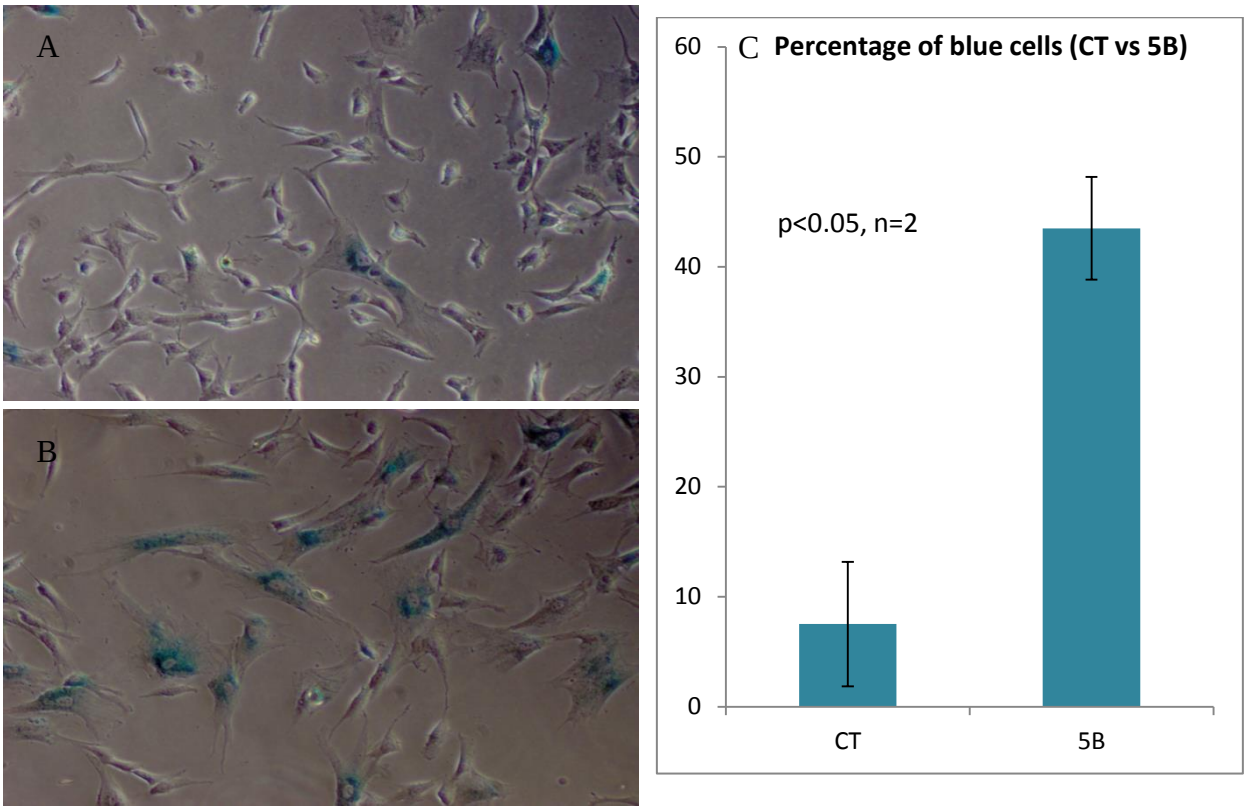
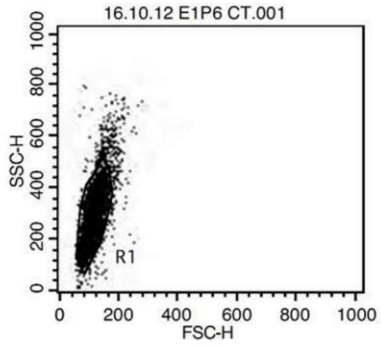


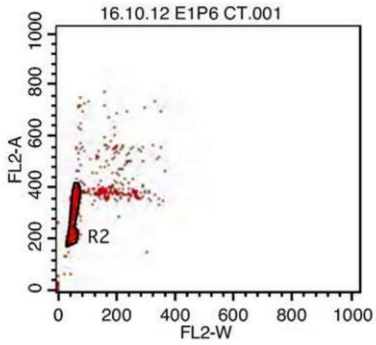
Fig 17: Cell senescence assay of WJMSCs. Blebbistatin treated cells (B) which are flattened out and developed blue color indicating cells have undergone early senescence when compared to control cells (A) which are in spindle shape with no development of blue color. Panel C: Cell with blue color and total number of cells is counted for each field and percent blue cell population is plotted (student's t-test p-value<0.05, n=2 times repeated with minimum 5 field counted in each case)

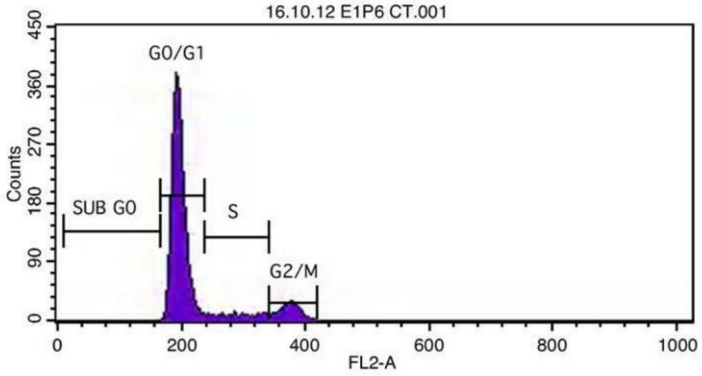
4.2.5 Cell cycle analysis by Flow cytometry

As from the above experiments, came to know that blebbistatin has some role in inhibiting the proliferation of WJMSCs and directing the cells towards early senescence. In order to know which phase of cell cycle is stalled by the action of blebbistatin, cell cycle analysis by flow cytometry was performed using propidium iodide. The cultured WJMSCs cells were exposed to blebbistatin (5µm conc.) for two passages and then treated with propidium iodide and run the flow according to the protocol mentioned in the material and methods. In the results obtained it has been observed that there is cell cycle arrest in G1 phase of cell cycle i.e., observed higher percentage of cells in G1 phase of cell cycle in blebbistatin treated culture compared to the control culture (Fig 18). The experiment was repeated for three different cord samples and data collected for the percent cell in G2/M phase and G1/G0 phase is plotted for control vs. treated (Fig 19).

A







Histogram Statistics

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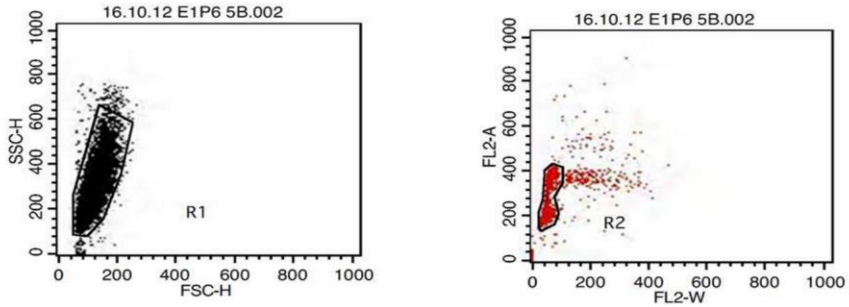
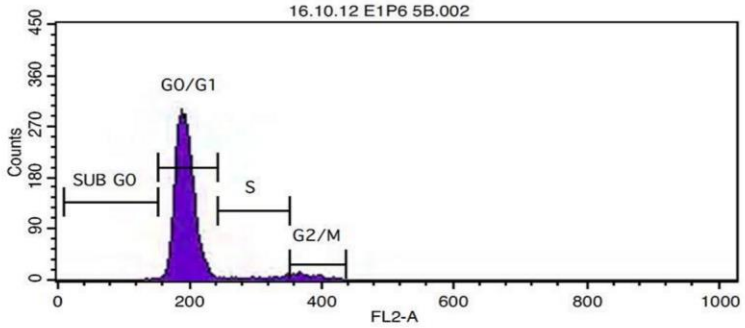
Gated Events: 9402
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G2
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	9402	100.00	94.02	218.11	212.88	25.25	197.00	193
G0/G1	167, 237	8001	85.10	80.01	196.67	196.41	5.23	195.00	193
G2/M	340, 420	853	9.07	8.53	373.11	372.82	3.95	374.00	375
S	237, 340	561	5.97	5.61	288.84	287.21	10.56	289.00	284
SUB G0	10, 167	0	0.00	0.00	***	***	***	***	***

B





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Patient ID:
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Panel: Untitled Acquisition Tube List
Acquisition Date: 16-Oct-12
Gate: G2
Gated Events: 9495
Total Events: 10000
X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	9495	100.00	94.95	202.19	199.25	20.18	193.00	188
G0/G1	152, 241	8899	93.72	88.99	192.68	192.25	6.72	192.00	188
G2/M	351, 434	338	3.56	3.38	377.58	377.15	4.83	374.00	365
S	241, 351	263	2.77	2.63	305.96	303.76	11.75	315.00	345
SUB G0	10, 152	9	0.09	0.09	145.78	145.64	4.60	149.00	152

Page 1

Fig 18: Cell cycle analysis by flow ctyometry using propidium iodide. Compared to control culture (A), blebbistatin treated culture (B) shows higher percentages of cells in G1/G0 phase (93.72% compared to control which is 85.10%) indicating cell arrest in G1 phase in the treated cultures. The data shown above is for one sample, like this flow run has been done for three other samples which also follow the same trend.

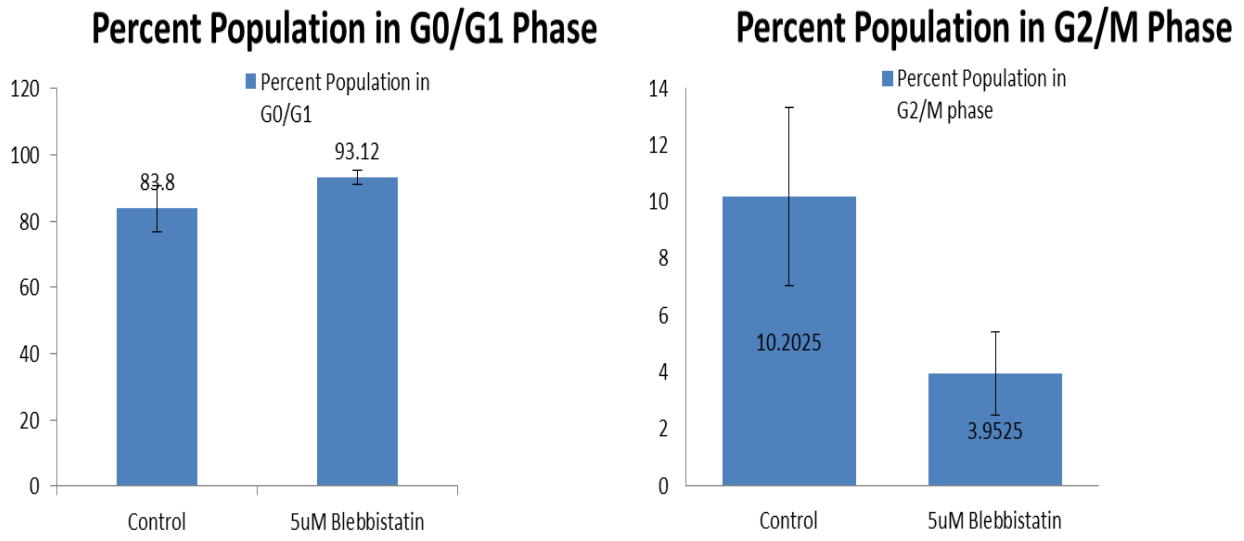

Fig 19: Cell cycle analysis by flow ctyometry using propidium iodide. Compared to control culture, blebbistatin treated cultures show higher percentages of cells in G1/G0 phase indicating cell arrest in G1 phase in the treated cultures. Consequently, lower G2/M phase population observed in case of treated cultures. (student's t-test p-value <0.05, n=4 times repeated)

4.3 Increase in stem cell marker expression when WJMSCs were cultured in serum deprived condition

4.3.1 Experiment:

Cells are plated at a density of 1000cells/cm^2 in case of control and 3000cells/cm^2 in case of serum deprived culture (SD).

Allowed the cells to attach and grow for two days. The serum deprived dish should be 80-90% confluent.

Media was aspirated from both dishes and the serum deprived dish was washed with D-PBS to remove traces of FBS.

Complete DMEM KO media added in case of control and complete DMEM KO media without FBS is added to the serum deprived dish.

The cells were monitored daily under phase contrast microscope.

Media change was given every 3rd day.

Culture stopped on the 11th day and cells were taken for RNA isolation by trizol method.

DNase treatment was given to RNA and cDNA was synthesized through RT-PCR.

Semi quantative PCR was carried out for 18S, OCT4, NANOG and ABCG2. Band intensity for 18S PCR was equalized by adjusting the cDNA volumes used in the reaction and then same volume used to set up semi quantitative or qRT PCR for other genes.

qRT PCR was carried out for 18S, OCT4, ABCG2 in duplicates.

The experiment was repeated for two samples: K_1P_2, L_1P_3

4.3.2 Cell Morphology

Day 4: Control → Cells growing normally like MSCs with healthy spindle shape morphology

Serum deprived → Cells showing elongated morphology with few cell bodies floating in the medium and fewer cells growing in the dish than control. (Possible speculation: cells are undergoing apoptosis)

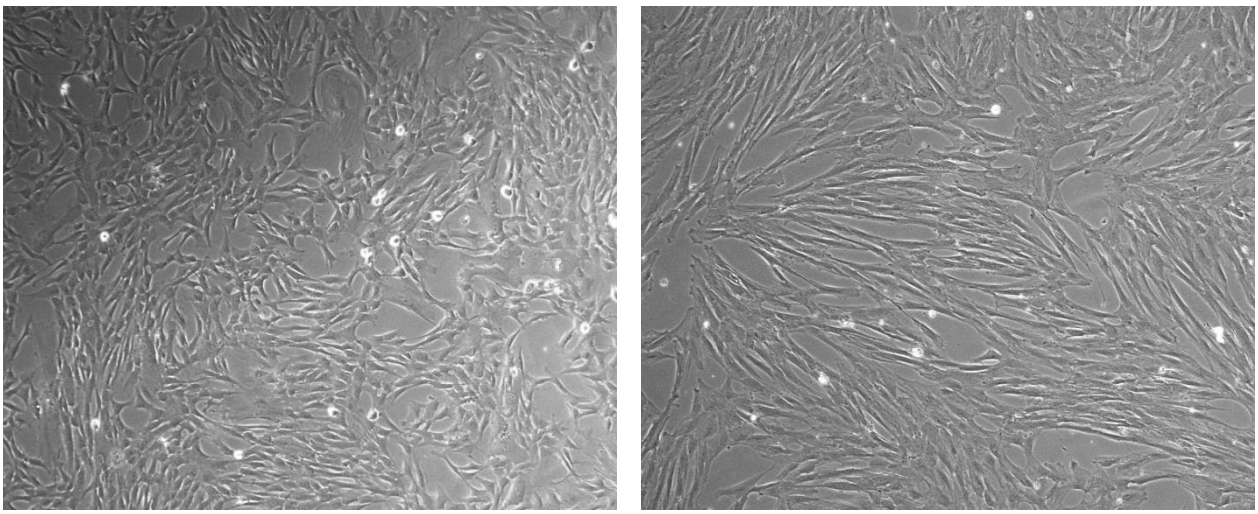

Fig 20: Day 4 Control and serum deprived culture

Day 6: Control → Cells growing normally like MSCs. Cells completely confluent.

Serum deprived → Cells showing elongated morphology with few cell bodies floating in the medium and fewer cells growing in the dish than control and day 4 serum deprived. (Possible speculation: cells are undergoing apoptosis)

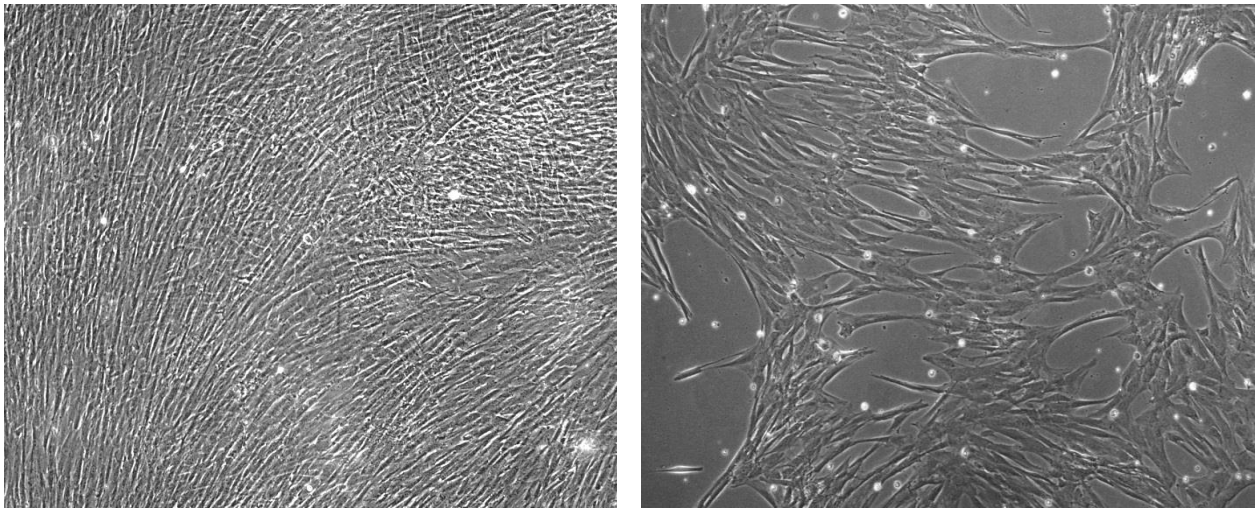

Fig 21: Day 6 Control and serum deprived culture

Day 10: Control → Cells growing normally like MSCs. Cells completely confluent.

Serum deprived → Cells showing elongated morphology with few cell bodies floating in the medium and fewer cells growing in the dish than control, day4 and day6 serum deprived. (Possible speculation: cells are undergoing apoptosis)

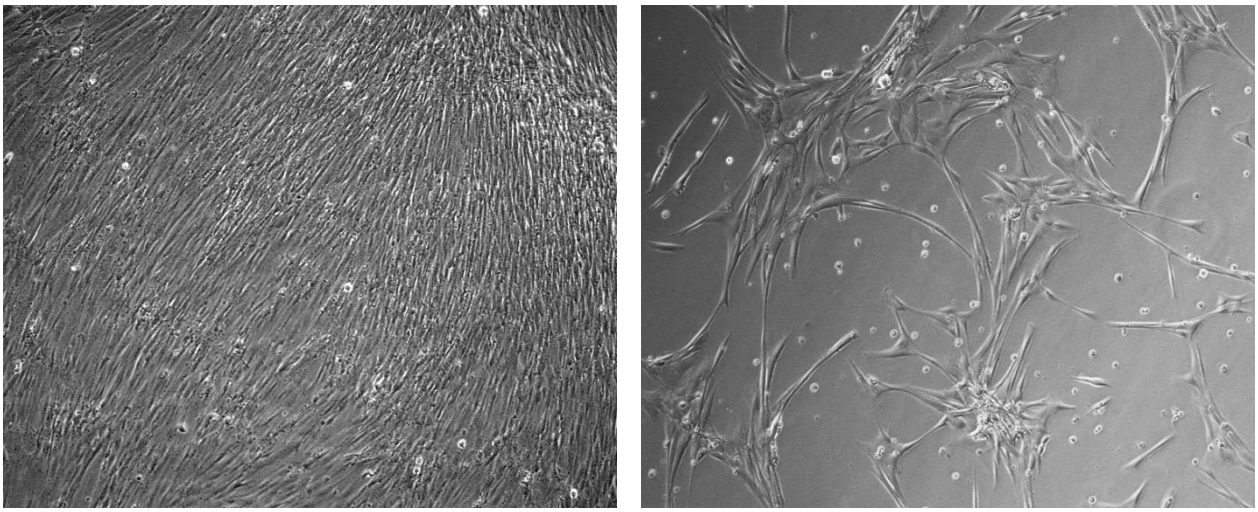

Fig 22: Day 10 Control and serum deprived culture

4.3.3 Semi-quantitative PCR Data (SD – Serum deprived)

The cDNA quantity in each sample is normalized by equalizing the expression level of 18S RNA (housekeeping gene). Once the normalization was done, PCR for other genes was repeated with the same amount of cDNA that was used for normalization. PCR product was then run on 0.8-1.5% agarose gel depending on the size of transcript.

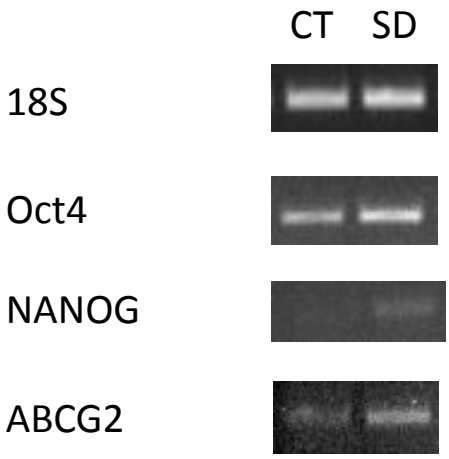

Fig 23: Agarorose gel electrophoresis images of PCR done to check for the upregulation/downregulation of pluripotent markers in serum deprived cultures with respect to the control. 18S, a house-keeping gene, expression level is used to normalize the amount of cDNA taken for setting up the PCR. All the bands were observed at correct base pair when compared to DNA ladder which was run simultaneously (not shown).

When 18S band intensity was equalized for control and serum deprived, it was seen that there was an increase in the levels of expression of Oct4, NANOG and ABCG2 in case of serum deprived cells.

4.3.4 qRT PCR Data

Through PCR, it was seen that there was an up-regulation of pluripotent markers like OCT4, NANOG and ABCG2. Hence, quantitative real time PCR was done through Taqman probe to estimate the fold upregulation of expression at the transcript level. The experiment was carried out for two samples. The reading for threshold cycle is as follows along with the calculation for Fold upregulation/downregulation. (For each reading, the qRT PCR was done in duplicates.)

Sample K1P2	**CT**		**SD**
18S	9.726389		10.90705
Average		10.31672	
normalizing value	0.590332		-0.59033
ABCG2	34.1519		32.47079
Normalize	34.74223		31.88046
Delta CT (CT - SD)		-2.86178	
Fold regulation (2^-delta CT)		**7.269108**	
OCT4	32.34771		29.88796
Normalize	32.93804		29.29763
Delta CT(CT - NS)		-3.64041	
Fold regulation (2^-delta CT)		**12.47019**	

Sample L1P3	**CT**		**SD**
18S	11.99723		11.59506
Average		11.79615	
Normalizing value	-0.20108		0.201084
ABCG2	34.05949		32.48049
Normalize	33.8584		32.68157
Delta CT (SD - CT)		-1.17683	
Fold regulation (2^-delta CT)		**2.260795**	
OCT4	32.18666		30.31709
Normalize	31.98558		30.51817
Delta CT(CT - SD)		-1.4674	
Fold regulation (2^-delta CT)		**2.765238**	

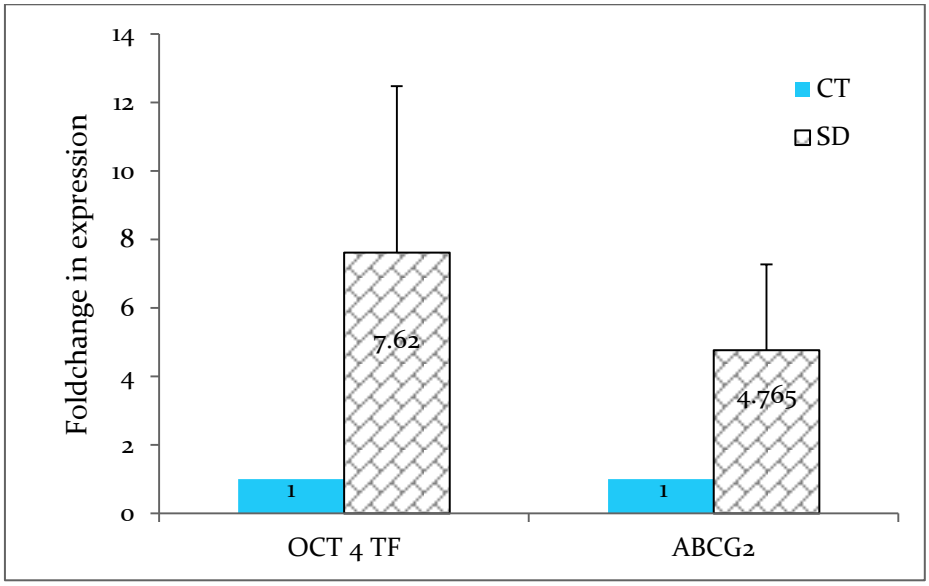

Fig 24: Representation of fold upregulation of OCT4 and ABCG2 through bar graph

Although sample to sample variations seems to be huge, but the trend of increase in the expression level of the pluripotent markers, OCT4, ABCG2 and NANOG remains the same. The experiment needs to be repeated with more samples to reduce the variation and to verify the result.

4.3.5 Immunofluorescence

Immunofluorescence analysis was done for serum deprived cells to check the effects of serum deprivation on the expression of OCT4 and cytoskeletal elements like vimentin, Non Muscle Myosin Heavy Chain IIA and IIB (NMHC-IIA, NMHC-IIB). The cells were grown in an eight-chamber slide. The experiment was repeated for two samples. The negative control data has not been shown.

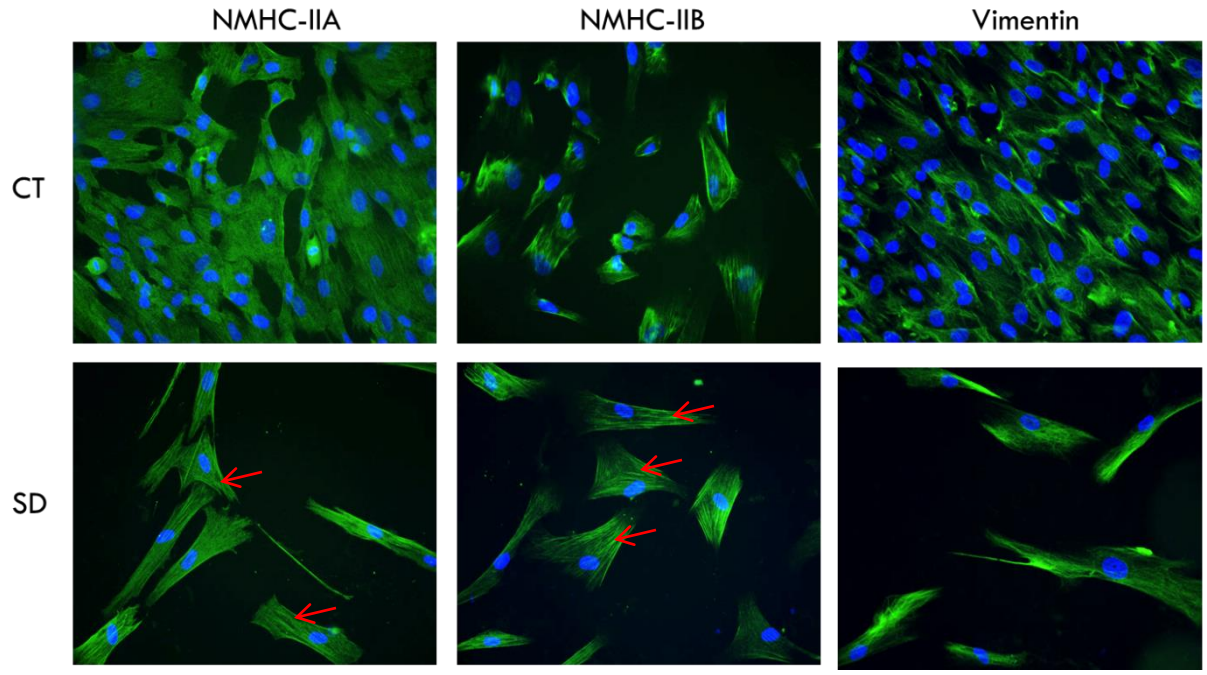

Fig 25: Fluorescence microscope images (magnification 400X) showing staining of NMIIA, NMIIB and Vimentin. Fluorophores used – Alexa Fluor 488 (green); DAPI (blue). Few of the stress fibers marked with red arrow.

It was observed that the cytoskeletal elements (actin filaments) organize themselves into distinct stress fibers in the serum deprived culture as compared to the control. In case of control, the localization of NMHC IIA, IIB and Vimentin is much more random. In case of Oct4, the antibody gave only background staining, hence images have not been shown. It is possible that antibody was not specific to the isoform of OCT4 found in WJMSCs.

5 DISCUSSION

5.1 Role of NMII in the cell cycle progression of WJMSCs

NMII is known to play critical roles in properties like cell motility, cell adhesion and cell division. Through Walker et al., it was shown that NMII inhibition in ESCs led to an increase in pluripotent marker expression level and a marked increase in proliferation. Knowing that WJMSCs are closer to ESCs in term of pluripotent marker expression and differentiation potential, we wanted to address the question whether NMII plays any significant role in increasing the proliferation rate of WJMSCs.

WJMSCs in presence of blebbistatin exhibit a profound change in cell morphology i.e. a loss in spindle shape, flattened out cell shape, formation of stress fibres. The size of the cells when treated with blebbistatin was significantly higher than the untreated cells. Immunofluorescence data also depicts the same with an indication towards the formation of stress fibres as vimentin is a cytoskeletal element (intermediary filaments). Hence when treated with 5µM blebbistatin and disrupting the actomyosin cytoskeleton, cells tend to exhibit stress and lose their morphology.

Further, it was shown through growth kinetics study the cells tend to become sluggish in terms of proliferation when treated with 5µM blebbistatin. The data clearly shows a significant difference between the population doubling time of control and treated samples. Hence, the treated cells proliferate slower than the control, which led to the next question: Is there a possibility of the active blockage of the cell cycle at one of the checkpoints? With the help of Flow cytometry Data and Senescence Data, it was confirmed that the WJMSCs grown in presence of blebbistatin show an increased population of cells in G0/G1 phase of the cell cycle.

In case of hPSCs, NMII acts as a marker for differentiated state and it is not expressed in the true stem cells population. Hence, inhibiting NMII activity led to the maintenance of the self-renewal property of hPSCs. Whereas, WJMSCs are much differentiated forms of stem cells than hPSCs and they express NMII in their normal state. Inhibition of NMII activity led to decreased proliferation of WJMSCs, indicating that NMII has much more vital roles to play in WJMSCs than to act as a marker of differentiated state.

In future, it should be noted that NMII activity is essential for growth and healthy proliferation of WJMSCs. While designing any technique with respect to better proliferation of WJMSCs, NMII activity should not be disrupted.

Through this set of experiments, it was shown that there is biasedness in the population distribution in cell cycle when treated with blebbistatin with respect to control. Further, we need to study what are the molecules that cause this biasedness. What is the exact role of NMII in the cell cycle inhibition of WJMSC? Whether NMII directly or indirectly influences cell cycle molecules or inhibitors? All these questions need to be addressed before anything conclusive can be said relating NMII and the cell cycle.

5.2 Effect of Serum Deprivation on WJMSCs

Ischemia is generally found in places where there is a lack of blood supply due to capillary rupture, infection, tumour, myocardial infarction etc. Prolonged ischemic condition may severely damage the tissue. MSCs are sometimes used to cure the effects of ischemia. However, MSCs tend to be sensitive towards it and fail to provide a long lasting effect. Serum deprivation is one of the conditions in ischemia along with hypoxia. What effects serum deprivation has on WJMSCs? Pochampally et al. have shown that BMMSCs in serum deprived conditions demonstrate a selection of smaller spindle shaped cells subpopulation which has a higher expression level of pluripotent markers.[22] *In vitro* experiments were done to test how WJMSCs respond to serum deprived condition.

Growing the WJMSCs in serum deprived conditions and then profiling their pluripotent markers, drives us in a new direction with few interesting data points. The cells are not able to proliferate in the absence of serum and a lot of floating cell debris was observed with less number of cells in the serum deprived dish. Although it has not been verified but the most probable cause of fewer cells in serum starved condition is that the cells are undergoing apoptosis. On 10^{th} day the cell extract was taken and RNA was isolated. When the expression level of 18S was equalized, it was observed that the expression level of pluripotent markers OCT4, NANOG, ABCG2 has increased. This data was further confirmed through quantitative real time PCR. qRT PCR clearly shows a significant fold increase in the expression level of OCT4 and ABCG2.

Pluripotent markers are associated with the function of self-renewal of the stem cell population. Is it possible that the stem cells having higher levels of expression of pluripotent marker are surviving longer or the stem cells are actively increasing the levels of expression of pluripotent markers to combat the stress conditions of no-serum?

This study has to be repeated for more samples to confirm the results and reduce the huge variation in the data. However, if the result is to be trusted, can a better cell based therapy be designed by acclimatizing cells to serum deprived condition before therapeutic usage? Can serum deprived conditions be used as selection pressure to extract healthier population of cells having higher pluripotent marker expression? Further careful investigations need to be carried out to answer these challenging questions.

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