Modulation of connective tissue stromal cell plasticity to generate cartilaginous phenotypes

Thesis submitted in fulfilment of the requirements of the degree of Master of Philosophy, University of Cardiff

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Abbreviations

ACT	Autologous chondrocyte transplantation
ASC	Adult Stem Cell
bFGFR	Basic Fibroblast Growth Factor Receptor
BMPs	Bone Morphogenic Proteins
BMP-4	Bone morphogenic protein – 4
Bb-MSCs	Bone marrow mesenchymal stem cells
C - ABC	Chondroitinase ABC
CD	Cluster of differentiation
CD29	Cluster of differentiation 29 (an integrin beta chain 1 unit)
CD34	Cluster of differentiation 34 (a cell surface glycoprotein)
CD44	Cluster of differentiation 44 (a cell-surface glycoprotein)
CD45	Cluster of differentiation 45 (Protein tyrosine phosphatase)
CD71	Cluster of differentiation 71 (Transferrin receptor)
CD73	Cluster of differentiation 73 (Activated leukocyte cell adhesion molecule)
CD90	Cluster of differentiation 90 (Thy-1 cell surface antigen)
CD105	Cluster of differentiation 105 (Endoglin)
CD106	Cluster of differentiation 106 (Vascular cell adhesion molecule-1)
CD120	Cluster of differentiation 120 (Tumour necrosis factor receptor)
CD124	Cluster of differentiation (Interleukin 4 receptor)
CD166	Cluster of differentiation 166 (Activated leukocyte cell adhesion molecule)
cDNA	complementary DNA
CILP	Cartilage intermediate layer protein
CM	Chondrogenic Medium
C-MSF	Chondrogenic Mesenchymal Stimulating Factor
COMP	Cartilage Oligomeric Matrix Protein
CRD	Carbohydrate Recognition Domain
CS	Chondroitin sulphate
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
DS	Dermatan sulphate

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated kinases
ESCs	Embryonic stem cells
EtOH	Ethanol
FACIT	Fibril associated collagens with an interrupted triple helix
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FGFs	Fibroblast Growth Factors
FGF – 2	Fibroblastic growth factor – 2
FITC	Fluorescin isothiocyanate
FLT3 ligand	Fms-related tyrosine kinase 3 ligand
GAG	Glycosaminoglycans
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
GlcN	Glucosamine
HA	Hyaluronan
Hep	Heparin
Hh	Hedgehog
HS	Heparan sulphate
HSC	Haematopoietic stem cells
ICAM-1	Intercellular adhesion molecule- 1
ICAM-2	Intercellular adhesion molecule -2
IGD	Interglobular domain
IGF – 1	Insulin-like growth factor – 1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ihh	Indian Hedgehog
IL – 1	Interleukin – 1
IL - 1β	Interleukin – 1 beta
IL-1R	Interleukin – 1 receptor
KS	Keratan sulphate
LFA-3	Lymphocyte function-associated antigen-3

LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
MAB 1740	Monoclonal antibody 1740
MAPK	Mitogen-activated protein kinase
MCP joint	Metacarpophalangeal joint
M-CSF	Macrophage colony-stimulating factor
MHC-II	Major Histocompatibility Complex II
M-MSF	Myogenic Mesenchymal Stimulating Factor
MM	Maintaining Medium
MMP-13	Matrix Metalloproteinase - 13
MSCs	Mesenchymal Stem Cells
MSF	Mesenchymal stimulating factor
Msx2	Msh homeobox 2
N-cadherin	Neuronal cadherin
N-CAM	Neural Cell Adhesion Molecule
OA	Osteoarthritis
O-MSF	Osteogenic Mesenchymal Stimulating Factor
P	Passage
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived Growth Factor Receptor
PFA	Paraformaldehdye
PGs	Proteoglycans
PMSF	Phenyl methyl sulfonyl fluoride
PRG - 4	Proteoglycan – 4 (Lubricin; Superficial Zone Protein, SZP)
Ptc	Patched
PTHrP	Parathyroid hormone-related protein
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
RT	Reverse transcription
SAGE	Serial analysis of gene expression
SCF	Skp, Cullin, F-box containing complex (or SCF complex)
SCR	Short Complement Repeat
SCFR	Skp, Cullin, F-box containing complex receptor

SH2	Src Homology 2 domain
SH3	Src Homology 3 domain
SH4	Src Homology 4domain
Shh	Sonic Hedgehog
SLRPs	Small Leucine Rich Proteoglycans
SMAD	C. elegans mothers against decapentaplegic protein
SOX – 9	Sry-related HMG box
SZP	Superficial zone protein
TAK1	TGF - β activated kinase 1
TCF	Transcription Factor
TGF - β	Transforming Growth Factor – beta
TGF - β2	Transforming Growth Factor – beta 2
TGFβIR	Transforming Growth Factor – beta I Receptor
TGFβIIR	Transforming Growth Factor – beta II Receptor
TNFIR	Tumour Necrosis Factors – I Receptor
TNFIIR	Tumour Necrosis Factors – II Receptor
t-MSCs	Tissue-derived stem cells
VCAM-1	Vascular Cell Adhesion Molecule -1
VEGF	Vascular endothelial growth factor
VWA	Von Willebrand factor A
WB	Western Blot

ABSTRACT

Stem cells are unspecialised cells found in the body which possess the ability to self renew and can be induced to proliferate and differentiate into more specialised cells. MSCs are adult stem cells that are capable of leaving the bone marrow and travelling in the bloodstream to a different site, where they may perform repair or regeneration processes of various mesenchymal tissues such as cartilage, bone and fat. Due to these properties of MSCs, it proves to be a useful source for the repair and regeneration of cartilage.

The aims of this study were to: 1) characterise primary bovine MSCs by assessing the gene expression of hyaline cartilage-specific genes (SOX-9, Aggrecan and Collagen type II) and non-specific genes (Collagen type I and type X); 2) investigate the effects of culture medium supplemented with FGF-2 or with the addition of an extra growth factor, TGF-β2, on bovine MSCs in a two dimensional culture system; 3) characterise the phenotypes of MSCs tissue grafts produced using MSCs pre-cultured in culture medium supplemented with FGF-2 or with the addition of TGF- β2 in a three dimension culture system (Transwell) seeded at high ($6x10^6$ cells) and low ($0.5x10^6$ cells) cell density.

Our study showed that different bone chamber size and thickness influenced the amount of marrow and cells harvested without interfering with the fibroblastic-shaped cell morphology and their adherence ability. The presence of stem/progenitor cell features is present in undifferentiated P0 BMSCs and P1 and P2 BMSCs cultured in FGF-2 or with the addition of TGF-β2. Gene expression analyses on undifferentiated P0 BMSCs and P1 and P2 BMSCs cultured in FGF-2 or with the addition of TGF-β2 suggested that the MSCs contain both fibroblastic and chondrogenic features. This implies that the MSCs are not fully committed towards a chondrogenic lineage. We have demonstrated that it is possible to generate a tissue graft using passaged BMSCs in a Transwell culture system but seeding density is important. The ideal seeding cell density is $0.5x10^6$ cells/well using P2 and P3 BMSCs pre-cultured in either FGF-2 or with the addition of TGF-β2. The tissue graft produced has a high expression level of aggrecan, collagens type I and II, which suggested that it has a fibro-cartilage phenotype.

CHAPTER 1: General Introduction

1.1 Stem cells

Stem cells are unspecialised cells found in the body which possess the ability to self renew and can be induced to proliferate and differentiate into more specialised cells (Barry and Murphy 2004; Adam *et al.,* 2005). Due to these properties, they can serve as reserve cells for damaged or compromised adult tissues requiring more extensive repair, regeneration or expansion. Stem cells can be derived from embryos and adults, which are referred to as embryonic and adult stem cells. They can also be categorised into three types, totipotent, pluripotent and multipotent. Totipotent means their potential is total with the capacity to give rise to any cell type and placenta and foetal membranes within the body, these are also referred to as the germ cells. Pluripotent (embryonic stem cells) can differentiate into virtually any cell type in the body. Multipotent cells (tissue specific adult stem cells) can give rise to only a limited number of cell types and these are generally known as adult stem cells (Collas & Hakelien 2003). The different types of stem cells are summarised in Figure 1.1.

Embryonic stem cells (ESCs) are totipotent cells that are derived from four or five days embryo, known as the blastocyst. They are developed from eggs that are fertilised *in vitro*. The blastocyst is composed of three structures; trophoblast (outer layer of the blastocyst), blastocoel (cavity of blastocyst) and inner cell mass (cells at the end of the blastocoel). Adult stem cells (ASCs) are multipotent, undifferentiated cells which may be found in mature tissues. The exact origin of ASCs is currently unknown. They appear to remain as quiescent cells in a specific area of each tissue until they appear to be activated by tissue injury or disease (Lorenzini *et al.,* 2007; Kim & Szele 2008).

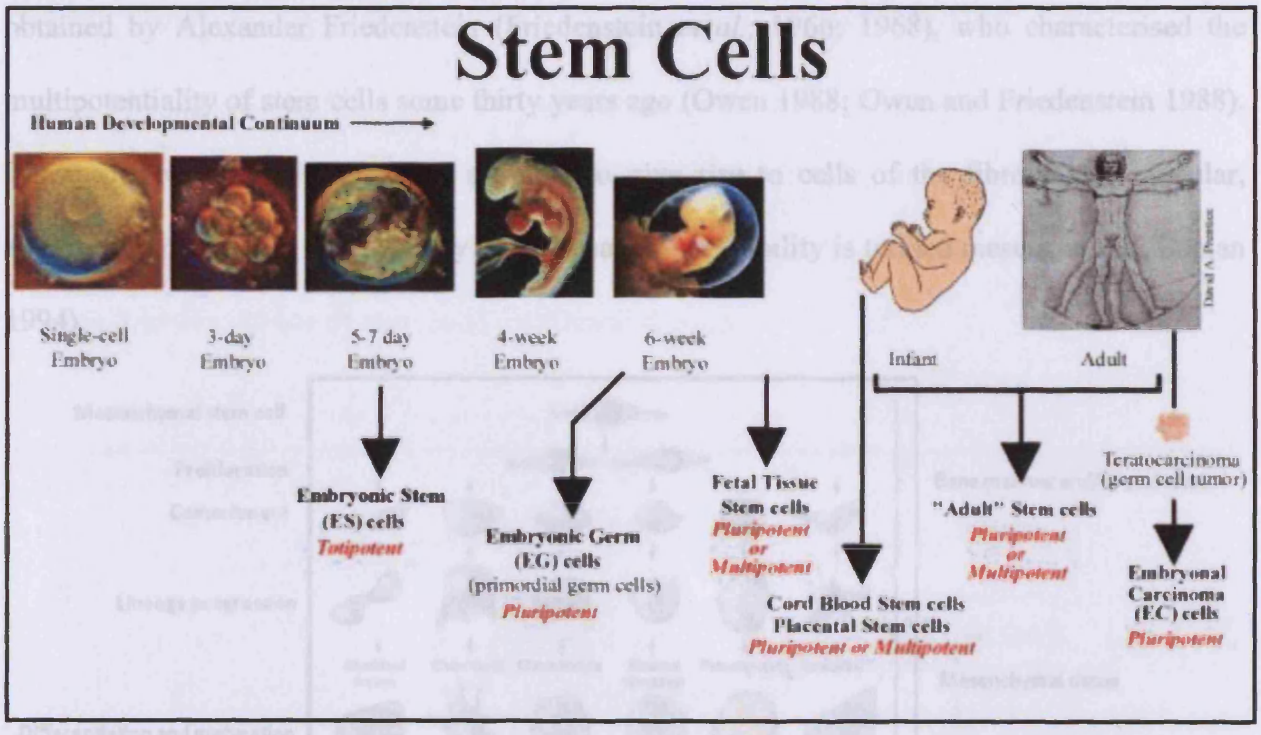


Figure 1.1: Flow diagram to illustrate the origin of the different categories of stem cells, totipotent, pluripotent and multipotent (www.stemcellresearch.org/images/prenti1.jpg).

The presence of stem cells in the bone marrow was first suggested by the observations of the German pathologist Cohnheim, 130 years ago (J Cohnheim 1867) through the study of wound repair. Later, more definitive evidence for the existence of stem cells in the bone marrow was obtained by Alexander Friedenstein (Friedenstein *et al.,* 1966; 1968), who characterised the multipotentiality of stem cells some thirty years ago (Owen 1988; Owen and Friedenstein 1988). These marrow stromal stem cells are able to give rise to cells of the fibroblastic, reticular, adipogenic, osteogenic and possibly other lineages, such ability is termed mesengenesis (Caplan 1994).

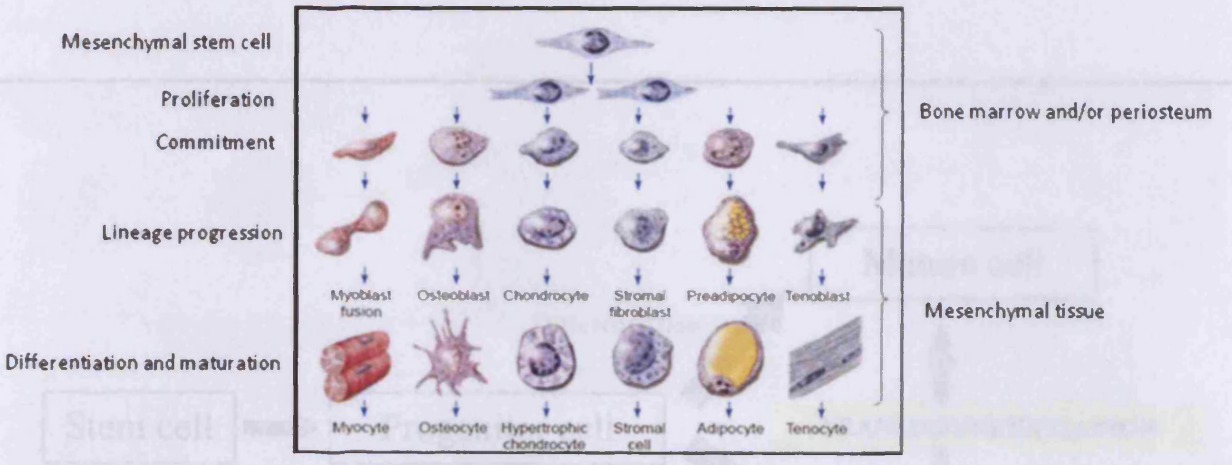


Figure 1.2: A schematic diagram to illustrate the transition changes of MSCs through a series of steps namely proliferation, commitment, differentiation and maturation, resulting in the formation of specific tissues such as bone, cartilage and muscle (Adapted from Risbud and Sittenger, 2002).

In addition to stem cells, other committed cells are also capable of undergoing transdifferentiation a process, in which cells from one lineage dedifferentiate into an intermediate cell type before redifferentiating into cells of an alternative lineage (Collas & Hakelien 2003). Due to their fascinating properties, there has been much interest in the use of stem cells for therapeutic use, for conditions such as paralysis, stroke, heart attack, musculoskeletal tissue injuries and neurodegenerative disease, and for diseases such as osteogenesis imperfecta and arthritis. However, further knowledge on stem cells is needed in order to improve the use of stem cells for clinical therapy.

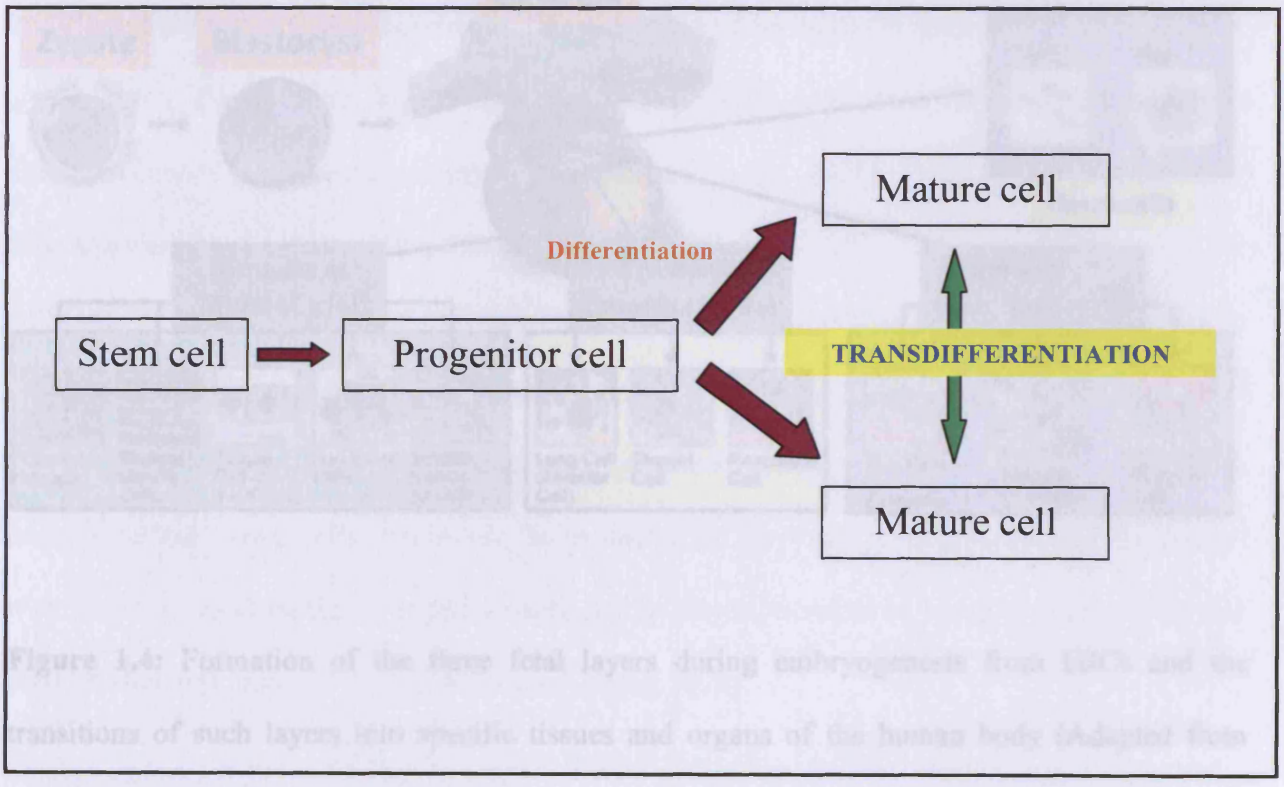


Figure 1.3: Flow diagram to show the process of transdifferentiation (Adapted from Collas & Hakelien 2003).

1.1.1 Differentiation of Embryonic stem cells (ESCs) to chondrocytes

The primary purpose of ESCs is to segregate and form unique morphologies and functional units that are integrated into the whole physiology of the organism (Dennis & Caplan 1993; Macdonald 2002). ESCs are able to differentiate into three layers, endoderm which later forms the interior gut lining; mesoderm, forming the muscle, bone, blood, cartilage; and ectoderm producing the epidermal tissues and the nervous system, which eventually specialise into any bodily tissue (Collas & Hakelien 2003; Lee & Hui 2006).

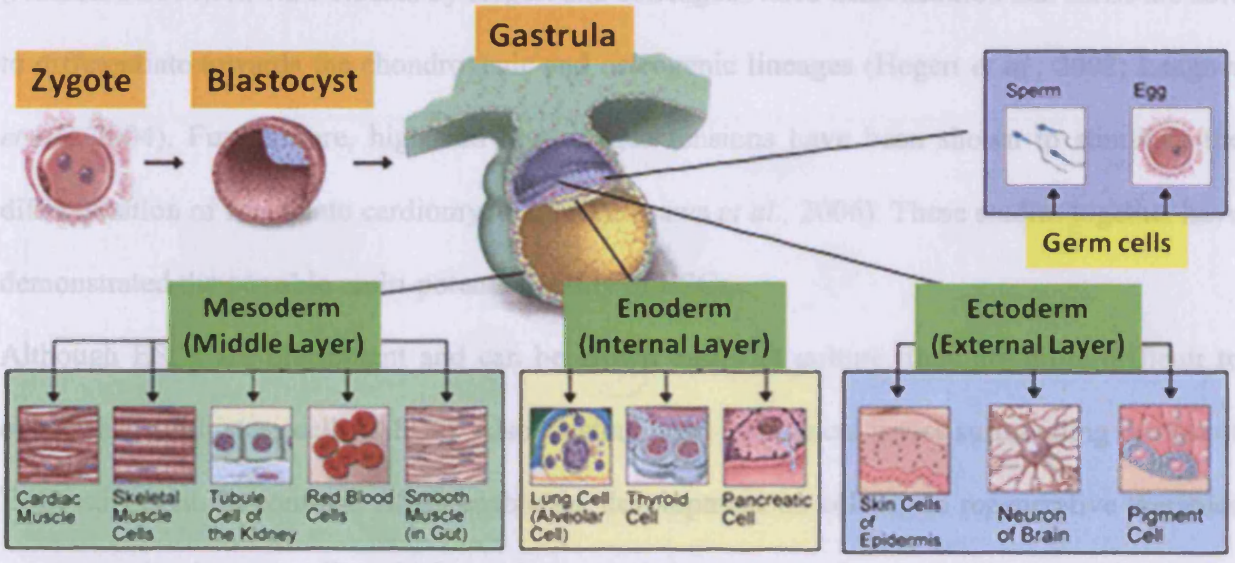


Figure 1.4: Formation of the three fetal layers during embryogenesis from ESCs and the transitions of such layers into specific tissues and organs of the human body (Adapted from www.stemcure.com).

In vitro cell culture of ESCs under certain conditions may allow the ESCs to remain undifferentiated (unspecialised). However, during the culture period, if the cells are clumped together and form embryoid bodies, this will enable the cells to differentiate spontaneously resulting in the formation of specific cell types such as muscle cells and nerve cells. Therefore, when using ESCs, it is important to control their differentiation behaviour by optimising the

composition of the culture medium. Multiple studies have shown the effect of limb bud progenitor on ESCs (Sui *et al.,* 2003), or murine ESCs exposed to dexamethasone (Tanaka *et al.,* 2004) and cultured on three dimensional hydrogels in the presence of glucosamine (Hwang *et al.,* 2006 a and b) contributed to the chondrogenesis of ESCs. In addition, other studies have also suggested the ability of ESCs to differentiate into other cell types such as those from the chondro-, osteo- and adipo- lineages (zur Niedeu *et al.,*. 2005). ESCs derived from early mouse embryo are able to form epidermis containing a dermal layer, similar to native skin when exposed to matrix from normal human fibroblast and bone morphogenic protein-4 (BMP-4) (Aberdam 2004). *In vitro* studies by Hegert and colleagues have demonstrated that ESCs are able to differentiate towards the chondrogenic and osteogenic lineages (Hegert *et al.,* 2002; Lengner *et al.,* 2004). Furthermore, high and low oxygen tensions have been shown to stimulate the differentiation of ESCs into cardiomyocytes (Kurosawa *et al.,* 2006). These studies together have demonstrated the possible multi-potential ability of ESCs.

Although ESCs are pluripotent and can be grown easily in culture, they are more difficult to obtain than adult stem cells (ASCs). Also existing legal and ethical issues surrounding the use of ESCs still exist. In contrast, ASCs enable the development on cell-based regenerative therapies using a patient's own cells, which can be expanded *in vitro* and re-introduced into the patient with minimal rejection risk. For this reason, ASCs demonstrated to be a better candidate for the regeneration and repair of damaged tissues (Caplan 2009).

1.1.2 Adult stem cells (ASCs)

Adult stem cells do not function in the embryonic microenvironment and do not respond to embryonic signalling molecules that specify tissue morphologies or those that induce an embryonic function (Caplan & Dennis 2006). They are undifferentiated cells that are responsible for the regenerative capacities of tissue going through numerous cycles of cell division while maintaining their undifferentiated state.

A common rich source of adult stem cells is found in the bone marrow; but these can also be found in other specific areas such as blood, skin, liver, bone, fat and intestine, where they may remain as quiescent (non-dividing) cells for many years (Ryans *et al.*, 2006). Adult stem cells from all of these tissues can differentiate into specialised lineage cells (see Figure 1.5) and for these reasons, such adult stem cells are referred to as multipotent. Adult stem cells are the preferred cell type for transplantation and tissue repairs because they have fewer ethical issues than ESCs. Also adult stem cells are hypoimmunogenic due to the lack of major histocompatibility complex II (MHC-II) and co-stimulatory molecule expression and are less likely to form teratomas (Ryans *et al.*, 2006).

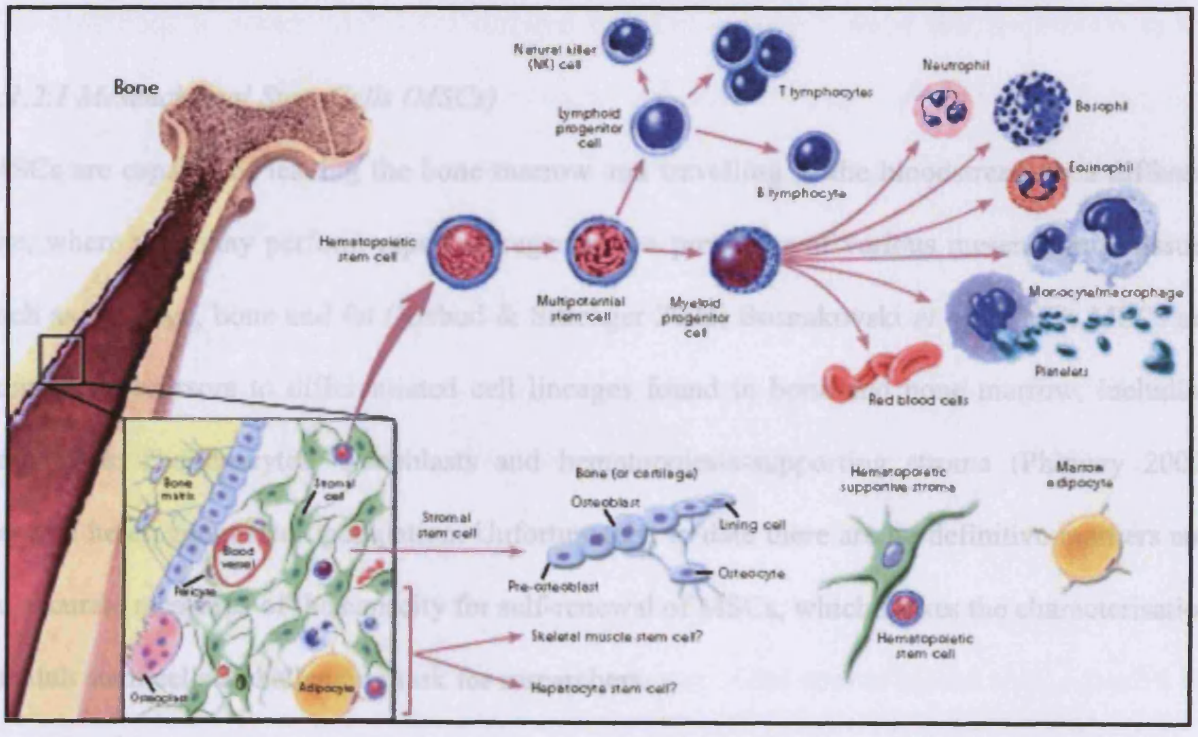


Figure 1.5: Diagram showing distinct niches that exist within the bone marrow (www.stemcell.nih.gov).

There are two main types of adult stem cells, haematopoietic stem cells (HSCs) and bone marrow mesenchymal stem cells (b-MSCs). HSCs from bone marrow can generate various blood

cells including red blood cells, platelets, and macrophages as shown in Figure 1.5., also they are being used currently for treating diseases such as leukaemia and multiple myeloma (Lee & Hui 2006). In contrast, b-MSCs are non-haematopoietic cells or stromal cells found in the bone marrow stroma. They provide a physical support for maturing precursors of blood cells and serve as a repository of a blood range of cell-derived cues and signals. This subsequently drives the commitment, differentiation and maturation of HSCs. MSCs are able to multi-differentiate into a wide range of musculoskeletal tissues such as bone, cartilage, muscle and fat. Since b-MSCs are capable of forming musculoskeletal tissues, they are a potential cell source for the repair and regeneration of cartilage for treating osteochondral defects and degenerative joint diseases, for example arthritis.

1.1.2.1 Mesenchymal Stem Cells (MSCs)

MSCs are capable of leaving the bone marrow and travelling in the bloodstream to a different site, where they may perform repair or regeneration processes of various mesenchymal tissues such as cartilage, bone and fat (Risbud & Sittenger 2002; Bosnakovski *et al.,* 2005). MSCs are common precursors to differentiated cell lineages found in bone and bone marrow, including adipocytes, chondrocytes, osteoblasts and hematopoiesis-supporting stroma (Phinney 2002) hence a heterogenous cell population. Unfortunately, to date there are no definitive markers and no accurate measures of the capacity for self-renewal of MSCs, which makes the characterisation of adult stem cells a challenging task for researchers.

Over time, the nomenclature of MSCs has evolved from fibroblast colony-forming cells, colony-forming unit fibroblasts, to mesenchymal progenitor cells or marrow stromal cells. Currently, they are referred to as mesenchymal stem cells, and were first identified some 40 years ago by Alexander Friedentsein as able to differentiate into cells of the connective tissue lineages (Owen 1988; Owen & Friedenstein 1988). Early studies by Friedenstein and colleagues suggested the presence of osteogenic precursors within the bone marrow (Petrakova *et al.,* 1963; Friedenstein

et al., 1961 and 1966). Subsequently their work has shown that marrow cells are capable of making new bone tissue at early passages, but lose such ability by the fifth passage, suggesting that prolonged passaging may cause the precursor cells to lose their multi-differentiation potential (Friedenstein *et al.,* 1968; Friedenstein & Kuralesova 1971). By 1970, Friedenstein had developed a simple isolation method and primary culture of stem cells from marrow which highlighted several findings:

1. rare population of b-MSCs in bone marrow,
2. MSCs do not enter S-phase until up to 60 hours after initial plating
3. MSCs have a high replicative capacity *in vitro*
4. MSCs are clonogenic and form colonies of irregular shape and density

The conditions in which MSCs are cultured are also important since this contributes to the variations in size, morphology, proliferation potential, alkaline phosphatase levels and osteogenic capacities *in vivo* (Friedenstein *et al.,* 1982). Subsequent groups identified the multi-lineage potentials of MSCs (Beresford *et al.,* 1992; Umezawa *et al.,* 1992; Dorheim *et al.,* 1993; Dennis *et al.,* 1999) and demonstrated that marrow stromal cell cultures are an admixture of cells, including reticular cells, adipocytes, osteogenic cells, smooth muscle cells, endothelial cells and macrophages, with varying developmental potentials and that culture conditions used to expand the cells *in vitro* do not support long-term maintenance of the phenotype of MSCs (see Figure 1.6). Several studies using different animal models in ovine, murine and rat have shown that bone marrow derived MSCs obtained from adult or foetal animal model have potential for cartilage repair (Seedhom *et al.,* 2007; Bernardo *et al.,* 2007).

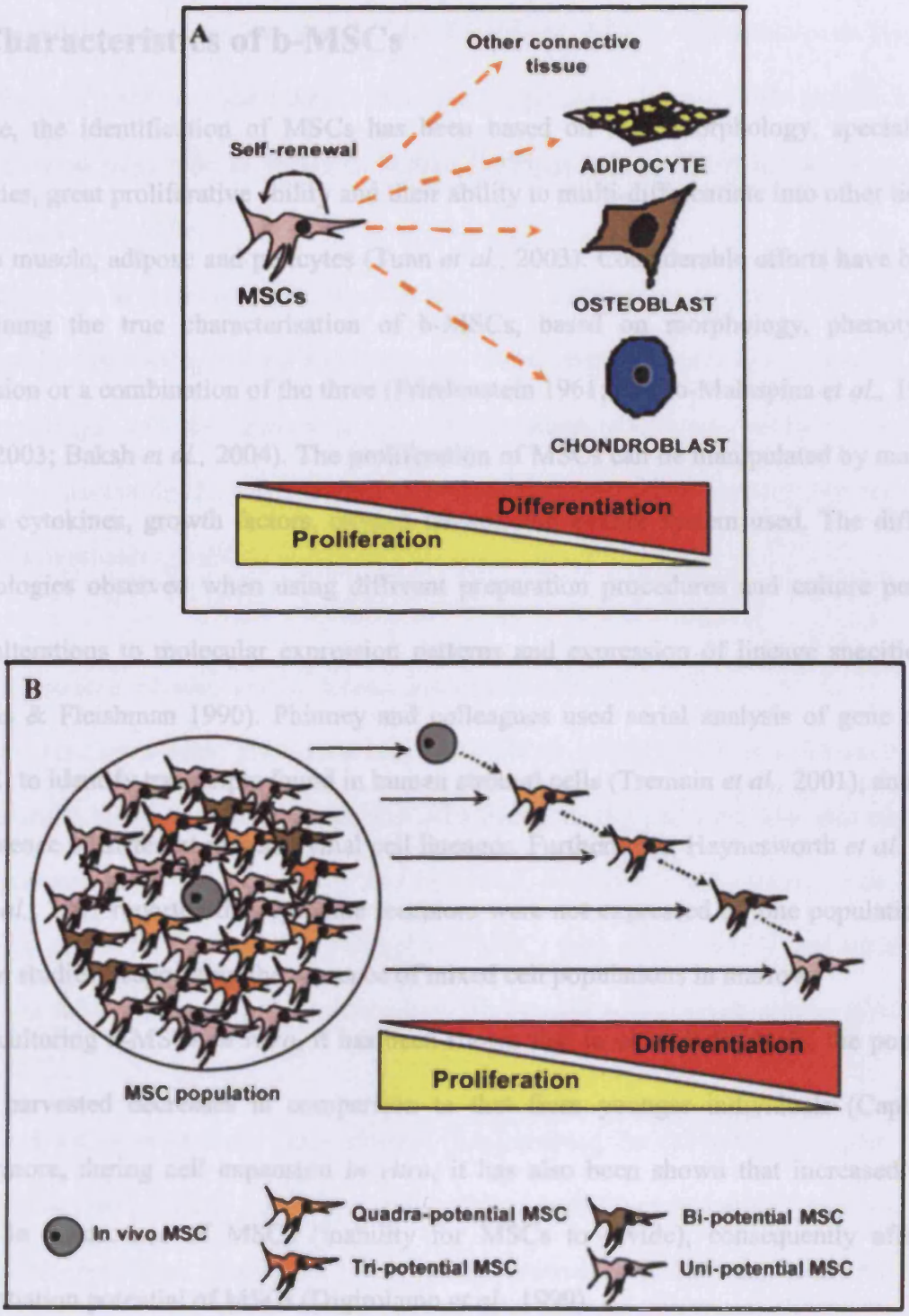


Figure 1.6: The potential of MSCs to differentiate towards different connective tissue types (Panel A) and the cell population with different differentiation potential (Panel B) (Adapted from Baksh *et al.*, 2004).

1.2 Characteristics of b-MSCs

To date, the identification of MSCs has been based on their morphology, special adherent properties, great proliferative ability and their ability to multi-differentiate into other tissue types such as muscle, adipose and pericytes (Tuan *et al.,* 2003). Considerable efforts have been made in defining the true characterisation of b-MSCs, based on morphology, phenotype, gene expression or a combination of the three (Friedenstein 1961; Castro-Malaspina *et al.,* 1980; Tuan *et al.,* 2003; Baksh *et al.,* 2004). The proliferation of MSCs can be manipulated by many factors such as cytokines, growth factors, oxygen tension and culture system used. The different cell morphologies observed when using different preparation procedures and culture periods also show alterations to molecular expression patterns and expression of lineage specific antigens (Perkins & Fleishman 1990). Phinney and colleagues used serial analysis of gene expression (SAGE) to identify transcripts found in human stromal cells (Tremain *et al.,* 2001), and indicated the presence of different mesenchymal cell lineages. Furthermore, Haynesworth *et al.,* 1996 and Lee *et al.,* 2001 reported that cytokine receptors were not expressed by one population of cells (*in vitro* studies), supporting the presence of mixed cell populations in marrow.

When culturing b-MSCs *in vitro*, it has been shown that in older individuals, the population of MSCs harvested decreases in comparison to that from younger individuals (Caplan 1994). Furthermore, during cell expansion *in vitro*, it has also been shown that increased passaging results in senescence of MSCs (inability for MSCs to divide), consequently affecting the differentiation potential of MSCs (Digirolamo *et al.,* 1999).

The proliferation of MSCs can be influenced by the addition of fibroblast growth factor (FGF) to the basal medium and by the initial seeding cell density (Solchaga *et al.,* 2005; Johnstone *et al.,* 1998; Satomura *et al.,* 1998; Digirolamo *et al.,* 1999). Together these factors contribute to the cell expansion profile and appear to govern the proliferation activity of MSCs in culture (Colter *et al.,* 2000). It is interesting to note that the addition of FGF-2 to the culture medium changes the cell morphology of MSCs from flatter, polygonal and spread out MSCs (referred to as type II

MSCs population) to smaller, spindle-like fibroblastic MSCs, (referred to as type I MSC population). In addition, type I MSCs has been suggested to have a faster growth kinetic and maybe derived from type II MSCs in human (Solchaga *et al.,* 2005; Johnstone *et al.,* 1998; Digirolamo *et al.,* 1999).

From the above, it is obvious that many factors affect the features and the behaviour of MSCs. Therefore further standardisation and improved characterisation methods are essential to further our knowledge on the behaviour of MSCs when they are subjected to different microenvironments. In the following section, we will discuss some methods that are commonly used for characterising MSCs.

1.2.1 Cell surface markers and molecules associated with MSCs

Different techniques were previously used to separate MSCs and HSCs. These include low forward angle scatter which separates the cells based on the cell size; low side angle scatter, separation method by cell granularity and by protein contents in cells (Hung *et al.,* 2002). To date, the common methods used to differentiate MSCs from HSCs involve cell surface markers to stain the cells, followed by separation using fluorescence activated cell sorting (FACS).

Continuous progress has been made towards characterising the cell surface antigenic profile of bone marrow-derived MSC population using various techniques including FACS and magnetic bead sorting techniques (Alsalameh *et al.,* 2004; Fickert *et al.,* 2004). An extensive number of antibodies have been raised to identify cell surface antigens, some of which exist on MSCs. To date the antigenic phenotype of b-MSCs has not been shown to be unique and they share features of cells found in mesenchymal, epithelial, endothelial and muscle tissues. However, it has been widely agreed that b-MSCs do not express CD45, CD34 and CD14, the typical markers for the characterisation of hematopoietic stem cells (Baddoo *et al.,* 2003). In contrast, markers such as CD29, CD44, CD71, CD90, CD106, CD120 and CD124 were found to be expressed strongly by

b-MSCs (Pittenger *et al.,* 1999). In addition to the above markers, the expression of other less MSCs – specific markers (including cytokines and growth factors together with their receptors), plus adhesion and extracellular matrix molecules such as collagens, proteoglycans and fibronectin have also shown to be expressed by MSCs (see Table 1.2) (Miguell *et al.,* 2001). Monoclonal antibodies raised against SH2, an epitope present on CD105 (or endoglin, which is part of the transforming growth factor beta receptor) (Haynesworth *et al.,* 1992; Barry *et al.,* 1999) and SH3 and SH4, epitopes present on CD 73 (activated leukocyte cell adhesion molecule, ALCAM) (Haynesworth *et al.,* 1992; Barry *et al.,* 2001a) have been used to characterise MSCs. It is interesting to note that SH2, SH3 and SH4 antibodies do not react with haematopoietic cells or osteocytes.

STRO-1 is a monoclonal antibody that reacts with non-haematopoietic, multipotent progenitor bone marrow stromal cells (Zannettino *et al.,* 2007; Song *et al.,* 2005, Dennis *et al.,* 2002; Gronthos *et al.,* 1994; Simmons & Torok-Storb 1991). Following cell isolation, monoclonal antibody raised against STRO-1 antigen is used to investigate the multi-lineage potential of MSCs (Simmons & Torok-Storb 1991; Gronthos *et al.,* 1994). Monoclonal antibody raised against the low-affinity nerve growth factor receptor was also used to characterise the multi-lineage potential of MSCs after isolation (Quirici *et al.,* 2002). SB-10 antibody reacts with an antigen on undifferentiated MSCs, which disappears once the cells embark along the osteogenic pathway and begin to express cell surface alkaline phosphatase (Stewart *et al.,* 2003; Bruder *et al.,* 1998; Bruder *et al.,* 1997). SB-10 antigen has been identified as CD166 (activated leukocyte-cell adhesion molecule). However, all these antigens do not provide the specificity needed for *in vivo* evaluation since they are also expressed on other cell types.

Analysis of cell surface molecules indicates that MSCs express a large spectrum of cell adhesion molecules, such as integrins, which have potential importance in cell binding and homing interactions (Docheva *et al.,* 2007). MSCs have a high expression of integrins alpha (α) -1, -5 and beta (β) 1 (Ip *et al.,* 2007; Lee *et al.,* 2004) and a low expression of integrins α -4, -L and β -

2. Expression of specific integrins by MSCs may contribute to the homing mechanisms for the sites of injury, binding to specific matrix molecules (Chang *et al.,* 2007) and improving their differentiation potential towards specific lineages (Varas *et al.,* 2007). Table 1.2 is a generalised table to summarise the types of markers, molecules and receptors that are currently found to exist on MSCs.

It is important to note that the expression of cell surface molecules may vary due to different culture length of time, culture conditions and sample preparations. Therefore it is important to maintain a standardised protocol when culturing MSCs *in vitro* in order to minimise the variation between batches of cell culture (Devine 2002; Jackson *et al.,* 2002). Currently, the commonly used *in vitro* conditions for chondrogenic differentiation of MSCs are high glucose, serum-free culture medium with the supplementation of Dexamethasone and selected pro-chondrocyte factors such as TGF-β3, FGF-2 and BMPs (Johnstone *et al.,* 1998; Mackay *et al.,* 1998; Yoo *et al.,* 1998; Pittenger *et al.,* 1999; Barry *et al.,* 2001b). In order to create a more standardised isolation protocol and a better optimised cell culture conditions for the *in vitro* study of MSCs, it is essential for us to gain a better understanding on the molecular signalling pathways for chondrogenesis. This will therefore enable us to improve the development on the conditions required for directing MSCs towards a chondrogenic lineage.

Markers	Designation
Specific antigens *(molecules that are on cell surface that stimulate an immune response)*	SH-2, SH-3, SH-4 STRO-1 A-smooth muscle actin MAB 1740
Cytokines and growth factors *(Proteins that act as signalling compounds that stimulate cellular proliferation and differentiation)*	Interleukins: 1α, 6, 7, 8, 11, 12, 14, 15 LIF, SCF, Flt-3 ligand GM-CSF, G-CSF, M-CSF
Cytokines and growth factor receptors	IL-1R, 3R, 4R, 6R, 7R LIFR, SCFR, IFNγR, TNFIR, TNFIIR, TGFβIR, TGFβIIR bFGFR, PDGFR, EGFR
Adhesion molecules *(cell surface proteins that are involved in binding with other cells or ECM)*	Integrins: αvβ3, αvβ5 Integrin chains: α -2, -3, -4, -5, αv, β3, β4 ICAM-1, ICAM-2 VCAM-1 LFA-3, L-selectin, CD44
Extracellular matrix	Collagen type I, III, IV, V, VI Fibronectin, Laminin, Hyaluronan, Proteoglycans

Table 1.2 Specific antigens, cytokines receptor, adhesion molecules and production of cytokines and matrix molecules on bone marrow - derived mesenchymal progenitors (Haynesworth *et al.*, 1992; Caplan & Bruder 2001; Miguell *et al.*, 2001; Dennis *et al.*, 2002).

1.3 The mesengenic process of chondrogenesis during embryonic development

During embryonic development, the initial differentiation of MSCs into each of the different lineages is controlled by unique 'bioactive' factors termed osteogenic (O-MSF), chondrogenic (C-MSF), myogenic (M-MSF) mesenchymal stimulating factor (Caplan 1994; Majumdar *et al.*, 1998). The cells undergo the processes of proliferation, differentiation and maturation where they eventually commit to and differentiate into specific lineage. During these stages, the cells undergo distinct changes in which they synthesize different ECM proteins at different stages. Figure 1.7 is a schematic diagram that outlines chondro- and osteo- genesis of b-MSCs and illustrates how b-MSCs proliferate, commit and differentiate into cartilage and bone respectively. The chondrogenesis of MSCs can be described in three different stages, 1) mesenchymal cell proliferation and condensation, 2) chondroprogenitor proliferation and differentiation and 3) chondrogenesis and terminal differentiation.

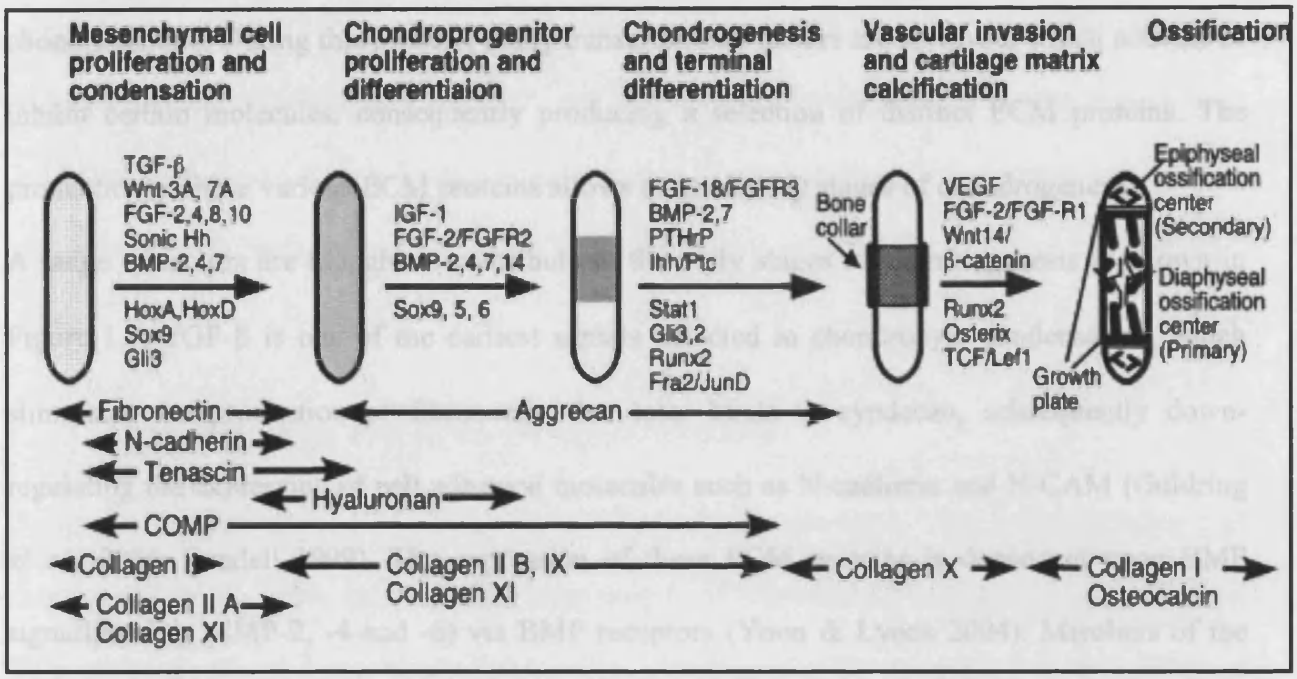


Figure 1.7: A diagram to illustrate the sequence of events that occur during chondrogenesis. The spatial growth patterns and differentiation factors (above the forward arrows) and the transcription factors (below the forward arrows) are shown. The extracellular matrix proteins that distinguish the different stages of chondrogenesis are also indicated (indicated by the reverse arrows) (Adapted from Goldring *et al.*, 2006).

1.3.1 Mesenchymal cell proliferation and condensation

Mesenchymal stem cell proliferation and condensation are the first events to occur during chondrogenesis. During this process, many transcriptional factors are involved, which activate or inhibit certain molecules, consequently producing a selection of distinct ECM proteins. The production of these various ECM proteins allows us to identify stages of chondrogenesis.

A range of factors are thought to contribute to the early stages of chondrogenesis as shown in Figure 1.7. TGF-β is one of the earliest signals detected in chondrocyte condensation, which stimulates the production of fibronectin that later binds to syndecan, subsequently down-regulating the expression of cell adhesion molecules such as N-cadherin and N-CAM (Goldring *et al.,* 2006; Sandell 1999). The expression of these ECM proteins is dependent upon BMP signalling (e.g. BMP-2, -4 and -6) via BMP receptors (Yoon & Lyons 2004). Members of the Wnt and FGF signalling families have been shown to interact to maintain expression of certain molecules such as FGF-10 and induce a positive feedback loop through Wnt 8 and 10, (Ornitz 2005). Other factors play a role in this stage of chondrogenesis, including Hox 11 and 13, which regulate cell proliferation, and Hox A and D, which support the expression of FGF-8 and Sonic Hedgehog genes (Shh) (Goldring *et al.,* 2006). Prior to condensation, prechondrogenic MSCs produce hyaluronan and collagens type I and IIA, which contributes to matrix assembly (Knudson 2003).

1.3.2 Chondroprogenitor proliferation and differentiation

During chondroprogenitor proliferation and differentiation, expression of many ECM molecules is turned on including collagen type IX, collagen type II, aggrecan, and link protein. Collagens type IIB, IX and XII appear to be distinct ECM proteins of chondroprogenitor cells. The expression of such ECM proteins is induced by the co-expression of Sox-5 and Sox-6 (de Crombrugghe *et al.,* 2001). Sox-9 and members of the FGF family are continuously expressed,

suggesting that they may be important for maintaining signalling pathways involved in cellular proliferation and differentiation. In contrast, Wnt signals are down regulated during this stage, suggesting that they are involved in earlier events of chondrogenesis. During chondroprogenitor proliferation and differentiation, cell-cell and cell-matrix interactions and the association with cell adhesion molecules contributes to the formation of gap junctions and changes that occur in cytoskeletal structures (Shum & Nuckolls 2002; Goldring *et al.,* 2006).

1.3.3 Chondrogenesis and terminal differentiation

During chondrogenesis, BMPs are involved in stimulating the differentiation of chondroprogenitor cells and regulate later stages of chondrocyte maturation and terminal differentiation to a hypertrophic phenotype. During chondrogenesis, the activation of transcription factors such as Runx, Stat and Fra2/JunD and PTHrP contributes to the production of and decline of ECM proteins such as collagen X and collagens IIB, IX and XII. At this stage, the hedgehog family instead of being regulated by Wnt, is now modulated by patched (Ptc), (a Shh receptor), this clearly shows that throughout chondrogenesis of MSCs, a constant change of transcription factors is occurring in order to result in production of the appropriate ECM proteins at each stage. Beyond this stage, the matured matrix will begin to calcify through the influence of other factors such as osterix and VEGF, which eventually cause the cells to further differentiate towards the bone lineage (Reddi 1998; Chung 2004).

Previous *in vitro* studies carried out using human MSCs have demonstrated some of the culture conditions necessary for the chondrogenesis of MSCs. Commonly, MSCs are cultured in serum-free medium with the presence of nutrient supplements and the pro-chondrogenic factors such as dexamethasone and TGF-βs (Yoo *et al.,* 1998; Johnstone *et al.,* 1998; Murdoch *et al.,* 2007). These published work suggested that in order to convert MSCs derived from various locations successfully into chondrocytes, it is necessary to create a microenvironment *in vitro* which is

similar to that during embryonic development. In the following section, the differentiation of MSCs into chondrogenic lineage will be discussed in detail.

1.4 Differentiation of MSCs to a chondrogenic lineage

In order to use stem cells successfully in regenerative medicine for the repair of cartilage, it is important to have a well-defined and efficient protocol, which helps to direct stem cells into this specific chosen lineage. This therefore will prevent stem cells differentiating spontaneously into any lineage other than the chondrogenic lineage. In addition, the defined media may help to improve the integration and engraftment within the recipient tissues. The conditions currently known to be required for the differentiation of MSCs towards a different chosen lineage are shown in Figure 1.8. Since this project relates to the differentiation of b-MSCs into chondrogenic lineage, therefore much of the culture conditions described will focus or relate to chondrogenesis of b-MSCs.

1.4.1 Use of cytokines and growth factors for chondrogenic differentiation of MSCs

Cytokines and growth factors are signalling proteins that regulate cellular events through binding to target cell surface receptors, consequently promoting cell proliferation, differentiation and maturation. At present, some cytokines have been identified as enhancers, which increase proliferation and differentiation potentials of b-MSCs on certain differentiation lineages (Banfi *et al.,* 2002; Bianchi *et al.,* 2003). In terms of chondrogenesis, numerous cytokines and growth factors have been implicated, such as transforming growth factors- beta (TGF-β) and its various isoforms, fibroblast growth factor-2 (FGF-2), Insulin-like growth factor-1 (IGF-1), bone morphogenic proteins (BMPs), and interleukin-1 beta (IL-1β) (Heng *et al.,* 2004; Solchaga *et al.,* 2005), many of which display a high degree of functional overlap.

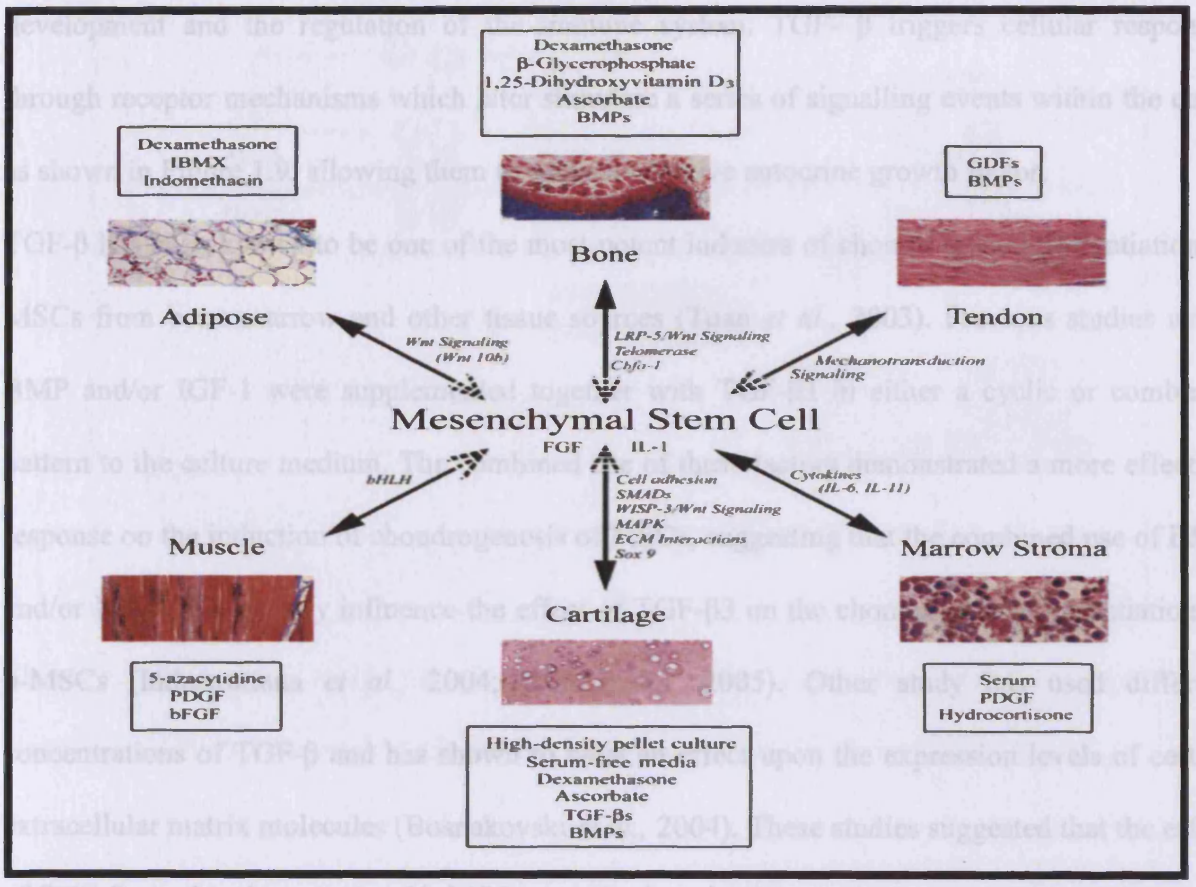


Figure 1.8: Conditions and factors necessary for differentiating MSCs towards specific lineages (Adapted from Tuan *et al.,* 2003).

During embryonic development, different factors are involved in order to induce chondrogenesis and osteogenesis (Shea *et al.,* 2003). Therefore it is important to understand the influence of different factors supplemented to the culture medium used for culturing MSCs *in vitro* for their differentiation towards a chondrogenic lineage. Common factors used for the chondrogenesis of MSCs and their effects during *in vitro* studies from previous publications will be discussed in the following sections.

1.4.1.1 Transforming growth factors – beta (TGF-β)

TGF- β are multifunctional peptides, which consist of three subtypes in human, TGF β-1, β-2 and β-3. These are responsible for tissue regeneration, cell differentiation, embryonic

development and the regulation of the immune system. TGF- β triggers cellular responses through receptor mechanisms which later stimulate a series of signalling events within the cells, as shown in Figure 1.9, allowing them to act as a negative autocrine growth factor.

TGF-β has been known to be one of the most potent inducers of chondrogenic differentiation in MSCs from bone marrow and other tissue sources (Tuan *et al.,* 2003). Previous studies using BMP and/or IGF-1 were supplemented together with TGF-β3 in either a cyclic or combined pattern to the culture medium. The combined use of these factors demonstrated a more effective response on the induction of chondrogenesis of MSCs, suggesting that the combined use of BMP and/or IGF-1 factors may influence the effect of TGF-β3 on the chondrogenic differentiation of b-MSCs (Indrawattana *et al.,* 2004; Kim *et al.,* 2005). Other study has used different concentrations of TGF-β and has shown to have an effect upon the expression levels of certain extracellular matrix molecules (Bosnakovski *et al.,* 2004). These studies suggested that the effect of TGF-β on chondrogenesis of b-MSCs may be increased by including more than one growth factor in the culture medium. Interestingly, Jin and co-workers showed that TGF- β3 appeared to have a different affect on chick leg bud mesenchymal cells where TGF- β3 seemed to inhibit chondrogenesis (Jin *et al.,* 2007). However, in their previous publication they have suggested that TGF- β3 stimulates chondrogenesis of chick wing bud mesenchymal cells (Jin *et al.,* 2006). These together indicate that cytokine TGF- β3 has different effects depending on the origin of the mesenchymal cells.

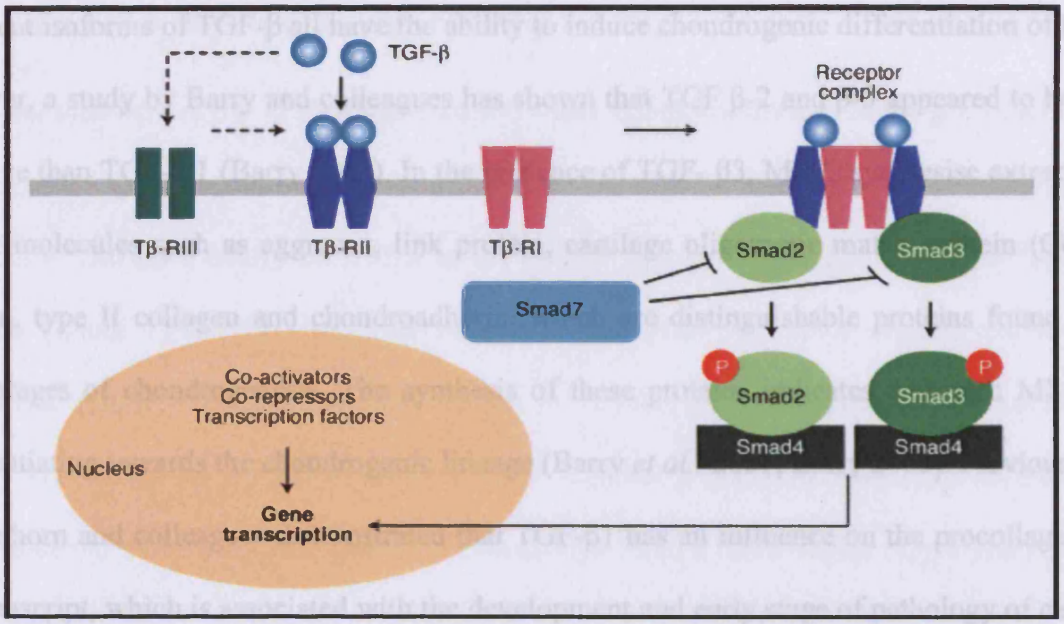


Figure 1.9: Key events within the transforming growth factor signalling pathway (Adapted from Expert Reviews in Molecular Medicine by Cambridge University Press 2003).

Different isoforms of TGF-β all have the ability to induce chondrogenic differentiation of MSCs, however, a study by Barry and colleagues has shown that TGF β-2 and β-3 appeared to be more effective than TGF- β1 (Barry 2003). In the presence of TGF- β3, MSCs synthesise extracellular matrix molecules such as aggrecan, link protein, cartilage oligomeric matrix protein (COMP), decorin, type II collagen and chondroadherin, which are distinguishable proteins found at the early stages of chondrogenesis. The synthesis of these proteins indicates that such MSCs are differentiating towards the chondrogenic lineage (Barry *et al.,* 2001; Barry 2003). Previous study by Melhorn and colleagues demonstrated that TGF-β1 has an influence on the procollagen type IIA transcript, which is associated with the development and early stage of pathology of cartilage in b-MSCs, but not in adipose-derived adult stem cells. However, TGF-β1 also induces the production of procollagen type IIB in b-MSCs only which is mainly found in mature chondrocyte cartilage (Mehlhorn *et al.,* 2006). The above studies suggested that the effect of different isoforms of TGF β on the chondrogenesis of MSCs may vary between species and the culture conditions that different research groups have used. Therefore further studies are necessary in order to have a better understanding on the effect of different isoforms of TGF-β towards the differentiation of MSCs to a chondrogenic lineage.

The effects of the TGF-β superfamily on chondrogenic differentiation are transduced through two major intracellular signalling pathways, SMAD family of signalling molecules and mitogen-activated protein kinase (MAPK) signalling, both of which are activated by TGF-β receptor complex, as shown in Figure 1.9 (Massague & Wotton 2000; Stanton *et al.,* 2003). On the other hand, other previous studies have also suggested that spontaneous chondrogenesis of bovine b-MSCs occurred without the addition of any external bioactive stimulators such as TGF- β family (Bosnakovski *et al.,* 2004). Therefore controversies exist on the necessity of such external pro-chondrogenic stimulator for triggering chondrogenesis of b-MSCs.

1.4.1.2 Bone Morphogenetic Proteins (BMPs)

BMPs are members of the TFG- β superfamily, which are synthesized as larger precursors with a hydrophobic signal sequence. They act on chemotaxis, mitosis and differentiation of progenitor stem cells. There are nearly 20 BMPs in the human genome which are crucial for biological function (Reddi 1998). *In vivo*, they have been identified to induce ectopic endochondral ossification (Urist 1965) and have been shown to be involved in stimulating the determination and differentiation of chondroprogenitor cells regulating the later stages of chondrogenic maturation and terminal differentiation of chondrocytes into a hypertrophic phenotype (Goldring *et al.*, 2006).

In vitro studies have demonstrated the ability of such protein to trigger chondrogenesis of b-MSCs through the activation of various signalling pathways such as TGF-β activated kinase (TAK1), SMAD and Ras/ERK1/2 or to cause an induction of certain transcription factors such as Jun B, D, ID and DLX family members (see Figure 1.10), which subsequently influence the downstream events that may regulate chondrogenesis (Yoon & Lyons 2004; Goldring *et al.*, 2006). BMPs exist in many isoforms and in particular, BMP-2 has been shown to be the most effective isoform which is able to give rise to a large amount of cartilage rich proteoglycans than other isoforms such as BMP-4 and -6 (Sekiya *et al.*, 2005). In addition, it is one of the commonly investigated isoforms since many studies have demonstrated its capability to trigger chondrogenesis through gene expression analysis.

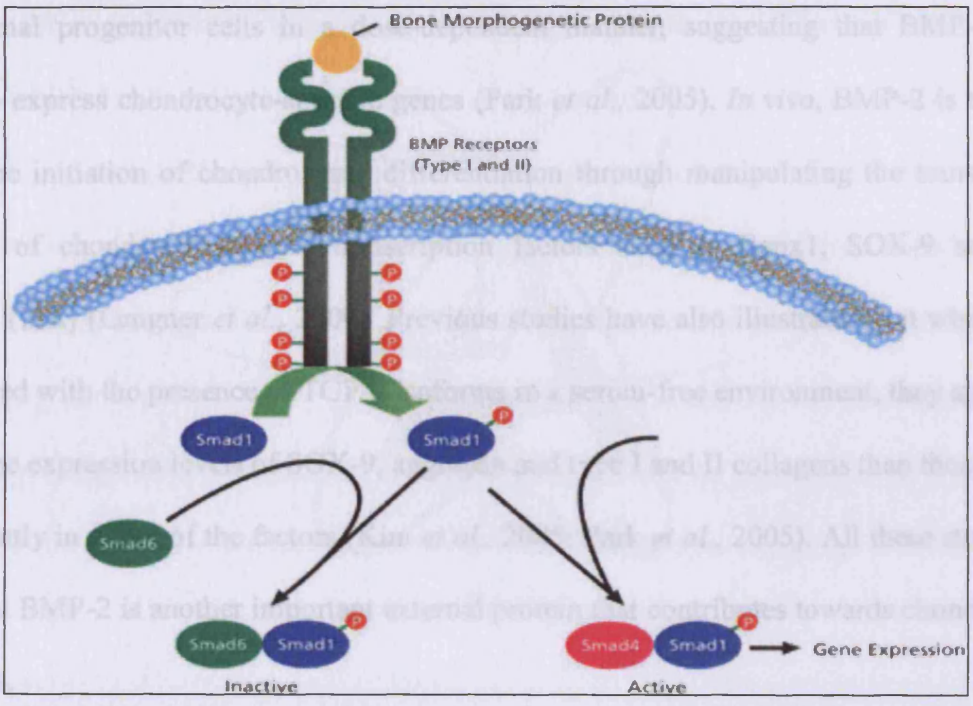


Figure 1.10: Signalling events for Bone Morphogenetic Protein. BMPs bind to BMP receptors present on the cell membrane. Signal transduction through BMP receptors result in the activation of Smad-1 via phosphorylation. Activated Smad-1 can either interact with Smad-4, which enables gene expression to occur, or Smad-6, which inactivate Smad-1 (Adapted from http://www.sigmaaldrich.com/img/assets/6460/bone_morph.gif).

BMP-2 has been shown to increase the production of SOX-9, type II collagen and aggrecan. This finding illustrated that BMP-2 contributes towards directing b-MSCs to a chondrogenic lineage. In addition, it has also been identified to regulate chondrogenesis of synovium-derived mesenchymal progenitor cells in a dose-dependent manner, suggesting that BMP-2 can be induced to express chondrocyte-specific genes (Park *et al.,* 2005). *In vivo*, BMP-2 is thought to regulate the initiation of chondrogenic differentiation through manipulating the transcriptional regulation of chondrocyte-specfic transcription factors such as Bapx1, SOX-9 and Indian Hedgehog (IHh) (Lengner *et al.,* 2004). Previous studies have also illustrated that when BMP-2 was cultured with the presence of TGF-β isoforms in a serum-free environment, they appeared to improve the expression levels of SOX-9, aggrecan and type I and II collagens than those cultured independently in either of the factors (Kim *et al.,* 2005; Park *et al.,* 2005). All these studies have proved that BMP-2 is another important external protein that contributes towards chondrogenesis of b-MSCs.

1.4.1.3 Fibroblast growth factors (FGFs)

FGFs are heparin-binding proteins that belong to the family of growth factors that are involved in wound healing and embryonic development. They are capable of interacting with cell-surface associated heparan sulphate proteoglycans (PGs) which are essential for FGF-signal transduction (Olsen *et al.,* 2003). In humans, there are 20 members within the FGF family, all of which are structurally related signalling molecules. FGF-2 (also known as basic FGF) has high and low molecular weight isoforms, which either induce and exert nuclear activities through an autocrine mechanism, or is cytoplasmic and functions in an autocrine manner, respectively. The main function of FGF-2 is to promote endothelial cell proliferation and physical organisation of endothelial cells into tube-like structures, hence promoting angiogenesis. It also associates with many developmental processes such as mesoderm induction, antero-posterior patterning and neural induction (Bötter & Niehrs, 2005). In addition, it contributes to the normal development

of both vertebrates and invertebrates, any irregularities in their function may lead to a range of developmental defects such as congenital diseases (Coumoul & Deng, 2003).

The addition of FGF-2 appeared to select the population of MSCs that is more likely to differentiate chondrogenically Bianchi *et al., 2003*. The expansion of human b-MSCs in the presence of FGF-2 will enhance their chondrogenic potential via specific regulatory molecules and signalling pathways (Solchaga *et al.,* 2005). Stevens and colleagues have demonstrated that the combined use of TGF-β with FGF-2 is able to differentiate perichondrium chondrogenically (Stevens *et al.,* 2004), generating neo-cartilage with a hyaline-like nature. The addition of the FGF-2 during the early stages of *in vitro* culture of periosteum in the presence of TGF-β significantly enhanced the cellular levels and chondrogenesis in later stages by selecting the number of committed cells that are programmed for chondrogenesis (Solchaga *et al.,* 2005).

1.4.1.4 Other components

In addition to TGF-β, BMPs and FGFs, other types of chemicals and biochemicals may also contribute to the promotion of MSCs towards a chondrogenic lineage (Heng *et al.,* 2004), described in the following sections

1.4.1.4.1 Non-proteinaceous chemicals

Dexamethasone, for example, is not a specific factor that stimulates chondrogenesis of MSCs, however, previous study by Johnstone has demonstrated that its addition to murine embryonic cell culture induced chondrogenesis and that it supports cell viability, and delays collagen type X appearance (Johnstone *et al.,* 1998). Ascorbic acid has been suggested to promote terminal differentiation of chondrocyte by increasing the production of vitamin D receptors (Farquharson *et al.,* 1998) which contributes to growth plate development and endochondral ossification. Vitamin D receptor also promotes osteoclastogenesis and regulates the production of FGF in osteoblast (Masuyam *et al.,* 2006). Altaf *et al.,* have suggested that the addition of ascorbate to

the culture medium may enhance chondrogenesis of certain cell line (Altaf *et al.,* 2006). In addition, ascorbate was also found to increase the cell numbers, protein levels and collagen content (Solursh & Jensen, 1982). The effects of these factors on chondrogenesis are induced through various signalling pathways such as SMAD (Massague & Wotton 2000) and MAP kinase (Stanton *et al.,* 2003) intracellular signalling pathways.

Glucosamine (GlcN) is a natural amino monosaccharide that is a constituent of glycosaminoglycans (GAG) found in hyaline cartilage. Recently it has been shown to influence the chondrogenesis of human MSCs (Derfoul *et al.,* 2007). Derfoul and colleagues have demonstrated that GlcN has a chondroprotective property on cartilage, but influences chondrogenesis in human MSCs. When GlcN was applied to human derived MSCs, expression of cartilage extracellular matrix components such as collagen type II and aggrecan was observed, therefore favouring chondrogenesis. Interestingly, GlcN treatment appeared to block IL-1β mediated regulations and inhibits the expression of MMP-13, which is involved in cartilage degradation (Derfoul *et al.,* 2007).

Another component that was found to have a direct effect on the chondrogenesis of b-MSCs is serum, which has shown to influence cell proliferation. Zuk and colleagues have shown that 1% serum in the media is enough to induce chondrogenic differentiation, indicating that different serum concentrations influence the lineage-specificity of b-MSCs (Zuk *et al.,* 2001) suggesting that serum does not solely affect the quality of cells but also the rate at which the cells grow. Other studies have also illustrated that the removal of serum reduces the rate of cell proliferation (Lennon *et al.,* 1995), but was still possible to culture MSCs in a serum-free environment (Schmitt *et al.,* 2003).

1.4.1.4.2 Biophysical parameters

As well as the supplements added to the culture medium that affects the behaviour and the lineage determination of MSCs, the environment to which they are exposed during culture also affects their differentiation outcome. Khan and colleagues have demonstrated the effect of oxygen tension on the chondrogenesis of human MSCs (Khan *et al.,* 2007). Elderly human MSCs derived from intrapatellar fat pad were cultured at 20% (normoxic) and 5% (hypoxic) oxygen. The different oxygen tension was shown to influence the overall ECM production, and the rate of cell proliferation (Khan *et al.,* 2007). In addition, the type of culture method used also needs to be considered since it may affect the cell-cell interaction, which promotes intercellular coupling consequently affecting the downstream events. At present, pellet and micromass culture systems have been the common systems used to study the molecular and cellular events that occur during chondrogenesis of MSCs *in vitro*. These culture systems restrict the cells to a confined space, enabling intimate contact between cells allowing cell-cell interactions (Bosnakovski *et al.,* 2004; Lengner *et al.,* 2004), which may help to induce chondrogenesis. The use of scaffolds to culture MSCs in animal models has also illustrated the potential of chondrogenesis of MSCs (Cai *et al.,* 2007). Recently, the use of Genzyme transwell model has proven the possibility of forming a tissue graft using human derived MSCs (Murdoch *et al.,* 2007).

As described above, numerous factors contribute towards directing MSCs into the chondrogenic lineage, Figure 1.11 summarises the factors that researchers have considered when attempting to differentiate MSCs into a variety of cell types for future stem cell therapies.

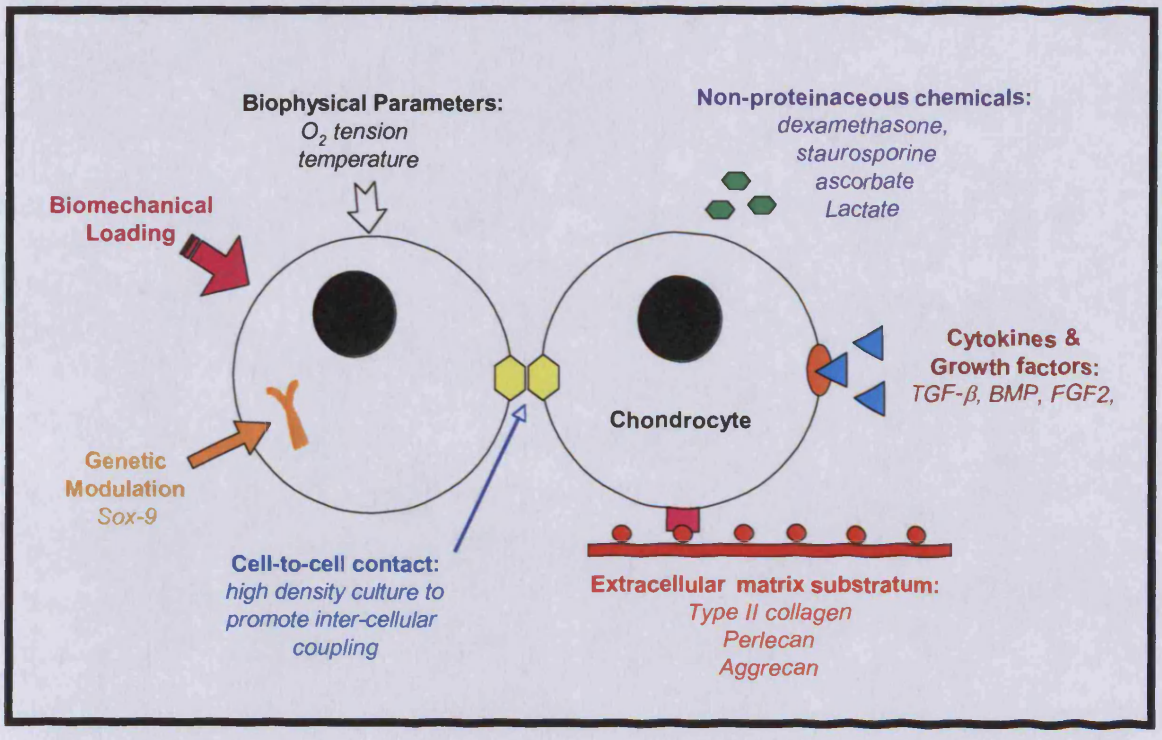


Figure 1.11: A diagram that summarises the factors that may contribute to chondrogenic differentiation of mesenchymal stem cells *in vitro*. Modified from (Heng *et al.*, 2004)

The influence of these parameters on the chondrogenesis of MSCs are commonly characterised by the expression of some chondrogeinc markers such as SOX-9, Collagen type II and Aggrecan. Other markers that were previously used are Notch receptors and their ligands such as Delta and Jagged (Oldershaw & Hardingham 2009).

1.4.1.4.2 SOX-9

SOX-9 is expressed in all chondroprogenitors during embryogenesis and its expression is directly controlled by BMP signalling. The Syr-type, high mobility group (HMG)-box containing transcription factor SOX-9, is a regulator of chondrocyte lineage (see Figure 1.12) through binding to the minor groove of the essential DNA sequence motifs in chondrocyte-specific enhancer elements of the type II and type IX collagen genes and the cartilage-derived retinoic-acid-sensitive protein (de Crombrugghe *et al.,* 2001; Lefebvre & de Crombrugghe 1998). Sox-9 is also capable of forming complexes with SOX -5 and -6 and may interact with other chondrocyte-associated transcription factors. Unlike SOX-9, SOX -5 and -6 do not contain a transcription domain. However they appear to cooperate with SOX-9 in DNA transfection experiments to activate a cartilage-specific enhancer of collagen type II and aggrecan genes (de Crombrugghe *et al.,* 2001). BMP has also been found to induce the expression of SOX-9 and Msx2, (a homeodomain transcription factor), which leads to the formation of ectopic cartilage (Shum 2002). A recent study has suggested that the overexpression of SOX-9 enhances chondrogenesis, by subsequently increasing other signalling pathways such as IHh/PTHrP and Wnt. These factors together contribute to the increased chondrogenic differentiation of MSCs (Tsuchiya 2003).

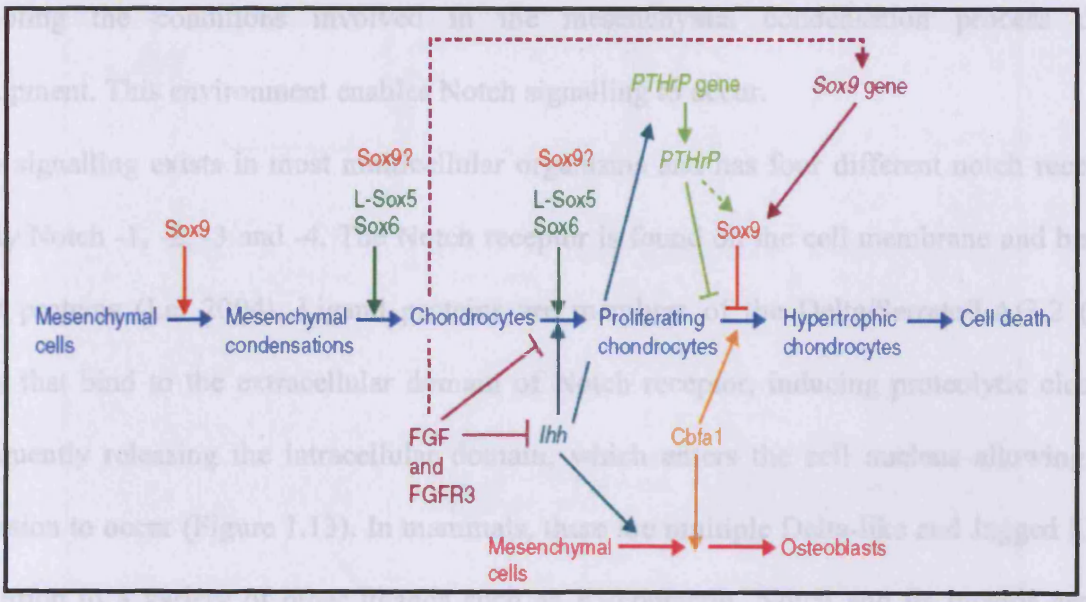


Figure 1.12: A flow chart to illustrate the possible contributions and effects of SOX-9 during cartilage development (adapted from de Crombrugghe *et al.*, 2001)

1.5 Notch signalling

When cells are cultured using a three dimensional system, this increases the cell-cell contacts resembling the conditions involved in the mesenchymal condensation process during development. This environment enables Notch signalling to occur.

Notch signalling exists in most multicellular organisms and has four different notch receptors, namely Notch -1, -2, -3 and -4. The Notch receptor is found on the cell membrane and binds to ligand proteins (Lai 2004). Ligand proteins are members of the Delta/Serrate/LAG-2 (DSL) family that bind to the extracellular domain of Notch receptor, inducing proteolytic cleavage, subsequently releasing the intracellular domain, which enters the cell nucleus allowing gene expression to occur (Figure 1.13). In mammals, there are multiple Delta-like and Jagged ligands in addition to a variety of other ligands such as F3/contactin. Notch and its ligands are both transmembrane proteins, so the cells expressing the ligands typically need to be adjacent to the Notch expressing cell for signalling to occur as shown in Figure 1.13. The Notch signalling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic development and throughout adult life.

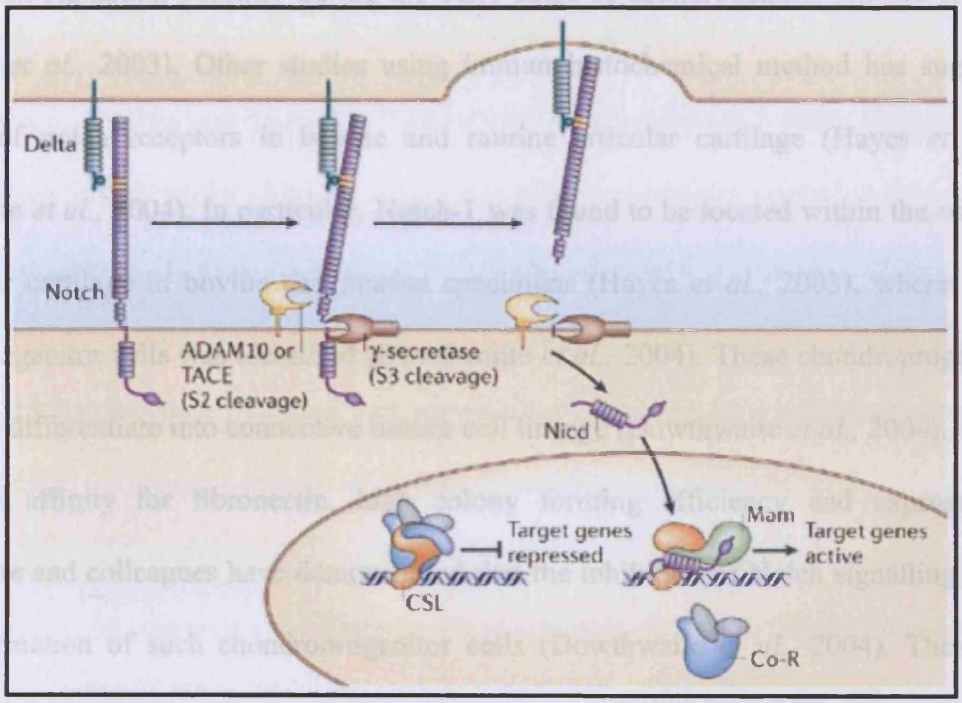


Figure 1.13: A schematic diagram to show the interaction between the Notch extracellular domain with a Delta ligand. The Tumour Necrosis Factor Alpha Converting Enzyme (TACE) cleaves the Notch protein just outside the membrane. The extracellular portion of the Notch protein is released and continues to interact with its ligand. The ligand/Notch complex is then endocytosed by the ligand-expressing cell. The enzyme gamma secretase (γ-scretase) cleaves the remaining intracellular portion of the Notch protein and this will migrate to the nucleus where it can regulate gene expression by activating the transcription factor CSL (Adapted from Bray 2006).

Previous studies carried out using murine and chick limb development have shown the activation of the Notch signalling pathway during the early stage of chondrogenesis (Crowe *et al.,* 1999; Watanabe *et al.,* 2003). Other studies using immunohistochemical method has suggested the presence of notch receptors in bovine and murine articular cartilage (Hayes *et al.,* 2003; Dowthwaite *et al.,* 2004). In particular, Notch-1 was found to be located within the surface zone of articular cartilage in bovine and murine specimens (Hayes *et al.,* 2003), where a niche of chondroprogenitor cells was identified (Dowthwaite *et al.,* 2004). These chondroprogenitor cells are able to differentiate into connective tissues cell lineage (Dowthwaite *et al.,* 2004). These cells have high affinity for fibronectin, high colony forming efficiency and express Notch-1. Dowthwaite and colleagues have demonstrated that the inhibition of Notch signalling affects the colony formation of such chondroprogenitor cells (Dowthwaite *et al.,* 2004). These together indicated the possible contribution of Notch signalling towards cartilage development and on the proliferation and differentiation of progenitor cells. Previous studies by Oldershaw and colleagues have suggested a general down regulation of Notch receptors on human MSCs cultured using a three dimensional cell aggregate system (Hardingham *et al.,* 2006). Further work from this group has demonstrated that Jagged-1 mediated Notch signalling contributes towards the initiation of chondrogenesis in human MSCs. However, such signalling must be inhibited in order to enable chondrogenesis to proceed (Oldershaw *et al.,* 2008). Another study carried out during murine skeletal development indicated that Notch-1 is strongly localised within the condensing mesenchyme during the early stages of chondrogenesis (Watanabe *et al.,* 2003).

1.6 Application of MSCs for the repair and regeneration of articular cartilage

When cartilage reaches maturity, the metabolism of chondrocytes drops dramatically. For cartilage tissue to perform its physiological function of dispersing compressive load during joint articulation, a balance between synthesis and degradation is vital. However, factors such as age, genetics, obesity, trauma, overload and instability may contribute to the biochemical changes within the matrix that cause the imbalance between the synthesis and degradation of the cartilage ECM, leading to the onset of degenerative joint diseases such as arthritis.

Arthritis is the most common joint disease affecting people aged over 65, therefore it is a major cause of pain and disability in the elderly. In the UK alone, approximately eight million people suffer from arthritis. It is a form of articular cartilage disease, which involves the loss of cartilage proteoglycan and catabolism of collagen. Together these cause erosion of the surface of the joint. In the early stages of the disease, aggrecan metabolites are released from the cartilage matrix and are detected in the synovial fluid (Lohmander 1991). Aggrecan catabolism is carried out by aggrecanases (Hardingham 1995), and is followed by breakdown of collagen type II (Hollander *et al.,* 1994) in the later stages of arthritis. Collagen breakdown results in irreversible damage to the cartilage. The two most common forms of arthritis are osteoarthritis (OA), usually caused by injury or trauma, and rheumatoid arthritis (RA) that is due to an influx of inflammatory cells (Feldmann *et al.,* 1996), releasing inflammatory cytokines and inducing the synthesis of matrix proteases that degrade the cartilage matrix molecules (Chu *et al.,* 1992).

1.6.1 Regeneration and repair of articular cartilage

Articular cartilage generally does not heal, or heals partially under certain biological conditions. Lesions usually lead to disability and symptoms such as joint pain. Treatments for lesions in the articular cartilage surface have historically included a variety of clinical procedures ranging from very conservative therapies to invasive surgery and including debridement, abrasion, perichondral grafting and mosaicplasty. These techniques reduce the necessity for open joint procedures, which can be risky particularly when performed on elderly patients. For patients with end stage OA or RA, total joint replacement may be the only option to relieve pain and prevent further damage to the subchondral bone. In some severe cases, replacement of an OA affected joint and replacement with an artificial joint to help mobility may be the only option (Hunziker 2001). However, there are disadvantages to using the above repair methods including expense, skeletal mal-alignment or changes in transarthrodial loading pattern, which may exacerbate the osteoarthritic condition, leading to the eventual amputation of the affected joint (Hunziker 2000 & 2001).

A recent approach to cartilage repair has involved the use of tissue engineering techniques. These have been used for a wide range of clinical applications for structural (such as cosmetic reconstruction), functional (such as musculoskeletal tissues) replacements, and for wound healing (such as autologous skin grafts in burn patients, chronic wound and corneal replacement). Tissue engineering involves the *ex vivo* production of replacement organs and tissues using i) cell lines, ii) natural products and / or iii) synthetic biomaterials as scaffolds to assist the repair, regeneration, remodelling and eventual structural and/or functional replacement of injured, diseased and pathological tissues. Cell precursors can be obtained from autologous and/or heterologous cells as well as from stem cells. Such stem cells can either be multipotent or pluripotent and are capable of forming a variety of mature cell types, derived from healthy or pathological tissue (Hunziker 2000 & 2001).

An example of cell-based technique is a method known as autologous chondrocyte transplantation (ACT), which was first described by Matts Brittberg and Lars Peterson in the early 1990's. This went on to be commercialised by Genzyme (Brittberg 1999; Brittberg *et al.*, 1994). ACT involves transplanting the autologous cultured chondrocytes harvested from a low load bearing region of the cartilage via an arthroscopic procedure, into a debrided articular cartilage defect void, which helps to repair any symptomatic cartilage defects of the femoral condyle caused by acute or repetitive trauma Figure 1.14 (Kim *et al.*, 2003). However, the removal of autologous cartilage sample from a healthy region may subsequently lead to the initiation of another site of pathology. Therefore it is important to seek an alternative tissue engineering method

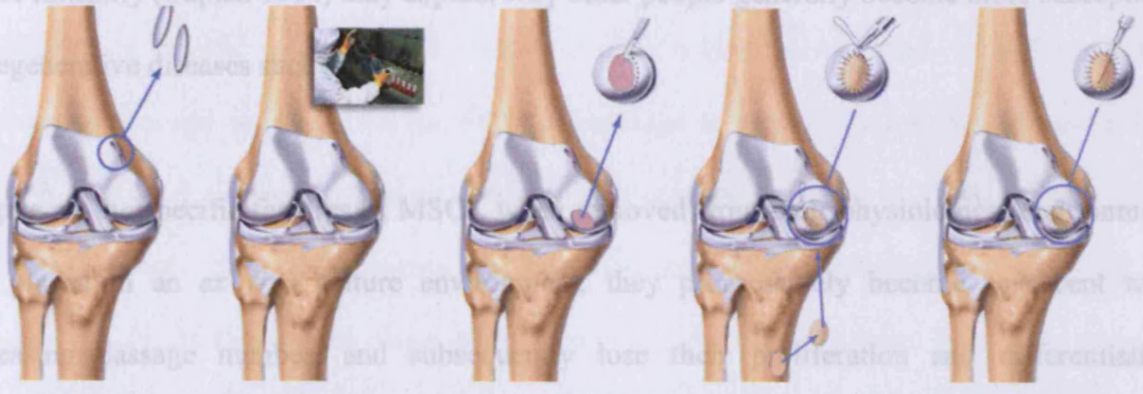


Figure 1.14: Autologous Chondrocyte Transplantation (ACT). Healthy chondrocytes are harvested from a low weight bearing region of the cartilage and undergo cell culture. Debridement is applied to the damaged region of the cartilage and a periosteal flap is sutured over the defect void. Cultured chondrocytes are then implanted into the debrided cartilage area (Adapted from www.genzymebiosurgery.com)

1.6.2 Stem cells and tissue engineering

Stem cells are interesting and attractive candidates for tissue engineering on various mesenchymal tissues including cartilage because they are hypoimmunogenic and can evade allorejection, in addition to their self-renewal and broad multi-lineage differentiation potentials. Due to their unique properties and developmental plasticity, a great interest has emerged in using b-MSCs to repair damaged tissues and for tissue engineering of different organs including heart, cartilage and bone in order to replace a faulty organ (Tsonis 2002; Ryans *et al.,* 2006). However, it is important to note that the overall performance of b-MSCs can have a dramatic impact on the overall health status of individuals. The number of b-MSCs controls the ability to remodel, repair and upon demand, rejuvenate various tissues naturally. The reducing number of b-MSCs with age or infirmity (Caplan 1994) may explain why older people generally become more susceptible to degenerative diseases such as OA.

Despite all the specific features of MSCs, when removed from their physiological environment and placed in an *ex vivo* culture environment, they progressively become senescent with increasing passage number, and subsequently lose their proliferation and differentiation potentials (Mackay *et al.,* 1998; Banfi *et al.,* 2002), hindering the development of MSC-based therapies (Conget & Minguell 1999; Digirolamo *et al.,* 1999). Hence the correct culture medium, the appropriate factors or stimulants and the timing at which these factors are added need to be identified in order to create an optimum culture environment for b-MSCs. In order to generate a tissue using MSCs that resembles articular cartilage, it is important to understand its morphology in further detail.

1.7 Morphology of Articular Cartilage

Articular cartilage is a hyaline cartilage only 2 – 5 millimeters thick that covers the subchondral bone in all diarthrodial joints. It is a highly complicated tissue that is hypocellular, avascular and aneural (Buckwalter & Mankin 1998; Poole *et al.,* 2001), which provides a low friction articulation surface by absorbing and distributing mechanical load (Akeson *et al.,* 2002). Following endochondral ossification, cartilage relies heavily on diffusion of nutrients and the exchange of metabolites from the articular surface, resulting in its low reparative potential (Archer *et al.,* 2003; Archer & Francis-West 2003; Ulrich-Vinther *et al.,* 2003).

1.7.1 Extracellular Matrix (ECM)

The ECM of articular cartilage is primarily composed of collagens, proteoglycans and water. The combination of specific collagen fibril orientation, distribution and 'location' of different types of proteoglycans and water, allows the ECM of cartilage to disperse any applied forces on the articular joint and provides a smooth, frictionless surface enabling joint movement. It is interesting to note that whilst matrix glycoproteins and plasma proteins are produced by chondrocytes during the various stages of differentiation, they are at a lower level of expression in terminally differentiated chondrocytes (Sandell 1999).

1.7.2.1 Collagens:

Collagen is a rod-shaped protein found to be most abundant in the ECM of all mammals and plays an important role in development and tissue homeostasis (Kielty *et al.,* 2002; Boot-Handford 2003). It contributes to the structure of all connective tissues by forming fibrous elements and also provides stability to other tissues and hence helps to maintain their structural integrity (Gelse 2003; Gustafsson 2003). Collagen contains at least 20 amino acid residues in the sequence of Glycine-X-Y where X and Y is proline or 4-hydroxyproline residues, occasionally, 3-hydroxyproline, rarely found elsewhere in the body (Kadler 1996). In each chain, this

sequence forms a left-handed helix and the chains wind around each other in a right- handed super triple helix. Twenty-eight members within the collagen family have now been identified and can be divided into separate classes according to their structures and functions as shown in Table 1.3 (Myllyharjn & Kivirikko 2001; Fitzgerald & Bateman 2001; Young *et al.,* 2000; Sato *et al.,* 2002; Eyre 2004; Veit *et al.,* 2006). Mutations of collagen genes have been linked to the onset of pathological diseases such as osteogenesis *imperfecta* and chondrodysplasia (Gustafsson 2003).

Collagens in Articular Cartilage

In articular cartilage, collagen is the major proteinacous component, occupying approximately two-thirds of the tissues dry weight. Generally, collagens are composed of protein monomers which are stacked in a quarter-staggered array. Each of the monomers is made up of three polypeptide chains arranged in a triple helix. In embryonic and young adolescent animals, the fibril diameter is 17 – 20 nm. In contrast, in older animals wider fibrils are found with many 20 nm fibrils protruding, suggesting an increase in fibril diameter with ageing (Bruckner & van der Rest 1994). Collagen types II, IX and XI are the key 'cartilage-specific' molecules which contribute to the mature matrix. In addition to these, types III, VI, X and XII are also present (Wotton & Duance 1994; Bruckner & van der Rest 1994; Gelse *et al.,* 2003).

Classes	Comments	Types	Tissue Distribution
Fibril-Forming collagen	These contain a large triple helical domain with around 330 Gly-X-Y repeats per chain; synthesised as large precursors; assemble into cross striated fibrils, with each molecule being displaced ¼ of its length along the axis of the fibril relative to its nearest neighbour (Burgeson, 1988)	*I*	Skin, bone, tendon, ligament, cornea, intervertebral disc and **cartilage**
		II	Vitreous, **cartilage**, intervertebral disc, body nucleus pulposus
		III	Skin, blood vessels, **cartilage**, reticular fibres of most tissues (e.g. lung, liver, spleen), intervertebral disc
		V	Skin, bone, tendon, intervertebral disc, **cartilage**,
		XI	Skin, bone, tendon, intervertebral disc, **cartilage**
		XXIV	Developing cornea, bone
		XXVII	Chondrocytes,developing stomach, lung, gonad, skin, tooth
Network Forming Collagen	Self assemble into networks with longer non-collagenous domains; tetramers formation; supercoiled structure (Hulmes, 2002)	*IV*	Basement membranes, stromal region of the cornea
		VIII	Descement's membrane, endothelial cells
		X	Calcifying **cartilage** (regulate EO of AC, Shen 2005)
Fibril associated collagens with an interrupted triple helix (FACITS)	No fibrils formed but attached to the surface of pre-existing fibrils of the fibril forming collagens (Shaw and Olsen, 1991).	*IX*	**Cartilage** and intervertebral disc
		XII	Skin, **cartilage**, intervertebral disc
		XIV	Skin, **cartilage**, intervertebral disc
		XV	Basement membranes, **cartilage**
		XVI	Skin, lung, arterial smooth muscle
		XVII	Most tissues, high levels in liver
		XIX	Most tissues, basement membranes
		XX	Corneal epithelium, tendon
		XXI	Blood vessels, smooth muscle
		XXII	Hair follicle
		XXVI	Testis, ovary
Other small groups	Beaded filaments	*VI*	Most tissues including **cartilage**
	Anchoring fibrils for basement membranes	*VII*	Anchor stroma to basement membranes in skin and cornea
	Transmembrane	*XXIII*	Prostate carcinoma
		XIII	Most tissues including skin

		XVII	Skin, muscle
Von Willebrand factor A (VWA) domain-containing proteins	Facilitate protein-protein interactions by presenting in the integrin and receptors responsible for the interaction with fibrillar collagens. It is also found in collagen types VI and VII. Several FACIT collagens also consist of VWA domains. (Veit *et al.*, 2006)	*XXVIII*	Skin, calvaria, doral root ganglia, peripheral nerves, Schwann cells in the peripheral nervous system

Table 1.1: Different classes of collagens, the functions and features, the name and location in which the collagens are distributed. (Modified from Dr Alison Rees thesis, 2004)

Collagen type II

The key collagen found in cartilage is collagen type II, which comprises 90% of the bulk collagen network in the matrix of articular cartilage (Wotton *et al.,* 1999). Collagen type II belongs to the fibril forming collagen family and is made up of three identical alpha-1 (α-1) polypeptide chains that form a triple helix from which very short amino terminal and carboxyl-terminal telopeptide domains extend at each end. Collagen type II fibrils cross-link with collagen types XI and IX to form heterotypic fibrils. Type II collagen fibrils are organised as quarter-staggered polymers, as shown in Figure 1.15. In mature cartilage, collagen type II forms fibrillar networks with finer fibrils located at the surface layer whilst the thicker fibrils lie in the deep zone. (Aydelotte & Kuetter 1988 a and b).

Collagen Type IX

Collagen type IX belongs to the FACIT class of collagens. It is present in fibrils and represents ~ 2% of the total collagen in articular cartilage. It is composed of a heterotrimer with its three alpha chains (α-1 [IX], α-2[IX] and α-3 [IX]) being products of different genes (Eyre & Wu 1995). Type IX collagen adds tensile strength to the tissue by cross-linking the type II collagen fibrils and acting to resist shear between fibrils (Akeson 2002). In addition, it facilitates fibril interactions with proteoglycan macromolecules. The globular domain, NC4 that projects out of the fibril, may be involved in molecular linkages with the inter-fibrillar matrix (Cremer *et al.,* 1998).

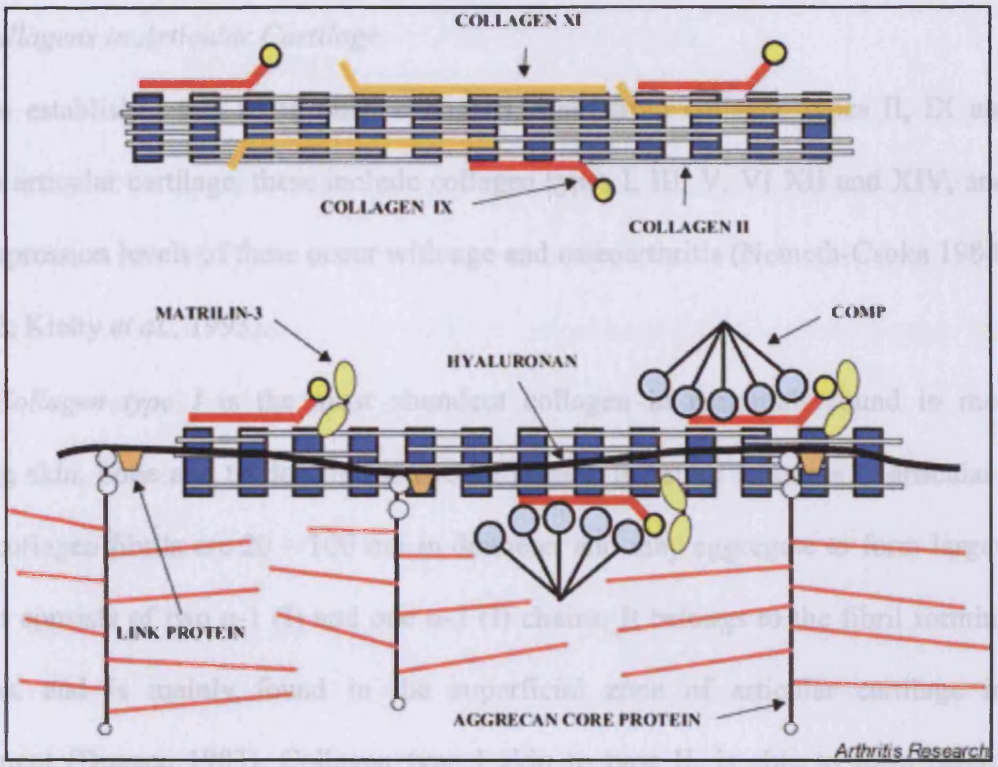


Figure 1.15: shows the interactions between collagen types II, IX and XI in articular cartilage and how the collagens interact with other ECM molecules in the matrix. (Adapted from Reginato & Olsen 2002)

Collagen Type XI

This member of the fibril forming collagen family contributes ~ 3% of the total collagen in articular cartilage tissue. It is made up of three distinct α-chains (α-1[XI], α-2[XI] and α-3[XI]) which co-assemble with collagen types IX and II (see Figure 1.15). Collagen type XI is present on the surface of, and within the heterotypic collagen fibrils.

Together, types II, XI and IX collagens form a cross-linked heteropolymer network during cartilage development (Eyre 1992; Eyre 2002a). Mutation of the genes encoding these collagens will affect the formation of a normal fibril which may lead to the onset of chondrodysplasia and destruction of joint cartilage (Eyre 1992; Cremer 1998).

Other collagens in Articular Cartilage

It is also established that some other collagens, apart from collagen types II, IX and XI, are found in articular cartilage, these include collagen types I, III, V, VI XII and XIV, and changes in the expression levels of these occur with age and osteoarthritis (Nemeth-Csoka 1984; Poole *et al.,* 1992; Kielty *et al.,* 1993).

Collagen type I is the most abundant collagen in mammals found in most tissues including skin, bone and tendon, but it is only present in minor amounts in articular cartilage. Type I collagen fibrils are 20 – 100 nm in diameter and may aggregate to form larger collagen fibrils. It consists of two α-1 (I) and one α-2 (I) chains. It belongs to the fibril forming class of collagens, and is mainly found in the superficial zone of articular cartilage in parallel arrangement (Duance 1983). Collagen type I akin to type II, is able to form heterofibrils. It associates with collagen types V and / or III (Kielty *et al.,* 1993).

Collagen type III is the second most abundant collagen in mammals and possesses elastic properties due to disulphide bonds and no lysyl oxidase dependent crosslinks in the carboxyl terminal. It consists of a homotrimer of three α -1 (III) chains and resembles other fibrillar collagens in its structure (Cheung *et al.,* 1983). This collagen copolymerises and links to collagen type II in adult human articular cartilage in significant amounts. These events lead to speculation that collagen type III may be produced by chondrocytes, in addition to collagen type II, in response to matrix damage similar to the wound healing role of collagen type III in type I collagen-based tissues (Eyre *et al.,* 2006).

Collagen type V belongs to the fibril forming collagen family and possesses high homology to collagen type XI (Gelse *et al.,* 2003).

Collagen type VI is a heteromeric and globular collagen. It forms a loose pericellular fibrillar meshwork in lacunae around the cells, known as the chondron that helps to anchor the cell membrane, maintain the integrity of the cell and protects against mechanical forces (Poole *et*

al., 1992; Poole *et al.,* 2002; Soder 2002). It has been found to be upregulated in osteoarthritic cartilage (Bruckner & van der Rest 1994; Chang & Poole 1996).

Collagen type X is a member of the network forming collagen family, which is found predominantly in the hypertrophic region of the growth plate during endochondral ossification (Nerlich *et al.,* 1992; Kielty *et al.,* 1985; Mayne & Brewton 1993; Bruckner & van der Rest, 1994; Mayne 1997). In disease states such as osteoarthritis (OA), chondrocytes appeared to synthesise an enhanced amount of collagen type X (Girkontaite *et al.,* 1996).

Collagen types XII, XIV and XV are members of the FACIT family identified in bovine articular cartilage (Watt *et al.,* 1992; Gelse *et al.,* 2003).

The function and contribution of collagens to the extracellular matrix is based on various factors including size, composition and interactions, which together create a functional matrix.

1.7.3.1 Proteoglycans:

Another important component of the matrix is proteoglycans (PGs), which occupy most of the non-collagenous area of the matrix and make up 22 – 38% of the dry weight of articular cartilage. They are complex macromolecules with high molecular weight polyanionic elements (Knudson 2001). A proteoglycan monomer consists of a central protein core with one or more negatively charged carbohydrate side-chain substituents, known as glycosaminoglycans (GAG) (Figure 1.16); common GAG include chondroitin sulphate (CS), keratan sulphate (KS) and dermatan sulphate (DS). Such GAG chains are variable in length and are made of repeating disaccharide subunits. The polyanionic nature of GAG contributes to the hydrophilic properties of PGs, enabling water retention within the ECM, creating the swelling pressure and turgidity necessary for the function of normal articular cartilage.

PG have no unifying features and they display great diversity of forms with regard to their size and the composition of their GAG side chains (Roughley PJ 1994). In addition, the biochemical composition of proteoglycan subunits has been shown to change with age (Roughley & White 1980). Abnormalities of PGs have shown to contribute to pathologies such as chondrodysplasia (Schwartz & Domowicz 2002). The different PGs found in articular cartilage is summarised in Table 1.2.

Name of PGs	Classes	Features	Reference
Aggrecan	Hyalectin	-Bind to hyaluronan & other matrix glycoproteins -KS & CS attachment between G2 and G3 domains -Loss of OA during joint degeneration	(Hardingham *et al.*, 1994; Watanabe *et al.*, 1998; Kiani *et al.*, 2002)
Decorin	Class I SLRPs (Small Leucine Rich Proteoglycans)	-Bind to TGF-beta, sequestering its mitogenic activity -A decorating PG that associate with collagen fibrils, regulating collagen fibrillogenesis -Most abundant out of all SLRPs -Detection is increased with age	(Roughley and Lee 1994)
Biglycan		-Binds to TGF-beta -Found in pericellular region of proliferating cells	
Fibromodulin	Class II SLRPs (Small Leucine Rich Proteoglycans)	-Regulate collagen fibril diameter by decorating collagen surface -Detection is increased with age	
Lumican		-In young cartilage, it exists as KS-PG -Treatment with IL-1, chondrocytes synthesise lumican devoid of GAG substitutions	(Benjamin and Ralphs 2004)
Keratocan		- Regulate collagen fibrillogenesis in tendon	
Proline arginine-rich and leucine rich repeat		-Has four potential N-linked glycosylation sites -Functions as a cartilage matrix protein with the capacity for matrix	

protein (PRELP)		organisation	
Epiphycan (PG-Lb)	Class III SLRPs	-Express during the development of growth plate -Excluded from presumptive articular cartilage and the hypertrophic zone	(Johnston 1997)
Mimecan		-Control cell growth, modulate by growth factors & cytokines	(Funderburgh *et al.*, 1997)
Perlecan	Large heparan sulphate proteoglycan	-Consists of five distinct domains -Enriched in the pericellular matrix - Accumulates during cartilage development	(Hassell *et al.*, 2003)
Superficial zone protein (SZP) or Proteogycan-4 (PRG-4)	Novel proteoglycan	Synthesised by superficial zone chondrocytes of articular cartilage -Proposed that it contribute to cell proliferation, cytoprotection, lubrication, self-aggregation & matrix binding	(Flannery *et al.*, 1999)
Syndecan	Cell surface transmembrane heparan sulphate proteoglycans	-Transmembrane heparan sulphate PGs -Interact with FGF through HS-chains -Different members of syndecans exist & are detected during various stages of chondrogenesis	(Bernfield *et al.*, 1992)
Glypican		-Glypican-1 is expressed in cartilage -Possess an extracellular region with a GAG attachment site	(Turnova *et al.*, 2000)

Table 1.2: A table that shows the types of proteoglycans found in articular cartilage

Glycosaminoglycans (GAG)

GAG are long linear polysaccharide chains, which vary in length and are made up of repeating disaccharide units. These repeating disaccharides are composed of a hexosamine and either uronic acid or galactose sugar. Such repeats also contain ester-sulphate on several of the sugar hydroxyls which contribute to the polyanionic properties of the GAG and ultimately the physiological functions of cartilage. The commonly recognised GAG include; chondroitin sulphate (CS), keratan sulphate (KS), dermatan sulphate (DS), heparan sulphate (HS), heparin (Hep) and hyaluronic acid (HA) (Paulsson & Heinegard 1984). The enrichment of GAG within cartilage creates an anion-rich environment, which allows water to be drawn in from the surrounding area, therefore enabling hydration of the ECM, subsequently allowing load to be dispersed throughout the cartilage. The GAG chains and their complex sulphation motifs also bind specific growth factors and thereby play important roles in cytokine or growth factor presentation in ECM and to cells.

Aggrecan

Aggrecan is the predominant proteoglycan present in articular cartilage (Kiani 2002). It belongs to the high molecular weight aggregating group of PG and is largely expressed in cartilage (Hardingham 1995). It is a member of the hyalectin family of proteoglycans, which has the ability to bind hyaluronan at the amino-terminus end whilst the carboxyl-end binds to other matrix glycoproteins via the G3 C-type lectin domain (Halberg *et al.,* 1988; Watanable *et al.,* 1998; Zhang *et al.,* 1998). Due to its structure, it provides the tissues' ability to resist compressive loads.

The core protein of 250 – 300 kDa contains three globular domains known as G1, G2 and G3 (Hardingham 1992; Roughley 2006). Between G1 and G2 is the interglobular domain (IGD) and glycosaminoglycans attachment region is located between G2 and G3. GAG, mainly keratan

sulphate (KS) and chondroitin sulphate (CS) are found to attach in two adjacent regions of the aggrecan core protein between G2 and G3 (Paulsson *et al.,* 1987).

G1 domain is 56kDa in weight and is located at the N-terminal of the aggrecan core protein where HA binds. It has an immunoglobulin (Ig) fold that contains similar sequences to those in the IgG superfamily of proteins. There are two PG tandem repeats (PTRs), which are also found in this domain that possess a similar sequence to that of the lymphocyte homing receptor, CD44, which is a glycoprotein that can bind to hyaluronan (Dudhia 2004).

G2 Domain has a molecular weight of 45kDa, which also has a tandem repeat region which closely resembles those in the G1. However, the G2 domain lacks HA binding activity (Watanabe *et al.,* 1997).

G3 Domain is composed of two epidermal growth factor-like domain (EGF1 and EGF2), a carbohydrate recognition domain (CRD) similar to the mammalian type C lectins and a complement regulatory protein B (short complement repeat, SCR). It is readily cleaved from secreted aggrecan. The G3 domain appears to provide the core protein with correctly folded C-terminal cap that is important for intracellular trafficking of aggrecan and preventing core protein degradation (Kiani 2002; Domowicz *et al.,* 2000).

In addition to collagens and proteoglycans, other ECM components found in lesser amounts includes, fibronectin, tenascins, cartilage intermediate layer protein (CILP), cartilage oligomeric matrix protein (COMP) and matrilins have been identified previously (Deak *et al.,* 1999). SLRPs such as decorin, biglycan and fibromodulin are also found in cartilage (see Table 1.2). The distribution, arrangements and expression levels of different collagens and PGs vary within different zones of the articular cartilage. In order to produce a tissue using MSCs that resembles articular cartilage, it is important to understand the zonal organisation of the native tissue. This will be discussed in the following sections.

1.7.2 Zonal organisation of Articular Cartilage

Mature articular cartilage is organised into histologically and biochemically distinct overlapping zones: i) superficial or tangential zone, ii) intermediate/middle or transitional zone, iii) deep or radial zone and, iv) calcified zone (see Figure 1.17). Each of the zones varies in terms of their cell morphology, collagen arrangement and which ECM molecules they contain giving unique properties to each zone (see Figure 1.18). In addition to these variations, chondrocytes located in different zones may respond differently to mechanical loading and to certain factors (Aydelotte *et al.,* 1992; Hauselmann *et al.,* 1996), suggesting that the development and maintenance of normal articular cartilage is dependent upon the unique phenotypic population of chondrocytes. Evidence has shown that the cartilaginous tissue composition, and possibly the mechanical properties may be modulated using cells from different zones of cartilage from native tissue and cultured *in vitro* within a three dimensional construct (Waldman *et al.,* 2003 a and b). Hayes and colleagues have illustrated that it is possible to reconstruct articular cartilage zonal organisation in Transwell cultures (Hayes *et al.,* 2007).

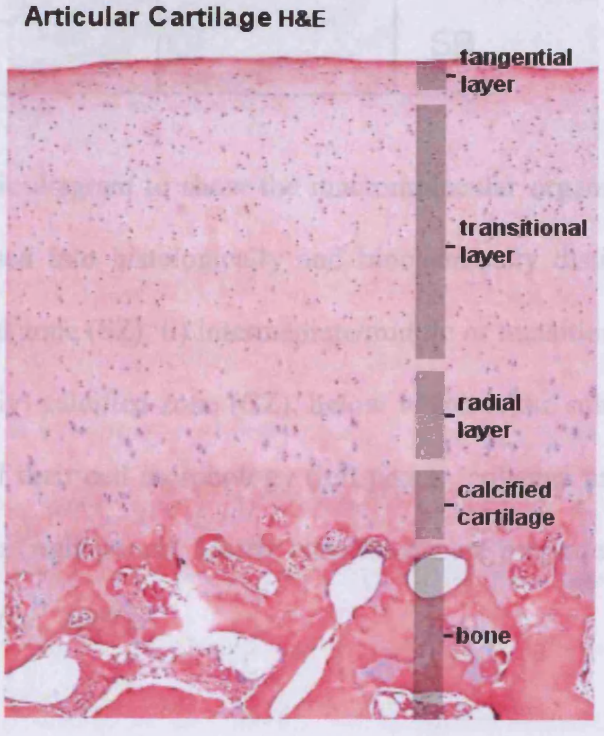


Figure 1.17: Diagram of different zonal organisation within an articular cartilage.

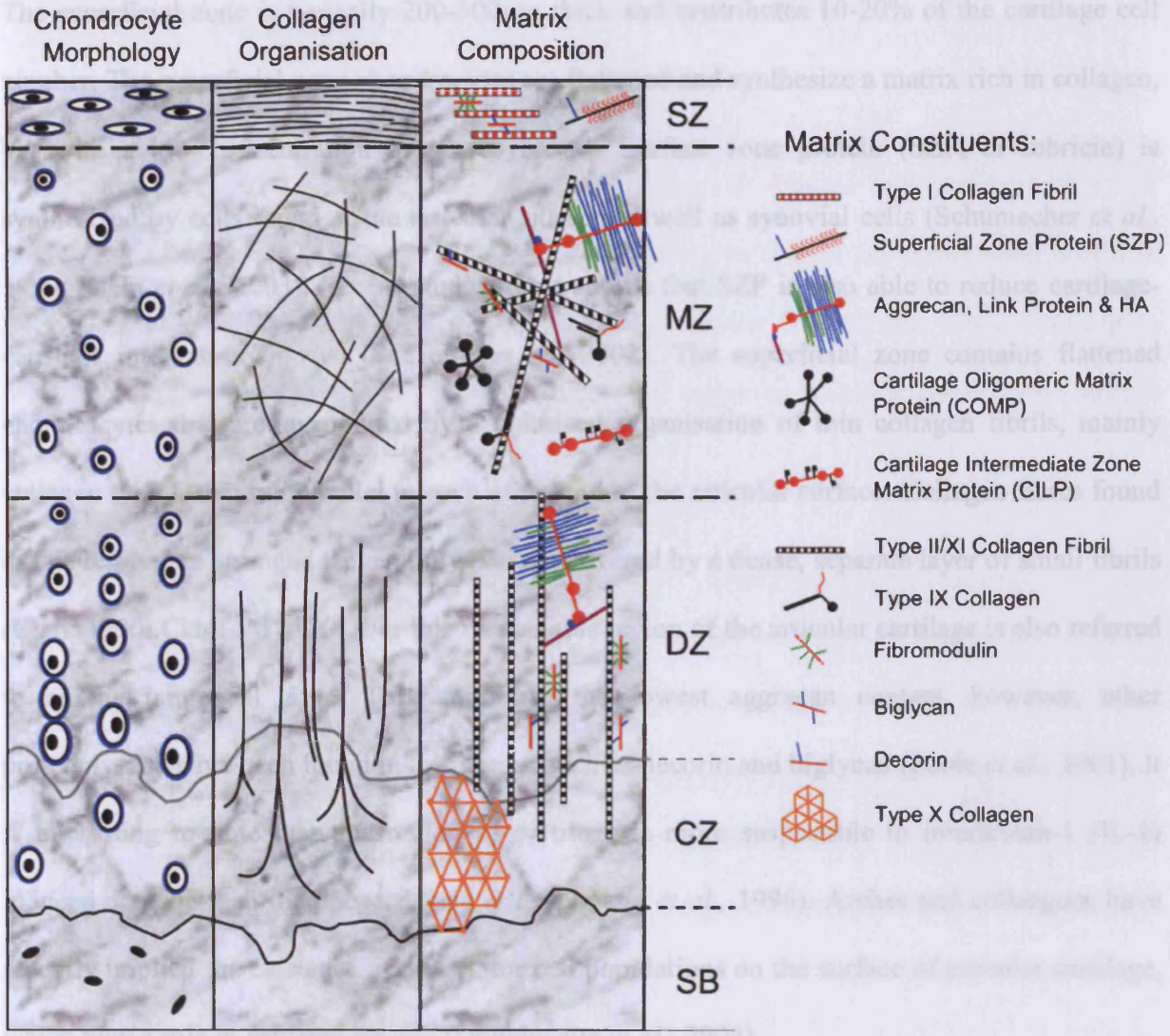


Figure 1.18: Schematic diagram to show the macromolecular organization of mature articular cartilage. It is organised into histologically and biochemically distinct overlapping zones: i) superficial or tangential zone (SZ), ii) intermediate/middle or transitional zone (MZ), iii) deep or radial zone (DZ) and, iv) calcified zone (CZ), below which is the subchondral bone (SB). Each zone varies in terms of their cell morphology (left panel), collagen arrangement (middle panel) and ECM compositions (right panel). Matrix constituents are presented as molecular schematics (adapted from Hayes *et al.*, 2007).

1.7.2.1 *Superficial Zone*

The superficial zone is typically 200-500μm thick and contributes 10-20% of the cartilage cell number. The superficial zone chondrocytes are flattened and synthesize a matrix rich in collagen, but with a low concentration of proteoglycans. Surface zone protein (SZP, or lubricin) is synthesized by cells found at the articular surface as well as synovial cells (Schumacher *et al.,* 1999; Klein *et al.,* 2003). Recent studies have shown that SZP is also able to reduce cartilage-cartilage integration *in vivo* (Schaefer *et al.,* 2002). The superficial zone contains flattened chondrocytes that are surrounded by a polarised organisation of thin collagen fibrils, mainly collagen type I, that run parallel to each other and to the articular surface. Collagen fibres found in this region are arranged tangentially and are covered by a dense, separate layer of small fibrils (Clark 1990; Clark JM 1990). For this reason, this region of the articular cartilage is also referred to as the tangential zone. This zone has the lowest aggrecan content, however, other proteoglycans have been found in this region such as decorin and biglycan (Poole *et al.,* 2001). It is interesting to note that this region of cartilage is more suspectible to interleukin-1 (IL-1) induced damage than the deeper layers (Hauselmann *et al.,* 1996). Archer and colleagues have recently implied the existence of progenitor cell populations on the surface of articular cartilage, which play a role in cartilage growth (Dowthwaite *et al.,* 2004).

1.7.2.2 Middle Zone

This is the intermediate region (or transitional zone) between the superficial and deep zones and contributes 40-60% of the articular cartilage cell number. The cells in this zone have a rounded, spherical morphology. It has the lowest cell density of all the zones, but the cells found in this region have a higher concentration of synthetic organelles than superficial zone cells (Buckwalter 1999; Hunziker 1999). Chondrocytes in the middle/deeper layers of the tissue have been found to express cartilage intermediate layer protein (CILP), which is then deposited in the interterritorial matrix (Lorenzo *et al.,* 1998). An abundant amount of collagen type II and aggrecan are found in this zone and collagen fibrils are larger and are arranged in a more random

pattern than the surface zone. In the pericellular region surrounding the chondrocytes in this zone, decorin and type VI collagens are also found (Poole *et al.,* 2001).

1.7.2.3 Deep Zone

The deep zone contributes 30% of the articular cartilage cell number and contains a low cell density but the aggrecan content and fibril diameter are higher than in any of the other zones. In contrast to the superficial and middle zones, the collagen content is very low. Within this region of the articular cartilage, the main ECM molecules are aggrecan, collagen type II and collagen type X.

1.7.2.4 Calcified Zone

The calcified zone provides a buffer, with intermediate mechanical properties, between uncalcified cartilage and subchondral bone. Chondrocytes in this zone are thought to have reached terminal differentiation and have a hypertrophic phenotype. The calcified matrix is not fully resorbed in development and resists vascular invasion. These properties provide an excellent structural integration with the subchondral bone (Buckwalter & Mankin 1998; Poole *et al.,* 2001).

1.8 Aim of this study

Current research has shown that it is possible to attach b-MSCs to an artifical matrix that can be implanted into full thickness osteochondral defects to later drive the formation of cartilage and bone, thereby generating and repairing the appropriate tissue (Qintavalla *et al.,* 2002). Previous studies by Dr Anthony Hayes and Amanda Hall within our laboratory have demonstrated the successful production of cartilage grafts using Millipore filter inserts (Kandel *et al.,* 1995). The grafts were initially produced from chondrocytes obtained from young bovine articular cartilage and seeded at high density onto filter inserts pre-coated with a selected ECM protein that helps cell attachment. After 4 weeks culture, a stratified hyaline-like tissue graft was generated, which closely resembles the *in vivo* features of young native articular cartilage, identified through histology and immunofluorescent labelling techniques. Thus, the focus and aims of my studies were:-

Aim 1: Characterisation of primary bovine MSCs by assessing the gene expression of cartilage-specific genes (SOX-9, Aggrecan and Collagen type II) and non-specific genes (Collagen type I and type X)

Aim 2: Investigate the effects of culture medium supplemented with FGF-2 or with the addition of an extra growth factor, TGF-β2, on bovine MSCs in a two dimensional culture system

Aim 3: Characterise the phenotypes of MSCs tissue grafts produced using MSCs pre-cultured in culture medium supplemented with FGF-2 or with the addition of TGF- β2 in a three dimension culture system (Transwell) seeded at high ($6x10^6$ cells) and low ($0.5x10^6$ cells) cell density

CHAPTER 2: General Materials and Methods

2.1 Materials

Histology: All commercial stains, histology slide holder, disposable blades and mounting medium were obtained from RA Lamb, Eastbourne, East Square, England. Xylene and powdered Alcian Blue were purchased from Fisher Scientific, Loughborough, Leicestershire, UK and Sigma-Aldrich, Poole Dorset, UK, respectively.

Immunohistochemistry: Immunomountant and HistoBond slides were purchased from RA Lamb, Eastbourne, East Square, England. All serum, negative controls, FITC-conjugated secondary antibodies and Dakocytomation pen were from Dako, Ely, Cambridgeshire, England. Chondroitinase ABC was obtained from Sigma-Aldrich, Poole, Dorset, UK. Keratanase and Vectashield Mountant with DAPI were obtained from Seikagaku, Tokyo, Japan and Vector, Orton Southgate, Peterborough, England, respectively.

DMMB: DMMB was obtained from SERVA and chondroitin sulphate C was purchased from Sigma-Aldrich, Poole, Dorset, UK. Multiwell plates were bought from Elkay.

SDS-PAGE/WB: 4-12% gradient gels were obtained from Invitrogen. Nitrocellulose membrane and filter papers were obtained from Whatman. NBT, BCIP and anti-mouse alkaline phosphatase were purchased from Promega.

2.2 Methods

2.2.2 Histological Techniques

2.2.2.1 Histology

Specimens were preserved in 4% (w/v) Paraformaldehyde (PFA) in Phosphate Buffered Saline (1 PBS tabet was dissolved in 100ml of water; each PBS tablet is composed of 8 M Sodium Chloride pH 7.4, 0.2 M Potassium Chloride, 1.5 M Di-sodium hydrogen phosphate, 0.2 M Potassium dihydrogen phosphate) overnight at 4°C. The specimens underwent an ascending alcohol series of 70% (v/v) ethanol (EtOH), 95% (v/v) EtOH and three times in 100% (v/v) EtOH, followed by xylene, a clearing fluid that is miscible with the dehydrating alcohol and wax embedding agent. This was followed by the impregnation of the specimen in molten wax for the final embedding stage.

Paraffin blocks containing the specimen were sectioned at 10μm using a Leitz microtome and processed for dewaxing, rehydration and staining using Alcian Blue at pH 2.5, which stains glycosaminoglycans (GAG) blue. Later counterstained with haematoxylin and eosin, as outlined in Table 2.1.

Chemicals	Time
Xylene	2 x 2 minutes
100% (v/v) EtOH	2 x 1 minutes
95% (v/v) EtOH	1 minute
70% (v/v) EtOH	1 minute
Wash in running tap water	1 minute
Stain with Alcian Blue, pH 2.5	20 minutes
Wash in running tap water	1 minute
Stain with Commercial Mayer's Haematoxylin	1 minute
Wash in running tap water	3 minutes
Stain in 1% (v/v) Commercial Eosin	2 minutes
Wash in running tap water	20 seconds
70% (v/v) EtOH	20 seconds
95% (v/v) EtOH	45 seconds
100% (v/v) EtOH	1 minute
100% (v/v) EtOH	2 minutes
Xylene	2 x 2 minutes
Mount in DPX mountant	
Leave in fumehood to dry overnight	
View under a brightfield microscope	

Table 2.1: Summary of the procedure for staining specimens using Alcian Blue, Haematoxylin and Eosin, which recognise sulphated glycosaminoglycans (GAG), cell nuclei and cytoplasm, respectively.

2.2.2.2 Immunohistochemistry

Harvested tissue was preserved in 0.5 – 1.0 ml of 95% (v/v) EtOH overnight, tissue was then transferred to ice-cold 5% (w/v) sucrose in PBS and incubated at 4°C for 1 – 3 hrs. Specimens were embedded and cryosectioned using a cryostat (Bright OTF 5000) at 10 μm and the sectioned specimen was collected onto HistoBond slides. The collected specimen was allowed to sit at room temperature for 1 – 2hrs before processing for immunostaining .

For immunohistochemical labelling, the sections were circumscribed with a water repellent ring using an ImmEdge pen to prevent cross contamination of immunoreagents. All incubations were at room temperature in a light-proof humidified chamber unless otherwise stated. The sections were soaked in PBS (pH 7.4) containing 0.1% (v/v) Tween 20® (PBS / Tween) for 10 mins prior to digestion with the appropriate enzymatic treatment(s) (refer to Table 2.2) for 1 hr at 37°C. The sections were washed in PBS / Tween20® three times for 5 mins. They were then blocked in the appropriate blocking serum (either goat or swine) at 1 in 20 dilutions in PBS / Tween20® for 30 mins. The serum was discarded and the selected primary antibody was applied using the dilution shown in Table 2.2, and incubated at 4°C overnight. The sections were washed in PBS / Tween20® 3 x 5 mins, then the appropriate FITC – conjugated secondary antibody, either Goat anti-mouse or Swine anti-rabbit, was applied at 1 in 50 dilution in PBS / Tween20® and incubated for 1 hr. Following the incubation, the sections were washed in PBS / Tween20® and mounted using Vectorshield Mountant containing 4', 6-Diamidino-2-phenylindole (DAPI), which stains cell nuclei blue. The edges of the coverslip were sealed with clear nail varnish, and the sections viewed under a fluorescent microscope (Olympus BX61).

Antibody	Dilutions	Ig	Species	Pre-treatment	Epitope	Reference
#70-XR90	1:200	Ascite	Rabbit (P)	C-ABC/K	Type I collagen	Fitzgerald Industries Int.
CIICI	1:5	IgG2a,κ	Mouse (M)	C-ABC/K	Type II collagen	DSHB/ Holmadhl *et al.*, 1986
αType X	1:100	-	Mouse (M)	C-ABC/K	Type X collagen	Gibson *et al.*, 1996
6B4	1:20	IgG1	Mouse (M)	C-ABC/K	Aggrecan (IGD domain)	Caterson *et al.*, 2000 Little *et al.*, 2002
12C5	1:5	IgG1	Mouse (M)	C-ABC/K	Versican (HA binding region)	DSHB/ Asher *et al.*, 1991
28.4	1:20	IgG1	Mouse (M)	C-ABC/K	Decorin (core protein)	Bidanset *et al.*, 1992
PR8A4	1:20	IgG	Mouse (M)	C-ABC/K	Biglycan (core protein)	Roughley *et al.*, 1993; 1994
1B5	1:500	IgG1κ	Mouse (M)	C-ABC/K	Unsulphated chondroitin (C-0-S) stub	
2B6	1:20	IgG1	Mouse (M)	C-ABC/K	Chondroitin-4-sulphate (C-4-S) stub	Couchman *et al.*, 1984 Caterson *et al.*, 1985
				C-ACII		
				C-B	Chondroitin-4-sulphate (C-4-S) stub in Dermatan sulphate	
3B3	1:20	IgM	Mouse (M)	C-ABC/K	Chondroitin-6-sulphate (C-6-S)	Caterson *et al.*, 1985
				None	Native Chondroitin sulphate chains	
5D4	1:20	IgG1	Mouse (M)	None	Native keratan sulphate chains	Caterson *et al.*, 1983
BKS-1	1:10		Mouse (M)	K	Keratan sulphate neoepitope	Dr B. Kerr
Negative Controls						
Mouse Ig	1:10		Mouse			
Rabbit Ig	1:200		Rabbit			

Table 2.2: Antibodies used for immunohistochemistry to identify extracellular matrix components within a specimen. P = Polyclonal antibody; M = Monoclonal antibody; C-ABC = Chondroitinase ABC; K = Keratanase

2.2.2 Biochemical Techniques

2.2.2.1 Extraction of Matrix Components from cartilage and tissue grafts

For extraction of PGs, samples were finely diced prior to extraction in 4 M guanidine-hydrochloric acid (pH 6.0) containing 0.05 M sodium acetate, 0.01 M sodium ethylenediaminetetraacetic acid (EDTA), 0.1 M 6-amino-hexanoic (caproic) acid, 0.005 M Benzamidine HCl, 0.5mM phenyl methyl sulfonyl fluoride (PMSF). 1 ml of extraction buffer was used per 100 mg of tissue and incubated at 4 °C for 24 – 48 hrs with continuous rolling. Following extraction, samples were centrifuged at 14000 rpm for 5 mins and the supernatant exhaustively dialysed against Milli-Q™ water. The dialysed supernatant was stored at -20°C for later analysis of matrix molecules using dimethylmethylene blue (DMMB) and hydroxyproline assays.

The remaining residue was treated with pepsin prepared in 0.5 M acetic acid (24.1 enzyme unit/mg tissue wet weight) overnight at 4°C with rolling. Then, the remaining residue was removed by centrifugation at 12,000 x g for 15 mins and the collagen content of the supernatant was determined using a hydroxyproline assay (see Section 2.2.3.4). The residue remaining following guanidine extraction and pepsin digestion was buffered in 0.005M sodium acetate pH 5.6 containing 0.025 M Na_2EDTA, 5 mM cysteine-HCl, and digested with papain (1 enzyme unit per ml of buffer) at 65°C for 18hrs with occasional mixing. Following digestion, the sGAG concentration of the supernatant was determined using the DMMB assay (refer to Section 2.2.3.2).

2.2.2.2 Dimethylmethylene Blue (DMMB) Assay

Proteoglycan (PG) concentration was measured as sGAG using the DMMB assay. This assay uses the principle of the metachromatic dye Dimethylmethylene Blue binding to the negatively charged sulphate groups on sGAG forming a dye-GAG complex. Formation of this complex produces a colour change from blue to pink, which can be read at 525 nm (Farndale *et al.,* 1986).

Standard solutions were prepared using chondroitin sulphate C sodium salt from shark cartilage. Five standards of 0, 10, 20, 30 and 40 µg/ml were prepared in Milli-Q water. 40 µl of each standard or test sample were pipetted in triplicate into the appropriate wells of a 96 well plate (Elkay). 200 µl of DMMB solution (32 mg 1,9-DMMB, 20 ml absolute ethanol, 59 ml 1M sodium hydroxide at 4 g / 100 ml, 7 ml of 98 % (v/v) formic acid and made up to 2 L with Milli-Q™ water) was added to the samples and standards, the plate was then read at 525 nm immediately on a Labsystem Multiscan MS spectrophotometer. The concentration of sGAG in the unknown sample was calculated from the linear regression standard curve using the software package Microsoft Excel and the statistical analyses were carried out using the computer software MiniTab.

CHAPTER 3:

Development of Quantitative Polymerase Chain Reaction for the quantification of cartilage-specific and cartilage non-specific genes involved in Mesenchymal Stem Cells (MSCs) differentiation

3.1 Introduction

The objectives of this chapter were to develop quantitative polymerase chain reaction (qPCR) for markers of MSCs differentiation in order to assess the gene expression of hyaline cartilage-specific (SOX-9, Aggrecan and Collagen type II) and cartilage non-specific genes (Collagens type I and X) for primary bovine MSCs (Chapter Four) cultured in different growth factors (Chapter Five) and for tissue grafts produced using passaged bovine MSCs (Chapter Six).

3.2 Materials

Primer pairs and plasmid DNA for aggrecan and collagen type II for qPCR were kindly provided by Dr Emma Blain. Collagen type I primers and plasmid DNA were kindly provided by Dr Siyuan Li at CTBL, Cardiff. Tri-reagent and Sybr Green mastermix were purchased from Sigma-Aldrich, Poole, Dorset, UK. All PCR buffers and reagents were obtained from Applied Biosystems Roche Molecular System Inc. Agarose and molecular markers were obtained from MP Biomedicals Inc., UK and BioVentures Inc., respectively.

3.3 Methods

3.3.1 Extraction of RNA

3.3.1.1 Extraction of RNA from old and young bovine articular cartilage

Total RNA was isolated from old and young bovine articular cartilage tissue harvested from the metacarpo-phalangeal (MCP) and metatarso-phalangeal (MTP) joint as positive controls for

SOX-9, aggrecan and collagen type II expressions (n=1). Approximately 100 mg of cartilage cuttings were initially weighed, snap-frozen in liquid nitrogen and homogenized for 1 minute at 2000 rpm in a liquid nitrogen chilled Braun Mikro-Dismembrator Vessel (Braun Biotech International GmbH). 1ml of Tri reagent per 100 mg tissue was added to the powdered cartilage and transferred to a sterile 1.5 ml Eppendorf tube. The vessel was washed in 5M sodium hydroxide and rinsed three times in Milli-Q^{TM} water.

3.3.1.2 Extraction of RNA from Bone Marrow harvested from young bovine metacarpal and metatarsal bones

Total RNA was isolated from bone marrow harvested from young bovine metacarpal and metatarsal bone. Approximately 5 g of bone marrow harvested from 3 – 6 separate carpo/tarsal bones was dispersed using 20 ml of sterile serum-free DMEM containing 0.05 mg/ml gentamicin, repeat this dispersal step twice. The cell suspension was transferred to a sterile 50 ml centrifuge tube and inverted gently to mix. The tube was allowed to stand until all the fatty deposits had separated. The fatty deposits and any debris were filtered through a 40 μm cell strainer (Falcon, VMR). The filtrate, containing a mixed population of red blood cells and stem cells, was centrifuged at 200 x g for 5 mins in order to obtain a cell pellet. The supernatant was discarded and the cell pellet, containing an unknown cell number of a heterogenous population of cells was resuspended in 1 ml of Tri reagent. The cell suspension was transferred to a sterile 1.5 ml Eppendorf tube.

3.3.2 RNA Isolation

RNA was phase separated by the addition of 200 μl of chloroform and the mixture was left to sit at room temperature for 15 mins. Samples were phase separated by centrifuging at 10,000 x g for 15 mins. The upper aqueous phase containing the RNA was removed and mixed with 375 μl of 70% (v/v) ethanol by gentle inversion. Qiagen RNeasy miniprep kit and reagents were used

according to the manufacturer's protocol to isolate total RNA from the sample. The mini-columns in the miniprep kit work by binding RNA to the silica-gel-based membrane allowing contaminants to be washed away by microspin technology. To remove DNA contaminants, the membranes were incubated with DNase I (80 µl, Qiagen) during the wash steps. Purified RNA was subsequently eluted in 30 µl of sterile RNase-free water (Qiagen) and quantified as shown in Section 3.3.6. The sample was then stored at -80 °C.

3.3.3 Quantification of RNA

RNA concentration and purity in each sample were measured using the Nanodrop (LabWare) at 260 nm and 280 nm. The concentration of RNA in a sample can also be calculated using the following formula:

(A260-A320) x *40 x Dilution factor x Final sample volume = RNA yield (ng)

A260 – A320 (OD readings)

40 (RNA extinction coefficient)

* The amount of DNA can be calculated by changing the coefficient to 50

3.3.4 Reverse Transcription of RNA (RT-PCR)

cDNA required for primer specific polymerase chain reaction amplification of a required target sequence was produced during the initial reverse transcription polymerase chain reaction (RT-PCR) procedure. For RT-PCR, 250 ng of RNA was used for cDNA amplification with a final reaction volume of 20 µl (See Table 3.2). The RT reaction mixture was gently mixed and incubated at room temperature for 10 mins. It was then subjected to the following cycle: 42°C for 30 mins, 99°C for 5 mins and incubated at 4°C for a short period of time in a Technegene thermal cycler. Once the RT was completed, the samples were stored at -20°C for later analysis.

RT mix	Volume
Magnesium-free 10xPCR Buffer (Final concentration: 1X)	2 µl
25 mM Magnesium Chloride (Final concentration: 2 mM)	1.6 µl
2.5 mM dNTPs (Final concentration: 0.2 mM)	1.6 µl
20 U/µl RNase inhibitor (Final concentration: 1 U/µl)	1 µl
50 U/µl Reverse transcriptase (Final concentration: 2.5 U/µl)	1 µl
50 µM Oligo d(T)'s (Final concentration:1 µM)	1 µl
Milli-Q™ water	11.8 µl – RNA volume
RNA sample *	Volume = 250 ng **Calculations:** **250 ng / RNA conc (ng/µl) = Volume for 250 ng RNA**

Table 3.2: RT-PCR reaction mixture.

3.3.5 Primer design for Quantitative Polymerase Chain Reaction (qPCR)

For amplification of SOX-9, aggrecan and collagens type I, type II and type X genes, primers were designed using sequences for each target gene obtained from Genbank (www.ncbi.nlm.nih.gov). Typically, they were designed using DNasis computer software for Macintosh. Primers were designed where sequences matched bovine SOX-9, aggrecan and collagens type I, type II and type X. Primer sequences used in this study for SOX-9, aggrecan and collagens type I, type II and type X are listed in Table 3.3 and were previously published (refer to Table 3.3).

A number of criteria had to be considered when designing the primers for qPCR (using Sybr Green assay), these included:

1. primers should be between 15 - 30 nucleotide in length
2. annealing temperature of the two primers should be within 2°C of each other, between 50 – 65°C
3. GC content of the primers should be between 40 – 60% and avoid G/C clumps at the 3'ends of the primers
4. self complementary sequences should be avoided to minimize primer dimer formation
5. the location of the primer (i.e. intron / exon boundaries) should also be considered
6. product size should range between 100 – 300 bp

Bovine glyceraldehydes-3-phosphate dehydrogenase (GAPDH, see Table 3.3) specific primers were also designed to be used as a endogenous control in RT-PCR analysis.

Proteins	Primers names	Primer Sequences	Product Size (bp)	Tm (°C)	Reference
Bovine SOX-9	7700 SOX9.F	5'-ACG CCG AGC TCA GCA AGA	71 bp	58	Shintani *et al.*, 2007
	7700 SOX9.R	5'-CAC GAA CGG CCG CTT CT			
Bovine Aggrecan	7700 aggrecan.F	5'-GCT ACC CTG ACC CTT CAT C	76 bp	60	Darling & Athansiou, 2005
	7700 aggrecan.R	5' AAG CTT TCT GGG ATG TCC AC			
Bovine Collagen Type I	7700 Col I.F	5'-TGC TGG CCA ACT ATG CCT CT	86bp	60	Mr Siyuan Li's Primers
	7700 Col.R	5'-TTG CAC AAT GCT CTG ATC			
Bovine Collagen type II	7700 col II.F	5'-AAC GGT GGC TTC CAC TTC	69 bp	60	Darling & Athansiou, 2005
	7700 col II.R	5'-GCA GGA AGG TCA TCT GGA			
Bovine GAPDH	7700 GAPDH.F	5'-GGC ATC GTG GAG GGA CTT ATG A	68 bp	60	Blain *et al.*, 2006
	7700 GAPDH.R	5'-GGG CCA TCC ACA GTC TTC TG			
Bovine Collagen type X	7700 ColX.F	5'-ACT TCT CTT ACC ACA TAC ACG TGA AAG	108 bp	60	Shintani *et al.*, 2007
	7700 ColX.R	5'-CCA GGT AGC CCT TGA TGT ACT CA			

Table 3.3: Oligonucleotide primers used in qPCR amplification of bovine SOX-9, aggrecan, collagens type I and type II. Bovine GAPDH was used as a positive control.

The primer pairs designed were assessed using a standard curve measurement generated from a serial dilution of plasmid DNA (pGEM-T vector containing primer product sequence for either bovine SOX-9, aggrecan, collagens type I and type II, and also GAPDH as a positive control) at 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and water. In this study, pGEM-T vectors containing GAPDH, aggrecan and collagen type II were already produced and kindly provided by Dr Emma Blain (Cardiff University, CTBL). pGEM-T vector containing collagen type I was kindly provided by Mr Siyuan Li (Cardiff University, CTBL).

3.3.6 Construction of pGEM-T vector containing the appropriate product sequence

pGEM-T vectors containing PCR products for SOX-9 and collagen type X were generated through a process of six steps:

1. RT-PCR to generate the PCR product and agarose gel electrophoresis to verify the product size
2. Purification to remove unwanted PCR products
3. Ligation of PCR product into a pGEM-T vector (Promega)
4. Transformation of the ligated PCR-product / vector into competent cells
5. Purification of plasmid DNA using Miniprep kit (Qiagen)
6. Restriction digest and agarose gel electrophoresis were used to confirm the size of the incorporated PCR product.

The following sections will outline the procedures carried out for each step. Figure 3.5 summarizes the steps involved to produce plasmid DNA.

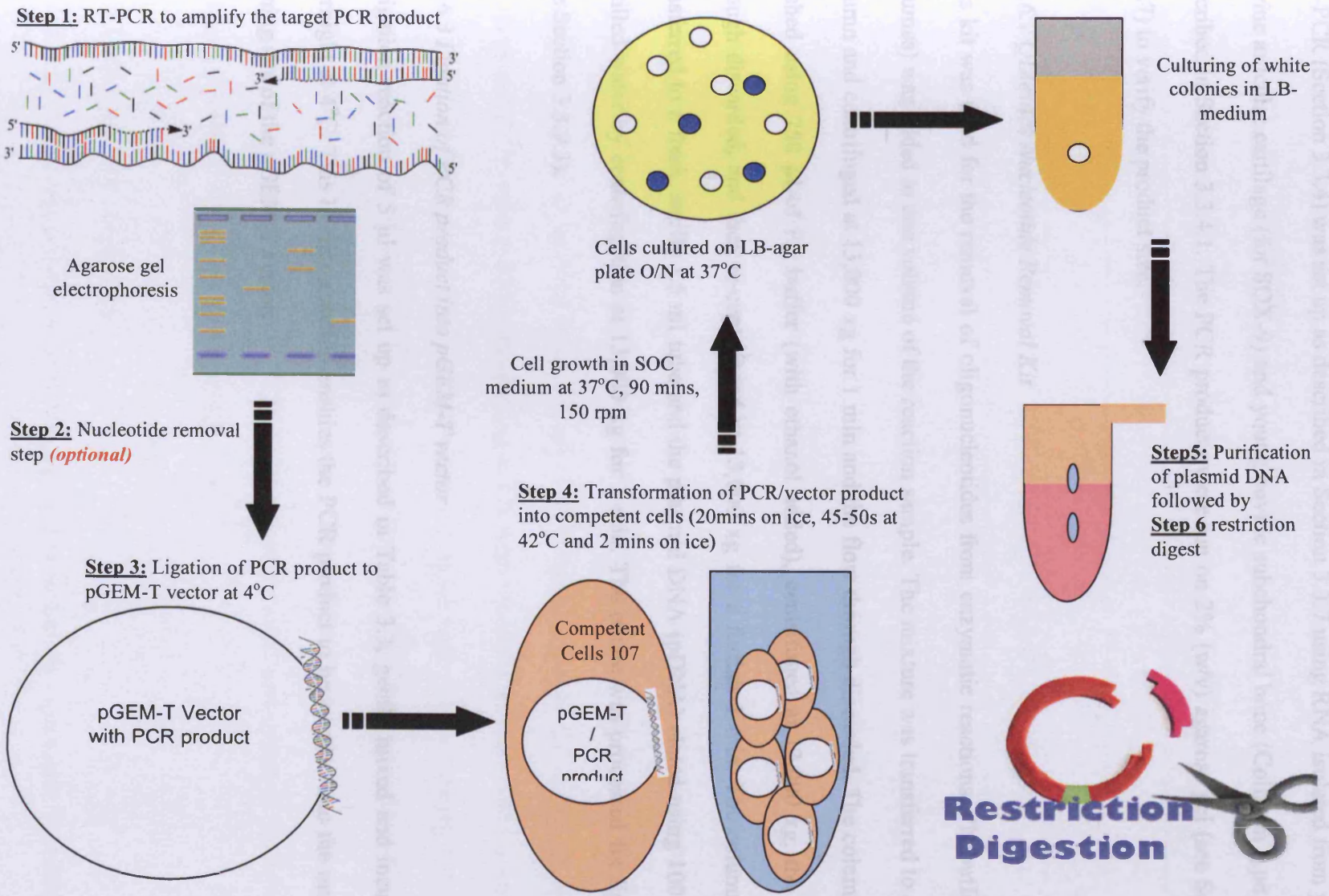


Figure 3.5: A summary diagram to methods used for gene cloning.

3.3.6.1 RT-PCR for SOX-9 and Collagen type X gel electrophoresis

RT-PCR (Section 3.3.4) was set up as described in Section 3.3.7 using RNA isolated from young bovine articular cartilage (for SOX-9) and young bovine subchondral bone (Collagen type X) as described in Section 3.3.4.1. The PCR products were run on 2% (w/v) agarose gel (see Section 3.3.7) to verify the product size.

3.3.6.2 QIAquick Nucleotide Removal Kit

This kit was used for the removal of oligonucleotides from enzymatic reactions. PN buffer (10 volumes) was added to one volume of the reaction sample. The mixture was transferred to a spin column and centrifuged at 13,000 xg for 1 min and the flow through discarded. The column was washed using 750 µl of PE buffer (with ethanol added), centrifuged at 13,000 xg, the flow-through discarded, and then re-centrifuged at 13,000 xg for a further 1 min. The column was transferred to a fresh, sterile 1.5 ml tube and the plasmid DNA (pDNA) eluted using 100 µl of distilled water by centrifugation at 13,000 xg for 1 min. The eluate was processed for ligation (see Section 3.3.9.3).

3.3.6.3 Ligation of PCR product into pGEM-T vector

A ligation reaction of 5 µl was set up as described in Table 3.3, gently mixed and incubated overnight at 4°C. This ligation reaction enables the PCR product to be inserted into the multiple cloning site of the pGEM-T vector.

Reagents	Volume for one reaction
2 X rapid ligation buffer, T4 DNA ligase **vortex briefly before use*	2.5 µl
pGEM-T vector (50 ng, *Promega*)	0.5 µl
PCR product	1.5 µl
T4 DNA ligase (3 weiss units / µl)	0.5 µl (1.5 weiss unit)

Table 3.3 Ligation reaction of PCR product

3.3.6.4 Transformation using pGEM-T vector ligation

The ligation reaction mix was centrifuged briefly and 2 µl of the mixture was transferred to a sterile bug tube containing 25 µl of JM109 competent cells (Promega), mixed and incubated on ice for 20 mins. The cell mix was heat shocked at 42°C in a waterbath for 45-50 secs, and immediately returned to ice for 2 mins. 475 µl of SOC medium was added to the cell mixture and incubated for 90 mins at 37°C with shaking at 150 rpm. When the incubation was completed, 100 µl of each transformation culture was streaked onto an LB agar plate containing 100 mg/ml of ampicillin, 500 µM of Isopropyl beta-D-1-thiogalatopyranoside (IPTG) and 50 µg/ml of X-gal. The plates were dried for 15-20 mins at room temperature, incubated overnight at 37°C. White colonies containing the ligated PCR product were selected and grown in 10 ml of LB medium overnight at 37°C with shaking at 220 rpm.

3.3.6.5 Miniprep DNA Purification

The bacterial culture was centrifuged at 10,000 x g for 5 mins and the supernatant discarded. The pellet was resuspended in 250 µl of cell resuspension solution and transferred to a sterile 1.5 ml tube. A further 250 µl of cell lysis solution was added and mixed by inversion. 10 µl of alkaline protease solution was added to the cell suspension, mixed by inversion and incubated at room

temperature for 5 mins. 350 µl of neutralisation solution was added and mixed by inversion. The mixture was centrifuged at 14,000 x g for 10 mins and the clear lysate transferred to a miniprep spin column and centrifuged at 14,000 x g for 1 min. The flow-through was discarded and the column washed in 750 µl of column wash solution (with ethanol) by centrifugation at 14,000 x g for 1 min. The flow-through was discarded and a further 250 µl column wash solution was added and centrifuged at 14,000 x g for 2 mins. The column was transferred to a clean 1.5 ml tube and the plasmid DNA was eluted using 100 µl of nuclease free water, by centrifugation at 14,000 x g for 1 min. The eluate containing pDNA was stored at -20°C. Concentration of the plasmid product was determined using the Nanodrop (Labware).

3.3.6.6 Restriction digest

The restriction enzymes Sal I and Nco I were used to cleave the multiple cloning site (which contains the cDNA sequence of interest) from the pGEM-T vector. The restriction digest was set up as described in Table 3.4. The mixture was incubated at 37°C for 2 hours and was either kept at 4°C overnight or run on a 2% agarose gel to verify the product size. Sequence analysis was also carried out using the core facilities at Cardiff University.

Reagents	**Volume required per reaction**
10 x Digest Buffer	2 µl
Sal I	1 µl
Nco I	1 µl
BSA	0.5 µl
Distilled water	10.5 µl
cDNA template	5 µl

Table 3.4 Restriction digest reaction contents.

3.3.7 Agarose Gel Electrophoresis

PCR reaction products were run for visualization on 2% (w/v) agarose gels made up in 30 ml of Tris Borate EDTA buffer (TBE; Sigma T-3913). The solution was microwaved to dissolve agarose and allowed to cool from boiling to warm. 2 µl of a 10 mg/ml ethidium bromide solution was added to the gel. The gel was poured into a PCR cassette and allowed to sit at room temperature for 10 mins and at 4°C for 10 mins. A DNA ladder of molecular weight standards was prepared by mixing 1 µl of dye solution (6 x tracking dye Cambio BV-BT-10) with 2 µl of 1x PCR buffer and 3 µl of DNA ladder (LMV 50 – 1000 bp Cambio BV-1159-01). 5 µl of all PCR reaction products were mixed with 1 µl of tracking dye. 5 µl of the DNA ladder and 5 µl of all samples were loaded into wells in the gel and run at 100 volts in 0.5x TBE until the dye front reached the bottom of the gel cassette. Bands were visualized on a UV light box and photographed.

3.3.8 Quantitative Polymerase Chain Reactions (qPCR)

PCR technology is a widely used method for quantifying DNA by amplifying the sequence of interest. In qPCR, the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule. In this study, the fluorescent signal was measured while the amplification was still progressing in order to calculate the initial template number. This measures the fluorescence at each cycle. This allows quantification of the template to be based on the fluorescence signal during the exponential phase of amplification.

In qPCR, a fluorescent DNA chelator is used to monitor the progress of the amplification reaction. With each amplification cycle, the fluorescence intensity increases proportionally with the increase in amplicon concentration, with the qPCR instrument system collecting data for each sample during each PCR cycle. Threshold cycle (Ct value) indicates the amount of initial DNA template in the sample, the earlier the Ct values for that sample, the greater the amount of initial DNA template.

Sybr green was used to measure the level of fluorescence of bound double-stranded DNA. As the amount of double stranded DNA increases, the binding sites for dye will also be increased therefore fluorescence increase is proportional to the concentration of DNA. As the target is amplified, the increasing concentration of double-stranded DNA in the solution can be directly measured by the increase in fluorescence signal. A limitation with using Sybr green is that it is based on the binding of any double-stranded molecule including primer dimers, therefore introducing errors into the analysis by inclusion of non-specific products. So primers should be designed to avoid non-specific binding. Such non-specificity can be detected by performing melting curve analysis (i.e. amplification curve) on the PCR product from every run.

qPCR reactions for the standards and the unknown samples were run in parallel with each other. qPCR reactions were prepared as shown in Table 3.5. The reaction mixture was mixed gently by vortexing, centrifuged and 24 µl of the reaction mix was placed into each of the 0.2 ml non-skirted wells on a 96 well plate (ABgene). 1 µl of cDNA was added to the mixture, mixed and when a row is completed, lids were placed over it. Typically the qPCR was set up as shown in Table 3.6.

Reagents	**Volume**
Sybr Green JumpStart™ Taq ReadyMix (Sigma)	12.5 µl
Forward and Reverse Primers (MWG; Final concentration of 200 nM)	0.5 µl
dH_2O (Sigma)	10.5 µl

Table 3.5: Reaction mix for quantitative PCR.

Segments	Time	Temperature	Cycles
One	2 minutes	95 °C	x 1
Two	2 minutes	95 °C	x 40
	40 seconds	60 °C	
	20 seconds	72 °C	
Three	1 minute	95 °C	x 1
Four	30 seconds	60 °C (heats up at every 1°C from 60°C until it reaches 95°C)	x 40

Table 3.6: A typical quantitative polymerase chain reaction (qPCR) thermal profile setup.

3.4 Results

3.4.1 Verification of newly designed primer pairs for SOX-9 and Collagen type X

Primer pairs specific for bovine SOX-9 and Collagen type X with a product size of 71 and 68 base pair (bp), respectively, were tested using young bovine full depth articular cartilage.Young bovine subchondral bone (1 µl cDNA of 500 ng) was used as a positive control for collagen type X. Young bovine articular cartilage was used as a postive control for SOX-9. Water was used as a negative control. RT-PCR mixture was prepared and carried out using Technegene PCR machine (Section 3.3.4) for 35 cycles with an annealing temperature of 60°C, other conditions were as described in Chapter 2, Section 2.2.4.5. PCR products were run on a 2% (w/v) agarose gel in order to verify the product size and the primers' function (Figure 3.15).

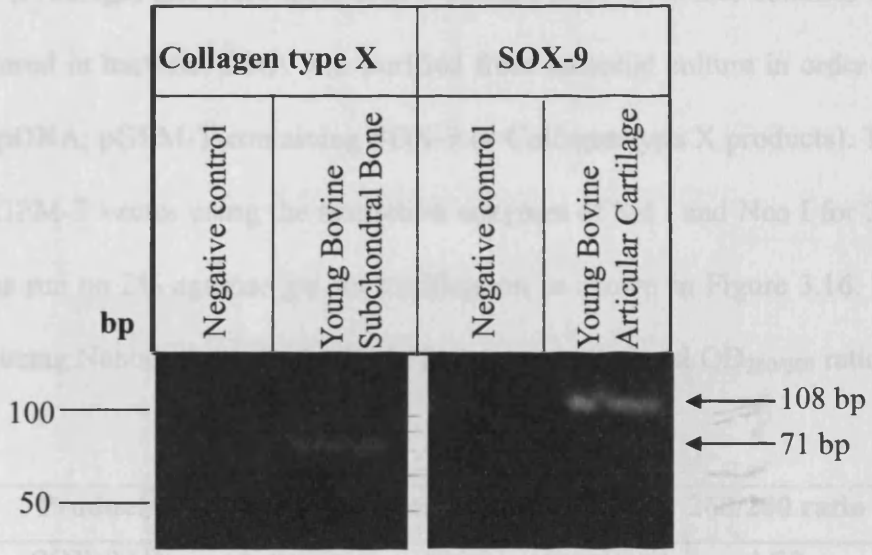


Figure 3.15: Analyses of RT-PCR products for bovine SOX-9 and Collagen type X using young bovine articular cartilage and subchondral bone cDNA. Primer pairs for bovine SOX-9 (7700SOX9.F/R), was used in lanes 1 and 2. Primer pairs for bovine collagen type X (7700ColX.F/R) was used in lanes 3 and 4. The migration positions of bSOX-9 and bovine collagen type X cDNAs are indicated by arrows on the right.

3.4.2 Generation of pGEM-T / SOX-9 vector and pGEM-T / Collagen type X and verifications

Once the PCR products were verified, the remaining PCR products were purified and processed for ligation into a pGEM-T vector. The ligated products were transformed using JM109 competent cells (Promega) and were incubated overnight at 37°C. White colonies were selected and further cultured in bacteria. DNA was purified from bacterial culture in order to obtain the plasmid DNA (pDNA; pGEM-T containing SOX-9 or Collagen type X products). The pDNA as cleaved from pGEM-T vector using the restriction enzymes of Sal I and Nco I for 2 hrs at 37°C. The product was run on 2% agarose gel for verification as shown in Figure 3.16. The products were measured using Nanodrop to obtain the DNA concentration and $OD_{260/280}$ ratio as shown in Table 3.8.

Products	DNA concentration (ng/ml)	260/280 ratio
SOX-9 (1)	105.9	1.88
SOX-9 (2)	89.0	1.91
Collagen type X (1)	90.8	1.93
Collagen type X (2)	96.8	1.90
Collagen type X (3)	52.6	1.77
Collagen type X (4)	76.1	1.89

Table 3.8: Quantification of DNA for SOX-9 and Collagen type X pDNA products.

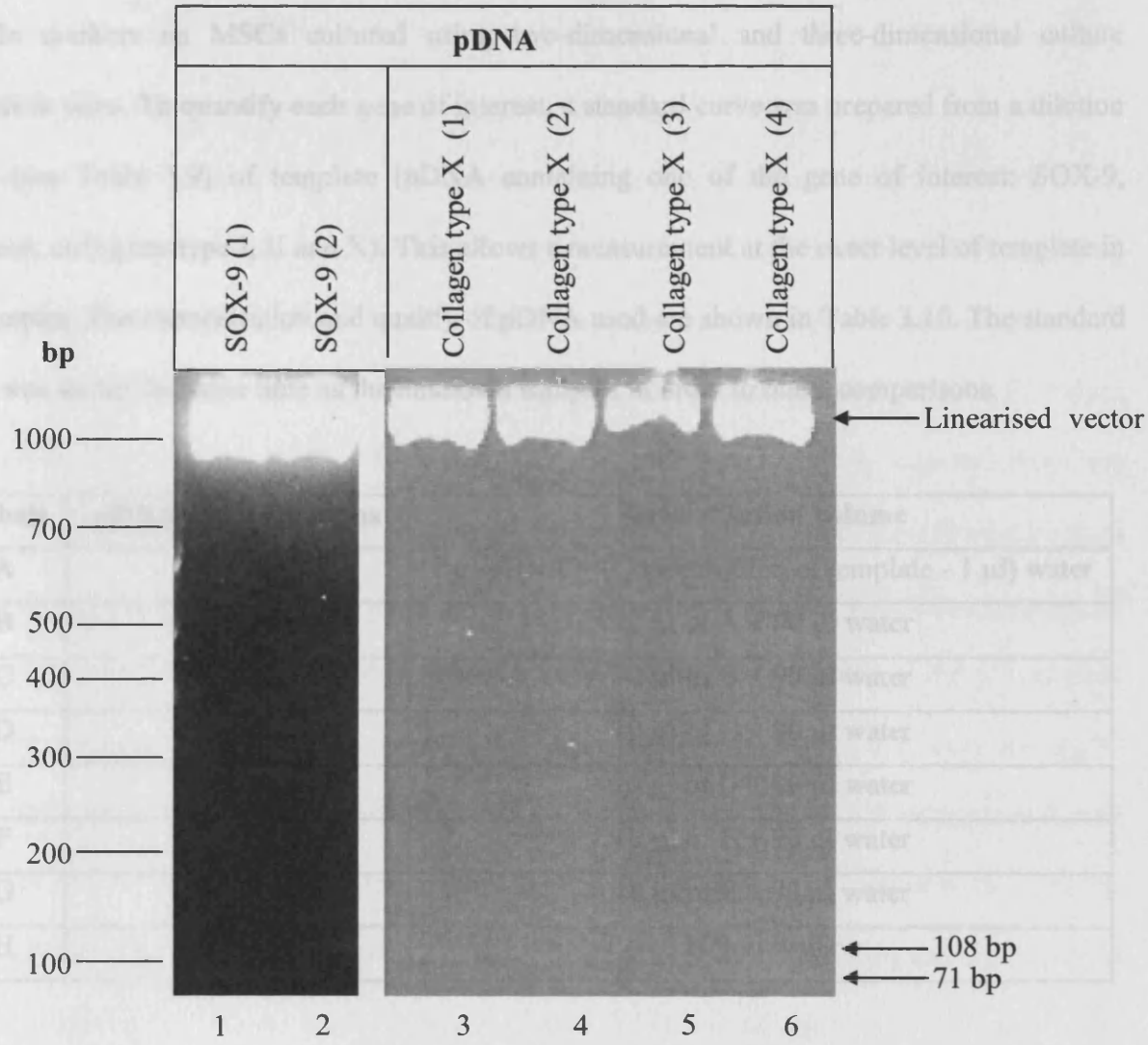


Figure 3.16: Restriction digestion analysis of bovine SOX-9 and Collagen type X expression constructs. Endonucleases Sal I and Nco I were used to insert PCR product into p-GEMT expression vector (3kb). Linearised vector can be seen after digestion. Excised pDNA for bovine SOX-9 (71 bp, lanes 1-2) and collagen type X (108 bp, lanes 3-5)can be seen after digestion with Sal I and Nco I, however, the level detected was much weaker than linearised vector. Migration of size standards is shown to the left.

3.4.3 Standard curves (Absolute quantification)

qPCR was used to assess the gene expression level of cartilage-specific and non-cartilage specific markers on MSCs cultured using two-dimensional and three-dimensional culture systems *in vitro*. To quantify each gene of interest, a standard curve was prepared from a dilution series (see Table 3.9) of template (pDNA containing one of the gene of interest: SOX-9, aggrecan, collagens type I, II and X). This allows a measurement at the exact level of template in the samples. The concentration and quality of pDNA used are shown in Table 3.10. The standard curve was set up the same time as the unknown samples in order to make comparisons.

Labels	pDNA concentrations	Serial dilution volume
A	1 ng	1 µl pDNA + (Concentration of template - 1 µl) water
B	100 pg	10 µl of A + 90 µl water
C	10 pg	10 µl of B + 90 µl water
D	1 pg	10 µl of C + 90 µl water
E	100 fg	10 µl of D + 90 µl water
F	10 fg	10 µl of E + 90 µl water
G	1 fg	10 µl of F + 90 µl water
H	Water	100 µl water

Table 3.9: Dilution series for the generation of a standard curve.

pDNA used for Standard curve	Concentrations	260/280 ratio
SOX-9	105.9 ng/µl	1.88
Aggrecan	1 ng *	1.80
Collagen type I	100 ng/µl	1.90
Collagen type II	110.7 ng/µl	1.95
Collagen type X	96.8 ng/µl	1.90
GAPDH	109.5 ng/µl	1.88

Table 3.10: Concentration and quality of each pDNA sample used for preparing the serial dilution for standard curve. *used 2 µl of pDNA with 18 µl of water to prepare 1 ng of pDNA

Standard curves on the gene expression using pDNA for Bovine SOX-9, aggrecan, collagens type I, II and X

pDNA for different genes of interest (SOX-9, aggrecan, collagens type I, II and X) were serially diluted to generate a standard curve using qPCR technology. Bovine GAPDH was used as a endogenous control and at least 2- 3 no template controls (NTC) were included in all the qPCR reactions. Amplifications of the standard dilution series of all the pDNA for SOX-9, aggrecan, collagens type I, II and X all produced a linear regression curve, which is denoted by the R squared value (R^2 or Pearson Correlation Coefficient). For all pDNA standards, the R^2 values were very close to 1 (± 0.007). Formation of primer dimers was only detected from the dissociation curve generated from pDNA for bovine collagen type II. All of the efficiency values generated from all the pDNAs were within the range of 90 – 110% with the exception of collagen type II (121.8%) and collagen type X (71.3%), which may be due to the primer pairs effect and suboptimal primer pairs, respectively. Table 3.11 summaries the efficiency and the R^2 values obtained from each standard curve. The R^2 values were all close to 1.0, suggesting that all standards showed a linear regression with high reaction efficiency of between 71 – 121%. Amplification plots, dissociation curves and the standard curves were generated for GAPDH (Figure 3.17), SOX-9 (Figure 3.18), aggrecan (Figure 3.19) and collagens type II (Figure 3.20), type I (Figure 3.21) and type X (Figure 3.22). The copy number for each gene of interest was calculated using the qPCR machine and these were plotted against the standards (in fg) as shown in Figure 3.23.

Genes	Efficiency (%)	R^2 values
GAPDH	92	0.999
SOX-9	106.5	0.993
Aggrecan	97.3	0.999
Collagen type II	121.8	0.998
Collagen type I	71.3	0.993

Table 3.11: Summary table to show the R^2 values and PCR efficiencies for each plasmid DNA (pDNA) designed to contain different gene if interest, namely, GAPDH, SOX-9, collagens type I, II and X and aggrecan.

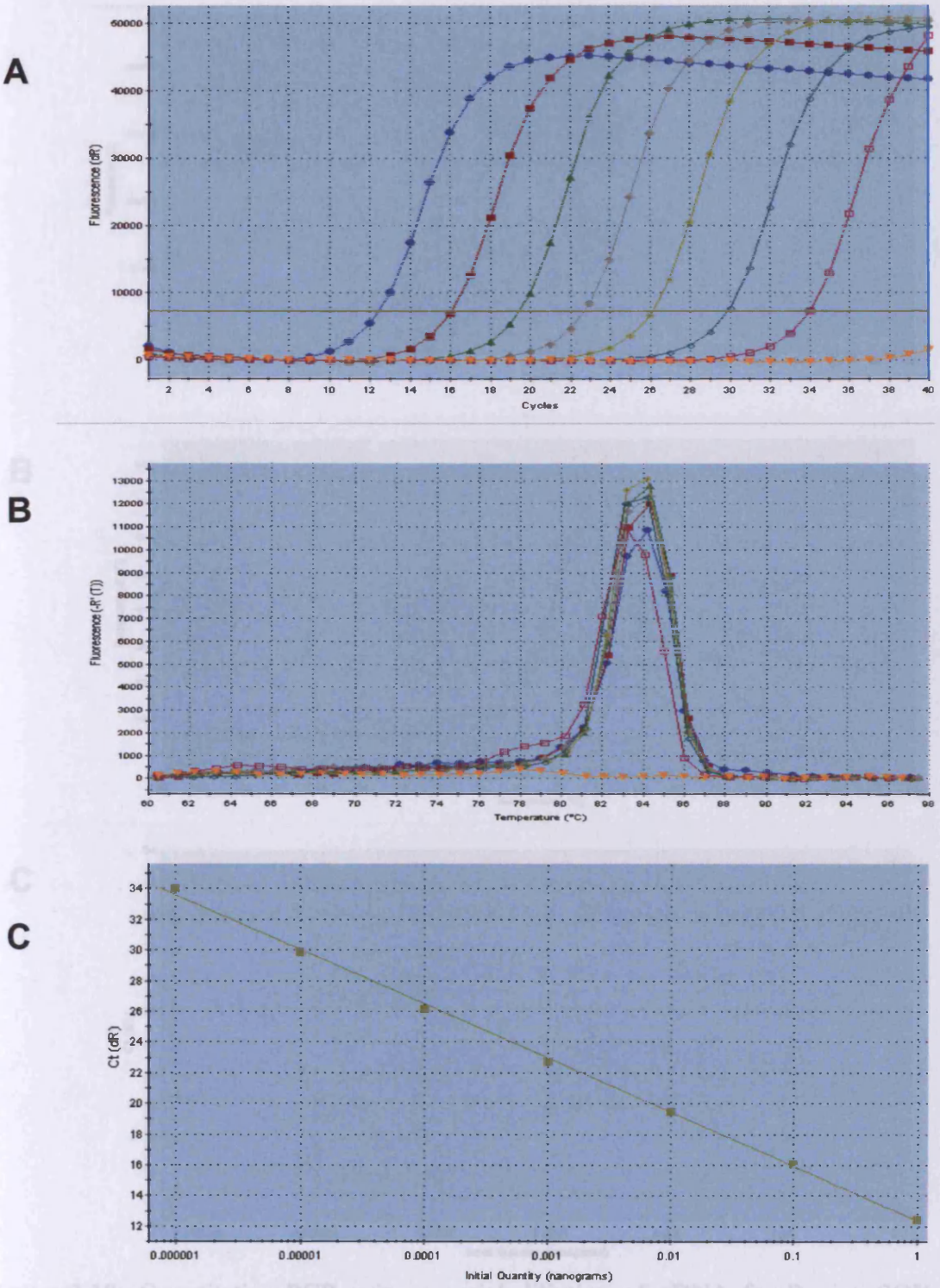


Figure 3.17: Quantitative PCR using a serial diliution of pDNA for Bovine GAPDH. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 92% and an R^2 value of 0.999.

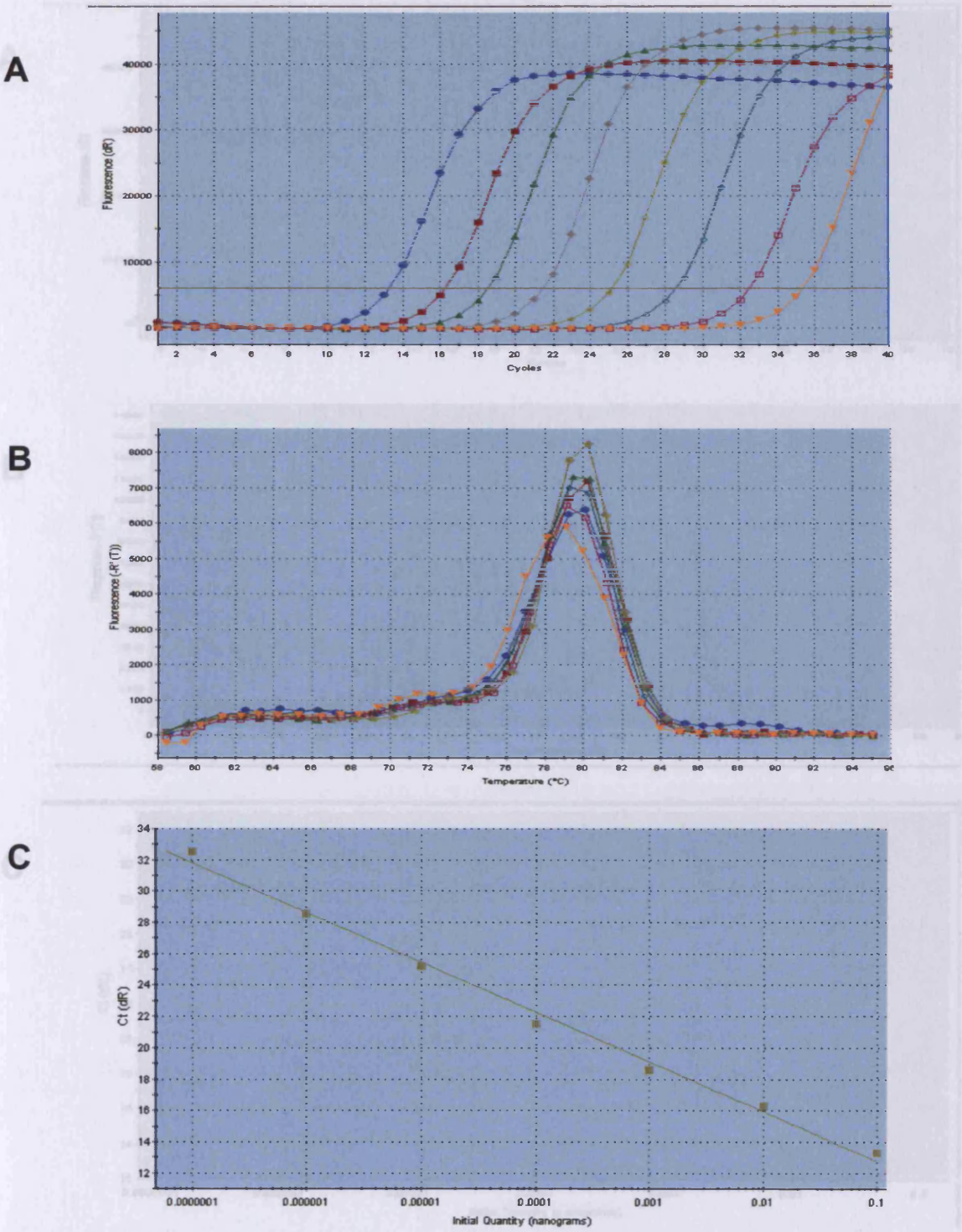


Figure 3.18: Quantitative PCR using a serial diliution of pDNA for Bovine SOX-9. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 92% and an R^2 value of 0.999.

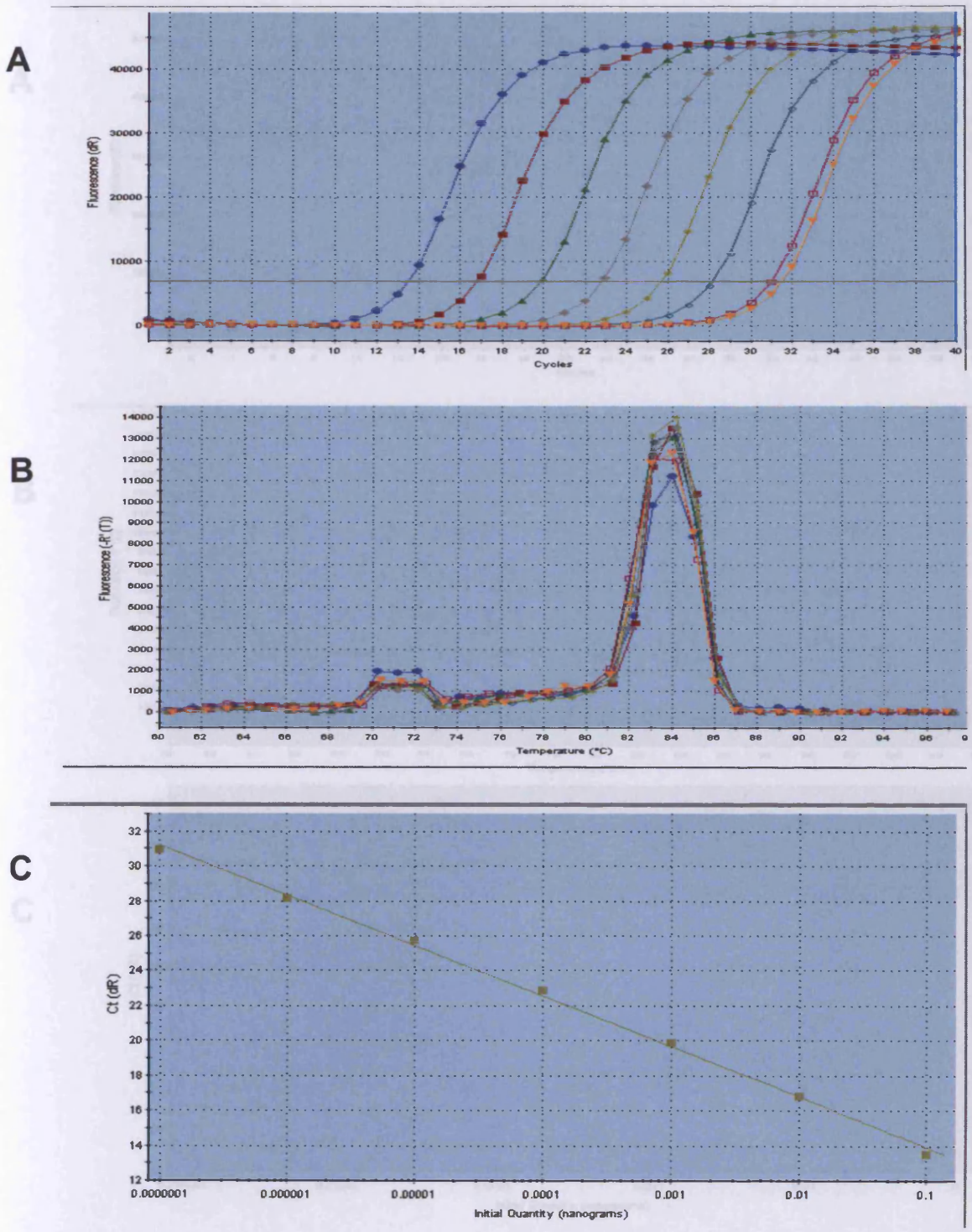


Figure 3.19: Quantitative PCR using a serial diliution of pDNA for Bovine Collagen Type II. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 121.8% and an R^2 value of 0.998.

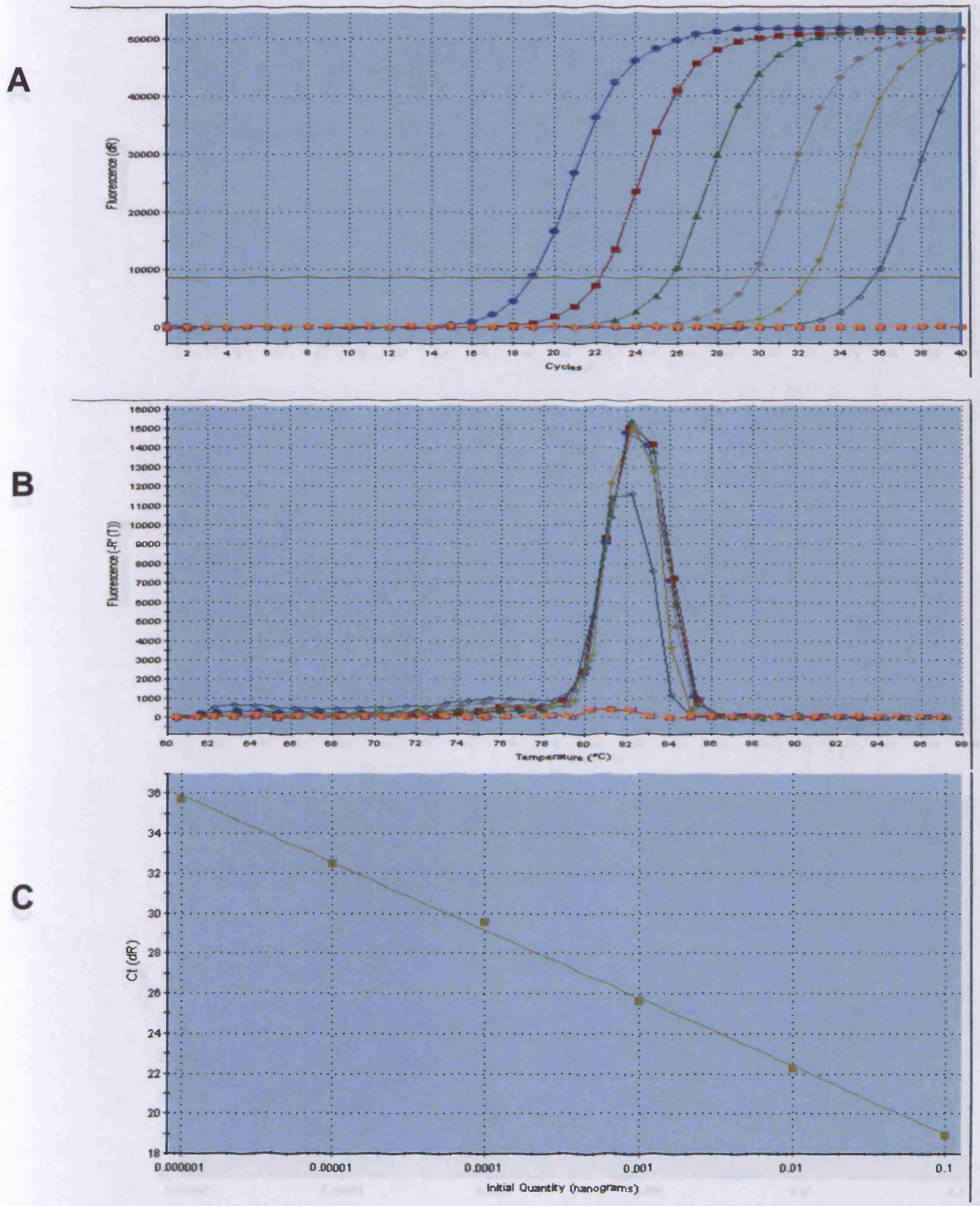


Figure 3.20: Quantitative PCR using a serial diliution of pDNA for Bovine aggrecan. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 97.3% and an R^2 value of 0.999.

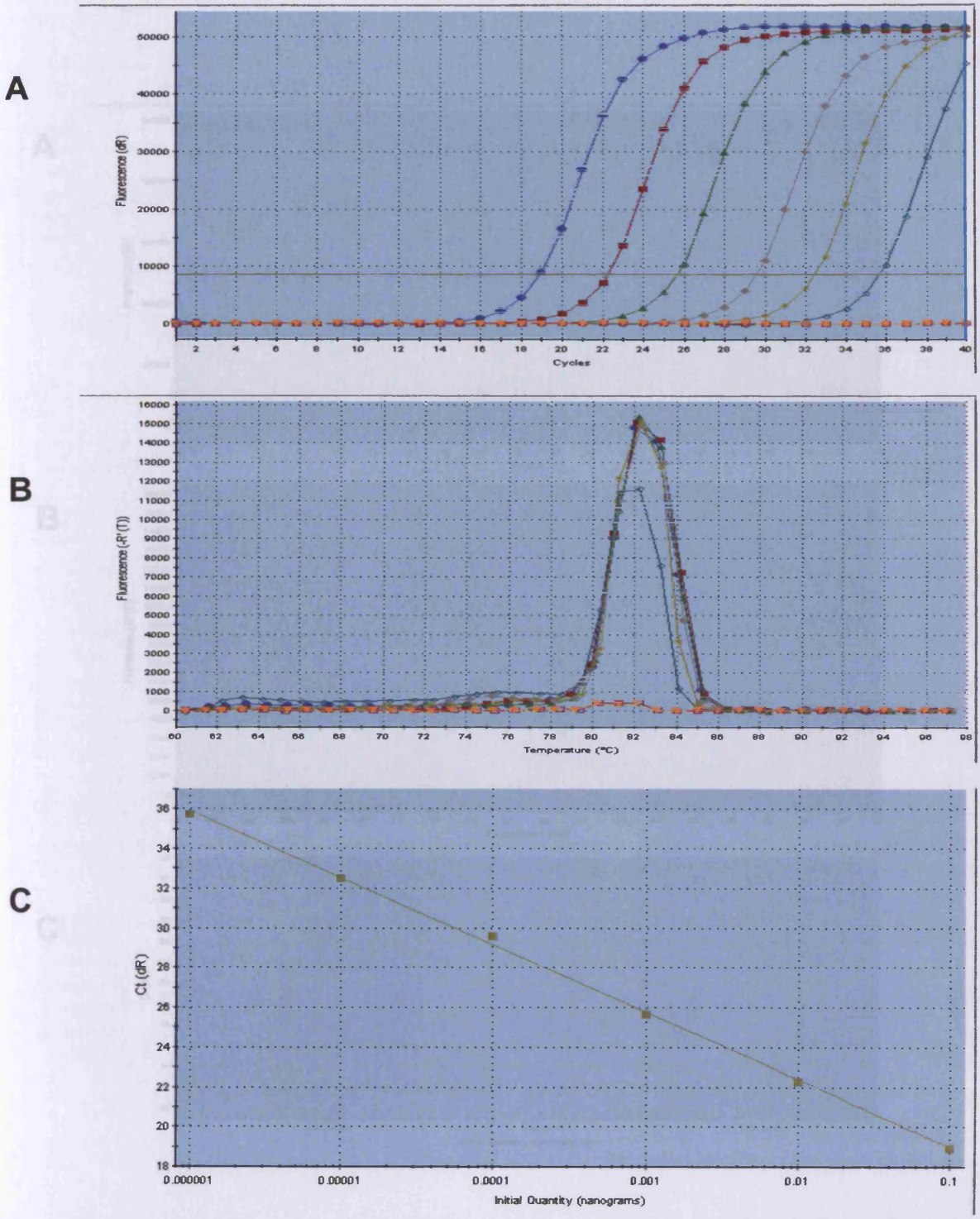


Figure 3.21: Quantitative PCR using a serial diliution of pDNA for Bovine Collagen Type I. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 71.3% and an R^2 value of 0.993.

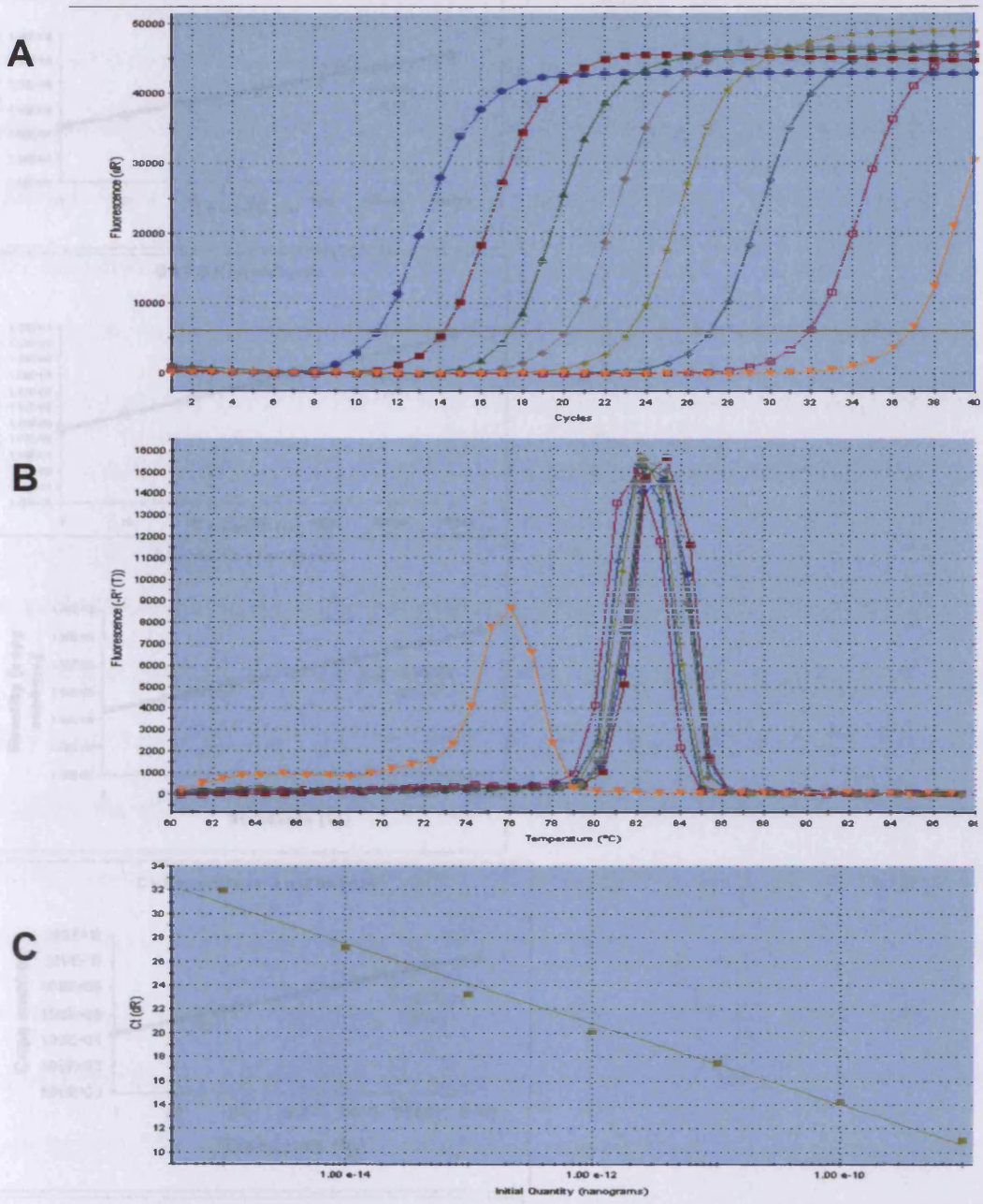


Figure 3.22: Quantitative PCR using a serial dilution of pDNA for Bovine Collagen Type X. The amplification plot (Panel A) and dissociation curve (Panel B) showed the initial quantity of the DNA and the quality of the product generated, respectively. Panel A and B showed 1ng (Blue), 100pg (Dark Red), 10pG (Green), 100fg (Mustard Yellow), 10fg (Light Blue), 1fg (Purple) and water (Orange). The standard curve (Panel C) gave an efficiency of 98.2% and an R^2 value of 0.992.

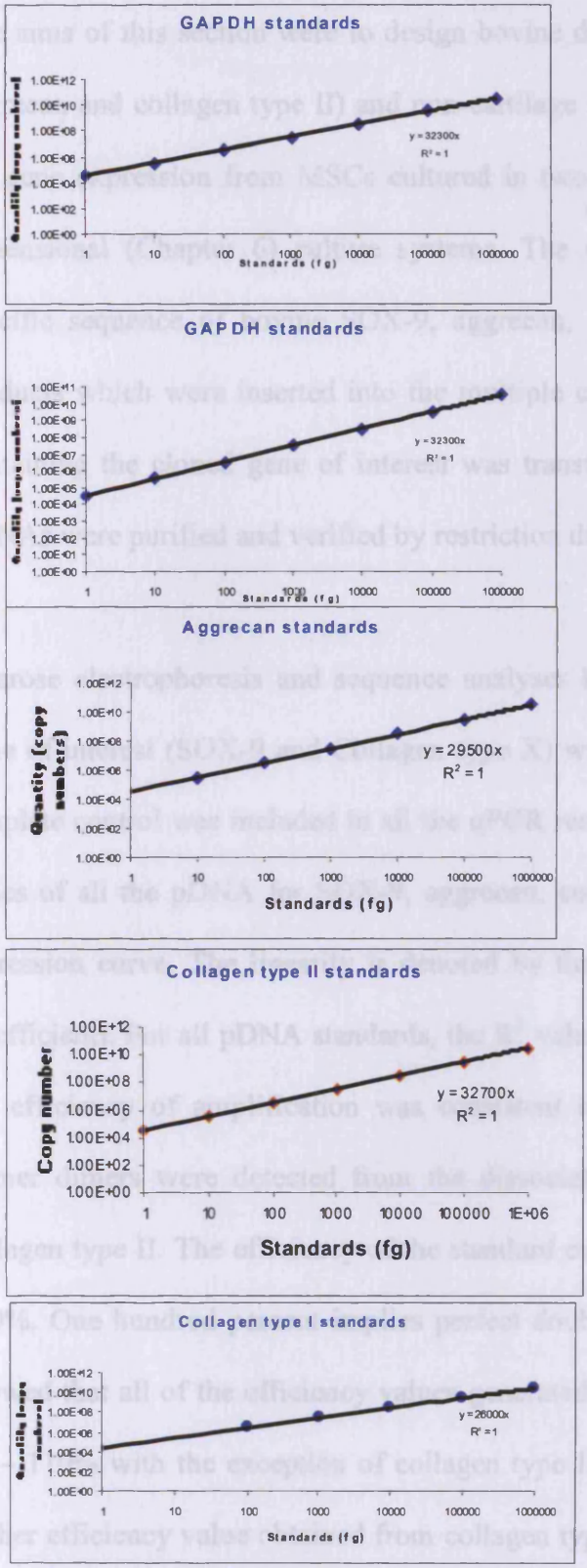


Figure 3.23: Standard curves generated using plasmid DNA containing the genes GAPDH, SOX-9, Collagen types I and II and aggrecan. The graphs were plotted on a log scale with copy numbers against standards of pDNA from different gene of interest. Expressed in femtogram (fg)

3.5 Discussion

The aims of this section were to design bovine derived cartilage-specific primer pairs (SOX-9, aggrecan and collagen type II) and non-cartilage specific primer pairs (Collagens type I and X) for gene expression from MSCs cultured in two-dimensional (Chapter 4 and 5) and in three-dimensional (Chapter 6) culture systems. The designed primer pairs were used to amplify specific sequence of bovine SOX-9, aggrecan, collagens type I, II and X to generate PCR products which were inserted into the multiple cloning site of pGEM-T plasmid. The plasmid containing the cloned gene of interest was transformed into competent cells, cultured and the pDNAs were purified and verified by restriction digestion using endonucleases Sal I and Nco I.

Agarose electrophoresis and sequence analyses have indicated that the correctly sized cloned gene of interest (SOX-9 and Collagen type X) was inserted into the plasmid. At leaset 2- 3 no template control was included in all the qPCR reactions. Amplifications of the standard dilution series of all the pDNA for SOX-9, aggrecan, collagens type I, II and X all produced a linear regression curve. The linearity is denoted by the R squared value (R^2 or Pearson Correlation Coefficient). For all pDNA standards, the R^2 values were all very close to 1, which implied that the efficiency of amplification was consistent at varying template concentrations. However, primer dimers were detected from the dissociation curve generated from pDNA for bovine collagen type II. The efficiency of the standard curves produced ideally should be between 90 – 110%. One hundred percent implies perfect doubling of amplicon number per cycle. Our data showed that all of the efficiency values generated from all the pDNAs were within the range of 90 – 110% with the exception of collagen type II (121.8%) and collagen type X (71.3%). The higher efficiency value obtained from collagen type II (121.8%) may be due to the formation of primer dimers as observed from the dissociation curve. In contrast, the low efficiency value obtained from collagen type X (71.3%) indicated that the reaction may be slowed either due to inhibitors present in the master mix or suboptimal primer pairs or reaction conditions.

3.6 Summary

- Bovine specific primer pairs were designed for GAPDH, SOX-9, aggrecan, collagens type I, II and X.
- pGEM-T 7 plasmids containing cloned gene of interest (SOX-9 and Collagen type X) were produced and were verified using agarose gel electrophoresis and sequence analysis.

Chapter 4:

Characterisation of primary cultured bovine bone marrow derived mesenchymal stem cells for specific markers associated with stem cells and chondrocytes

4.1 Introduction

The objectives of this section were to establish primary cultures of young bovine bone marrow mesenchymal stem cells (BMSCs) by adherence to tissue culture plastic and to establish methods for the identification and quantification of the stem cell markers (Notch-1, Delta and Jagged-2). In addition, the molecular expression of chondrogenic markers such as SOX-9, aggrecan, and collagen type II were analysed. The expression levels were compared between freshly isolated heterogenous population of bovine bone marrow cells and undifferentiated BMSCs in primary culture, referred to as P0 BMSCs.

In this study, the method used to extract bone marrow from young bovine legs was direct extraction from an open carpo bone. The cell suspension obtained from bone marrow consisted of a mixture of cell types, including red blood cells, unidentified nucleated cells of the hematopoietic lineage, monocytes, macrophages and fibroblast-like cells. The cell mixture was plated into culture flasks and BMSCs are isolated by adherence to plastics, non-adherent cells were removed by regular changes of culture medium. When cells reached confluency, the cultures were trypsinized and further cultured (refer to Chapter 4 and Chapter 5). The following analyses were carried out on the trypsinised cells:

- Cell number determination, as compared to original material
- Analysis of protein expression in the isolated stem / progenitor cells for Notch-1, Delta and Jagged-2 using FACS

- Analysis of gene expression in the freshly isolated P0 BMSCS stem cells for SOX-9, aggrecan, collagens type I and II markers to investigate if these genes are already expressed before further culturing.

The outline of the experiment and the analyses carried out for this chapter are summarised in Figure 4.1.

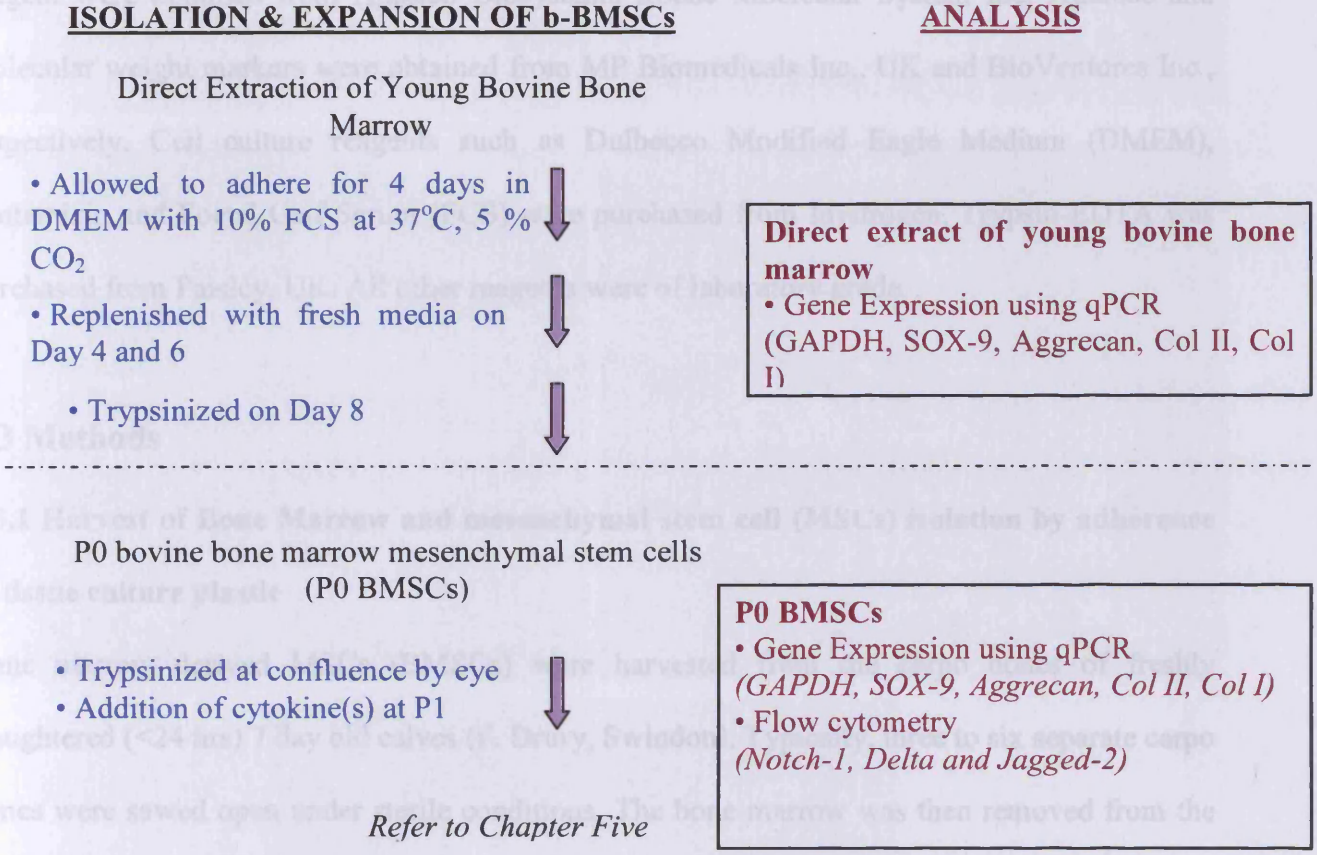


Figure 4.1: A flow chart to show the isolation of BMSCs from young bovine bone marrow and the conditions used to culture the isolated BMSCs. Confluent cells were trypsinised by trypsin-EDTA. Analyses carried out on direct extracts of bovine bone marrow and on P0 BMSCs are highlighted on the right in purple.

4.2 Materials

Polyclonal antibodies for FACs analyses were obtained from Santa Cruz Biotechnology Inc., Mile Elm, Caln, Wiltshire, UK. Primer pairs and plasmid DNA for aggrecan and collagen type II for quantitative PCR were kindly provided by Dr Emma Blain. Collagen type I primers and plasmid DNA were kindly provided by Dr Siyuan Li at CTBL, Cardiff University, Cardiff. Tri-reagent and TBE were purchased from Signma-Alrich, Poole, Dorset, UK. All PCR buffers and reagent were obtained from Applied Biosystems Roche Moelcular System Inc. Agarose and molecular weight markers were obtained from MP Biomedicals Inc., UK and BioVentures Inc., respectively. Cell culture reagents such as Dulbecco Modified Eagle Medium (DMEM), gentamicin and Foetal Calf Serum (FCS) were purchased from Invitrogen. Trypsin-EDTA was purchased from Paisley, UK. All other reagents were of laboratory grade.

4.3 Methods

4.3.1 Harvest of Bone Marrow and mesenchymal stem cell (MSCs) isolation by adherence to tissue culture plastic

Bone marrow derived MSCs (BMSCs) were harvested from the carpo bones of freshly slaughtered (<24 hrs) 7 day old calves (F. Drury, Swindon). Typically, three to six separate carpo bones were sawed open under sterile conditions. The bone marrow was then removed from the two chambers using a surgical spatula and transferred to a pre-weighed Petri-dish (Figure 4.2).

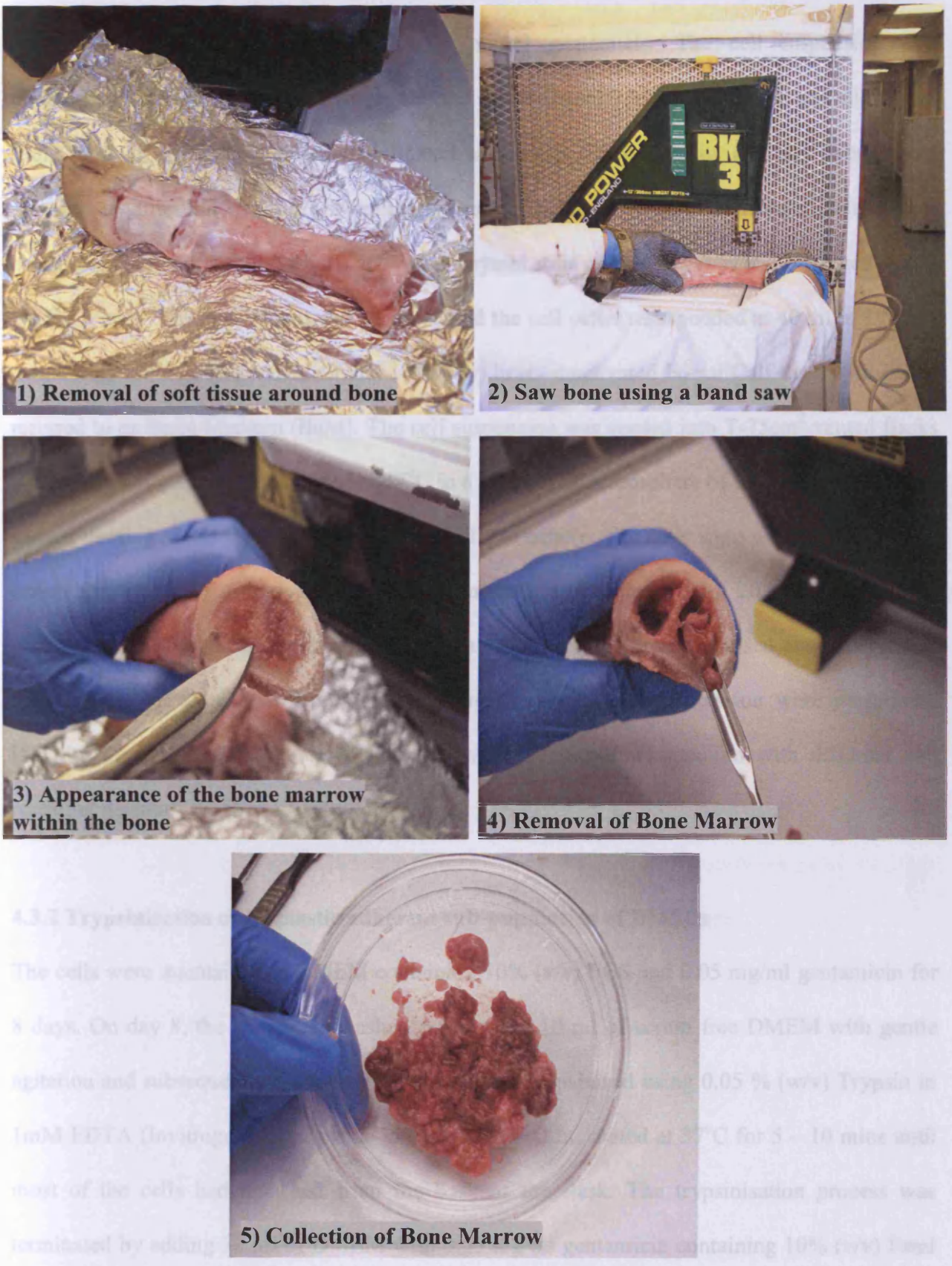


Figure 4.2: Extraction of Bone Marrow

The marrow removed was dispersed twice in 20 ml of sterile serum-free Dulbecco's Modified Eagles Medium (DMEM) containing 0.05 mg/ml gentamicin. The cell suspension was transferred to a sterile 50 ml centrifuge tube and inverted gently to mix. The tube was allowed to stand until all the fatty deposits had floated to the top. Fatty deposits and any debris were removed by filtration through a 40 μm cell strainer (Falcon, VWR). The filtrate, containing a mixed population of red blood cells and mesenchymal stem cells (MSCs), was centrifuged at 200 x g for 5 mins. The supernatant was removed and the cell pellet resuspended in 40 ml of DMEM containing 0.05 mg/ml gentamicin and 10% (v/v) heat –inactivated Foetal Calf Serum (hi-FCS), referred to as Basic Medium (BaM). The cell suspension was seeded into T-75cm^2 vented flasks (Corning). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% (v/v) carbon dioxide (CO_2) for 4 days to allow the cells to adhere. The cells were washed twice using serum free DMEM to remove any non-adherent cells and replaced with 20 ml of BaM. This washing step was repeated after 6 days. On Day 8, the cells were trypsinised and analyses on protein expression of stem / progenitor cell markers and gene expression were performed. P0BMSCs were subcultured up to Passage two (P2) in culture medium with different cell replating densities (refer to Chapter 5).

4.3.2 Trypsinisation of P0 plastic adherent sub-population of BMSCs

The cells were maintained in DMEM containing 10% (v/v) FCS and 0.05 mg/ml gentamicin for 8 days. On day 8, the cells were washed twice using 10 ml of serum free DMEM with gentle agitation and subsequently photographed. Cells were trypsinised using 0.05 % (w/v) Trypsin in 1mM EDTA (Invitrogen), 5 ml per T-75 cm^2 flask, and incubated at 37°C for 5 – 10 mins until most of the cells had detached from the base of the flask. The trypsinisation process was terminated by adding 30 ml of DMEM with 0.05 mg/ml gentamicin containing 10% (v/v) Fetal Calf Serum (FCS). The cells were pelleted by centrifugation at 200 x g for 5 mins and the cell pellet resuspended in 10 ml of DMEM with 10% (v/v) FCS and 0.05 mg/ml gentamicin. The

cells were counted using a haemocytometer (Sigma). The number of BMSCs per flask was calculated as shown below:

1) Formula for calculating cells / ml:

   Average cell number per square x 10^4 x *Dilution factor*

2) Formula for calculating the total number of cells

   Cells / ml x *original volume of fluid*

4.3.3 Fluorescence-activated cell sorting (FACS) analyses of adhered MSCs using antibodies recognising the cell surface marker Notch-1 and its ligands, Delta and Jagged-2

FACs analysis was performed using a FACS Canto (BD Bioscience). Cells were harvested by trypsinisation (see Section 3.3.2), 1 x10^6 cells were retained after tryspinisation and were pelleted by centrifugation at 2000 rpm for 5 mins. The cells were fixed in 1 ml of 2% (w/v) paraformaldehyde (PFA) at room temperature for 2 mins, the cell suspension was divided into 4 times 0.2 ml, containing approximately 0.2x10^6 cells for each analysis. The cell suspensions were centrifuged at 2000 rpm for 5 mins and the supernatant discarded. Non-specific binding sites were blocked using 5% (v/v) rabbit serum in PBS / 1% Tween20® for 15 mins at 4°C, followed by incubation in appropriately diluted primary polyclonal antibodies (refer to Table 4.1) for 30 mins at 4 °C. FACs analysis was carried out using 30,000 events.

Antibodies	Dilutions	Isotype	Species	Epitope	References
Goat IgG	1:200	IgG	Goat Polyclonal	-	Invitrogen
Notch-1 C-20	1:40	IgG		C-terminus of Notch-1 of human origin	Santa Cruz Biotechnologies, Inc.
Delta F-15	1:40	IgG		N-terminus of Delta of human origin	
Jagged-2 NOTCH-19	1:40	IgG		N-terminus of Jagged-2 of human origin	

Table 4.1: A table to show the Notch-1, Delta and Jagged-2 specific antibodies used for FACs analyses.Goat immunoglobulin (goat IgG) was used as a negative control. Note no positive controls were used for FACs analyses.

4.3.4 Immunohistochemistry of BMSCs using Notch-1, Delta and Jagged-2 and, the co-localization with cell membrane using fluorescently labeled Wheat Germ Agglutinin (WGA)

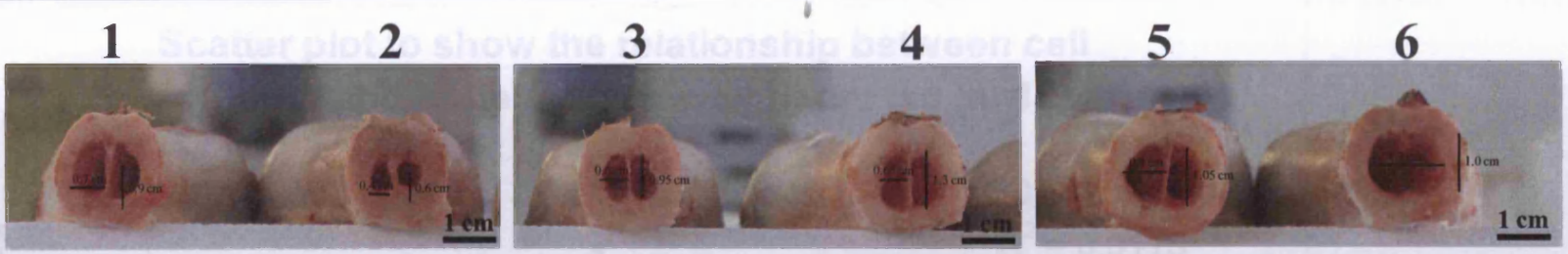
WGA is a carbohydrate - binding protein that selectively recognizes non-reducing terminal sialic acids and N-acetylglucosaminyl sugar residues on molecules (e.g. glycoproteins and glycolipids) which are predominantly found in the plasma membrane of cells. Hence, WGA is often used as a tool for co-localization to identify membrane associated proteins (Wright 1984).

Trypsinized cells from Day 8 (750,000 cells) were allowed to adhere to HistoBond slides in a humidified atmosphere of culture media (DMEM containing gentamicin and 10% [v/v] hi-FCS) overnight at 37°C 5 % (v/v) CO_2. Sterile PBS and wheatgerm agglutinin Alexa – 647 were pre-chilled on ice. The cells were chilled on ice for 45 mins. The media was removed and 1 ml of 200 μg/ml of WGA – Alexa 647 in PBS, was incubated on the cells on ice for 20 mins. Following incubation, the WGA was removed and the cells were washed 3 times using pre-chilled sterile PBS and fixed for 20 mins using 2ml of pre-chilled 2% (w/v) paraformaldehyde (PFA). The PFA was removed and the cells were washed 3 times in PBS and blocked in rabbit serum (1:20 dilution in PBS) for 15 mins at room temperature. After blocking, the cells were transferred to a humid chamber and incubated with appropriately diluted primary antibodies (refer to Table 3.1) for 20 mins at room temperature. The cells were washed 3 times in PBS, followed by incubation in goat anti-mouse secondary antibodies for 15 mins at room temperature. The cells were washed three times in PBS, then mounted in ProlongGold™ (Invitrogen) and were viewed under a confocal microscope.

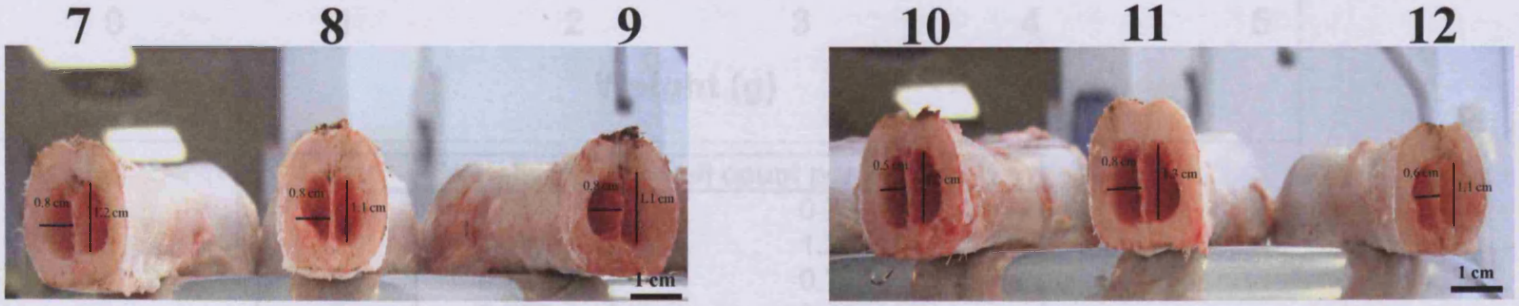
4.4 Results

4.4.1 Comparison of bone marrow wet weight with cell number obtained at Day 8 of culture

Variations in the bone marrow chamber size and the presence of a mid-bone separating the two chambers were observed to be visually different for all young bovine legs that were processed. The dimension of each chamber and the wet weight of bone marrow harvested were recorded from 12 individual legs from different bone marrow harvests, to ascertain whether bone marrow wet weight influenced the number of cells obtained after 8 days of culture in DMEM containing 10% (v/v) FBS and gentamicin. Figure 4.3 illustrates the diversity of chamber dimensions from 12 legs obtained from different animals, the bone marrow wet weight and cell count following 8 days of primary culture in serum-containing medium. From the 12 legs obtained from different animals, a broad range of cell count per leg ranging between $0.25x10^6$ to $1.74x10^6$ cells counted on Day 8 was obtained (see Figure 4.3, 'Cell count per leg'). From Figure 4.3, it shows that the heaviest bone marrow sample (Leg 10, 3.956g with 0.68 $x10^6$ cells) did not generate the highest cell count (Leg 9, 2.021g with 1.74 $x10^6$ cells). In some cases, no BMSCs were harvested from the bone marrow sample at all, as observed from Leg 11 (3.177g bone marrow, with no cells). These data were further analyzed using a scatter plot of cell number against gram per wet weight of bone marrow. Normally, an R^2 value close to 1 indicates a linear relationship between two variables. The R^2 value for wet weight and cell number was 0.0115 as shown in Figure 4.4 suggesting no correlation. Furthermore, scatter graphs were plotted to investigate whether a correlation existed between 1) cell number and the area of the bone marrow chamber (Figure 4.4) and 2) weight of bone marrow harvested and the area of the bone marrow (Figure 4.5). The R^2 values obtained were 0.0092 (Figure 4.5) and 0.2463 (Figure 4.6) suggesting no correlation between these factors.

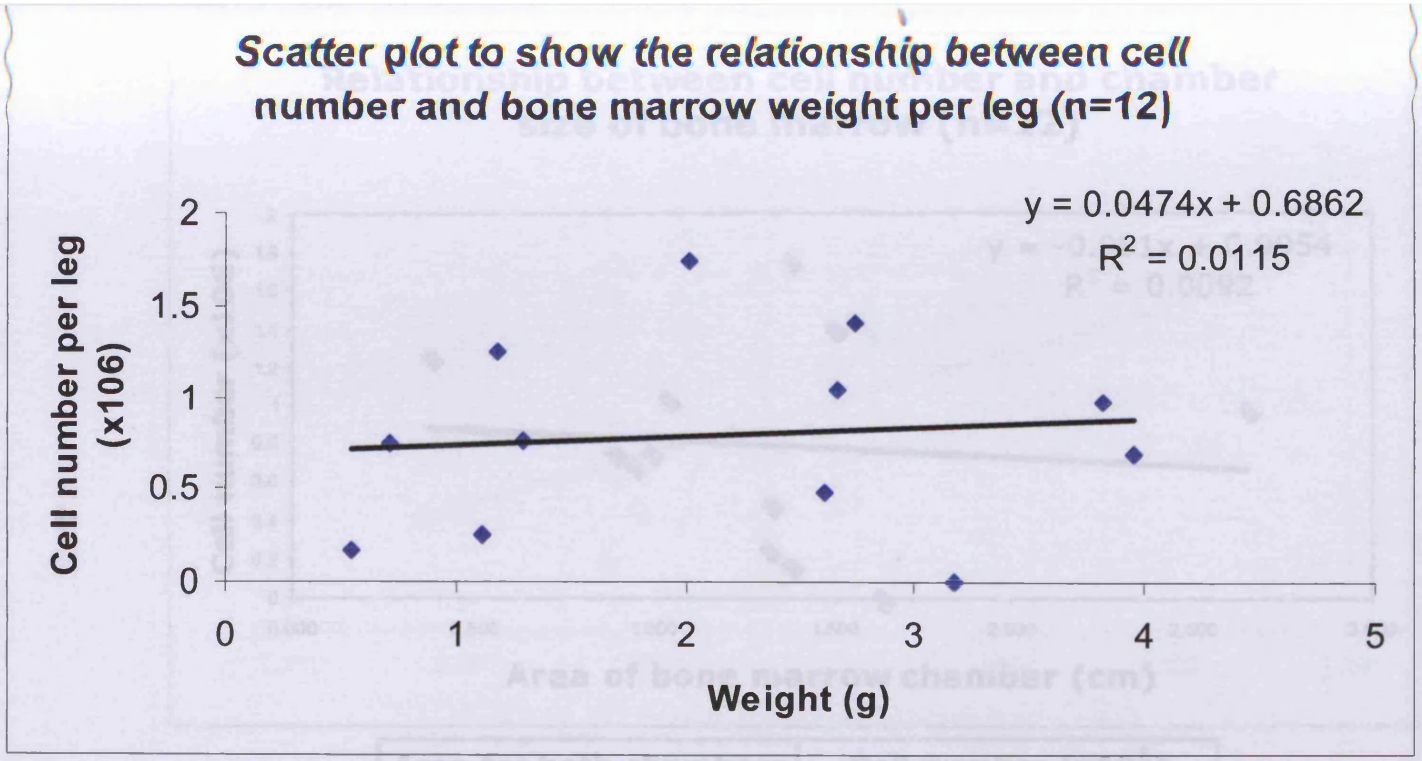


Bone Marrow Weight (g)	1.288	1.183	0.717	2.607	1.116	3.82
Cell count per leg (x 10^6 cells)	0.75	1.25	0.74	0.48	0.25	0.97



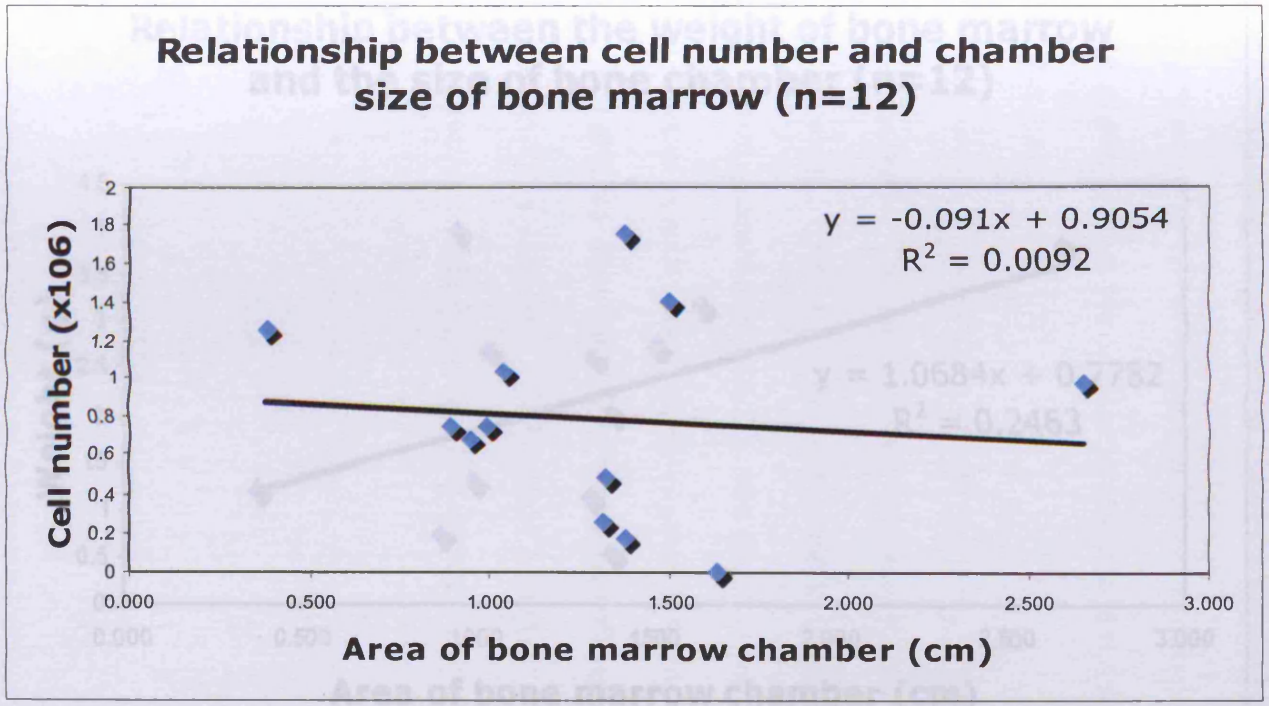
Bone Marrow Weight (g)	2.743	0.544	2.021	3.956	3.177	2.665
Cell count per leg (x 10^6 cells)	1.4	0.17	1.74	0.68	0	1.03

Figure 4.3: Photographs to show the variations of exposed bone marrow chambers between different young bovine mid-bone by eye. The weight of bone marrow and cell count obtained per bone are shown to the corresponding bone from the images.



Legs	Weight (g)	Cell count per leg (x10 ^{6})
1	1.288	0.75
2	1.183	1.25
3	0.717	0.74
4	2.607	0.48
5	1.116	0.25
6	3.82	0.97
7	2.743	1.4
8	0.544	0.17
9	2.021	1.74
10	3.956	0.68
11	3.177	0
12	2.665	1.03

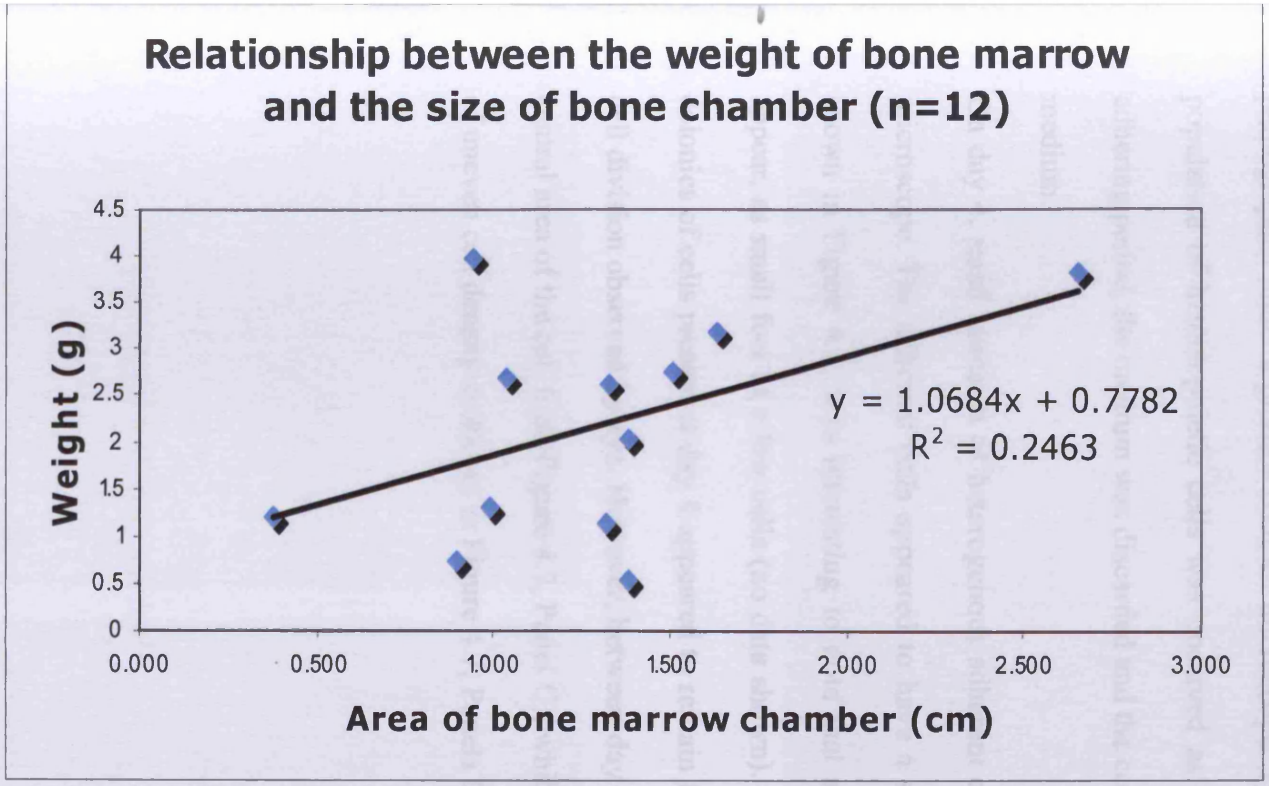
Figure 4.4: A scatter plot to identify a relationship between the cell number and raw bone marrow weight per leg



Area for both chambers	Cell number (x10^6)
0.989	0.75
0.377	1.25
0.895	0.74
1.327	0.48
1.319	0.25
2.653	0.97
1.507	1.4
1.382	0.17
1.382	1.74
0.942	0.68
1.633	0
1.036	1.03

Figure 4.5: Graphs to show the relationship between the area of bone marrow chamber with cell number and weight of the bone marrow from 12 young bovine legs. The area of the chambers was calculated using the formula of: *area = 0.5 length x 0.5 breath x pi (∏)*

A



B

Area for both chambers	Weight (g)
0.989	1.288
0.377	1.183
0.895	0.717
1.327	2.607
1.319	1.116
2.653	3.82
1.507	2.743
1.382	0.544
1.382	2.021
0.942	3.956
1.633	3.177
1.036	2.665

Figure 4.6: Relationship between the area of none marrow chamber with weight

4.4.2 Cell morphology of adherent Mesenchymal stem cells (MSCs) from day 0 to day 8 of culture

The method used for isolation of BMSC was a simple selection based upon the adherence of BMSC to plastic (Oreffo *et al.,* 1997). The initial plating of the cell inoculum was observed and photographed under a phase – contrast microscope. Typically, within one hour of plating, a rich population of hematopoietic cells was observed as shown in Figure 4.7. Following a 4 day adhering period, the medium was discarded and the cells were washed and replenished with fresh medium.

On day 4, small numbers of heterogenous adherent cells were observed using a phase contrast microscope. The adherent cells appeared to have a spindle-shaped fibroblastic morphology as shown in Figure 4.7. It is interesting to note that small colonies of cells were beginning to appear, as small foci of a few cells (no data shown). Visual observation suggests that the small colonies of cells present at day 4 appeared to remain fairly static up to Day 6, followed by rapid cell division observed by eye. However, between day 6 and 8, rapid cell division occurred in the central area of the cell foci (Figure 4.7, Panel C), whilst the periphery of the foci contained areas of uneven cell density as shown in Figure 4.7, Panels D and E.

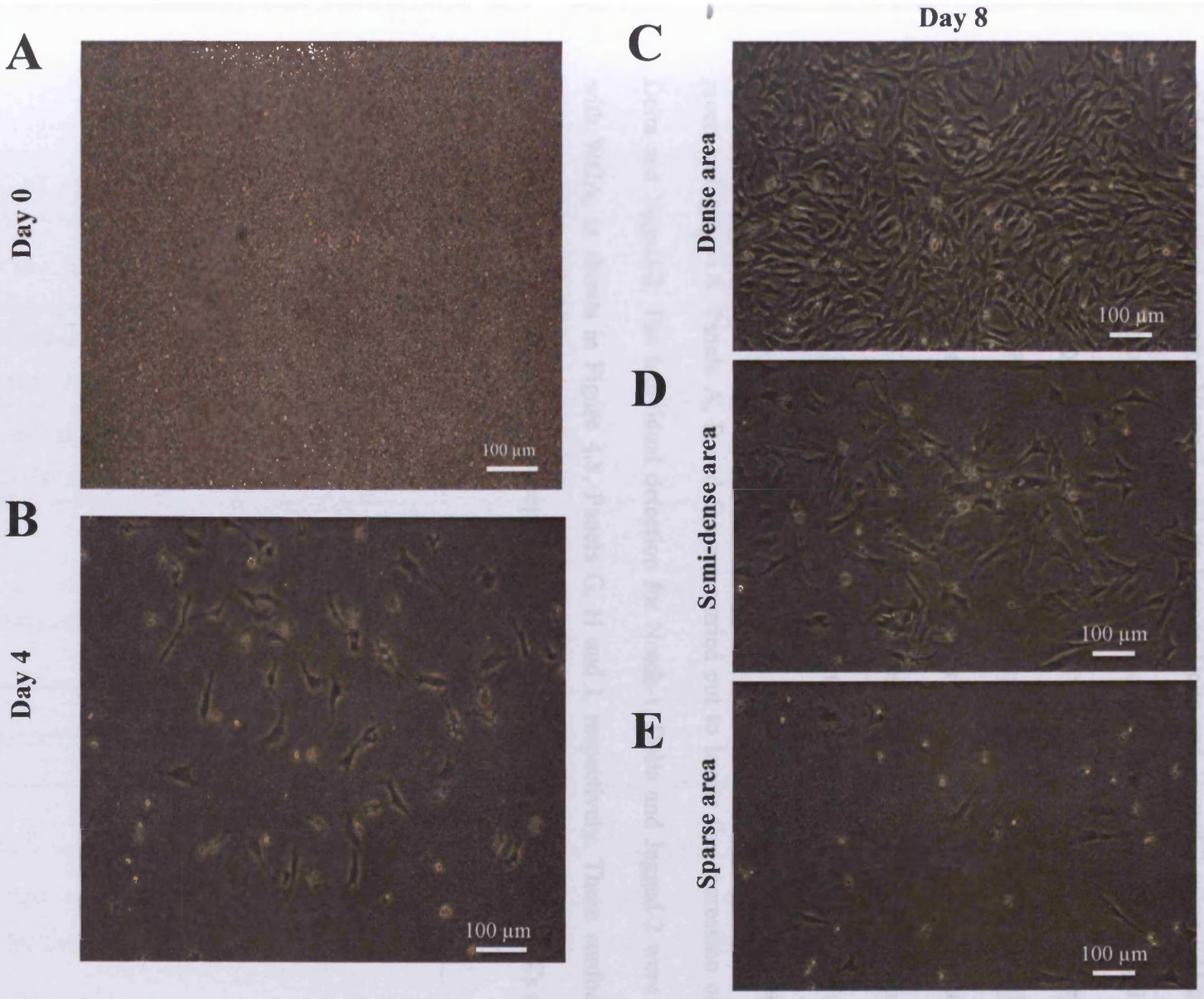


Figure 4.6: Cell morphology of freshly isolated young bovine bone marrow-derived mesenchymal stem cells (b-BMSCs) at Day 0 (Panel A), after 4 days of adhering period (Panel B) and at Day 8 (Panel C) for before trypsinisation. Panel C, D and E highlighted the different population density (dense, semi-dense and sparse areas) observed throughout the base of a T-75 flask.

4.4.3 Cell surface expression of Notch-1, Delta and Jagged-2 on stem / progenitor cell population at day 8 of primary cultures

Monolayers of cells were trypsinised at day 8 (herein named P0) and analyzed for the expression of the cell surface receptor Notch-1, and its ligands Delta and Jagged-2 using FACs analyses. Table 4.2 summarised the detection of trypsin-treated cells harvested on Day 8 against young bovine articular chondrocytes (n=1), which acted as a positive control. The average detection for P0 BMSCs for Notch-1 (90.3%), Delta (78.9%) and Jagged-2 (92.8%) were calculated as shown in Table 4.2. An n = 1 was used for the cell surface expression of Delta due to cell shortage. The graph shown in Figure 4.7 shows a shift in the fluorescence of the cells towards 10^5 for Notch-1, Delta and Jagged-2, away from the control, suggesting a positive expression. Colocalisation between WGA (shown in red, Figure 4.8, Panels D, E and F) and surface antibodies shown in green in Figure 4.8, Panels A, B and C were carried out to locate the expression of Notch-1, Delta and Jagged-2. The individual detection for Notch-1, Delta and Jagged-2 were combined with WGA, as shown in Figure 4.8, Panels G, H and I, respectively. These confocal images demonstrated the detection of these receptor and ligands on the surface of P0 BMSCs cultured in serum-containing medium for 8 days.

	Percentage of positive cells (%)			
	Goat Immunoglobulin (GIg)	**Notch-1**	**Delta**	**Jagged-2**
Young Bovine Articular Cartilage	0.9	98.4	84.7	44.1
Trypsin-treated P0-bovine BMSCs (from Experiment 1)	0.5	90.9	No data due to lack of cells	98
Trypsin-treated P0-bovine BMSCs (from Experiment 2)	0.5	89.7	78.9	87.5
Average between Experiment 1 & 2 data	0.5	90.3 (n=2)	78.9 (n=1)	92.75 (n=2)
Standard errors	0	0.6	n/a since n =1	5.25

Table 4.2: FACs analyses on trypsin-treated cells harvested on Day 8 (Passage 0, P0). Young bovine full depth articular cartilage was used as a positive control for the receptor protein Notch-1 and its ligands Delta and Jagged-2. Protein expression for GIg, Notch -1 and Jagged-2 on P0 cells from Day 8 was repeated twice, whereas the ligand, Delta only has an n-number of 1 due to shortage of cells.

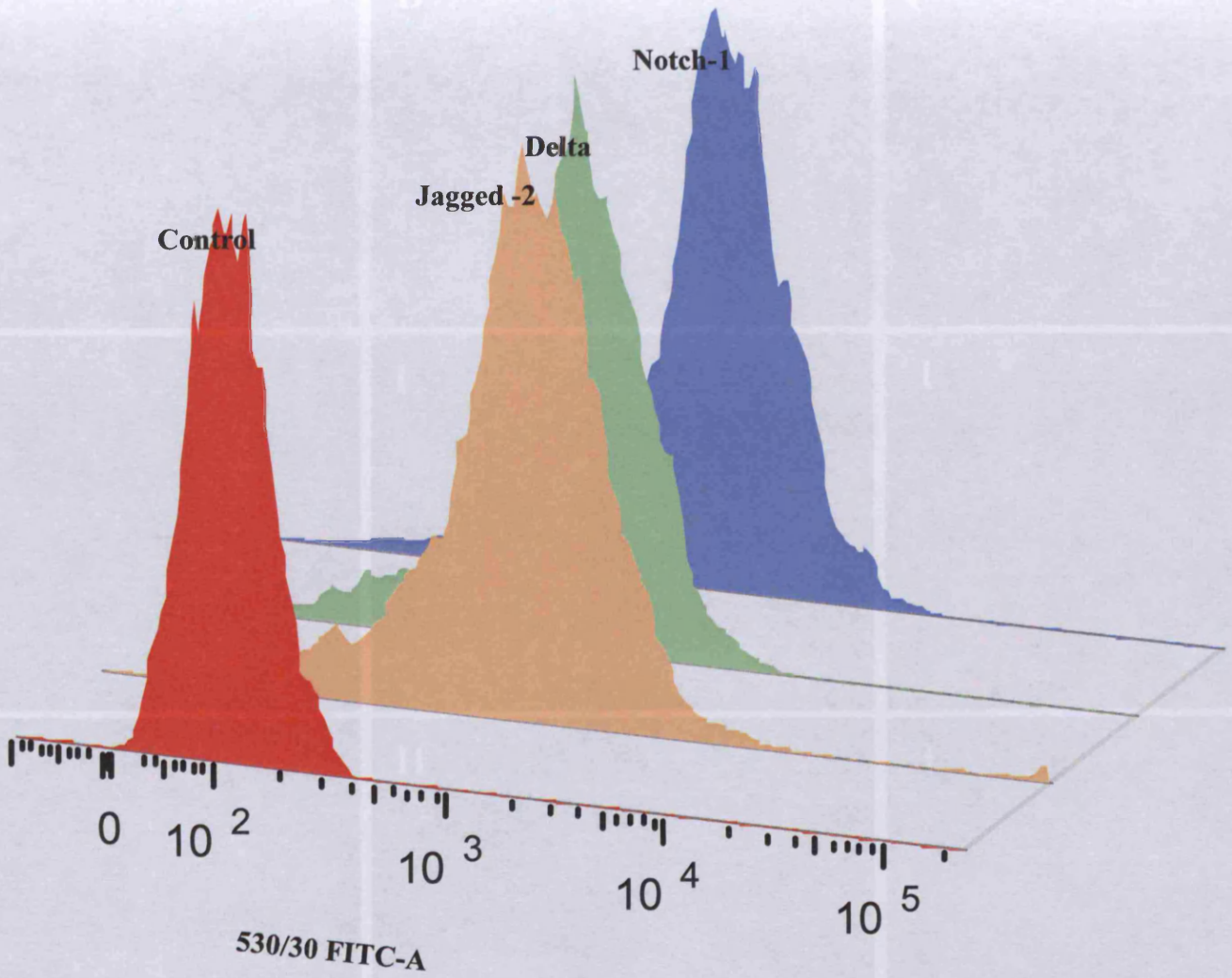


Figure 4.7: FACs data on the expression of Notch-1, Delta and Jagged-2 on the surface of trypsin-treated young bovine BMSCs on Day 8. Negative control used is goat immunoglobulin (Goat IgG), no positive control was used.

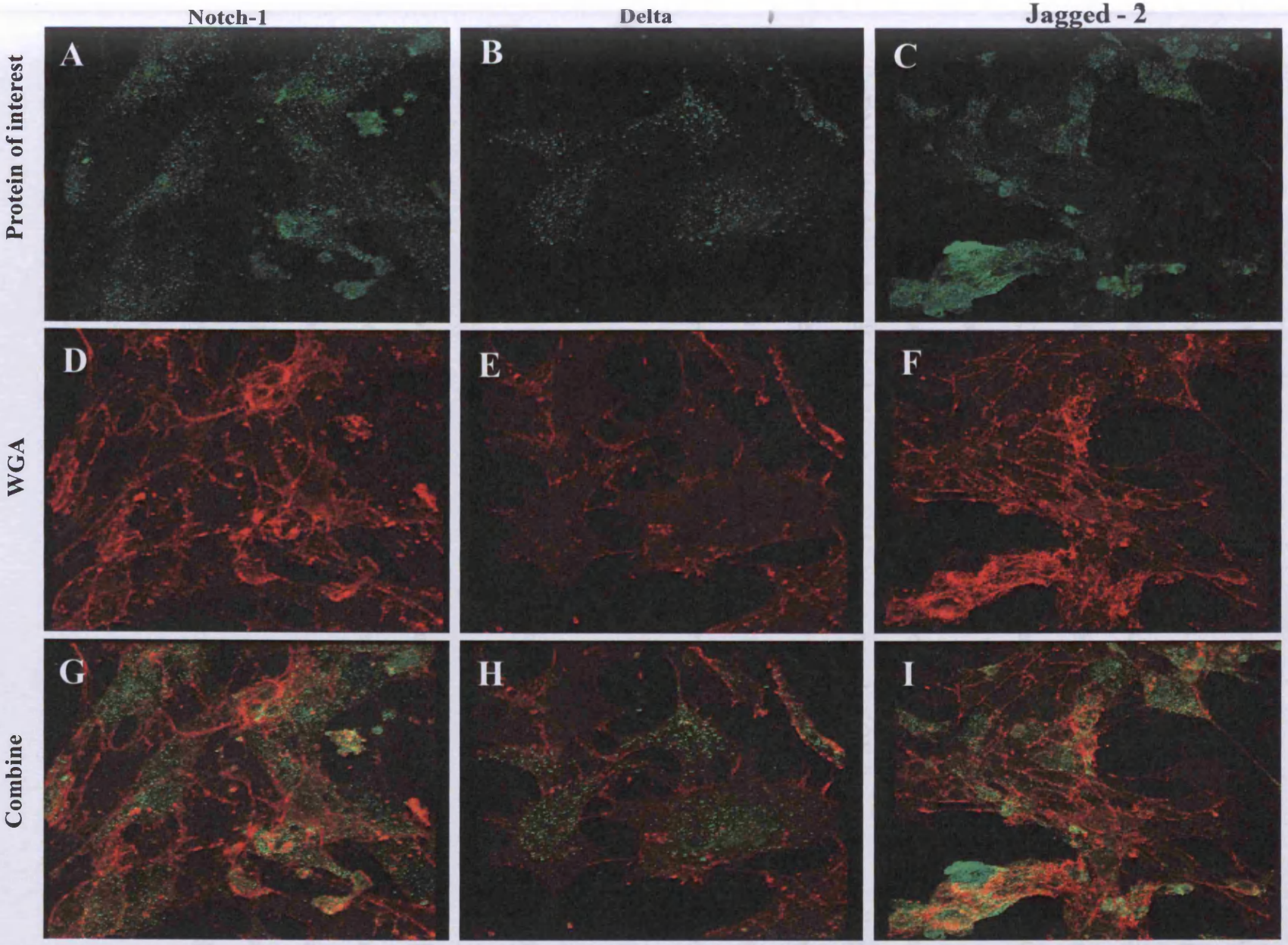


Figure 4.8: Detection of the presence of Notch – 1 (A), Delta (B) and Jagged – 2 (C) using FACs analyses on selected population of BMSCs cultured for 8 days in DMEM with 10% (v/v) FBS and 0.05 mg/ml gentamicin. Cells were labelled with FITC-conjugated antibodies (counterstained with wheat germ agglutinin, D-F) and visualised using scanning confocal microscopy. Panels G, H and I are co-localisation of the two fluorophores.

4.4.4 Quantitative Polymerase Chain Reaction for SOX-9, aggrecan, collagens type I and II of trypsin-treated BMSCs obtained from Day 8

Quantitative PCR was used to assess the gene expression level of P0 MSCs after eight days of culture *in vitro*. The common cartilage specific markers of SOX-9, aggrecan, and collagen type II were used to investigate if these markers were already expressed by direct extract of young bovine bone marrow and from P0 BMSCs cultured for 8 days in DMEM containing gentamicin and 10% (v/v) FBS. Collagen type I was also used to investigate if P0 BMSCs were differentiating a fibroblastic/fibrocartilage phenotype.

For each gene of interest, a standard curve was prepared from a dilution series of template of pDNAs containing the selected cloned gene of interest, namely SOX-9, aggrecan, collagens type I and II (refer to Chapter 3 for details). The qPCR for the dilution series of the template was carried out at the same time as the unknown samples. Ct values of the unknown were calculated from the standard curve plot, which allowed the determination of the copy number for the unknown samples.

4.4.4.1 Comparisons of the gene expression between direct tissue extracts and passage 0 (P0) BMSCs cultured for 8 days in serum containing medium

Direct tissue extracts obtained from young bovine articular cartilage (YBAC), old bovine articular cartilage (OBAC) and young bovine bone marrow (YBBM) were used to compare the gene expression levels of SOX-9, aggrecan, collagens type I and II with trypsin-treated P0-BMSCs cultured in DMEM containing 10% FCS with gentamicin for 8 days. The qualities of RNA extracted from direct tissue extracts (n=1) and from the P0-BMSCs (n=3) were in the range of 1.97 – 2.10 (see Table 4.3).

Samples	260/280 ratio	Amount of RNA (ng/µl)
Old Bovine Articular Cartilage extracts	2.1	141.5
Young Bovine Articular Cartilage extracts	2.08	288.0
Young Bovine Bone Marrow extracts	1.97	262.4
P0 BMSCs (1)	2.08	275.7
P0 BMSCs (2)	1.99	32.6
P0 BMSCs (3)	2.07	295.3

Table 4.3: Summary of the quality of RNA etracted from three separate batches of native tissue extracts and P0 BMSCs.

	SOX-9	COLLAGEN II	COLLAGEN I	AGGRECAN
Old Bovine AC (n=1)	0.058	600.382	0.030	20.650
Young Bovine AC (n=1)	0.048	521.678	31.678	48.042
Young Bovine BM (n=1)	0.000	0.000	0.078	0.000
P0MSCs (n=3)	0.076	0.000	2.785	1.119

Table 4.4: Summary of gene expression levels of GAPDH, SOX-9, collagen type I, collagen type II and aggrecan from different native tissues (old bovine articular artilage, young bovine articlar cartilage and young bovine bone marrow) and from three sets of P0MSCs cells.

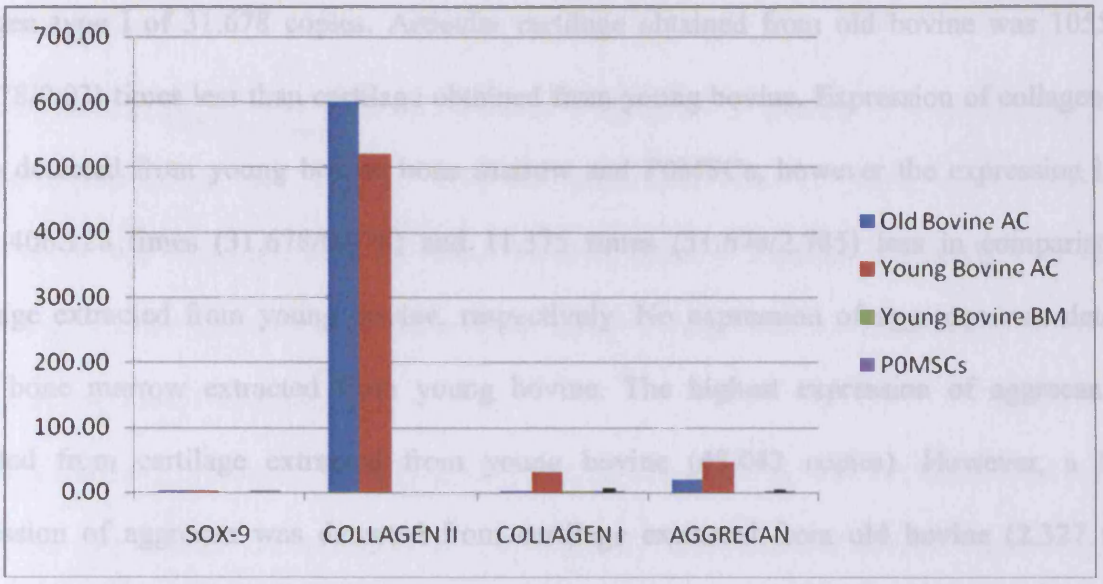


Figure 4.10: Histogram to show the expression pattern of GAPDH, SOX-9, collagen type I, collagen type II and aggrecan of different tissue extracts (old bovine articular cartilage [n=1], young bovine articular cartilage [n=1] and young bovine bone marrow [n=1]) and compared these tissue extracts to P0MSCs (n=3). The y-axis represents the number of copies each gene was expressing, and the x-axis represents the different genes. Error bars were generated from a triplicate of P0MSCs.

No expression of SOX-9 was detected from bone marrow extracted from young bovine. P0MSCs expressed the most of SOX-9 (0.076 copies), followed by old bovine cartilage (0.058 copies) then from young bovine cartilage (0.048 copies). The expression of SOX-9 detected from P0MSCs was 1.340 times (0.076/0.058) higher than old bovine cartilage and 1.583 times higher than young bovine cartilage. Highest expression of collagen type II was observed from articular cartilage extracted from old bovine (600.382 copies). This is interesting because normally, a higher amount of collagen type II would be expressed in articular cartilage extracted from young bovine rather than from old bovine. Here, articular cartilage obtained from old bovine was 1.151 (600.382/521.678) times more expressed than articular cartilage obtained from young bovine. No expression of collagen type II was detected from P0MSCs and from bone marrow extracted from young bovine. Cartilage extracted from young bovine generated the highest expression of

collagen type I of 31.678 copies. Articular cartilage obtained from old bovine was 1055.933 (31.678/0.03) times less than cartilage obtained from young bovine. Expression of collagen type I was detected from young bovine bone marrow and P0MSCs, however the expression levels were 406.128 times (31.678/0.078) and 11.375 times (31.678/2.785) less in comparison to cartilage extracted from young bovine, respectively. No expression of aggrecan was detected from bone marrow extracted from young bovine. The highest expression of aggrecan was detected from cartilage extracted from young bovine (48.042 copies). However, a lower expression of aggrecan was detected from cartilage extracted from old bovine (2.327 times (48.042/20.650)) than young bovine cartilage. A low expression of aggrecan was detected from P0MSCs. The level detected was 42.933 times (48.042/1.119) less than the detected generated from young bovine cartilage.

From this study, it suggested that bone marrow extracted from young bovine did not express SOX-9, Collagen type II and aggrecan. A sound expression of collagen type I was detected in this study, however, this result needs to be further verified since this data is based on an n-number of one. Despite the fact that collagen type I and aggrecan were detected from triplicates sample of P0MSCs, in comparison to the detection obtained from cartilage extracted from young and old bovine, these genes were expressed at a much lower level. These suggested that P0MSCs do not possess a chondrogenic phenotype at such an early cell culture stage.

4.5 Discussion

The aim of this chapter was to isolate primary BMSCs by adherence to tissue culture plastic through aspiration of bone marrow from the metacarpal bone of young bovine. This was followed by the characterisation of these adhered primary BMSCs, P0 BMSCs in serum-containing medium for 8 days by observation using a phase contrast microscope, protein and molecular expression analyses.

Methods that have commonly been used to extract bone marrow include i) aspiration of the iliac crest from live animals (Johnstone *et al.,* 1998) and ii) direct removal of bone marrow from its chamber using sterile culture medium (Zangi *et al.,* 2006). In the later method, bone marrow is aspirated into a syringe containing heparin which prevents blood coagulation. This method has been used in different animal models including rabbit (Johnstone *et al.,* 1998) human (Lennon *et al.,* 1996) and bovine (Bosnakovski *et al.,* 2004). Once the bone marrow is obtained, the BMSCs are isolated based on their; i) adherence to plastics (Friedenstein *et al.,.,* 1976; Prockop 1997; Murdoch *et al.,* 2007;) and ii) differential adhesion to fibronectin (Archer *et al.,* 2007). It is widely agreed that MSCs can adhere to plastic and HSCs cannot (Castro-Malaspina *et al.,* 1980). Furthermore, attachment of cells to negatively charged culture dishes is in fact considered a selector for fibroblastoid cells (Phinney 2002). Usually, bone marrow in culture medium is plated into culture flasks and incubated at 37°C with 5% (v/v) carbon dioxide for 1 - 5 days (Murdoch *et al.,* 2007; Zangi *et al.,* 2006; Mauck *et al.,* 2006). After the adhering period, the medium is changed and the cells are grown to confluence.

An alternative approach to the selection of BMSCs from an enriched bone marrow exudate is based on their adhesion to fibronectin. Differential adhesion to fibronectin utilises the high affinity of stem or progenitor cells for fibronectin, a glycoprotein which binds to integrins on the cell surface. The cells are subjected to serial incubations in order to select for colony-forming cells. The colony – forming unit fibroblast (CFU - F) assay can be used to quantify the adherent

cell population by scoring colonies presumed to be derived from a single precursor (Friedenstein *et al.,* 1976; Deans *et al.,* 2000; Perkins & Fleischman 1990).

In addition to the above purification methods, separation between nucleated MSCs and non-nucleated HSCs can be achieved using a Lymphoprep™ kit from Nycomed Pharma (Oreffo *et al.,* 1997) or by density gradient centrifugation to obtain mononucleated cells (Majumdar *et al.,* 1998). The initial method for the extraction of bone marrow utilised in this study was adapted from Dr Richard Oreffo's laboratory in Southampton University (Oreffo *et al.,* 1997).

This study has demonstrated that differences in bone marrow chamber size and bone thickness influence the amount of bone marrow and subsequently the number of cells harvested. The variations in chamber size could be due to many factors including arrival age, front and back leg (carpo versus tarso), arrival breed, sex of the animal, and time after slaughter which may influence cell viability. Previous study has shown that the isolation of BMSCs from crude BM, by adherence to plastic, enabled a separation of hematopoietic and non-hematopoietic cells as described by other research groups (Oreffo *et al.,* 1997). The morphology of the cells at Day 4 and Day 8 showed the typical spindle-shaped fibroblastic features of undifferentiated MSCs and colony-forming MSCs that were previously observed by Friedenstein and Caplan.

FACs analyses indicated that undifferentiated MSCs (harvested at Day 8) expressed the stem / progenitor markers of Notch-1, Delta and Jagged-2 (Dowthwaite *et al.,* 2004). This suggested that the cells isolated initially exhibit stem cell like features. Hardingham and co-workers recently demonstrated that Notch signalling through Jagged-1 is necessary to initiate chondrogenesis of MSCs using a human model. However, the expression of Jagged-1 must be switched off in order to enable MSCs to complete chondrogenesis (Oldershaw *et al.,* 2008).

From this study, it suggested that bone marrow extracted from young bovine did not express SOX-9, Collagen type II and aggrecan. A sound expression of collagen type I was detected in this study, however, this result needs to be further verified since this data is based on an n-number of one. Despite the fact that collagen type I and aggrecan were detected from triplicates sample of P0MSCs, in comparison to the detection obtained from cartilage extracted from young and old bovine, these genes

were expressed at a much lower level. These suggested that P0MSCs do not possess a chondrogenic phenotype at such an early cell culture stage.

4.6 Summary

- MSCs isolated possessed a spindle–shaped fibroblastic morphology as observed in previous published work on similar systems.
- Protein expression of Notch-1, Delta and Jagged-2 by primary MSCs suggests they possess some stem/progenitor cell features.
- No expression of SOX-9, aggrecan, type I and type II collagens were detected from P0MSCs.

4.7 Future work

- Use Lymphoprep to separate hematopoietic and non-hematopoietic cells, enabling a clear number of the mononucleated cells to be obtained.
- Carry out cell viability testing to assess the viability of the cells after the first trysinisation on Day 8.
- Jagged-1, as well as Notch-1, Delta and Jagged-2, should be used as markers for the differentiation of MSCs towards a chondrogenic lineage.
- Perform qPCR analyses using primer pairs designed for Notch-1, Delta and Jagged-2 in order to quantify the amount of these markers being expressed by undifferentiated MSCs.

Chapter 5:

Investigate the effects of Fibroblast Growth Factor alone and in the presence of Transforming Growth Factor Beta-2 on the differentiation of BMSCs to a chondrogenic phenotype

5.1 Introduction

In this chapter, a selected sub-population of BMSCs (described in Chapter 4) were cultured in serum-containing medium described in Chapter 4 in the presence of 5ng/ml of fibroblast growth factor 2 (FGF-2) and in the presence of both FGF-2 and transforming growth factor beta-2 (TGF-β2) to ascertain proliferation properties of these cytokines on primary BMSCs and their abilities to initiate differentiation of the BMSCs to a chondrogenic lineage.

Many factors such as cytokines and growth factors contribute to the cellular events of proliferation, differentiation and maturation of cell types through binding to target cell surface receptors and initiating intracellular events (as described in Chapter 1 Section 1.4.1).

Many reports have shown that FGF-2 contributes to the selection of MSCs that are more likely to differentiate chondrogenically Bianchi *et al., 2003*. Previous studies have suggested that addition of FGF-2 during the subculture phase enhances the chondrogenic potential of human derived MSCs (Solchaga *et al.,* 2005). As well as FGF-2, TGF-β has been found to be one of the most potent inducers of chondrogenic differentiation of BMSCs isolated from bone marrow in many different species and other tissue sources (Bosnakovski *et al.,* 2004). Alterations in the concentration of TGF-β have been shown to affect the expression levels of certain extracellular matrix compounds in a bovine model (Bosnakovski *et al.,* 2004). Stevens *et al.,* have demonstrated that the combined use of TGF- β and FGF-2 influenced perichondrium to differentiate towards a chondrogenic lineage (Stevens *et al.,* 2004).

In this study, the initial BMSCs cell isolates were plated and maintained in serum-containing medium for 8 days (see Chapter 3). On day 8, the cells were trypsinized and subcultured at different cell densities in serum-containing medium with 5 ng/ml of FGF-2 and in the presence of 5ng/ml FGF-2 and 5ng/ml TGF-β2. The cells were cultured until they were confluent on plate under a phase contrast microscope, passaged, and the BMSCs were taken for the following analyses:-

- Cell number and cell morphology
- At each passage the protein expression of the stem/progenitor cell markers Notch-1, Delta and Jagged-2 were assessed by FACS analysis to determine whether the cells remained in an undifferentiated state.
- At each passage molecular expression of SOX-9, aggrecan, collagens type I and type II were determined in order to ascertain whether the potential stem cells had undergone chondrogenic differentiation

The outline of the experiment and the analyses carried out for this chapter are summarised in Figure 5.1.

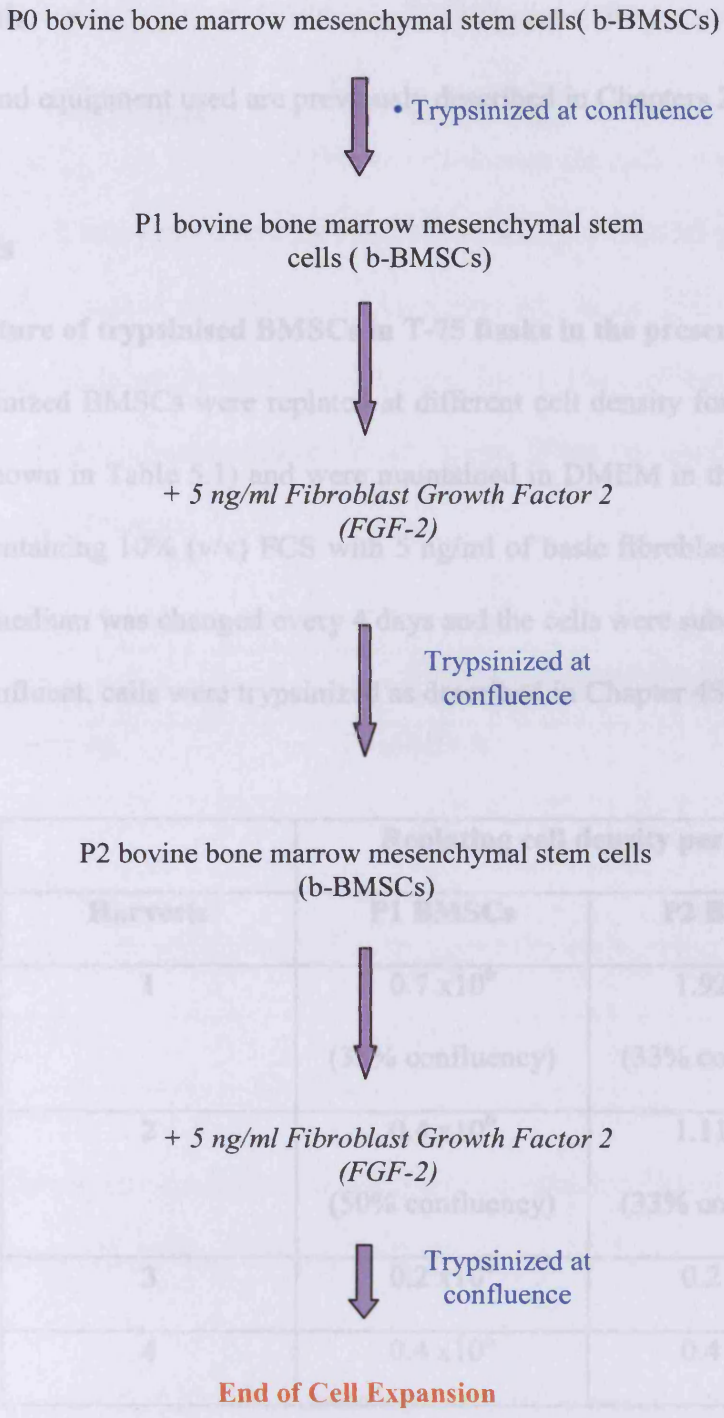


Figure 5.1: A flow chart to illustrate the processing of Passage One (P1) and Passage Two (P2) bovine bone marrow mesenchymal stem cells (b-BMSCs). Analyses carried at different passages were summarized as above.

5.2 Materials

All reagents and equipment used are previously described in Chapters 2 and 3

5.3 Methods

5.3.1 Sub-culture of trypsinised BMSCs in T-75 flasks in the presence of FGF-2

Briefly, trypsinized BMSCs were replated at different cell density for each harvest and at each passage (as shown in Table 5.1) and were maintained in DMEM in the presence of 0.05 mg/ml gentamicin containing 10% (v/v) FCS with 5 ng/ml of basic fibroblastic-growth factor (FGF-2, Sigma). The medium was changed every 4 days and the cells were subcultured until confluent by eye. When confluent, cells were trypsinized as described in Chapter 4Section 4.3.2)

	Replating cell density per flask	
Harvests	**P1 BMSCs**	**P2 BMSCs**
1	0.7×10^6 (33% confluency)	1.92×10^6 (33% confluency)
2	0.4×10^6 (50% confluency)	1.11×10^6 (33% confluency)
3	0.2×10^6	0.2×10^6
4	0.4×10^6	0.4×10^6

Table 5.1: A table to summarise the replating cell densities for P1 and P2 BMSCs cultured in the presence of 5ng/ml of FGF-2.

5.3.2 Sub-culture of trypsinised BMSCs in T-75 flasks in the presence of TGF-β2 and FGF-2

Trypsinized BMSCs were plated at different cell density for each harvest and at each passage as shown in Table 5.2 and were maintained in DMEM with 0.05 mg/ml gentamicin containing 10% (v/v) FCS with 5 ng/ml of basic fibroblastic-growth factor (FGF-2, Sigma) and 5 ng/ml transforming growth factor beta-2 (TGF-β2, PeproTech). The medium was changed every 4 days and the cells were subcultured until confluent by eye. When confluent, cells were trypsinized as outlined in Chapter 4 Section 4.3.2)

	Replating cell density per flask	
Harvests	**P1 BMSCs**	**P2 BMSCs**
1	0.7×10^6	3.3×10^6
2	0.4×10^6	4.31×10^6
3	0.2×10^6	0.2×10^6
4	0.4×10^6	0.4×10^6

Table 5.2: A table to summarise the replating cell densities for P1 and P2 BMSCs cultured in the presence of 5ng/ml of FGF-2 and 5ng/ml TGF-β2.

At each passage, a total of 2×10^6 cells were retained for analyses of protein expression of Notch – 1, Delta and Jagged – 2 using FACs analyses (see Chapter 4 Section 4.3.3) and for quantitative PCR for the expression of chondrogenic markers, namely, SOX-9, aggrecan, collagens type I and II (see Chapter 3 Section 3.3.11).

5.4 Results

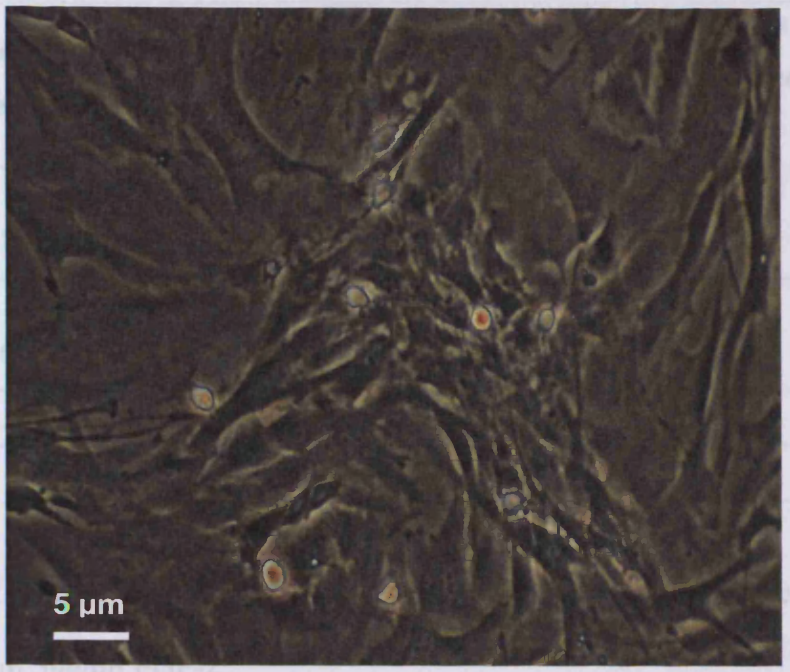
5.4.1 Cell morphology and cell numbers of Passage One (P1) and Two (P2) BMSCs cultured in Basal Medium supplemented with 5 ng/ml of Fibroblast Growth Factor - 2 (FGF-2)

BMSCs from four harvests were cultured in serum containing medium for 8 days on tissue culture plastic. The selected population of BMSCs were trypinised on Day 8 and replated at different cell density for each of the harvest as shown in Table 5.3 and the cells are referred to as Passage One (P1). The cells were fed every 2 days with 20 ml of fresh medium.

The cell morphology was recorded by photographing under a phase contrast microscope. Unlike P0 BMSCs, P1 BMSCs cultured in the presence of 5 ng/ml of FGF-2 had no distinct cell foci or colonies and were distributed evenly throughout the base of the tissue culture flask. The morphology of P1 BMSCs were fibroblastic with long and thin cell shape, and appeared to be more granular and more fibroblastic than P2 BMSCs as can be seen on Figure 5.2. Even though harvests 2 and 4 were replated at the same replating cell density of 0.4 $x10^6$ cells / flask (Table 5.3), the number of days for the cells to become confluent differed (Table 5.3). Harvest 2 took 10 days to become confluent, whilst harvest 4 required half the time to reach confluency. Individual cell count from each flask from the four experiments were recorded as shown in Table 5.4. The replating cell density and cell count from each flask of Passage two (P2) BMSCs cultured in serum-containing medium with the addition of 5 ng/ml FGF-2 were recorded in Table 5.5 and 5.6, respectively. The data (Table 5.5) showed that the cell count per flask at confluence from P2 BMSCs was lower than P1 BMSCs (Table 5.3). The lowest difference was 0.5-fold from harvest 3 and the highest difference observed was 4-fold from harvest 1. The number of days the cells used to reach confluence was similar between P1 and P2 BMSCs, with the exception of harvest 4, where there were 6 days difference between P1 and P2 BMSCs (see Table 5.3 and 5.5).

+FGF-2

P1 BMSC



P2 BMSC

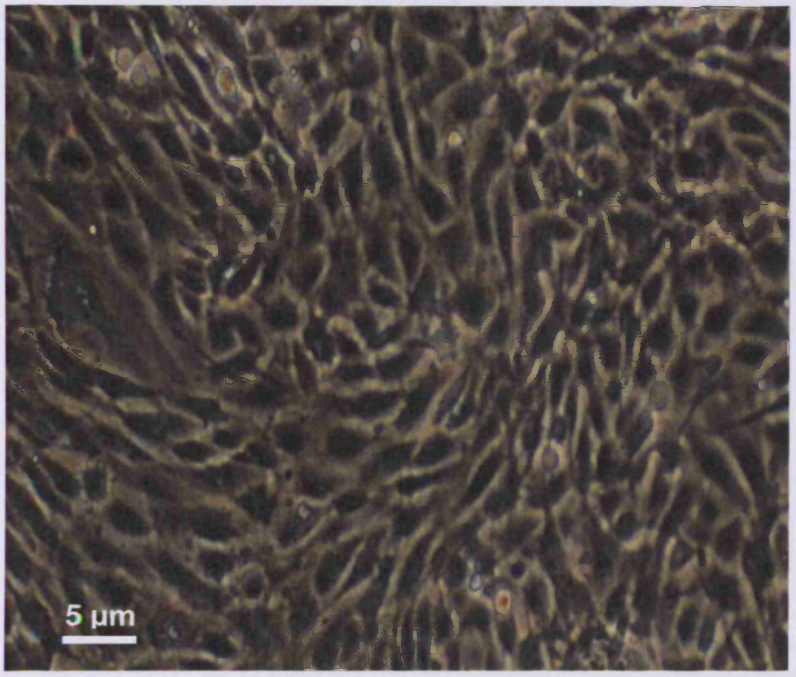
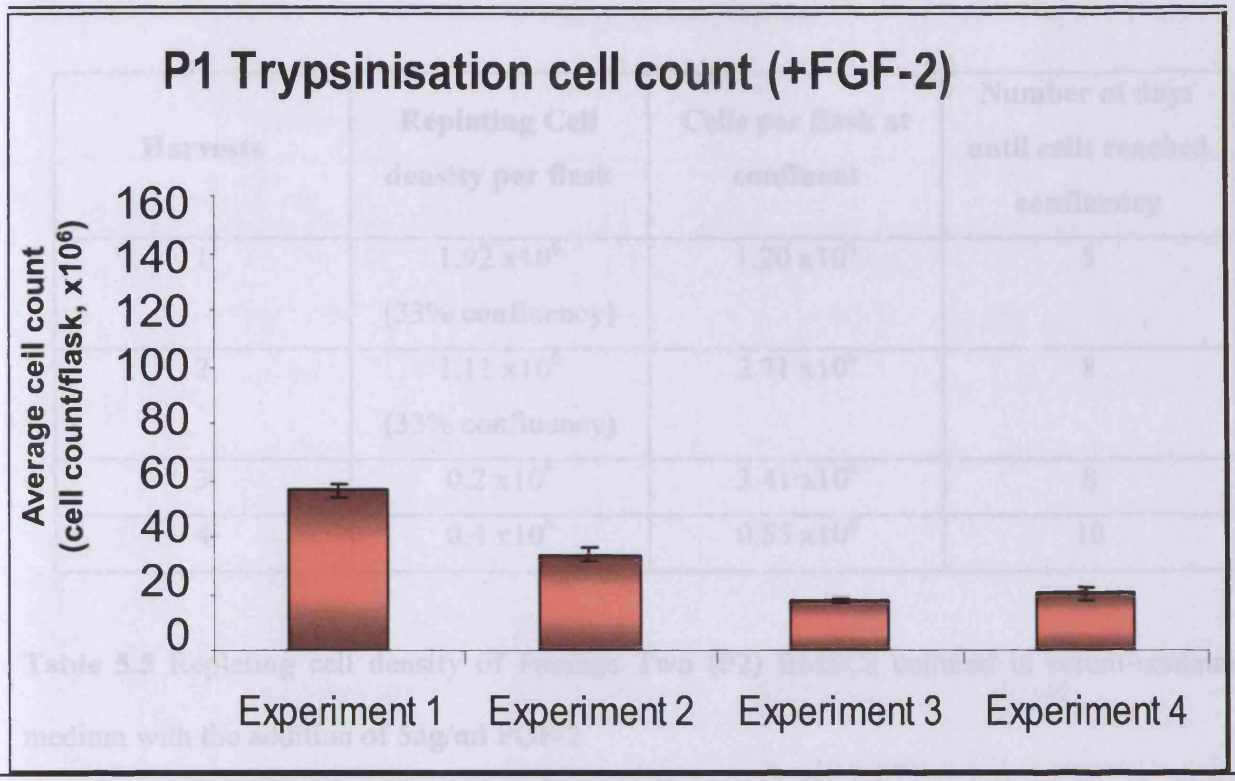


Figure 5.2: Cell morphology of bovine derived mesenchymal stem cells expanded in monolayer in the presence of 5 ng/ml FGF-2 at passage 1 (P1) and passage 2 (P2). The scale shown represents 5µm.

Harvests	Replating Cell density per flask	Cells per flask at confluent	Number of days until cells reached confluency
1	0.7×10^6 (33% confluency)	5.65×10^6	4
2	0.4×10^6 (50% confluency)	3.38×10^6	10
3	0.2×10^6	1.77×10^6	11
4	0.4×10^6	2.01×10^6	4

Table 5.3: Replating cell density of Passage One (P1) BMSCs cultured in serum-containing medium with the addition of 5ng/ml FGF-2.



P1 trysinisation cell count (x10⁶)				
	Experiment 1 n=6	Experiment 2 n=8	Experiment 3 n=6	Experiment 4 n=3
	4.600	4.540	1.620	2.300
	5.560	2.220	1.890	1.540
	6.480	3.220	1.510	2.200
	5.400	3.700	1.620	
	5.640	2.980	1.890	
	6.160	3.380	2.100	
		4.100		
		2.920		
Total cell	33.840	27.060	10.630	6.040
Av. cells/flask	**5.640**	**3.383**	**1.772**	**2.013**
Std dev.	0.651	0.728	0.224	0.413
Error	0.266	0.257	0.091	0.238

Table 5.4: Cell count per flask of P1 BMSCs cultured in the presence of 5 ng/ml of FGF-2 from four harvests. Top panel shows the average cell count per flask obtained from different harvests in a histogram format. Bottom panel is a tabulated format of the cell count data.

Harvests	Replating Cell density per flask	Cells per flask at confluent	Number of days until cells reached confluency
1	1.92 x10^6 (33% confluency)	1.20 x10^6	5
2	1.11 x10^6 (33% confluency)	2.71 x10^6	8
3	0.2 x10^6	3.41 x10^6	8
4	0.4 x10^6	0.55 x10^6	10

Table 5.5 Replating cell density of Passage Two (P2) BMSCs cultured in serum-containing medium with the addition of 5ng/ml FGF-2.

P2 trypsinisation cell count (x10^6)			
Experiment 1	Experiment 2	Expperiment 3	Experiment 4
n=14	n=3	n=10	n=7
1.140	2.780	3.600	0.760
2.480	2.520	3.340	0.440
2.300	2.840	2.340	0.600
1.440		3.320	0.500
0.820		3.720	0.440
1.110		5.500	0.500
1.110		3.400	0.620
1.110		2.820	
1.110		3.280	
0.836		2.760	
0.836			
0.836			
0.836			
0.836			
Total cell 16.800	8.140	34.080	3.860
Av. cells/flask 1.200	**2.713**	**3.408**	**0.551**
Std Dev. 0.537	0.170	0.846	0.116
Error 0.144	0.098	0.268	0.044

Table 5.6: Cell count per flask of P2 BMSCs cultured in the presence of 5 ng/ml of FGF-2 from four harvests. Top panel shows the average cell count per flask obtained from different harvests in a histogram format. Bottom panel is a tabulated format of the cell count data.

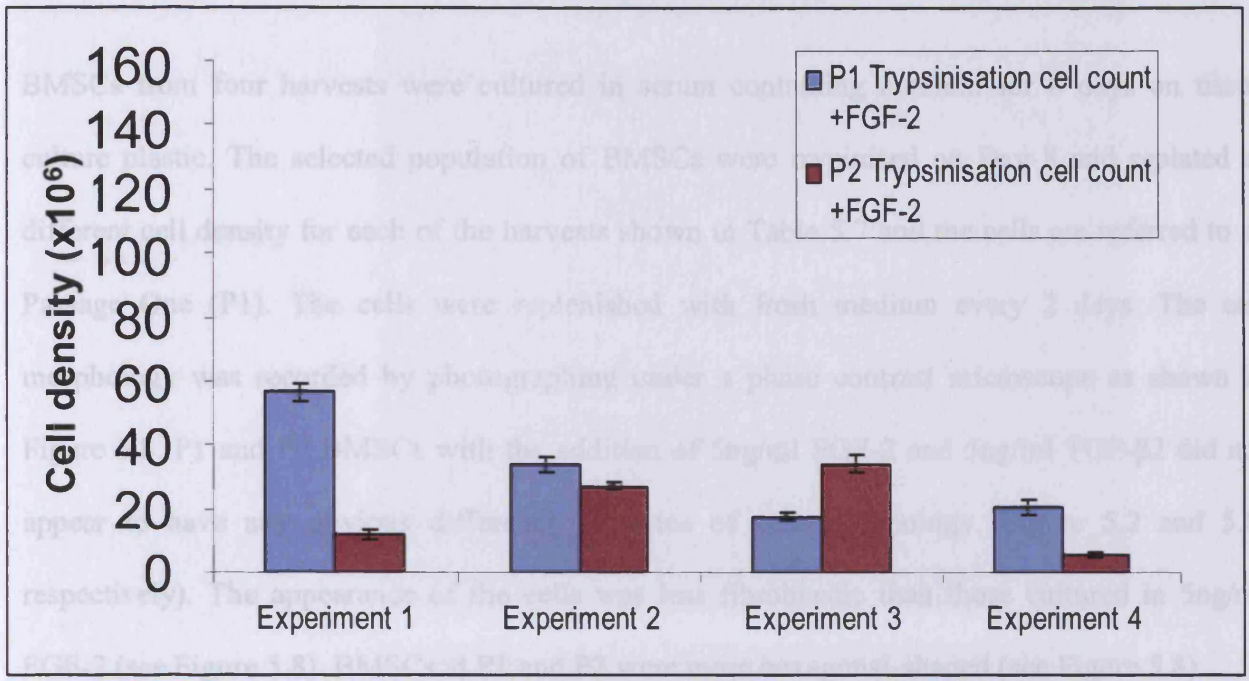


Figure 5.3: Comparison between the average cell count from P1 and P2 BMSCs trypsinisation after culturing in serum containing medium with the addition of 5 ng/ml FGF-2.

Table 5.5 summarised the number of cells obtained per flask and the number of days for cells to reach confluence for P2 BMSCs. At this point, the trypsinised cells were seeded at different cell density into Transwell inserts (refer to Chapter 6). Figure 5.3 compares the average cell numbers obtained from the average cell density per flask for each harvest at different passage number (P1, blue and P2, purple) with different replating cell density, cultured in serum containing medium with the addition of 5 ng/ml FGF-2. P1 BMSCs have a higher overall cell count than P2 BMSCs with the exception of harvest 3, the greatest difference was observed between harvests 1 and 4 for P1 BMSCs with a difference of 6- and 5- fold, respectively (see Figure 5.3).

5.4.2 Cell morphology and cell numbers of Passage One (P1) and Two (P2) BMSCs cultured in 5 ng/ml of FGF-2 and 5ng/ml TGF β-2

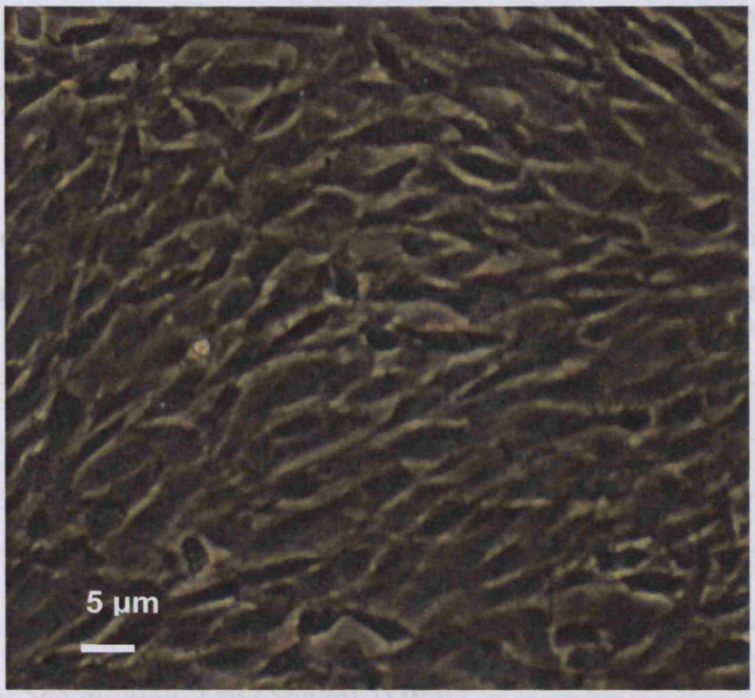
BMSCs from four harvests were cultured in serum containing medium for 8 days on tissue culture plastic. The selected population of BMSCs were trypinised on Day 8 and replated at different cell density for each of the harvests shown in Table 5.7 and the cells are referred to as Passage One (P1). The cells were replenished with fresh medium every 2 days. The cell morphology was recorded by photographing under a phase contrast microscope as shown in Figure 5.8. P1 and P2 BMSCs with the addition of 5ng/ml FGF-2 and 5ng/ml TGF-β2 did not appear to have any obvious difference in terms of cell morphology (Figure 5.2 and 5.8, respectively). The appearance of the cells was less fibroblastic than those cultured in 5ng/ml FGF-2 (see Figure 5.8). BMSCs at P1 and P2 were more hexagonal-shaped (see Figure 5.8).

Harvests	Replating Cell density per flask	Cells per flask at confluence	Number of days until cells reached confluency
1	0.7×10^6 (33% confluency)	9.64×10^6	4
2	0.4×10^6 (50% confluency)	13.57×10^6	10
3	0.2×10^6	6.0×10^6	7
4	0.4×10^6	5.26×10^6	3

Table 5.7: Replating cell density of Passage One (P1) BMSCs cultured in serum-containing medium with the addition of 5ng/ml FGF-2 and 5ng/ml TGF-β2.

+FGF-2 & TGF-β 2

P2 BMSC



P3 BMSC

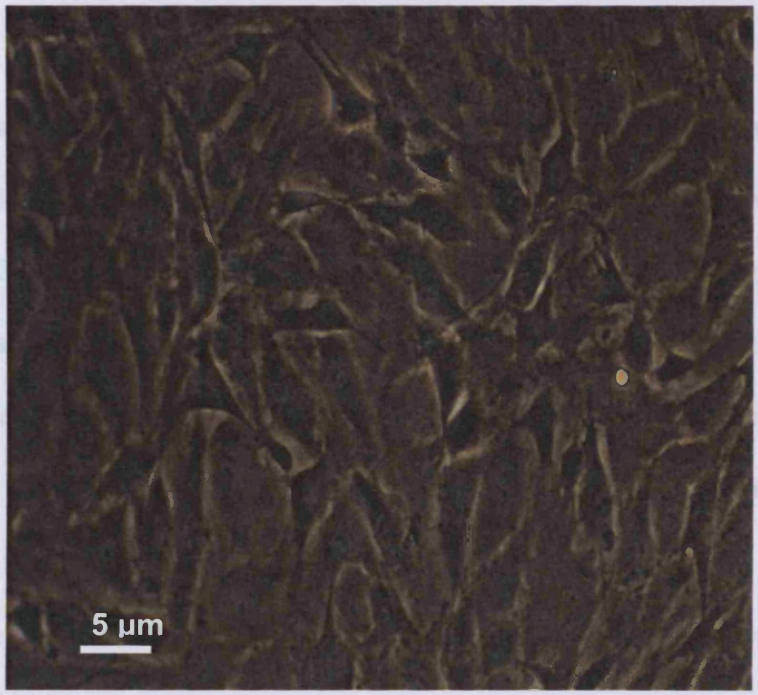


Figure 5.8: Cell morphology of bovine derived mesenchymal stem cells expanded in monolayer in the presence of 5 ng/ml FGF-2 with the addition of 5 ng/ml of TGF-beta 2 at passage 1 (P1) and passage 2 (P2).

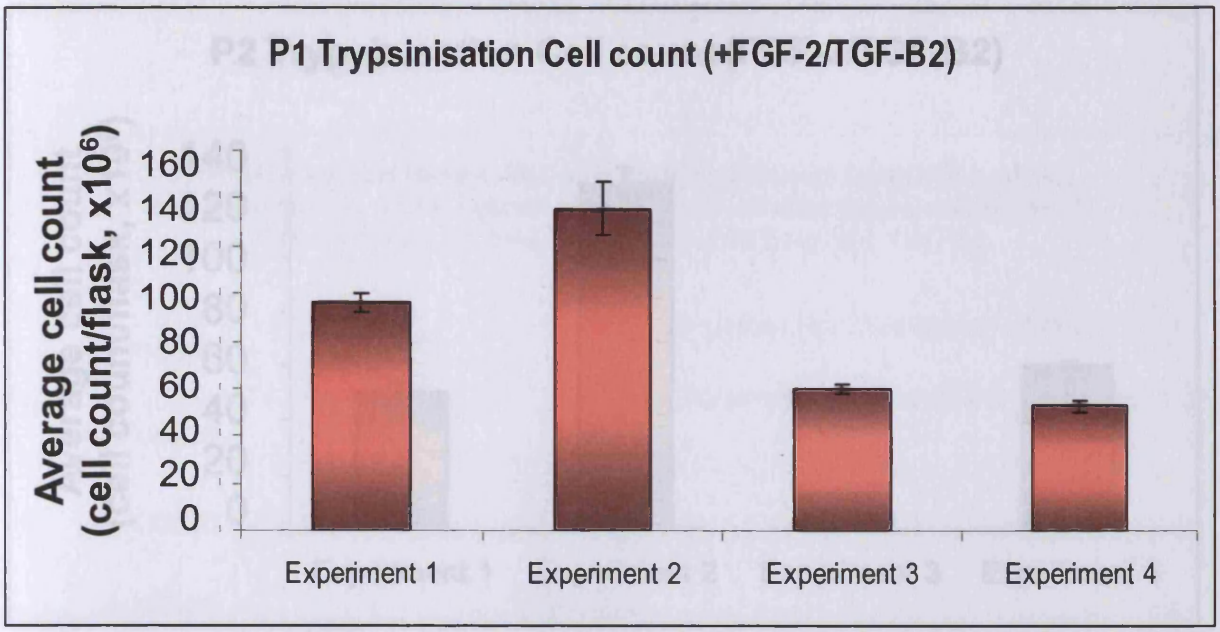
When P1 BMSCs had reached confluence, the cells were trypsinised as before. The average cell number in each flask and the number of days the cells took to reach confluence were recorded and the results were summarised in Table 5.8 and 5.9.

Harvests	Replating cell density per flask	Number of cells per flask at confluence	Days for cells to reach confluence
1	3.3×10^6	5.088×10^6	5
2	4.31×10^6	12.73×10^6	3
3	0.2×10^6	0.62×10^6	6
4	0.4×10^6	6.04×10^6	4

Table 5.8: Cell count from each flask at Passage One (P2) BMSCs cultured in serum-containing medium with the addition of 5ng/ml FGF-2 and 5ng/ml TGF-β2.

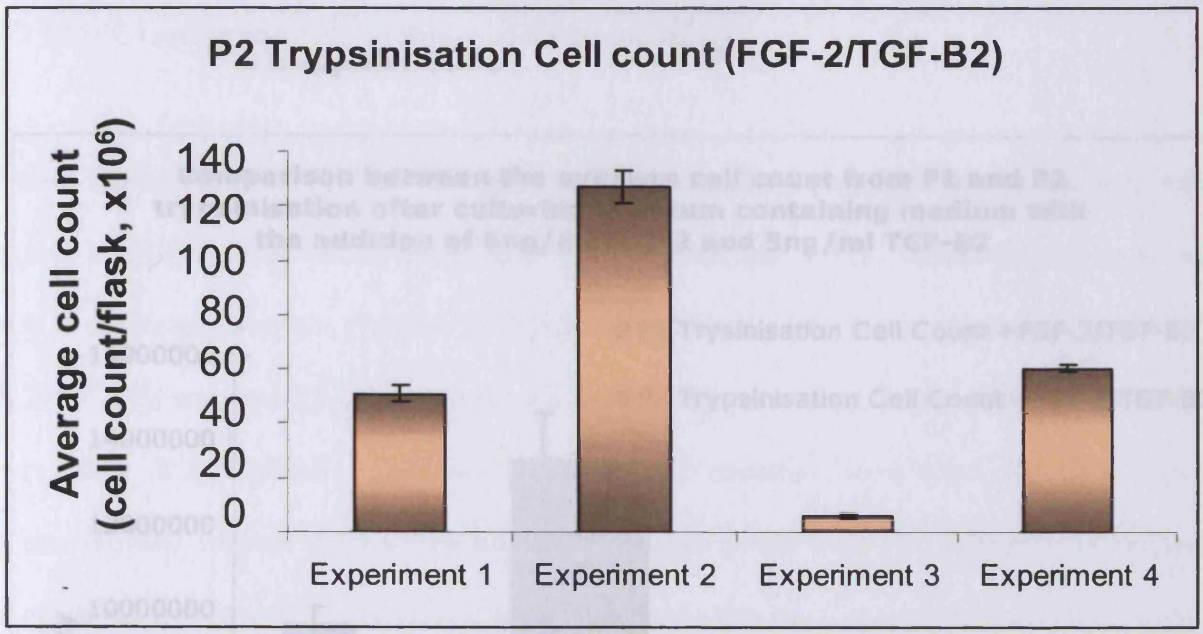
The P2 trypsinised BMSCs cells were counted from each flask and all results were recorded as shown in Table 5.10. At this stage, the trypsinised cells were seeded at different cell density into Transwell inserts (refer to Chapter 6). The cell count data obtained from P1 and P2 trypsinisation were plotted on a histogram to compare the results as shown in Figure 5.9. Out of the four harvests, the average cell count obtained from P1 stage exceeded P2 BMSCs, with the exception of harvest 4. A difference of 50% and 80% can be seen from Harvests 1 and 3, respectively, whilst harvest 2 only had a minute difference of about 10%.

Our data shown that cells cultured in FGF-2 and with the addition of TGF-β2, cells were evenly distributed and no formation of cell foci was formed. In FGF-2, P1 BMSCs appeared to be more fibroblastic than P2 BMSCs. Cell count obtained was higher from P1 than P2 BMSCs cultured in either FGF-2 or with the addition of TGF-β2. The cell number obtained was generally higher from BMSCs cultured in FGF-2/TGF-β2 than in FGF-2 alone. From these data, it has suggested that culturing BMSCs in the presence of two growth factors gave an increased number of cells per flask, i.e. increased cell proliferation.



	P1 trypsinisation cell count +FGF-2/TGF-beta 2 (x10^6)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	10.26	18.60	6.46	4.58
	10.16	11.00	5.82	6.02
	10.20	13.00	6.80	5.44
	8.40	13.40	5.70	5.00
	8.40	11.40	5.70	
	10.40	14.00	5.50	
		14.20		
		9.00		
Average	9.64	13.57	6.00	5.26
Std Deviation	0.96	2.73	0.51	0.62
Std error	0.40	1.11	0.21	0.25

Table 5.8: Cell count per flask of P1 BMSCs cultured in the presence of 5ng/ml of FGF-2 and 5ng/ml TGF-β2 from four harvests. Top panel shows the average cell count per flask obtained from different harvests in a histogram format. Bottom panel is a tabulated format of the cell count data.



	P2 trypsinisation cell count +FGF-2/TGF-beta 2 (x10^6)			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	3.94	12.20	0.52	5.98
	3.74	14.80	0.70	5.74
	4.34	11.20	0.35	6.34
	6.64		0.42	5.26
	6.22		0.64	5.60
	5.20		1.09	5.78
	5.20			6.48
	5.20			6.18
	5.20			6.90
	5.20			6.14
Average	5.09	12.73	0.62	6.04
Std Deviation	0.91	1.86	0.26	0.47
Std error	0.29	0.59	0.08	0.15

Table 5.9: Cell count per flask of P2 BMSCs cultured in the presence of 5ng/ml of FGF-2 and 5ng/ml TGF-β2 from four harvests. Top panel shows the average cell count per flask obtained from different harvests in a histogram format. Bottom panel is a tabulated format of the cell count data.

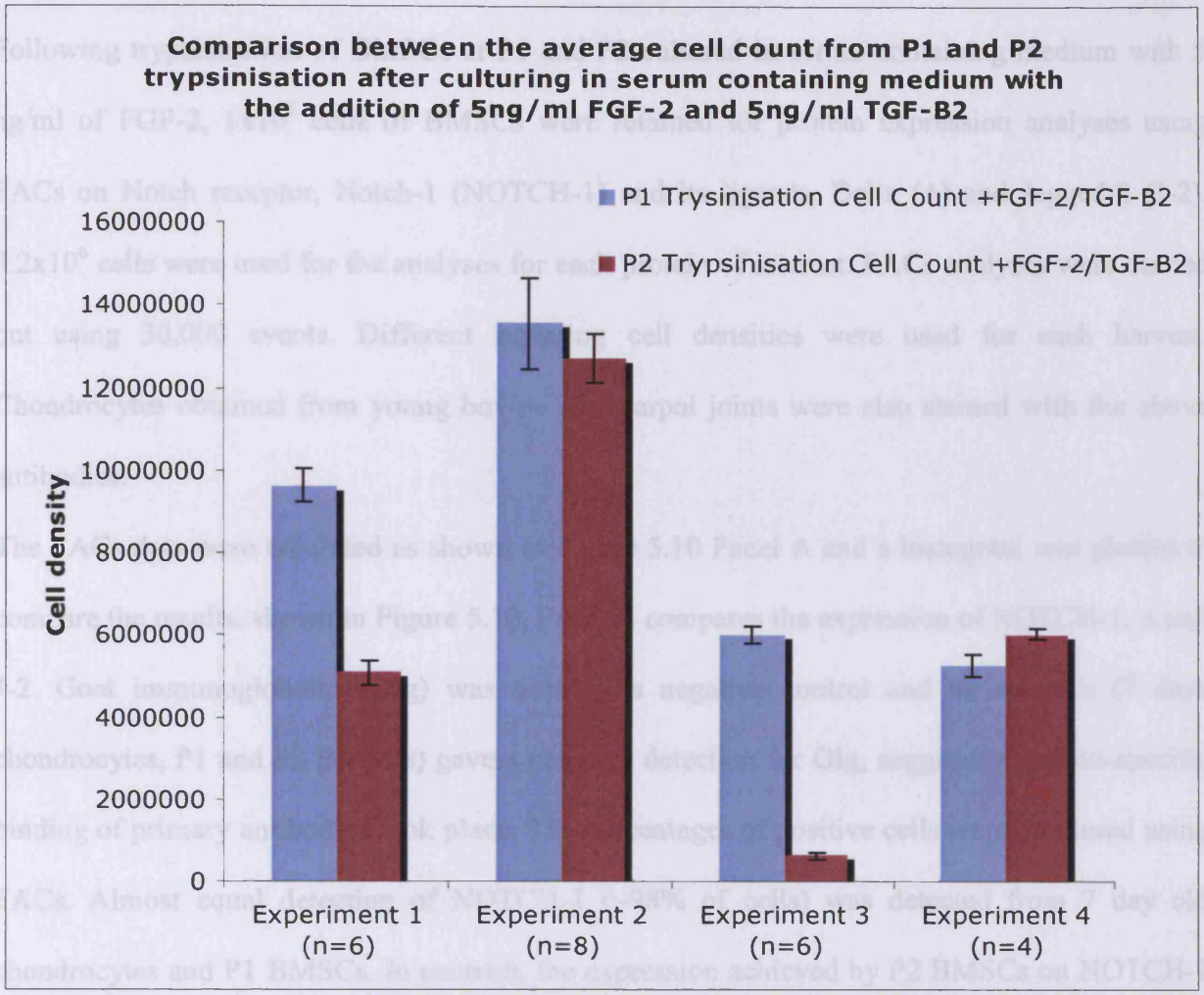


Figure 5.9: Comparison between the average cell count from P1 and P2 BMSCs trypsinisation after culturing in serum containing medoium with the addition of 5 ng/ml FGF-2 and TGF-β2.

5.4.3 Protein expression of Notch-1 receptor and its ligands Delta and Jagged-2 by P1 and P2 BMSCs cultured in the presence of 5 ng/ml FGF-2

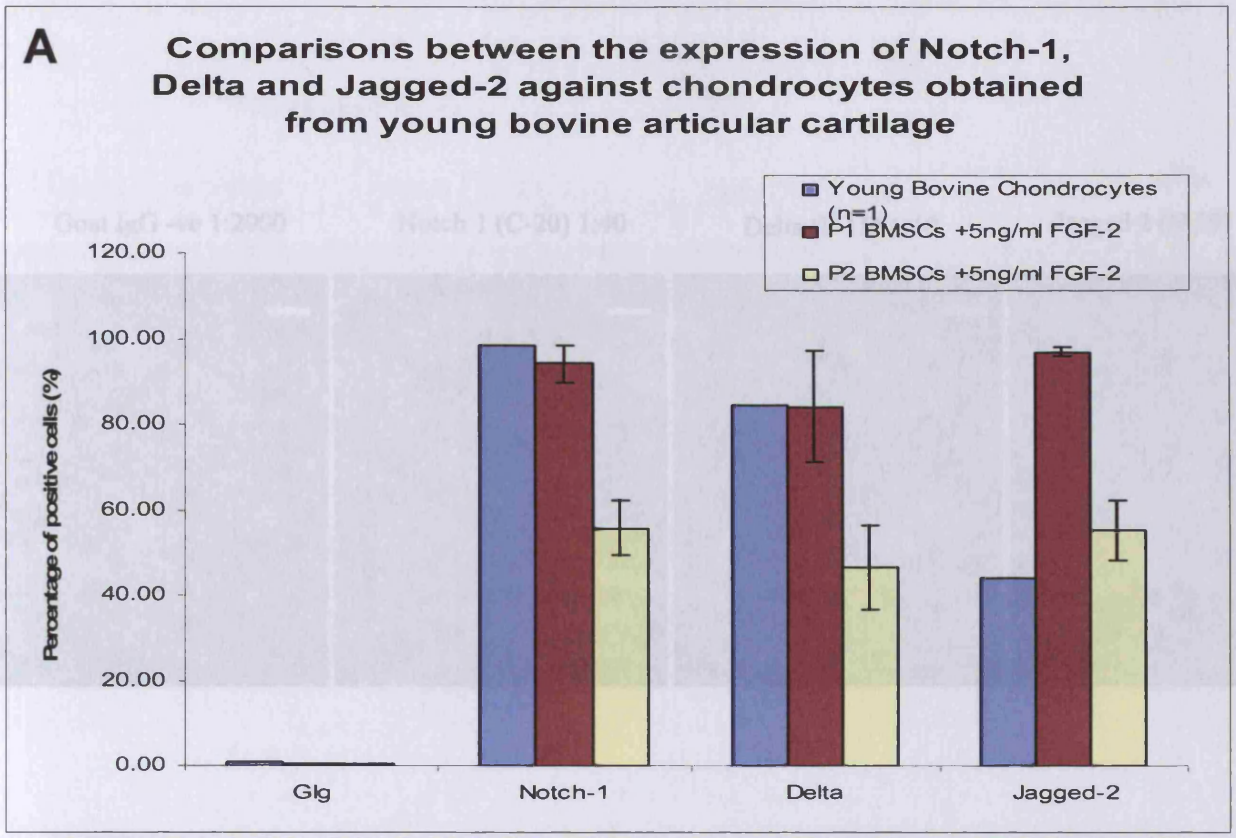
Following trypsinisation of BMSCs at P1 and P2 cultured in serum-containing medium with 5 ng/ml of FGF-2, $1x10^6$ cells of BMSCs were retained for protein expression analyses using FACs on Notch receptor, Notch-1 (NOTCH-1) and its ligands, Delta (Δ) and Jagged-2 (J-2). $0.2x10^6$ cells were used for the analyses for each protein of interest. FACs analyses were carried out using 30,000 events. Different replating cell densities were used for each harvest. Chondrocytes obtained from young bovine metacarpal joints were also stained with the above antibodies.

The FACs data were tabulated as shown in Figure 5.10 Panel A and a histogram was plotted to compare the results, shown in Figure 5.10, Panel B compares the expression of NOTCH-1, Δ and J-2. Goat immunoglobulin (GIg) was used as a negative control and all samples (7 days chondrocytes, P1 and P2 BMSCs) gave a negative detection for GIg, suggesting no non-specific binding of primary antibodies took place. The percentages of positive cells were measured using FACs. Almost equal detection of NOTCH-1 (~98% of cells) was detected from 7 day old chondrocytes and P1 BMSCs. In contrast, the expression achieved by P2 BMSCs on NOTCH-1 was 50% less than chondrocytes and P1 BMSCs. The expression pattern for Delta was similar to that of NOTCH-1. These data suggested that the expression of NOTCH-1 and Delta were reduced with increased passage number of BMSCs in a bovine model. The expression pattern for Jagged-2 was different to NOTCH-1 and Delta. P1 BMSCs expressed the highest amount of Jagged-2 (97%), whilst the expression by P2 BMSCs (55%) exceeds chondrocytes (44%) by approximately 10%.

When FACs analyses were completed, P1 and P2 BMSCs were counter-stained with DAPI and were photographed as shown in Figures 5.11 and 5.12 for P1 and P2 BMSCs, respectively. GIg

was used as a negative control. NOTCH-1, delta and Jagged-2 appeared to be distributed uniformly throughout the surface of P1 and P2 BMSCs.

A



B	Percentage of Positive cells (%)			
	Glg	Notch-1	Delta	Jagged-2
Young Bovine Chondrocytes	0.90	98.40	84.70	44.10
P1 BMSCs (1)	0.40	90.10	71.30	96.00
P1 BMSCs (2)	0.50	98.50	97.30	98.20
Average	*0.45*	*94.30*	*84.30*	*97.10*
Std Dev	*0.07*	*5.94*	*18.38*	*1.56*
Std Error	*0.05*	*4.20*	*13.00*	*1.10*
P2 BMSCs (1)	0.20	43.80	43.70	48.30
P2 BMSCs (2)	0.50	73.40	67.70	73.00
P2 BMSCs (3)	0.10	56.20	21.00	40.60
P2 BMSCs (4)	0.50	49.40	54.80	59.40
Average	*0.33*	*55.70*	*46.80*	*55.33*
Std Dev	*0.21*	*12.84*	*19.80*	*14.09*
Std Error	*0.10*	*6.42*	*9.90*	*7.04*

Figure 5.10: Protein expression of Notch-1, Delta and Jagged-2 by young bovine articular cartilage (YBAC, positive control), P1 and P2 BMSCs cultured in serum-containing medium in the presence of 5ng/ml FGF-2 (Panel A).

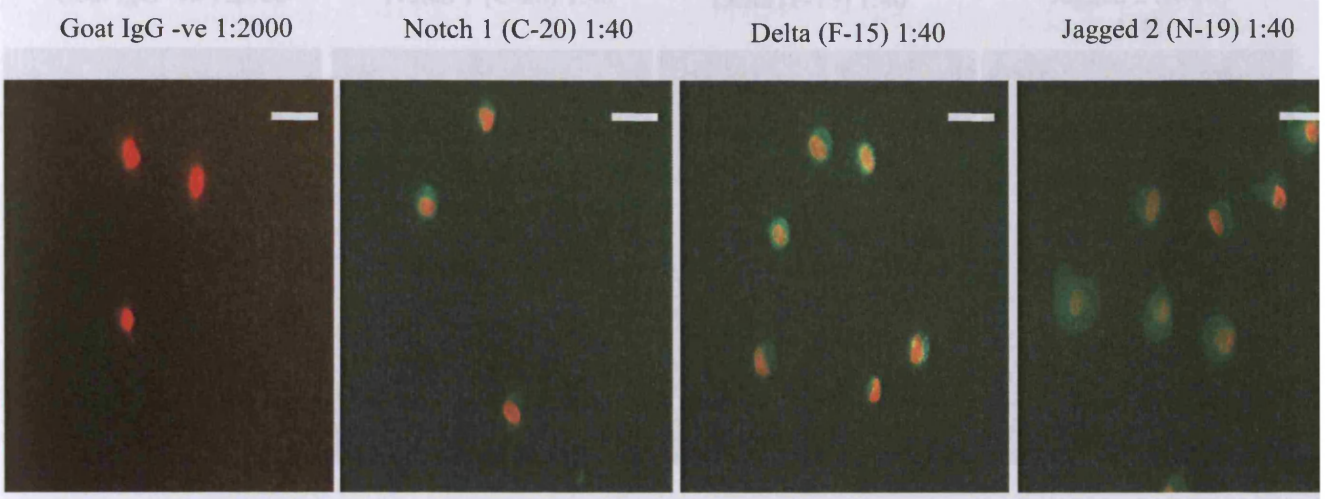


Figure 5.11: Cell smear images for P1 BMSCs cultured in serum containing medium with the presence of 5 ng/ml FGF-2. DAPI as a counter stain for staining the nucleus (shown in red), FITC staining is shown as green. Scale bar represents 2μm and images were taken at x40 magnification

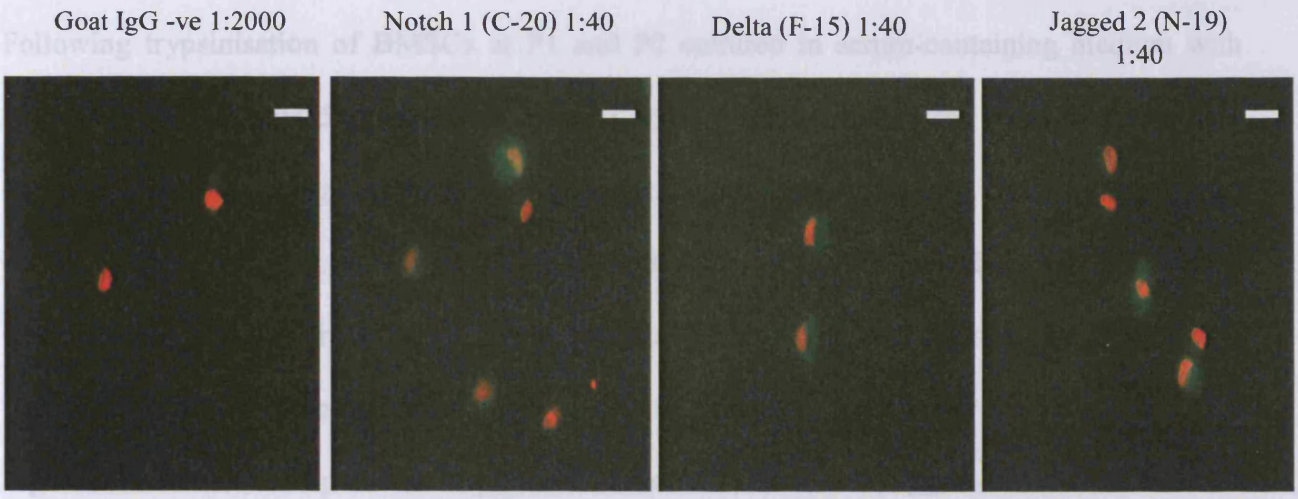


Figure 5.12: Cell smear images for P2 BMSCs cultured in serum containing medium with the presence of 5 ng/ml FGF-2. DAPI as a counter stain for staining the nucleus (shown in red), FITC staining is shown as green. Scale bar represents 2μm and images were taken at x40 magnification

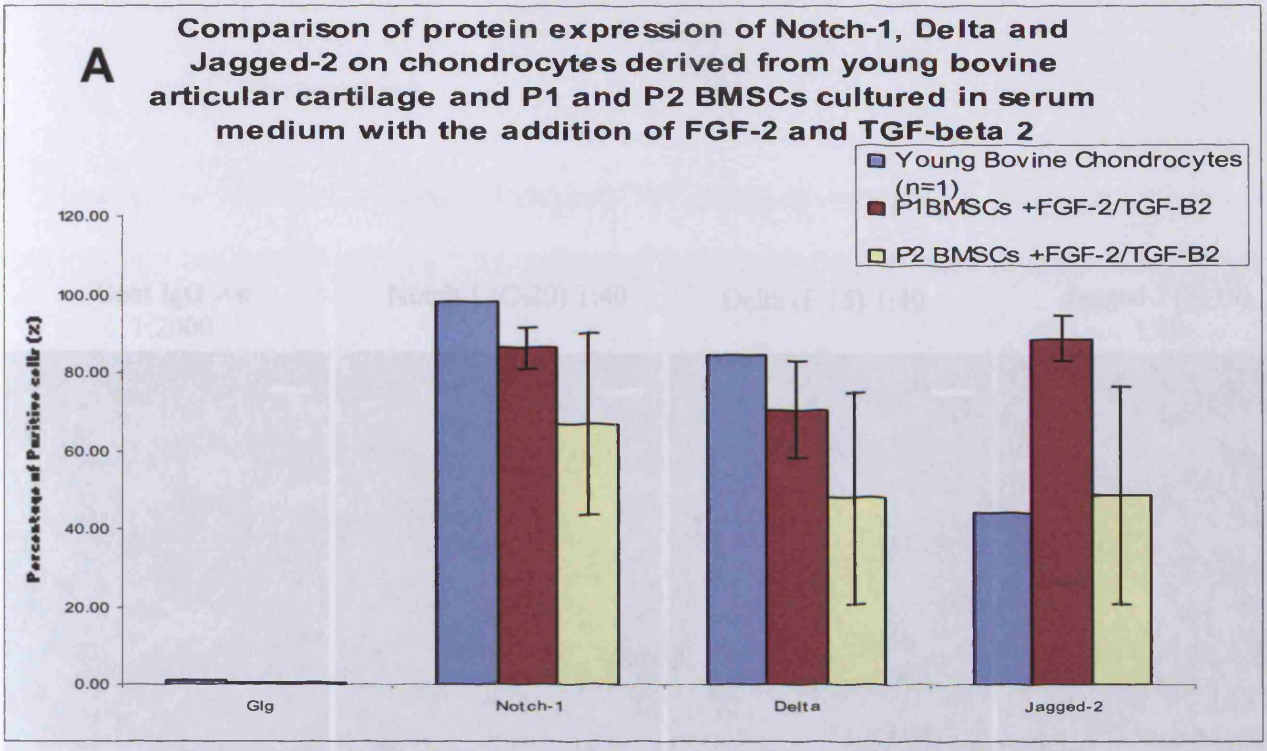
5.4.3 Expression of Notch-1 receptor and its ligands Delta and Jagged-2 by P1 and P2 BMSCs cultured in the presence of 5ng/ml FGF-2 AND 5ng/ml TGF-β2

Following trypsinisation of BMSCs at P1 and P2 cultured in serum-containing medium with 5ng/ml of FGF-2 plus 5ng/ml TGF-β2, $1x10^6$ cells BMSCs cells were retained for protein expression analyses using FACs on Notch receptor, Notch-1 and its ligands, Delta and Jagged-2 , before further passaging. $0.2x10^6$ cells were used for the analyses of each protein of interest. Chondrocytes obtained from 7 days bovine articular cartilage (n=1) of the same amount of cells ($0.2x10^6$) for each protein was used. GIg was used as a negative control.

Figure 5.13 Panel B compares the expression of Notch-1, Delta and Jagged -2. All samples had a negative detection of GIg suggesting no non-specific binding. Notch-1 was highly expressed by 7 day old chondrocytes (98%), followed by P1 BMSCs (79%) and P2 BMSCs (67%). A similar pattern was observed in the expression of Delta but with a lower general expression level of 85% (chondrocytes), 70% (P1 BMSCs) and 48% P2 BMSCs. These data suggest that an increase in passage number for bovine BMSCs may result in a reduction in the expression of Notch-1 and Delta. For Jagged-2 expression, P1 BMSCs generated the highest expression level of 89%, whilst similar detection was obtained from chondrocytes (44%) and P2 BMSCs (49%).

When FACs analyses were completed, the cells were counter-stained with DAPI and for P1 and P2 BMSCs, cell smears images (Figure 5.14 and 5.15 respectively) were obtained to show the visual staining of each protein of interest. All Notch-1, Delta and Jagged-2 appeared to be distributed uniformly throughout the surface of P1 and P2 BMSCs.

A



B

FGF-2 & TGF-beta 2	Percentage of Positive cells			
	Glg	Notch-	Delt	Jagged
Young Bovine Chondrocytes	0.9	98.40	84.7	44.10
P1 BMSCs (1)	0.3	89.10	69.8	97.50
P1 BMSCs (2)	0.4	99.70	99.0	99.70
P1 BMSCs (3)	0.6	77.30	74.1	76.10
P1 BMSCs (4)	0.5	79.30	39.2	81.70
Average	*0.4*	*86.35*	*70.5*	*88.75*
Std Dev	*0.1*	*10.29*	*24.5*	*11.64*
Std Error	*0.0*	*5.14*	*12.2*	*5.82*
P2 BMSCs (1)	0.6	70.50	2.00	1.10
P2 BMSCs (2)	0.4	97.90	95.8	97.70
P2 BMSCs (3)	0.0	0.00	0.00	0.00
P2 BMSCs (4)	0.5	99.00	94.2	95.50
Average	*0.3*	*66.85*	*48.0*	*48.58*
Std Dev	*0.2*	*46.48*	*54.2*	*55.46*
Std Error	*0.1*	*23.24*	*27.1*	*27.73*

Figure 5.13: Expression of Notch-1, Delta and Jagged-2 are plotted on a histogram (Panel A) and Panel B is a table to summarise the data.

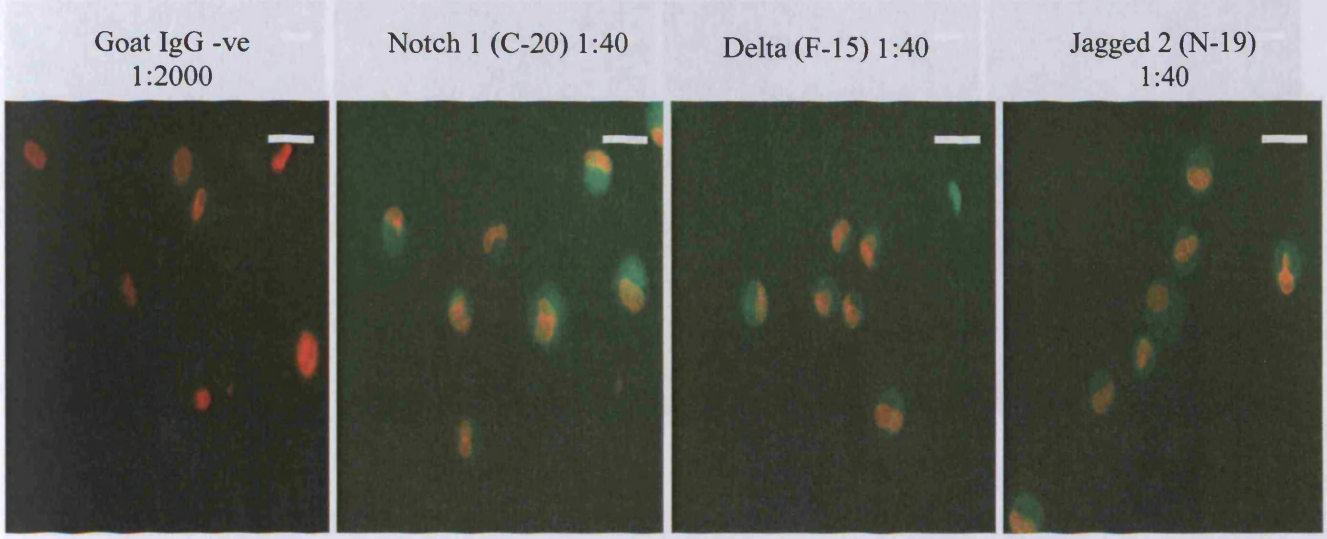


Figure 5.14: Cell smear images on P1 BMSCs after trypsinisation cultured in serum containing medium with the addition of 5ng/ml FGF-2 and 5ng/ml TGF-β 2. DAPI was used as a counter stain (shown in red) for staining the nucleus, FITC staining is shown as green. Scale bar is at 2μm, images taken at x40 magnification

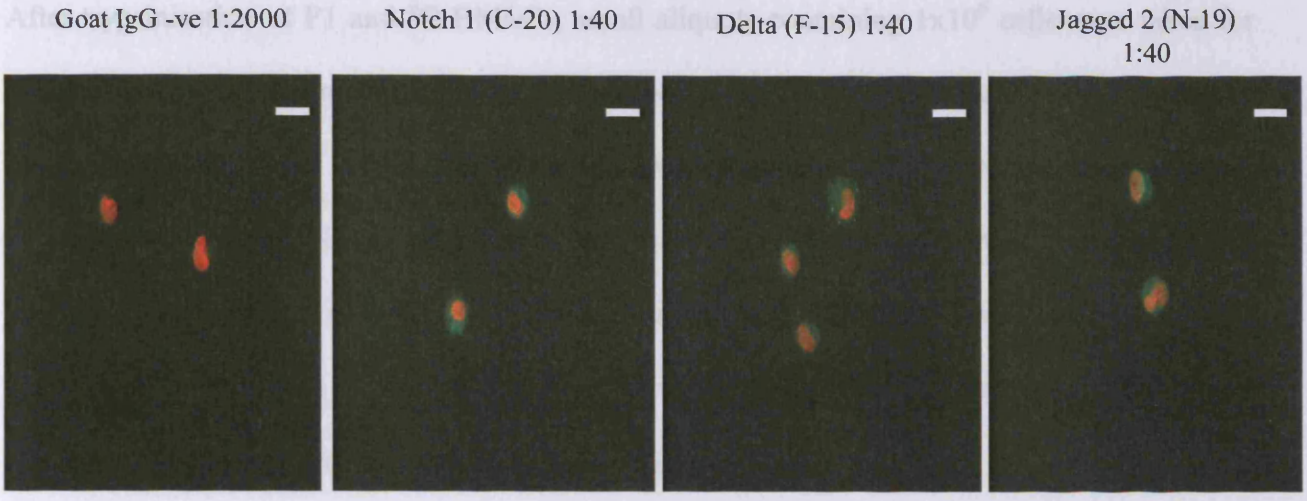


Figure 5.15: Cell smear images on P2 BMSCs after trypsinisation cultured in serum containing medium with the addition of 5ng/ml FGF-2 and 5ng/ml TGF-β 2. DAPI was used as a counter stain (shown in red) for staining the nucleus, FITC staining is shown as green. Scale bar is at 2μm, images taken at x40 magnification

5.4.5 Gene expression of SOX-9, aggrecan, collagens type I and II by P1 and P2 BMSCs cultured in the presence of 5ng/ml FGF-2 and in combination with 5ng/ml TGF-β2

After trypsinisation of P1 and P2 BMSCs, small aliquots containing $1x10^6$ cells were taken for the analysis of the commonly reported chondrogenic marker genes, namely SOX-9, aggrecan and collagens type I and II at the mRNA level. As a comparison, RNA was also harvested from young (7 days) and old (18 months) bovine articular cartilage and young bovine bone marrow. The quality and quantity of RNA harvested from tissues and $1x10^6$ cells from P1 and P2 BMSCs cultured in the presence of FGF-2 and in combination with TGF-β2 were measured using a Nanodrop. The results are summarised in Table 5.10 and 5.11.

Samples	260/280 ratio	RNA (ng/µl)
Young Bovine Articular Cartilage	2.08	288
Old Bovine Articular Cartilage	2.10	141.5
Young Bovine Bone Marrow	1.97	262.4
P1 BMSCs (1)	2.10	657.8
P1BMSCs (2)	2.11	257.0
P1BMSCs (3)	2.09	202.7
P2 BMSCs (1)	2.07	385.3
P2 BMSCs (2)	2.11	697.0
P2 BMSCs (3)	2.07	229.6

Table 5.10: Quality and quantity of RNA extracted from tissue samples and BMSCs at P1 and P2 cultured in the presence of FGF-2.

Samples	260/280 ratio	RNA (ng/µl)
Young Bovine Articular Cartilage	2.08	288
Old Bovine Articular Cartilage	2.10	141.5
Young Bovine Bone Marrow	1.97	262.4
P1 BMSCs - Harvest2	2.09	754.7
P1 BMSCs – Harvest3	2.08	115.3
P1 BMSCs – Harvest4	2.07	145.5
P2 BMSCs – Harvest2	2.07	417
P2 BMSCs – Harvest3	2.10	638.9
P2 BMSCs – Harvest4	2.09	222.8

Table 5.11: Quality and quantity of RNA extracted from tissue samples and BMSCs at P1 and P2 cultured in the presence of 5ng/ml FGF-2 and 5ng/ml TGF-β2.

The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid extracted from all the unknown samples. RNA preparations for tissues and P1 and P2 BMSCs samples have OD_{260}/OD_{280} values of ~ 2.0 (± 0.1) as shown in Table 5.10 and 5.11. The cDNA was used for quantitative PCR (qPCR) in order to assess the molecular expression of a series of reported chondrogenic marker genes, namely SOX-9, aggrecan and collagens type I and II using primers designed specifically for qPCR (refer to Chapter 3). Bovine GAPDH was used as a endogenous control and all genes of interest were normalised against GAPDH.

Mesenchymal stem cells (MSCs) at passage 1 (P1) and 2 (P2) were treated with 5ng/µl of FGF-2 and with the addition of 5ng/µl TGF-β2. The gene expression levels for SOX-9, collagens type I and II, and aggrecan were examined using qPCR. GAPDH was used as an endogenous control.

	SOX-9	COL. II	COL. I	AGGRECAN
P1 MSCs +FGF-2 (n=3)	0.000	0.000	3.096	0.010
P2 MSCs +FGF-2 (n=3)	0.000	0.000	1.436	0.001
P1 MSCs +FGF-2/TGF-B2 (n=3)	0.000	0.000	0.093	0.009
P2 MSCs +FGF-2/TGF-B2 (n=3)	0.000	0.000	0.445	0.001

Table 5.12: A summary table showing the average copies of genes generated from two dimensional cultures of Passage 1 and 2 MSCs maintained in culture medium with 5ng/µl of FGF-2 and with the combined supplementation of 5ng/µl TGF-beta 2.

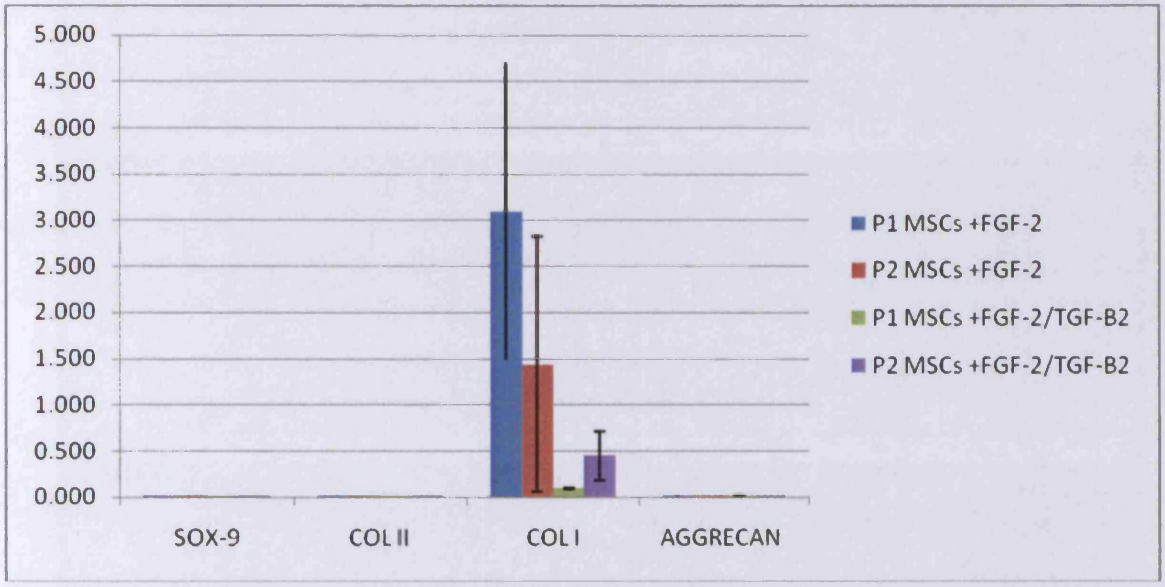


Figure 5.17: Histogram showing the different expression levels of SOX-9 collagen type I, collagen type II and aggrecan from Passage 1 and 2 MSCs cultured in 5ng/µl of FGF-2 and 5ng/µl TGF-beta supplemented medium.

No expression of SOX-9 and collagen type II was detected from all the different cultures. Collagen type I was detected from all the cultures, the highest expression was detected from P1 MSCs cultured in medium with the addition of 5ng/µl FGF-2 (3.096 copies). P2MSCs cultured in FGF-2 supplemented medium was 2.156 (3.096/1.436) times less than P1MSCs cultured in the same medium. Such decrease in collagen type I may be due to the effect of an increase in passage number (?). In contrast, P1 MSCs cultured in two growth factors, FGF-2 and TGF-B2 expressed 4.785 times less than P2 MSCs cultured in the same medium. Equal detection of aggrecan was generated from P2 MSCs cultured in FGF-2 supplemented and with the addition of TGF-β2 (0.001 copies). P1 MSCs cultured in FGF-2 and with the addition of TGF-β 2 generated similar expression of aggrecan of 0.01 and 0.009 copies, respectively.

This study suggested that culturing MSCs in one or two growth factors do not elevate or influence the expression of SOX-9, collagens type I and II, and aggrecan. Early passage number and one passage difference did not have much influence on varying the expression levels of the genes of interest. The purity and the size of the qPCR products were verified by the dissociation curve summerised in Figure 5.19.

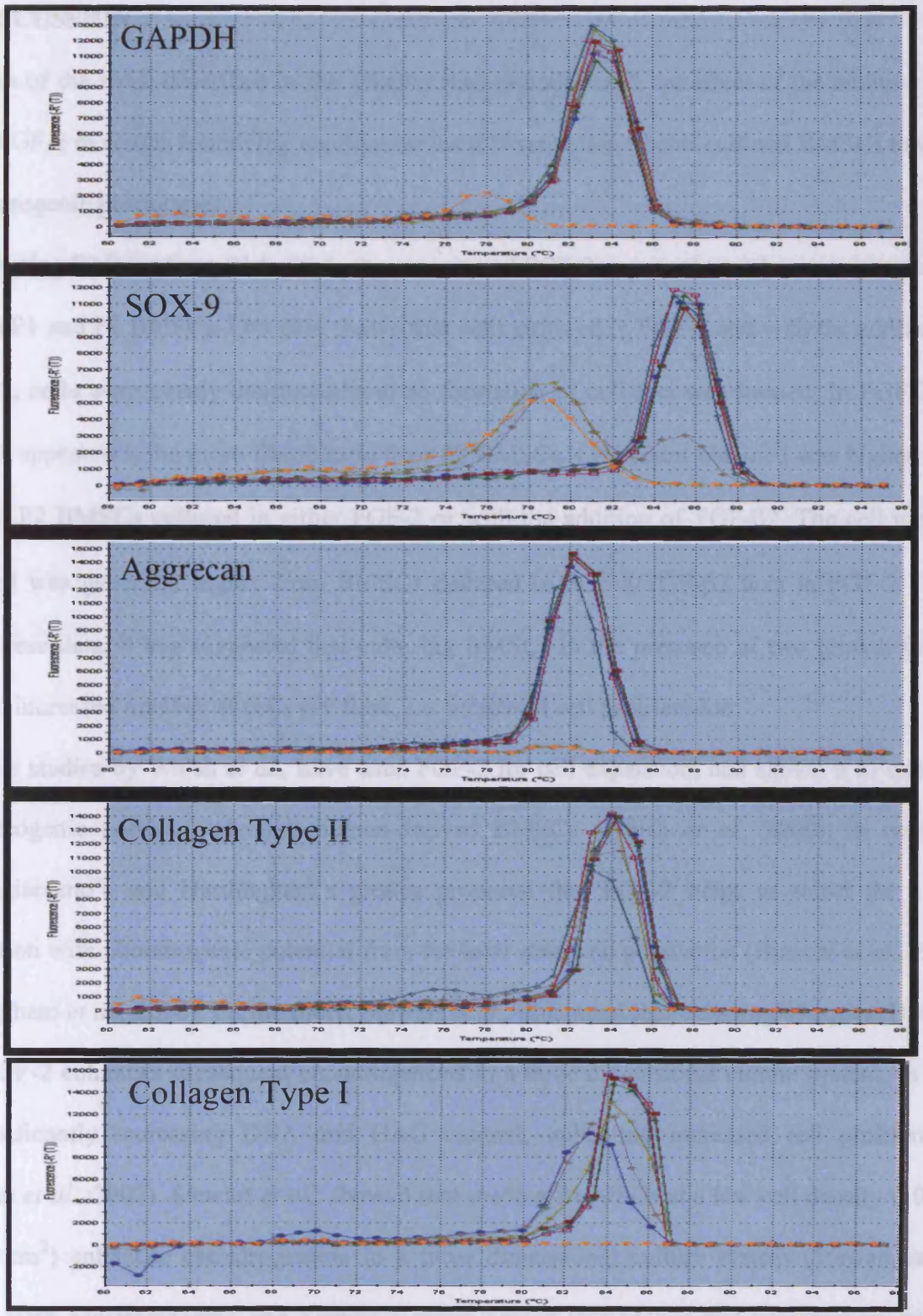


Figure 5.19: Dissociation curves on GAPDH (housekeeping gene), SOX-9, aggrecan, collagens type II and I. Young Bovine Bone Marrow (Grey), Old Bovine Articular Cartilage (Blue), Young Bovine Articular Cartilage (Maroon Red), P1 BMSCs +FGF-2 (Pale Turquoise), P1 BMSCs +FGF-2 TGF-B2 (Green), P2 BMSCs +FGF-2 (Mustard Yellow), P2 BMSCs +FGF-2 TGF-B2 (Purple), Water NTC (Orange).

5.5 DISCUSSION

The aim of the work described in this chapter was to understand the effect of the addition of 5 ng/ml FGF-2 in serum-containing medium on the differentiation of sub-cultured BMSCs towards a chondrogenic phenotype.

Subculturing BMSCs from P1 to P2 in the presence of FGF-2 appeared to reduce the growth rate of both P1 and P2 BMSCs. Our data shown that cells cultured in FGF-2 and with the addition of TGF-β2, cells were evenly distributed and no formation of cell foci was formed. In FGF-2, P1 BMSCs appeared to be more fibroblastic than P2 BMSCs. Cell count obtained was higher from P1 than P2 BMSCs cultured in either FGF-2 or with the addition of TGF-β2. The cell number obtained was generally higher from BMSCs cultured in FGF-2/TGF-β2 than in FGF-2 alone. From these data, it has suggested that culturing BMSCs in the presence of two growth factors gave an increased number of cells per flask, i.e. increased cell proliferation.

Previous studies by Walsh *et al.,* have used FGF-2 for cell expansion, and shown it to enhance the osteogenic potential of adult human-derived BMSCs (Walsh *et al.,* 2000). In contrast, Mastrogiacomo's and Hardingham's groups proposed that FGF-2 helps to select for a cell population with chondrogenic potential from the total stem cell population (Bianchi *et al.,* 2003; Hardingham *et al.,* 2006). Furthermore, Stewart *et al.,* illustrated that culturing of equine BMSCs with FGF-2 enhances subsequent chondrogenesis in a three dimensional culture system, as well as significantly increasing DNA and GAG content, indicating increased cell proliferation (Stewart *et al.,* 2007). Stewart *et al.,* showed that seeding P1 MSCs at a low cell density (10,000 cells / cm^2) enhances chondrogenesis in a three dimensional culture system (Stewart *et al.,* 2007). Both Mastrogiacomo and Stewart's group agreed that FGF-2 had no effect on colony formation, however, FGF-2 markedly increased the cell proliferative potential. Worster *et al.,* demonstrated that treatment of cells with FGF-2 increased the expression of the developmental marker STRO-1 and alkaline phosphatase (AP), a major marker for osteoblast lineage (Worster *et al.,* 2001). Interestingly, supplementation with ascorbate, or the combined use of FGF-2 with

dexamethasone, modulates the effect of FGF-2 on AP expression and consequently the lineage which the stem cells will differentiate into. This suggests that since in this study FGF-2 was not used in conjunction with another 'FGF-2 effector', such as ascorbate or dexamethasone, no effects on protein expression were observed. Cell morphology observed in the current study replicated previous published data (Murdoch *et al.,* 2007). Solchaga *et al.,* has previously shown that in the absence of FGF-2, MSCs appeared to be flatter, polygonal and more spread out than MSCs cultured in the presence of FGF-2, which gave a smaller, spindle-like fibroblastic morphology (Solchaga *et al.,* 2005). It is interesting to note that as well as FGF-2, recently, FGF-18 has also been shown the ability to promote chondrogenesis of MSCs (Davidson *et al.,* 2005). Grayson *et al.,* illustrated that sub-culturing human-derived MSCs in the absence of any growth factors is sufficient to maintain the viability of MSCs. But oxygen tension, as well as growth factors, contributes to the behaviour of MSCs influencing their final differentiation lineage (Grayson *et al.,* 2006).

FACs analyses showed positive staining of Notch-1, Delta and Jagged-2 from P1 and P2 BMSCs cultured in FGF-2 or with the addition of TGF-β2. P1 BMSCs cultured in FGF-2 or with the addition of TGF-β2 have similar pattern on the percentage of positive cells for the three proteins of interest. The pattern was, Jagged-2 > Notch -1 > Delta. In contrast, P2 BMSCs cultured in FGF-2 have a pattern of: Jagged-2 ≥ Notch -1 > Delta. P2 BMSCs cultured in FGF-2 and TGF-β2 have a pattern of Notch -1 > Delta ≥ Jagged-2. These imply that P1 and P2 BMSCs both express these three proteins, but the different pattern observed from P2 BMSCs in comparison to P1 BMSCs suggested that the passage number may have an influence on the expression of these proteins. On the other hand, the culturing conditions (i.e. FGF-2 or with the addition of TGF-β2) did not appear to contribute to the expression of these proteins.

Collagen type I was highly expressed by P1 and P2 MSCs cultured in FGF-2 alone or with the addition of TGF-β2. The order of expression was P1MSCs +FGF-2>P2MSCs+FGF-2/TGFβ2>P2MSCs+FGF-2/TGFβ2>P1MSCs+FGF-2/ TGFβ2. SOX-9, collagen types II and

aggrecan were not highly expressed. These data suggested that the culturing conditions used to maintain MSCs at P1 and P2 did not contribute to the expression of these genes.

5.6 SUMMARY

- Cell morphology was similar to primary isolated cells but no colonies or cell foci were formed.
- The use of two growth factors showed an increase in the number of cells per flask, which implies that two growth factors enhanced cell proliferation of BMSCs in a bovine model.
- The different protein expression pattern observed from P2 BMSCs suggested that this change may be influenced by passage number rather than by the addition of one or two growth factors.

CHAPTER 6:

INVESTIGATING THE CHARACTERISTICS OF TISSUE GRAFTS PRODUCED FROM PASSAGED BOVINE BONE MARROW DERIVED STEM CELLS USING A TRANSWELL CULTURE SYSTEM

6.1 Introduction

The objective of this chapter was to determine if passaged BMSCs harvested at P2 and P3 are capable of forming a tissue graft when cultured in defined chondrogenic medium using the Transwell culture system (Kandel *et al.,.*, 1995). The purpose of exposing BMSCs to chondrogenic medium was to determine if this would further differentiate the passaged BMSCs towards a chondrogenic lineage, by causing the cells to produce an extracellular matrix resembling hyaline cartilage, thus allowing them to be used as a cell-based strategy for cartilage repair.

Adult mesenchymal stem cells (b-MSCs) are basic cells that have the unique ability to self-renew and under certain conditions, differentiate multi-potentially into specialized cells such as cartilage, muscle and bone (Ashton *et al.,* 1980; Ferrari *et al.,* 1998; Jiang *et al.,* 2002). Their unique ability to repair and regenerate tissues, plus their ready availability from a variety of tissue sources make them an attractive candidate for tissue engineering of connective tissues such as articular cartilage. Damaged articular cartilage has a limited capacity for self repair and often the repaired tissue has a fibrocartilaginous phenotype, which does not possess the properties of native normal healthy articular cartilage (Hunziker 2000; Hunziker 2001). Currently, limited treatments are available for cartilage repair due to increase prosthesis failure. This limits the treatment that can be carried out for individuals under the age of 50 therefore, a

desirable treatment for repairing and regenerating cartilage lesions is the use of cell-based strategies, rather than the usual methods of debridement, abrasion, perichondral grafting and mosaicplasty (Hunziker 2001). Tissue engineering of cartilage using a cell-based strategy, such as autologous chondrocyte transplantation (ACT; Kim *et al.,* 2003) is a recent novel approach to cartilage repair. Commonly, an autologous cell source, normally primary chondrocytes, are used however, MSCs have now become a viable alternative. The cells are usually expanded in culture until sufficient cells are obtained. The cells are then delivered into the defected area and localised by a suitable method.

Typically, MSCs are harvested from bone marrow, followed by cell expansion in high glucose DMEM medium containing foetal bovine serum (FBS) or in high glucose DMEM medium supplemented with growth factors, such as FGF-2, which has been shown to select for a cell population of b-MSCs that has an increased chondrogenic potential (Bianchi *et al.,*. 2003). Usually, MSCs are passaged, until the required cell number or passage number is achieved before being seeded into a chosen culture system where they are maintained in a medium containing all the necessary factors for MSCs to differentiate towards a chondrogenic lineage. However, prolonged passaging may result in cell senescing and losing their differentiation potential (Digirolamo *et al.,* 1999).

Many previous studies (refer to Chapter 1 Section 1.4.1) have indicated that a range of cytokines, growth factors and different culture methods contribute to the chondrogenic differentiation of b-MSCs. However, most of these data are based on studies using pellet or micromass culture systems, which do not show the *in vivo* organization evident in native bovine articular cartilage. Recent studies have demonstrated the use of a Transwell culture system to generate a cartilage-like tissue graft using young bovine articular chondrocytes in DMEM supplemented with 20% FBS, 5μg/ml TGF-beta 2 and 100 μg/μl ascorbic acid over a 4 week

culture period (Kandel *et al.,* 1995; Hayes *et al.,* 2007). Such cartilage grafts have been shown to produce varying zonal morphology, resembling those observed in native cartilage (Hayes *et al.,* 2007). Recently, Hardingham and colleagues have illustrated the use of the Transwell system to form a cartilage-like tissue graft using human MSCs (hMSCs) *in vitro* (Murdoch *et al.,* 2007).

This chapter describes the culture of young bovine bone marrow MSCs (Bb-MSCs) using the Transwell system. Briefly, Bb-MSCs were expanded in culture, seeded into inserts at passages 2 and 3 (P2 and P3, respectively), and maintained for 4 weeks in chondrogenic medium (CM), (containing TGF-β3 and dexamethasone [refer to Methods Section for details]). The tissue grafts produced were characterized using a range of analytical methods as follows:-

- Determined the wet mass of the tissue grafts produced
- Compared the phenotype of successful versus unsuccessful grafts and related this to the initial phenotypic state of the cells before seeding into the Transwell system
- Histological analyses were also carried out to assess the PG and collagen type II and type X contents within the matrix of the grafts produced.
- The GAG content of the outer and insert media were analyzed to assess the GAG release profile of the tissue grafts produced
- Analyzed the molecular expression of GAPDH as the endogenous control, investigate the expression of cartilage-related genes, SOX-9, aggrecan and collagen type II, and used collagen type X as a marker for osteogenesis.

6.2 Materials

Dulbecco's Modified Eagle Medium (DMEM), foetal Bovine Serum (FBS), gentamicin and 10x Trypsin / EDTA were purchased from Gibco, Paisley, UK. Basic fibroblast growth factor (bFGF), type II collagen from chick sternum, and ascorbate were purchased from Sigma-Aldrich, Poole, Dorset, UK. Pronase from *Streptomyces griseus* was obtained from Roche Applied Science, Lewes, East Sussex, UK. Collagenase type II from *Clostridium histolyticum* was obtained from Worthington, Reading, Berkshire, UK. Millipore filter inserts was purchased from Millipore (U.K.) Limited, Watford, England, UK. Transforming growth factor beta 2 (TGF-β2) was obtained from Peprotech EC. Ltd., London, UK. All plastic wares were obtained from Greiner Bio-one, Stonehouse, Gloucestershire, UK and Corning Ltd., Artington, Surrey, UK.

6.3 Methods

6.3.1 Extraction of Bone Marrow and Isolation of Bb-MSCs

Bone marrow was extracted from 7 day old bovine, metatarsel bone several hours after slaughter. One end of the bone was sawed open under sterile condition and a small amount of exposed bone marrow was removed. The unexposed marrow was scooped out using a surgical spatula and transferred to a pre-weighed Petri dish.

The extracted bone marrow was dispersed using 50 ml of sterile serum free DMEM containing 0.05 mg/ml gentamicin and transferred to a sterile 50 ml centrifuge tube and inverted gently. The tube was allowed to stand until all the fatty deposits had floated to the top, the fatty deposits were filtered through a 0.4 μm cell strainer placed over a sterile 50 ml centrifuge tube and discarded. A filtrate was obtained and was centrifuged at 200 x g for 5 minutes and the supernatant was removed gently, leaving behind a red cell pellet. The cell pellet was resuspended in 40 ml of Basic Medium (DMEM containing 0.05 mg/ml gentamicin and 10% (v/v) heat-

inactivated FBS). The 40 ml of cell suspension was seeded at equal volume into two T-75 cm^2 vented flask and incubated at 37°C with 5% carbon dioxide (CO_2) for 4 days to allow the cells to adhere. The media was changed on the fourth day and the cells were washed in serum free media to remove any non-adherent cells. The cells were fed every 2 days with 20ml of Basic Medium until day 8. When the cells reached confluence they were trypsinized and passaged until the target cell number was reached. On day 8, the Basic medium was supplemented with 5ng/ml of FGF-2.

6.3.1.1 Passaging Bb-MSCs

When the Bb-MSCs reached confluency, they were washed three times using 10 ml serum free DMEM containing 0.05 mg/ml gentamicin with gentle agitation. The Bb-MSCs were photographed and then trypsinised by adding 2ml of 1x (v/v) Trypsin / EDTA to each T-75 cm^2 vented flask. They were then incubated at 37°C until the cells started to detach from the bottom of the flask. 10ml of cell suspension was transferred to a sterile 50 ml centrifuge tube containing 30ml of Basic medium and centrifuged at 200 x g for 5 minutes. The cell pellet was resuspended in 10 ml of basic medium and the cells were counted using a haemocytometer. Cells were plated into T-75 cm^2 vented flask at 6 x 10^6 cells per flask (33% confluent) for further expansion.

6.3.2 Dissection of cartilage and isolation of chondrocytes

Cartilage was dissected from seven day old bovine metacarpophalangeal (MCP) joints obtained from a local abattoir. After washing, the feet were skinned, and the joint was opened under sterile conditions in a laminar flow hood. Full depth articular cartilage was removed from the joint using a sharp scalpel blade and was transferred to a tube containing 30 ml of DMEM containing 0.05 mg/ml gentamicin and 10% (v/v) FBS (Basic Medium).

Cartilage pieces harvested from MCP joint were digested in 0.1 % (w/v) pronase (at 7.5 ml per given weight of tissue) for 3 hours at 37°C with gentle mixing followed by overnight digestion in 300U / mg (120,000 U / 100ml) collagenase type II. The digested cell suspension was filtered through a cell strainer (4 μm pore size) to remove any undigested debris. Cell suspensions were pelleted by centrifugation at 200 x g for 5 minutes and resuspended in 10 ml of basic medium and the cell number counted using a haemocytometer. Cells were plated into T-75 cm^2 vented flask.

6.3.2.1 Passaging of chondrocytes

When chondrocytes reached confluency, they were washed three times using 10 ml of serum free DMEM containing 0.05 mg/ml gentamicin with gentle agitation, and subsequently photographed. Cells that were left for 4 days or more were treated with 300 U / mg of collagenase type II for 5 minutes at 37°C followed by three washes in serum free medium. Chondrocytes were trypsinised using 2 ml of 1x (v/v) Trypsin/EDTA per T-75 cm^2 vented flask, and incubated at 37°C until they were detached from the bottom of the flask. 10 ml of cell suspension was transferred to a sterile 50 ml centrifuge tube containing 30 ml basic medium, and centrifuged at 200 x g for 5 minutes. The cell pellet was resuspended in 10 ml of basic medium and the cells were counted using a haemocytometer. Cells were plated at low cell density (6 x 10^6 cells per flask, 33% confluent) for further expansion.

6.3.3 Seeding Bb-MSCs and chondrocytes in Transwell inserts

Briefly, the filter insert are placed into individual wells of a 24 well plate, coated with chick type II collagen (in 0.1 N acetic acid), dried overnight under sterile conditions, UV irradiated for 10 minutes and washed extensively with serum free DMEM before cell seeding. When the appropriate passage number was obtained from the monolayer expansion, the Bb-MSCs and chondrocytes were detached using methods described in Sections 3.3.1.1 and 3.3.2.1

respectively, pelleted, resuspended in fresh media and counted. Cells were then resuspended in an appropriate volume of DMEM plus 0.05 mg/ml gentamicin and 20% (v/v) heat inactivated FBS. The cells were seeded at 12 x 10^6 cells per 500 µl into filter inserts pre-coated with 0.5 mg/ml chick type II collagen. After seeding, the cells were incubated at 37°C for 5 minutes before adding 600µl of DMEM containing 0.05 mg/ml gentamicin and 20% (v/v) FBS to the outer chamber (see Figure 3.1).

These high cell density cultures were maintained for 4 weeks in two different media: i) maintaining medium (MM), composed of DMEM (with 4.5 g/L glucose, L-glutamine, no sodium pyruvate) containing 0.05 mg/ml gentamicin, 20% (v/v) FBS, 5 µg/ml TGF-β2 and 100 µg/ml ascorbate (sodium salt), and ii) chondrogenic medium (CM) that has DMEM (with 4.5 g/L glucose, L-glutamine, no sodium pyruvate) containing 0.05 mg/ml gentamicin, 50 µg/ml ascorbic acid 2-phosphate (magnesium salt), 40 µg/ml L-proline, 100nM dexamethasone, 10 ng/ml TGF-β3 and 1% (v/v) ITS+1. The outer and inner media was replaced three times a week (600 µl in the outer and 400 µl in the inner wells (refer to figure 6.1).

At the end of the culture period, the grafts produced were harvested, weighed and subdivided for histological (refer to Chapter Two, Section 2.2.1.1), immuno-histochemical (refer to Chapter 2, Section 2.2.1.2), biochemical (refer to Chapter Two, Section 2.2.2) and molecular (refer to Chapter Two, Section 2.2.3) analyses, summaries in Figure 3.1b.

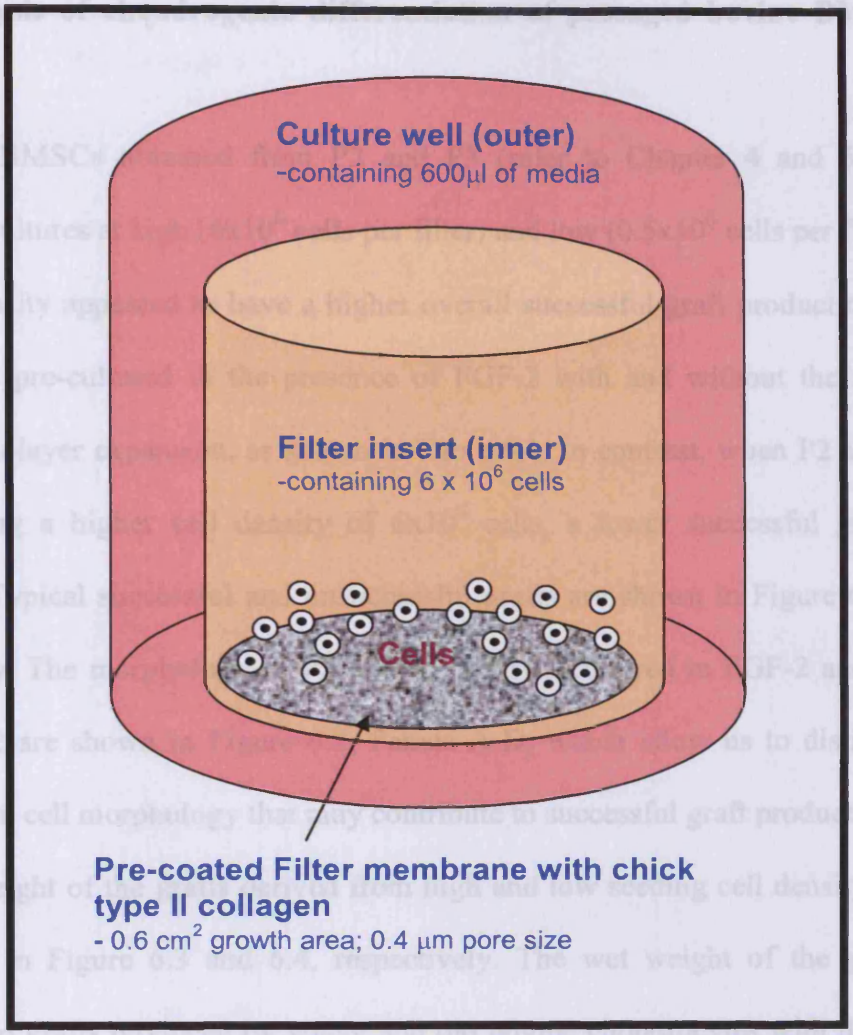


Figure 6.1 Diagram showing the layout of a typical Transwell culture system

6.4 Results:

6.4.1 Analysis of chondrogenic differentiation of passaged bovine BMSCs in Transwell cultures

Passaged bBMSCs obtained from P2 and P3 (refer to Chapter 4 and 5) were seeded into Transwell cultures at high ($6x10^6$ cells per filter) and low ($0.5x10^6$ cells per filter) density. Lower seeding density appeared to have a higher overall successful graft production rate using P2 and P3 BMSCs pre-cultured in the presence of FGF-2 with and without the addition of TGF-β2 during monolayer expansion, as shown in Table 6.1. In contrast, when P2 and P3 BMSCs were seeded using a higher cell density of $6x10^6$ cells, a lower successful graft production was observed. Typical successful and unsuccessful grafts are shown in Figure 6.2, Panels E and F, respectively. The morphology of P2 and P3 BMSCs cultured in FGF-2 and in the presence of TGF-beta 2 are shown in Figure 6.2, Panels A-D, which allow us to distinguish any obvious difference in cell morphology that may contribute to successful graft production.

The wet weight of the grafts derived from high and low seeding cell density were recorded and are shown in Figure 6.3 and 6.4, respectively. The wet weight of the grafts produced was compared to grafts produced by young and old bovine chondrocytes with the same cell seeding densities. MSCs pre-cultured in FGF-2 with the addition of TGF-β2 were able to produce tissue grafts than MSCs pre-cultured in FGF-2. The passage number of BMSCs (either P2 or P3) did not affect the generation of a tissue graft. However, the seeding cell density appeared to affect the weight of tissue grafts produced. At $6x10^6$ cells/well, grafts produced using cells pre-cultured in FGF-2 were lighter than grafts produced by cells pre-cultured in FGF-2 with the addition of TGF-β2. A similar pattern was observed from tissue grafts generated using $0.5x10^6$ cells/well. From Harvest3 (replating density of 0.2 $x10^6$ cells), grafts produced using P3 BMSCs pre-cultured in FGF-2 was approximately 4 times heavier than those produced using P2 BMSCs. No grafts were produced using P3 BMSCs passaged in FGF-2/TGF-β2, 12 times heavier grafts were produced by P2 BMSCs passaged in FGF-2/TGF-β2. In contrast, from Harvest 4 (with a

	Fraction of successful graft			
	P2 +FGF-2	P3 +FGF-2	P2 +FGF-2/TGF-B2	P3 +FGF-2/TGF-B2
$6x10^6$ ($0.2x10^6$)	0/1	0/4	0/4	not enough cells
$6x10^6$ ($0.4x10^6$)	not enough cells	not enough cells	0/2	0/4
$6x10^6$ (50%)	3/3 *	not enough cells	0/6	not enough cells
$0.5x10^6$ ($0.2x10^6$)	1/1	0/4 *	4/4	2/2
$0.5x10^6$ ($0.4x10^6$)	2/2	2/2	2/2	4/4
$0.5x10^6$ (50%)				

* hard cell lump

* characterised by staining

Table 6.1: Overall successful grafts produced using different initial seeding cell density. * represents the presence of a hard cell lump after 4 weeks of culture.

* indicates that the graft has been characterised by staining.

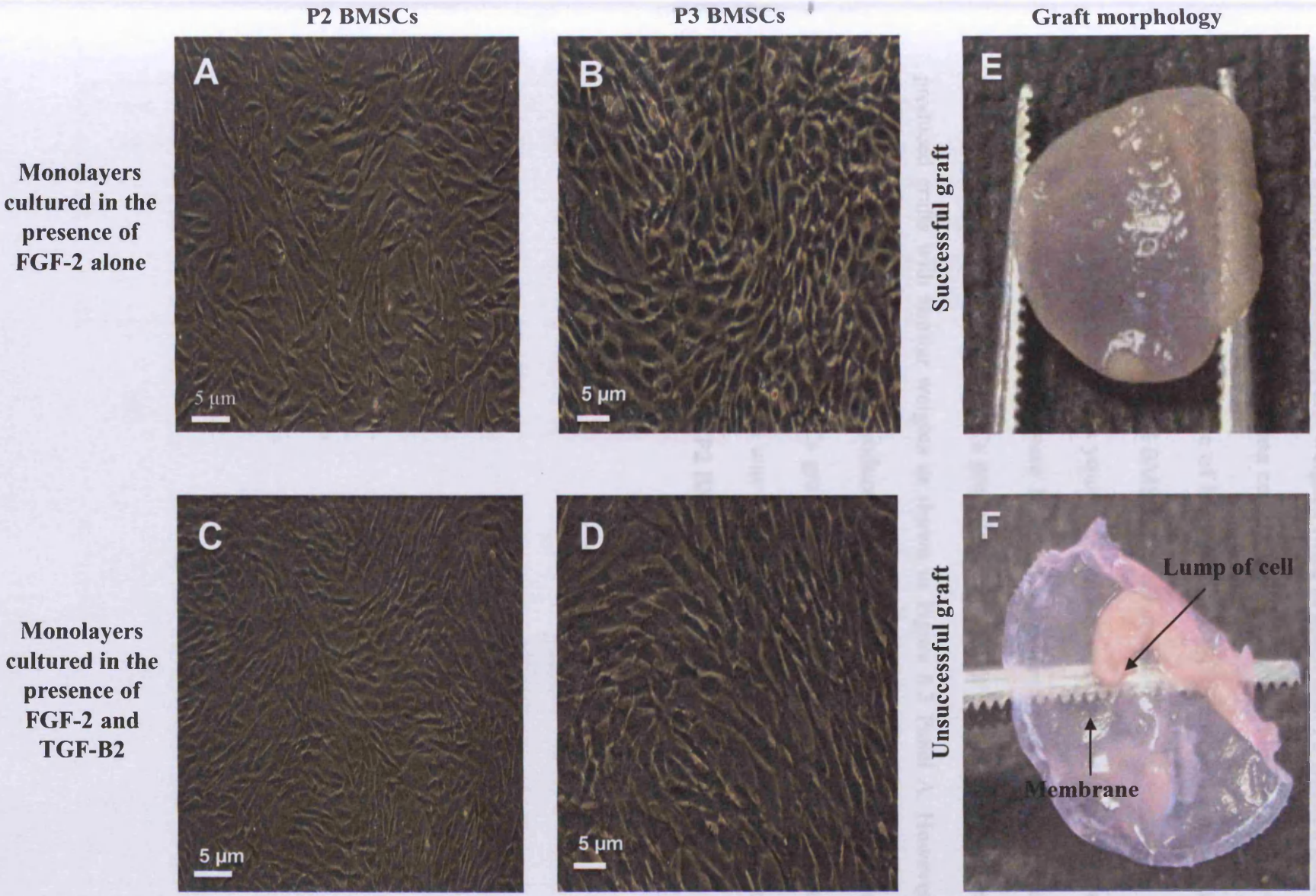


Figure 6.2: Cell morphology for P2 and P3 BMSCs cultured in the presence of 5 ng/ml FGF-2 alone (Panels A and B) and with the addition of 5 ng/ml TGF- β2 (Panels C and D). The grafts produced using the Transwell culture system were photographed as shown in Panel E, indicating a successful graft production and Panel F shows the morphology of an unsuccessful grafts with a transparent filter membrane attached to it for support.

replating density of 0.4 x10^6 cells), no grafts were obtained using P2 and P3 BMSCs pre-cultured in FGF-2. BMSCs passaged in the presence of both FGF-2/ TGF-β2 is able to produce tissue grafts. P2 BMSCs generated grafts weighed twice as much than P3 BMSCs generated grafts. In conclusion, the use of a lower seeding cell density per Transwell insert (0.5x10^6 cells/well) and BMSCs pre-cultured in the presence of FGF-2 and TGF-β2 generated a more successful tissue graft than using 6x10^6 cells/well and BMSCs pre-cultured in FGF-2.

Similar patterns were observed from young and old bovine chondrocyte grafts seeded at 0.5 x10^6 cells/well, however, their weights were 16 and 3 times less (respectively) than those produced using 6x10^6 cells. P2 and P3 BMSCs pre-cultured in FGF-2/ TGF-β2 from Harvests 3 and 4 all produced grafts with similar weights as shown in Figure 6.3 Panel A. However, BMSCs that were pre-cultured in FGF-2 also produced graft except harvest 4, P2 BMSCs, and Figure 6.3 Panel B. From harvest3, P2 BMSCs grafts were 5 times heavier in mass than those produced using P3 BMSCs, and the opposite was observed from harvest 4 graft production can only be seen using P3 BMSCs, but not with P2 BMSCs.

A

6x10⁶ cells/insert (+FGF-2)	Average	Std Error	6x10⁶cells/insert (+FGF-2/TGF-B2)	Average	Std Error
Young Bovine chondro graft in CM	16.8	0.46	Young Bovine chondro graft in CM	16.8	0.46
Old Bovine chondro graft in CM	6.25	0.35	Old Bovine chondro graft in CM	6.25	0.35
Harvest 3 P2BMSCs	0.8	0	Harvest 3 P2BMSCs	12.83	0.98
Harvest 4 P2BMSCs	0	0	Harvest 4 P2BMSCs	3.9	0.5
Harvest 3 P3BMSCs	4.43	1.4	Harvest 3 P3BMSCs	0	0
Harvest 4 P3BMSCs	0	0	Harvest 4 P3BMSCs	1.55	0.13

B

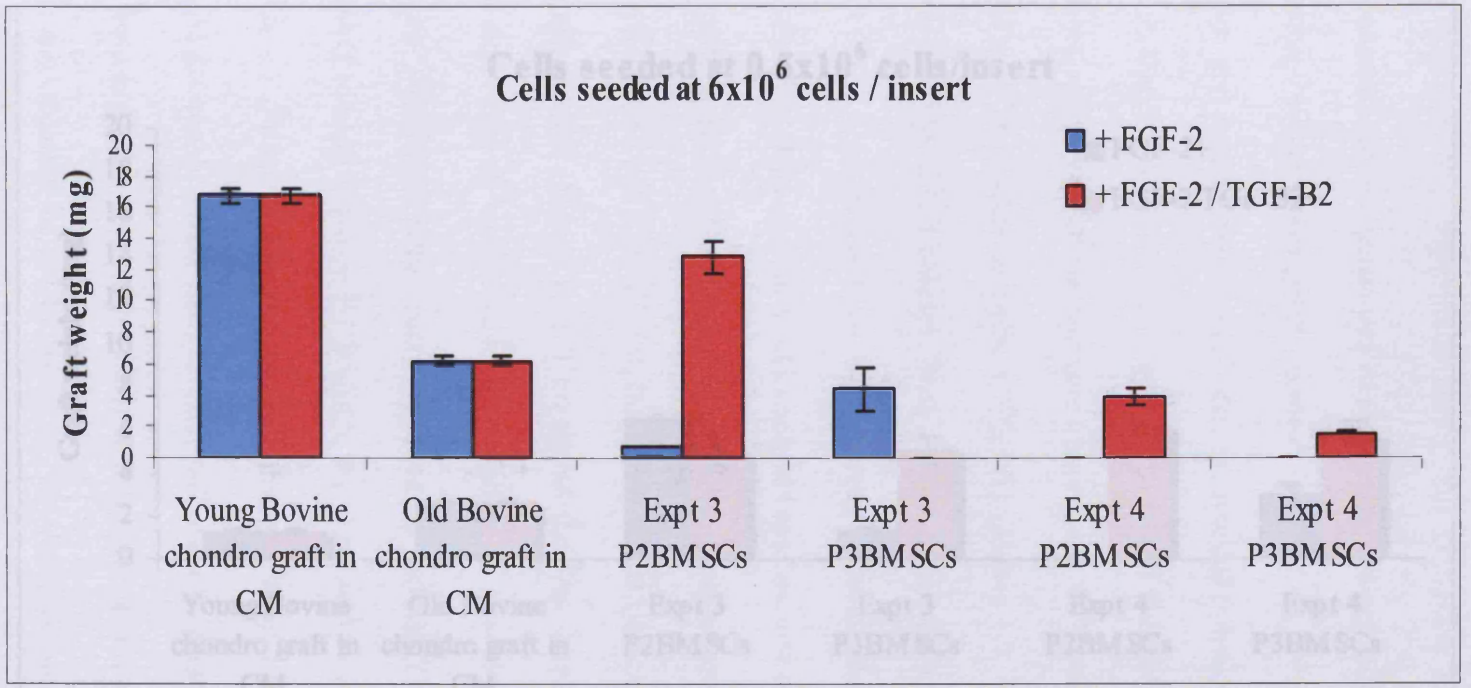


Figure 6.3: Graft weight obtained from plating 6 x10⁶ cells into each of the Transwell system. The average weights and standard errors for grafts cultured using chondrocytes derived from young bovine articular cartilage (n= 6), old bovine articular cartilage (n= 6) and from P2 and P3 BMSCs were tabulated (Panel A) and the overall results were plotted on a histogram (Panel B). Note that these results are based on harvests 3 and 4 (refer to Chapter 4 and 5 for details).

A

0.5x10⁶ cells/insert (+FGF-2)	Average	Std Error	0.5x10⁶cells/insert (+FGF-2/TGF-B2)	Average	Std Error
Young Bovine chondro graft in CM	1.3	0.1	Young Bovine chondro graft in CM	1.3	0.1
Old Bovine chondro graft in CM	2.7	0	Old Bovine chondro graft in CM	2.7	0
Harvest 3 P2BMSCs	6.6	0	Harvest 3 P2BMSCs	5	0.62
Harvest 4 P2BMSCs	0	0	Harvest 4 P2BMSCs	6.2	0
Harvest 3 P3BMSCs	1.4	0.12	Harvest 3 P3BMSCs	5.1	0.6
Harvest 4 P3BMSCs	3	0.4	Harvest 4 P3BMSCs	5.7	0.18

B

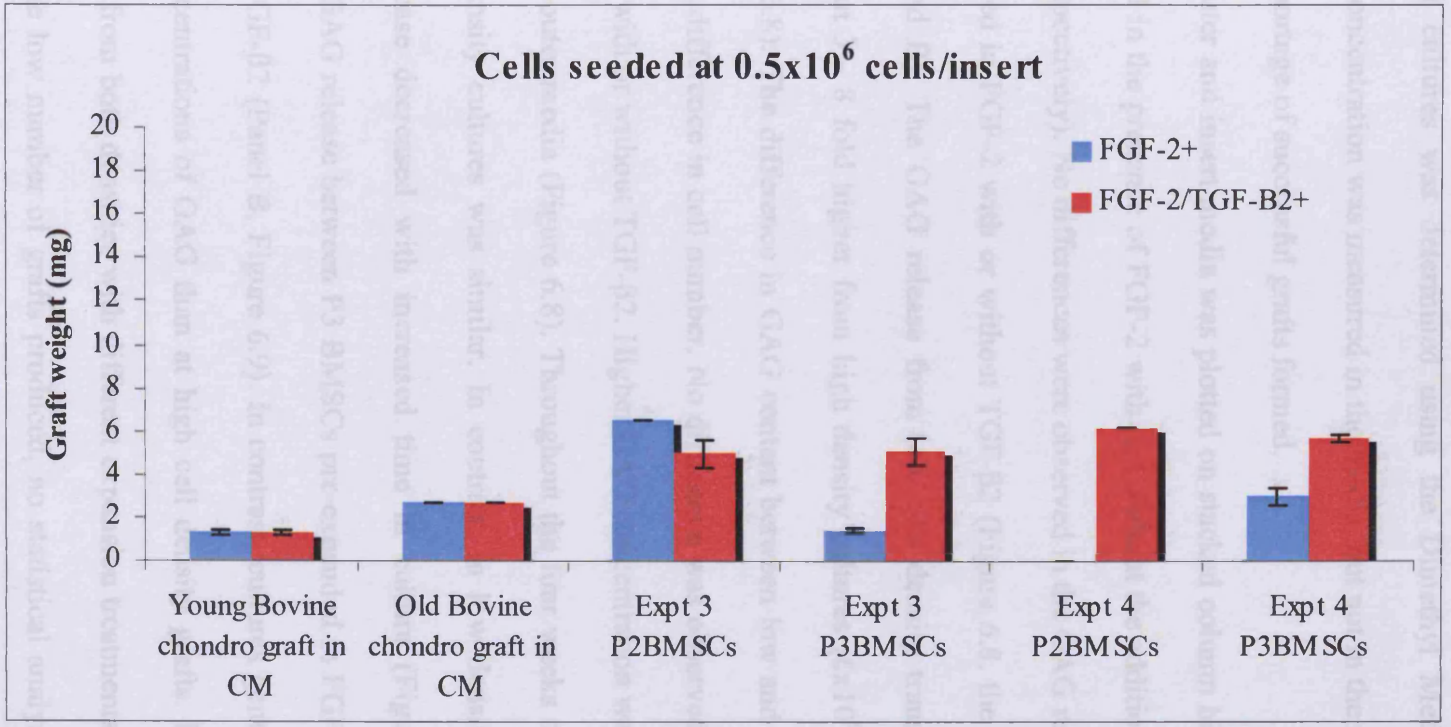


Figure 6.4: Graft weight obtained from plating 0.5 x10^6 cells into each of the Transwell system. The average weights and standard errors for grafts cultured using chondrocytes derived from young bovine articular cartilage (n= 6), old bovine articular cartilage (n= 6) and from P2 and P3 BMSCs were tabulated (Panel A) and the overall results were plotted on a histogram (Panel B). Note that these results are based on harvests 3 and 4 (refer to Chapter 4 and 5 for details).

6.4.2 Analysis of sulphated GAG release from media obtained from passaged BMSCs in Transwell culture system

The amount of sulphated GAG released to the medium collected at weekly intervals from passaged BMSCs transwell cultures was determined using the Dimethyl Methylene Blue (DMMB) assay. The GAG concentration was measured in the media, but not in the matrix of the transwell cultures due to a shortage of successful grafts formed.

The GAG content of both outer and insert media was plotted on stacked column histograms for P2 and P3 BMSCs expanded in the presence of FGF-2 with and without the addition of TGF-β2 (refer to Chapter 4 and 5, respectively). No differences were observed in the GAG release pattern between P2 BMSCs expanded in FGF-2 with or without TGF-β2 (Figure 6.8, the high density data shown in Panels A and B). The GAG release from low cell density transwell culture ($0.5x10^6$ cells/insert) is about 3 - 8 fold higher from high density cultures ($6x10^6$ cells/insert) (Panels C and D of Figure 6.8). The difference in GAG content between low and high density grafts is probably due to the difference in cell number. No difference was observed between P2 BMSCs expanded in FGF-2 with or without TGF-β2. Higher GAG concentration was detected in the insert media than in the outer media (Figure 6.8). Throughout the four weeks of culture the GAG release from high density cultures was similar. In contrast, in low density grafts the concentration of GAG release decreased with increased time in culture (Figure 6.9). No difference was observed in GAG release between P3 BMSCs pre-expanded in FGF-2 (Panel A, Figure 6.9) or FGF-2 and TGF-β2 (Panel B, Figure 6.9). In contrast, cultures generated at low density released higher concentrations of GAG than at high cell density grafts. However, no obvious trend was observed from both densities with different expansion treatments (Figure 6.9, Panels C and D). Due to the low number of grafts produced, no statistical analyses could be carried out to further investigate the difference between different passage numbers of BMSCs, different plating density and different expansion treatment and their effects on GAG release. Our

GAG analyses showed that a higher GAG level was detected from low density tissue graft than high density tissue graft.

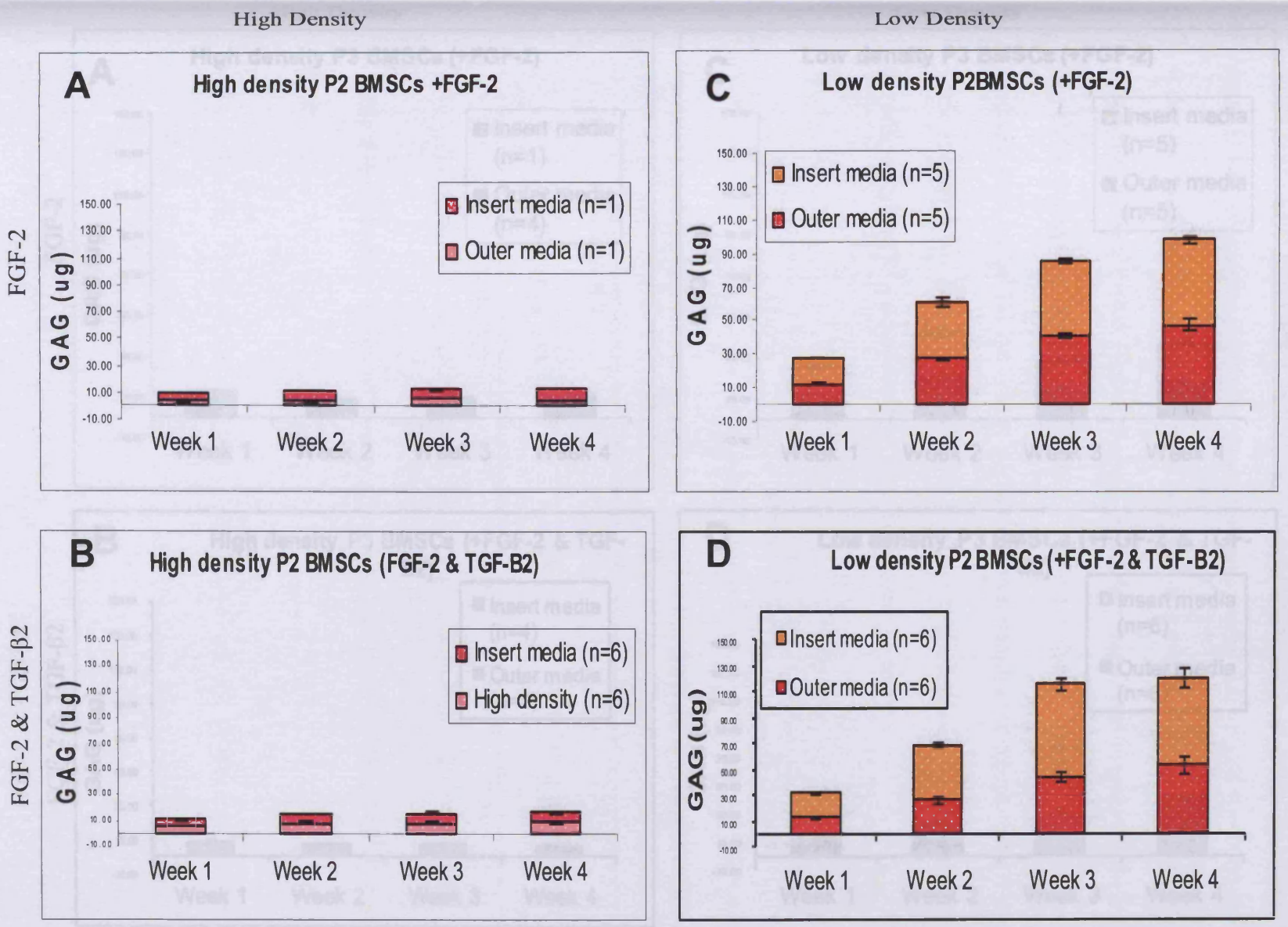


Figure 6.12: GAG contents obtained from high and low density Transwell cultures produced from Passage 2 (P2) BMSCs. Comparisons were made between P2 BMSCs pre-expanded in the presence of FGF-2 alone and with the addition of TGF-beta 2 to investigate their possible effects on graft formation using the Transwell system.

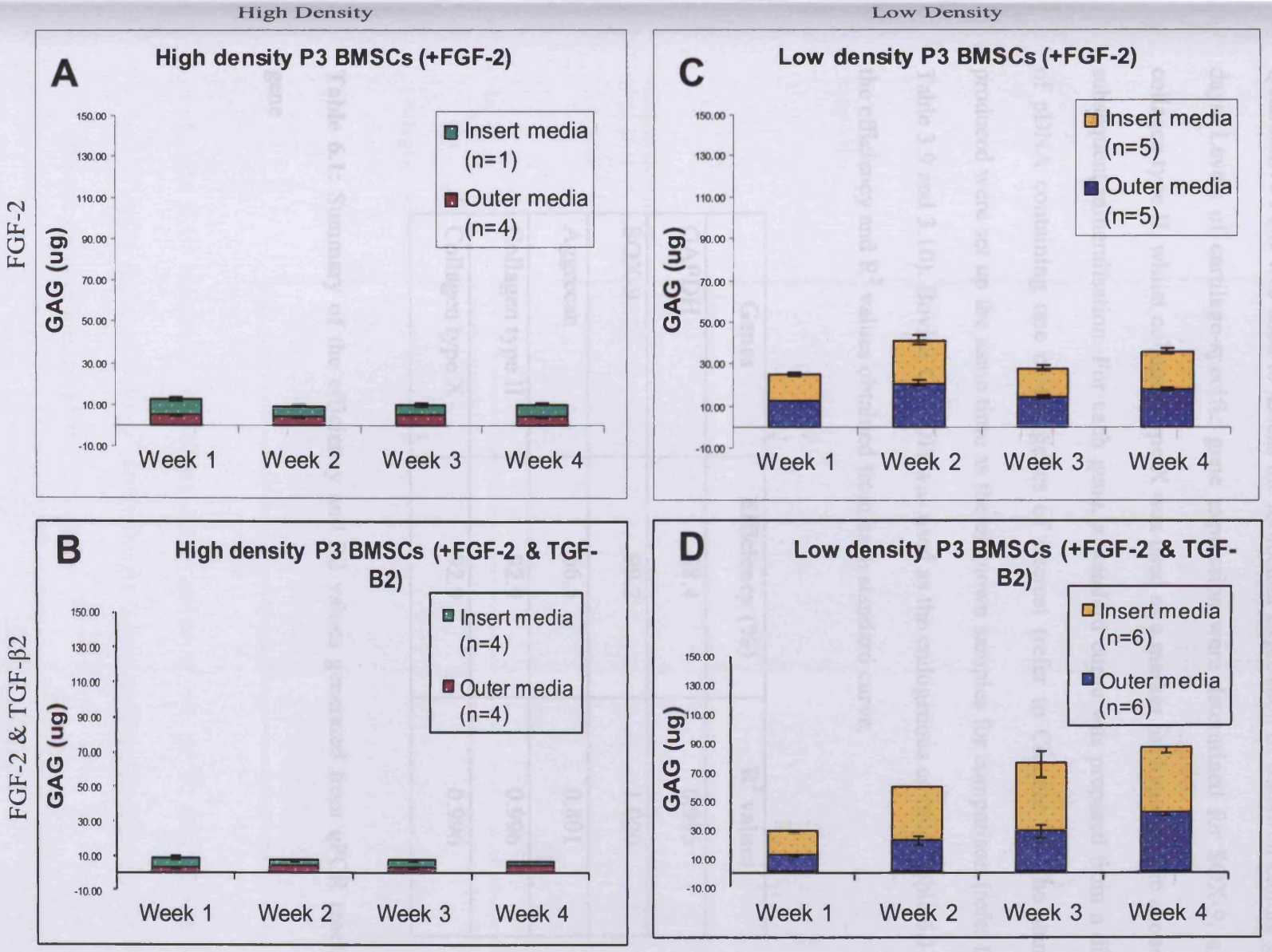


Figure 6.13: GAG contents obtained from high and low density Transwell cultures produced from Passage 3 (P3) BMSCs. Comparisons were made between P2 BMSCs pre-expanded in the presence of FGF-2 alone and with the addition of TGF-beta 2 to investigate their possible effects on graft formation using the Transwell system.

6.4.3 Expression of cartilage-specific genes from BMSCs tissue grafts produced using Transwell culture system

Quantitative PCR was used to assess the molecular expression of transwell culture graft after 28 days. Levels of cartilage-specific gene expression were determined for SOX-9, aggrecan and collagen type II, whilst collagen type X was used as a marker for hypertrophic chondrocytes and subsequent mineralisation. For each gene, a standard curve was prepared from a dilution series of pDNA containing one of the genes of interest (refer to Chapter 3). The standard curves produced were set up the same time as the unknown samples for comparison (refer to Chapter 3, Table 3.9 and 3.10). Bovine GAPDH was used as the endogenous control. Table 6.1 summarizes the efficiency and R^2 values obtained from each standard curve.

Genes	Efficiency (%)	R^2 values
GAPDH	88.4	0.995
SOX-9	99.7	1.000
Aggrecan	66.5	0.801
Collagen type II	92.9	0.996
Collagen type X	92.9	0.996

Table 6.1: Summary of the efficiency and R2 values generated from qPCR reaction for each gene

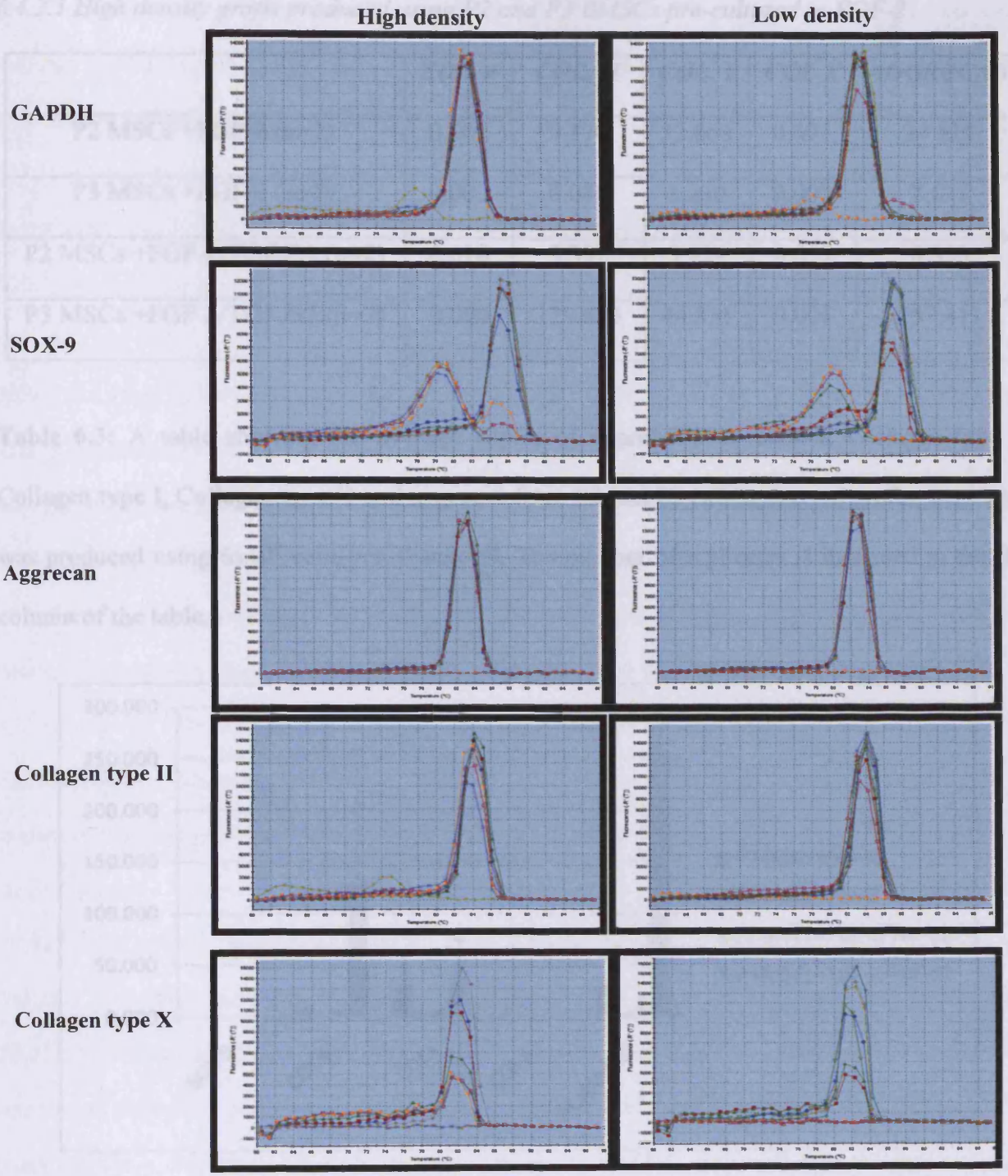


Figure 6.10 summaries the dissociation curves of the qPCR data obtained from high ($6x10^6$ cells) and low ($0.5x10^6$ cells) density tissue grafts using primer pairs designed for different genes of interest (left).

6.4.2.1 High density grafts produced using P2 and P3 BMSCs pre-cultured in FGF-2

	SOX-9	*COL. II*	*COL. I*	*COL X*	*AGGRECAN*
P2 MSCs +FGF-2 (n=2)	0.003	9.176	32.068	0.071	24.525
P3 MSCs +FGF-2 (n=2)	0.002	0.070	3.230	0.003	2.121
P2 MSCs +FGF-2/TGF-B2 (n=2)	0.016	3.392	3.924	0.015	8.300
P3 MSCs +FGF-2/TGF-B2 (n=7)	0.090	129.853	44.831	0.005	257.411

Table 6.3: A table showing the average copies of expression of SOX-9, Collagen type II, Collagen type I, Collagen type X and aggrecan from P2 and P3 MSCs-derived grafts. Each graft was produced using $6x10^6$ cells per Transwell. The number of replicates is indicated in the first column of the table.

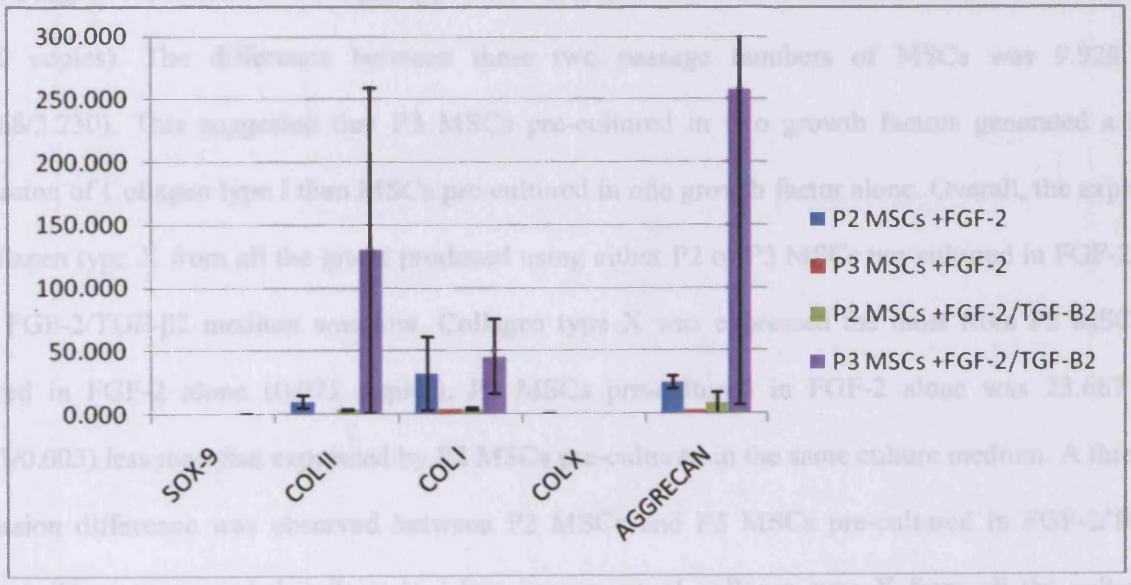


Figure 6.5: A graphical summary of the gene expression levels for SOX-9, Collagen type I, II and X and aggrecan from P2 and P3 MSCs pre-cultured in the either 5ng/µl of FGF-2 alone or in combination with 5ng/µl of TGF-β2. Each graft was produced using $6x10^6$ cells per Transwell.

The highest detection of SOX-9 was generated from P3 MSCs pre-cultured in FGF-2/TGF-β2 medium with 0.090 copies. P2 MSCs pre-cultured in the same culture medium was 5.625 times (0.090/0.016) less than P3 MSCs. In contrast, the levels of SOX-9 expression detected from P2 and P3 MSCs pre-cultured in

FGF-2 alone was 30 times (0.090/0.003) and 45 times (0.090/0.002) less than that of P3 MSCs pre-cultured in FGF-2/TGF-β. This suggested that pre-culturing MSCs in two growth factors gave a higher expression of SOX-9 than those cultured in only one growth factor. P3 MSCs pre-cultured in FGF-2/TGF-β2 generated the highest detection of Collagen type II, of 129.853 copies. P2 MSCs pre-cultured in the same culture medium was 38.282 times (129.853/3.392) less than the expression generated from P3 MSCs. However, when MSCs were pre-cultured in FGF-2 alone, a higher expression of Collagen type II was detected from P2 MSCs than P3 MSCs. The difference of collagen type II expression between P2 MSCs and P3 MSCs pre-cultured in FGF-2 alone was 131.086 times higher (9.176/0.070). This indicates that pre-culturing MSCs in two growth factors gave a higher expression of Collagen type II than those cultured in just one growth factor. The highest detection of collagen type I was generated from P3 MSCs pre-cultured in FGF-2/TGF-β2 of 44.831 copies. P2 MSCs cultured in the same culture medium expressed 11.425 times (44.831/3.924) less than P3 MSCs. When MSCs were pre-cultured in FGF-2 alone, a higher expression of Collagen type I was detected from P2 MSCs (32.068 copies) than P3 MSCs (3.230 copies). The difference between these two passage numbers of MSCs was 9.928 times (32.068/3.230). This suggested that P3 MSCs pre-cultured in two growth factors generated a higher expression of Collagen type I than MSCs pre-cultured in one growth factor alone. Overall, the expression of collagen type X from all the grafts produced using either P2 or P3 MSCs pre-cultured in FGF-2 alone or in FGF-2/TGF-β2 medium was low. Collagen type X was expressed the most from P2 MSCs pre-cultured in FGF-2 alone (0.071 copies). P3 MSCs pre-cultured in FGF-2 alone was 23.667 times (0.071/0.003) less than that expressed by P2 MSCs pre-cultured in the same culture medium. A three-fold expression difference was observed between P2 MSCs and P3 MSCs pre-cultured in FGF-2/TGF-β2 medium. These suggested that there is a low expression of collagen type X from all the cultures. A decrease in collagen type X was observed with increase passage number (one passage number difference). No obvious differences were observed between MSCs cultured in FGF-2 alone or in FGF-2/TGF-β2. Aggrecan was highly expressed by P3 MSCs pre-cultured in FGF-2/TGF-β2 medium with 257.411 copies. P2 MSCs pre-cultured in the same culture medium was 31.013 times (257.411/8.3) less than that detected from P3 MSCs. A reverse pattern of aggrecan expression was observed from MSCs pre-cultured in FGF-2 alone. P2 MSCs pre-cultured in FGF-2 alone was 11.563 times higher than P3 MSCs. These

suggested that the expression of aggrecan was more favourable in MSCs pre-cultured in the presence of two growth factors. An increase in passage number appeared to generate a higher expression of aggrecan.

6.4.2.3 Low density grafts produced using P2 and P3 BMSCs pre-cultured in FGF-2

	SOX-9	COL II	COL I	COL X	AGGRECAN
P2 MSCs +FGF-2 (n=3)	0.012	17.223	1.280	0.016	179.582
P3 MSCs +FGF-2 (n=9)	0.207	1.784	4.085	0.017	19.906
P2 MSCs +FGF2/TGFB2 (n=5)	0.012	1.320	3.413	3.164	11.990
P3 MSCs +FGF-2/TGF-B2 (n=6)	0.077	5.113	2.445	1.099	52.873

Table 6.4: A table showing the average copies of expression of SOX-9, Collagen type II, Collagen type I, Collagen type X and aggrecan from P2 and P3 MSCs-derived grafts. Each graft was produced using $0.5x10^6$ cells per Transwell. The number of replicates is indicated in the first column of the table.

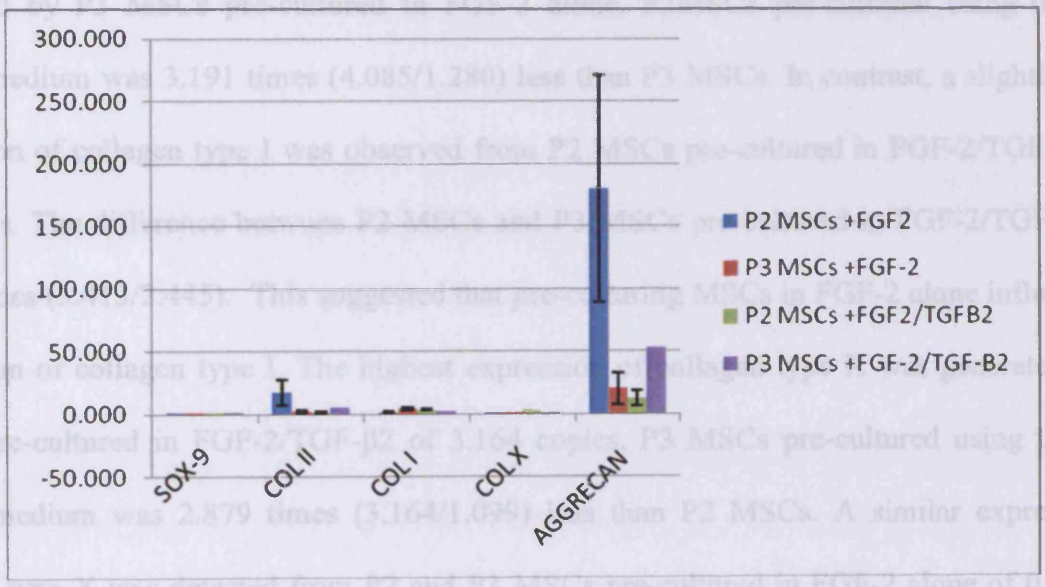


Figure 6.6: A graphical summary of the gene expression levels for SOX-9, Collagen type I, II and X and aggrecan from P2 and P3 MSCs pre-cultured in the either 5ng/µl of FGF-2 alone or in combination with 5ng/µl of TGF-β2. Each graft was produced using $0.5x10^6$ cells per Transwell.

The overall expression of SOX-9 from all the cultured was low. The highest expression of SOX-9 was generated by P3 MSCs pre-cultured in FGF-2 alone, with 0.207 copies. P2 MSCs pre-cultured using the same medium was 17.25 times (0.207/0.012) less than P3 MSCs. A similar

pattern was observed from MSCs pre-cultured in FGF-2/TGF-β2, in which P3 MSCs has a higher expression of SOX-9 than P2 MSCs. The difference between P2 and P3 MSCs pre-cultured in FGF-2/TGF-β2 was 6.417 times (0.077/0.012). This suggested that for tissue graft produced using $0.5x10^6$ cells/Transwell, MSCs pre-cultured in FGF-2 alone with a higher passage number, in this case, P3 generated more expression of SOX-9. Collagen type II was expressed by all the cultures, however, P2 MSCs pre-cultured in FGF-2 alone generated the highest expression of 17.233 copies. P3 MSCs pre-cultured in the same culture medium was 9.665 times less (17.223/1.782) than P2 MSCs. A reverse pattern was observed from MSCs pre-cultured in the presence of FGF-2/TGF-β2, in which P3 MSCs expressed 3.873 times higher than P2 MSCs. These suggested that pre-culturing MSCs in the presence of FGF-2 alone is more favourable for the expression collagen type II. The highest expression of collagen type I was generated by P3 MSCs pre-cultured in FGF-2 alone. P2MSCs pre-cultured using the same culture medium was 3.191 times (4.085/1.280) less than P3 MSCs. In contrast, a slightly higher expression of collagen type I was observed from P2 MSCs pre-cultured in FGF-2/TGF-β2 than P3 MSCs. The difference between P2 MSCs and P3 MSCs pre-cultured in FGF-2/TGF-β2 was 1.396 times (3.413/2.445). This suggested that pre-culturing MSCs in FGF-2 alone influence the expression of collagen type I. The highest expression of collagen type X was generated by P2 MSCs pre-cultured in FGF-2/TGF-β2 of 3.164 copies. P3 MSCs pre-cultured using the same culture medium was 2.879 times (3.164/1.099) less than P2 MSCs. A similar expression of collagen type X was detected from P2 and P3 MSCs pre-cultured in FGF-2 alone of 0.016 and 0.017 copies, respectively. This indicated that the expression of collagen type X was more favourable when MSCs were pre-cultured in the presence of two growth factors than one. P2 MSCs pre-cultured in FGF-2 alone generated the highest expression in comparison to other genes of interest. A detection of 179.582 copies was obtained from P2 MSCs pre-cultured in FGF-2 alone. Interestingly, a drastic reduction in the expression of aggrecan was observed from P3 MSCs pre-cultured under the same medium. A difference of 159.676 copies (179.582-19.906)

was obtained, a 9.022 times difference was detected. In contrast, a higher expression of aggrecan was generated from P3 MSCs pre-cultured in FGF-2/TGF-β2 than P2 MSCs was observed. A 4.4- fold difference between P3 and P3 MSCs pre-cultured in the presence of two growth factors was detected. This suggested that early passage number was more favourable for the expression of aggrecan when MSCs was pre-cultured in FGF-2 alone. However, when MSCs were pre-cultured in the presence of two growth factors, in this case, FGF-2 and TGF-β2, one passage number difference appeared to have an influence on the expression of aggrecan. These together indicate that the passage number and the presence of different growth factors have a combined influence on the expression of various chondrogenic-associated genes.

6.5 DISCUSSION

The objective of this study was to determine if passaged BMSCs harvested at P2 and P3 are capable of forming a tissue graft when cultured in defined chondrogenic medium using the Transwell culture system (Kandel *et al.,* 1995). The exposure of BMSCs to chondrogenic medium was to determine if this would further differentiate the passaged BMSCs towards a chondrogenic lineage, allowing the cells to produce an extracellular matrix resembling hyaline cartilage that can be used as a cell-based strategy for cartilage repair.

Bovine-derived BMSCs seeded into Transwell system are capable of forming a uniformly distributed tissue graft throughout the dried, porous polycarbonate membrane, pre-coated with chick collagen type II. Transwell culture filter inserts, established using inserts of 6.5 mm in diameter with 0.4 μm pore size polycarbonate membrane, were used by Murdoch *et al.,* to produce tissue grafts using 0.5 $x10^6$ hMSCs per insert and cultured in chondrogenic medium (Murdoch *et al.,* 2007). This study demonstrated the feasibility of forming a flexible, shallow tissue graft of passage 2 and 3 (P2 and P3) bovine derived BMSCs seeded at $0.5x10^6$ cells/insert. However, successful grafts when formed less often when BMSCs were seeded at $6x10^6$ cells/insert. Those seeded using a higher cell density appeared to fail to stay as multi cell layers, instead they contract to form a lump of cell mass. This may be due to the effects of residual serum left with the cells before resuspending in chondrogenic medium as suggested by Tew *et al.,* 2008. In addition, uneven sedimentation of the BMSCs during the settling period without the use of centrifugation to produce more uniform cell sedimentation may cause a cell mass to form. The wet mass recorded from Hardingham's group indicated an average of 20-25 mg for tissue grafts produced over a 4 week culture period, with a matrix composed of mainly aggrecan and collagen type II, which are hallmarks of chondrogenesis (Tew *et al.,* 2008; Murdoch *et al.,* 2007). Our grafts produced using bovine derived BMSCs are 3-4 times lighter than those produced by using hMSCs as shown by Hardingham's group (Murdoch *et al.,* 2007). This difference in graft weight may be due to the use of a bovine model instead of a human model.

Our data demonstrated that the use of a lower seeding cell density per Transwell insert ($0.5x10^6$ cells/well) and BMSCs pre-cultured in the presence of FGF-2 and TGF-β2 generated a more successful tissue graft than using $6x10^6$ cells/well and BMSCs pre-cultured in FGF-2.

Gene expression study at high seeding density ($6x10^6$ cells/well) suggested that pre-culturing MSCs in the presence of both FGF-2 and TGF-β2 favoured the expression of chndrogenic-associated genes such as collegn type II and aggrecan. An increase in one passage number lso favours the expression of such genes. From low seeding density ($0.5x10^6$ cells/well), similar results were obtained. This indicated that the passage number and the presence of different growth factors have a combined influence on the expression of various chondrogenic-associated genes. In the review by Tew *et al.,* 2008, they suggest that the addition of FGF-2 to the expansion medium prior to the differentiation of hMSCs in Transwell culture system improved the ability of hMSCs to differentiate towards a chondrogenic lineage (Tsutsumi *et al.,* 2001; Bianchi *et al.,* 2003; Bianchi *et al.,* 2001; Solchaga *et al.,* 2005). In contrast, our data suggested that the presence of FGF-2 and TGF-β2 gave a better expression of SOX-9, aggrecan and collagen types I and II. However, our data suggests that the addition of TGF-β2 to the expansion medium did not affect the differentiation process. It is interesting to note that in a human model, the absence of FGF-2 in the medium appeared to affect the ability of hMSCs to undergo chondrogenesis using the Transwell culture system, in comparison to those pre-expanded in medium with the addition of FGF-2. It has also been demonstrated by Hardingham's group that in a human model, MSCs can be passaged continuously to at least P7 in FGF-2 supplemented medium (Tew *et al.,* 2008; Bianchi *et al.,* 2003).

In conclusion, this chapter illustrated that it is possible to generate a successful tissue graft using the Transwell culture system. The ideal seeding cell density is $0.5x10^6$ cells/well, using P2 or P3 BMSCs pre-cultured in either FGF-2 and TGF-β2. The tissue graft produced has a high expression level of aggrecan, collagens type I and II, which suggested that it has a fibro-cartilage phenotype.

6.6 Summary

- Low seeding cell density ($0.5x10^6$ cells/well) is able to generate more successful tissue graft than high seeding cell density ($6x10^6$ cells/well).
- BMSCs pre-cultured in FGF-2 with the addition of TGF-β2 (see Chapter 5) contributed to the generation of successful graft.
- The gene expression levels of aggrecan, collagens type I, II and X and SOX-9 were similar between low and high cell density Transwell grafts.
- One passage number did not influence any changes on the expression levels or the trends of expression.
- Pre-culturing cells in FGF-2 and TGF-β2 did not affect the expression pattern of ggrecan, SOX-9 and collagens type I and II.
- Higher GAG content was detected from tissue grafts produced using a low cell seeding density ($0.5x10^6$ cells/well).

6.7 Future work

- Further examine the effect of passage number on the gene expression pattern by increasing the passage number to P10or beyond
- Use more chondrogenic markers such as Jagged-1, and -2, Notch -1 and Delta in addition to aggrecan, SOX-9, collagens type I, II and X, in order to further understand the lineage that the tissue grafts is differentiating into.

CHAPTER 7: General Discussion

Stem cells are unspecialised cells found in the body which possess the ability to self renew and can be induced to proliferate and differentiate into more specialised cells. MSCs are adult stem cells that are capable of leaving the bone marrow and travelling in the bloodstream to a different site, where they may perform repair or regeneration processes of various mesenchymal tissues such as cartilage, bone and fat. Due to these properties of MSCs, they prove to be a useful source for the repair and regeneration of cartilage. Before the attempt to differentiate MSCs towards a particular lineage, it is important to first optimise a method for the harvesting of bone marrow and for the isolation of MSCs using a chosen model.

In this study, a bovine model was used. The establishment of primary cultures of BMSCs by adherence to tissue culture plastic of bone marrow stem cells through aspiration of bone marrow from the metacarpal or metatarsal phalangeal joints of young bovine calves proved to be a viable method for the isolation of primary BMSCs. These findings reiterate previously published methods by Caplan *et al.,* 1999 and Oreffo *et al.,* 1997, using human and rat models, respectively. During extraction of the bone marrow, we discovered that there was variation in the bone chamber size and bone thickness, and that this influenced the amount of bone marrow and cells that could be harvested. The variations in chamber size could be due to many factors including arrival age (at abattoir), front and back leg (carpo versus tarso), breed, sex of the animal, and time after slaughter which may influence cell viability later on. However, these differences did not affect the morphology of the cells isolated or their adherence ability. These data suggest that freshly harvested stem cells retain their original 'status' despite being processed and washed several times, with serum free medium, prior to plating and adhering to plastic. The isolated BMSCs possessed a spindle-shaped fibroblastic morphology as shown previously in publications by Caplan & Bruder 2001, and Owen *1988.* The method used to extract bone marrow in this study using young bovine legs was direct extraction from an open carpal or tarsal bone. The extracted bone marrow underwent a series of washes and was plated into culture

flasks. Primary cultures of young bovine bone marrow mesenchymal stem cells (BMSCs) were established by adherence to tissue culture plastic. After the initial 8 days of culture, cells were trypsinised and the cell number was determined. The protein expression of Notch-1, Delta and Jagged-2 was analysed. In addition, the gene expression of aggrecan, SOX-9, collagens type I and II was determined using quantitative PCR. From Chapter 3 data, it has been found that different chamber size and bone thickness influenced the amount of bone marrow and cells harvested. However, this did not affect the morphology of the cells and their adherence ability. The morphology of the BMSCs cultured in serum-containing medium for the initial 8 days of culture, possess a spindle-shaped fibroblastic morphology. This feature resembles that of other previously published work (Murdoch *et al.,* 2007). Wakitani and co-workers have also recently studied the correlation between cell morphology and aggrecan gene expression level during differentiation of mesenchymal stem cells to chondrocytes using a human model. They found that the cell morphology of MSCs tended to change from fibroblastic-like to polygonal shape and such change resulted in an upregulation of aggrecan mRNA expression level during the differentiation of MSCs to chondrocytes (Takagi *et al.,* 2008). FACs analyses indicated that undifferentiated MSCs (P0BMSCs) harvested at Day 8 expressed the stem / progenitor markers of Notch-1, Delta and Jagged-2 These markers were previously described to be expressed on the surface of progenitor cells of young cartilage in a chick model (Dowthwaite *et al.,* 2004). This suggested that the cells isolated initially exhibit stem cell like features. Hardingham and co-workers recently demonstrated that Notch signalling through Jagged-1 is necessary to initiate MSCs to differentiate towards a chondrogenic lineage using a human model. However, the expression of Jagged-1 must be switched off in order to enable MSCs to complete chondrogenesis (Oldershaw *et al.,* 2008). Gene expression analyses of SOX-9, aggrecan, collagens type I and II suggested that the primary MSCs do not contain any chondrogenic features.

When P0BMSCs underwent trypsinisation, they were further cultured in serum containing medium with the supplementation of one or two growth factors (FGF-2 or with the addition of TGF-β2). Many factors such as cytokines and growth factors contribute to the cellular events of proliferation, differentiation and maturation of cell types through binding to target cell surface receptors and initiating intracellular events. Several reports have shown that FGF-2 contributes to the selection of MSCs that are more likely to differentiate chondrogenically Bianchi *et al., 2003*. Previous studies have suggested that the addition of FGF-2 during the subculture phase enhances the chondrogenic potential of human derived MSCs (Solchaga *et al.,* 2005). However, Walsh et al., have used FGF-2 for cell expansion and have shown that FGF-2 enhanced the osteogenic potential of adult human-derived BMSCs (Walsh *et al.,* 2000). These studies imply that the application of FGF-2 on the chondrogenic differentiation of MSCs is still controversial. In addition to FGF-2, TGF-β has also been found to be one of the most potent inducers of chondrogenic differentiation of BMSCs isolated from bone marrow in many different species and other tissue sources (Bosnakovski *et al.,* 2004). Stevens *et al.,* have demonstrated that the combined use of TGF-β and FGF-2 influenced perichondrium to differentiate towards a chondrogenic lineage (Stevens *et al.,* 2004). Subculturing BMSCs from P1 and P2 in the presence of FGF-2 or with the addition of TGF-β2 did not change the cell morphology. Both P1 and P2 BMSCs possess a fibroblastic phenotype. The use of two growth factors FGF-2 with TGF-β2 showed to increase the number of cells per flask, which implies that the use of two growth factors enhanced cell proliferation of BMSCs in a bovine model. FACs analyses showed positive binding of Notch-1, Delta and Jagged-2 from P1 and P2 BMSCs cultured in FGF-2 or with the addition of TGF-β2. This implied that P1 and P2 BMSCs still have some progenitor stem cells features, which suggest that they were not fully committed to a specific lineage. Quantitative PCR on P1 and P2 BMSCs cultured in FGF-2 showed no expression of SOX-9, aggrecan and collagen type II. Collagen type I was detected but at a lower expression level in comparison to those observed from articular cartilage extracted from young and old bovine.

Passaged BMSCs were harvested and re-seeded as P2 and P3 BMSCs into Transwell inserts. The Transwell culture system was first described by Kandel *et al.,* (Kandel *et al.,* 1995), where chondrocytes were seeded into a Transwell insert, culture for certain period of time (usually a few weeks) and upon tissue grafts are generated. The cells in the Transwell insert formed a shallow flat disc on the permeable support, enabling cell-cell contact that other studies have shown to be necessary for efficient chondrogenesis (Johnstone *et al.,* 1998; Yoo *et al.,* 1998). The advantage of using the Transwell culture system is that it allows all the cells to have a close contact to the nutrient supply with easy diffusion access from above and below. Murdoch *et al.,* have illustrated that using Transwell culture system results in a five times greater surface area than the commonly used three dimensional pellet culture systems. Also they have demonstrated that the matrix deposited by the stem cells derived from human tissue has a higher retention of PG than pellet culture (Murdoch *et al.,* 2007).

Previous work by Hayes and co-workers have demonstrated the ability to generate a tissue graft using young bovine articular chondrocytes cultured in DMEM supplemented with FBS, TGF-β2 and ascorbic acid, over a 4 week culture period (Hayes *et al.,* 2007). Such cartilage grafts have shown to produce varying zonal morphology, resembling those observed in native cartilage. Recently, Hardingham and colleagues have illustrated the use of Transwell system to form a cartilage-like tissue graft using human MSCs *in vitro* (Murdoch *et al.,* 2007). Our aim was to attempt to produce a tissue graft using BMSCs instead of young bovine chondrocytes. The passaged BMSCs were seeded into Transwell cultures at two seeding densities, $6x10^6$ and $0.5x10^6$ cells/well. These BMSCs were cultured in chondrogenic medium containing TGF-β3 and dexamethasone. Our study demonstrated that bovine-derived BMSCs seeded into Transwell system are capable of forming a uniformly distributed tissue graft throughout the dried, porous polycarbonate membrane, pre-coated with chick collagen type II. Transwell culture filter inserts, established using inserts of 6.5 mm in diameter with 0.4 μm pore size polycarbonate membrane, were used by Murdoch *et al.,* to produce tissue grafts using 0.5 $x10^6$ hMSCs per insert and

cultured in chondrogenic medium (Murdoch *et al.,* 2007). This study demonstrated the feasibility of forming a flexible, shallow tissue graft of passage 2 and 3 (P2 and P3) bovine derived BMSCs seeded at 0.5 $x10^6$ cells/insert. However, successful grafts when formed less often when BMSCs were seeded at 6 $x10^6$ cells/insert. Those seeded using a higher cell density appeared to fail to stay as multi-cell layers, instead they contract to form a lump of cell mass. This may be due to the effects of residual serum left with the cells before re-suspending in chondrogenic medium as suggested by Tew *et al.,* 2008. In addition, uneven sedimentation of the BMSCs during the settling period without the use of centrifugation to produce more uniform cell sedimentation may cause a cell mass to form. The wet mass recorded from Hardingham's group indicated an average of 20-25 mg for tissue grafts produced over a 4 week culture period, with a matrix composed of mainly aggrecan and collagen type II, which are hallmarks of chondrogenesis (Tew *et al.,* 2008; Murdoch *et al.,* 2007). Our grafts produced using bovine derived BMSCs are 3-4 times lighter than those produced by using hMSCs as shown by Hardingham's group (Murdoch *et al.,* 2007). This difference in graft weight may be due to the use of a bovine model instead of a human model. Our data demonstrated that the use of a lower seeding cell density per Transwell insert ($0.5x10^6$ cells/well) and BMSCs pre-cultured in the presence of FGF-2 and TGF-β2 generated a more successful tissue graft than using $6x10^6$ cells/well and BMSCs pre-cultured in FGF-2.

Gene expression studies also suggested that our bovine tissue grafts are mainly composed of aggrecan and collagen type I and type II with a low expression of SOX-9 and collagen type X. Similar gene expression patterns were observed from grafts produced using both high (6 $x10^6$ cells) and low (0.5 $x10^6$ cells) seeding densities. These imply that one passage difference did not have any influence on the expression levels or their trend. Also pre-culturing BMSCs in FGF-2 or with the addition of TGF-β2 did not affect the expression pattern. In the review by Tew *et al.,* 2008, they suggest that the addition of FGF-2 to the expansion medium prior to the differentiation of hMSCs in Transwell culture system improved the ability of hMSCs to

differentiate towards a chondrogenic lineage (Tsutsumi *et al.,* 2001; Bianchi *et al.,* 2003; Mastrogiacomo *et al.,* 2001; Solchaga *et al.,* 2005), which corresponds to our findings on the effects of the addition of growth factors, FGF-2 and in the presence of TGF-β2. However, our data suggests that the addition of TGF-β2 to the expansion medium did not affect the differentiation process.

In conclusion, our study has shown that it is possible to produce tissue grafts using Transwell culture system by using a cell seeding density of $0.5x10^6$ cells/well. It has shown that the use of passaged BMSCs pre-cultured in FGF-2 and TGF-β2 favours the ability to generate a tissue graft. Further optimisation on the culture conditions is necessary in order to differentiate passaged BMSCs towards a chondrogenic lineage, subsequently generating tissue grafts that resemble close to hyaline cartilage, which can be used for the repair and regeneration of cartilage. For future experimentation, it would be interesting to isolate BMSCs using different methods such as Lymphoprep which enables the separation between hematopoietic and non-hematopoietic cells, this allows us to obtain a clear number of mononucleated cells. It would be beneficial that further characterisation of passaged BSCs (P0, P1 and P2) in order to further understand the expression pattern of BMSCs at different stages of culture. Therefore more primer pairs (such as Notch-1, Delta and Jagged-1 and -2) designed specifically for bovine are essential to carry out further gene expression analyses. During each stage of passage, cell viability tests should be carry out in order to assess the viability o the cells after each trypsinisation in order to monitor the cell viability at different stages. A standardised replating cell density would be useful for studying the effect of cell density on the gene and protein expression. The proliferation rates of BMSCs at different passage number can also be studied by recording the cell doubling time at each passage step. Furthermore, it would be interesting to further investigate the effect of passage number on the gene expression pattern by in creasing the passage number to P10 or beyond.

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