INFLUENCE OF CULTURE CONDITIONS ON THE MOLECULAR SIGNATURE OF MESENCHYMAL STEM CELLS

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For the light of my life my beloved *Master*

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KEYWORDS

Adult tissue derived cells, biomarkers, cellular reprogramming, clonal population, cMyc, cytoskeleton, differentiation, induced pluripotent stem cells, mesenchymal stem cells, Oct4, pluripotency, proliferation, regenerative medicine, reprogramming, Sox2, subtractive immunization, vimentin.

ABSTRACT

Cell based therapies require cells capable of self renewal and differentiation, and a prerequisite is the ability to prepare an effective dose of *ex vivo* expanded cells for autologous transplants. The *in vivo* identification of a source of physiologically relevant cell types suitable for cell therapies is therefore an integral part of tissue engineering.

Bone marrow is the most easily accessible source of mesenchymal stem cells (MSCs), and harbours two distinct populations of adult stem cells; namely hematopoietic stem cells (HSCs) and bone mesenchymal stem cells (BMSCs). Unlike HSCs, there are yet no rigorous criteria for characterizing BMSCs. Changing understanding about the pluripotency of BMSCs in recent studies has expanded their potential application; however, the underlying molecular pathways which impart the features distinctive to BMSCs remain elusive. Furthermore, the sparse *in vivo* distribution of these cells imposes a clear limitation to their *in vitro* study. Also, when BMSCs are cultured *in vitro* there is a loss of the *in vivo* microenvironment which results in a progressive decline in proliferation potential and multipotentiality. This is further exacerbated with increased passage number, characterized by the onset of senescence related changes. Accordingly, establishing protocols for generating large numbers of BMSCs without affecting their differentiation potential is necessary.

The principal aims of this thesis were to identify potential molecular factors for characterizing BMSCs from osteoarthritic patients, and also to attempt to establish culture protocols favourable for generating large number of BMSCs, while at the same time retaining their proliferation and differentiation potential.

Previously published studies concerning clonal cells have demonstrated that BMSCs are heterogeneous populations of cells at various stages of growth. Some cells are higher in

the hierarchy and represent the progenitors, while other cells occupy a lower position in the hierarchy and are therefore more committed to a particular lineage. This feature of BMSCs was made evident by the work of Mareddy et al., which involved generating clonal populations of BMSCs from bone marrow of osteoarthritic patients, by a single cell clonal culture method. Proliferation potential and differentiation capabilities were used to group cells into fast growing and slow growing clones. The study presented here is a continuation of the work of Mareddy et al. and employed immunological and array based techniques to identify the primary molecular factors involved in regulating phenotypic characteristics exhibited by contrasting clonal populations. The subtractive immunization (SI) was used to generate novel antibodies against favourably expressed proteins in the fast growing clonal cell population. The difference between the clonal populations at the transcriptional level was determined using a Stem Cell RT^2 Profiler ^{TM} PCR Array which focuses on stem cell pathway gene expression. Monoclonal antibodies (mAb) generated by SI were able to effectively highlight differentially expressed antigenic determinants, as was evident by Western blot analysis and confocal microscopy. Co-immunoprecipitation, followed by mass spectroscopy analysis, identified a favourably expressed protein as the cytoskeletal protein vimentin. The stem cell gene array highlighted genes that were highly upregulated in the fast growing clonal cell population. Based on their functions these genes were grouped into growth factors, cell fate determination and maintenance of embryonic and neural stem cell renewal. Furthermore, on a closer analysis it was established that the cytoskeletal protein vimentin and nine out of ten genes identified by gene array were associated with chondrogenesis or cartilage repair, consistent with the potential role played by BMSCs in defect repair and maintaining tissue homeostasis, by modulating the gene expression pattern to compensate for degenerated cartilage in osteoarthritic tissues. The

gene array also presented transcripts for embryonic lineage markers such as FOXA2 and Sox2, both of which were significantly over expressed in fast growing clonal populations.

A recent groundbreaking study by Yamanaka et al imparted embryonic stem cell (ESCs) -like characteristic to somatic cells in a process termed nuclear reprogramming, by the ectopic expression of the genes Sox2, cMyc and Oct4. The expression of embryonic lineage markers in adult stem cells may be a mechanism by which the favourable behaviour of fast growing clonal cells is determined and suggests a possible active phenomenon of spontaneous reprogramming in fast growing clonal cells. The expression pattern of these critical molecular markers could be indicative of the competence of BMSCs. For this reason, the expression pattern of Sox2, Oct4 and cMyc, at various passages in heterogeneous BMSCs population and tissue derived cells (osteoblasts and chondrocytes), was investigated by a real-time PCR and immunoflourescence staining. A strong nuclear staining was observed for Sox2, Oct4 and cMyc, which gradually weakened accompanied with cytoplasmic translocation after several passage. The mRNA and protein expression of Sox2, Oct4 and cMyc peaked at the third passage for osteoblasts, chondrocytes and third passage for BMSCs, and declined with each subsequent passage, indicating towards a possible mechanism of spontaneous reprogramming. This study proposes that the progressive decline in proliferation potential and multipotentiality associated with increased passaging of BMSCs *in vitro* might be a consequence of loss of these propluripotency factors. We therefore hypothesise that the expression of these master genes is not an intrinsic cell function, but rather an outcome of interaction of the cells with their microenvironment; this was evident by the fact that when removed from their *in vivo* microenvironment, BMSCs undergo a rapid loss of stemness after only a few passages.

One of the most interesting aspects of this study was the integration of factors in the culture conditions, which to some extent, mimicked the *in vivo* microenvironmental niche

of the BMSCs. A number of studies have successfully established that the cellular niche is not an inert tissue component but is of prime importance. The total sum of stimuli from the microenvironment underpins the complex interplay of regulatory mechanisms which control multiple functions in stem cells most importantly stem cell renewal. Therefore, well characterised factors which affect BMSCs characteristics, such as fibronectin (FN) coating, and morphogens such as FGF2 and BMP4, were incorporated into the cell culture conditions. The experimental set up was designed to provide insight into the expression pattern of the stem cell related transcription factors Sox2, cMyc and Oct4, in BMSCs with respect to passaging and changes in culture conditions. Induction of these pluripotency markers in somatic cells by retroviral transfection has been shown to confer pluripotency and an ESCs like state. Our study demonstrated that all treatments could transiently induce the expression of Sox2, cMyc and Oct4, and favourably affect the proliferation potential of BMSCs. The combined effect of these treatments was able to induce and retain the endogenous nuclear expression of stem cell transcription factors in BMSCs over an extended number of *in vitro* passages. Our results therefore suggest that the transient induction and manipulation of endogenous expression of transcription factors critical for stemness can be achieved by modulating the culture conditions; the benefit of which is to circumvent the need for genetic manipulations.

In summary, this study has explored the role of BMSCs in the diseased state of osteoarthritis, by employing transcriptional profiling along with SI. In particular this study pioneered the use of primary cells for generating novel antibodies by SI. We established that somatic cells and BMSCs have a basal level of expression of pluripotency markers. Furthermore, our study indicates that intrinsic signalling mechanisms of BMSCs are intimately linked with extrinsic cues from the microenvironment and that these signals appear to be critical for retaining the expression of genes to maintain cell stemness in long

term *in vitro* culture. This project provides a basis for developing an "artificial niche" required for reversion of commitment and maintenance of BMSC in their uncommitted homeostatic state.

LIST OF ABBREVIATIONS

Ab	Antibody
ASC	Adult stem cells
BMP4	Bone morphogenetic protein 2
BMSC	Bone marrow stromal cells
BSA	Bovine serum albumin
CDC2	cell division cycle 2
CFU-F	Colony forming unit fibroblast
DLL3	Delta- like 3
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribo nucleic acid
ESC	Embyonic stem cells
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor
FN	Fibronectin
FOXA2	Forkhead box a 2
HMG	High-mobility-group
HSC	Hematopoietic stem cells
IF	Immunoflourescence
IGF	Insulin-like growth factor
iPS	Induced pluripotent stem cells
kDa	kilo Daltons
LIF	Leucocyte inhibitory factor
mAb	Monoclonal antibody
MSC	Mesenchymal stem cells
NOTCH1	Notch homolog 1
OA	Osteoarthritis
OPN	osteopontin
PBS	Phosphate buffer salin
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid

RT-PCR	Reverse transcriptase ploymerase chain reaction
SI	Subtractive immunization
Sox2	SRY (sex determining region Y)-box2
TBE	Tris-borate EDTA
TBS	Tis buffered saline
TBST	Tis buffered saline with Tween20
TEMED	Tetramethylethylenediamine
WB	Western blot

STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: _____

Date: _____

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CHAPTER 1

INTRODUCTION

Stem cells are considered to be the "holy grail" of cell-based tissue engineering owing to their capacity for self-renewal and pluripotency. Stem cells are postulated as having a number of uses in applications in regenerative medicine, immunotherapy, gene therapy, but it is in the area of tissue engineering that they generate the most excitement [1]. In an effort to harness the potential of these cells a number of studies have been conducted using both embryonic/foetal and adult stem cells. The use of embryonic stem cells (ESCs), despite of the perceived versatility due to their pluripotency, has been restricted by strong ethical and political concerns; and their potential in reparative therapy has been found to be limited due to immunological incompatibilities and *in vivo* teratoma formation from administered ESCs [2]. Adult stem cells, by contrast, can be readily used in autografts since immune rejection is not an issue, and these cells do not attract the ethical concerns as it is the case with the use of ESCs. The major limitation to their use, however, is the fact that adult stem cells are exceedingly rare in most tissues, which makes the process of identifying and isolating these cells problematic, bone marrow perhaps being the most notable exception [3].

Bone marrow is a heterogeneous population of cells, containing two predominant populations: (i) HSCs which give rise to all the major blood cell lineages and (ii) MSCs, which forms the supporting structure for HSCs [4]. MSCs are capable of differentiating into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma [4, 5]. Recent findings indicate that adult bone marrow may also contain cells that can differentiate into other mature, nonhematopoietic cells of a number of tissues

including epithelial cells of the liver, kidney, lung, skin, gastrointestinal (GI) tract, and myocytes of heart and skeletal muscle [6]. MSCs can be easily expanded *in vitro*, genetically modified by use of viral vectors, as well as, they can be stimulated to differentiate into different cell lineages by changing the microenvironment properties, collectively making them ideal vehicles for cellular gene therapy [7].

Despite many attractive features associated with MSCs, there are still many hurdles that must be overcome before these cells are readily available for use in clinical applications. The main concern relates to their *in vivo* characterization and identification. Unlike HSCs, which are well characterized and have been successfully isolated using specific biomarkers, there are no universally expressed biomarkers for characterizing and identifying MSCs, and the biomarkers that are known are expressed at particular stages of the cell cycle. This problem is further exacerbated by the sparse distribution of these cells. These quiescent cells are cryptically present among the differentiated cells, and are only activated by certain factors such as injury, disease or stress. The lack of a universal biomarker, sparse distribution *in vivo*, and decline in their number with age points toward an obvious need to decipher the critical molecular players which govern the unique character of MSCs. A recent breakthrough study identified some key players involved in reprogramming mouse tail tip fibroblasts to behave as pluripotent stem cells; these somatic cells were termed induced pluripotent stem cells (iPS) [8]. This study demonstrated that retrovirus-mediated introduction of as few as four transcription factors (Oct4, Sox2, cMyc, and KLF4) into mouse embryonic or adult fibroblasts, and selection for the expression of Fbx15, a target of Oct4 and Sox2, resulted in the generation of iPS cells, which were similar to ESCs with respect to morphology, proliferation, and teratoma formation [8]. These same factors were capable of generating iPS cells from adult human dermal fibroblasts [9]. The finding that only four transcription factors were needed to reprogram

an adult cell into an iPS cell has revolutionized the field of generating patient and disease specific stem cells, as well as disease modelling, drug screening, toxicology tests and, ultimately, autologous cell-based therapies [10] . One of the major concerns with iPS is the presence of a retroviral integration site in the clone cells; the potential reactivation of retroviral inserted cMyc may increase the risk of tumorigenesis. This challenge can be overcome by using recombinant techniques to generate proteins or small molecules which can penetrate into the cell and induce the target gene expression which mimics retroviral gene transfer; alternatively, a genetically modified adenovirus might be used for gene transfer. It is clear that cellular reprogramming is a naturally occurring phenomenon; however the molecular mechanisms and the sequence of events accompanying reprogramming of somatic cells to iPS are yet to be fully understood.

The phenomenon of reprogramming somatic cells to iPS is closely linked with dedifferentiation of fully committed fibroblast cells to their embryo-like state. The differentiation capabilities, multipotentiality and self-renewal ability of stem cells are controlled by intrinsic genetic pathways that are subject to regulation by extrinsic signals emanating from the stem cell niche [11]. This niche provides a microenvironment composed of cellular structures, growth factors and extracellular matrix in which stem cells are maintained in an undifferentiated state [11]; it also holds the key signals that organize fate decision of early embryonic development and differentiation of specific cell types.

It is a well established that differentiation of stem cells can be controlled by modulating the cellular microenvironment in culture conditions. An interesting approach to the reprogramming study would be to mimic the niche specific for reversion of commitment and develop an “artificial niche”. Defined culture conditions capable of altering *in vitro* cell fate would alleviate the need for forced expression by transgenesis, thus eliminating the risk of mutagenic effect of genetic manipulations [12]. The principal

aim of my doctoral work has been to identify the molecular signatures of MSCs, and also to explore crucial factors of the stem cell niche that can be modulated to maintain the high expression of these molecular signatures without the need for transgenic manipulation.

Specific Aims of the Study

Three specific aims have been addressed in this study.

The first aim was to investigate and identify the molecular players that are altered in conjunction with the change of phenotypic characteristics of BMSCs. This was accomplished by producing antibodies capable of identifying differentially expressed proteins in two contrasting clonal populations from the same BMSC sample by the method of subtractive immunization and PCR Array technique.

The second aim was to investigate the expression pattern of the key molecular markers Oct4, Sox2 and cMyc, which are responsible for spontaneous reprogramming phenomena at various passages of *in vitro* cultured tissue derived osteoblasts, chondrocytes and BMSCs.

The third aim was to induce and manipulate the endogenous expression of these genes by modulating the culture conditions. We were able to demonstrate that it was possible to achieve changes in BMSCs that would be beneficial for the development of patient specific cell therapy by manipulating the culture conditions.

Thesis Outline

Chapter 1 of this thesis is a short introduction which gives a brief background to the scientific problems addressed, the research questions being investigated and the specific aims of the study.

Chapter 2 is the literature review section and gives a more in-depth introduction to the central themes of this project SCs. It then goes on to a present an overview of the developments that has occurred in BMSCs biology and identifies the knowledge gap in this field. It also presents a detailed background of reprogramming related phenomena and iPS, discussing in detail some of the current developments and limitations of iPS and the implications of iPS on the regenerative medicine and cell therapy research

Chapter 3 describes the pilot study of the project in which attempts to identify a robust biomarker for MSCs by the technique of subtractive immunization, this study outlines the generation of novel antibodies capable of identifying differentially expressed antigens in contrasting clonal populations from the same BMSC sample. In addition 84 genes related to stem cell pathways were screened and quantified by using The Stem Cell RT^2 Profiler ^{TM} PCR Array.

Chapter 4 identifies the existing pattern of spontaneous reprogramming phenomena in the *in vitro* cultured tissue and culture expanded osteoblasts, chondrocytes and BMSCs. It encompasses the expression pattern of reprogramming markers Oct4, Sox2 and cMyc and presents an insight into their interaction with each other.

Chapter 5 expands on the work described in Chapter 4, encompassing what effects the manipulation of cell culture conditions has on the endogenous expression of genes related to the phenomenon of spontaneous reprogramming. In this part of the work we attempt to bypass the need for retroviral induced gene expression, and retain the native gene expression by modulating the cellular niche.

Chapter 6 summarizes the results from the previous three chapters, presents a detailed discussion on these outcomes, and concludes with a brief discussion of future perspective.

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CHAPTER 2

LITERATURE REVIEW

A brief introduction to this review

The primary goal of stem cell research is to produce cells that are both patient specific, as well as having properties suitable for the specific conditions they are intended to remedy. From a purely scientific point of view, stem cells allow scientists to gain a deeper understanding of developmental biology and regenerative therapies [1]. Stem cells serve as biological repair system, and have the potential to differentiate into a number of specialised cell types in the body [2]; they therefore represent the most useful candidates for cell based therapies.

An extensive amount of research has already been conducted using embryonic stem cells (ESC) to generate potential treatment strategies for replacement of damaged or dead tissue. Ethical issues arise as to their use given that the only source of ESCs is from embryos during early stage embryogenesis [3]. The means of obtaining these cells have therefore been surrounded by much ethical and political debate, something which severely hampered their use in stem cells research [4]. Besides the ethical issues concerning ESC, the possibility of tumorigenesis, immune rejection and dangers of immunosuppressive therapies also adds layers of complications to the application ESC in research, which has led to the search for alternative sources for stem cells [5].

The adult tissues in higher organisms harbour cells that are of the reminiscent of the embryonic unprogrammed cell which are termed adult stem cells (ASCs) [6]. A number of

ASCs sources have been described [7] and bone marrow is by far the most easily accessible source of two potent population of ASC namely: haematopoietic stem cells (HSCs) and bone marrow stem cells (BMSCs). BMSCs, also known as mesenchymal stem cells (MSCs), are multipotent in nature and are precursors for skeletal tissue components such as bone, cartilage, haematopoiesis-supporting stroma, and adipocytes. MSCs have been used in regenerating tissues of mesodermal origin, as well as tissues of different embryological derivation [8]. Clonal studies of the BMSC populations have reported them as being a heterogeneous pool comprising of cells at different stage of differentiation and putative progenitor cells [9]. BMSCs can exert profound immunosuppressive effects via modulation of both cellular and innate immune pathways [10], and this property allows them to overcome immune rejection, which is a clear limitation with ESCs. The isolation of homogenous populations of progenitors from BMSC still remains a challenge however; a problem all the more so given the lack of as set of universal biomarkers for these cells. This project uses the method of subtractive immunization to identify potential biomarkers for the progenitor cells.

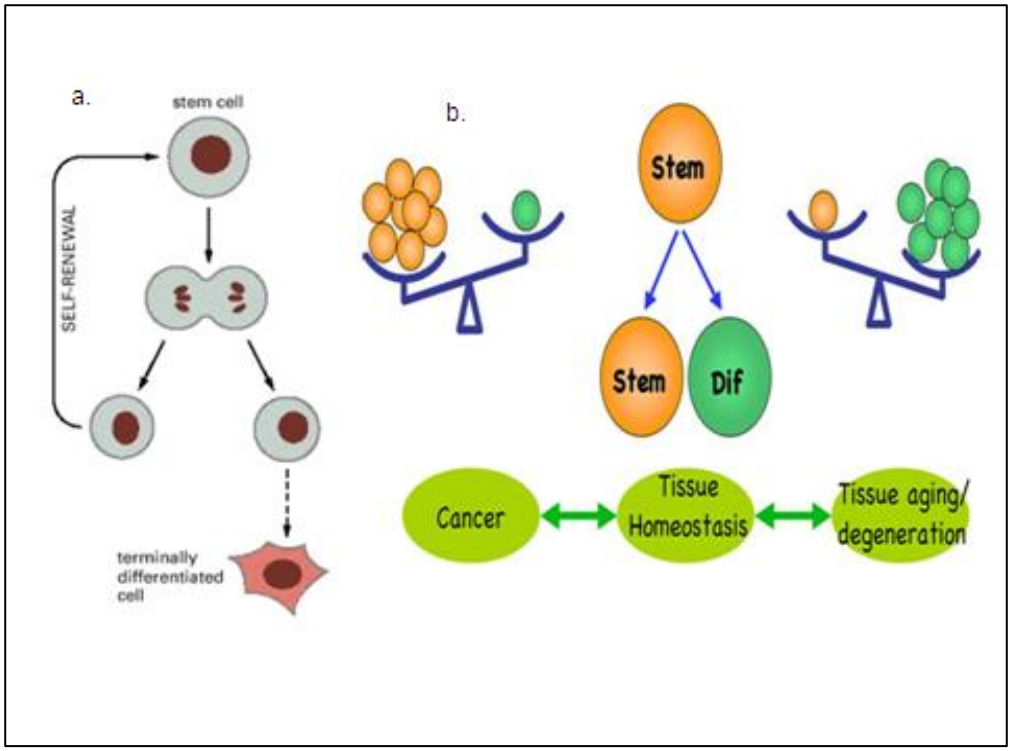
Studies have been conducted to identify genes responsible for assigning peculiar characteristics to these cells. One such study has recently demonstrated that the transient expression of as few as four particular transcription factors can reprogram somatic cell to behave like embryonic stem cells. [11]. These cells have been termed induced pluripotent cells (iPS) and their discovery has generated much interest in the scientific community as they represent a genuine alternative source to ESCs. The ethical and immune concerns associated with ESCs are not an issue with iPS; there are, however, issue related to teratoma formation when iPS cells are transplanted *in vivo*. Another issue is the use of retroviral vectors for the ectopic expression of the transcription factors, which may randomly integrate into the genome, thus increasing the risk of insertional mutagenesis

[12], which may result in malignant transformation of iPS. These are major challenges which need to be addressed, either by the use of recombinant technology to produce small drugs that could trigger a controlled transient expression of target transcription factors in somatic cell, or alternatively an artificial cellular niche could be created by using modified culture conditions that mimics the *in vivo* triggers which stimulates the target transcription factors that prompt somatic cells to behave as ESCs. Although the induction of somatic cells is an interesting and promising approach, the main challenge is to understand the molecular biology which controls cellular reprogramming. The aim of this study is therefore to investigate the events which govern spontaneous reprogramming and to determine the effect of cellular niche on reprogramming and also to identify which factors can be manipulated to trigger a stem cell like phenotype.

Stem cells

Early pioneers in the study of stem cells were Till and McCulloch who effectively laid the foundation for this field of biology [13]. They observed the formation of nodules arising from single bone marrow cells which had been injected into the spleens of irradiated mice and which were thought to be stem cells. The term stem cell was first used by Theodor Boveri and Valentin Hacker to describe cells committed to give rise to germline cells, and Arthur Pappenheim, Alexander Maximo, Ernest Newmann and others used it to describe proposed progenitor cells of the blood system [14]. A unique feature of stem cells is their ability to remain quiescent *in vivo* in an uncommitted state, and then to be triggered by certain conditions caused by disease, injury or aging, thus forming a reservoir and natural support system to replenish lost cells. They retain the plasticity to differentiate into various tissues; however, being able to control this differentiation process

still remains one of the biggest challenges in stem cell research [2]. The cell division of stem cells is a distinct aspect of their biology, since this division may be either symmetric or asymmetric **(Figure 1)**. Symmetric division takes place when the stem cells divides and forms two new daughter cells. Asymmetric division is thought to take place only under certain conditions where stem cells divides and gives rise to a daughter cell which remains primitive and does not proliferate, and one committed progenitor cell, which heads down a path of differentiation. Asymmetric division of stem cells helps reparative process, and also ensures that the stem cells pool does not decrease, whereas symmetric division is responsible for stem cells undergoing self renewal and proliferation. The factors which prompt the stem cells to undergo asymmetric division are, however, not well understood, but it is clear that the delicate balance between the self renewal and differentiation is what maintains tissue homeostasis**.**



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b. www.bioscience.org/2009/v14/af/3430/figures.htm

Figure 1: Schematic diagram of the symmetric and asymmetric cell division of stem cells, parent stem cell divides asymmetrically to produce a daughter stem cell and a progenitor cell succeeded by terminally differentiated cells. b. Balance between symmetric and asymmetric division maintains the stem cell pool and replenishes loss of tissue in turn providing homeostasis in a healthy tissue.

Stem cell niche

The dual property of self renewal and differentiation into plethora of specialized daughter cells makes stem cells imperative for regenerating tissues. Complicated array of molecular signals from the microenvironment which anatomically hosts these cells, in concert with internal signalling pathways dictate the stem cell behaviour. These anatomically defined locations along with extracellular cues are termed as cellular niche. The concept of niche was proposed by Schofield in year 1978 to describe the psychologically limited microenvironments that support stem cells. He suggested that "the stem cell is seen in association with the other cells which determine its behaviour. It becomes essentially a fixed tissue cell. Its maturation is prevented and, as a result, its continued proliferation as a stem cell is assured. Its progeny, unless they can occupy a similar stem cell 'niche' are first generation colony-forming cells which proliferate and mature to acquire a high probability of differentiation."[15]

Niche shelters and sequesters stem cells to maintain their quiescent state by shielding them from differentiation or apoptotic signals, also ensures its long term survival in an uncommitted non cancerous state. It is critical in safeguarding against the cancerous state of stem cells as a result of excessive proliferation [16]. It maintains a delicate balance between quiescent and active state of proliferation to maintain a life-long non-cancerous source of stem cell which may play a critical role in maintaining tissue homeostasis. Spatial and temporal specified presentations of stem cells in niche stipulate stem cell division to be symmetrical or asymmetrical [17].In response to stress or injury stem cell undergo asymmetrical division to produce a transient amplifying cell and a quiescent daughter stem cell to replenish the stem cell pool. The clear indicator of role played by stem cell niche is evident in ESC, when introduced in a foreign environment in a syngeneic animal, teratoma formed has cells of all three germ layers indicating towards a complete

disarray of the differentiation control mechanism [18]. In the presence of appropriate niche such as suitable blastocyst, ESC are capable of forming developmentally normal blastocyst [19].Components of the stem cell niches can be broadly classifies into static and dynamic factors, static factors include the effect of direct physical contact of membrane associated proteins, extra cellular matrix. The dynamic factors are the soluble niche effectors such as morphogens fibroblast growth factors and bone morphogenic proteins[20]. These factors of niche create a morphogenetic gradient which controls critical features of SC such as self renewal and differentiation. However, identifying the morphogens and establishing morphogenetic gradient remains the key challenge and can be utilize to generate artificial niche for cells, deconstruction of the cell culture using proteomic approach can be explored to gain vital insight into the role of microarchitecture in maintenance of static state. Numerous studies have successfully established that the microarchitecture is not an inert component of tissue; it is of prime importance, sum total of stimuli from microenvironment dictates the complex interplay of regulatory mechanism which determines cell fate. For example all the culture conditions designed for sustaining human ES cell potential require supplementation with basic fibroblast growth factor (bFGF), it has been demonstrated that bFGF does not act on ESC rather it plays a critical role in modulating the gene expression profile of the supporting cells to produce factors such as IGF II which in turn effect the signaling pathways required for maintenance of a pluripotent state [21].

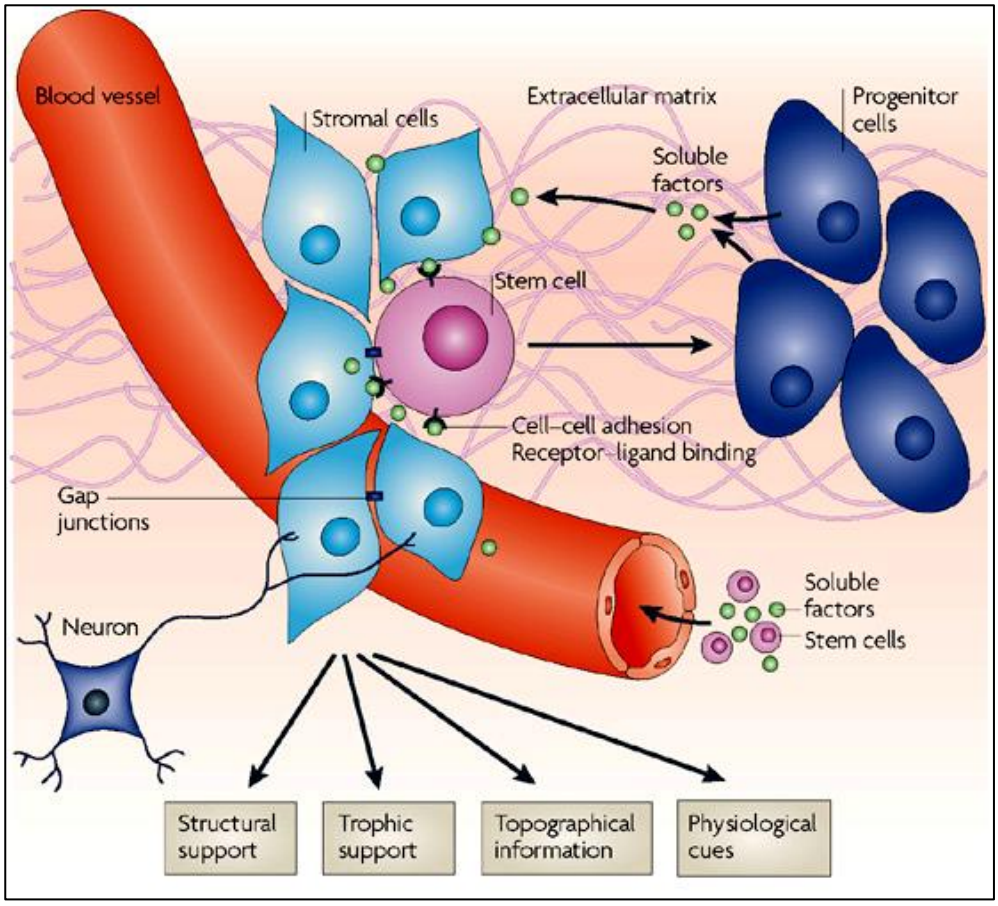

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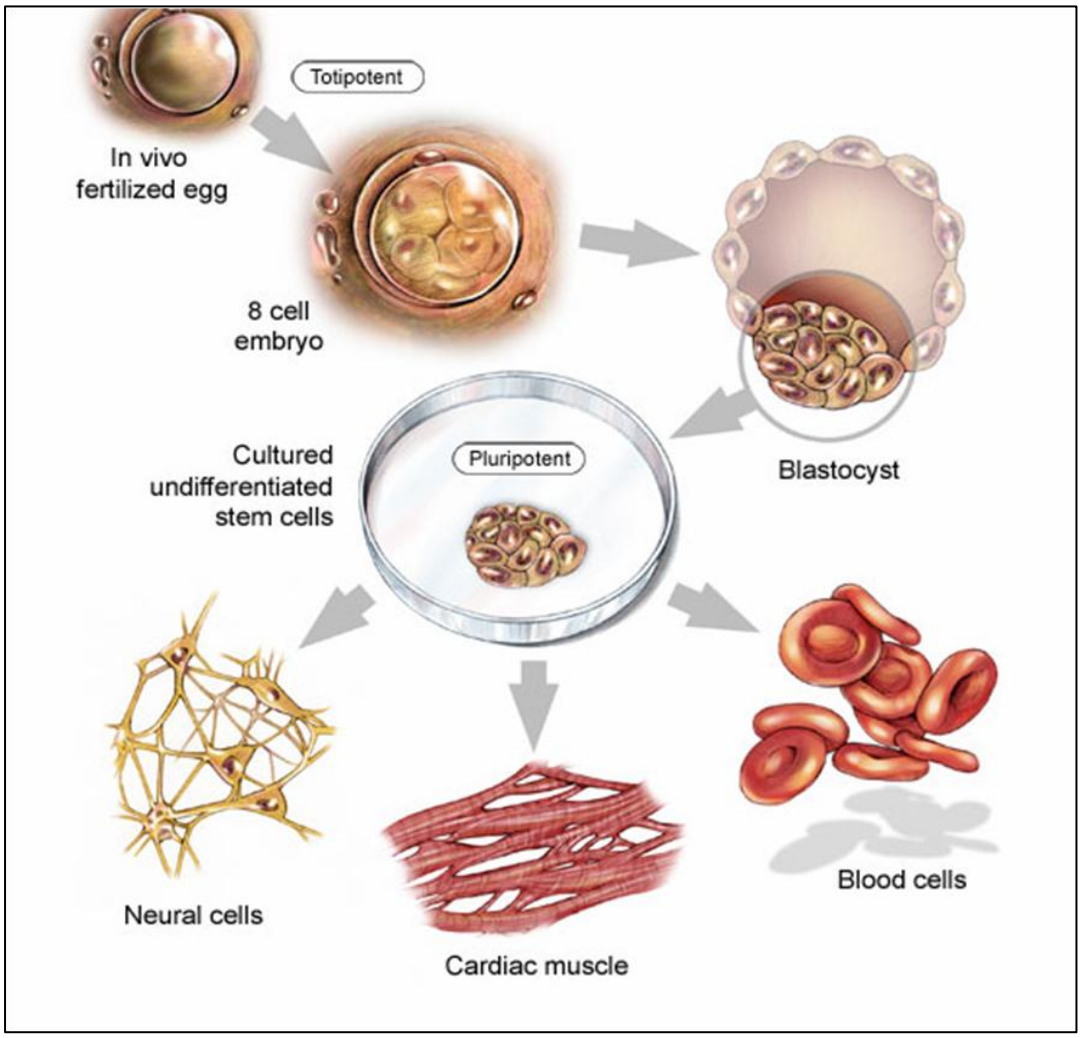
Figure 2: A schematic representation of components of stem cell niche. The figure depicts static factors such as neighbouring stromal cells, stem cell itself and extracellular matrix and dynamic factors such as soluble growth factors. Niche shelters and sequesters stem cell and is a dynamic structure controlling the intrinsic signalling pathways responsible for stem cell behaviour.

Nomenclature and Classification

Various classification schemes for stem cells have been used in order to better understand these cells. The earliest classification was done as early as 1894 by Bizzozero; this classification system recognized three basic types of tissues: renewing, static and expanding, and was related adult tissues rather than the embryo.

Based on the stage of development, the versatility of stem cells can be broadly classified as *totipotent, pluripotent and multipotent* (Figure 3). Every totipotent cell is a self contained entity capable of creating an entire organism: zygotes are perfect examples of totipotent cells. The totipotential nature of the zygote is maintained until the 'morula' stage of development after which the zygote forms a hollow sphere of cells called 'blastocyst'. The 'inner cell mass' (ICM) of blastocysts are pluripotent, meaning that individual cells in the ICM are capable of generating tissue from all the three embryonic germ layers, but lack the capacity to form an entire organism [22]. Pluripotent cells are capable of forming multiple germ layers *in vitro* or are able to survive after transplantation in more than one germ layer. With the commitment of cells to the lineage there is a decrease in their developmental plasticity. Multiopotent cells are lineage committed progenitor which can yield a more restricted subset of cell lineages [23]: mesenchymal stem cells (MSCs) is an example of a multiopotent stem cell type.



Adapted from www.molecularstation.com

Figure 3: An illustration of the developmental stages and differentiation potential of cells in the process of ontogeny. Starting from zygote till specialized tissue formation, zygote and each cell of morula is totipotent, followed by pluripotent state of the inner cell mass. Pluripotent cells are precursors for progenitor cells which are multipotent capable of forming specialized tissues. Development is a process leading to gain of specialization and loss of plasticity.

One of the most commonly used method for stratifying stem cells takes into account the stage of development and tissues from which they were harvested [1]. Based on these criteria stem cells are said to be either embryonic stem cells (ESCs), foetal stem cells (FSCs) or adult stem cells (ASCs). This classification system, based on development potential and stages of development, can be interlinked to present a comprehensive insight for the nomenclature of stem cells**.**

Embryonic stem cells

A groundbreaking discovery in the field of regenerative medicine was the generation of cell lines from the inner cell mass (ICM) of blastocyst from the preimplantation embryo, these cells were pluripotent and are termed as embryonic stem cells (ESC) [24]. The developmental fate of these cells has not yet been defined; hence they are capable of forming all the three germs layers when grown under certain *in vitro* conditions. Normal preimplantation mouse embryos were used for the first time in 1981 to isolate mouse ESC [25, 26]. Human ESC were produced by embryos generated by *in vitro* fertilization for clinical purposes, these embryos were cultured to the blastocyst stage and the ICM was isolated for generation of ES cell lines [24]. It is the loss of embryos in the process of generation of ES cell line that is the most controversial aspect of ESC. Somatic cell nuclear transfer (SCNT) is an alternative source of ESC that involves the removal and replacement of nucleus of an egg with that of the somatic cell; Dolly the Sheep is the most celebrated example of SCNT [27].

Foetal stem cells or embryonic germ cells

Eight weeks into its development, the embryo is referred to as a foetus, and foetal stem cells are responsible for development of all tissue before birth. As is the case with ESCs, foetal stem cells are also pluripotent. Embryonic germ cells form from the primordial germ cells of the gonadal ridges in the 5-9 week old foetus and are capable of producing all the three germ cell types. Foetal stem cells are abundantly found in placenta, foetal blood and umbilical cord. Various stem cells such as neural crest stem cell, foetal haematopoietic stem cells and pancreatic stem cells have also been found in the foetus. These are the primitive cell types which exist in the organs of the foetus. Umbilical cord blood cells have also been found to contain many circulating stem cells and the matrix cells of the umbilical cord, known as Whartson's jelly, is a potential source of foetal stem cells.

Adult stem cells

Adult stem cells are the primitive cells which exist cryptically within differentiated tissues. The main function of these cells in a living organism is for tissue maintenance and repair and the origin of these cells is unknown, unlike that of embryonic stem cells. Adult stem cells are found very sparsely in organs and tissues and may remain quiescent for many years until they are activated by injury or disease. Adult stem cells have been found in brain tissue, bone marrow, peripheral blood, skeletal muscles, blood vessels, liver and skin. The most common source of adult stem cell is the bone marrow.

The mesoderm is responsible for the formation of the haematopoietic system in the foetus. Haematopoiesis occurs in the yolk sac and liver of the foetus, but later in life is sustained by bone marrow. Bone marrow is a complex tissue harbouring heterogeneous

population of cells composed of haematopoietic and supporting stromal cells Apart from providing a support system stromal cells also play a role in maturation of blood cells through cell signalling, and the stromal compartment is influenced by haematopoietic cells; the two compartments are both interdependent and indispensable to the other. Haematopoietic stem cells are rare in that they are pluripotent bone marrow stromal cells responsible for the production of blood cells continuously throughout life and are easily isolated using antibodies. Bone marrow has a subclass of stem cell like cell which are referred to as fibroblastic colony–forming units (F-CFU)[28], and these cells are precursors for non haematopoietic tissues. Bone marrow stromal tissues are composed of different kinds of cells such as fibroblasts, reticulocytes, endothelial cells and adipocytes which accounts for its heterogeneous nature. The cells present in stromal tissues have different proliferation and differentiation capacity and some of the stromal cells are found to be multipotent and can give rise to adipoblast, chondroblast and osteoblasts.

Bone marrow stromal cell

Mammalian bone marrow consists of three main cellular systems: hematopoietic, endothelial, and stromal, with the stromal cells loosely referred to as the non hematopoietic cells of mesenchymal origin [29]. The stromal tissue of adult bone marrow (BM) has traditionally been seen as having a supporting role in haematopoiesis; that is, its principal function is to provide a microenvironment within the BM to support the tightly regulated proliferation, differentiation and maturation of hematopoietic stem cells (HSC) into each of the eight distinct lineages that comprise the hematopoietic system [30].

The German pathologist Cohnheim made the observation, in the 1880s, of the presence of stem cells other than hematopoietic cells in bone marrow, [31]. Evidence that

the bone marrow cells were capable of differentiating into mesenchymal cell linages, as well as fibroblast, came from the pioneering work of Friedenstein and co-workers [32], who observed that when bone marrow cells adhered to tissue culture plastics a rare cell population developed into colony forming units with a fibroblastic appearance, labelled CFU-F for short [33, 34]. Bone Marrow Stromal Cells (BMSCs), are also referred to as MAPCs (Multipotent Adult Progenitor Cells), MSCs (Mesenchymal Stem Cells), bone marrow stromal stem cells (BMSSC) and MPCs (Mesodermal Progenitor Cells) [35]. The marrow stromal tissues consists of a heterogeneous population of non-hematopoietic cells and include all cell types that are located between the outer surfaces of marrow blood vessels and the bone surfaces which encase the hematopoietic space and tissue, such as marrow adipocytes, Westen-Bainton cells, bone-lining cells (inactive osteoblasts), and osteoblastic cells [36]. The heterogeneous nature of the population is immediately evident by the observation of BMSC single colonies; these colonies appear different in shape and size, reflecting differences in proliferation and growth rate among the CFU-Fs [35].

In vitro multilineage potential of MSC

Single cell clonal population studies of bone marrow derived cells has demonstrated that the BMSCs are a heterogeneous cell population consisting of cells at different commitment stages [9]. BMSCs can be induced to differentiate into bone, cartilage and fat *in vitro*, therefore the differentiation potential of BMSC represents an important criterion for characterizing these cells.

Chondrogenic differentiation is achieved by growing a monolayer of expanded BMSCs in serum free medium containing transforming growth factor-beta 3 (TGF-β3) [37]. Chondrogenic differentiation is confirmed by Alcian blue staining or alternatively,

type 2 collagen staining can be performed since this protein is characteristic of articular cartilage formation.

Osteogenic differentiation is achieved by growing the cells in media supplemented with dexamethasone, beta-glycerol phosphate, ascorbic acid and 10% v/v foetal bovine serum (FBS) [38]. Osteogenic differentiation is confirmed by the calcium deposition, expression of bone matrix protein mRNA expression and a transient increase in the expression of alkaline phosphatase. In addition to this von kossa staining can be performed as a confirmatory test for osteogenic differentiation.

Adipogenic differentiation can be promoted by treatment with 1-methyl-3-isobutylxanthine, indomethacin, insulin and dexamethasone [39]. The presence of lipid rich vacuoles by Oil red O staining is a positive indicator for adipogenic differentiation.

5-azacytidine is an unspecific DNA methyltransferase inhibitor and can induce human MSCs to differentiate *in vitro* into cells with characteristics commonly attributed to cardiomyocytes [40].

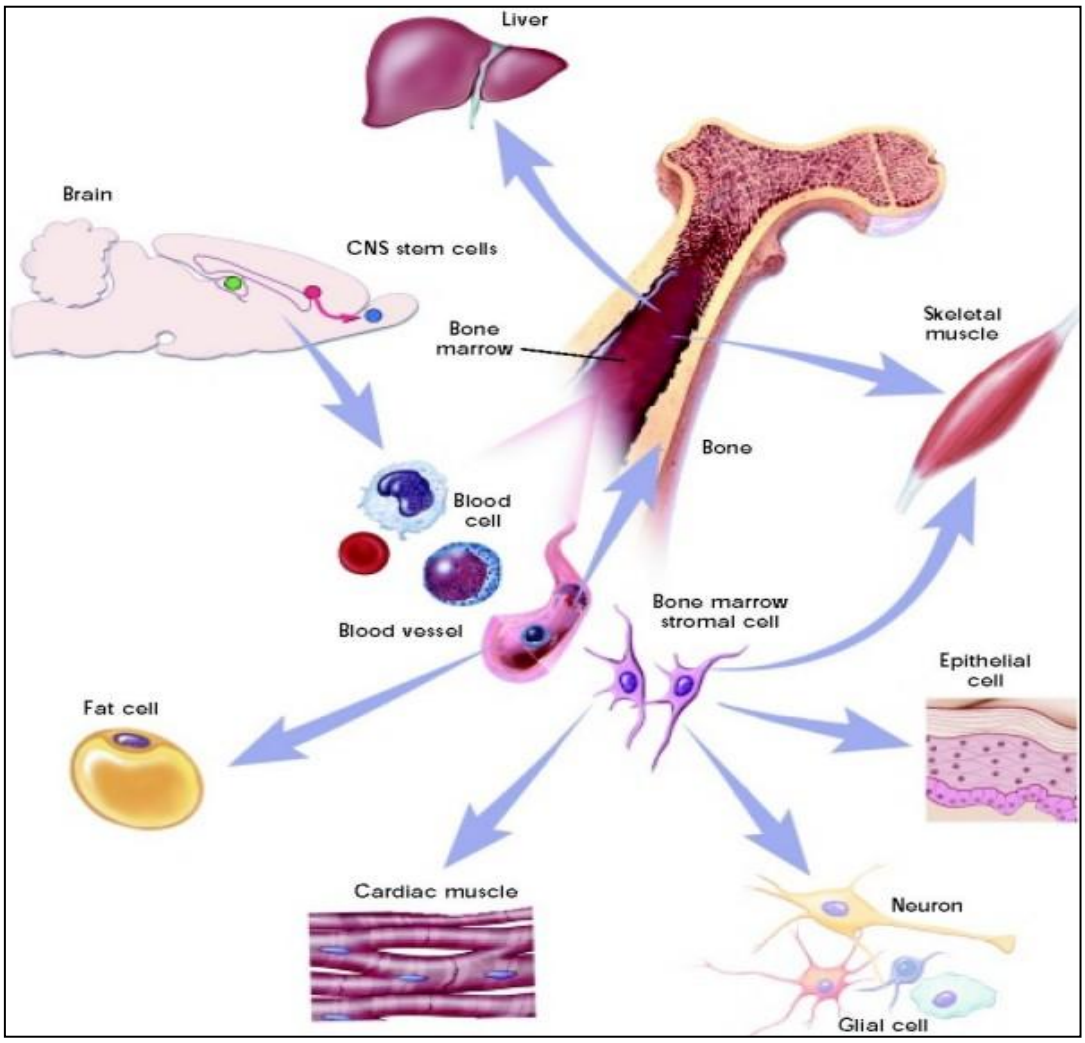
It has been observed that a combination of acidic fibroblast growth factor (αFGF), basic fibroblast growth factor (βFGF) and hepatocyte growth factor (HGF) with type IV collagen coating may induce hepatic differentiation of BMSCs [41]

Media supplemented with EGF (epidermal growth factor), NGF (neural growth factor) and BDNF (Brain-derived neurotrophic factor) is capable of stimulating the formation of neuron like cells from MSC [42]. Beta-mercaptoethanol exposure for 3hrs to the MSC resulted in the change in the morphology of the fibroblast like to neuron like cells [43].

The unspecialized nature of BMSCs, with a capacity for self-renewal, plasticity, and differentiation, makes these cells potentially useful source for cell based treatments of an

increasing number of conditions which challenge more traditional approaches in medicine. Stem cells, unlike many other cells such as blood cells or nerve cells, maintain a population pool by undergoing division and proliferation. Symmetric and asymmetric cell division is an important aspect of the stem cell life cycle. Symmetric division takes place when a stem cell divides and forms two new daughter stem cells or give rise to two progenitor cells. Asymmetric division, on the other hand, is said to take place under certain conditions whereby a stem cell divides and gives rise to one daughter stem cell and one non stem cell which is a progenitor cell, which the proceeds down the path of differentiation. The factors which prompt some stem cells to undergo asymmetric division are not well understood. The asymmetric division of stem cells assists in the reparative process and also ensures that the stem cell pool does not decrease; symmetric division helps stem cells to undergo self renewal, proliferation and replication.

Bone marrow stromal cells are unspecialized and lack the tissue specific structures that are necessary to perform specialized tasks in the cells. BMSCs are multipotent cells that can replicate to produce undifferentiated daughter cells with the potential to differentiate into mesenchymal tissue lineages, including bone, cartilage, fat, tendon, muscle and marrow stroma [7, 39].



Adapted from chroma.med.miami.edu/micro/images/faculty-jurecic.html

Figure 2: The figure illustrates the bone marrow as a source of bone marrow stromal cells. The stromal cell network comprises a heterogeneous population of progenitor cells capable of generating bone, cartilage, fat and fibrous connective tissue.

Plasticity or trans-differentiation is another stem cell characteristic which makes these cells ideal candidates for cell-based regenerative therapies Trans-differentiation is the phenomenon in which stem cells from one organ, when engrafted into another damaged organ, changes into the cell type of the damaged organ. Trans-differentiation has been

well documented in bone marrow stromal cells. Recent findings indicate that adult BM also contains cells that can differentiate into additional mature, non-hematopoietic cells of multiple tissues including epithelial cells of the liver, kidney, lung, skin, gastrointestinal (GI) tract, and myocytes of heart and skeletal muscle [44]. This lineage conversion was proposed to occur directly, by activation of an otherwise dormant differentiation program to alter the lineage specificity of the cell. It has also been proposed that lineage conversion can occur whereby tissue-specific cells dedifferentiate back to a more primitive, multipotent state and then re-differentiation along a new lineage pathway [45].

Selection and proliferation strategies for MSC

Bone marrow is considered as an ideal source of MSC. Other sources include tissues such as skeletal muscle [46], adipose tissue [47], trabecular bone, synovial membrane [48] and periosteum [49]. MSC are very sparsely distributed in the bone marrow and their number declines with age; the number of MSC in a newborn is about 1/10,000 which decreases to about 1/ 20,00,000 in an 80 years old person [50]. The scarcity in the presence of these cells *in vivo* imposes a limitation on their study *in vitro*. Also, the low number of the MSC illustrates an obvious need for *ex vivo* expansion in order to obtain sufficient number of cells useful for transplantation. However, there is an orderly loss of differentiation potential, parallel to the proliferation capacity [51]. A number of strategies have been developed to improve the ability of the cells to proliferate while retaining their differentiation plasticity following numerous passages [52]. The preferential plastic adherent nature of the MSC has been used to separate them from the hematopoietic stem cells. The bone marrow has a low amount of extra cellular matrix, such that a single cell suspension can be obtained by gentle mechanical disruption[53]. When plated at low

density the BMSC rapidly adhere and repeated washing separates them from non adherent hematopoietic stem cells [53, 54]. Morphological characters such as size of the MSC has been used to isolate and purify MSCs from bone marrow, culture device-a plastic culture dish comprising a plate with 3-microm pores were used to sieve out a homogeneous population of cells from bone marrow aspirates. Size-sieved cells that adhered to the upper porous plate surface were a relatively homogeneous population as indicated by morphology and other criteria, such as surface markers [55]. Many sophisticated approaches such as magnetic activated cell sorting [56]or fluorescence activated cell sorting complemented with microbeads [57]have been successfully used in isolation of the MSC. Also, magnetic beads conjugated with the relevant monoclonal antibodies specific for membrane antigens such as Sca-1, STRO-1, NGF receptor, CD73 or CD105 has been used for selection on MSC [58-62]. The immunodepletion of the hematopoetic cell using anti CD11b/CD34/CD45, forms the fundamental concept for negative selection method of MSC purification [63, 64].

However, there is an orderly loss of differentiation potential, parallel to the proliferation capacity [51]. A number of strategies have been developed to improve the ability of the cells to proliferate while retaining their differentiation plasticity following numerous passages [52]. It was observed by Martin *et al* that the size of colonies formed in clonal conditions was approximately 2.5 times larger in presence of FGF-2. The stable transfection of MSC with the catalytic subunit of telomerase abolishes senescence associated phenotype and maintains cell functions including unlimited proliferative ability, capacity to differentiate into multiple cell lineages, and *in vivo* bone forming ability [65]. Alternatively; *ex vivo* expansion of BMSCs on a denatured collagen (DC) matrix appears to reduce the rate of morphological changes and preserves the potential for osteogenic differentiation [66]. The preferential binding capability of the MSC to fibrin has been used

to develop fibrin microbeads system which gives about one fold higher MSC as compared to plastic adherence [52]. It has been observed that MSC when expanded *ex vivo* on fibronectin coated plates could be grown for more than 50 population doublings without obvious signs of differentiation or senescence [67].

Despite the dedicated efforts of previous researchers the major drawback of these methods of purification is the fact that isolated MSC populations are heterogeneous in respect to surface marker profile, phenotypic characteristics and morphology. The expression of membrane antigens is inconsistent and not uniquely expressed on MSC, necessitating the need to find a potential biomarker with the desired characteristic to differentially and specifically identify these potent cells *in vivo* and *in vitro*. The low yield of the initial number of cells, already mentioned above, is another hurdle which makes it necessary to expand the number of these cells before any further application.

Biomarkers

Biomarkers are defined by the Biomarkers Definitions Working Group as: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Biomarkers are strong indicators of the underlying physiological pathways activated in a cell. The combination of biomarkers in specific conditions can form a strong parameter for the identification and classification of disease condition and for therapeutic response assessment. The emerging science of biomarkers has applications to diagnosis, staging, prognosis and monitoring of disease progression, as well as in the monitoring of clinical responses to a therapeutic intervention and the development and delivery of personalized treatments to reduce attrition in clinical trials [68]. Within the field of

genomics, the challenge of biomarkers is to identify unique molecular signatures in complex biological mixtures that can be unambiguously correlated to biological events in order to validate novel drug targets and predict drug response [69]. Applications of biomarkers in clinical research are (1) as a diagnostic tool for the identification of disease or abnormal conditions; (2) in determining the stage or classification of extent of a disease; (3) as a disease prognosis indicator; and (4) as a tool for prediction of clinical response on intervention.

Apart from its important role in clinical research, biomarker studies hold an important place in basic research as well. The expression of biomarker profiles is an important tool for purification, characterization and identification of cell populations. The reason the expression of biomarker is critical for the identification, classification and characterisation of cells is that the presence of biomarkers gives an insight into the signalling pathways activated in a cell. These cell signalling pathways in turn alter the gene expression of the cells which causes alternative phenotypic characteristic. Biomarker studies continue to attract much attention in the field of stem cell, since signalling pathways controlling the self renewal, differentiation or pluripotency may be identified with the help of biomarker, making it possible, in theory, to utilize these features under controlled conditions in regenerative therapy. However, it is important to note that differences in cell surface expression of many markers may be influenced by factors secreted by accessory cells in the initial passages, and that the *in vitro* expression of some MSC markers does not always correlate with their *in vivo* expression pattern [70]. The biomarker expression change as the cell moves from one stage to the next in its life cycle. That is why several marker combinations are necessary to describe a cell population.

Biomarkers for mesenchymal stem cells

Mesenchymal stem cells lack any typical markers for haematopoietic and endothelial cell lineages such as CD11b [63], CD31 [63] CD34 [71], CD45 [71]and CD117. The absence of CD14, CD34 and CD45 antigens on mesenchymal stem cells is what specifically differentiates them from haematopoietic stem cells. Mesenchymal stem cells shows are positive for CD105 [71] ,CD44 [71], CD73 [71], CD166, CD117, Sca-1, STRO-1 [72], CD90 (Thy-1) [71], CD29, CD106 (Vascular cell adhesion molecule V CAM-1), ICAM-2, VCAM-1, CD72, and LFA-3 [73]. The systemic analysis of cell surface molecules of MSCs has revealed that these cells also express, at various levels, a group of cell adhesion molecules such as high expression of integrin α1, α5, and β1; low expression of α2, α3, α6, αV, β2, and β4; and no expression of α4, αL, and β2 [73] This specific expression pattern of adhesion molecules suggest *in vivo* cell adhesion and homing interactions.

Various antibodies have been generated in the past by the hybridoma method of antibody production which can specifically identify the biomarkers of mesenchymal stem cells. The murine monoclonal antibody STRO-1 was generated by Paul J. Simmons and Beverly Torok –Strob by lymphocyte hybrid fusion between NS1-Ag4-1 murinemyeloma cells and BALBlc spleen cells from an animal immunized with a population of CD34 positive (CD34+) BM cells [72]. STRO 1 is by far the best known antibody for identification of MSCs. The monoclonal antibody, SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105), the TGF-β receptor III present on endothelial cells, syncytiotrophoblasts, macrophages, and connective tissue stromal cells [59]. This antibody was used by Barry et al. for magnetic selection of MSCs. The SH-3 and SH-4 monoclonal antibodies recognize epitopes present on the surface of human MSCs; analysis of peptides derived from this protein by mass spectrometry and

sequencing identified it as CD73 (ecto-5'-nucleotidase) [58]. The SB10 antibody recognises activated leukocyte cell adhesion molecule (ALCAM), more commonly known as CD166. SB10 was shown to be present on undifferentiated MSCs and its expression disappears when the MSCs moves towards the osteogenic differentiation pathway as evident by the presence alkaline phosphatise [74]. Despite the expression of all the above mentioned biomarkers, there are none yet found that are consistent and specific for MSCs [75]. Currently available cell surface markers are not sufficient to identify the mesenchymal stem cells *in vivo* or in a heterogeneous population because the putative markers may also be found on non stem cells, or a particular marker may only be expressed on stem cells at a particular cell cycle stage or under certain conditions. The problem is aggravated by the fact that these cells are very sparsely distributed *in vivo* [76] and there are no strict parameters of identifying and characterizing these cells. Factors secreted by the accessory cells in the initial passages may affect the surface marker expression and expression of markers *in vitro* may not reflect the true picture of *in vivo* marker expression [70]. From the discussion above, the desired characteristics of MSC biomarkers would be: (1) identifying the MSC distribution sites *in vivo*; (2) pinpointing the molecular pathways that trigger the differentiation of MSC to progenitor cells; (3) discerning the MSC subtypes; (4) differentiating MSC from the progenitor cells; and (5) monitoring the MSC homing after the MSC based clinical trials.

Subtractive immunization

In contrast to, traditional techniques of monoclonal antibodies production [77, 78], which usually result in generation of monoclonal antibodies to immunodominant molecules, subtractive immunization provides a very potent alternative method for

production of monoclonal antibodies against the epitopes which are poorly immunogenic. SI is also capable of producing mAb against closely resembling proteins, such as proteins derived from protein engineering. Subtractive immunization is a technique proven to facilitate efforts to produce monoclonal antibodies specific for antigens that are present in low abundance in a protein mixture, poorly immunogenic and/or similar in sequence or structure to other proteins [79].

Concept of subtractive immunization

Subtractive immunization involves two major steps, the first step known as tolerization involves the immunization of the animal with the antigen (the tolerogen). The animal is then tolerized against the tolerogen. The tolerization can be induced by neonatal tolerization[80], high zone tolerance [81], chemical immunosuppression [82] .

The immunological definition of self is established in an organism shortly before and briefly after the birth [83]. This aspect of the embryo is used to achieve the tolerization against non self in **neonatal tolerization**. On exposing the tolerogen properly to the embryo any B cell produced against the tolerogen is eliminated by clonal inactivation, also T suppressor cells with similar specificity are produced[84, 85]. On subsequent exposure of the tolerogen to the animal it is treated as self so no immune reaction against tolerogen is generated. This is one of the most extensively used method for antibody production against minor immunogenic determinant. Neonatal tolerization with testicular sperm proteins followed by immunization with epididymal sperm proteins has been successfully used to enhance the production of antibodies to proteins exclusively of epididymal origin[86]. A monoclonal antibody luminal epithelial antigen (LEA.135) generated by subtractive immunization has been successfully used as a prognostic marker for breast

cancer owing to its ability to specifically recognise a luminal epithelial antigen exclusively in normal condition and not under the malignant condition[87].

High zone tolerance is achieved by injecting the animal with a very large quantity of antigen without the adjuvant[81]. In the absence of the adjuvant, B cells specific for the antigen are suppressed. Also, the corresponding T cells fail to trigger a secondary response upon contact with such antigens; this further prevents the activation of corresponding B cells. The humoral response specific for the tolerogen is prevented, consequently on subsequent exposure of desired epitopes; the antibody is exclusively for target epitopes. The method is used by some of the viruses to manipulate the host response to avoid elimination by the immune system and to persist in the host[88]. Lebron et. al. used high zone tolerization used to generate monoclonal antibodies (mAbs) against one polypeptide chain of a heterodimeric protein when the other chain was more immunogenic[89].

Cyclophosphamide mediated tolerization Matthew and Patterson were the pioneers in using the cyclophosphamide to modulate the immune response to augment the desired antibody production [90]. Cyclophosphamide is a cytostactic as well as a cytotoxic drug. It has been used to induce chemical immunosuppression because it can preferentially eliminate the antigen stimulated B cells and T cells. On the co-injection of cyclophosphamide and antigen into the animal the drug eliminates the B cells and T cells produced in antigen response, leaving the animal tolerized to immunodominant epitopes. On subsequent exposure of the animal with the desired antigen the animal produces antibodies only against the epitopes which are not expressed immunodominantly. Chemical immunosuppression with cyclophosphamide is the most effective subtractive immunization technique and the cyclophosphamide regime employed was a critical determinant in the success of chemical immunosuppression[91] antibodies generated by

chemical induced immunization was successfully used to generated monoclonal antibodies which could specifically inhibit metastasis.

The next step in SI involves the immunization of the animal with the second set of antigen which is closely related but not identical to the tolerogen. The animal preferentially produces antibodies against the desired epitopes owing to the suppressed immune response against tolerogen. This is closely followed by the hybridoma production.

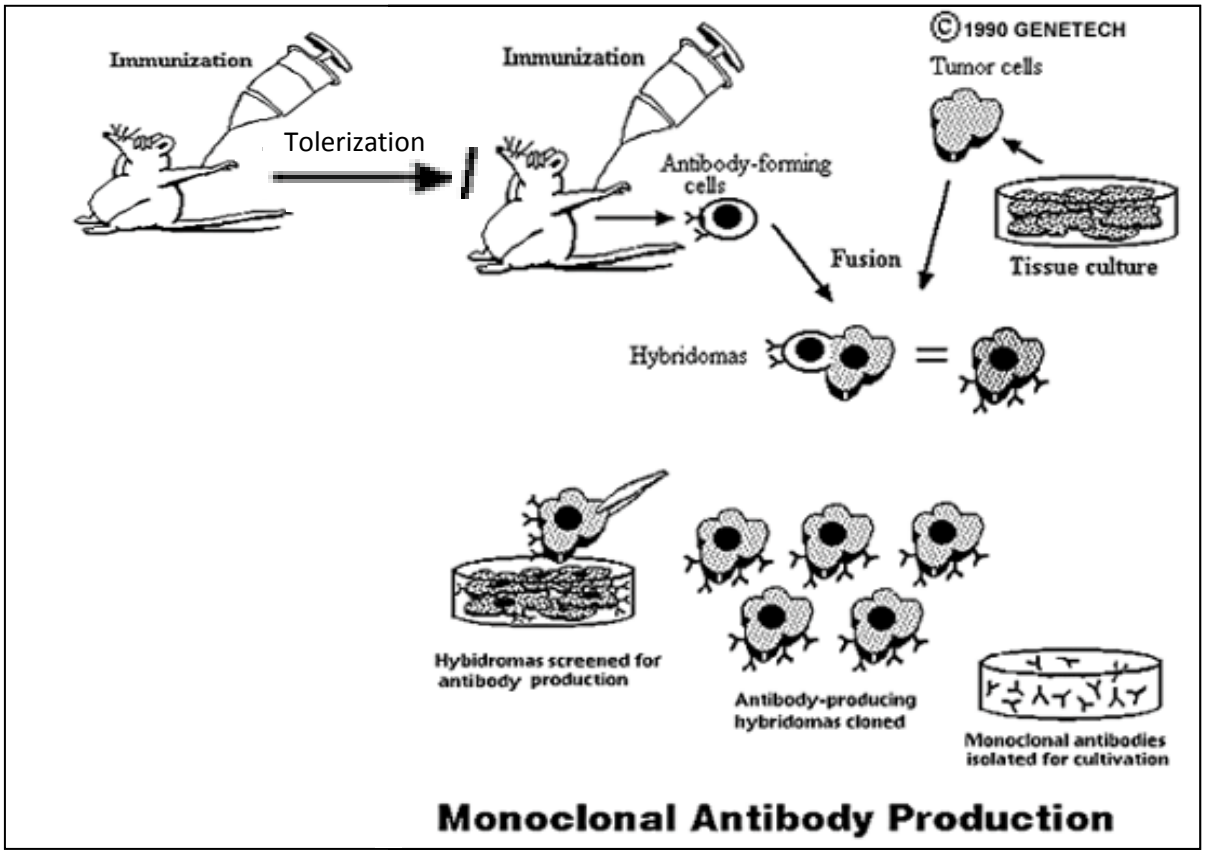


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Figure 3: Diagrammatic representation of the method of subtractive immunization. The animal is tolerized with the immunodominant epitopes followed by immunization with the target epitope. The spleen cells from immunized animal are fused with the tumour cells to produce hybridoma. Antibody cells (known as hybridomas) are produced and cultured to get target antibody in the media suspension.

This project is an attempt towards finding a potential therapeutically useful biomarker and cues that determine the role played by BMSCs in diseased state of OA, using the method of subtractive immunization; where two closely related homogenous populations of cells generated by clonal expansions were used.

Reprogramming

As a fertilized egg develops into an adult organism, specialized cells are formed by a one-way process (differentiation), and they become increasingly, and normally irreversibly, committed to their fate [92]. Every cell of an organism is genetically identical, therefore ideally all the cells are capable of acting in the totipotent manner, however the specialized state of a cell is an outcome of transitional programming of the genetic material by reversible epigenetic changes that are gradually imposed on the genome during development [93]. It is generally believed that the fate of a differentiated cell is stable; however, naturally occurring and experimental evidence indicate that dedifferentiation events, or return to pluripotency, can take place[94]. Some lower vertebrates such as teleost fish and some amphibians are able to compensate for the loss of body parts by regenerating a nearly perfect copy of the original part by dedifferentiating cells *in vivo* to facilitate regeneration [95]. The mechanism responsible for dedifferentiation known as nuclear reprogramming, involves reversal of genomic modifications that are imprinted on cells during differentiation and development [96]. Nuclear reprogramming presumably involves many complex cellular changes, including activation and silencing of specific genetic pathways, chromatin remodelling, and changes to DNA methylation and packaging [97]. The process is of interest as it can identify the mechanism responsible for cell differentiation and the maintenance of specialized state, it can provide an unremitting source of patient specific therapeutic cell. Additionally, it can explain the process of

disease onset by establishing the cell lines from disease tissue and can be useful to screen for therapeutic drugs [92].

The term reprogramming is interchangeably used for dedifferentiation or transdifferentiation; however, the phenomena of dedifferentiation and transdifferentiation are different degrees of direct reprogramming. Partially committed progenitor cells which have not yet determined their lineage fate are capable of undergoing dedifferentiation to produce a different cell type by the process of transdetermination. On the contrary, a fully committed cell can completely change its lineage-this process is termed as transdifferentiation [98]. Reprogramming phenomena can be mimicked *in vitro* by experimental procedures such as nuclear transfer, cell fusion, cell explantation and direct reprogramming [96].

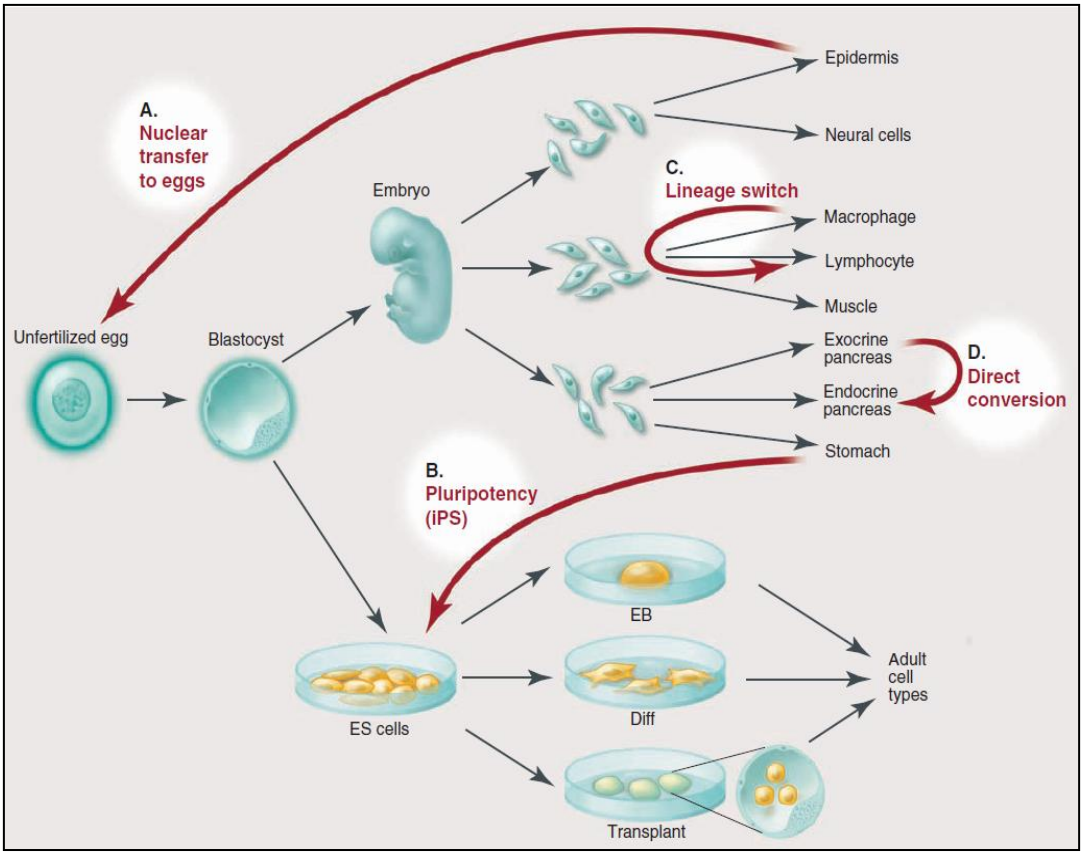

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Figure 4: An illustration for normal developmental process and experimental methods for inducing nuclear reprogramming. Unfertilized egg upon fertilization divides to forms blastocyst and results in embryo formation by cell differentiation and specialization. Nuclear reprogramming can be induced by (A) transfer of nucleus of a differentiated cell into unfertilized egg, a process termed as SCNT (B) by induced pluripotency iPS by transfection of stem cell factors into somatic cells, (C) by lineage switch and (D) by direct conversion.

Nuclear Transfer

Nuclear transfer, commonly known as cloning or somatic cell nuclear transfer (SCNT), is a classic example of the genetic totipotency demonstrated by terminally differentiated cells. In contrast to earlier theories, which suggests that each cell fate

decision during development involved the progressive loss of genes that would not be used by the more differentiated progeny, SCNT has proved beyond doubt that cells maintain the full complement of genomic information and developmental potential as they become differentiated[99]. Also, the genomic information can be modulated to erase the modifications associated with developmental programming of cells, under certain conditions to return the differentiated genome to its pluripotent state [100]. SCNT is a technique wherein the nucleus from somatic cells (non-gamete cells) is implanted into an enucleated egg cell which can then be implanted into, and develop in, a surrogate mother, and potentially become an adult organism[101]. Pioneers study by Hans Spemann laid the foundation for SCNT, his study demonstrated that an entire adult salamander can be created by implanting a nucleus from a fertilized salamander egg that had already undergone cell division several times into a cell from a newly fertilized enucleated salamander egg [101]. This concept later was applied by Briggs and Kings to introduced the concept of SCNT, which involved transplanting the nuclei of blastula cells into the enucleated frog oocyte to successfully produce a viable normal swimming tadpole of Rana pipiens[102].Similar studies using nuclei from fully differentiated cells into enucleated frog(Xenopus laevis) oocyte, was able to generate completely normal fertile offspring[103]. Using the concept of SCNT the nuclei of cultured sheep mammary cell line was transplanted into enucleated sheep egg to generate probably the most famous and clonally produced 'Dolly' the [104]sheep.

Most recent advancement in this field is the isolation of two ES cell lines from clonally produced embryos, generated by a modified SCNT approach to produce rhesus macaque blastocysts from adult skin fibroblasts [105]. Nuclear transfer approach can be widely used for drug discovery, toxicology testing and serve as an undiminishing reservoir for ESC, however, SCNT has never been practised successfully with human cells, in large

part because of the difficulty of obtaining adequate numbers of human oocytes [100],additionally nuclear transfer can be a bridge between regenerative medicine and its academic counterpart development biology. Despite the challenges and limited achievements in human, nuclear transfer remains the “gold standard” in nuclear reprogramming with clear demonstrations of the production of both healthy clones and pluripotent stem cells identical to those derived from fertilized embryos[99].

Cell fusion

Direct differentiation and phenotypic dominance at cellular level of one lineage to another has been demonstrated by experimental procedure of cell fusion. The process involves the fusion of two somatic cells and forcing them to be in tetraploid state using cell division inhibitor to form a heterokaryon. In these heterokaryons, the dominant cell, usually the larger and more actively dividing partner imposes its own pattern of gene expression on the other partner 1, the differentiated state of the cells is also a determining factor for dominance, it has been observed that the higher a cell in differentiation hierarchy more dominance it expresses on other cell type.

The cellular dominance of one cell type over other anticipated that this system could be used to investigate the mechanism for this fate respecification as a proxy for understanding the effectors of cell fate decisions normally made in the process of development 2. Cell fusion is another attempt towards inducing pluripotentency in differentiated cells, in year 1976 Miller and Ruddle produced pluripotent hybrids by the fusion of pluripotent teratocarcinoma cells with primary thymocytes, these hybrids were identical (potential to induce tumour) to the parent embryonal carcinoma cells. Similar experiments using somatic cell with murine embryonic germ and embryonic stem revealed the dominance of pluripotent cells over differentiated cells.

Somatic cell fusion seems to be a potentially attractive technique to induce pluripotency in somatic cell to generate customized cell for therapy, however, along with difficulties with proliferation capacity and tetraploid state of the cells is the most significant hindrance in its *in vivo* application. The efficiency of cell fusion and reprogramming is problematic, limiting its usefulness in the study of genetic and epigenetic of reprogramming.

Cell explantation

Also, known as culture induced reprogramming is the phenomena of induction of dedifferentiation or trans-differentiation of cells in response to certain physiological conditions. Three recent studies proposed the generation of multipotent adult germ line stem cells or multipotent adult spermatogonial-derived stem cells from neonatal and adult, testes in the presence of appropriate growth factors and culture conditions. These pluripotent cells share hallmark characteristics such as multilineage differentiation, marker expression, etc with ESC, however, their epigenetic imprinting status differs extensively from that of the ESC, making them an inherently unattractive source for therapeutically useful cells.

Direct reprogramming

Direct reprogramming of somatic cells into pluripotent cell is achieved by transient ectopic expression of certain transcription factors. The effect of transcription factor in controlling the lineage of cells was explained for the first time in year 1989 by Weintraub, the pioneer work involved the forced expression of MyoD into fibroblast or adipoblast cell lines to direct them towards forming muscle cells[106]. Likewise, the enforced expression

of C/EBP alpha and C/EBP beta in differentiated B cells leads to their rapid and efficient reprogramming into macrophages.[107]. These experiments established that certain master gene can lead to lineage switch in differentiated cells.

A spectacular advance in this field was the concurrent, though independent, work of two groups, the first lead by the Yamanaka [11] and the second lead by James Thomson [108], demonstrated that differentiated cells can be effectively reprogrammed genetically and epigenetically to their embryonic pluripotent state. Retroviral mediated transfection of fibroblast by a cocktail of four transcription factors can revert their lineage specifically to embryonic state. The major difference between these two studies was the combination of transcription factors used; Yamanaka group used Oct3/4, Sox2, cMyc and KLF4 to reprogramme mouse fibroblasts [11] where as Thompson group used Oct4, Sox2, NANOG, and LIN28 in the reprogramming of human somatic cells [108]. Soon after, same concept was used to generate human pluripotent cells from the bone marrow cells, spermatogonial cells, and parthenogenetic embryos[109], these reprogrammed cells were termed as induced pluripotent cell (iPS) by Yamanaka group.

This innovative study (by the Yamanaka group) begin with a hypothesis that the factors that play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells. Based on this assumption 24 candidate genes were identified as candidates for factors that induce pluripotency in somatic cells [11], a rigorous selection criterion was implemented for subsequent selection for Fbx15 expression. Fbx15 is specifically expressed in mouse ES cells and early embryos; Fbx15 is dispensable for the maintenance of pluripotency and mouse development [11]. iPS generated were similar in their morphology , growth properties and cell marker gene expression to ESC, however, no live chimera could be produced also, the global gene-expression patterns and DNA methylation status of these induced cells vary

remarkably from the ESC. Subsequent study using same four transcription factors employed for selection for Nanog expression results in germline-competent iPS cells with increased ES-cell-like gene expression and DNA methylation patterns compared with Fbx15 iPS cells [110], however, a significant shortcoming of this study was high rate of tumour formation in experimental mice (about 20%). Nanog iPS resemble ESC in their morphology, proliferation, feeder dependence, surface markers, gene expression, promoter activities, telomerase activities, *in vitro* differentiation[111], transcription profile [112], epigenetic status [113] and ability to form high-grade germ line chimeras[99].

It has been proposed that reprogramming is the sequence of stochastic events involving reestablishment of an autoregulatory loop of the four endogenous pluripotency factors Oct4, Nanog, Sox2 and Tcf3by the exogenous factors, on activation the exogenous genes are silenced by endogenous gene expression [114]. This benchmark study is a new stepping stone towards the primary goal of cell therapy i.e. generation of patient specific therapeutic cells. The generation of iPS could have important implications for understanding development, disease pathogenesis, drug testing and toxicology; however, lower efficiency (0.02% to 0.002% in human cell) of the procedure is a concern [111]. Additionally, the retroviral vector method of transfection is a major impediment, as multiple integration sites in the iPS clone may increase the risk of tumorigenesis making these cells unfit for *in vivo* application. cMyc transcription factor used in the study is closely linked to cancerous state of cells, retroviral vector integration into host genome may result in insertional mutagenesis.

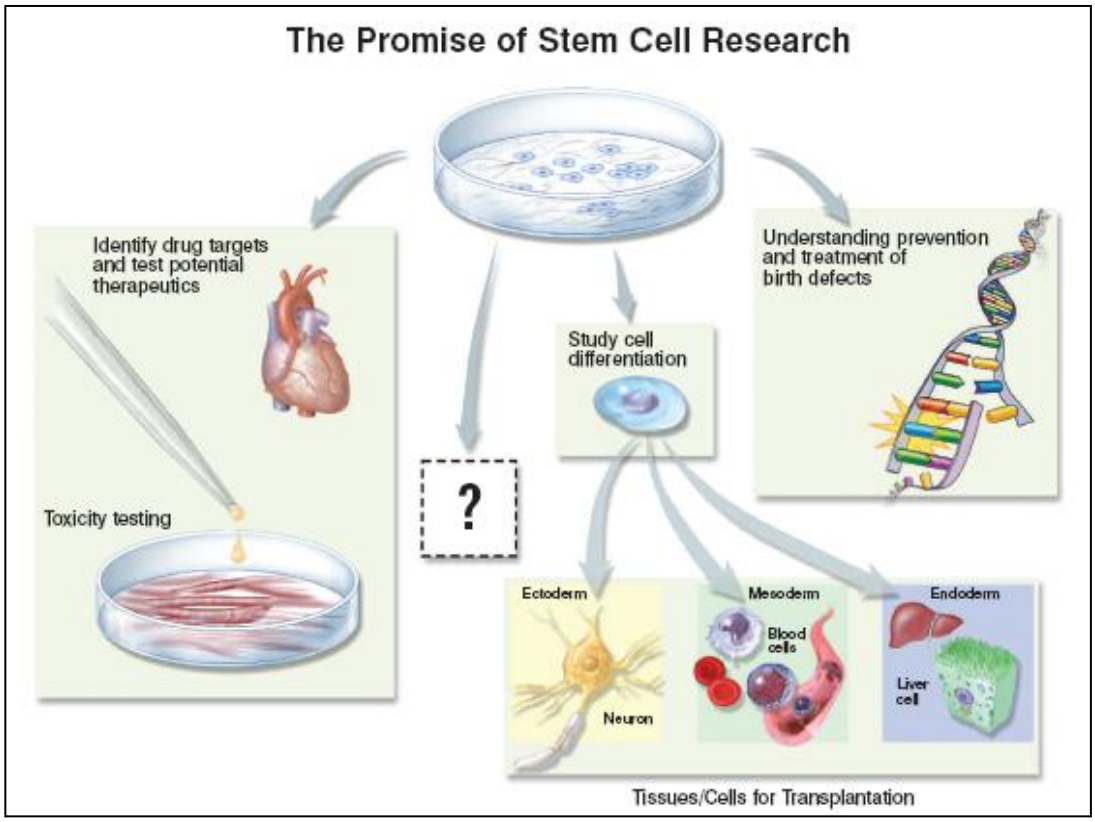
The future studies in generation of iPS focuses on using alternate methods of transcription factor induction as Nanog iPS cells indicate that factors are required for induction but not for maintenance. Less virulent, non-integrating and transient expression

of gene delivery method like adenovirus –mediated system and episomal vectors[115], have been used to fabricate mouse induced pluripotent stem (iPS) cells from fibroblasts and liver cells [116]. Alternate safer methods of iPS generation involve employing doxicycline inducible lentivirus [114] and using single virus efficient polycistronic expression from a single promoter instead of multiple vectors, hence reducing the chances of mutagenesis [117]. An alluring approach is vector free method of transfection such as by direct protein induction by recombinant proteins and small molecule based or by modulating the culture conditions to direct cells towards the induction of the reprogramming.

Potential Applications of Stem Cell Research

Conceivably, the most exciting and important application of stem cell is in the field of tissue engineering. Multilineage potential and self renewing capacity along with their capability to migrate in preferential manner to the defect site makes these cells an ideal candidate for cell based therapies [118]. Stem cell therapy may be used in cardiac repair [119] and to cure diseases such as osteoarthritis[120] or connective tissue disorder [121], infarcted myocardium [122], haematological disorders and neural disorders [123, 124]. In 1968, the first bone marrow transplant was used for the treatment of severe combined immunodeficiency syndrome [125]. Marrow transplants have been widely used for the treatment of immunodeficiency syndrome and malignancies since 1970. Phase II, III studies are in process to check if the improvement shown by ischemic heart condition patients was due to stem cell [126]. Autologous CD34+ stem cell transplantation may be safely administered and appears to offer some therapeutic benefit to patients with both viral and autoimmune-induced end-stage liver disease[127]. The *in vitro* expansion and culture of neural stem cells from brain and spinal cord has given new direction towards stem cells based therapy in Alzheimer's, Parkinson's and Spinal Cord and brain

injury[128]. Autologus application of stem cells with fibrin spray accelerates acute cutaneous wound healing in patients with skin cancer [129]. In addition to the clinical applications stem cells have an important role in developmental biology, the molecular mechanism of normal development remains unclear, stem cells present the prospect of understanding the formation of tissues by the growth and differentiation of cells. This may result in devising new therapeutic strategies for treatment of medical conditions such as cancers and birth defects which arise due to abnormality in cells functions such as division and differentiation. Another important application for stem cells is in the drug screening and toxicity testing of new drugs, pluripotent cells can be used to generate a wide variety of differentiated cells which can be used to test the new drugs prior to introducing them into clinical trials [130], thereby making the drug discovery process quicker and efficient. ES cells have been differentiated under controlled environment to generate functional cardiac, neuronal and pancreatic cells to analyse the *in vitro* testing of cytotoxic potential of chemical factors, drugs and xenobiotics [131]. Hence substantial advances in basic cell biology techniques are required to gain a comprehensive insight into the molecular mechanisms and genetic controls that regulate cell division and specialization.



Adapted from stemcells.nih.gov (© 2006 Terese Winslow)

Figure 5: An illustration for the promise of stem cell research. Stem cell research has a potential to identify drug target, effectiveness and toxicity testing. It is critical in understating the mechanism of tissue formation by cell differentiation and most important of all is generation of differentiated cells for therapeutic applications.

Summary

BMSCs are self renewing cells with multipotential nature, hence are attractive candidates for cell based therapies. However, BMSCs are sparsely distributed *in vivo* and their identification *in vivo* poses major challenge due to lack of putative markers capable of identifying them. Currently available cell surface markers are not sufficient to discriminate mesenchymal stem cells. Also, there is a decline in stemness associated features of BMSCs when cultured extensively *in vitro*. Hence, generation of sufficient number of *ex vivo*

expanded therapy potent cells remains the most crucial challenge. Stemness features associated with BMSCs are output of the internal signalling mechanism and external cues from the niche. Hence, this project attempts to highlight critical molecular players associated with stemness, determines the effect of *in vitro* culture on these molecular signatures and employs changes in culture conditions to stimulate proliferation potential and retain the expression of stemness markers over extensive passaging.

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CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF THE MOLECULAR SIGNATURES OF MESENCHYMAL STEM CELLS

Overview

BMSCs, owing to their multipotential nature and proliferative capacity, have been considered as an important source of therapeutically potent cells for cell based therapy. However, BMSCs are heterogeneous population of cells, unlike HSCs which have been well characterized; there is no dedicated biomarker for identifying these cells *in vivo*. Hence, this experimental section was designed to identify molecular signatures for clonal population of BMSCs by methods of subtractive immunization (SI) (Part I) and gene array analysis (Part II).

Abstract

BMSCs are promising candidate cell type for use in restorative and regenerative medicine [1-7]. A complex interplay of transcription factors and gene expression controls the unique multi-lineage developmental potential and self- renewal attributes of stem cell [8]. A comprehensive protein expression picture of BMSCs has been presented by numerous researchers using advanced proteomic methods to highlight the underlying mechanism governing these distinct features [6, 9, 10]. In the present study we have used an immunological and array based approach to identify the primary molecular factors responsible for maintenance of multipotentiality and self renewal characteristics associated with BMSCs. This study is a continuation of previous work that demonstrated the occurrence of fast growing multipotential cells from the bone marrow samples of patients undergoing elective knee surgery using cell clonal culture method [11]. The fast growing cell clones were subsequently used to generate novel antibodies against slow growing clones by the method of SI. This study aims to investigate potential molecular factors associated with the stemness by using these novel antibodies. A study of stem cell pathway-specific differential gene expression was performed between fast and slow growing clones using Stem Cell RT^2 Profiler ^{TM} PCR Array. The monoclonal antibodies (mAbs) generated by SI were used to highlight the differentially expressed antigenic determinants in the clonal populations by Western blot analysis. The cellular location of the unknown antigen was further determined by confocal microscopy. Finally, co-immunoprecipitation, followed by mass spectrometry analysis, was employed to identify the antigens highlighted by the novel antibodies and further validated with quantitative polymerase chain reaction. The mass spectroscopy revealed cytoskeletal protein Vimentin as the differential protein in contrasting clonal populations, suggesting the probable role of cytoskeletal protein in conferring stem cell like characteristics to BMSCs. The gene array

studies involved analysis of 84 stem cell related genes by quantitative polymerase chain reaction. The fast growing clonal cell population showed higher expression of a group of ten genes, nine of which were found to be associated with chondrogenesis or cartilage repair, indicating that BMSCs play a critical role in defect repair and maintaining tissue homeostasis by modulating gene expression patterns to compensate for degenerated cartilage in osteoarthritic tissues. A number of genes were found to be associated cell fate determination, growth factors, maintenance of embryonic and neural stem cell renewal. In summary, this study revealed a higher expression of vimentin, FGF-2, FOXA2, and Sox2 in the fast growing clone cells in comparison to the slow growing clones. The differential expression of these markers in the clonal populations may be associated with stem cell properties such as self renewal and differentiation potential.

Introduction

An integral aspect for developing cell-based therapy is to identify and generate physiologically relevant patient-specific cells. BMSCs are a potential source of clinically useful cells given their intrinsic self-renewal and multipotential nature and easy accessibility from the bone marrow. The morphological and phenotype changes associated with the BMSCs maturation and differentiation are tightly regulated by a complex interplay of cell cytoskeleton, transcription factors and controlled gene expression. These cells are readily expanded *in vitro* and can be genetically altered by viral vectors, making them an ideal vehicle for cellular gene therapy [9, 13, 14]. The major challenges associated with BMSCs are their sparse *in vivo* distribution and the sequential loss of multipotency and proliferation capacity on successive passaging during *ex vivo* culture. In order to utilize their obvious potential it is necessary to establish protocols for harvesting these cells

from bone marrow followed by *ex vivo* expansion to generate sufficient number while at the same time maintaining their unique characteristics. The molecular mechanisms controlling the distinct features of BMSCs remain elusive. Research is therefore required which focuses on identifying the factors that regulate and control BMSCs fate decisions. Such an effort would lead to a better understanding of the molecular, biological and physiological characteristics of this potentially useful stem cell type [15].

This study is to identify potential molecular pathways that are altered synchronously with changes to phenotypic characteristics of BMSCs. The study presented here is a continuation of the work in our research group by Mareddy et.al. [12]. Mareddy's work has identified and characterized 14 clonally expanded populations of BMSCs that were isolated from bone marrow samples from there osteoarthritic patients undergoing elective knee surgery [12]. The clonal populations generated were assessed for proliferative capacity, differentiation efficiency and cellular aging. The populations were classified as either fast growing or slow growing clones based on their proliferation capacity. Marked changes were found in different clonal populations, the fast clone cells were found to be pre-senescent, tripotential and fast growing. The slow growing clones showed a limited differentiation potential (unipotent), changes in cell morphology associated with signs of cell senescence and limited proliferation potential as the population doubling time for these cells was very high [12]. Clonal populations were employed in the current study to generate potential stem cell related antibodies using SI methods and to explore stem cell related gene expression profile. This study has been divided into two sections.

- **Part I** of this study highlights the differentially expressed proteins by the employing novel antibodies generated by SI.
- **Part II** highlights differentially expressed genes by Stem Cell RT^2 Profiler ^{TM} PCR Array.

Two contrasting clonal BMSC populations from the same patient samples were used to generate novel antibodies by SI. This technique generates antibodies against weakly immunogenic epitopes, as opposed to the traditional technique of mAbs production [16,17], that usually result in generation of monoclonal antibodies to immunodominant molecules. SI is a two-step process: 1) The experimental animal is first immunized with undesired epitopes known as tolerogen; the animal is then 'tolerized' against the tolerogen using tolerizing agent, such as cyclophophamide, to suppress the animal's immune system. 2) The second step involves the inoculation of animals with the desired antigen, known as the immunogen. In the previous study that has been completed by our group, mice were immunologically tolerized using the slow clone cells; this was followed by immunization of the mice with the fast clone cells. Hybridomas were generated from the immunoresponsive mice and screened for mAbs that were differentially reactive to the fast growing clone cells. In the present study, we describe the characterization, purification and identification of antigens recognised by two novel antibodies 12D10H1E2A and 12D10H1B9; these antibodies were reactive with elevated expression of unknown antigenic determinants on fast growing clonal cell populations.

Three fast and three slow growing clonal populations were used for quantification and screening of a set of 84 genes related to stem cell pathways. Stem Cell RT^2 Profiler ^{TM} PCR Array is an integrated approach for screening multiple stem cell pathways focused on gene expression and quantification of genes that confer self-renewal and multipotentiality associated with BMSCs. Our study revealed an elaborate picture of gene expression associated with stem cell fate, maintenance and self renewal in BMSCs.

PART I

Material and Methods

Western blot analysis

Fast and slow growing clone cells were grown to confluence in T75 flasks, and a crude cell extract was harvested with a lysis buffer (0.5 mL 1 M Tris, 1.5 mL% NaCl, 2.5 mL 20% triton X, 0.5mL of 0.5 M EDTA in 45 mL of distilled water). The protein concentration was quantified using a bicinchoninic acid protein assay. Equal concentration of the cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12% separating gel was prepared by adding 6mL of 30% acrylamide/0.8% bisacrylamide, 3.75mL of 1.5 M Tris-Cl, 0.4% SDS, 5.25mL of dH_20, 0.05mL of 10% ammonium persulfate and 0.01mL of TEMED and a 3.9% stacking gel prepared by adding 0.65mL of 30% acrylamide/0.8% bisacrylamide, 1.25 mL 0.5 M Tris-Cl, 0.4% SDS, 3.05 mL H_2O, 25µl of 10% ammonium persulfate and 5 µL TEMED. The electrophoresis was run in an SDS electrophoresis buffer (0.025M Tris base, 0.192M glycine , 0.1% SDS and the pH adjusted to 8.3) at 75V until the dye front reached the stacking gel, and then the voltage raised to 100V and run until the dye front reached the base of separating gel. The protein was transferred to a nitrocellulose membrane at 100V for 60mins in transfer buffer (100 mL 10 X running buffer, 200mL methanol dissolved in 800mL of dH_2O) and the membranes blocked with Tris buffered saline containing 0.1% Tween20 and 5% non-fat milk, followed by incubation with hybridoma of primary antibodies 2D10H6, 12D10H1E2A and 12D10H1B9 at 1: 1 dilutions for overnight at 4^0C. Membranes were then washed thoroughly in Tris buffered saline containing Tween TBS-T buffer (20mM Tris-Cl at pH 7.5, 500mM NaCl and 0.05% Tween-20) three times, each for

10mins. After primary antibody incubation the membranes were incubated with a secondary goat anti-mouse or goat anti-rabbit -horseradish peroxidase conjugated antibody (diluted in blocking buffer at 1:1000 dilution) for 60 mins and washed. SuperSignal West Femto Western Blotting Detection Reagents (Thermo Fisher Scientific, VIC, Australia) was used for detection according to the manufacturer's instructions and visualized by exposure to X-ray film (Fujifilm, Stafford, Australia) and developed in a AGFA CP 1000 automatic film processor (AGFA-GEVAERT Limited, Burwood, Australia).

Confocal microscopy

Confocal microscopy was performed to visualise the location of the unknown protein in the cells. Fast and slow growing clones were grown to confluence in chamber slides. The cells were washed with PBS at RT (room temperature) and fixed with 4 % PFA for 20mins. This was followed by three washes with PBS for 5 min each and then the cells were permeabilized with 0.2% Tween 20 in PBS for 20 min. Non specific binding was blocked by incubating the cells with 0.2%BSA in PBS for 1hr at RT. The 12D10H1E2A and 12D10H1B9 was diluted 1:1 in 0.2%BSA in PBS and incubated with the cells for 2.5 hours at RT. The excess of the primary antibody was removed by washing three times in PBS for 5mins. The green-fluorescent Alexa Fluor 488-conjugate secondary antibody was diluted in 1:1000 concentration using 0.2% BSA in PBS and the slides incubated in the dark at RT for 1 hr then washed three times with PBS for 5 min and mounted in mounting media (Prolong Gold, 1:1 glycerol: PBS). The coverslips were sealed with clear nail varnish and observed under confocal microscope (Leica TCS SP5, Leica Microsystems Pty Ltd, Mount Waverley, Australia).

Immunoglobulin M purification from the conditioned hybridoma media

Dialysis followed by gravity flow method was employed for Immunoglobulin M (IgM) purification from the conditioned hybridoma media. 100 mL of the hybridoma media was poured into the dialysis tube and placed in 4 L of dialysis buffer (20mM Tris/1.25 sodium chloride pH 7.4) with a magnetic stirrer for 4 hrs at 4^0C. This step was repeated four times each time using fresh dialysis buffer. The dialysed media was removed from the tube and equal volume of the dialysis buffer was added to it. This dialysed media was then subjected to the column chromatography to purify the antibody.

Immunolobulin isotyping of the hybridoma indicated that the antibody generated was of the IgM isotype. IgM purification possess challenges with the traditional method of antibody purification, therefore Immobilized Mannan Binding Protein (MBP) combined with an optimized buffer system was used to purify the IgM antibodies. MBP covalently attached to an agarose support produces an excellent tool for affinity purification of IgM. Column was carefully packed with the Immobilized MBP provided with the columns. Four gel-bed volumes of Binding Buffer to the column added and allow the solution to drain through. An extender (funnel) placed on the top of the column which allowed application of the Binding Buffer in larger amounts. Added 100 mL of cold (4^0C) dialysed hybridoma solution to the column, the flow through was collected. The solution was passed through resin repeatedly five times, maintaining a continuous flow through the column. The elution procedure was performed at RT. The concentration of the eluted fraction was measured using a Nanodrop instrument (Biolab Ltd, Victoria, Australia). Added 2 ml of Binding Buffer per 5 ml of gel bed to the column and incubate at 4^0C for 30 min. Washed the column with nine gel-bed volumes of the Binding Buffer to remove non-bound protein, the wash were monitored by collecting fractions and measuring their absorbance at 280 nm. These purified IgM were employed in immunoprecipitation of the unknown antigen.

Co-Immunopurification of the unknown protein

The immunoprecipitation of the unknown protein of interest was done by using ProFound™ Co-Immunoprecipitation (Co-IP) Kit (Pierce, Thermo Fisher Scientific Rockford, IL. U.S.A). This kit allows the isolation of native protein complexes from a lysate or other complex mixture. Immunoprecipitation is an approach to study protein-protein interaction as it uses a specific antibody to pool the protein of interest. This experimental procedure involves immobilization of the antibody on antibody coupling gel, followed by co-immunoprecipitation of protein complex and subsequent elution of co-immunoprecipitated complex.

The Antibody Coupling Gel and reagents were equilibrated to room temperature. Coupling Buffer was dissolved in the appropriate quantity of ultrapure water and gently swirled to obtain an even suspension. 50% gel slurry was added into a Handee™ Spin Cup Column (Pierce, Thermo Fisher Scientific, Rockford, IL. U.S.A). The column was centrifuged in a microcentrifuge tube, the supernatant was discarded and the spin cup was placed back into the tube. The gel was washed by adding 0.4 mL of Coupling Buffer to the spin cup. The tube was capped and gel was suspended by inverting and gently shaking the tube followed by centrifuging the tubes. The supernatant discarded and the spin cups placed into new microcentrifuge tube. The purified antibody specific for the unknown protein was diluted in Coupling Buffer in 1:4 ratios. 100µL of purified antibody was added to the spin cup containing the gel. 1µl of 5M sodium cyanoborohydride was added to each sample in a safety fume hood and incubated overnight with gentle end-over-end mixing. The following day the tubes were centrifuged and the spin cup placed in a new tube. 0.4mL of Coupling Buffer was added and the tube was capped and inverted tube end-over-end 10 times. The tubes were centrifuged, 0.4mL Quenching Buffer added to each tube which were inverted 10 times and centrifuged. The flow through buffer was discarded.0.4mL

Quenching Buffer was added to the gel. Inside a safety fume hood 4µl 5M Sodium Cyanoborohydride was added to each tube and inverted five times. This was followed by incubation for 30mins with end-over-end mixing after which the tubes were centrifuged. The gel was washed four times with 0.4mL Wash Solution and two times with 0.4mL Coupling Buffer. Spin cup conjugated with purified antibodies were employed for co-immunoprecipitation of unknown protein, 400µL of the cell lysate from the fast and slow clones were added to the respective spin cups. The spin cups were incubated with gentle end-over-end mixing for 1 to 2 hrs. The tubes were centrifuged and the spin cups placed in new tubes to which 0.4mL of Coupling Buffer was added. The tubes were inverted 10 times and centrifuged. Spin cup containing gel antibody and unknown antigens were then subjected to elution steps to elude the co-immunoprecipitated proteins.200 µl of Elution Buffer was added to the gel in the spin cup, gently tapped to mix and the tubes centrifuged. This was repeated five times to elute all the protein. The eluted sample was separated on 12% SDS-PAGE and the proteins highlighted in the gel by silver staining method.

Silver staining of the eluted samples

The silver staining is one of the most sensitive methods available for protein staining. The proteins eluted by co-immunoprecipitation were run on 12% SDS-PAGE. The gel was placed in silver stain fixing solution (40% absolute ethanol, 10% acetic acid and made up to 1L in de-ionized water) overnight. The following day the gel was placed in sensitizing solution (30% absolute ethanol, 6.8% sodium acetate, 0.5% sodium thiosulphate volume made up to 1L in deionized water) for 30 mins, then washed three times 10 mins with deionized water. The gel was incubated in silver staining solution (0.25% silver nitrate made in 1L with deionized water) for 20mins, then washed with deionized water for 1min.

The developing solution (2.5% sodium carbonate made up in 1L with deionized water) was poured over the gel for 5 mins. The gel was left in the stopping solution (1.46% of disodium ethylenediaminetetraacetate made up to 1L with deionised water) for 1 hr. and then washed three times, each for 5mins with deionised water.

Mass spectroscopic analysis of the unknown protein

An EMBL protocol was used for destaining and in-gel digestion of the silver stained gel. The gel was washed with deoionized water for 15 mins and the bands were cut out and place in eppendorf tubes. The samples were dried with a Speedi-Vac for approximately 15 mins. De-staining was performed by incubating the silver stained bands in destaining solution (freshly prepared 1:1 solution of 30mM potassium ferricyanide and 100mM sodium thiosulphate) for 8 mins. The gel was washed four times 8 mins with 1 mL of mill-Q water and 100% acetonitrile was added for 15mins and the solution discarded. The samples were dried with a Speedi-Vac for 30 mins. Alkylation or reduction of the gel was not performed as the main aim was the identification of unknown samples.

For In-Gel Digestion, 12.5 ng/μL of Sequence-Grade Modified Porcine Trypsin (Promega) was added to the bands. 50μL of ice cold solution (50mM ammonium bicarbonate and 5mM $CaCl_2$, pH between 8 and 9) was added to the spots so they were just covered and incubated on ice for 1hr, the spots were checked every 10 mins to ensure that they have not absorbed all of the liquid. After 1hr excess liquid was discarded and 30μl of 50mM ammonium bicarbonate/5 mM $CaCl_2$ was added, these samples were then incubated at 37°C overnight. The following day the supernatant was removed and kept in a siliconized microcentrifuge tube on ice. 30μl of 20mM ammonium bicarbonate was added to the gel pieces and incubated for 20mins after which the supernatant was removed and

pooled with the supernatant from previous step. This was followed by addition of 30 μL of solution (50% acetonitrile and 5% formic acid) and incubation at RT for 20 mins. The supernatant was removed and pooled with the supernatant from the two previous steps. The samples were then analysed by MALDI-TOF.

Semi-quantitative real time polymerase chain reaction (qRT-PCR) analysis

Real-time quantitative polymerase chain reaction analysis was used to validate the expression of vimentin in three contrasting clonal populations. The detailed protocol for the procedure has been mentioned in Part II experimental section.

Results

12D10H1E2A and 12D10H1B9 mAb generated by SI recognises a 54 kDa antigen

Western blot analysis was performed to detect the antigen recognised by the novel antibodies isolated from hybridoma media. The expression of the antigen highlighted by novel antibodies was higher in fast growing clones compared to the slow growing clones **(Figure 1a.)**. In the present study, we initiated the identification and characterization of the antigen recognized by the novel antibodies. Western blot studies showed that 12D10H1E2A and 12D10H1B9 highlighted an unknown antigen of about 54 kDa in fast and slow growing clonal cell lysate, however different staining pattern for both lysates were observed. The differential immunoreactivity of the 12D10H1E2A and 12D10H1B9 indicated the differential expression pattern of the cognate antigen between the two clonal

populations. However, 2D10H6 mAb did not present any differential binding capacity to either of the cell lysates. GAPDH antibody was used as the loading control in the experiment.

Expression pattern of the unknown antigen identified by confocal microscopy

Confocal microscopy was used to identify the expression of unknown antigens in the fast and slow growing clonal cell populations **(Figure 1b.)**. Cells were stained with the novel mAb 12D10H1E2A or 12D10H1B9, and secondary antibody Alexa-Flour 488 was used. The stained cells were observed under confocal microscope and in both the clonal populations the expression of target antigen was cytoplasmic. The expression pattern of the unknown antigen matched closely with the expression pattern of cytoskeletal protein actin hence, to determine the expression pattern of unknown cytoskeletal protein with respect to actin filaments, the contrasting clonal populations were further stained with 12D10H1B9 mAb, with Alexa-Flour 488 conjugated secondary antibody used against 12D10H1B9 mAb. Actin filaments were stained by using Alexa-Flour 568 conjugated antibody and 4', 6-diamidino-2-phenylindole (DAPI) was used for nuclear staining **(Figure 1c.)**. No overlap in the staining pattern of unknown antigen and actin protein was observed.

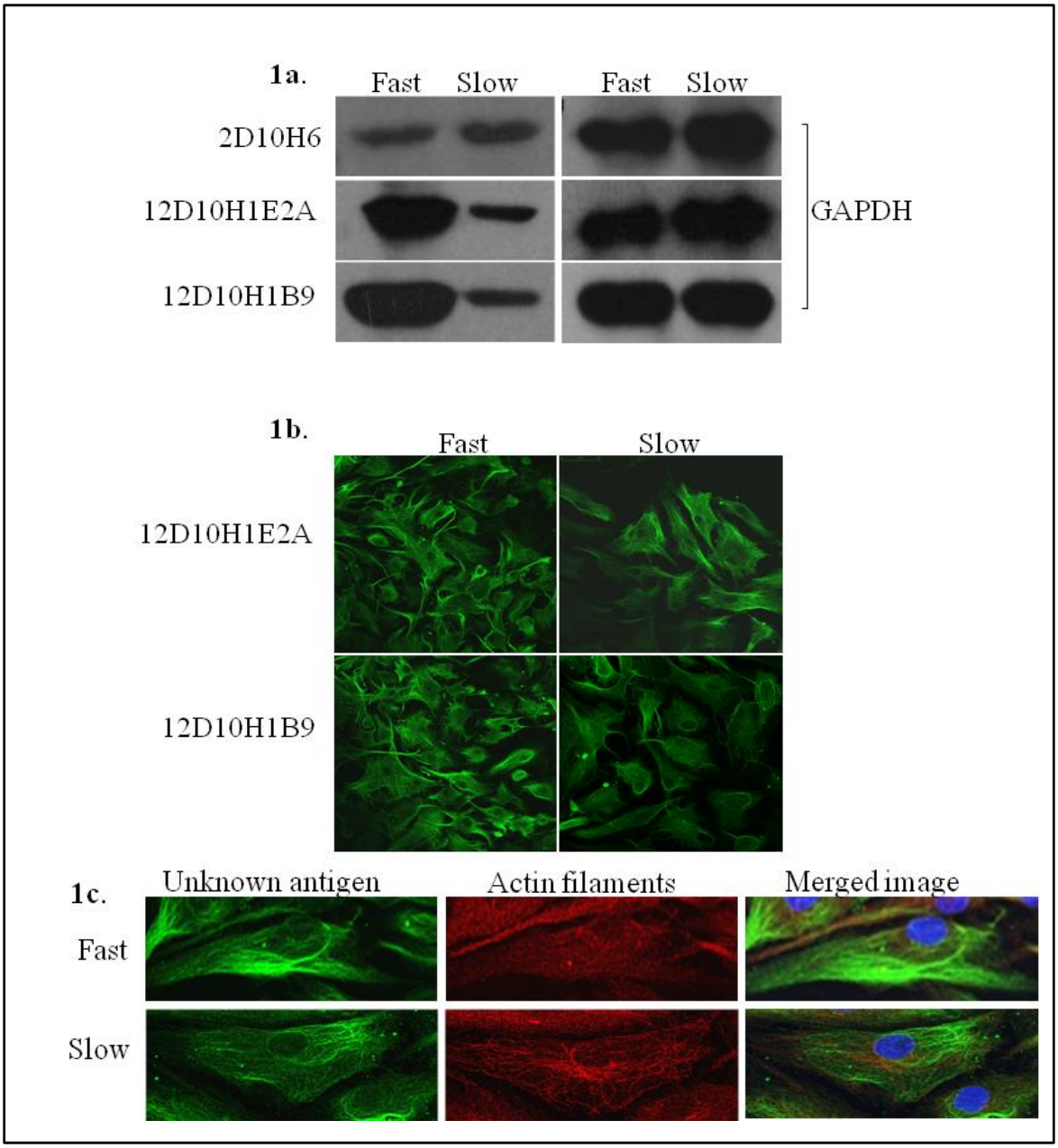

Figure 1: Differential reactivity of novel antibodies. **1a.** Western blot analysis revealed antibodies 12D10H1E2A and 12D10H1B9 showed differential binding capacity to the slow and fast clone cell lysate; whereas antibody 2D10H6 did not illustrate any differential binding capacity, GAPDH was the loading control. **1b.** Confocal microscopy was performed to identify the location of the antigen, cells were stained with primary antibody (12D10H1E2A, 12D10H1B9) and secondary antibody green-fluorescent Alexa Fluor 488-

conjugate was used to highlight the proteins of interest. **1c.** Confocal microscopy analysis of fast and slow growing clonal cells was performed. Cells were stained with primary antibody (12D10H1E2A) and the secondary antibody green-fluorescent Alexa Fluor 488-conjugate was used to highlight the proteins of interest. Phalloidin Alexa flour 568 was used to stain actin the nucleus was stained with DAPI.

Purification of 12D10H1E2A antibody from conditioned hybridoma media

Immobilized Mannan Binding Protein (MBP) combined with an optimized buffer system was used to purify the IgM antibodies from the conditioned hybridoma media as per the experimental procedure. Antibodies were purified from the conditioned hybridoma media, which were then used for immunoprecipitation of the unknown antigen. The dialysed conditioned hybridoma media was subjected to column purification and to validate the purification of the IgM the eluted fractions were separated on 12 % SDS PAGE and the separated proteins were transferred on nitrocellulose membrane. Western blot was performed using HRP conjugated primary goat anti-mouse anti- IgM antibody to highlight purified IgM antibody from hybridoma **(Figure 2a.)**. Bands corresponding to heavy and light chain of immunoglobulin were seen at 50kDa and 25kDa respectively. A band at 150 kDa indicates towards the intact IgM complex which consists of two heavy and two light chains of immunoglobulin. The presence of these bands indicated towards the successful elution of the IgM complex from the conditioned hybridoma media.

Mass spectrometric analysis of the immunoprecipitated antigen

Immunoprecipitation of the unknown antigen was performed on cell lysates from fast and the slow clone cells using the antibodies 12D10H1E2A. The immunoprecipitated proteins were examined on a 12% SDS PAGE gel and the proteins were highlighted with silver stain **(Figure 2b.)**. The highlighted bands were excised and subjected to in-gel tryptic digestion and mass spectroscopy analysis. The amino acid sequence of the proteins was deduced and Basic Local Alignment Search Tool was employed to compare the amino acid sequence deduced, with NCBI database of sequence available. The corresponding sequence presented 97% homology to the intermediate filament protein Vimentin **(Figure 2c.)**.

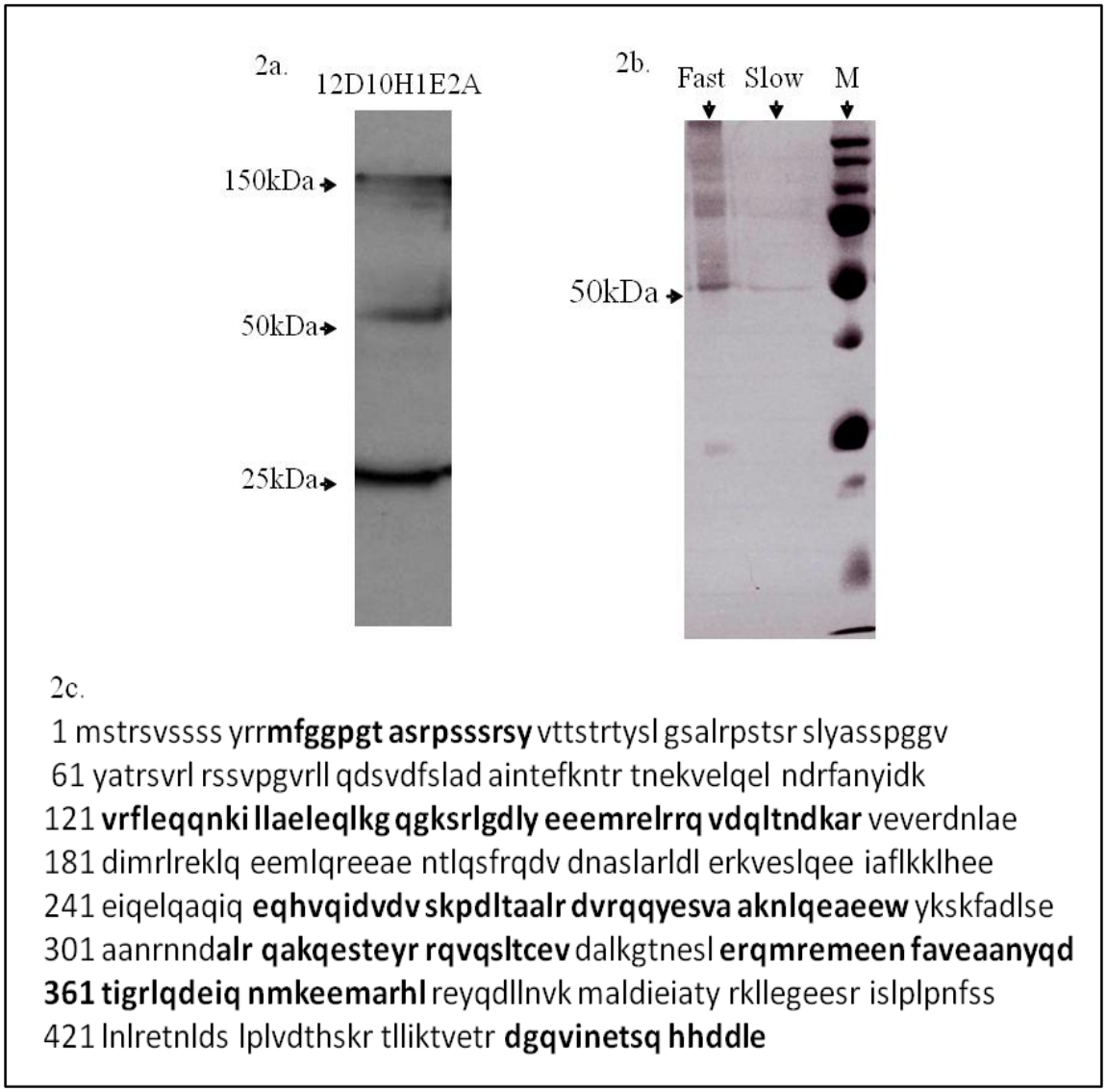

Figure 2: Identification of the unknown antigen. 2a. The conditioned hybridoma media was dialysed and purified using gravity flow method for IgM purification. The fraction with the highest of protein concentration was subjected to western blot analysis; the purified antibodies were detected using primary antibody (goat anti-mouse IgG, IgM and IgA) specific for IgM. Western blot analysis of the eluted fractions shows the presence of heavy and light chain of antibodies at 50kDa and 25kDa respectively. **2b.** Purified antibodies were used to immunoprecipitate of the unknown protein of interest. The eluted proteins were separated from the immobilized antibodies by subsequent elution steps.

Silver stained gel of purified unknown protein complex MW ~50kDa. The immunoprecipitated protein bands were excised from the gel and in gel digestion of the protein sample was performed. **2c.** The mass spectroscopic analysis provided the amino acid sequence of the unknown protein of interest unknown protein was vimentin (amino acid sequence).

Vimentin mRNA expression in contrasting clonal populations

To further validate the results obtained by the mass spectroscopic analysis, the mRNA expression of vimentin in six contrasting clonal populations from three patient samples was performed by qRT-PCR. These results showed greater vimentin expression in the three fast growing clonal populations compared to slow growing clonal populations. Vimentin mRNA expression was approximately three times higher in the fast clones of patient sample #1 compared to slow clones, whereas, in samples #2 and #3 expression levels were significantly higher in fast compared to slow clones. The vimentin expression in slow clone #2 and fast clone #1 were comparable, but this was most likely due to patient to patient variation of the samples **(Figure 3)**. The trend, however, of greater vimentin expression in fast compared to slow clonal populations was the same in all the patient samples.

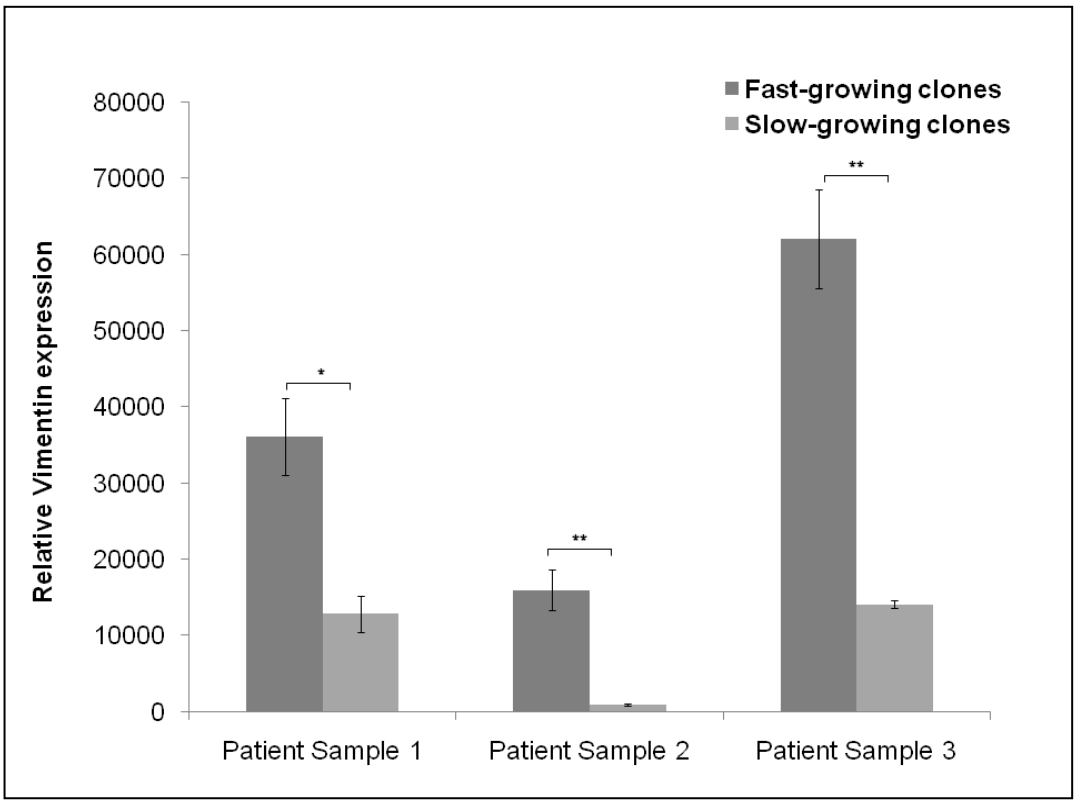

Figure 3: Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis for the validation of the expression of Vimentin was performed on the fast and slow clonal populations at the tenth passage. Student's *t*-test was used to test for difference in the absolute expression levels between fast- and slow-growing clones, and statistical significance was accepted at $p \leq 0.05$ (*). Error bars reflect the mean (±standard error of mean) across three fast-growing versus three slow-growing clones from three patient samples. All of the three fast clone depicted a marked increase in the expression of Vimentin when compared to corresponding slow clonal population.

Discussion

BMSCs are a multipotent cell population and their easy accessibility from bone marrow has broadened their potential use in therapeutic applications. There is a complex interaction of unknown molecular players and diverse signalling mechanism which leads to

BMSCs maturation and proliferation, and a number of studies have been conducted to identify the key molecular components involved in determining BMSCs specific phenotypes.

We employed the method of SI to investigate the molecular basis for this differential behaviour of contrasting clonal populations originating from the same patient sample. SI is a proven technique to produce monoclonal antibodies specific to antigens present in low quantity in a protein mixture, poorly immunogenic and/or similar in sequence or structure to other proteins [18]. The SI approach has been used successfully to yield mAbs that specifically inhibit metastasis, highlight a cell surface phosphorylated glycoprotein antigen and identify differentially expressed proteins in metastatic human tumour cell lines. We used this approach of immuno-suppression / tolerization treatments to generate mAbs specific for differentially expressed antigens in selected clonal populations of BMSCs. In our study the tolerogen was a slow growing, unipotent, senescent and clonally expanded population of BMSCs. A fast growing, tripotent and pre-senescent contrasting clonal population was used as immunogen. A rigorous criterion for the screening of the antibodies was used to identify the novel antibodies which showed differential reactivity between the tolerogen and immunogen. SI in conjugation with the sequential screenings identified the antibodies which showed differential reactivity with the tolerogen and immunogen. The efficacy of the SI approach was demonstrated by the differential reactivity of the antibodies 2D10H6E2A and 12D10H1B9 towards the cell lysate of the fast and slow growing clone cells.

Here, we report the use of mAbs generated by SI, to purify and identify the differentially expressed antigenic determinants in clonal populations. Western blot analysis using the two antibodies highlighted a band at 54kD in fast and slow growing clone cells with a strong difference in the intensity of the band. This differential reactivity confirmed

that SI can be successfully used in the generation of the antibodies against antigens that are significantly up-regulated in contrasting clonal population of primary cells. The cellular distribution of the unknown antigen was determined using fluorescence confocal microscopy. The cells incubated with the antibodies 2D10H1E2A and 12D10H1B9 showed strong cytoplasm staining in fast and slow clone cells. The mAb 12D10H1E2A was employed in immunoprecipitation of the unknown target antigen from the cell lysate of the clone cells. Tryptic digestion of immunoprecipitated protein was performed to obtain peptide sequence information by employing mass spectrometric analysis, interestingly the unknown antigen was found to be the intermediate filament (IF) protein vimentin. Changes in the cytoskeleton are known to play an important role in differentiation, embryogenesis and carcinogenesis [19]. The cytoskeleton, specifically IF, act as signal transducers, relaying information from the extracellular matrix to the nucleus [20]. During differentiation [21], transdifferentiation, morphological development [22] and neoplastic transformation [23], there are dramatic changes in IF expression and organization. Vimentin is a type III IF, and is dynamic in its organization, providing a flexible intracellular scaffold, providing structure to the cytoplasm and enables resistance to stresses externally applied to the cell [24]. In response to various stimuli, vimentin can undergo dissociation and reformation in a cell cycle-dependent manner [24]. Growth factors such as platelet derived growth factors (PDGF), can lead to reorganisation of vimentin IF network through tyrosine phosphorylation [25]. Vimentin is required in the process of normal embryonic and adult wound healing [26], additionally, vimentin is thought to have *in vivo* functional importance in regulating chondrogenic differentiation of MSCs [27] and Blain et al. have demonstrated that an intact vimentin network is necessary for adult chondrocytes homeostasis [28]. An intact vimentin IF network contributes to the maintenance of the chondrocyte phenotype, therefore, an imbalance favouring filament

disassembly can disturb the integrity of the articular cartilage, and may ultimately lead to the development of pathologies such as osteoarthritis [28]. Vimentin has been implicated directly in the regulation of various signalling events [29], phosphorylated vimentin has been found to sequester 14-3-3 proteins, resulting in the failure of association of 14-3-3 with the Raf and Wee1 kinase signalling components [30]. Previous studies have demonstrated that vimentin acts especially as an organiser of proteins, involved in both structural and signalling association [31]. We observed that the vimentin expression was very high in the fast growing clone cells, the expression declined in the slow growing clone cells. The elucidation of vimentin and cytoskeletal dynamics is therefore necessary for understanding the mechanisms by which the cytoskeleton contributes to these fundamental processes [19]. However, it remains to be investigated if the observed differences in the vimentin network of fast clone cell and the slow clone cell are a cause or an effect of changes in phenotypic characteristics of the clonal BMSCs populations. It would be of vital importance to detrmine the role that vimentin plays in stem cell morphogenesis.

PART II

Material and Methods

Stem Cell RT^2 Profiler ™ PCR Array

Stem cell RT^2 Profiler PCR Array system (Super Array, Jomar Bioscience, Kensington,SA, Australia) was used to determine stem cell properties of the fast growing clones compared to the slow growing clones. Total RNA was extracted from clonal populations at passage 6 by Trizol, using the manufacturer's protocol. The RNA was run on a non denaturing 1.5% agarose Tris borate EDTA buffered gel to determine the quality, which was indicated by the integrity of the 18 s and 28 s ribosomal bands. The Super Array RT^2 First Strand Kit was used synthesize template cDNA from 1 µg of total RNA following manufacturer's protocol. cDNA template was then mixed with 2X RT^2 qPCR Master Mix and 25 µL of this cocktail was pippetted into each well of PCR array plate containing pre-dispensed gene-specific primer sets. Each array contained 84 stem cell pathway genes, 5 housekeeping genes, 3 RNA and 3 PCR quality control genes. Each sample was run in triplicate. Reactions were carried out using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Scoresby, VIC, Australia). PCR amplification consisted of two-step cycling program: 10 min denaturizing at 95ºC, 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Analysis of the expression of the panel of genes was performed using the manufacturer's data analysis template and instructions (www.superarray.com=pcrarraydataanalysis.php). Relative expression of the panel of genes was determined by using ΔΔCt method, where ΔΔCt = ΔCt of Fast clones - ΔCt of

Slow clones. The statistical significance between the expression levels of fast and slow growing clones was set at $p \leq 0.05$.

qRT-PCR analysis for senescence associated genes

Total RNA was isolated from clonal populations using Trizol reagent (Invitrogen, VIC, Australia) following the manufacturer's protocol. Quality and concentration of the total RNA samples were assessed by gel electrophoresis and spectrophotometry respectively. First strand cDNA synthesis was carried out in a total volume of 20μL from 1μg of RNA for each sample. Conditions for qRT-PCR were 65°C for 5 minutes, 70°C for 15 minutes and 50°C for 1 hour. Then, 2.5μL of the reaction mixture was incubated with PCR master mix with double-stranded DNA dye SYBR Green I (Applied Biosystem, Queensland, Australia) in a total volume of 25 μL. The primers used for detection are listed in Table 1. The conditions for PCR were as follows: 95^{o}C for 10 min for activation of HotStart DNA polymerase, followed by 40 cycles of denaturation at 95^{o}C for 15 s each, and finally, primer extension at 60^{o}C for 1 min. Quantification of the senescence associated genes p53, p16, TERT, Rb1 and 18s control mRNAs were performed by using an ABI Prism 7000 sequence detection system (Applied Biosystems, CA, US) according to the reported method. Each plate contained 18s housekeeping gene to normalize the PCR array data. All experiments were repeated three times. Raw data were acquired and processed to calculate the threshold cycle (Ct) value and relative gene expression values. △△Ct method was performed to analyze mRNA expressions from BMSCs.

Table 1: List of Primer pairs used for Quantitative Reverse Transcriptase-Polymerase chain reaction analysis

Gene	Primers
p53	Forward: 5'- TGC GTG TGG AGT ATT TGG ATG -3'
	Reverse : 5'- TGG TAC AGT CAG AGC CAA CC -3'
p16	Forward: 5'- CAA CGC ACC GAA TAG TTA CGG TC-3'
	Reverse : 5'- CAG CGT CGT GCA CGG GTC-3'
TERT	Forward: 5'- CGT ACA GGT TTC ACG CAT GTG -3'
	Reverse : 5'- GAG GCC GTG TCA GAG ATG AC-3'
Rb1	Forward: 5'- GGA CCT GCC TCT CGT CAG G -3'
	Reverse : 5'- CCT CCC AAT ACT CCA TCC ACA G -3'
Vimentin	Forward : 5'-ACA CCC TGC AAT CTT TCA GAC A-3'
	Reverse : 5'-GAT TCC ACT TTG CGT TCA AGG T-3'
18s	Forward: 5'-TCG GAA CTG AGG CCA TGA TTA AG-3'
	Reverse : 5'-TCT TCG AAC CTC CGA CTT TCG-3'

Results

Stem cell pathway-specific gene expression profiling

The Super Array Stem cell RT^2 Profiler PCR Array system was used to compare the stemness of fast growing clones with slow growing clones. Expression of 84 stem cell-pathway related genes were analysed by using a defined template developed by the SA Bioscience company following the instruction from the Super Array Web site. Relative expression of the panel of genes was determined by the ΔΔCt method, where ΔΔCt=ΔCt of Fast clones-ΔCt of Slow. A total of 24 differentially expressed genes with a fold change of 1.5 were identified, 17 of these were up-regulated in the fast-growing clones and 7 in the slow-growing clone. Ten of the 17 genes up-regulated in the fast growing clone showed a minimum fold change of 2 ($p \leq 0.05$). The genes up-regulated in the fast growing clone were associated with stem cell maintenance, self-renewal and lineage determination **(Table 2)**. Genes corresponding to three morphogens FGF2, IGF1 and BMP2 were also significantly up-regulated in the fast growing clones. The genes NOTCH1, DLL3 and SOX2, all highlighted by the array, are associated with the neural stem cell renewal and maintenance **(Figure 4.)**. Chondrogenesis associated markers, such as ACAN and COL2A1, were also significantly up-regulated in fast growing clones. Other up-regulated genes such as FOXA2 and CDC2 are known to be associated with endodermal organogenesis and cell-fate determination. Interestingly, nine of the ten genes highlighted in the fast growing clones were associated with repair of skeletal tissue, maintenance of chondrocytes or chondrogenesis in embryonic or postnatal stages, indicating the role played by these fast growing cells in restoring the defects of osteoarthritic tissues.

Table 2: List of differentially expressed genes in contrasting clonal populations identified by gene array analysis and grouped on the basis of their function:

Symbol	Description	p value	Fold Change
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	0.0984	-1.42
ACTC1	Actin, alpha, cardiac muscle 1	0.8618	-1.21
ADAR	Adenosine deaminase, RNA-specific	0.3920	6.09
ACAN	Aggrecan	0.0084	7.14
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	0.3731	-3.48
ALDH2	Aldehyde dehydrogenase 2 family (mitochondrial)	0.3943	1.74
ALPI	Alkaline phosphatase, intestinal	0.4196	2.03
APC	Adenomatosis polyposis coli	0.6850	-1.11
ASCL2	Achaete-scute complex homolog 2 (Drosophila)	0.5827	1.38
AXIN1	Axin 1	0.8080	-1.13
BGLAP	Bone gamma-carboxyglutamate (gla) protein (osteocalcin)	0.5856	1.08
BMP1	Bone morphogenetic protein 1	0.2804	-1.17
BMP2	Bone morphogenetic protein 2	0.0087	4.92
BMP3	Bone morphogenetic protein 3 (osteogenic)	0.1511	1.48
BTRC	Beta-transducin repeat containing	0.8135	1.08
CCNA2	Cyclin A2	0.1628	1.47
CCND1	Cyclin D1	0.3370	-1.66
CCND2	Cyclin D2	0.0662	-2.95
CCNE1	Cyclin E1	0.5506	-1.22
CD3D	CD3d molecule, delta (CD3-TCR complex)	0.9843	1.00
CD4	CD4 molecule	0.3429	-1.39
CD44	CD44 molecule (Indian blood group)	0.0144	-1.59
CD8A	CD8a molecule	0.2116	1.26
CD8B	CD8b molecule	0.2292	1.39
CDC2	Cell division cycle 2, G1 to S and G2 to M	0.0249	2.07
CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	0.9728	-1.01
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.2171	2.39
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	0.9117	1.09
COL1A1	Collagen, type I, alpha 1	0.2534	2.53
COL2A1	Collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	0.0472	5.11
COL9A1	Collagen, type IX, alpha 1	0.0892	1.77
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	0.6160	1.05
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	0.4132	1.87
DHH	Desert hedgehog homolog (Drosophila)	0.0688	1.96
DLL1	Delta-like 1 (Drosophila)	0.5168	1.30
DLL3	Delta-like 3 (Drosophila)	0.0164	4.10
DTX1	Deltex homolog 1 (Drosophila)	0.2945	-2.40
DTX2	Deltex homolog 2 (Drosophila)	0.6146	1.19
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	0.4791	2.59
EP300	E1A binding protein p300	0.4062	1.60
FGF1	Fibroblast growth factor 1 (acidic)	0.1301	-2.40
FGF2	Fibroblast growth factor 2 (basic)	0.0242	4.64
FGF3	Fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homolog)	0.6135	1.73
FGF4	Fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)	0.8801	1.07
FGFR1	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	0.5834	-1.12
FOXA2	Forkhead box A2	0.0465	4.25
FRAT1	Frequently rearranged in advanced T-cell lymphomas	0.5740	1.59
FZD1	Frizzled homolog 1 (Drosophila)	0.1115	-1.32
GCN5L2	GCN5 general control of amino-acid synthesis 5-like 2 (yeast)	0.3568	-1.16
GDF2	Growth differentiation factor 2	0.2605	5.88
GDF3	Growth differentiation factor 3	0.3657	1.27
GJA1	Gap junction protein, alpha 1, 43kDa	0.9011	-1.03
GJB1	Gap junction protein, beta 1, 32kDa	0.8413	-1.07
GJB2	Gap junction protein, beta 2, 26kDa	0.3332	-4.03
HDAC2	Histone deacetylase 2	0.8088	1.04
HSPA9	Heat shock 70kDa protein 9 (mortalin)	0.4364	-29.00
IGF1	Insulin-like growth factor 1 (somatomedin C)	0.0009	2.97
PDX1	Pancreatic and duodenal homeobox 1	0.6464	1.24
ISL1	ISL1 transcription factor, LIM/homeodomain, (islet-1)	0.7604	1.32
JAG1	Jagged 1 (Alagille syndrome)	0.0526	2.23
KRT15	Keratin 15	0.8169	-1.25
MME	Membrane metallo-endopeptidase	0.9258	1.20
MSX1	Msh homeobox 1	0.1607	-4.77

Symbol	Description	p value	Fold change
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	0.5004	-1.73
MYOD1	Myogenic differentiation 1	0.8970	-1.03
MYST1	MYST histone acetyltransferase 1	0.8278	1.08
MYST2	MYST histone acetyltransferase 2	0.5845	1.15
NCAM1	Neural cell adhesion molecule 1	0.9137	1.12
NEURO(	Neurogenin 2	0.2619	2.30
NOTCH1	Notch homolog 1, translocation-associated (Drosophila)	0.0007	2.93
NOTCH2	Notch homolog 2 (Drosophila)	0.3399	1.29
NUMB	Numb homolog (Drosophila)	0.7065	-1.08
OPRS1	Opioid receptor, sigma 1	0.6377	1.05
PARD6A	Par-6 partitioning defective 6 homolog alpha (C. elegans)	0.1020	1.64
PPARD	Peroxisome proliferator-activated receptor delta	0.8742	1.07
PPARG	Peroxisome proliferator-activated receptor gamma	0.9288	1.06
RB1	Retinoblastoma 1 (including osteosarcoma)	0.6460	-1.40
S100B	S100 calcium binding protein B	0.2412	1.39
SOX1	SRY (sex determining region Y)-box 1	0.4377	1.50
SOX2	SRY (sex determining region Y)-box 2	0.0374	3.14
T	T, brachyury homolog (mouse)	0.8397	-1.10
TERT	Telomerase reverse transcriptase	0.0769	1.45
TUBB3	Tubulin, beta 3	0.5526	-2.01
WNT1	Wingless-type MMTV integration site family, member 1	0.4606	-1.66
B2M	Beta-2-microglobulin	0.4679	-1.40
HPRT1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	0.0171	1.34
RPL13A	Ribosomal protein L13a	0.4128	1.28
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.0118	-1.09
ACTB	Actin, beta	0.6083	-1.12

Table 3: List of differentially expressed genes in fast clonal populations identified by gene array analysis and grouped on the basis of their function

Functional grouping of genes	Fast Clones
Cell cycle regulator	Cell division cycle 2
Embryonic cell lineage marker	Forkhead box 2 SRY (sex determining region Y)-box 2
Mesenchymal cell lineage marker	Aggrecan Collagen, type II, alpha 1
Stem cell maintenance	Delta-like 3 Notch homolog 1
Cytokines and growth factor	Fibroblast growth factor 2 (basic) Insulin-like growth factor 1 Bone morphogenetic protein 2

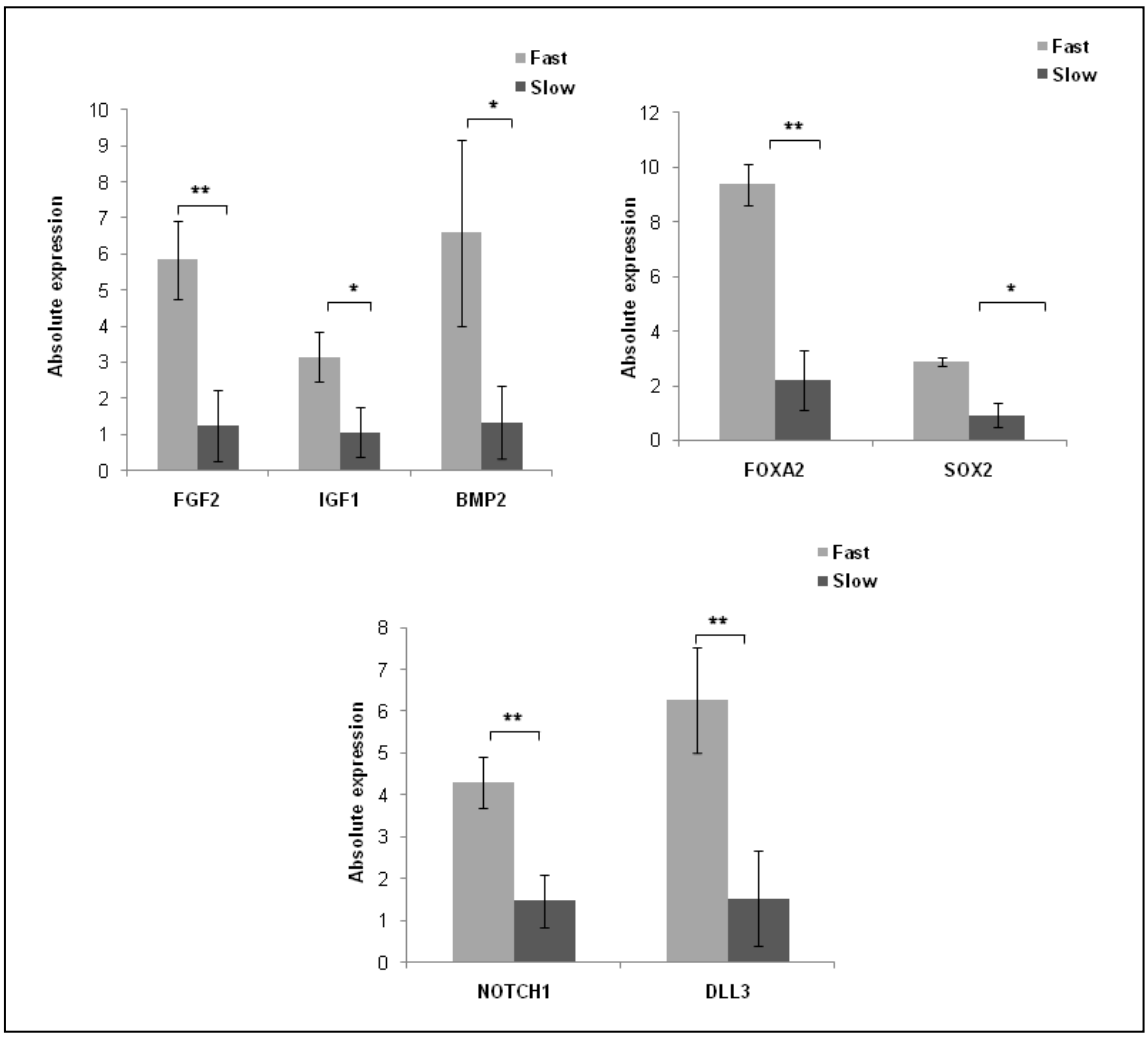

Figure 4: The over-expressed genes transcripts were grouped based on their function in fast-growing clones (n=3) compared with slow-growing clones (n=3). Student's t-test was used to test for difference in the absolute expression levels between fast- and slow-growing clones, and statistical significance was accepted at $p \leq 0.05$ (*). Error bars reflect the mean (±standard error of mean) across three fast-growing versus three slow-growing clones from three patient samples. The genes for growth factors fibroblast growth factor 2 (FGF2), insulin-like growth factor 1 (IGF1) and bone morphogenetic protein 2 (BMP2). Embryonic lineage markers forkhead box a2 (FOXA2) and sex-determining region Y-box 2 (SOX2) were significantly upregulated. Factors for NOTCH signalling pathway delta-like 3 (DLL3), notch homolog 1 (NOTCH 1) were also significantly over represented.

qRT-PCR analysis for senescence associated genes

Expression of cell senescence–related gene expression in three fast and three slow clone populations was determined by qRT-PCR analysis. Slow-growing clone populations presented a predominant expression of p53, Rb1, and p16 **(Figure 5)**. Human telomerase reverse transcriptase (hTERT) was not significantly expressed in both types of cell clones.

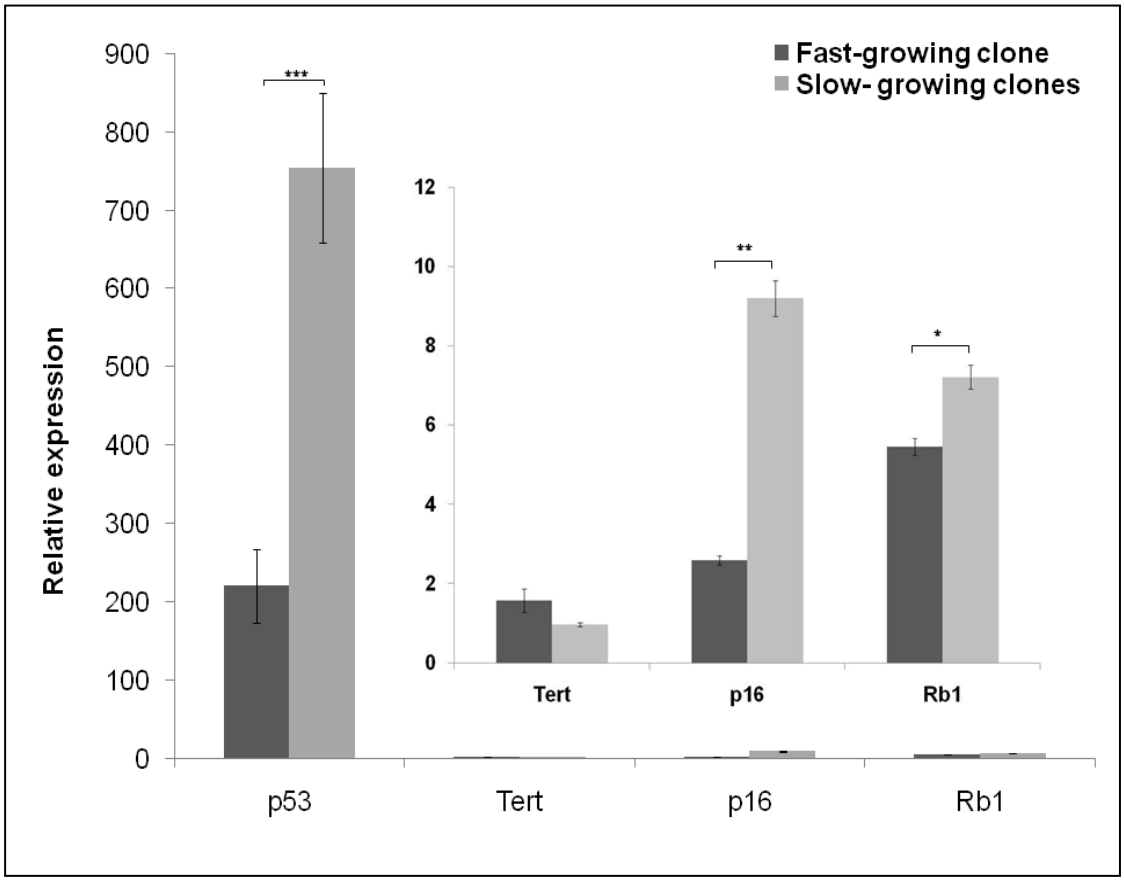

Figure 5: qRT-PCR analysis revealed elevated expression of cell senescence–related genes p53, p16, and Rb1 expression in slow-growing clones. No obvious expression of hTERT genes could be detected in both cell clones. Student's t-test was used to test for difference in the absolute expression levels between fast- and slow-growing clones, and statistical significance was accepted at $p \leq 0.05$ (*).

Discussion

Studies involving DNA microarray-based, genome wide differential gene expression analysis have been performed to gain comprehensive insight into molecular signatures of bone marrow stromal cells [32]. This study has sought to detect gene transcripts potentially unique to BMSCs by using stem cell specific gene arrays.

A panel of 10 genes were found to be significantly up-regulated in the fast growing clonal population with the p value of ≤ 0.05, and these genes were related to the maintenance, self renewal and lineage markers. By contrast, in slow growing clonal population the up-regulated genes were senescence related such as p53, Rb1 and p16. Chondrogenesis associated markers ACAN (aggrecan) and COL2A1 (collagen2A1) were also up-regulated in the fast growing clonal population. DLL3 and NOTCH1 were two genes up-regulated in the fast growing clones, and these are members of the Notch signalling pathway [33] which are associated with stem cells regulation and maintenance in diverse niches [34-36]. DLL3 is important in neurogenesis and skeletal development; mutations in DLL3 lead to developmental and axial skeletal defects [37]. NOTCH signalling pathway is active in hematopoietic stem cells (HSCs) existing in their native microenvironment, contrastingly it is down-regulated with HSCs differentiation [38]. Constitutive expression of active NOTCH1 in hematopoietic progenitors allows the establishment of immortalized cells [38].

The gene transcripts of the growth factors IGF 1, BMP2 and FGF2 were found to be significantly up-regulated in fast growing clonal populations. IGF1 is known to play a pivotal role in central nervous system development; it also promotes differentiation, survival and growth of glial cells [39-41], and is involved with the transformation of mesenchyme into chondrocytes, supporting chondrocyte proliferation [42, 43]. The growth factors IGF1 and BMP2 pivotal roles in skeletal pattern formation and embryonic

development, and also regulate apoptosis, cell differentiation and proliferation [44]. BMP2 has an indispensable role in embryogenesis and organogenesis of many tissue types [45, 46], it is known to play major role in the developmental bone morphogenesis, repair and development of mesenchymal skeletal tissue [47]. Induction of osteogenic media with BMP2 increases calcium deposition and bone nodule formation, and MSCs can be induced *in vitro*, into an osteogenic lineage by a combination of BMP2 and FGF2 [48, 49]. Cultures supplemented with FGF2 increases the longevity of MSCs by selecting cells with longer telomeres [50] and delays their senescence by down regulating TGFβ2 expression [51]; it also, increases the size of colonies formed in clonal conditions by approximately 2.5 times [52]. Previous studies also suggest that FGF2 prolongs the immature uncommitted state of MSCs, thereby maintaining the stemness [51, 52]. Along with FGF2, cell division cycle 2 (CDC2) [53] transcripts were up-regulated in the fast growing clone cells and these factors are known cell cycle regulators. CDC2 expression is restricted to proliferating cells; it regulates the orderly progression of cell from S-phase to mitosis [54]. The favourable proliferation potential of fast growing clonal population may therefore be attributed to the up-regulated expression of the FGF2 and CDC2 genes.

Interestingly, the embryonic lineage markers FOXA2 and Sox2 were also significantly upregulated. FOXA2 (forkhead box a2) is an endothelial lineage marker and master gene in fetal lung development [55]; it is also required in generation of dopamine neurons from embryonic stem cells during foetal development [56]. Sox2 is an embryonic self-renewal marker expressed in ESCs and neural stem cell progenitors; the constitutive expression of Sox2 inhibits neuronal differentiation and maintains the progenitors in an undifferentiated state [34, 45, 57]. It belongs to the group of SRY-related high-mobility-group (HMG) box transcription factors. Sox2 is indispensible for maintaining pluripotency in early embryo and ESCs [58]. The ectopic expression of Sox2 along with

few other transcription factors can effectively reprogram differentiated cells to their embryonic pluripotent state [59, 60]. The gene array study demonstrated that the complex interplay of a number of molecular factors responsible for activation of signalling pathways specific for stem cell properties of BMSCs clones. Also, the expression of embryonic lineage markers may be the potential reason for favourable behaviour of fast growing clonal population.

Conclusion

In conclusion, this part of the study has demonstrated that novel antibodies could specifically recognize and highlight the differentially displayed cytoskeletal protein vimentin in the two populations. Interestingly, a diverse range of signalling pathways regulating stem cell renewal, proliferation, fate determination and maintenance were identified which may be critical for regulating stemness state of fast growing clones. A significantly upregulated Sox2 expression indicates that fast growing clone population may have the capacity to maintain some degree of pluripotency markers. Stem cell behaviour is closely regulated by its niche which consists of both intrinsic and extrinsic cues from the microenvironment. The expression of these factors indicate that the control for activation of stem cells and cues for fate determination remains within its microenvironment. Factors such as extra cellular matrix, cytoskeleton (vimentin) and dynamic factors such as morphogens (FGF2, IGF1, BMP2) dictate the fate determination of BMSCs. Future studies probing the role of critical propluripotency markers may present us with unique markers making it possible to identify *in vivo*, potent fast growing clone population which can be sourced for *ex vivo* expansion.

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CHAPTER 4

EFFECT OF *IN VITRO* CULTURE AND PASSAGING ON EXPRESSION OF MOLECULAR SIGNATURES

Overview

In previous chapter the molecular signatures of contrasting clonal populations were identified by gene array analysis. Amongst all the significantly over represented gene transcripts, most interesting was the presence of embryonic lineage markers Sox2 and FOXA2. The over representation of these markers indicates a possible role of embryo lineage pathways in assigning fast clonal population with favourable features. A recent study has presented evidences that Sox2 along with Oct4 and cMyc/Klf4 can assign pluripotency in somatic cells, indicating Sox2 as a key mediator in regulating the stemness-associated features. We hypothesized that the loss of multipotency and proliferation potential associated with culturing BMSCs *in vitro* might be associated with decline in the expression of these key markers. Hence this experimental section was designed to study the changes in the expression of Sox2 and associated molecular factors, and to determine the effects of *in vitro* culture on their expression pattern.

Abstract

Terminally differentiated somatic cells transfected with Sox2, Oct4, Nanog/Klf4 transcription factors can be reprogrammed genetically to behave as ESCs; these induced somatic cells are termed induced pluripotent stem cells (iPS). Generation of iPS is a major high point in scientific community as it holds the promise for undiminishing source of patient-specific therapeutically potent cells, also because it proposes that cell fate determination is a bidirectional process which may proceed from progenitor cells to the terminally differentiated cells or vice versa under specific trigger. Although it has been demonstrated that reprogramming is not a rare event in nature, spontaneous reprogramming phenomenon in tissue-derived cells and the underlying regulatory mechanisms involved in the process remain poorly understood. Here we investigated the expression of the critical transcriptional factors regulating reprogramming, including Sox2, Oct4 and cMyc in the *in vitro* cultured tissues and culture expanded osteoblasts, chondrocytes and BMSCs at various passages at transcriptional and translational levels. A noticeable change of nuclear to cytoplasmic translocation of stemness related transcription factors was observed in association with extensive passaging of somatic and BMSCs in our study. At transcriptional level the expression for Sox2, Oct4 and cMyc peaked at the third passage for osteoblasts, BMSCs and chondrocytes with the levels subsequently decreasing along passages afterwards, with an exception of cMyc expression in chondrocytes which peaked at passage one and decreased subsequently. For the first time the expression pattern of reprogramming related genes was unveiled in the tissue-derived MSCs and BMSCs during their expansion, which provides new insight in the MSCs culture and expansion in the field of regenerative medicine and stem cell therapies.

Introduction

MSCs are critical for tissue maintenance and regeneration, however during serial subcultivation, MSCs undergo severe stress by enzymatic dispersion and sustain cumulative damages [1]. These cumulative damages are evident by increase in cell size, reduction in proliferation rate and decline in differentiation potential with successive passaging in culture. It has also been observed that competency of MSCs decrease with the age of the donors [2]. MSCs from young donors present spindle morphology in very early cultivations and a gradual loss of these features has been observed over cultivation time [3]. These observations are leading impediment in harvesting sufficient doses of *ex vivo* expanded cells for reparative therapy. We hypothesized that the loss of multipotency and proliferation potential associated with culturing BMSCs *in vitro* might be associated with decline in the expression of key molecular markers associated with pluripotency. A recent breakthrough study successfully identified pluripotency associated markers Oct4, Sox2, cMyc and Klf4, these factors were capable of assigning pluripotent state in somatic cells [4-7]. In the previous chapter we observed over expression of Sox2 in fast clone cell population indicating towards possible activation of associated molecular players Oct4 and cMyc in these populations. Oct4 and Sox2 along with Lin28 and Nanog can also assign pluripotency to human cells [8], indicative of the central role played by Oct4-Sox2 complex, and cMyc often can be left out, albeit with a precipitous drop in efficiency [9, 10]. The reprogrammed somatic cells share biological similarities with ESCs, including self-renewal capacity and pluripotency. Reprogramming somatic cells to behave like ESCs is a significant objective of stem cell therapy as it offers an undiminishing source of patient-specific cells and thus has enormous potential for the treatment of degenerative diseases [11]. The efficiency for the process of generation of iPS is a major limitation only 1 in 1,000 and 1 in 10,000 cells are reprogrammed. Also, use of retroviral vector may lead

to insertional mutagenesis, however, non integrating virus [12, 13], transposon [14] or plasmids [13] can be a potent alternative to retroviruses.

Some somatic cells such as epidermal keratinocytes present a better reprogramming efficiency [15] and efficiency can also be improved by addition of small molecules affecting the epigenetic markers [16]. Indeed, the addition of such molecules allows the reprogramming of neural stem cells, which already express Sox2 and Klf4, to be accomplished by adding only Oct4 [17]. Nonetheless the generation of iPS is highly significant as it proposes that every cell is genetically equipped to undergo nuclear reprogramming and behave as pluripotent cells. Previously in an attempt to achieve pluripotency in somatic cells numerous techniques have been employed [18-20]. The effect of transcription factor in controlling the lineage of cells was explained for the first time in year 1989 by Weintraub, the pioneer work involved the forced expression of MyoD into fibroblast or adipoblast cell lines to direct them towards forming muscle cells [21]. However, until now there are as many questions as discoveries remained unanswered, and little is known about the mechanism and sequence of molecular events accompanying nuclear reprogramming process. Recent studies have identified a stable, partially reprogrammed state in which genes associated with pluripotency as well as those associated with the differentiated fibroblast state were active [22]. Partially and fully reprogrammed states of cells have been studied. In one such study, the location of Nanog, Oct4 and Sox2 binding to chromosomal DNA in fully reprogrammed cells, in partially reprogrammed cells and in ESCs was determined [23]. Consequently it is presumed that the Oct4/Sox2/cMyc pluripotency network is active in ESCs, and can be re-established in somatic fibroblasts through viral transduction of the transcriptional factors. Techniques to reprogram human cells without leaving in the introduced genes [14, 24-26], is a promising advancement and critical for unraveling the mechanism for the generation of iPS.

However, it is possible that expression of key pluripotency players may be associated with change in cellular behaviour with respect to successive passaging, to address this question the present study was designed to elucidate the status of stemness related transcription factors in the *in vitro* cultured tissues and culture expanded BMSCs, osteoblasts and chondrocytes at various passages.

Material and Methods

Isolation and expansion of BMSCs, osteoblasts and chondrocytes

The QUT Human Ethics Committee (QUT 3099H), the Prince Charles Hospital Human Ethics Committee (EC2310) and the Holy Spirit North Side Hospital Human Ethics Committee of Brisbane provided ethics clearance for this research project. Bone marrow, bone and cartilage tissue were obtained from patients undergoing elective knee replacement surgery after informed consent. BMSCs, osteoblasts and chondrocytes were isolated as previously reported. Briefly, the bone marrow samples were centrifuged at 400 g without acceleration or brake for 35 min at 20^0C. Cells located at the interface between the bone marrow sample and lymphoprep were collected and further re-suspended in 1ml of Dulbecco's modified Eagle medium with low glucose (DMEM-LG) (GIBCO, Invitrogen Corporation) supplemented with batch-tested 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT), 10 U/ml penicillin G, and 10 mg/ml streptomycin (GIBCO, Invitrogen Corporation). The cartilage and sponge bone tissues were collected and cultured in 6-well plates in the same culture condition of BMSCs. The cells were incubated at 37^0C in 5% CO_2. The medium was changed every 3 days.

Quantitative Real-time reverse-transcription polymerase chain reaction

Total RNA was isolated from osteoblasts, chondrocytes and BMSCs at passage 1, 3 and 7 by using Trizol reagent (Invitrogen, VIC, Australia) following the manufacturer's protocol. Assessment of the concentration and quality of the total RNA samples were carried out by spectrophotometry and gel electrophoresis. One microgram of each RNA sample was used as template for first strand cDNA synthesis in a total volume of 20μL. Conditions for reverse transcription RT-PCR were 65^0C for 5 minutes, 70^0C for 15 minutes and 50^0C for 1 hour. Then, 2.5μL of the reaction mixture was incubated with PCR master mix containing double-stranded DNA dye SYBR Green I (Applied Biosystem, Queensland, Australia) in a total volume of 25 μL. The primers used for detection were listed in Table 1. The conditions for polymerase chain reaction(PCR) were as follows: 95^0C for 10 min for activation of HotStart DNA polymerase, followed by 40 cycles of denaturation at 95^0C for 15 s each, and finally, primer extension at 60^0C for 1 min. PCR was performed by an ABI Prism 7000 system (Applied Biosystems, CA, US). All experiments were repeated three times. Raw data were acquired and processed to calculate the threshold cycle (Ct) value and relative gene expression values. △△Ct method was performed to analyse the level of mRNA expressions from BMSCs, osteoblasts and chondrocytes at various passages.

Immunohistochemical analysis

Human bone and cartilage tissue were obtained from patients as mentioned above. Immunoflourescence staining was done with deparaffinised and rehydrated paraffin sections. The sections were rinsed in PBS, permeabilized with 0.1% Triton for 20 min, and then incubated with 10% swine serum (Dako, Australia) for 1 hour at room temperature to

block non-specific binding. Sections were then transferred to a humidified chamber and stained with anti-Sox2 (1:100, Santa Cruz Biotechnology, CA USA), cMyc (1:100, Santa Cruz Biotechnology, CA, USA), Oct4 (1:100, Santa Cruz Biotechnology, CA, USA) antibodies overnight at 4ºC. Samples were washed three times in PBS and incubated with fluorochrome labelled secondary antibody (1:150, invitrogen, CA, USA) for 3 hours RT. Following this the sections were thoroughly washed in three changes of PBS for 5 min each, and stained with DAPI (1:1400, Invitrogen, CA, USA), and then mounted for analysis. The images were captured on Axion software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) under a fluorescent microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Immunofluorescent staining of cells

BMSCs at passage 0, 1, 3, and 7, osteoblasts and chondrocytes at passage 0, 1, 3 and 7 were culture in chamber slides (Nunc, NY, USA) until 80% confluence, and fixed with 3% paraformaldehyde for 15 min. The slides were rinsed in PBS three times for 5 min respectively, and then permeabilized with 0.1% Triton for 20 min, and incubated with 10% swine serum (Dako, NSW, Australia) for 1 hour at room temperature to block non-specific binding. Slides were then transferred to a humidified chamber and stained with anti-Sox2 (1:100, Santa Cruz Biotechnology, CA USA), cMyc (1:100, Santa Cruz Biotechnology, CA USA), Oct4 (1:100, Santa Cruz Biotechnology, CA USA) antibodies overnight at 4ºC. Samples were washed three times in PBS and incubated with fluorochrome labelled secondary antibody (1:150, Invitrogen, CA, USA) for 3 hours room temperature. Following this the sections were thoroughly washed in three changes of PBS for 5 min each, and stained with Phalloidin (1:500, Invitrogen, CA, USA) and DAPI (1:1400,

Invitrogen, CA,USA), and then mounted for analysis. The images were captured on Axion software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) under a fluorescent microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

Western Blot

Cell lysates from chondrocytes and osteoblasts in passage 3 and 7 were extracted as per the protocol mentioned in Chapter 3. Protein concentration was determined by bicinchoninic acid protein assay. Equal concentration (30µg) of the total cell lysate was separated on a 12% SDS-PAGE. The gel run and western blot analysis was performed on protocol similar to previous experimental section. Primary antibodies, anti-Sox2 (1:1000, Santa Cruz Biotechnology, CA USA) anti-cMyc (1:1000, Santa Cruz Biotechnology, CA USA), anti-Oct4 (1:1000, Santa Cruz Biotechnology, CA USA) were used to detect the proteins. Goat anti-rabbit IgG-horseradish peroxidase conjugated antibody (diluted in blocking buffer at 1:1000 dilution was used as secondary antibody. Super Signal West Femto Western Blotting Detection Reagents (Thermo Fisher Scientific, VIC, Australia) was used for detection according to the manufacturer's instructions and visualized by exposure to X-ray film (Fujifilm, Stafford, Australia) and developed in a AGFA CP 1000 automatic film processor (AGFA-GEVAERT Limited, Burwood, Australia).

Statistical analysis

All experiments were repeated thrice, and the representative results were presented. The quantitative data were presented as mean standard deviation. One-way analysis of variance (ANOVA) was applied to compare means and post hoc multiple comparison tests

were chosen according to the equality of variances among each compared group. Student t test was used to compare two means (analysis for the expressions of mRNAs). For any hypothesis test, the difference was considered as being of statistical significance at $p \leq 0.05$. The SPSS12.0 software package (SPSS Inc, Chicago, IL) was used for the statistical tests.

Result

Localization and distribution of Sox2, Oct4 and cMyc in the *in vitro* cultured tissues

Immunoflourescence staining for Sox2, cMyc and Oct4 was performed on *in vitro* cultured bone and cartilage tissue, also on culture expanded osteoblasts and in chondrocytes generated from the tissues **(Figure 1a, 1b and 1c)**. No obvious staining for Sox2, cMyc and Oct4 could be observed in the *in vitro* cultured tissue samples. Sox2, Oct4 and cMyc expression was hardly detected in the matrix of tissues. However, *in vitro* expanded osteoblast and chondrocytes demonstrated that strong staining for Sox2, Oct4 and cMyc.

Intense staining of Sox2 was markedly present in the nucleus of the outgrowing osteoblasts lining the surface of the human bone tissue and outgrowing chondrocytes in human cartilage tissue cultured *in vitro* for 4 weeks. No obvious staining of Sox2 was found in human bone tissue and human cartilage tissue without *in vitro* culture.

Intense staining of cMyc was observed in the nucleus of the outgrowing osteoblasts lining the surface of the human bone tissue and chondrocytes in human cartilage tissue *in vitro* cultured for 4 weeks. No staining of cMyc was found in human bone tissue and human cartilage tissue without *in vitro* culture.

Nuclear staining for Oct4 was observed in outgrowing osteoblasts lining the surface of the human bone tissue and human cartilage tissue in-vitro-cultured for 4 weeks. No staining for Oct4 was found in human bone tissue without *in vitro* culture.

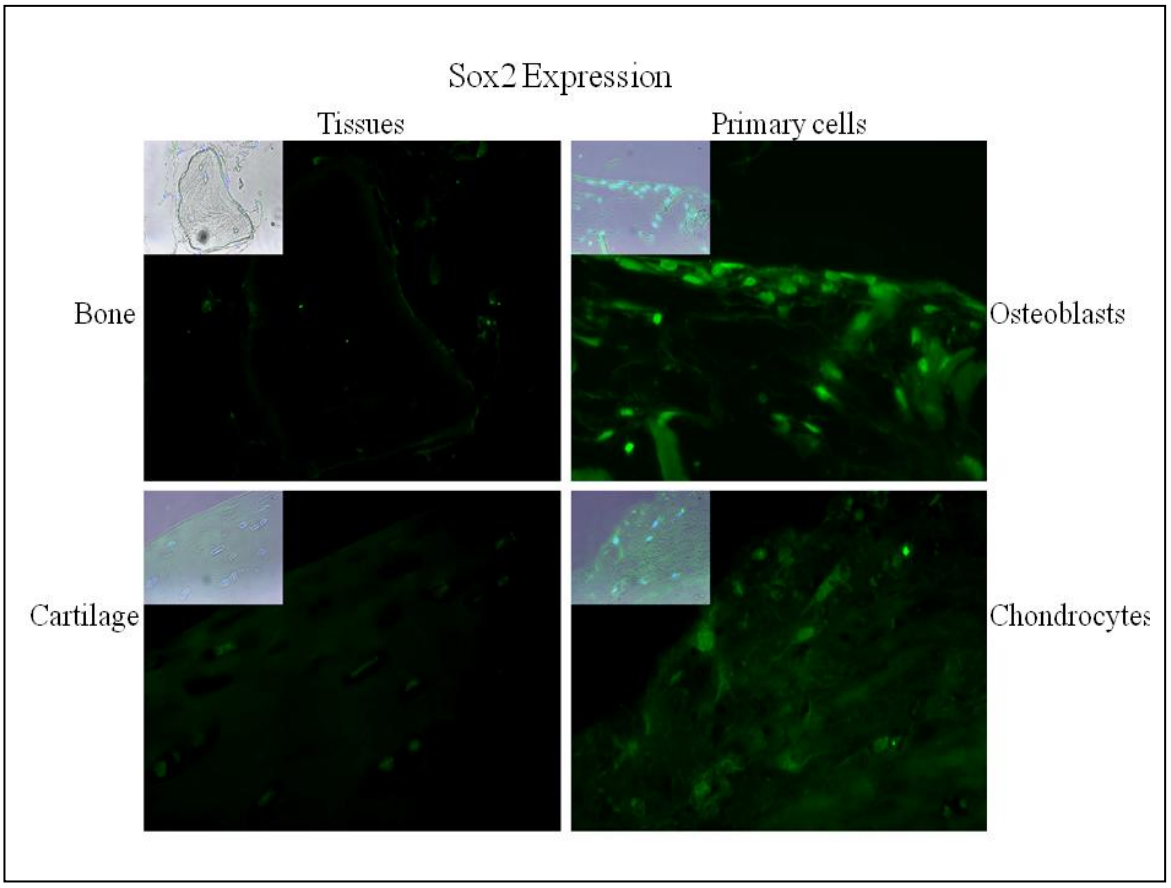

Figure 1a: Expression and distribution of Sox2 in human bone and cartilage cultured *in vitro* for 4 weeks. Intense staining of Sox2 is markedly present in the nucleus of the outgrowing osteoblasts lining the surface of the human bone tissue *in vitro*-cultured for 4 weeks. (400X). Rare staining of Sox2 was found in human bone tissue without *in vitro* culture (400X). Strong staining of Sox2 is detected in the nucleus of the outgrowing chondrocytes in human cartilage tissue *in vitro* cultured for 4 weeks (400X). Rare staining of Sox2 was found in human cartilage tissue without *in vitro* culture (400X), insets fluorescent staining combines bright field.

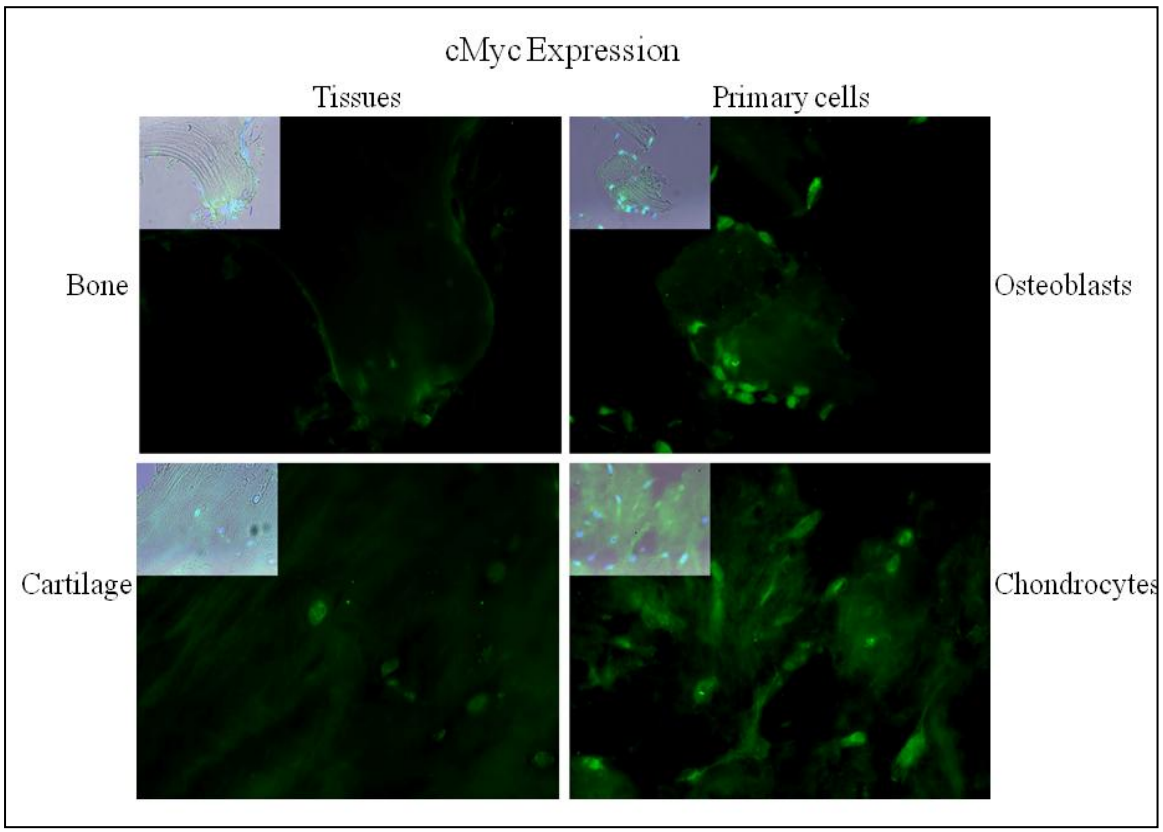

Figure 1b: Expression and distribution of cMyc in human bone and cartilage cultured *in vitro* for 4 weeks. Intense staining of cMyc is markedly present in the nucleus of the outgrowing osteoblasts lining the surface of the human bone tissue cultured *in vitro* for 4 weeks (400X). Rare staining of cMyc was found in human bone tissue without *in vitro* culture (400X). Strong staining of cMyc is detected in the nucleus of the outgrowing chondrocytes in human cartilage tissue cultured *in vitro* for 4 weeks (400X). Rare staining of cMyc was found in human cartilage tissue without *in vitro* culture (400X), insets the fluorescent staining combines bright field.

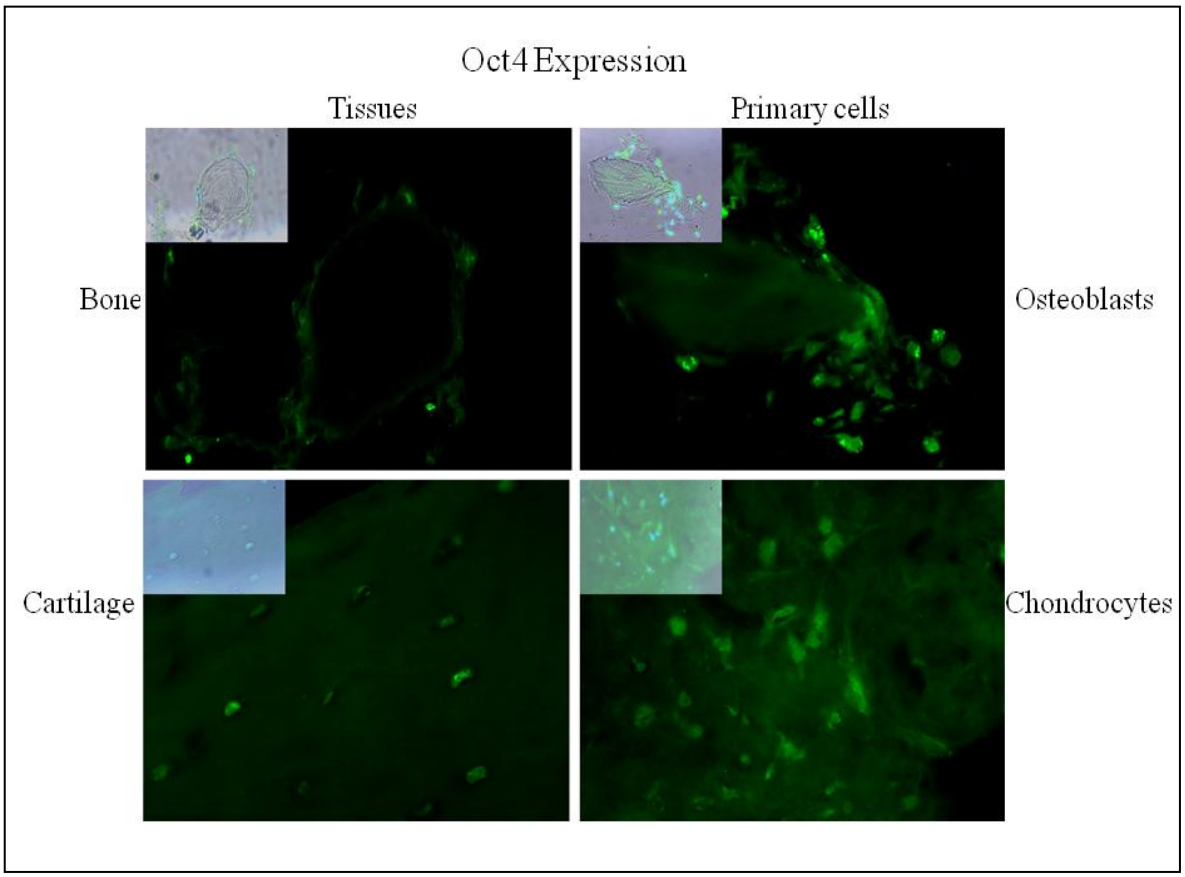

Figure 1c: Expression and distribution of Oct4 in human bone and cartilage cultured *in vitro* for 4 weeks. Intense staining of Oct4 is markedly present in the nucleus of the outgrowing osteoblasts lining the surface of the human bone tissue cultured *in vitro* for 4 weeks (400X). Rare staining of Oct4 was found in human bone tissue without *in vitro* culture (400X). Strong staining of Oct4 is detected in the nucleus of the outgrowing chondrocytes in human cartilage tissue cultured *in vitro* for 4 weeks (400X). Rare staining of Oct4 was found in human cartilage tissue without *in vitro* culture (400X), insets the fluorescent staining combines bright field.

Subcellular localization and expression level of Sox2 in osteoblasts, chondrocytes and BMSCs from various passages

The cellular localization of Sox2 in osteoblast, chondrocytes and BMSCs from various passages was examined by immunoflourescence microscopy. Sox 2 expression was detected in the nucleus of osteoblasts, chondrocytes and BMSCs as early as the primary culture and the first passage **(Figure 2)**, whereas the cytoplasm showed relatively weak expression. With subsequent passaging the nuclear expression for Sox2 was lost and cytoplasmic expression was evident in later passages. Along with the passages, the expression pattern altered as evidenced by the cytoplasmic translocation of Sox2 transcription factor.

The expression level of Sox2 gene transcripts were determined by qRT-PCR analysis **(Figure 2)**. Chondrocytes, osteoblast and BMSCs showed a similar expression trend and the Sox2 expression altered in a passage-dependent manner, with passage one and three retaining the peaked expression and decrease afterwards. The differences of the Sox2 expression in BMSCs, osteoblasts and chondrocytes among various passages were statistically significant ($p \leq 0.05$) for each transcript.

The expression of Sox2 protein was verified by western blot analysis. Equal amount of cell lysates were loaded onto 12% SDS PAGE transferred on to nitrocellulose membrane and probed with anti-Sox2 primary antibody. Tubulin was used for loading control. Bands corresponding to Sox2 proteins could be seen at 40kD. Consistent with the qRT-PCR analysis, the expression of Sox2 was evidently higher in passage 3 of chondrocytes and osteoblasts evident by the band strength, no bands could be observed in passage 7 of osteoblasts and chondrocytes.

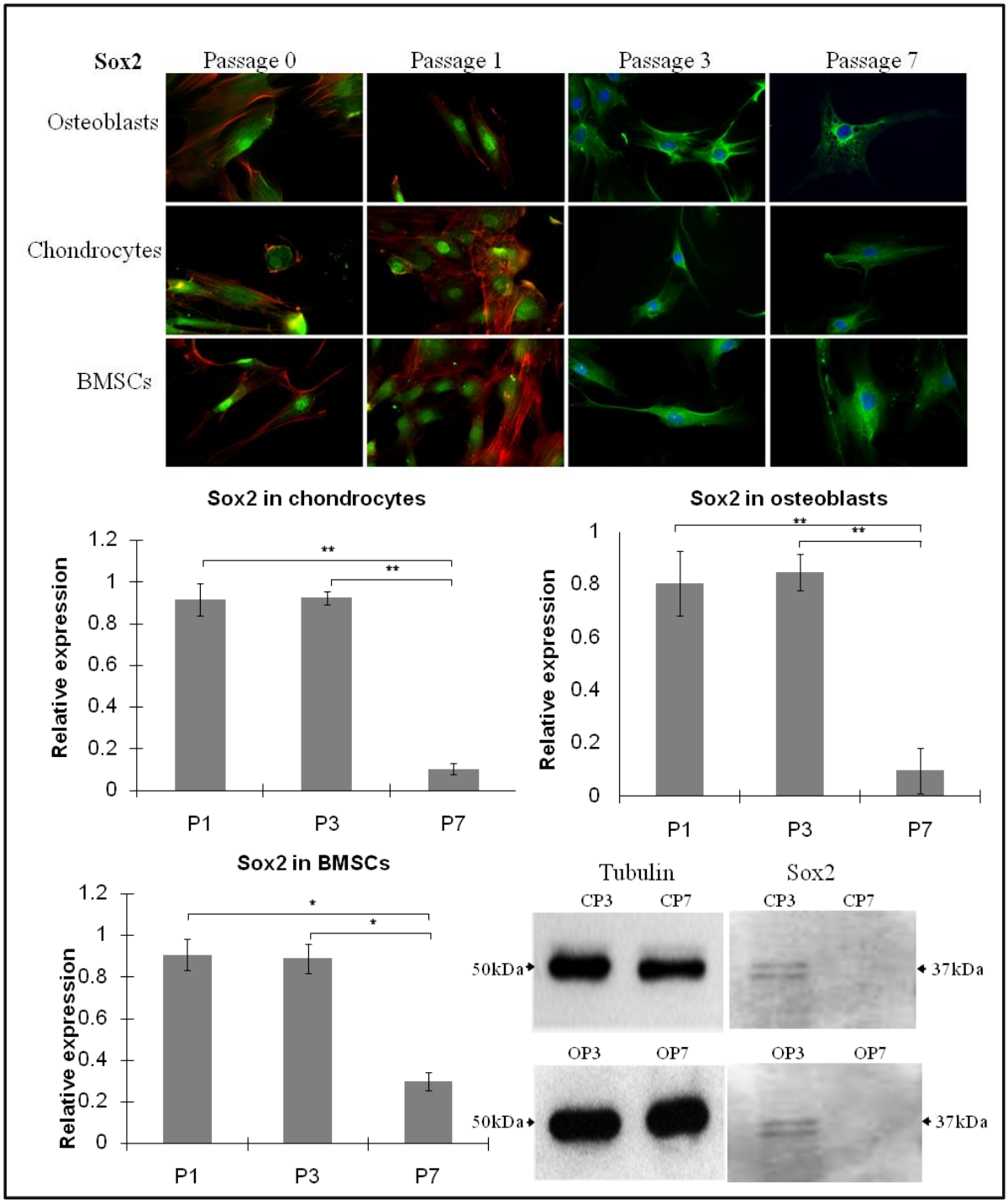

Figure 2: Subcellular localization and expression level of Sox2: Immunoflourescence assay was performed to determine the translocation of Sox2 protein in osteoblast, chondrocytes and BMSCs at various passages. qRT-PCR analysis was performed to quantitate the expression of Sox2 gene transcripts in chondrocytes, osteoblasts and BMSCs, statistical significance was accepted at $p \leq 0.05$ (*). Western blot was performed to

verify the expression of Sox2 in chondrocytes passage 3 and 7 (CP3 and CP7), also in osteoblasts passage 3 and 7 (OP3 and OP7). Bands corresponding to Sox2 were observed at 40kD in CP3 and OB3.

Subcellular localization and expression level of cMyc in osteoblast, chondrocytes and BMSCs from various passages

The cellular localization of cMyc in osteoblast, chondrocytes and BMSCs from various passages was identified by immunoflourescence assay. cMyc expression was detected in the nucleus of osteoblast until passage 3. Nuclear expression for cMyc could be seen only in passage 0 in chondrocytes every passage there on presented cytoplasmic expression. In BMSCs nuclear expression was lost after passage 1 and cytoplasmic location was evident for later passages **(Figure 3)**.

The expression level of cMyc gene transcripts was determined by qRT-PCR analysis **(Figure 3)**. In chondrocytes, cMyc expression was highest in passage 1 and declined sharply in passage 3 and almost no expression was seen in passage 7. In osteoblasts and BMSCs similar expression pattern for cMyc was observed, passage one and three presented higher expression and expression decreases in passage 7. The differences of the cMyc expression in BMSCs, osteoblasts and chondrocytes among various passages were statistically significant ($p \leq 0.05$) for each transcript.

cMyc protein expression of was validated by western blot analysis. Equal amount of cell lysates were loaded onto 12% SDS PAGE transferred on to nitrocellulose membrane and probed with anti-cMyc primary antibody. Tubulin was used for loading control. Bands corresponding to cMyc proteins could be seen at 57kD. Consistent with the qRT-PCR analysis, the expression of cMyc was similar in passage 3 and passage 7 of osteoblasts. No

band corresponding to cMyc protein could be observed in either of the passages for chondrocytes.

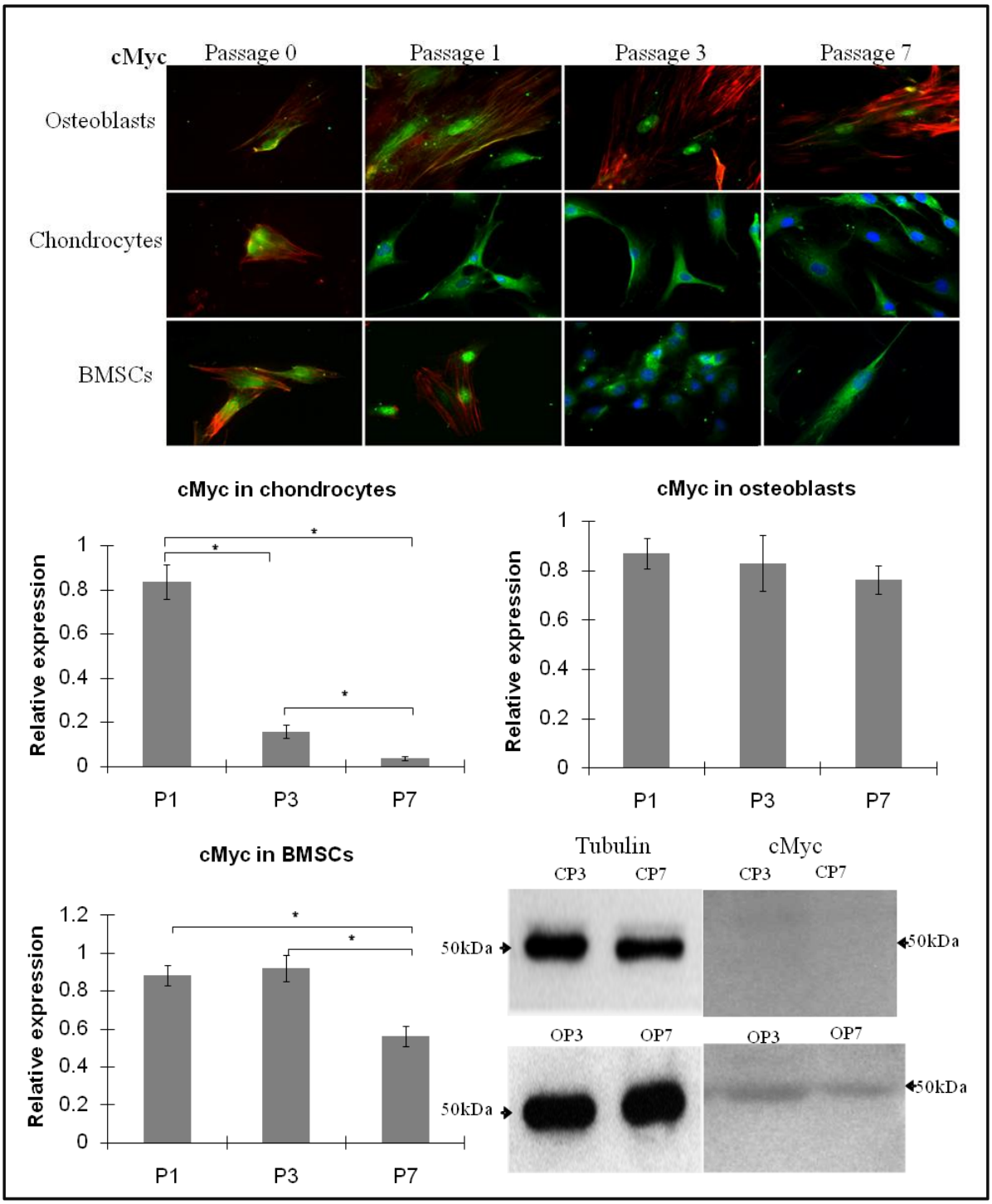

Figure 3: Subcellular localization and expression level of cMyc: Immunoflourescence assay was performed to determine the translocation of cMyc protein in osteoblast, chondrocytes and BMSCs at various passages. qRT-PCR analysis was performed to

quantitate the expression of cMyc gene transcripts, statistical significance was accepted at $p \leq 0.05$ (*) and western blot analysis on chondrocytes and osteoblast passage 3 and 7 was performed to validate the expression of cMyc. No staining could be observed in chondrocytes in passage 3 or 7, bands corresponding to cMyc protein could be observed in passage 3 and 7 of osteoblasts.

Subcellular localization and expression level of Oct4 in osteoblast, chondrocytes and BMSCs from various passages

The cellular localization of Oct4 in osteoblast, chondrocytes and BMSCs from various passages was identified by immunoflourescence assay **(Figure 4)**. Oct4 expression was detected in the nucleus of osteoblast until passage 3. Nuclear expression for Oct4 could be seen only in passage 0 in chondrocytes every passage there on presented cytoplasmic expression. In BMSCs nuclear expression was lost after passage 1 and cytoplasmic location was evident in later passages.

The expression level of Oct4 gene transcripts was determined by qRT-PCR analysis **(Figure 4)**. In chondrocytes, Oct4 expression was highest in passage 1 and declined sharply in passage 3 and almost no expression was seen in passage 7. For osteoblasts Oct4 expression remained same until passage 3 and then decline in passage 7 and in BMSCs expression at passage 1and passage 3 was similar. The differences of the Oct4 expression in BMSCs, osteoblasts and chondrocytes among various passages were statistically significant ($p \leq 0.05$) for each transcript.

Western blot analysis was performed to validate the expression of Oct4 protein. Equal amount of cell lysates were loaded onto 12% SDS PAGE transferred on to nitrocellulose membrane and probed with anti-Oct4 primary antibody. Tubulin was used for loading control. Bands corresponding to Oct4 proteins could be seen at 47kD. Consistent with the qRT-PCR analysis, the expression of Oct4 was evidently higher in

passage 3 of osteoblasts evident by the band strength; faint band for Oct4 could be observed at passage 3 for chondrocytes and no bands could be observed in passage 7 of osteoblasts and chondrocytes.

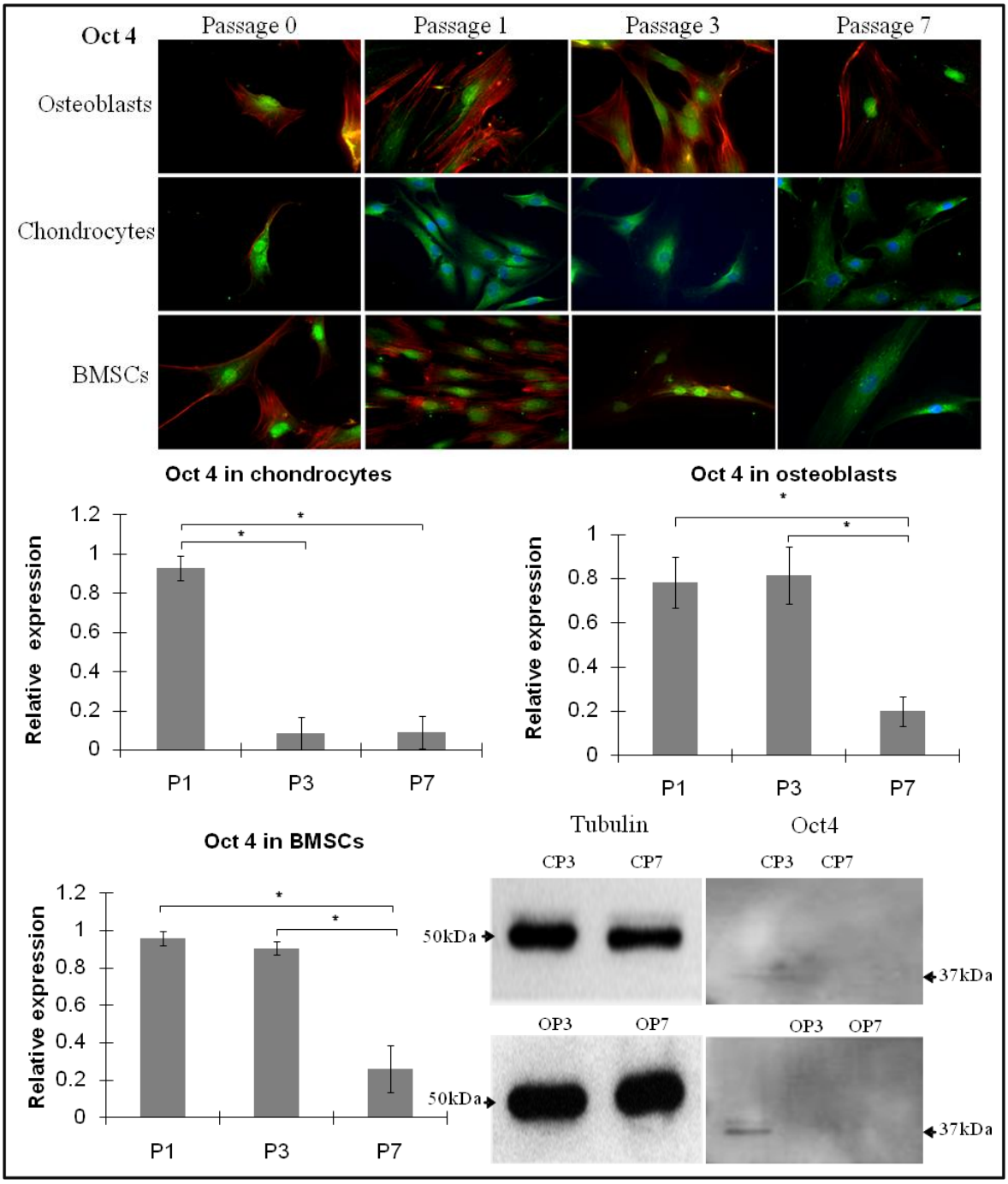

Figure 4: Subcellular localization and expression Oct4: Immunoflourescence assay was performed to determine the translocation of Oct4 protein in human osteoblast,

chondrocytes and BMSCs at various passages. qRT-PCR analysis was performed to quantitate the expression of Oct4 gene transcripts in chondrocytes, osteoblasts and BMSCs, statistical significance was accepted at $p \leq 0.05$ (*). Protein expression was determined by western blot analysis cell lysates for Chondrocytes and osteoblasts at passage 3 and 7 (CP3, CP7 and OP3, OP7) were run on 12% SDS Page transferred on to nitrocellulose membrane and probed with anti-Oct4. Antibody bands corresponding to Oct4 protein could be observed at 47kD.

Discussion

Previous studies regarded ESCs as the ideal source for replacement therapies owing to the unlimited proliferation and pluripotency maintenance capabilities [27]. However, the derivation of ESCs using human embryos involved with ethical considerations and concerns of immunological incompatibility, whereas tissue derived somatic cells can be found in many fully developed organism are immuno privileged and does not involve ethical concerns. BMSCs are potential alternative to ESCs, however there is a marked decline in differentiation and proliferation potential with successive passaging, hence limiting their therapeutic application. Previous chapter presented that molecular factors such as Sox2 and FOXA2 were significantly over-expressed in fast clone BMSCs population. Indicating the role played by embryonic pathways in maintaining competency of BMSCs. Reprogramming leading to pluripotency is a slow and gradual process involving the sequential reactivation of pluripotency and ESCs markers such as Oct4, Sox2, cMyc, SSEA1 and Nanog [5, 28]. In this study we studied the expression of these critical factors in the *in vitro* cultured tissue and expanded cultured osteoblasts, chondrocytes and BMSCs at various passages. The Oct4 protein encoded by octamer-

binding transcription factor 4 gene (Oct4, Oct3/4 and Pou5F1) belongs to a transcription factor family. Since its discovery as an essential regulator of pluripotency in ESCs and iPS cells, Oct4 has become a marker for pluripotency and self-renewal capability maintenance [29]. Oct4 levels in human ESCs would be characteristic of the undifferentiated state, and loss expression of Oct4 result in differentiation and progressive loss of potency, suggesting that Oct4 functions as a critical switch during differentiation by modulating cells with pluripotent potential [30].

Besides Oct4, self renewal and embryonic cell lineage markers Sex determining region Y bos-2 (Sox 2) and V-Myc myelocytomatosis viral oncogene homolog (cMyc), were both reported to play essential roles in the regulation of pluripotency and self-renewal of ESCs and somatic stem cells [31]. Sox2 belongs to the SRY-related HMG box transcription factor family, and exhibits a similar expression pattern to Oct4 in early embryonic development [32]. Knockdown of Sox2 by RNAi causes differentiation to multiple lineages [33]. Other studies indicated Sox2 may down-regulate Wnt target genes such as CCND1 and cMyc through interfere with β-catenin, and inhibit osteoblasts differentiation [34]. Furthermore, Oct4 and Sox2 can interact cooperatively to activate the expression of several pluripotency factors like cMyc, FGF4 and Nanog and regulate pluripotency, implying the top role of the hierarchy that Oct4-Sox2 complex played at the genetic regulatory network in differentiation and reprogramming [35] .

cMyc, belongs to a family of transcription factors containing helix-loop-helix and leucine zipper domains, is a frequent contributor oncogene in tumours. cMyc can activate the Wnt pathway, and operates in a positive regulatory loop to promote breast cancer [36]. Since the promise of reprogramming is the possibility to generate patient-specific pluripotent stem cells for transplantation therapy [11], and reactivation of the cMyc retrovirus can increase tumorigenicity in animals [5], thus this represents a major safety

concern for clinical applications to human being. Hence, some recent studies generated the human iPS cells from adult dermal fibroblasts the absence of this particular oncogenes.

In this study, we investigated the expression of Oct4, Sox2 and cMyc in the *in vitro* cultured tissue and expanded cultured osteoblasts, chondrocytes and BMSCs at various passages. Detection of Oct4 and Sox2 in BMSCs is consistent with previously reported data by Beltrami et.al. and Greco et.al. respectively [37]. Notably, our results demonstrated the expression of Oct4 and Sox2 up-regulated and maintain the nuclear location from primary to certain passages, subsequently down-regulated and lost their nuclear location afterwards. Studies in mouse and human cells indicate that Oct4 is a component of a network of transcription factors, including the homeo box protein NANOG and Sox2 that co-operatively maintain pluripotency in ESCs [38]. Interestingly, Oct4 and Sox2 showed a similar expression pattern during *in vitro* culture, implying the top role of the hierarchy that Oct4-Sox2 complex played at the genetic regulatory network in differentiation and reprogramming [39]. Furthermore, osteoblasts, chondrocytes and BMSCs showed different expression pattern of the three factors, suggesting this process may involve in a cell type specific mechanism.

Importantly, it has been revealed that the human Oct4 show different expression patterns and functional capability to maintain self-renewal and pluripotency. The data generated by our study is consistent with previous studies which revealed that there are two isoforms of oct4 transcription factor, namely Oct4A in nucleus and Oct4B in cytoplasm have been detected in peripheral blood mononuclear cells and the stem cells associated properties were attributed to Oct4A [40]. The splice variant Oct4A is known to sustain stem cell property confers self-renewal and has a nuclear localization. Oct4 along with Sox2 and cMyc mainly localized in the nucleus, and gradually lost their nuclear

localization while being expressed in the cytoplasm of the cell after several passages, indicating the loss of their functional role during this process.

Previous genome-wide studies showed that Sox2 is a direct target of Oct4 in both human and mouse ESCs, whereas others argues that Sox2, in contrast to Oct4, may play an independent role in adult somatic cells reprogramming or even influence the ability of Oct4 to act as activator or repressor [35]. Some recent studies reported that Oct4 expression is not required for mouse somatic stem cell self-renewal [41], which raise the question whether Oct4 is indispensable in somatic stem cell function. From our result of immunoflourescence staining, after several passages of *in vitro* culture, Sox2 firstly translocates from the nucleus to the cytoplasm as early as passage 2 or 3 and, followed by the translocation of Oct4 and cMyc with the same pattern, which happened at passage 3 or even later. Based on these results, we conclude that activation of the transcription factor such as Sox2, Oct4 and cMyc was detected along with *in vitro* culture, then followed by the subsequent decrease with cell passaging, indicating the lost of stemness in concert with several passages. Sox2 is the earliest detectable switch change in cells during this process. Thus, we conclude Sox2, changed prior to Oct4, may have an independent role for the pluripotency maintenance of human tissue derived cells. Therefore, our data revealed a central role of Sox2 instead of Oct4 in maintaining stemness of tissue derived cells during *in vitro* culture.

Little is known about the functional role of these pluripotency markers in adult stem cells it has been shown that knockdown of Oct4 in human BMSCs induces change in cell morphology, decrease growth rate and shifts cells from a cycling to non-cycling stage [42]. Therefore, a similar regulatory mechanism has been suggested for Oct4 in both ESCs and BMSCs. Notably, reactivation of these markers is not yet indicative of a fully reprogrammed phenotype because the surface markers, multi-differentiation capabilities of

the cells at various passages need further confirm. The importance of this finding is revealing the potential possibility that tissue derived somatic cells can achieve pluripotency even without force expression of ESCs markers, which is an important step in identifying a defined medium for culture. The system described here should be useful for providing potent population of somatic cells for generating the large number of iPS for therapeutic and other applications. Numbers of stem cells and their stemness property are tightly regulated by the extrinsic and intrinsic signals from the microenvironment during *in vitro* or *in vivo*. Thus, the major unresolved issue that remains is to identify alternative strategies, such as further modification of the microenvironment and culture system that could activate relevant pathways and thus would maintain the expression of factors required for reprogramming.

Taken together, the present study demonstrated the expression pattern of Sox2, Oct4 and cMyc in the *in vitro* cultured tissue derived somatic cells, which holds much promise in the regenerative medicine and the clinical application of stem cell therapies. To the best of our knowledge, this is the first study demonstrating that expression pattern of stem cell related factors in the *in vitro* culture of tissue derived cells and BMSCs with respect to passaging. However, future studies are required to demonstrate the pluripotency property of tissue derived cells at various passages and the effective way to maintain the stemness property with long term *in vitro* culture.

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CHAPTER 5

EFFECT OF MODULATION OF CULTURE CONDITIONS ON THE MOLECULAR SIGNATURE OF MESENCHYMAL STEM CELLS

Overview

In the previous chapter the effect of *in vitro* culture on Sox2, Oct4 and cMyc expression pattern was identified. It was found that *in vitro* expansion of MSCs resulted in the loss of expression and cytoplasmic translocation of Sox2, cMyc and Oct4 transcription factors. These results suggest the importance of microenvironment for MSCs expansion and the loss of niche results in loss of pluripotency associated markers as evident by the previous experimental sections. It is hypothesised that microenvironment for MSCs expansion may be able to maintain the expression of pluripotent markers in MSCs, which will result better proliferation and differentiation properties of MSCs for cell-based therapy. This experimental section was designed to incorporate important factors of microenvironment into the culture conditions and aimed to establish an ideal culture condition which would maintain BMSCs in a competent state reflected by their proliferation and differentiation potential also by maintaining the expression of Sox2, cMyc and Oct4. A marked decline in the stemness features associated with BMSCs in

current culture conditions presents a major hurdle in developing sufficient *ex vivo* expanded MSCs and this study may provide cues towards overcoming this limitation.

Abstract

The generation of iPS cells by reprogramming terminally differentiated somatic cells holds great promise as a means of producing patient and disease-specific physiologically relevant cells for regenerative therapy. The generation of iPS has stirred much interest in scientific community as this protocol is seen as a potential alternative to ESCs; it also seemingly defies the dogma that cell fate determination is a unidirectional process which proceeds from progenitor cells to the terminally differentiated cells. The procedure for generating iPS involves retroviral transfection of somatic cells with a cocktail of early developmental transcription factors. Retroviral transfection, however, is seen a major impediment to the clinical use of iPS since multiple integration sites in the iPS clone may increase the risk of insertional mutagenesis, making these cells unfit for *in vivo* applications. An interesting approach to the reprogramming study is to modulate cellular niche to maintain high endogenous expression of the reprogramming related genes without resorting to retroviral transfection. Transcription factors such as Sox2 and Oct4 are expressed at high levels in ESCs, they are considered to form a transcriptional regulatory circuitry for pluripotency and self renewal of ESCs [1]. Down-regulation in ESCs of these transcription factors correlates with the loss of pluripotency and self-renewal, and the beginning of subsequent differentiation steps [2]. In the previous chapter, we demonstrate that primary MSCs in their early culture have basal endogenous expression of Sox2, cMyc and Oct3/4, which peaks at the third passage, then decreases with subsequent passages thereafter under current cell culture conditions. The proliferation potential and differentiation capability of MSCs is known to follow the similar trend of property losing

in vitro. Interestingly, we also found that the endogenous expression levels of these critical factors can be retained through later passages by altering the cell culture conditions, i.e. manipulation of culture conditions by coating the culture surface with extracellular matrix protein (ECM) such as fibronectin (FN) and supplementation of media with morphogens such as BMP4 and FGF2; these treatments can significantly up-regulate the expression of these pluripotency related genes. The cellular location of these factors and morphological characteristics of MSCs also appear to be affected by culture conditions. We demonstrate that the *in vitro* proliferation potential of MSCs can be significantly increased by manipulating the culture conditions. Proliferation assays indicated that the proliferation potential of treated cells at passage five was similar to control cells at passage three. An increase of proliferative capacity is of significance since it is necessary to culture an adequate number of *ex vivo* expanded MSCs for patient specific cell based therapies. Our results suggest that it is possible to both stimulate and retain the endogenous expression of pluripotency related genes in MSCs over an extended number of passages by manipulating the cellular niche. To summarize: this study presents a comprehensive insight into the expression pattern of stem cell related transcription factors in MSCs with respect to passaging and change in culture conditions. The results suggest that transient induction and manipulation of endogenous expression of transcription factors critical for stemness can be achieved by modulating the cellular niche and may therefore circumvent the need for genetic manipulations. Future molecular studies involving the effect of these factors on MSCs culture can promote the formulation of ideal culture conditions capable of retaining pluripotency markers for longer duration *in vitro*.

Introduction

Multipotentiality and self renewal capacity of adult MSCs derived from bone marrow and other mesenchymal tissues make such cells a promising source for cellular therapy. In addition, MSCs possess immune-suppressive or immunomodulatory properties which obviate the potential risks of immune rejection and complications associated with allografts. However, their sparse *in vivo* distribution coupled with a marked decline in the multipotentiality and self renewal capability with successive *in vitro* passaging imposes limits to their therapeutic applications. The distinct characteristics of MSCs are not autonomously achieved but are rather attributed to complex signalling interactions that are controlled by the niche in which these cells reside. The interaction between local and systemic cues within the microenvironment determines stem cell fate, leading to self-renewal or differentiation [3]. Cellular microenvironment consists of morphogens (soluble or dynamic factors) and morphostats (static fields). Soluble factors include growth factors, cytokines and chemokines, whereas static factors include cell-cell interaction and extracellular matrix proteins. In the absence of cues for activation, the niche harbour and maintains stem cells in a quiescent state, whereas, under triggers such as stress, injury or disease the niche regulates proliferation or differentiation also, dedifferentiation of progenitor cells as suggested by the previous chapter and others [3]. Pluripotency in ESCs is regulated by transcriptional regulatory circuitry involving transcription factors such Sox2 and Oct3/4, which are considered to be responsible for pluripotency and self renewal of ESCs [4-6]. Interestingly, these factors, along with others such as NANOG and LIN28 [7], cMyc and KLF4 [8] have been used in a recent landmark study to generate induced pluripotent cells (iPS) from lineage committed somatic cells (fibroblasts) [8-10]. The viral transfection of genes encoding these transcription factors was capable of inducing pluripotency in both mouse [8] and human fibroblasts [11]. These studies elucidate the

process of reprogramming is highly conserved and somatic cells under specific conditions will override the developmental program, in effect defying dogma that cell fate determination is a unidirectional process. Numerous studies have shown that Sox2 and Oct4 are indispensible for transforming fibroblasts into iPS [7, 12]. This scientific breakthrough has had major implication for stem cell research since iPS type cells have properties in common with the ESCs "gold standard" in terms of morphology, growth properties, pluripotency and cell surface marker expression. However, no live chimera have as yet been produced, and importantly, the global gene-expression patterns and DNA methylation status of these induced cells show a marked disparity in quality from ESCs [13], indicating towards partial reprogrammed status of somatic cells. Another shortcoming of iPS is the low efficiency (0.02% to 0.002% in human cell) of the procedure for reprogramming somatic cells [11]. Also, the viral transduction of oncogenes such cMyc and KLF4 may integrate into host genome or reactivation of these retrovirus may lead to insertional mutagenesis contributing to tumour formation making iPS unfit for clinical application [12].

An interesting approach would be to produce 'genetically clean' iPS by designing artificial niches and defined culture conditions which can stimulate and maintain endogenous expression of critical transcription factors capable of inducing reprogramming in cells; this would eliminate the need for viral transfection. This objective can be realised by revealing which factors are closely linked with maintenance and self renewal. Interestingly, for generation of iPS from human fibroblasts Yamanaka's group cultured cells in hESCs culture medium supplemented with FGF2 [14]. It is known that continuous exposure to FGF2 is a prerequisite for maintenance of pluripotency in ESCs or iPS [15] and that its absence triggers spontaneous cell differentiation [16]. It has been demonstrated that FGF2 does not act on ESCs but rather plays a critical role in modulating the gene

expression profile of the supporting cells to produce factors such as IGF2, which in turn affect the signalling pathways required for maintenance of a pluripotent state [17].

In addition, BMP4, which inhibits the mitogen-activated protein kinase pathway (MAPK), is known to support ESCs self-renewal and activation of the MAPK pathway leads to differentiation of ESCs, conversely its inhibition prevents ESCs differentiation [18]. Combining BMP4 with leukaemia inhibitory factor increases the self renewal of ESCs and BMP4 also to induction of the inhibition of differentiation (Id) gene which in turn suppresses ESCs differentiation [19]. Furthermore, vimentin, a mesenchymal lineage marker, has been identified to be highly expressed in the fast growing clones of MSCs in the previous study. Cell adhesion and spreading are regulated by complex interactions involving the cytoskeleton and extracellular matrix proteins. It has been reported that MSCs, when expanded *ex vivo* on fibronectin coated plates, could be maintained for more than 50 population doublings without obvious signs of differentiation or senescence [20]. However, the effect of these critical microenvironment factors on the expression and localization of pluripotency associated markers and *ex vivo* proliferation potential of MSCs have not been investigated. This study was therefore designed to investigate BMSCs response to culture medium supplemented with these factors over extensive culture periods at various passages. The effect was determined by measuring cell proliferation and assessing morphological features. In addition to this quantitation at mRNA level of these transcription factors was assessed by Real-time PCR assay and immunoflourescence staining was performed to identify the cellular location of transcriptional factors regulating reprogramming (Oct4, Sox2 and cMyc). These results demonstrate that BMSCs niche can be modulated effectively to direct these potent cells to retain the basal expression and even over express reprogramming factors for extended periods under *ex vivo* conditions.

Materials and methods

Isolation and expansion of BMSCs

Human Ethics Committees of QUT (QUT 3099H), Prince Charles Hospital (EC2310) and Holy Spirit North Side Hospital of Brisbane provided ethics clearance for this project. Bone marrow samples were obtained from patients undergoing elective knee replacement surgery after informed consent. Bone marrow samples were collected in falcon tubes containing 5mL of phosphate buffer saline solution (PBS) supplemented with 200U/mL heparin. This solution was filtered through a 100-mm filter, and mixed with Hanks buffer (Invitrogen Australia Pty Ltd, Victoria, Australia), 15 ml of lymphoprep (Aix-Shield PoC AS, Oslo, Norway) was gently layered (15 ml Lym:30 ml sample) on the solution. Samples were then centrifuged at 400 g without acceleration or brake for 35 min at 20^{o}C. The intermediate layer between the bone marrow sample and lymphoprep was collected and resuspended in 1 mL of Dulbecco's modified Eagle medium with low glucose (DMEM-LG) (GIBCO, Invitrogen Corporation, Melbourne, Auustralia) supplemented with batch-tested 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT), 10 U/ml penicillin G, and 10 mg/ml streptomycin (PS) (GIBCO, Invitrogen Corporation, Melbourne, Australia). Cell suspension was plated on T75 culture flask and cultured at 37^{o}C in 5% (v/v) carbon dioxide. Cells were grown till confluence and passaged for setting up experiments using 0.05% (w/v) trypsin-ethylenediaminetetraacetic acid (EDTA) (GIBCO, Invitrogen Corporation, Melbourne, Australia); at this stage some cells were stored in Liquid nitrogen for future use.

Sample groups

Six different experimental groups were set up and investigated in this study, namely:

- A control group where BMSCs were cultured with DMEM supplemented with 10%FCS and 1% PS.
- In the second group BMSCs were cultured on **FN coated** plates with DMEM supplemented with 10%FCS and 1% PS.
- Third group consisted of BMSCs culture with DMEM supplemented with **2ng/mL FGF2**, 10% FCS and 1% PS.
- In the fourth group BMSCs were cultured on **FN coated** plates and DMEM supplemented with **2ng/mL FGF2**, 10% FCS and 1% PS.
- Fifth group had BMSCs cultured on **FN coated** plates and DMEM supplemented with **10ng/mL BMP4**, 10%FCS and 1% PS.
- Sixth group had BMSCs cultured on **FN coated** plates and DMEM supplemented with **2ng/mL FGF2**, **10ng/mL BMP4**, 10%FCS and 1% PS.

FN Coating procedure: Diluted FN stock 5mg/mL to 5µg/mL using serum free culture medium (DMEM). Aliquots of 1mL working concentrations were stored in 4^{o}C. Added 1ml of diluted FN to the T25 culture flask, incubate the flask at room temp for 1hr. aspirated the remaining material. Rinsed the flask carefully with sterile dH_2O twice avoiding scraping the bottom surface.

Analysis of cell response

BMSCs at passage 1 were trypsinized and an equal number ($1x\ 10^6$) of the cells (after counting cells using haemocytometer) was seeded on the culture plates as per the experimental plan. These cells were grown till confluence, trypsinized for obtaining cells at passage 3 and cells at passage 2 were stored in liquid nitrogen for future use. BMSCs at passage 3 were used to set up experimental groups over several passages; light microscopy

was employed to determine the effect of culture conditions on cell morphology. Still photography via inverted microscopy was used to assess the effect of modulated cultures on BMSCs phenotype; at regular intervals day 3, 5 and 7 the pictures of the experimental groups was taken to determine the phenotypical changes in the cells in response to the treatment.

Cell Proliferation

Proliferation potential of BMSCs grown under culture conditions as mentioned in experimental groups was determined using the CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen Corporation). This assay uses a proprietary green fluorescent dye, CyQUANT® GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Equal numbers of BMSCs at passage 3 were seeded on 96 well plates as per the experimental groups. A reference standard curve was created using BMSCs for converting sample fluorescence values into cell numbers. 11mL of 1XHBSS buffer was prepared by diluting 2.2 mL of 5X HBSS buffer with 8.8 mL deionized water. Prepared 1X dye binding solution by adding 22 µL of CyQUANT® NF dye reagent to 11 mL of 1X HBSS buffer. The culture density was determined using a hematocytometer. Standards and cells were plated on a microplate. These cells were then left over night in incubator for adhesion. The growth medium from cells was removed by gentle aspiration using a manual pipettor. 100 µL of 1X dye binding solution was dispensed into each microwell of the plate. Covered the microplate and incubated at 37°C for 60 minutes. This incubation period is required for equilibration of dye–DNA binding, resulting in a stable fluorescence endpoint. The fluorescence intensity of each sample on day 1, 3 and 5 was measured using a fluorescence microplate reader with excitation at ~485 nm and emission detection at ~530 nm. Similar set up was employed to determine the proliferation potential of passage 5 and 7 BMSCs.

Quantitative Real Time Polymerase Chain Reaction

Total RNA was isolated from BMSCs of treatment groups at passage 3, 5 and 7 by using Trizol reagent (Invitrogen, Melbourne, Australia) following the manufacturer's protocol. Quality and concentration of the total RNA samples were assessed by gel electrophoresis and spectrophotometry respectively. First strand cDNA synthesis was carried out in a total volume of 20μL from 1μg of RNA for each sample. Gene expression assays were used to quantitate the expression of Sox2, c Myc, Oct4 and verify lineage specific differentiation of the BMSCs. To determine the expression of differentiation relevant genes of osteopontin for osteogenic differentiation: aggrecan for chondrogenic differentiation and PPAR2 for adipogenic differentiation. The primers used for detection are listed in Table 1. Detailed procedure for qRT-PCR experimental has been mentioned in previous chapter. The relative gene expression values were calculated by the △△Ct method. All results were generated from triplicate studies.

Immunofluorescent staining of cells

BMSCs at passage 3 and 5 of different treatment groups at were culture in chamber slides (Nunc, NY, US) until 60% confluence. Experimental protocol for immunofluorescent staining mention in Chapter 5 was employed for staining of the cells.

In vitro differentiation of BMSCs populations

Differentiation capabilities of the FN+FGF2+BMP4 treatment group and untreated control group in passage 5 was determined by stimulating cells in selective differentiation media for osteogenic, chondrogenic and adipogenic lineage.

Chondrogenic differentiation

Cells at passage 5 for both groups were differentiated into chondrocytes according to a previously described micro-mass pellet culture [21]. Briefly, cells ($2.5x10^5$) were induced by replacing regular medium with serum-free high glucose DMEM supplemented with 10 ng/ml transforming growth factor-β3, 100 nM dexamethasone, 50 mg/ml ascorbic acid 2-phosphate, 100 mg/ml sodium pyruvate, 40 mg/ml proline, and a commercial preparation of insulin transferrin selenious acid-plus (final concentration: 6.25 mg/ ml insulin, 6.25 mg/ml transferrin, 6.25 mg/ml selenious acid, 5.33 mg linoleic acid, and 1.25 mg/ml bovine serum albumin)(All reagents from Sigma-Aldrich). Cell pellets were formed by centrifuging at 500g for 15 min, these pellets were cultured in chondrogenic medium for 21 days and the medium was changed twice weekly. The pellets were stained with Alcian blue stain for evidence of proteoglycan deposition.

Alcian Blue staining: After 21days, the cell pellets were fixed with 4% PFA and matrix deposition of proteoglycan was detected by staining cells for 15 min with 1% Alcian Blue in 3% acetic acid. Excess stain was washed off with distilled water, samples were air dried, observed under a light microscope and images captured.

Osteogenic differentiation

Osteogenic differentiation was induced on a monolayer of confluent populations with complete media supplemented with 50 mM ascorbic acid, 10 mM b-glycerol phosphate

and 100 nM dexamethasone (Sigma-Aldrich, Castle Hill, NSW, Australia). The cells were cultured for 21 days in this medium, which was change twice weekly. Aggregates or nodules were observed after two weeks, and these nodules progressed to form mineralized bone matrix.

Alizarin red Staining: After 21 days, the cells were fixed with 4% (w/v) paraformaldehyde (PFA) and incubated with Alizarin red stain to observe calcium depositions.

Adipogenic differentiation

Adipogenic induction media was made up of DMEM containing 10% FBS, 0.5 mM isobutylmethyl xanthein, 100 to 200 mM indomethacin, 1 mM dexamethasone, and 10 mg/ml insulin in DMEM with 10% FBS and insulin. Confluent monolayer of cells were grown in adipogenic induction medium, after completion of 3 cycles of adipogenic induction, cells were kept in adipogenic maintenance medium (10 mg/ml insulin in DMEM with 10% FBS) for 21 days and the medium changed twice weekly. Lipid droplets were detected and stained with oil red-O stain.

Oil red-O staining: Cells were washed with PBS and fixed with 4% PFA, and stained with oil red-O stain. The staining solution was prepared by mixing 2 parts of water with 3 parts solution (0.5% in isopropanol) the mixture was filtered 0.4 μm filter. Excess of stain was washed with 60% isopropanol in water. Lipid droplets were detected under light microscope and images were captured.

Statistical analysis

All experiments were repeated three times, and the representative results were presented. The quantitative data were presented as mean standard deviation. One-way analysis of variance (ANOVA) was applied to compare two more means and different kinds of post hoc multiple comparison tests were chosen according to the equality of variances among each compared group. Student's *t*-test was used to compare two means (analysis for the expressions of mRNAs). For any hypothesis test, the difference was considered as being of statistical significance at $p \leq 0.05$. The SPSS12.0 software package (SPSS Inc, Chicago, IL) was used for the statistical tests.

Table 3: RT-PCR primers used in quantiation of SOX2, cMyc, OCT4 and characterize differentiation

Gene	Primers
Sox2	Forward: 5'-CAC TGF CCC TCT CAC ACA TG-3'
	Reverse : 5'-CCC ATT TCC CTC GTT TTT CTT-3'
cMyc	Forward: 5'-CGT CTC CAC ACA TCA GCA CAA-3'
	Reverse : 5'-TCT TGG CAG CAG GAT AGT CCT T-3'
Oct4	Forward: 5'-GCT CGA GAA GGA TGT GGT C-3'
	Reverse : 5'-ATC CTC TCG TTG TGC ATA GTC G-3'
18s	Forward: 5'-TCG GAA CTG AGG CCA TGA TTA AG-3'
	Reverse : 5'-TCT TCG AAC CTC CGA CTT TCG-3'
Osteopontin	Forward: 5'-CTG AGG CTG AGA ATA CCA CAC TT-3'
	Reverse : 5'-GGT GAT GTC CTC GTC TGT A-3'
PPAR2	Forward: 5'-CTG TTG ACT TCT CCA GCA-3'
	Reverse : 5'-GTC AGC GGA CTC TGG A-3'
Aggrecan	Forward: 5'-AGA CTT GGT GGG GTC AG-3'
	Reverse : 5'-GAT GTT TCC CAC TAG TG-3'
Vimentin	Forward : 5'-ACA CCC TGC AAT CTT TCA GAC A-3'
	Reverse : 5'-GAT TCC ACT TTG CGT TCA AGG T-3'

Results

Morphological changes and proliferation rates during culture

BMSCs were cultured in supplemented culture conditions. The morphological changes were assessed by light microscopy over passage 3, 5 and 7 on regular intervals **(Figure 1)**. It showed that BMSCs in all the groups at passage 3 maintained their spindle and fibroblast shape with no observed difference amongst different experimental groups. Similar morphological characteristics were observed at passage 5 with an exception of FN+FGF2+BMP4 group in this group the cells appeared elongated in comparison to the other groups. At passage 7 cells of the control group demonstrated flattened polygonal morphology associated with ageing. Cells in all the other groups retained their fibroblast elongated shape and morphological characteristics lacked any signs of senescence associated phenotype.

The proliferative capacities of experimental groups was analysed using CyQUANT® NF Cell Proliferation Assay Kit **(Figure 2)**. All the groups in passage 3 demonstrated higher proliferation rate than the control group. FN+FGF2 treatment group showed three fold higher potential rate compared to the control group. FN+FGF2+BMP4 treatment group showed two times higher where as other groups showed about 20- 30% higher proliferative rate when compared to control. BMSCs at passage 3 grown on culture media supplemented with BMP4 only presented flattened morphology and the proliferation rate was less than control group indicating a decline in population hence this treatment was not included in future experiments (results not shown). At passage 5, FN+DMEM and FN+FGF2+BMP4 treated groups showed two fold higher proliferation rate, however the proliferation rate of FN+FGF2 treated cells declined significantly from passage 3 and other groups showed higher potential than control. At passage 7, BMSCs grown in FN coated,

FGF2 supplemented and FN+FGF2+BMP4 treated showed two fold increase in proliferation rate compared to control. These results indicated that the proliferation potential of BMSCs were positively affected by treatment conditions, FN+FGF2 treatment group showed marked increase proliferation rate at passage 3 and 7, contrastingly at passage 5 there was a incongruity in this trend. Moreover, all other groups showed a decline in potential with passaging but the proliferation rate was always higher than the untreated control group. Interestingly, FN+FGF2 +BMP4 treatment group indicated nearly consistent two fold higher proliferation potential compared to control samples.

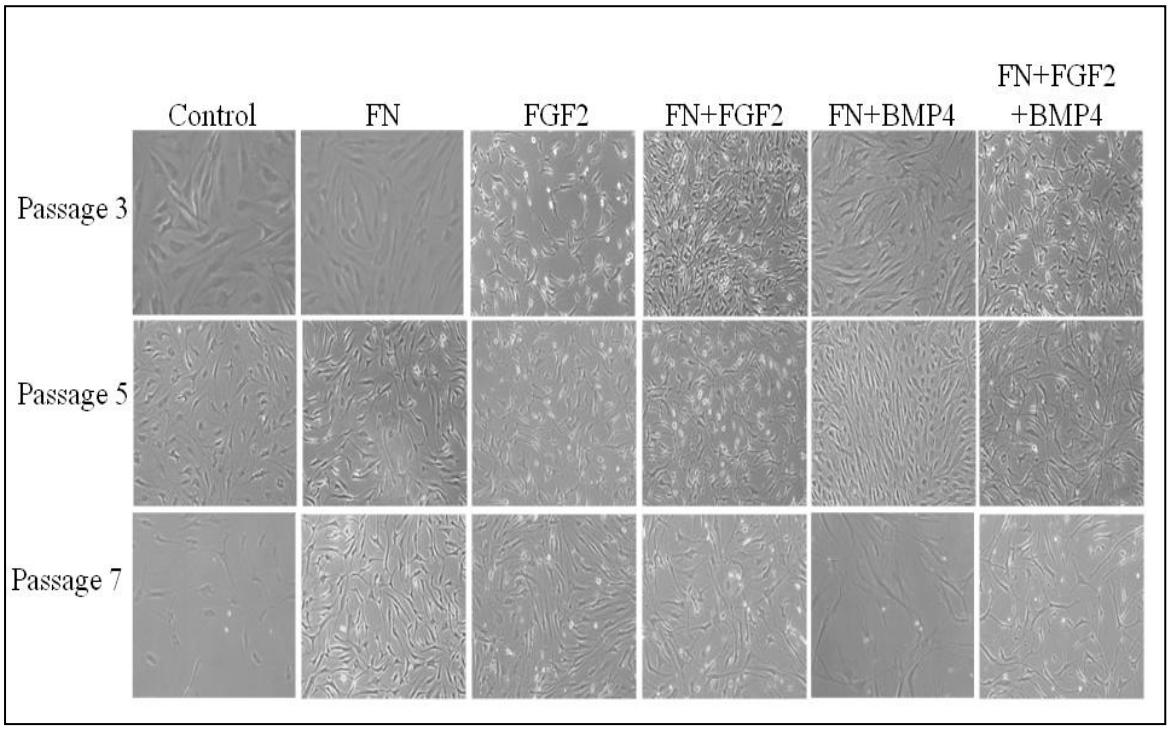

Figure 1: Light microscopy micrographs of BMSCs at passage 3, 5 and 7, after 5 days of culture. Morphological feature of BMSCs grown under different culture conditions were observed at regular intervals by light microscopy at regular intervals.

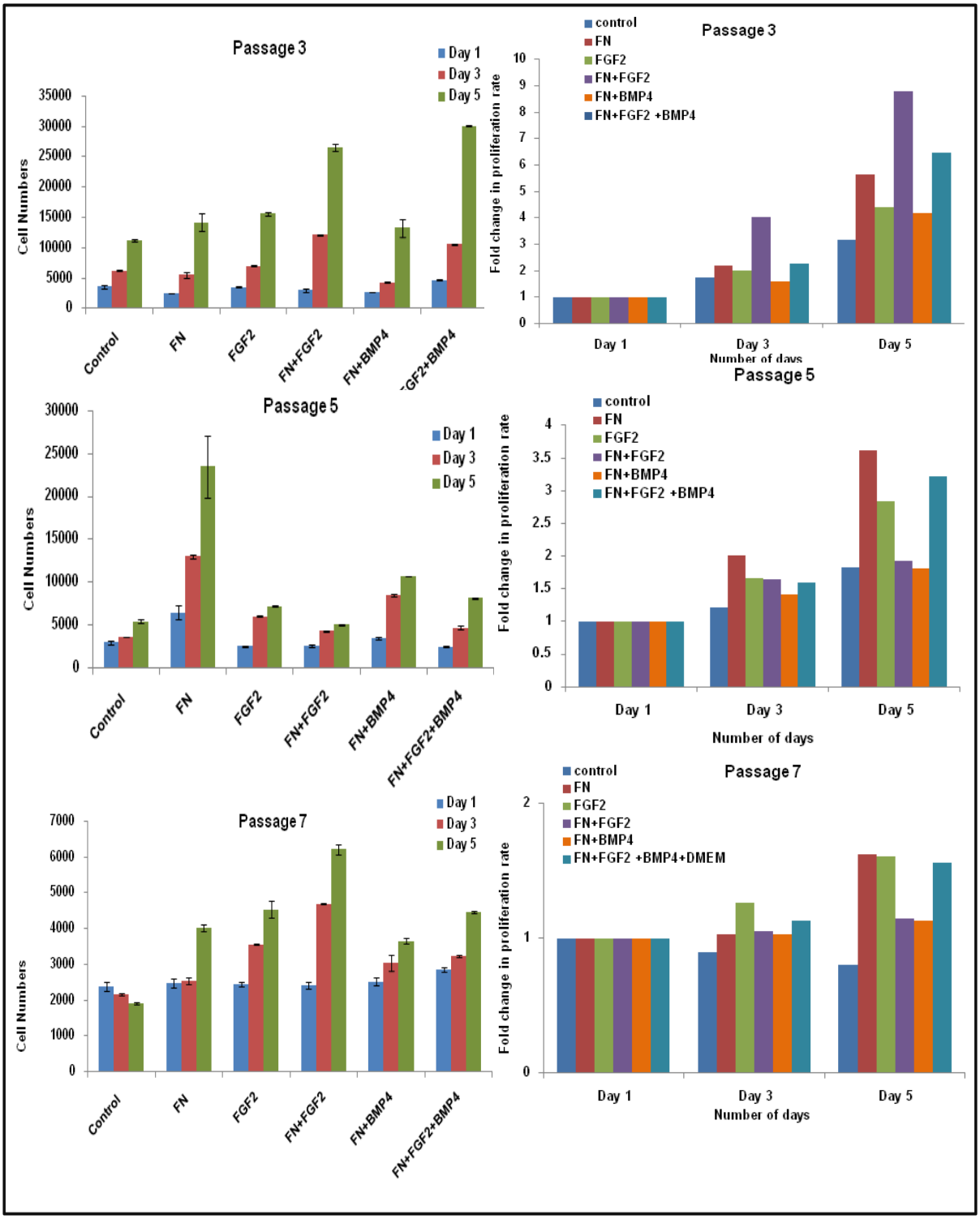

Figure 2: Proliferation potential of BMSCs estimated by CyQUANT assay on the passage 3, 5 and 7 cells. The absorbance values were determined as the average of the results from three experiments. An increased trend of proliferation potential was observed in all treatment groups in every observed passage with respect to untreated control group.

There was a marked decline in proliferation potential in all experimental groups from passage 3 to passage 5 and passage 7. Error bars reflect the mean (±standard error of mean) and statistical significance was accepted at $p \leq 0.05$.

Effect of treatments on Sox2 gene transcripts and protein expression in BMSCs

The transcriptional expression level of stem cell gene transcripts Sox2 in experimental groups and control group in passage 3, 5 and 7 **(Figure 3)** was determined by qRT-PCR analysis. The expression level of gene transcripts in all the groups was compared with the control group. For FGF2, FN+FGF2 and FN+BMP4 treatment group Sox2 gene transcript expression level peaked at passage 3, it gradually declined from there on to passage 5 and the expression was lowest in passage 7. Interestingly, treatments in these groups increased the expression of Sox2 by two fold in passage 3 compared to control group. The expression level of Sox2 in passage 5 in these groups was similar to passage 3 of control group. However, in passage 7 these groups showed a marked decline in the Sox2 expression in comparison to control groups. For BMSCs grown in FN coated plates the expression of Sox2 peaked at passage 5, and became comparable to expression level of Sox2 in passage 3 control group. For FN+FGF2+BMP4 treatment group the expression of Sox2 in passage 3 was similar to control group, however the expression in passage 5 increased twelve folds compared to control in passage 5. Moreover, FN+FGF2+BMP4 treatment group retained the increased expression of transcripts in passage 7. FN+FGF2+BMP4 treatment successfully induces and maintains increased Sox2 transcript expression over passage 5 to passage 7.

Immunoflourescence staining for Sox2 protein was performed and protein detected in cytoplasm of BMSCs in control group in passage 3 and 5; remarkably cytoplasmic expression of human Sox2 was detected in passage 3 and 5 cells in FGF2 and FN+FGF2

groups. In FN+FGF2+BMP4 treatment group Sox2 protein expression presented cytoplasmic localization in passage 3 and in fifth passage nuclear expression was observed **(Figure 4)**, indicating towards nuclear translocation of Sox2 protein.

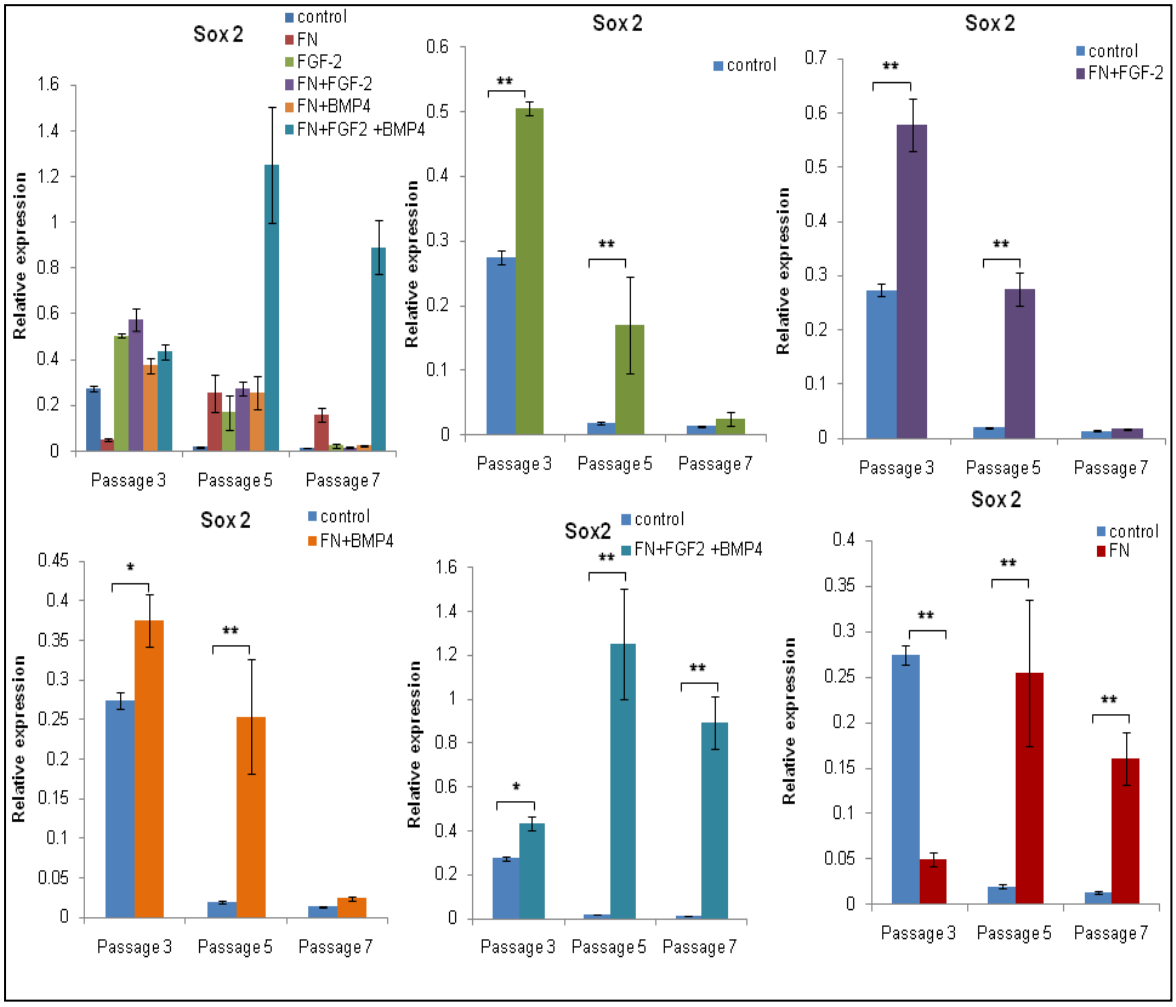

Figure 3: qRT-PCR analysis for quantitation of Sox2 gene transcript expression in control and treated BMSCs samples. All the treatments transiently induced the expression of Sox2 transcript in early passage, however, only FN+FGF2+BMP4 treatment retained induced expression in passage 7. In all other treatment groups transcript expression in passage 5 was comparable with passage 3 in control. Student's t-test was used and statistical significance was accepted at $p \leq 0.05$ (*).

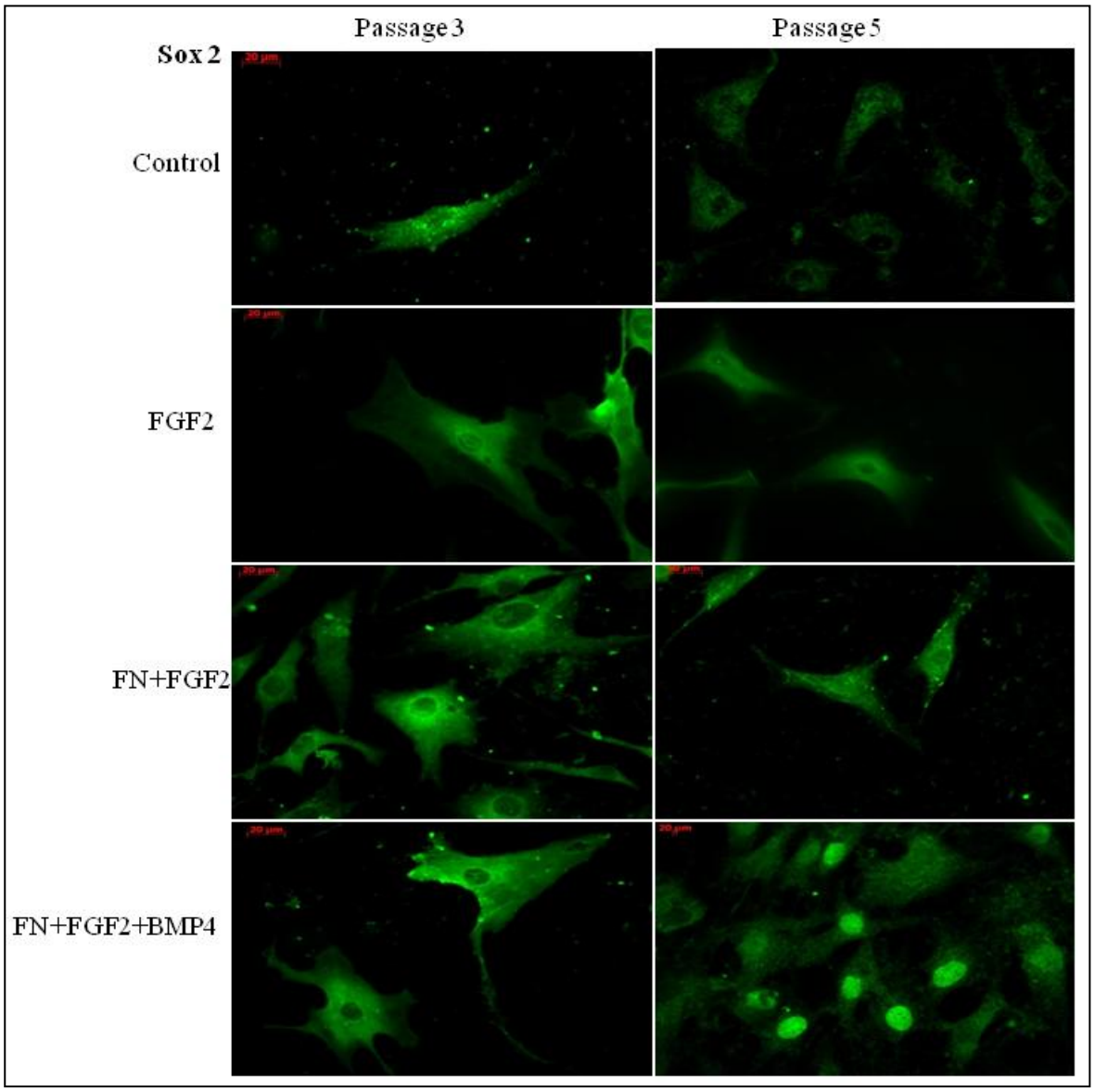

Figure 4: Immunoflourescence assay was performed to observe the expression pattern and translocation of Sox2 protein. Anti-Sox2 primary antibody was detected with Alexa flour 488 (green stain) nuclear expression of Sox2 protein was observed in passage 5 of FN+FGF2+BMP4 treated cells (400X).

Effect of treatments on cMyc gene transcripts and protein expression in BMSCs

cMyc gene transcript expression **(Figure 5)** for FGF2, FN+FGF2 and FN+BMP4 treatment groups gradually increased from passage 3; peaked expression in passage 5 and declined in passage 7. The expression of cMyc transcript in all the tested passages was significantly higher than the control group; however expression in passage 7 was comparable to the control group. For BMSCs cultured on FN coated plates the expression of cMyc gene transcript was similar in passage 3 and passage 5 and slightly increased in passage 7. However, in comparison with the control group the expression was approximately three fold higher in passage 3 and 5. For FN+FGF2+BMP4 treatment group the expression of cMyc peaked in passage 5. Interestingly, for FN+FGF2+BMP4 treatment group the expression of cMyc gene was several fold higher than the control group and the expression level in passage 3 and 5 were comparable, in passage 7 the expression decreased by two fold compared passage 3 and 5, however the expression was about three fold higher than the control groups. In this treatment group expression of cMyc in passage 7 was three fold higher than the control group. This group could effectively retain increased expression of cMyc over extended periods in culture from passage 3 to passage 7.

Immunoflourescence staining was **(Figure 6)** performed to identify localization of cMyc protein in various treatment and control group. In control group cMyc protein was present in the cytoplasm in passage 3 and 5; similar expression was observed for FGF2 and FN+FGF2 treatment groups. However, for FN+FGF2+BMP4 treatment group cMyc protein was presented cytoplasmic location in passage 3 and nuclear expression was observed in passage 5, indicating towards the translocation of cMyc protein from cytoplasm to the nucleus.

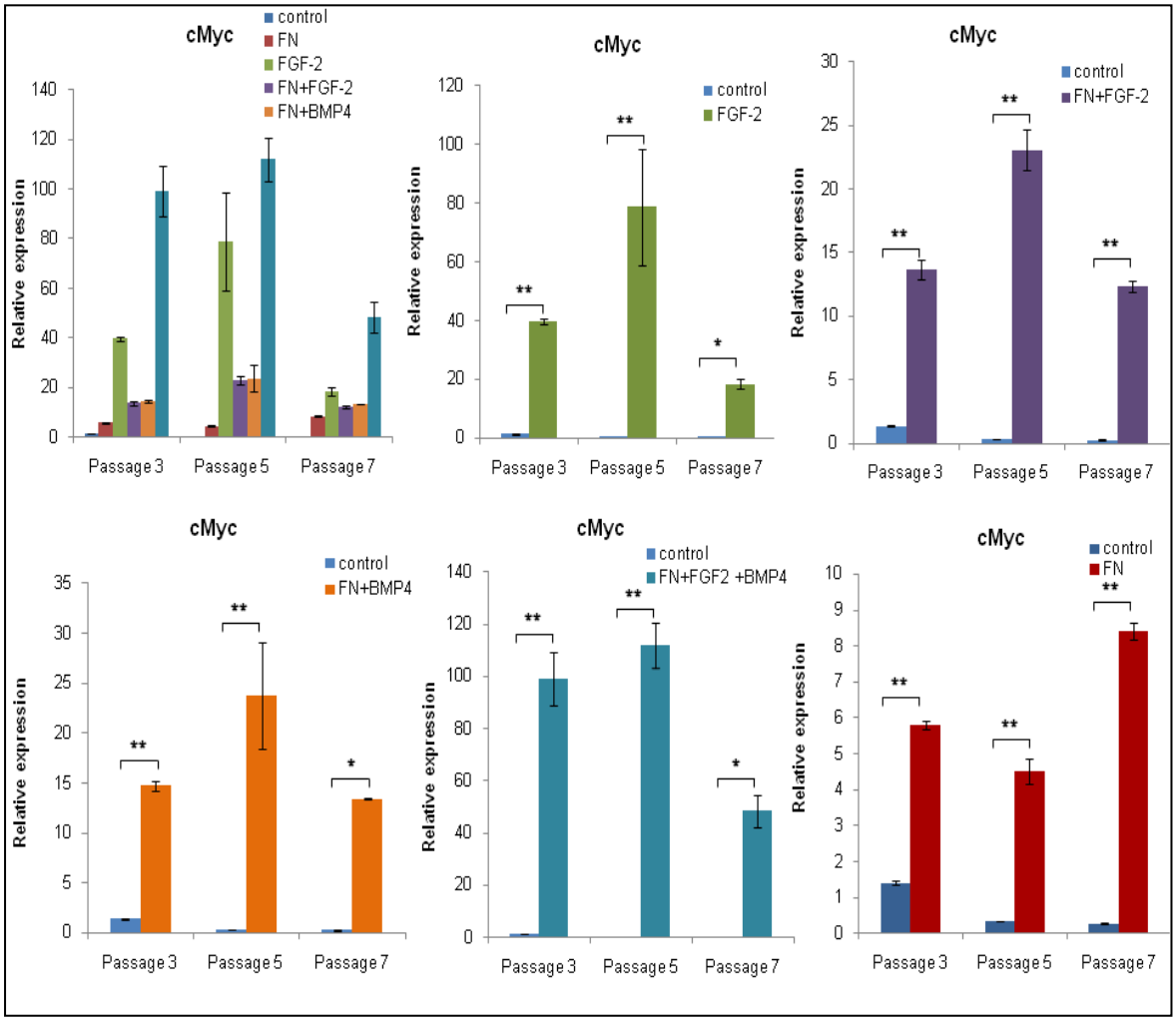

Figure 5: Quantitation of cMyc gene transcripts by qRT-PCR analysis. A comparative analysis of gene expression pattern for cMyc in all experimental groups and control is presented; all treatments significantly induced the expression of cMyc gene transcript. The expression was comparable in passage 5 of FGF2 treated and combinatorial treatment group. Student's t-test was used to test for difference in the absolute expression levels between treated and control groups, and statistical significance was accepted at $p \leq 0.05$ (*). Error bars reflect the mean (±standard error of mean) across three treatment group cells versus three control group cells from three patient samples.

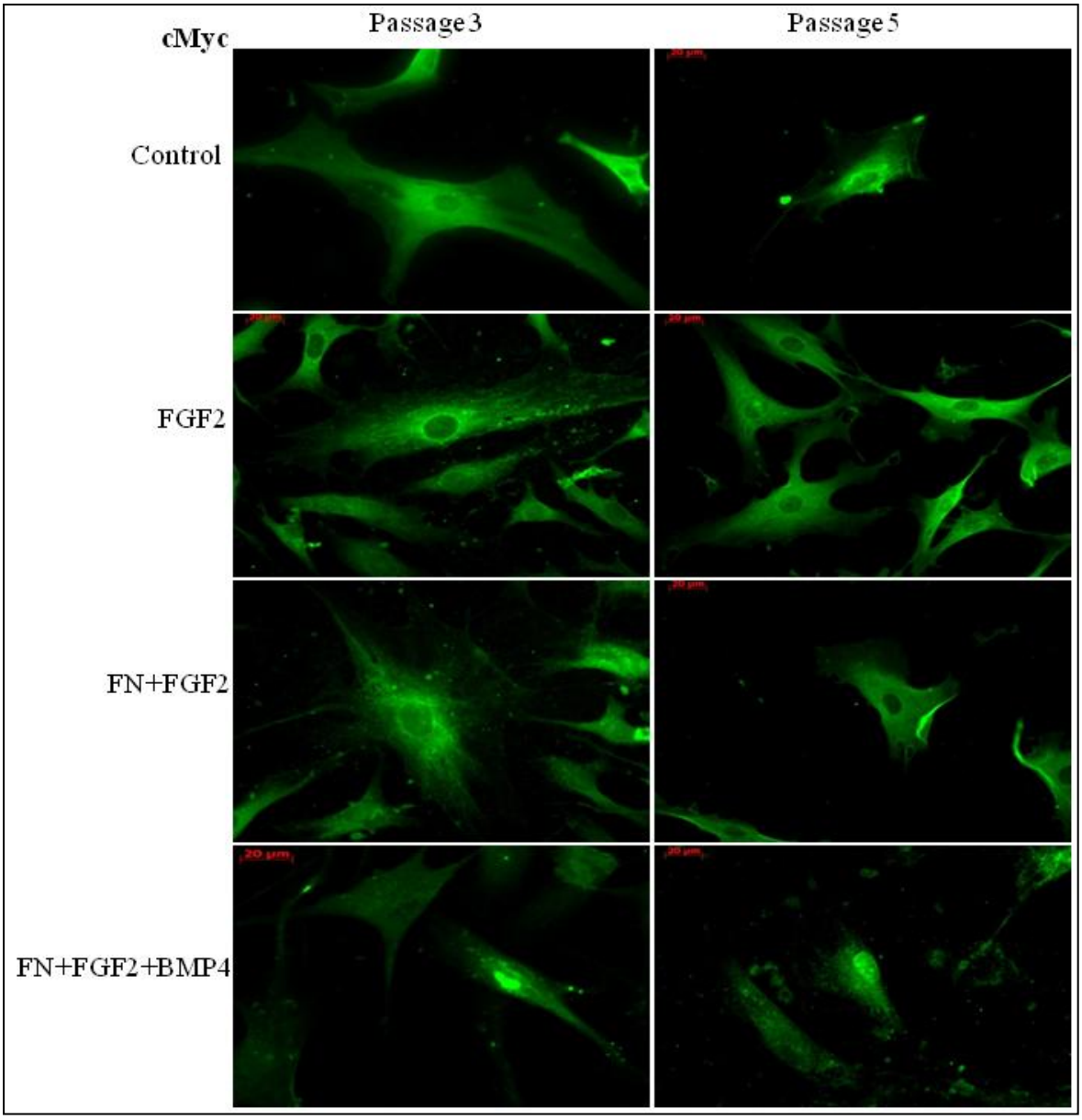

Figure 6: Immunoflourescence results for expression pattern and translocation of cMyc protein. Anti-cMyc primary antibody was detected with Alexa flour 488 (green stain) secondary antibody, nuclear expression was observed in passage 3 and 5 of FN+FGF2+BMP4 treated cells (400X).

Oct4 gene transcripts and protein expression in BMSCs

Oct4 transcript expression **(Figure 7)** in FGF2, FN+FGF2 and FN+BMP4 treatment groups peaked at passage 3, it gradually declined from there on to passage 5 and the expression was lowest in passage 7. Interestingly, treatments in these groups the increased the expression of Oct4 by seven, six and four fold respectively, in passage 3 compared to control group. The expression level of Oct4 in passage 5 in these two treatment group was twofold higher than passage 3 of control group. However, in passage 7 these two treated groups showed one fold decline in the Oct4 expression in comparison to control groups. For BMSCs grown in FN coated plates the expression of Oct4 peaked at passage 3 and 7, and declined in passage 5. Treatment increased the expression level of Oct4 in passage 3 by four fold compared to control group, however in passage 5 the expression of Oct4 was higher in control than the treated group, in passage 7 the expression was comparable in control and treated group. For FN+FGF2+BMP4 treatment group the expression of Oct4 peaked in passage 5, the expression was four fold higher in comparison to the control group. In passage 3 Oct4 expression was six fold higher in FN+FGF2+BMP4 treatment group compared to untreated group. In passage 7, Oct4 expression was 3 fold higher in FN+FGF2+BMP4 treatment group. Moreover, FN+FGF2+BMP4 treatment group retained the increased expression of transcripts from passage 5 to passage 7.

Immunoflourescence assay **(Figure 8)** revealed a cytoplasmic location of Oct4 protein in passage 3 and 5 in control; FGF2 and FN+FGF2 treated samples. In FN+FGF2+BMP4 treatment group cytoplasmic to nuclear translocation of Oct4 protein could be observed from passage 3 to passage 5, however, only a few cells could demonstrate this staining pattern.

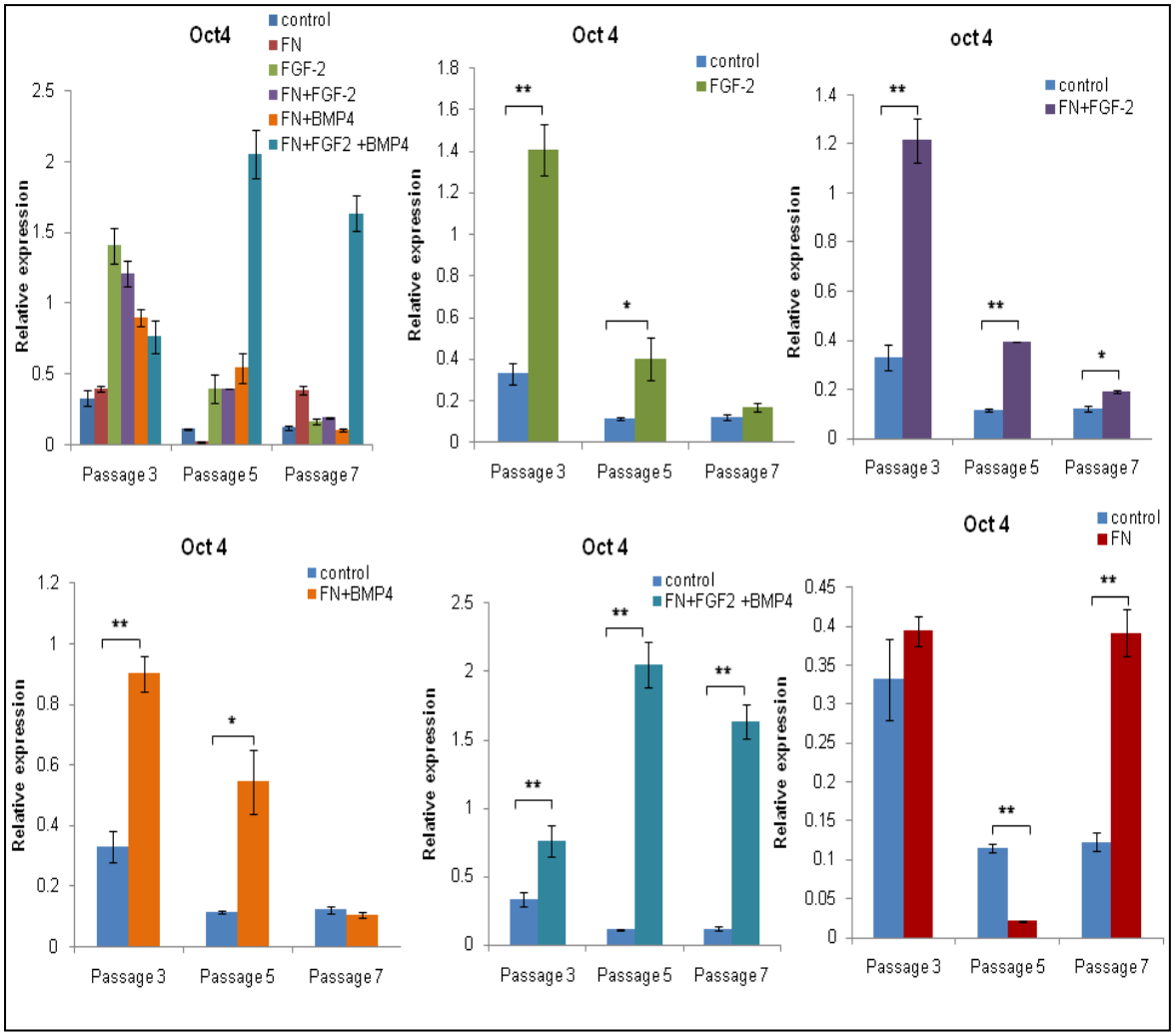

Figure 7: Quantitation of Oct4 gene transcripts expression in BMSCs. qRT-PCR analysis was performed to quantitate the expression of Oct4 gene transcript. A comparative analysis of gene expression pattern for cMyc in all experimental groups and control is presented. Oct4 declined with passaging in the control and all the treatment groups with an exception in FN coating group expression declined in passage 5 and increased in passage 7. In FN+BMP4+FGF2 treatment group the expression of Oct4 transcript was similar in passage 5 and 7. Student's t-test was used to test for difference in the absolute expression levels between treated and control cells, and statistical significance was accepted at $p \leq 0.05$ (*).

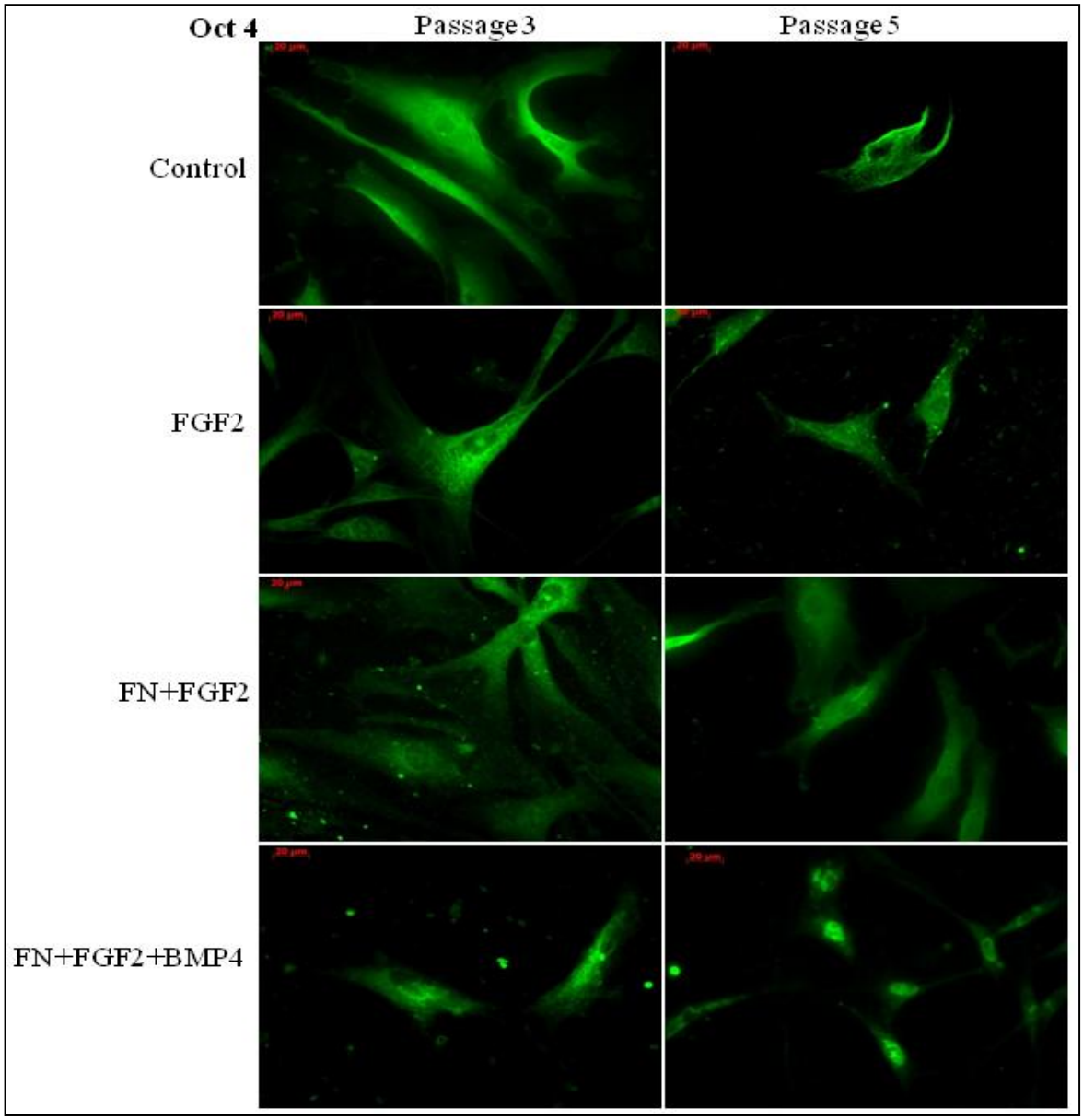

Figure 8: Immunoflourescence assay was performed to observe the expression and translocation pattern of Oct4 protein. Anti-Oct4 primary antibody was detected with Alexa flour 488 (green stain) nuclear expression was observed in passage 5 of FN+FGF2+BMP4 treated cells. Cytoplasmic expression was observed in passage 3 and 5 in FN, FGF2, FN+FGF2 treated samples.

Differentiation potential

A considerable variation in the extent of differentiation potential between untreated controlled and FN+FGF2+BMP4 treated group in fifth passage was observed by differentiation and qRT-PCR assay **(Figure 9)**. The treated BMSCs appeared to be more efficient as evident by the staining pattern and differentiation transcript expression. Pellets culture of control and treated with same cell numbers were differentiated into chondrocytes. Alcian blue staining was more prominent in treated samples. The ACAN gene transcript expression was significantly higher in treated samples.

When cultured in osteogenic media both groups showed mineralization and calcium deposition. The treated samples were almost completely covered with calcium deposits as revealed by Alizarin red staining. Osteopontin expression corresponding to osteogenic differentiation was higher for the treated samples.

For adipogenic differentiation treated BMSCs after 21 days in culture showed a larger number of clusters of lipid droplets in comparison with the untreated controls also, PPAR2 expression was higher for treated samples. Evidently FN+FGF2 and BMP4 treatment of BMSCs improves the differentiation potential of BMSCs

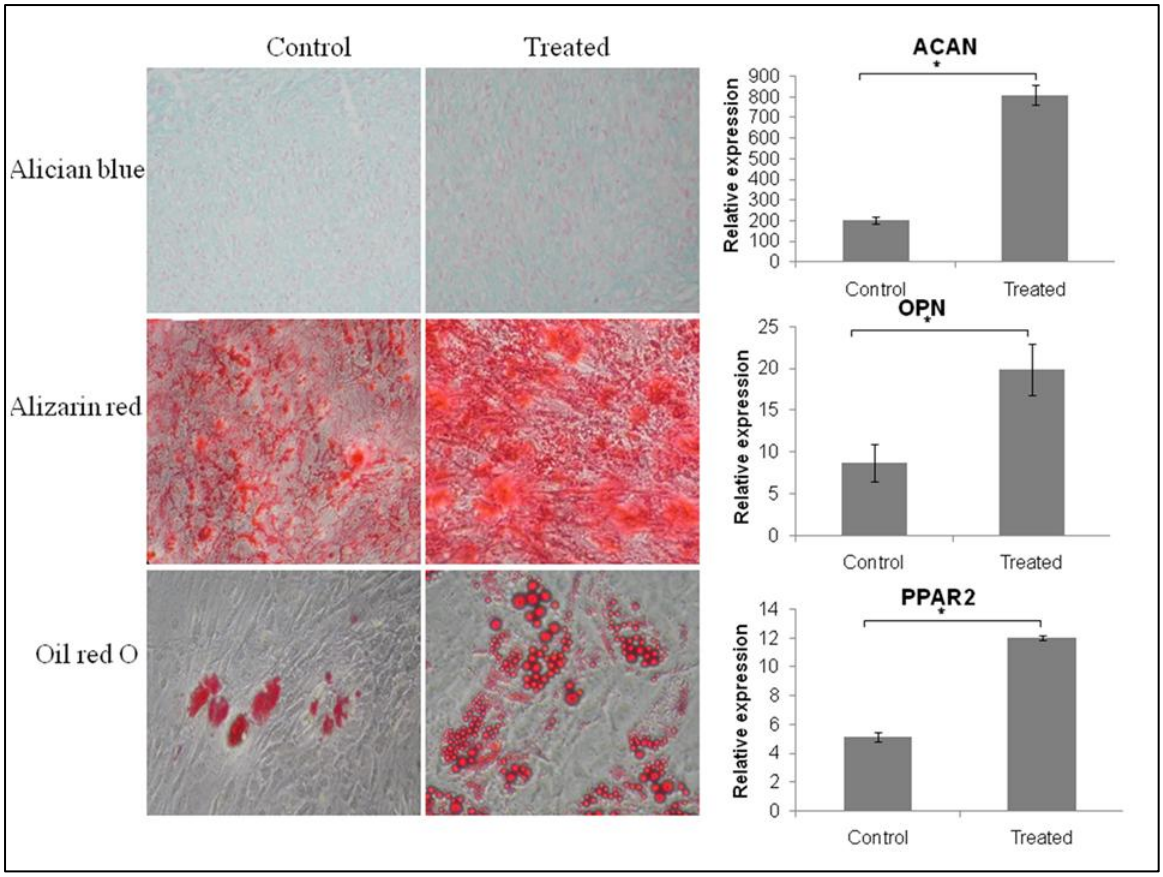

Figure 9: Staining and qRT-PCR analysis to determine the differentiation potential of treated and control compared. Treated (FN+FGF2+BMP4) and control BMSCs in fifth passage was induced in chondrogenic, osteogenic and adipogenic medium for 21 days, followed by staining and qRT-PCR analysis. Strong staining for all the three lineages was observed in treated BMSCs. qRT-PCR analysis for ACAN, OPN and PPAR2 revealed higher expression of lineage specific markers in treated BMSCs, statistical significance was accepted at $p \leq 0.05$(*).

Vimentin expression in control and treated samples

Real time PCR array was performed to determine the expression of vimentin gene transcript in untreated control and FN+FGF2+BMP4 treated samples. The treatment increased vimentin expression in BMSCs; vimentin expression declined with an increase in passage number. The trend of vimentin expression was similar to the relative proliferation rate in treated and control samples.

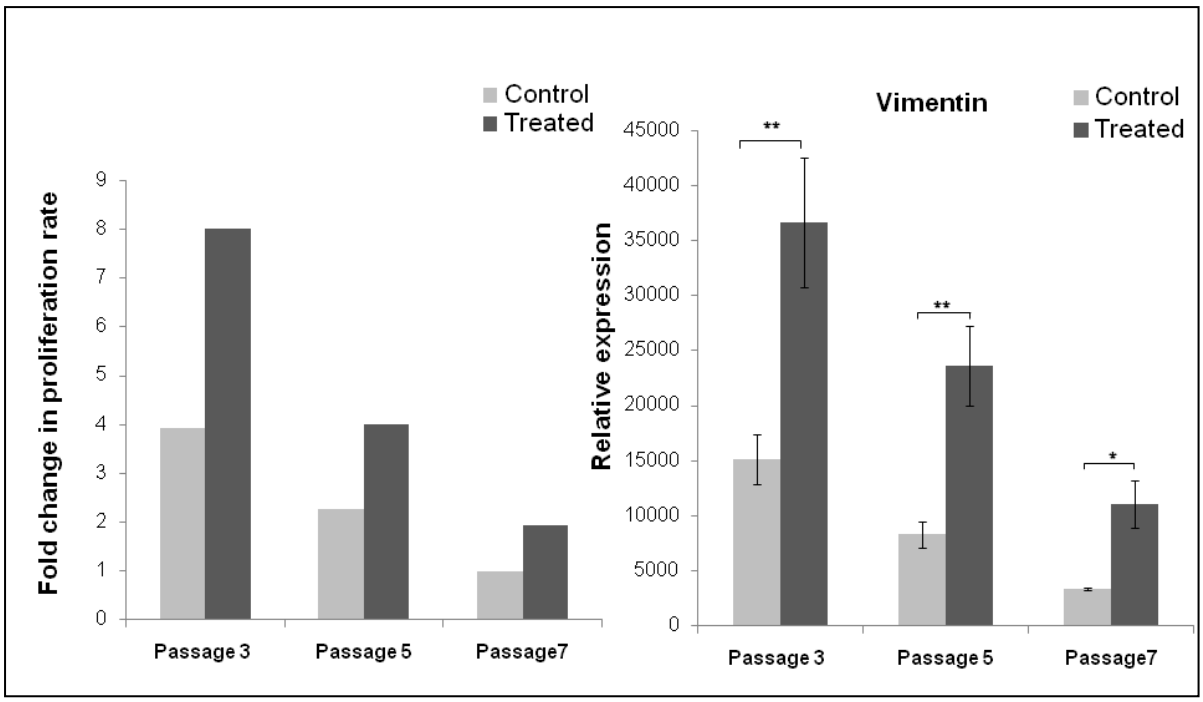

Figure 10: Vimentin expression in treated and control samples. The proliferation potential of control and treated samples were compared in all the three passages. The treatment of BMSCs increased the proliferation rate in all the studied passages by two fold in comparison to the control. It was evident that treated samples in passage 5 presented similar proliferation rate of control samples in passage 3. Vimentin expression in treated and control samples was compared. Treatment significantly increased the expression of Vimentin. Expression in treated samples was significantly higher than the control in all the three passages

Discussion

The generation of iPS by transfection of somatic cells with transcription factors has unlocked fascinating opportunities for developmental and stem cell research [9]. Inducing pluripotency in terminally differentiated cells indicates that the cellular programming from the embryonic state to the committed state can effectively be reversed to generate cells that closely resemble ESCs. The present study has demonstrated that endogenous expression of these developmental genes can be induced by modulation of culture conditions and this may circumvent the use of viral transduction–a procedure that can potentially lead to insertional mutagenesis resulting tumour formation *in vivo*. We chose to use human BMSCs since these cells have a basal endogenous expression of the factors which predispose them to preferentially favour reprogramming. Experiment was designed to modulate the crucial static and dynamic factors of BMSCs niche *in vitro* and the effect of these treatments were assessed over several passages by observing cellular morphology, changes in the proliferation potential, transcriptional expression and cellular localization of these crucial developmental factors.

A complex interplay of multiple signalling pathways regulate stem cell behaviour and is the consequence of cross talk between stem cells with their microenvironment [22]. The stem cell microenvironment provides the extrinsic regulatory cues which interacts with intrinsic genetic programs to effect stem cell physiology [23]. Microenvironmental factors of the stem cell niche such as ECM protein FN and FGF2 and BMP4 morphogens are known to favour the maintenance and pluripotency of stem cells; hence in our study we incorporated these factors into the culture conditions and observed the changes conferred upon BMSCs *in vitro* behaviour. It has been reported that MSCs when expanded *ex vivo* on FN coated plates could be cultivated for more than 50 population doublings with no obvious signs of differentiation or senescence [20]. Likewise, it has been seen that cultures

supplemented with FGF2 increase the size of colonies formed in clonal conditions by approximately 2.5 times [24]. Proliferation assays in our study presented results consistent with earlier studies, the proliferation potential of BMSCs was significantly affected when cultured on FN coated plates and in medium supplemented with FGF2. At passage 3 and 5, FN coating increased proliferation potential of BMSCs approximately two-fold and FGF2 supplementation approximately 1.5 fold; when combined the two factors increased the proliferation rate nearly threefold, in contrast with passage 3 cells, the combined treatment of these two factors in passage 5 did not produce any significant change compared to controls. In seventh passage, two fold increase in proliferation potential was observed for FN coating group and FGF2 supplementation group; the combined treatment increased proliferation potential about 1.5 fold. Interestingly, for every experimental group which complemented FN coating and FGF2 supplementation with BMP4 consistently resulted in two fold higher proliferation of BMSCs in all the three tested passages.

FGF2 increases MSCs longevity by selecting cells with longer telomeres [25] and delay cell senescence by down regulating TGFβ2 expression [26]. Previous studies also suggest that FGF2 prolongs the immature uncommitted state thereby maintaining MSCs stemness [24, 26]. FGF2 signalling has been shown to alienate BMP signalling in maintaining the pluripotency of human embryonic stem cells [27]. BMP4 is a morphogen produced by extra-embryonic ectoderm and stimulate the growth and development of early germ cells [28]; it has also been recognised as an extracellular propluripotency cue [18]. BMP4 primarily contributes to stem cell pluripotency by shielding them from differentiation inducing signals such as, activating inhibitor of differentiation (Id) gene, via the Smad signalling pathway to maintain stem cells in an undifferentiated state [19, 29]. Furthermore, it has been suggested BMP4 acts synergistically with LIF and may play a role in regulation of self-renewal of stem cells by mediating inhibition of p38 and ERK,

MAPKs [18, 29, 30]. Moreover it has been reported that BMP4 is capable of assigning re-differentiation capacities to keratinocytes making these cells developmentally more potent [31]. The effects of FN, FGF2 and BMP4 on differentiation potential, self renewal capabilities and proliferation potential of stem cells has been the subject of numerous studies but their role as transcriptional activators associated with cellular reprogramming has not been investigated. Our study presents an insight into the role played by these microenvironmental factors on transcription factors frequently associated with reprogramming. In the process of reprogramming, introduction of exogenous Sox2 and Oct4 by retroviral transfection leads to sequential reactivation of self sustaining autoregulary loop of the same endogenous transcription factors [32]. Sox2 belongs to the sex-determining region Y (SRY) related high mobility group (HMG) box transcription factor family and is an embryonic self-renewal marker expressed in embryonic stem cells and neural stem cell progenitors; the constitutive expression of Sox2 inhibits neuronal differentiation and maintains the progenitors in an undifferentiated state [33-35]. Sox2 is indispensible for maintaining pluripotency in early embryo and ESCs [36]. Knockdown of Sox2 by RNAi causes differentiation to multiple lineages. Our study revealed that the basal transcriptional expression and localization of Sox2 could be induced by microenvironmental changes. Cultured BMSCs have basal Sox2 expression at early passages but subsequent passages results in marked decline of Sox2 gene transcription; moreover, throughout the culture these transcription factors remain in the cytoplasm. Treatment of BMSCs with FGF2 and FN+FGF2 can transiently increase Sox2 gene expression at passage 3 up to two fold, however, this induction in expression was not observed in subsequent passaging. Interestingly, BMSCs cultured on FN coated plates presented an elevated expression of Sox2 at passage 5 comparable to passage 3 of control group. It was noteworthy that the experimental group FN+FGF2+BMP4 presented some

interesting results: Sox2 expression at passage 3 was similar to control group in this group and the expression at passage 5 increased several folds compared to controls. The FN+FGF2+BMP4 treatment group retained an elevated Sox2 expression even at passage 7. In treatment group FN+FGF2+BMP4 cytoplasmic localization of Sox2 protein was observed at passage 3 and nuclear expression at passage 5. Our study demonstrate that it is possible to increase transcriptional expression of Sox2 over several passages *in vitro* culture by supplementation of growth media by morphogens such as BMP4 and FGF2 complemented with FN coating of the culture surface. Also, the elevated transcriptional expression was complemented with nuclear translocation of Sox2 transcription factor.

It is thought that highly conserved non-coding DNA elements are rich in binding site for Sox2 and POU factors [37]. Oct4 exhibits similar expression pattern to Sox2 in early embryonic development. Oct4 is a master switch for pluripotency and may prevent the expression of genes activated during differentiation and is also a pivotal player in maintaining pluripotency of ESCs [38]. The Oct4 transcription factor (alternatively known as Oct3/4 and Pou5F1) is a maternally expressed protein [39], normally expressed in the totipotent and pluripotent stem cells of pregastrulation embryos[40], unfertilized oocytes, the ICM (inner cell mass) and in primordial germ cells [41], its expression is down-regulated during differentiation. Oct4 is indispensable for development–this has been demonstrated in knock down experiments which showed that the absence of Oct4 results in lethality due to the absence of ICM formation [42]. FGF2 and FN+FGF2 treatment of BMSCs transiently induced a seven and six fold Oct4 transcript expression at passage 3 respectively compared to controls; it gradually declined at passage 5 and 7. For FN+FGF2+BMP4 treatment group the expression pattern of Oct4 transcript was similar to Sox2 transcript expression. The Oct4 protein was present in the cytoplasm of control group and other treatment group at passage 3 and 5. The FN+FGF2+BMP4 treatment group,

interestingly, could retain the induced expression beyond passage 5, indicating that this treatment successfully induces and maintains increased Oct4 transcript expression over extended passages in BMSCs during *in vitro* culture conditions. This treatment also resulted in a translocation from the cytoplasm to the nucleus of Oct4 protein from passage 3 to passage 5; however, only few cells demonstrated this staining pattern. Oct4 and Sox2 interact cooperatively to activate the expression of several genes such as cMyc, FGF4 and Nanog to regulate pluripotency [43].

cMyc is another key transcription factor in the regulation of pluripotency and self-renewal of embryonic stem cell and enforced expression of cMyc confers LIF-independent growth in ESC [44]. It is thought that cMyc supports pluripotency by modifying epigenetic patterning which blocks additional differentiation or, alternatively, promotes dedifferentiation. It has also been suggested that cMyc induces cell programs necessary for self renewal and selects a rare population of cells favourable for induced pluripotency and self renewal [45]. In our study cMyc gene expression in the FGF2 and FN+FGF2 treatment groups gradually increased from passage 3, peaked at passage 5 and declined by passage 7. Gene expression of cMyc in BMSCs cultured on FN coated plates was similar at passage 3 and 5 and only slightly increased at passage 7. For FN+FGF2+BMP4 treatment group the expression of cMyc was several fold higher than in the control group and the expression level at passage 3 and 5 were comparable, at passage 7 the expression decreased by two fold compared passage 3 and 5. In the control group cMyc protein was present in the cytoplasm at passage 3 and 5, however, in the FN+FGF2+BMP4 treatment group cMyc protein was present in the cytoplasm at passage 3 interestingly at passage 5 the nuclear staining was evident.

Also, FN+FGF2+BMP4 treatment group increased the proliferation rate, differentiation potential and vimentin expression as evident by the proliferation assay,

differentiation assay studies and RT-PCR analysis. The proliferation potential of BMSCs in the treated group (FN+FGF2+BMP4) was consistently higher by a two-fold magnitude , as compared to the control group, in all of the passages tested. These finding indicate that this treatment efficiently increases the competency of BMSCs, and consequently it brings forth the hypothesis that the level of vimentin expression may be an indicator of the proliferative potential of the BMSCs. Our previous study on clonal population revealed a higher expression of vimentin in fast clone population, and this study presented that BMSCs with higher proliferative rate presented higher vimentin expression and vice versa.

Taken together, data from the present study clearly demonstrate that the endogenous level of reprogramming related factors can be significantly induced by modulating the culture conditions. All treatment groups employed in our study could successfully induce the stem cell genes, however, only a few groups could retain the induced expression over extensive passaging in culture. The ability of FN coating and media supplemented with FGF2 and BMP4 to induce, and maintain over extensive passaging, the expression of stem cell genes in human BMSCs indicates that this type of treatment of BMSCs may prove a way of keeping cells in a state of active reprogramming and prove useful for generating sufficient numbers of therapeutically potent cells. The microenvironment factors used in our study should be investigated further, as they may play an important role when formulating the experimental parameters required to modify culture medium, and they should also be considered as a valid alternative to the use of retroviral transfection in generating iPS. This study also indicates that the intrinsic signalling mechanisms of the BMSCs are intimately linked to extrinsic cues from the microenvironment, and that these signals are critical for maintaining gene expression to support the stemness of such cells during extended *in vitro* culture.

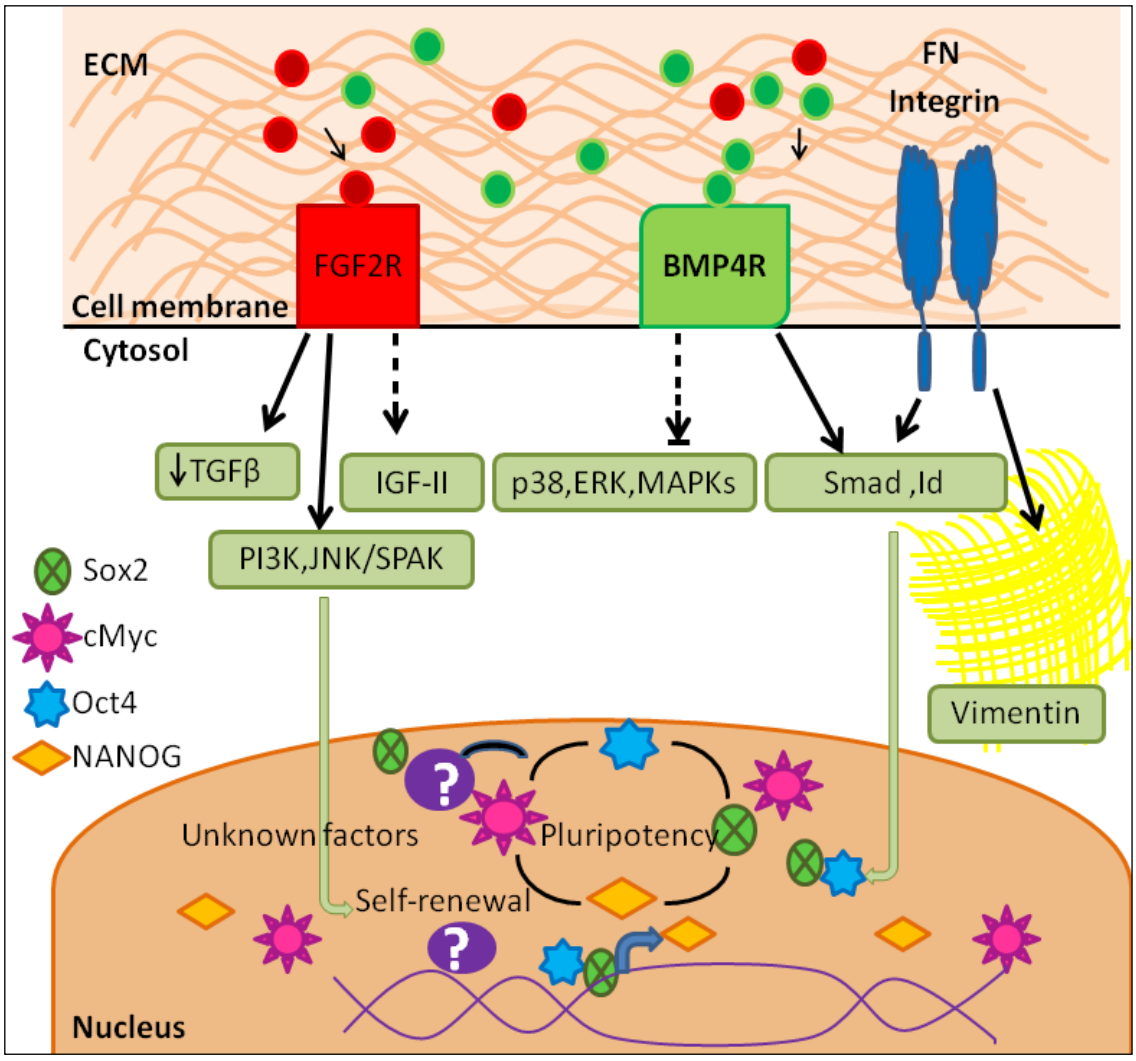

Figure 11: Effect of culture conditions on BMSC competency. A diagrammatic illustration of the effect of the microenvironment components, such as FGF2, BMP4 and FN coating on the behavioural mechanisms of BMSCs. Cell surface receptor in response to morphogens in the culture media initiates cascade of signalling pathways directly or through unknown molecular players. These pathways affect the multipotency and self renewal property of BMSCs by triggering transcription factors such as Sox2, Oct4, cMyc and NANOG. FGF2 delays cell senescence by down regulation of TGFβ and indirectly regulate IGF-II signalling. BMP4 primarily contributes to multipotency by activating Id gene which shields them from differentiation inducing signals via Smad signalling

pathways. BMP4 mediates inhibition of p38, Erk and MAPK pathways. FN coating in the ECM leads to activation of Integrin and may affect cytoskeletal components vimentin. All these signals are conveyed to the nucleus and interpreted by the cells by recruiting Oct4, Sox2 complex on the binding site for NANOG. Interaction of these key transcription factors leads establishment of regulatory loop which controls the proliferation/self renewal and differentiation potential of stem cells.

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CHAPTER 6

SUMMARY

The ease with which BMSCs can be accessed from bone marrow, coupled with their multipotent nature, has put them centre stage within the field of regenerative therapy. BMSCs, in particular, are an attractive source for patient specific therapeutic cells; these cells have the ability to self-renew and, given certain *in vitro* triggers, are capable of undergoing differentiation into osteogenic [1], chondrogenic [2], adipogenic [3], cardiomyocytes [4], hepatocyte [5] and neuronal [6], lineages suggesting an intrinsic capability of plasticity and transdifferentiation. Despite these promising properties, their characterization and the signalling mechanisms controlling their unique features remains elusive. Furthermore, BMSCs are very sparsely distributed in bone marrow and their numbers decline with age; the number of BMSCs in a neonate is estimated at 1 in every 10,000 bone marrow cells and this decreases to an estimated 1 in every 2,000,000 in a person 80 years old [7]. This *in vivo* scarcity imposes a limitation on their study *in vitro*; also, the low number of the BMSCs highlights the obvious need for *ex vivo* expansion in order to obtain sufficient number of cells useful for cell transplantation. However, when cultured *in vitro* over several passages, there is an orderly loss of differentiation potential, in parallel with a loss of proliferation capacity [8]. A number of strategies have been developed to improve the ability of the cells to proliferate, while retaining their differentiation plasticity, following extended passages [9]. Another issue concerns the heterogeneous nature of BMSCs populations [3], and the lack of biomarkers presents a further hurdle to realizing their potential applications. The currently available cell surface markers are insufficient to identify the mesenchymal stem cells *in vivo*, or in a

heterogeneous population, since the markers used are also present in non-stem cells. A particular marker may also only be expressed on stem cells at a particular stage of the cell cycle, or under certain conditions. In order to harness the full potential of these sparsely distributed, but therapeutically potent cells, the sequential loss of multipotency and proliferation capacity on successive *ex vivo* passaging must be addressed by devising methods of harvesting these cells from bone marrow, followed by *ex vivo* expansion to generate sufficient cell number, while maintaining their unique characteristics in culture.

The present study has addressed a number of questions related to BMSCs by employing sophisticated molecular techniques to gain an understanding of the role played by these cells *in vivo*. This study aimed to identify a potential biomarker and unravel the molecular pathways activated in BMSCs. SI and stem cell specific gene array study was performed on contrasting clonally expanded populations of BMSCs generated by limiting dilution method. The SI study identified the cytoskeletal protein vimentin as being more highly expressed in fast growing clonal populations compared to the slow growing populations. The gene array study, on the other hand, quantified the transcription profile of eighty four stemness genes. Ten genes were significantly over represented in the target population: these genes could be grouped based on functions into cell cycle regulators (CDC2), cell lineage markers (COL2A1, ACAN), growth factors (FGF2, BMP2 and IGF1), and maintenance of embryonic and neural stem cell renewal (Fox A2, Sox2, Notch1 and DLL3).

As was mentioned before, there is a marked decline in the multipotentiality and proliferation potential of these cells when cultured over extensive passages *in vitro*. Hence, the preparation of large numbers of *ex vivo* expanded cells may be a problem. This study revealed the pluripotency marker Sox2 as being a significantly up-regulated gene in the potent clonal population, indicating that down regulation of these markers and other

associated molecular players, such as Oct4 and cMyc which are known to assign pluripotency, may be responsible for age associated phenotypic changes in BMSCs.

With this hypothesis in mind, the next phase of our study was designed to determine the effect of *ex vivo* culture on pluripotency associated marker expression. These markers, in a recent groundbreaking study, have been shown to assign ESCs-like characteristics to somatic cells suggesting that pluripotency is not the exclusive domain of stem cells, but the result of activation of specific signalling pathways activated in response to cellular niche in which BMSCs reside. The pluripotent state in somatic cells was achieved by stimulating the exogenous expression of pro-pluripotency factors in somatic cells. We hypothesised that these exogenous factors were triggered by the stimulation of endogenous genes by a self sustaining auto regulatory loop. Our data strongly suggest that the rapid decline of multipotency and proliferation potential of BMSCs, when passaged *in vitro* under current culture conditions, occur in conjunction with a loss of gene expression and cytoplasmic translocation of these critical transcription factors. Our study showed that, when cultured *in vitro*, BMSCs and somatic cells such as chondrocytes and osteoblasts during early passages still express Sox2, c-Myc, and Oct4 genes and maintain nuclear location; however, with subsequent passages there is a gradual decline of phenotypic gene expression coupled with migration of these transcription factors into the cytoplasm. This is manifested as a pronounced loss of multipotentiality and proliferation potential, most evident by the presence of senescence associated phenotypes. Our conclusions from this part of the study is that loss of multipotency of BMSCs with subsequent *ex vivo* passaging may be due to the loss of stem cell specific gene expression, caused by the loss of the natural physiological niche in which BMSCs reside *in vivo*. It is this loss of niche that is thought to trigger the loss of multipotentiality in favour of commitment to a more specialized lineage. We therefore hypothesized that if cues from the BMSCs niche could

be mimicked in the *in vitro* culture conditions, it might be possible to maintain the expression of critical transcription factors over extended passages in culture conditions, making it possible to harvest a sufficient number of therapeutically potent cells expanded *ex vivo*.

The microenvironment of BMSCs encompasses a range of dynamic factors, some of which are growth factors such as FGF2 and BMP4. These growth factors have been shown to play a role in stem cell maintenance and their role was investigated subsequently in this project, as was the effect of static factors, such as FN. Although the role of these factors has been studied extensively with respect to stem cells proliferation and maintenance, their role with respect to reprogramming related genes has not been demonstrated. An experimental setup was designed in order to study the effects on BMSCs of these microenvironmental factors, both individually and in combination. We found in all the treatment groups that it was possible to transiently induce stem cell genes expression, but that only a limited number of groups could maintain this gene expression over extended periods in culture. In particular, BMSCs plated out on FN coated plates, in media supplemented with FGF2 and BMP4, were capable of inducing and maintaining an elevated expression of stem cell genes over extensive passages, indicating that this treatment may hold the key to keeping cells in an active reprogramming state and therefore be useful for generating sufficient numbers of therapeutically potent cells. Also, this treatment could significantly affect proliferation and differentiation potential of BMSCs evident by the proliferation and differentiation experiments. This study has illustrated that over expression and nuclear location of pluripotency related genes by modulating culture conditions has the potential to maintain the stemness of BMSCs, without the need for genetic manipulations.

Also, the vimentin expression in the treated samples was significantly higher than the control samples, and vimentin expression declined with a decline in proliferation rate. Similar results regarding vimentin expression were obtained by SI study. SI performed on clonal populations indicated that vimentin protein presented a significantly higher expression in fast-growing clonal populations. Though, a trend in the vimentin expression and proliferation rate of BMSCs is observed, the functional role of vimentin, particularly with respect to stem cells remains unclear. It remains to be investigated if the observed differences in the vimentin network of fast clone cell and the slow clone cell are a cause or an effect of changes in morphological and phenotypic characteristics of the clonal BMSCs populations. It would be of vital importance to determine the role that vimentin plays in stem cell morphogenesis.

In summary, this study presents new insights into the molecular signature of BMSCs. The study of the transcriptional profile of a number of stemness related genes has been integrated with a novel approach of generating monoclonal antibody against a favourably expressed protein, by the method of subtractive immunization. As such, this is a pioneering study by utilizing primary cells to generate novel antibodies by subtractive immunization. The differentially expressed protein vimentin, and the over-expressed genes we identified, are closely associated with mesenchymal lineage and pluripotency related markers. The pro-pluripotency factor, Sox2, was up-regulated in the fast clone cell populations and, interestingly, this is one of the factors identified as being vital for stem cell pluripotency markers, together with cMyc, Klf4 and Oct4 [10]. We found that somatic and BMSCs have a basal expression level of these factors when grown *in vitro*, and that the expression level declines precipitously with passaging. These findings indicate that the native microenvironment of these cells is critical in regulating the level of propluripotency factors. These findings formed the basis for our hypothesis that when cultured *ex vivo*, the

loss of niche leads to a loss these critical markers. When exposed *in vitro* to morphogens such as FGF2, BMP4 and the static factor fibronectin, the basal expression level of stem cell factors were induced and maintained over an extended number of passages. Our study indicates that the intrinsic signalling mechanisms of BMSCs are intimately linked with the extrinsic cues from the microenvironment and these signals are critical for maintaining gene expression necessary for cellular stemness when subject to long term *in vitro* culture.

LIMITATION OF THIS STUDY

The major drawback of this study is the lack of *in vivo* experiments, this is a preliminary study and in an attempt to understand the trend of expression of marker the study has been carried out *in vitro*. The study has presented culture technique to improve the proliferation and differentiation potential of BMSCs, however, it remains to be established if treated BMSCs when seeded into scaffold and implanted in animal defect model can show and improved performance. Also, the effect of the treatments on BMSCs homing and recruiting mechanism needs to be established using *in vivo* experiments.

Future Directions

- This investigation provides strong preliminary evidence that BMSCs behaviour is intimately linked with their native *in vivo* niche. This was evident by their transcription profile which was indicative of these being triggers naturally occurring in the microenvironment (Chapter 3). A more detailed molecular study, to identify the key signalling molecules present in the microenvironment of BMSCs, will be valuable to deepen the understanding of underlying signalling pathways activated in controlling cell fate determination.
- Induced pluripotent stem cells have been generated from somatic cells by using viral vectors which render them unfit for clinical applications. Our pilot study

opens up the possibility to generate "genetically clean" therapy potent cells by mimicking, *in vitro*, the niche in which stem cells normally reside (Chapter 5). The microenvironment is indispensable in dictating the future fate of adult stem cells, hence microenvironmental cues need to be closely studied and employed in order to harvest a sufficient number of *ex vivo* expanded therapeutically potent cells.

- Future molecular studies involving the effect of these factors on BMSCs can promote a growth medium formulation ideal for culture conditions capable of retaining pluripotency markers for longer duration *in vitro*. The factors explored in this study may be employed in designing artificial niche capable of retaining BMSCs characteristics over extensive passaging.
- Age-dependent and growth stage related regenerative capabilities needs to be explored via RNA interference technique of blocking protein function of the stemness factors and subsequent tests on expression and localization of these factors needs to be studied.
- Finally molecular studies involving cytoskeletal proteins primarily Vimentin may provide a better insight into assigning physiological and functional role of vimentin in maintenance of the pathways activated in highly proliferating populations of BMSCs.
- Further *in vivo* study on tissue forming capacity of treated BMSCs needs to be evaluated by employing these cells in defect models and the bone forming efficiency of these cells needs to be tested. Live animal tracing, lineage tracing and real time imaging of the proliferative and regenerative potential of engrafted BMSCs over a period of time.

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