The Characterization of Bovine Adipose-Derived Stem Cells in Conventional and Co-culture Environments for Tissue Engineering

by

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A thesis submitted to the Department of Chemical Engineering

in conformity with the requirements for

the degree of Master of Applied Science

Queen's University

Kingston, Ontario, Canada
February, 2011

Abstract

Adipose-derived stem cells (ASCs) have been extensively investigated for their applicability in the field of tissue engineering due to their multi-lineage differentiation potential and the convenience of cell acquisition. To date, conventional inductive media have been used to induce lineage-specific differentiation of ASCs; however, this general approach has limitations in terms of high costs and unstable differentiation responses. In native tissues, mesenchymal stem cells (MSCs) interact with their surrounding cells (i.e. mature cells) through paracrine and autocrine signaling, which can regulate their metabolism and cell function. Therefore, the author developed *in vitro* co-culture models to study the interactions between ASCs and three different mature cell types: adipocytes, chondrocytes and osteoblasts.

In this work, bovine ASCs (bASCs) from the interdigital fat pad were first isolated and characterized, in terms of *in vitro* proliferation and multi-lineage (bone, cartilage, fat) differentiation with conventional inductive media and culture conditions. Doubling time calculations and gene expression analysis of stem cell markers indicated a threshold existed for stem cell degeneration at passage 5 (P5) for bASCs when expanded extensively *in vitro*. The multi-lineage differentiation potentials were compared between passage 2 (P2) and P5. Interestingly, while the P5 bASCs presented significantly lower levels of adipogenesis and chondrogenesis, osteogenesis was maintained or even improved with serial passaging.

In the designed indirect co-culture systems, adipogenesis and chondrogenesis were investigated in growth medium without key differentiation factors, whereas osteogenesis was induced in conventional osteogenic medium, to maintain the stable phenotype of the mature osteoblasts in culture. The results were used to demonstrate the general feasibility of mature cell-induced or -enhanced bASC differentiation through soluble, cell-secreted paracrine signaling. When compared to growth factor (GF)-stimulated differentiation, the bASCs in co-culture presented an earlier, but potentially stronger, level of differentiation.

Among these paracrine factors, Wnt proteins are known to play essential roles in mediating stem cell self-regulation and fate determination. In the current thesis, the Wnt inhibitors WIF-1 and DKK-1 were used to explore the involvement of the Wnt canonical and non-canonical signaling pathways in the designed co-culture environments. The data showed a strong correlation with the literature, indicating the canonical pathway was upregulated during osteogenesis, but inhibited during adipogenesis. The inhibition of chondrogenesis through the canonical pathway was suggested on a genetic level.

Acknowledgements

First, I would like to thank my supervisors Drs. Lauren Flynn and Stephen Waldman, for their never-ending support and encouragement during my time in Queen's University. Their invaluable knowledge and constant feedback helped clarify my ideas and guide the development of this project. I am truly honored to be one of their graduate students and it was a pleasure to work with them for the past 2 years. In addition, I would like to thank Drs. Brian Amsden, Stephen Waldman, Steve Pang, Tim Bryant and Yat Tse for their outstanding teaching during my studies at Queen's University. Their courses helped me to gain a better understanding into my topic of research. I would like to give many thanks to the Flynn Lab Group and Waldman Lab Group for their assistance with the project. In particular, I would like to thank Jackie Fan, Jake Kaupp, and Jill Brenner for their help in the acquisition of my cell sources. A big thank you should also be given to Abby Sukarto, Aasma Khan, Hoi Ki Cheung, and Denver Suarro for their friendship and being the best company.

My deepest thanks to my fiancé, Ziqiu Chen, who is always patient with me during this important phase in my life. His understanding and dedication helped me make this work possible. Finally, a special thanks goes towards my parents for their ongoing encouragement. Their presence and their constant faith in me were a source of strength that I continue to draw from on a day-to-day basis. This thesis is dedicated to my grandmother, Mrs. Xunxia Liu, who passed away in January of 2011 from heart failure. Her love and support will never be forgotten.

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Commonly Used Acronyms

2-D	Two-dimensional
ABCG2	ATP-binding cassette superfamily G member 2
ADD-1	Adipocyte determination and differentiation dependent factor-1
ALDH	Aldehyde dehydrogenase
A2P	Ascorbic acid 2 phosphate
AGC	Aggrecan
ALP	Alkaline phosphatase
APC	Adenomatosis polyposis coli
(b)ASC	(Bovne) adipose-derived stem cell
bMSCs	Bone marrow stem cell
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CAMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CK	Casein kinase
DEX	Dexamethasone
DMMB	Dimethylene blue
DVL	Dishevelled
DKK1	Dickkopf-related protein 1
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FIHC	Fluorescence immunohistochemical
Fz	Frizzled receptors
GC	Glucocorticoid

GSK3	Glycogen synthase kinase 3	PKA (C)	Protein kinase A (C)
GF	Growth factor	PG	Proteoglycan
GPDH	Glycerol-3-phosphate dehydrogenase	PPAR	Peroxisome proliferator activated receptor
HSC	Hematopoietic stem cell	RHOA	Rho-family GTPases
IBMX	Isobutylmethylxanthine	RT-PCR	Reverse transcription polymerase chain reaction
IGF	Insulin growth factor		
IHC	Immunohistochemistry	Ryk	Receptor tyrosine kinase
JNK	C-Jun N-terminal kinases	sFRP	Secreted frizzled-related protein
LEF	Lymphoid enhancer-binding protein transcription factor	sGAG	Sulfated glycosaminoglycan
LPL	Lipoprotein lipase	SREBP-1c	Sterol regulatory element binding protein-1c
MAPK	Mitogen activated protein kinase	SVF	Stromal vascular fraction
MSC	Mesenchymal stem cell	TCF	T-cell factor
NLK	Serine/threonine protein kinase	TCPS	Tissue culture poly(styrene)
OCN	Osteocalcin	TGF	Transforming growth factor
P2	Passage 2	TZD	Thiazolidinediones
PBS	Phosphate buffered saline	WIF1	Wnt inhibition factor-1
PCP	Planar cell polarity		

Chapter 1

Introduction

1.1 Cell Therapy and Adipose-Derived Stem Cells

Tissue and organ damage due to trauma, disease, or tumor resection can dramatically impact a patient's quality of life, and can be associated with high costs to the Canadian healthcare system. Many tissues in the body are not capable of self regeneration after a significant amount of loss, including cartilage, bone, and adipose tissue (fat) [1]. These deficits often result in functional impairments that can broadly impact the health and wellbeing of the patient. In 2006, there were more than 1 million hip and knee replacements conducted in the United States alone [2]. In 2008-2009, over 78,000 of these procedures were performed in Canada [3]. Further, in 2009, approximately 3.95 million individuals underwent reconstructive surgery associated with tumor removal, which represented a 5% increase from 2008 [4]. These statistics indicate that a high demand exists for tissue-engineered substitutes.

Several different approaches have been studied in the field of regenerative medicine including: (i) Cell-based therapies, (ii) Biomaterials-based approaches, and (iii) Combination methods involving cell-seeded biomaterials. In general, biomaterials have been used to create tissue replacements that closely mimic natural tissues and activate normal body healing. Ideally, the regenerating tissues would eventually replace the biomaterials, as tissue function is restored [5]. Cell-based therapies utilize

isolated living cells for tissue restoration [6]. Combining the two approaches to maximize wound healing and tissue regeneration, cells and customized biomaterial scaffolds have been incorporated together for a broad range of applications in soft tissue engineering. As one famous example, in 1997, Cao *et al*. seeded chondrocytes onto polymers to produce tissue-engineered cartilage in the shape of the human ear on the backs of mice [7]; Ogawa used human adipose-derived stem cells (ASCs) in 2006 to reconstruct the human ear using similar methods [8]. Hyaluronic acid-collagen scaffolds seeded with human ASCs have been shown to retain a predefined shape and dimension for use in soft tissue augmentation and the reconstruction of defects [6]. These exciting results demonstrate the great potential of the tissue-engineering field.

Cell-based therapies in tissue engineering seek to repair and regenerate tissues and organs using a combination of cells, biomaterials and cytokines. The cell source candidates for cell-based therapies include terminally-differentiated cells (i.e. mature cells such as osteoblasts and chondrocytes) and stem cells. Mature cells are generally limited in availability and may require painful harvesting procedures; however, stem cells, which are capable of self renewal and multi-lineage differentiation [9-12], hold great promise in this field. In some cases, stem cells may be harvested autologously to completely bypass the potential for immune responses. While embryonic stem cells (ESCs) theoretically exhibit unlimited differentiation potential, the application of ESCs in cell-based therapies is limited by ethical and legal issues [8, 13]. Mesenchymal stem cells (MSCs), especially the regenerative cells derived from fat, termed ASCs, also show great potential in the field. ASCs can be obtained

autologously by simple liposuction, and can be expanded *in vitro* to obtain a desired cell number. Due to their ease of harvesting and relative abundance, ASCs are an attractive and readily-available adult stem cell source, which is becoming increasingly popular for many applications in tissue engineering [14, 15].

However, there are unresolved questions and limitations associated with the basic biology and understanding of ASCs for clinical applications. For example, instead of replacing damaged cells *in vivo*, in many cases ASCs can fuse with these damaged cells to support their survival [8, 16]. Some studies indicate that pre-differentiating the cells *in vitro* towards a desired lineage may promote more complete cellular regeneration *in vivo*. In the differentiation protocols developed to date, cultured ASCs have been induced towards various lineages by using stimulatory supplements, including growth factor and cytokine combinations, which target specific cellular pathways involved in lineage specification and differentiation. Therefore, by studying the signaling pathways involved in differentiation, optimized medium formulations and improved culture methods may be developed. Further, within the body, there is significant crosstalk between mature cells and local stem cells through paracrine-mediated effects and cell-cell contact, and these interactions are crucial for stem cell regulation [17, 18]. By studying these interactions, it may be possible to more closely simulate the natural physiology of the tissues in culture, which could contribute to improving the cellular responses and the applicability of ASCs in the field of tissue engineering.

1.2 Thesis Overview

To date, various combinations of cytokines and factors have been used in differentiation formulations for ASCs [11, 13, 19, 20]. Within the body, there is evidence that some cytokines that mediate stem cell differentiation are secreted by the neighboring populations of mature cells [17, 18]. Thus, a key focus of this thesis was to develop *in vitro* co-culture models to study the interactions between mature cells and ASCs. Moving towards this goal, the first objective was to isolate and characterize a new cell source, bovine adipose-derived stem cells (bASCs) from bovine interdigital fat, in terms of *in vitro* proliferation and multilineage (bone, cartilage, fat) differentiation with inductive media. In addition, it was determined that a threshold existed for bASCs when expanded extensively *in vitro*, after which there was degeneration of the stem cell capacity to proliferate and differentiate. This work demonstrates the importance of ensuring the cell quality in the development of cell-based therapies, and provided a well-characterized stem cell source for the co-culture models.

The second phase of the thesis involved the design of a unique co-culture environment to study the interactions between mature cells (bovine adipocytes, chondrocytes, osteoblasts) and bASCs in terms of adipogenic, chondrogenic and osteogenic potential. This research established that the mature cells had a positive influence on bASC differentiation, mediated by soluble factors through paracrine signaling. The positive results were similar for all 3 lineages studied (bone, fat, cartilage), and could possibly extend to other lineages. In terms of the specific soluble

factors, Wnt proteins and their associated signaling pathways are known to play essential roles in MSC differentiation [21, 22]. As such, the designed co-culture systems were also used to probe the role of the Wnt signaling pathways in the interactions between the mature cells and the bASCs through the use of Wnt inhibitors.

1.3 Hypotheses

1. To assess whether ASCs from bovine interdigital fat are capable of self renewal, as well as multi-lineage (fat, cartilage, bone) differentiation in culture.
2. To determine whether co-culturing multipotent bovine ASCs with mature adipocytes, chondrocytes or osteoblasts can be used as an alternative to differentiation media for the induction of lineage-specific differentiation.
3. To understand whether multilineage differentiation of bASCs in indirect co-culture with mature cells is mediated through the Wnt signaling pathways.

1.4 Research Objectives

1.4.1 Isolation and Characterization of bASC

- To develop methods for the isolation and culture bASCs from the interdigital fat pad of the hoof.

- To study the proliferation potential of bASCs by characterizing the growth kinetics and trends with serial passaging, through doubling time calculations and assessment of stem cell marker expression.
- To develop traditional culture medium formulations to stimulate multilineage (bone, cartilage, fat) differentiation of bASCs. For each lineage, compare the level of differentiation by 3 independent assays (histology and immunohistochemistry, protein expression, gene expression) at passage 2 (P2) and passage 5 (P5).

1.4.2 Co-culture Models of bASCs with Mature Cells

- To isolate mature bovine osteoblasts, chondrocytes and adipocytes.
- To develop indirect co-culture models of bASCs with the mature cells.
- To characterize the impact of co-culture on the differentiation of the bASCs without growth factor stimulation.
- To assess the effects of the Wnt inhibitory factors WIF-1 and DKK on multilineage differentiation of the bASCs in the designed co-culture systems, and to probe the involvement of the Wnt signaling pathways for each lineage.

Chapter 2

Literature Review

2.1 Tissue Engineering

Tissue engineering is a quickly developing branch of science that incorporates cell biology, molecular biology, and materials science to design functional tissue substitutes using living cells, chemical or mechanical signaling and engineered cellular micro-environments [9]. The long-term aim is to help people regenerate or reconstruct tissues, such as following traumatic injury, tumor resection or for cosmetic purposes. The prime candidates are those tissues that are not capable of extensive self regeneration after a significant amount of loss or damage, such as: cartilage, bone, and adipose tissue [1].

Three key factors being investigated in tissue engineering are: (i) the cell source, (ii) stimulation including chemical signaling and physical factors, and (iii) the extracellular matrix (ECM) environment [5]. Each of these factors needs to be highly incorporated *in vitro* or *in vivo* to mimic the natural tissue reparation processes within specific tissues or organ systems. In order to be an ideal cell source, the cell population must be readily available using minimal-invasive techniques, which have low impact on the cell donor, and the cells must be generally abundant. To avoid the requirement for long-term immunosuppression, the cells should ideally be autologous or trigger minimal immune responses *in vivo* [20, 23]. Perhaps most importantly, the

cells need to possess a high degree of plasticity in order to be able to differentiate to the appropriate cell type(s) for the particular application. The cells also require a degree of stimulation to proliferate and differentiate and typically, hormones and growth factors are commonly used *in vitro* to help in both of these processes [10]. Physical stimulation is desirable for certain lineages, such as bone and cartilage, with biomechanical signaling acting as a potent stimulator for cartilage and bone metabolism [24]. Lastly, the three-dimensional (3-D) micro-environment, including the substrate on which the cells attach, also has a great impact on stem cell behavior and fate determination. Modification of the micro-environmental properties, such as chemical composition, matrix stiffness, porosity and elasticity, can influence cell behavior on both microscopic and macroscopic levels [25].

2.2 Comparison between Embryonic Stem Cells and Mesenchymal Stem Cells

Stem cells, with rapid and theoretically infinite self renewal capacities, combined with broad differentiation potentials, are emerging as promising cell sources for tissue engineering. Self-renewal and multi-lineage differentiation potential are the minimal set of features that all stem cells have in common. These common properties are also referred to as “stemness” [26]. In general, there are two predominant types of stem cells that are being investigated for applications in connective tissue regeneration: embryonic stem cells (ESC) and mesenchymal stem cells (MSC) isolated from adult tissues.

ESCs are derived from the inner cell mass of pre-implantation embryos, and have been investigated as a cell source for tissue engineering since 1981, when they were first extracted in the murine system [27]. These cells can be stimulated to differentiate towards all cell types of the body when treated with specific stimulating factors. In addition, methods have been well-established for culturing ESCs, and studies have shown that it is possible to obtain a relatively uniform cell population *in vitro* in a relatively short period of time [27]. However, the potential for immune rejection in allogenic transplantation is a major disadvantage of these cells. Another obvious problem is the debate about "when life begins", and the ethical concern that harvesting ESCs may not respect the value of human life [28]. In addition, there are potential risks associated with tumor formation, especially teratomas or teratocarcinomas, resulting from uncontrolled cell growth and/or differentiation within the body [8].

Another option for cell-based therapies is to utilize MSCs isolated from adult tissues. MSCs normally reside in mesenchymal tissues, which are derived from the mesoderm during embryonic development [29]. These regenerative cells function in wound healing and normal tissue turnover, sustaining the tissues for their entire lifetime. Following injury, MSCs are responsible for secreting a broad array of paracrine factors that promote the recruitment of stromal cells to replace the damaged or lost tissues [18]. MSCs are typically rare in mature tissues, with the population declining with age. Emerging research has suggested that the cells are often found in perivascular niches. Similar to ESCs, MSCs are capable of differentiating into

numerous cell types given the appropriate biological cues [29]. However, one major difference is that multipotent MSCs typically have a more limited differentiation capacity than pluripotent ESCs [30]. Despite this limitation, for many applications, and in particular for connective tissue engineering, MSC are believed to be more promising as a cell source for cell-based therapies [20] .

The use of MSCs is associated with fewer ethical problems and there is the potential to avoid immune responses with autologous cell harvesting. Since 1976 [31], many studies have confirmed that with proper stimulation, MSCs, such as bone marrow-derived mesenchymal stem cells (bMSCs) and adipose-derived stem cells (ASCs), are capable of differentiation into various cell types, including bone, cartilage, fat, and muscle cells, as well as nerve-like populations, epithelial cells, and endothelial cells [32-34]. After reintroduction into the body, autologous cells would significantly reduce the potential for immune rejection, and there is also evidence to suggest that allogenic MSCs have an "immuno-privileged" status, downregulating T-cell activation and mediating the inflammatory response [22, 35]. Eliminating the requirement for long-term immunosuppressive therapy would represent a significant advantage in terms of cost and quality of life.

However, MSCs also have drawbacks that currently limit the broad-scale clinical application of these unique regenerative cells. Unlike ESCs, which are theoretically capable of infinite replication, MSCs tend to lose their proliferation and differentiation capacities after long term *in vitro* subculture [36, 37]. This degeneration is undesirable and often unpredictable, and may limit their use,

especially for older patients who have naturally diminished MSC populations. In addition, MSC isolation typically requires *in vitro* culture to eliminate contaminating cell types, and it is difficult to guarantee the homogeneity of the extracted cell population [12]. Despite their noted weaknesses, MSCs are a viable potential cell source for tissue engineering applications.

2.3 Adipose-derived Stem Cells

While bone marrow has been investigated for a long time as the major source of MSCs, Zuk *et al*. were the first to characterize MSCs in human adipose tissue as a multipotent cell source [12], which were later termed "adipose-derived stem cells" (ASCs). As agreed upon by the International Fat Applied Technology Society (iFATS), the term ASC encompasses both the multipotent stem cell population, as well as the more committed adipogenic progenitors, which are found in fat (sometimes referred to as preadipocytes). ASCs are similar in size and morphology to fibroblasts, and are difficult to identify within histological sections. More specifically, ASCs have long, spindle cell bodies and contain a large, round nucleus, with a prominent nucleolus. These cells are widely dispersed throughout mature adipose tissue, embedded within the basement membrane of the adipose ECM. ASCs are usually surrounded by a network of reticular fibrils, collagen IV and laminin. [38]

As a stem cell source, fat is ideal in many aspects. In particular, ASCs can be easily harvested from the human body by non-invasive liposuction or biopsy methods. The morphological differences between mature adipocytes and ASCs are favorable

for ASC isolation *in vitro*. Adipocytes have a high volume to mass ratio and are easily separated from the stromal vascular fraction (SVF), including the ASCs, based on their density and high intracellular lipid content. The proliferation and differentiation potentials of ASCs are comparable to bone marrow stem cells (bMSCs) [32]. In addition, some studies have suggested that the potential for allogenic immune response is even lower for ASCs than for MSCs from other tissue sites. Due to the large quantity of cells required for most tissue engineering applications, it is ideal to have a large primary cell population within a single harvest. For ASCs, approximately 2×10^6 stem cells can be isolated from 5 mL of well-minced adipose tissue using collagenase digestion, which is order of magnitude higher in terms of yield when compared to other MSC isolations. In fact, up to 2 % of the cells within the SVF of adipose tissue are capable of multilineage differentiation, relative to 0.002 % within the SVF of bone marrow [39]. The higher cell density facilitates less stringent culture conditions, as well as reduced time and costs before transplantation. There have been several recent human clinical trials where ASCs have been applied in cell-based therapies using a closed cell extraction system within the operating room, bypassing the requirement for *ex vivo* culturing [40, 41].

2.3.1 The Cellular Composition of the Adipose Stromal Vascular Fraction

The standard method for ASC isolation is to use collagenase to degrade the collagen fiber framework and release all cell types from the adipose ECM. As discussed, adipocytes, which are the most abundant cell type in adipose tissue, are

easy to separate from the other cell types in the tissue by gravity separation. Before isolation, large, visible blood vessels are removed as much as possible to reduce contaminating blood cell populations. However, some cells associated with the vascular and neural systems will remain within the processed tissues. Thus, the SVF may include mast cells, erythrocytes, endothelial cells, pericytes (Relatively undifferentiated cell serves to support small blood vessels and be able to differentiate into fibroblast, smooth muscle cell or macrophage [42]), fibroblasts and ASCs [43]. Compared to ASCs, the doubling time of the contaminating populations is typically longer, and their cell adhesion on tissue culture polystyrene (TCPS) is lower. Thus, stem cell selection can occur naturally with *in vitro* passaging. Macrophages, leukocytes and blood cells are non-adherent, and are eliminated after the first medium change. Thus, a relatively homogeneous population remains after the first passage. Zuk *et al.* [12] analyzed cell isolates from human adipose tissue using flow cytometry in order to determine the composition of the SVF. Their results suggested that the cultured SVF incorporated a relatively homogenous population of mesodermal or mesenchymal cells, with low contamination by endothelial cells, pericytes, and smooth muscle cells.

2.3.2 ASC Immunophenotype

MSCs display specific patterns of expression of cell surface markers that can be used to help identify them within an isolated primary cell population. In general, ASCs have been shown to be very similar to MSCs derived from the bone marrow in

terms of both morphology and immunophenotype [44]. The characteristic markers expressed within the ASC population are summarized in Table 2.1. Most of these proteins are also commonly associated with bMSCs, with the exception of CD34, Sca-1 and CD49d. While Sca-1, aldehyde dehydrogenase (ALDH) and the ATP-binding cassette superfamily G member 2 (ABCG2) are some of the most recognized hematopoietic stem cell (HSC) markers [45], some studies have indicated that they are also expressed by specific regenerative sub-populations of ASCs [14]. Several research groups have explored the patterns of surface protein expression of ASCs, yielding conflicting results with certain markers, such as CD106. These differences may be due to variations in the methods of ASC isolation and culture, or due to intrinsic donor variation, which will be discussed in section 2.4.3. There have also been large differences documented between species, as well as with ASCs isolated from different depots (i.e. subcutaneous versus visceral fat).

The cell immunophenotype also changes with *in vitro* passaging. Among all the markers that ASCs express, several stromal cell–associated markers (CD13, CD29, CD44, CD63, CD73, CD90, CD166), which initially have low expression on primary isolated SVF cells, increase significantly with passaging and time in culture. However, the stem cell–associated marker CD34 is most highly expressed in the primary isolates, and reduces with increasing passages. The HSC surface markers ALDH and ABCG2 are expressed by both primary isolated SVF and cultured ASCs at low levels. Some endothelial cell–associated markers including CD31, CD144 or VE-cadherin, vascular endothelial growth factor receptor 2 (flk-2), and von Willebrand factor (vWF)

are expressed by subpopulations within the SVF, and remain detectable at low levels with serial passaging [9, 23].

Table 2.1 Immunophenotype of ASCs

Adhesion Molecules	Tetraspan protein (CD9)	[10, 13]
	Integrin β1 (CD29)	
	Integrin α4 (CD49d)	
	Endoglin (CD105)	
	Vascular cell adhesion molecule (VCAM; CD106)	
	Intracellular adhesion molecule 1 (ICAM-1; CD54)	
	Activated leukocyte cell adhesion molecule (ALCAM; CD166)	
Receptors	Hyaluronate receptor (CD44)	
	Transferrin receptor (CD71)	
	Insulin receptor	
	Glucocorticoid receptor	
	Triiodothyronine (T3) receptor	
	Retinoic acid receptor	
Surface enzymes	Neutral endopeptidase (CD10)	
	Aminopeptidase (CD13)	
	Ecto 5' nucleotidase (CD73)	
	Aldehyde dehydrogenase (ALDH)	
ECM proteins and Glycoproteins	Collagen I	
	Collagen III	
	Osteopontin	
	Osteonectin	
	Thy-1 (CD90)	
	MUC-18 (CD146)	
Complementary proteins	Decay accelerating factor (CD55)	
	Complement protein (CD59)	
Cytoskeletal Proteins	Intracellular α-smooth muscle actin	
	Vimentin	
Other	HLA-ABC (Major histocompatibility complex class I antigen)	
	Stem cell antigen-1 (Sca-1)	
	Sialomucin protein (CD34)	
	ATP-binding cassette superfamily G member 2 (ABCG2)	

2.4 Proliferation and Differentiation Potential of ASCs

2.4.1 *In vitro* Self Renewal and Subcultivation

One of the defining characteristics of stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended time periods [30]. The absolute self-renewal potential of MSCs remains an open question, due to the variety of different harvesting and isolation approaches, and various methods of characterization. As a similar, but more extensively-studied type of cell, methods have been developed for the long term subcultivation of bMSCs *in vitro* in the presence of serum [46]. Similarly, ASCs are normally cultured in growth medium containing 10% bovine serum, which contains factors that are required for cell adhesion and proliferation [15]. The *in vitro* doubling time for ASCs has been measured to be within 1-4 days, depending on the specific cell source, any additional supplementary factors, and the number of passages [9].

In serum-free culture medium, ASCs display low levels of attachment to treated TCPS. The cells do not spread or proliferate well under these conditions, indicating the inhospitality of the serum-free environment. Due to the complex and variable composition of fetal bovine serum (FBS), which is commonly used in these cell cultures, supplemented growth factors, such as insulin-like growth factor 1 (IGF1) and fibroblast growth factor 2 (FGF2), can be used to help stimulate proliferation [47-49]. Parker et al. demonstrated that human ASCs have a doubling time of 5.79 days in serum free medium with the presence of additional growth factors. With the

addition of very low serum concentrations (0.5% human serum), a significant decrease in the doubling time (1.86 days) was observed [46].

Studies have shown that ASCs are capable of maintaining their proliferative potential during extended culture periods (up to 15 passages or 165 days) without an observable reduction in doubling time [12]. However, Wall [37] found that although human ASCs are capable of proliferating up to 10 passages, the doubling time significantly increases after passage 6. Similar results have been observed in bMSCs [50] suggesting that both ASCs and bMSCs might experience a degeneration in proliferation potential after extensive *in vitro* subcultivation. Therefore, degradation of stem cell properties may be common for MSCs after extensive in vitro subcultivation.

2.4.2 Differentiation Potential of Cultured ASCs

The multi-lineage differentiation potential of MSC populations derived from a variety of different species has been extensively studied *in vitro* since their first discovery in the 1960s [51]. These studies demonstrated that bMSCs from human, canine, rabbit, rat and mouse have the capacity to differentiate into mature cell types associated with mesodermally-derived tissues and organs, such as bone, cartilage, tendon, muscle, and adipose tissue [52, 53]. Similarly, the differentiation potential of ASCs from various species has been investigated, as summarized in Table 2.2. Although all lineages have not been investigated in most of the species, it is commonly accepted that ASCs are capable of multi-lineage differentiation. There is

evidence to suggest that subpopulations of ASCs can not only differentiate along the classic mesenchymal lineages (adipogenesis, chondrogenesis, osteogenesis, and myogenesis), but can also be stimulated to differentiate into non-mesenchymal cell types, such as into cells associated with the vascular or neural systems (Table 2.2) [33, 54].

Several models have been developed to help understand the MSC differentiation process. In 1994, Caplan [55] suggested that self-renewing MSCs initially proliferate to produce a large pool of regenerative cells. These cells then commit to differentiate along a specific lineage pathway, and ultimately become a terminally differentiated mature cell of that lineage. In this model, the MSCs produce lineage-restricted progenitors, which are not able to change pathways after commitment. In 1998, Aubin [56] suggested a different hypothesis, based on the observation of trans-differentiation between adipogenic-osteogenic and osteogenic-chondrogenic lineages. She proposed that expanding multipotent MSCs gradually lose their differentiation potential to become more restricted tri- or bi-potential progenitors, and finally shift into a default osteogenic lineage. In 2009, Sarugaser et al. [57] suggested the final lineage would instead be fibroblastic, stating that MSCs eventually lose their osteogenic differentiation capacity after extensive *in vitro* subcultivation. These hypotheses provide different ideas of how stem cells might degenerate in culture, and there is evidence to support each model in the literature [37, 57].

Table 2.2 Multilineage ASC differentiation potential investigated to date, as reported in the literature

Species	Lineages Differentiation	References
Human	Fat, Cartilage, Bone, Skeletal muscle, Smooth muscle, Neuron-like, Glia, Cardiomyocytes, Epithelial tissue, Endothelium.	[12, 32, 33, 58]
Murine	Fat , Cartilage, Bone, Neuron-like, Epithelial tissue	[14, 59-61]
Rabbit	Fat , Cartilage, Bone	[62]
Canine	Fat , Cartilage, Bone, Skeletal muscle	[19, 63]
Rat	Fat , Cartilage, Bone, Neuron-like, Epithelial tissue	[62, 64, 65]
Porcine	Fat , Cartilage, Bone	[66]
Bovine	Fat , Cartilage	[67, 68]
Equine	Cartilage, Bone	[69]

2.4.3 Variability Between Donors, Depot, and Species

In humans, adipose tissue is located beneath the skin (subcutaneous fat), around internal organs (visceral fat), in the bone marrow (yellow bone marrow) and in the breast. These locations each contain a substantial quantity of adipose tissue, and are thus referred to as “adipose depots”. To date, most human ASC studies have used cells isolated from the subcutaneous or visceral depots [15, 29, 70]. Among all adipose tissue throughout the body, the cells from different depots display different metabolic properties. For example, visceral fat is more strongly linked to the development of insulin resistance than subcutaneous fat [71]. The ASCs in the subcutaneous and visceral depots are more strongly correlated to the specific levels of sex hormones, as well as overall body metabolism, while intramuscular adipose tissue and the structural fat pads in the limbs are more insensitive to these effects [38].

There may exist regional differences in adipose tissue with regards to ASC proliferation, even if there are no observable differences in *in vitro* differentiation

capacity [72]. In a canine model, cells derived from subcutaneous fat tended to become confluent in 5–6 days, as compared to ASCs derived from omental fat, which took 11–12 days to reach confluency when seeded under identical conditions on TCPS. However, no significant variations in the differentiation capacity were observed between the ASCs isolated from the two depots [63].

ASCs derived from individual patients also display significant differences in the measured *in vitro* proliferation and differentiation capacities. In one study, only 66% of the ASC surface markers in the gene expression profile measured using flow cytometry were in concordance between various donors [73], which could translate into significant variability in the effectiveness of cell-based therapies. Age and sex can also greatly influence the observed ASC behavior. Increasing age tends to be associated with a decrease in the general proliferative activity of ASCs [72]. Interestingly, ASCs isolated from pregnant women showed greater proliferation potential and more consistent potency, when compared to cells isolated from non-pregnant women [74]. Various methods of tissue harvesting may also result in cellular variability. Compared to bulk tissue explants obtained from abdomoniplasty or breast reduction surgery, liposuction techniques can cause a sustained and concentrated tissue insult, resulting in inconsistency of the cell population composition [75, 76].

In addition to these uncertainties within a single species, variations between species are also present. Although comparisons amongst species are difficult to make due to variations in the assessment methods used in literature, Ni et al. [62]

demonstrated that between human, rabbit and rat ASCs, rabbit ASCs showed the lowest osteogenic potential, while human and rat ASCs could be induced to have a similar level of response in culture using a specific inductive culture medium. However, in that study, all three species displayed a similar adipogenic capacity.

2.5 Multilineage (Adipogenic, Chondrogenic, Osteogenic) Differentiation and Related Transcriptional Control

2.5.1 Adipogenesis

Adipogenic media employed in the literature have various formulations. The most common components include insulin, a glucocorticoid (GC), such as dexamethasone (DEX) or hydrocortisone, and isobutylmethylxanthine (IBMX). Other supplements selectively used also include the insulin-sensitizing thiazolidinediones (TZDs), triiodothyronine (T3), biotin and pantothenate. These supplements are closely associated with lipolysis and lipogenesis of adipose tissue [77, 78]. For example, increasing levels of the secondary messenger cyclic adenosine monophosphate (cAMP) are favorable for lipogenesis. Therefore, IBMX, a phosphodiesterase inhibitor that protects cAMP, is favorable for adipogenesis. TZDs are cell-surface ligands of the peroxisome proliferator-activated receptor-γ (PPARγ) [79]. As PPARγ and CCAAT/enhancer-binding protein α (CEBP/α) have been identified as the master co-regulators of adipogenic differentiation, TZDs, including troglitazone and rosiglitazone, are promoters of *in vitro* adipogenesis [80]

ASCs from different species and depots demonstrate variable responses to different formulations and concentrations of each supplement. For example, a stronger adipogenic response is observed for bovine ASCs when the formulation used by Hirai et al. [67] is used, which incorporates 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), Bovine insulin and troglitasone, rather than the medium developed by Ohsaki et al. [68], which contains biotin, sodium octanate, bovine serum albumin (BSA) and insulin – transferrin – selenium (ITS). Although ASCs themselves do not proliferate well in the absence of serum, as previously discussed, the short-term serum starvation may be necessary for some species to induce the adipogenic differentiation pathways [63, 81], as serum contains a number of anti-adipogenic mitogenic factors.

Transcriptional control of adipogenesis

Adipogenic differentiation is closely associated with the C/EBP protein family and PPARγ [81]. During induction, ASCs are stimulated by the supplements mentioned in the previous section. C/EBPβ and C/EBPδ are then expressed endogenously by the cells [82]. These two genes activate PPARγ by a direct transcriptional effect through C/EBP binding sites in the PPARγ promoter. The elevated PPARγ expression then induces the expression of C/EBPα. This is evidenced by the observation that PPARγ knock-out ESCs are unable to differentiate and have a low level of CEBPα expression, despite having normal levels of CEBPβ and CEBPδ [83]. The increasing level of CEBPα also raises the level of PPARγ in a positive feedback loop. Another parallel initiator is ADD1/SREBP1c, which is

thought to work as a ligand upregulator of PPARγ [84]. C/EBPα and PPARγ then work as co-regulators to activate and upregulate differentiation related gene expression, such as the fatty acid binding protein aP2, glycerol-3-phosphate dehydrogenase (GPDH), lipoprotein lipase (LPL), leptin, adiponectin and glucose transporter-4 (Glut4) (See Figure 2.1), potentially mediated through a shared promoter with the co-regulators [79, 85]. In addition, there is also evidence that ADD-1/SREBP-1 directly activates LPL expression through another pathway [86].

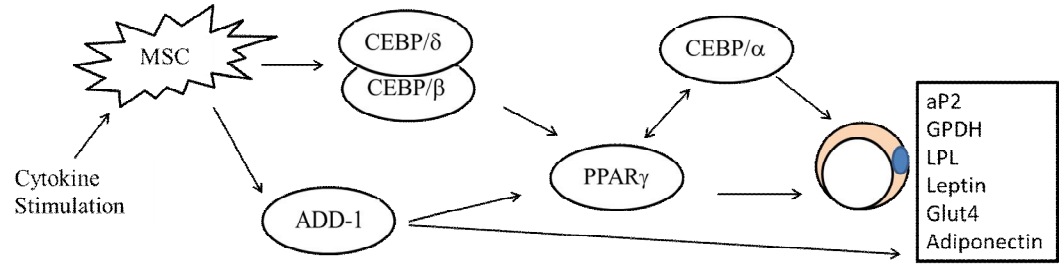

Figure 2.1 Transcriptional control illustration of adipogenic differentiation.

2.5.2 Chondrogenesis

Native cartilage does not have a penetrating blood supply, and the nutrient source for mature chondrocytes is mostly the synovial fluid. Many chondrogenic ASC studies have used serum-free media formulations for *in vitro* conditioning in order to mimic the native environment, as well as to avoid variability associated with the inconsistencies observed in serum composition. However, in most studies to date, serum has been essential for the optimal proliferation and chondrogenesis of ASCs [87]. For chondrogenesis, a 3-D network of cells is advantageous in order to obtain the natural rounded morphology of mature chondrocytes. In the literature, 3-D

cultures have been achieved by various means, such as by micromass culturing [63], centrifugation [88] or through encapsulation within biomaterial scaffolds [89].

Growth factors, such as the transforming growth factor (TGF) super-family and bone morphogenic protein (BMP) protein family are commonly used to induce chondrogenic differentiation. IGF is also effective, when used in combination with members of the previous growth factor families [90, 91]. In addition, combinations of multiple growth factors [90, 92] or increased dosages of these factors [91, 93] have been used to more precisely tune chondrogenic differentiation.

Ascorbic acid is commonly used in osteogenic and chondrogenic media as a stimulator of collagen synthesis [94]. However, ascorbic acid is readily decomposed by oxidation in aqueous media, causing a reduction in activity (half-life of approximately 12 hours). As an alternative, ascorbate-2-phosphate (A2P) is a commonly used as a substitute, as it can be effectively maintained in aqueous medium for days [95]. The glucocorticoid DEX also promotes chondrognesis [96], by supporting cell viability through mediating cell aggregation, and delaying the appearance of collagen type X, which is associated with hypotrophy [97].

Transcriptional control of chondrogenesis

Chondrogenesis is a multi-step process involving proliferation, condensation, early stage differentiation and finally, maturation. The proliferation stage can be shortened using 3-D culturing methods, as the structure of the cell pellet helps in ASC condensation.

Sox9 is the master gene involved in the initiation of chondrogenesis, and its expression is required for the commitment of osteochondro-progenitors, chondrogenic mesenchymal condensation and proper chondrocyte proliferation, differentiation, maturation and hypertrophic conversion during embryonic development [98]. Further, Sox9 controls the proliferation and differentiation of non-hypertrophic chondrocytes, and acts as a negative regulator of chondrocyte hypertrophy. This activity is regulated by the phosphorylation of Sox9 by protein kinase A (PKA), a downstream intracellular signaling molecule of the parathyroid hormone-related peptide (PTHrP)/PTHrP receptor [99]. Sox5 and Sox6, which belong to the same protein family as Sox9, can bind to Sox9 and enhance its function *in vitro* [100]. Despite Sox5 and Sox6 are not able to induce chondrogenesis by themselves, they are required for the expression of components of the cartilage ECM, such as collagen IX, aggrecan, and cartilage link protein [101]. Sox9 gene expression is also required for the expression of signature cartilage markers, including collagen XI, prior to matrix deposition in the cartilage [100]. Over-expression of Sox9, Sox5, and Sox6 in cultured cells can induce the expression of collagen II and proteoglycans (PGs), which are the major constituents of the cartilage ECM [100, 101].

Members of TGF-β superfamily (i.e. TGF-β1, TGF-β2, TGF-β3, and BMP-6) have all proven to readily induce the sequential expression of chondrocytic markers [102]. Several studies have reported that FGF, IGF-I, human cartilage glycoprotein 39, transient receptor potential vanilloid 4 (TRPV4), retinoic acid receptor (RAR) agonists, and Src inhibitor increase Sox9 expression [103-108]. CCAAT-binding

factor, Sp1, CREB, Sonic hedgehog, and hypoxia-inducible factor 1a directly transactivate the Sox9 proximal promoter [109-113]. Other transcription factors including Pax1, Pax9, Nkx3.1, Nkx3.2, and Barx2 control the level of Sox9 expression [114]. The stability and degradation of Sox9 protein are determined by the ubiquitin-proteosome pathway [115] In chondrogenesis, Smad3, a downstream signaling molecule of transforming growth factor-β, (TGF−β) stimulates the Sox9-dependent transcriptional activation by modulating the interaction between Sox9 and CBP/p300 [116]. Overall, control of chondrocyte-specific Sox9 expression is a complex process that is not yet fully characterized.

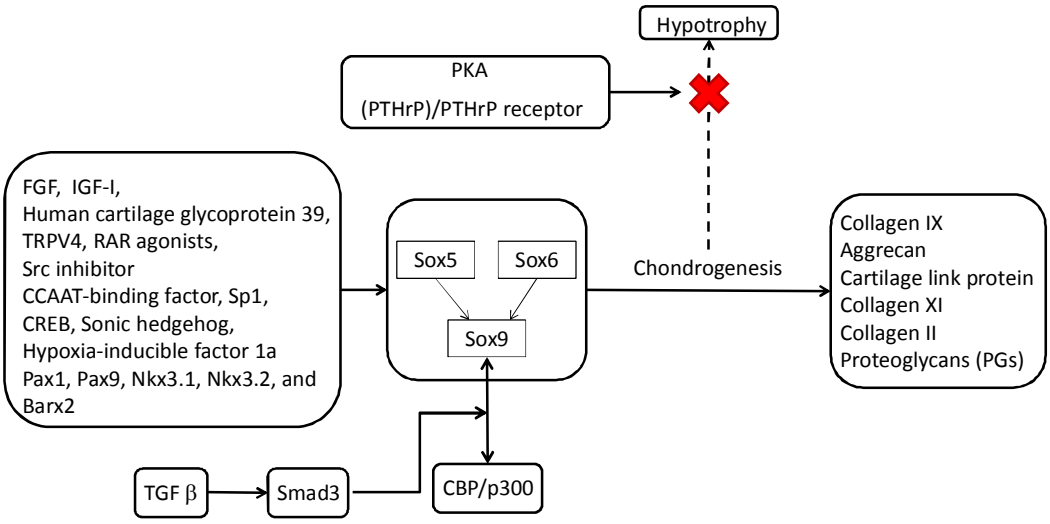

Figure 2.2 Transcriptional control illustration of chondrogenic differentiation.

2.5.3 Osteogenesis

The osteogenic differentiation of ASCs from multiple species and depots has been investigated using a variety of inductive media formulations. As with adipogenesis and chondrogenesis, osteogenesis is a complex process that is associated

with key changes in gene and protein expression. In general, MSCs express low levels of factors from each of the lineages, which cross-regulate one another to maintain the undifferentiated state. During osteogenic differentiation, the expression of the controlling transcription factors associated with the osteogenic lineage are upregulated, while those involved in adipogenesis and chondrogenesis are simulatenously repressed.

Alkaline phosphatase (ALP) is a "bone enzyme" which is required for bone synthesis. In the presence of ALP, inorganic phosphate can be released from a phosphate donor, such as β-glycerophosphate or possibly A2P, in the medium in order to form calcium phosphate, which is deposited in the maturing ECM [117]. The enzyme also effectively releases ascorbic acid from A2P, in a process which helps collagen I synthesis in the bone ECM.

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal cortex. In particular, DEX has complex stimulatory and inhibitory effects on skeletal metabolism and bone synthesis [118], and is commonly used to promote MSC osteogenic differentiation at low concentrations (e.g. 10^{-7} M). At physiological concentrations, DEX regulates the mitogen activated protein kinase (MAPK) signaling pathway through a GC-receptor mediated transcriptional mechanism [119]. MAPK has been proven to play an important role in stress responsive signaling in cells. The C-Jun N-terminal kinases/stress-activated protein kinase (JNK) pathway is one of three members of the MAPK superfamily. The activation of the JNK pathway

promotes skeletal development, and will be discussed in more detail in section 2.7.2. [120]

Transcriptional control of osteogenesis

Cbfa1/Runx2, a key transcription factor involved in osteogenesis, is composed of α and β subunits. Unit α is the "runt domain", which binds with DNA to transcriptionally-activate target genes. Unit β stimulates the affinity of the α unit with DNA, without direct binding. Runx2 is a key transcriptional factor and initial switch for osteogenic differentiation of MSCs, and skeletal morphogenesis [121]. Runx2 interacts with a variety of co-regulating transcription factors and signaling proteins to determine whether cells are going towards osteogenic lineage [121].

BMP2 is a growth factor which is necessary for osteogenic commitment and differentiation [122]. Activation of BMP receptors initiates the BMP signaling pathway through the receptor-regulated Smad group (R-Smad) and MAPK-signaling pathways, which respond to exterior physical stimulation. The common Smad group (Co-Smad) in the cytoplasm forms a complex with phosphorylated R-Smad, which enters the nucleus to trigger Runx2 gene expression (Figure 2.2). Another Runx2 activation pathway is triggered by excess β-catenin in the nucleus through the Wnt canonical pathway, which is explained in more detail in Section 2.6.

Runx2 is one of the earliest specific markers of osteogenesis, and a key activator of osteogenic differentiation via both the endochondral and intramembrane pathways [123]. As discussed, Runx2 interacts with variety of co-regulating transcription factors and signaling proteins, and these interactions guide differentiating MSCs

through the early stages of osteogenic differentiation [124, 125]. The knockdown of Runx2 in osteoblasts results in trans-differentiation towards the adipogenic and chondrogenic lineages. Osterix, a gene controlled by Runx2, becomes dominant in the later stages of osteogenesis and is involved in osteoblast maturation. Both Runx2 and osterix control the expression of the main structural proteins of the bone ECM, such as collagen I, osteocalcin (OCN), osteopontin, bone sialoprotein, and fibronectin (see Figure 2.3) [125]. These two genes also upregulate the levels of ALP in the differentiation process. Only OCN is regarded as a highly specific marker of mature osteoblasts, which is expressed in the later stages of osteogenesis and the initial stages of bone ECM production [125].

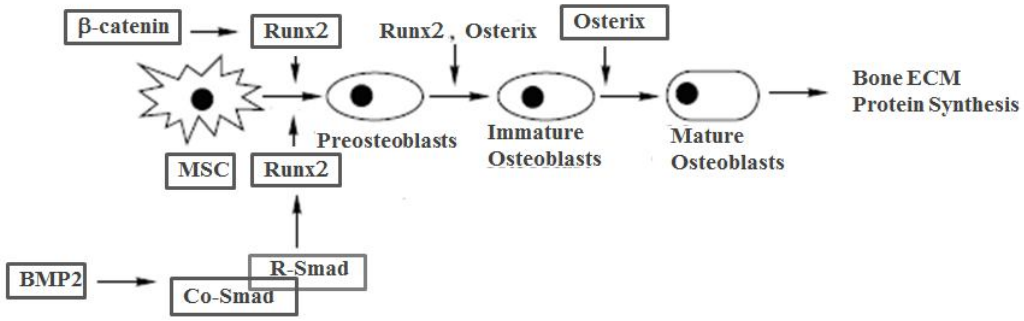

Figure 2.3 Transcriptional control of osteogenic differentiation.

2.6 The Wnt Signaling Pathways

2.6.1 Overview of Wnt Proteins:

Wnt proteins are a family of secreted glycoproteins that function in autocrine and paracrine signalling to regulate adult tissue homeostasis and remodeling [126, 127]. To date, 19 Wnt proteins have been identified in human system and occur throughout

the animal kingdom [126]. Wnt proteins are highly involved in embryonic development and have also been found to play important roles in cell polarity, adhesion, migration and cell fate (including proliferation, differentiation, and apoptosis) [128, 129]. Recent studies have indicated that Wnt signaling plays an important role during lineage specification and differentiation of MSCs, including ASCs, although the process is not yet fully understood [22, 129].

2.6.2 The Canonical and Non-canonical Pathways:

To date, two main categories of Wnt signaling pathways have been identified. The first, which is termed the Wnt canonical (Wnt/β-catenin) pathway, is also the better characterized signaling pathway.

2.6.2.1 Wnt canonical pathway

In the canonical pathway, which involve the cell surface frizzled (Fz) receptors, the Wnt protein binds with the Fz-LRP5/6 receptor complex on the cell surface (Figure 2.4). The cytoplasmic protein Dishevelled (DVL) is then activated, which interacts with the degradation compartment, including adenomatosis polyposis coli (APC), Axin and glycogen synthase kinase 3 (GSK3), by binding with the Fz-LRP5/6 complex on the internal side of the cell membrane, inhibiting β-catenin degradation. In the absence of canonical Wnt signaling, β-catenin in the cytoplasm binds with APC, Axin, casein kinase 1 (CK1) and GSK3 to form a complex. This complex induces the phosphorylation of β-catenin, which stimulates its degradation. Therefore,

the amount of β-catenin that enters the nucleus is significantly reduced in the absence of Wnt signaling. [22, 128-130]

The canonical Wnt signaling complex results in reduced degradation and an increase in free β-catenin in the cytoplasm, which allows a larger amount of β-catenin to transfer across the nuclear membrane and accumulate inside the nucleus [128, 130]. Within the nucleus, the β-catenin can activate target genes, in association with T-cell factor (TCF) and lymphoid enhancer-binding protein transcription factor (LEF) (See Figure 2.4). The activation of these genes commonly triggers the pathways involved in cell fate determination, as discussed in Section 2.5. DKK1 is a Wnt inhibitor that specifically targets the Wnt canonical pathway, which will be discussed in detail in Section 2.6.3.

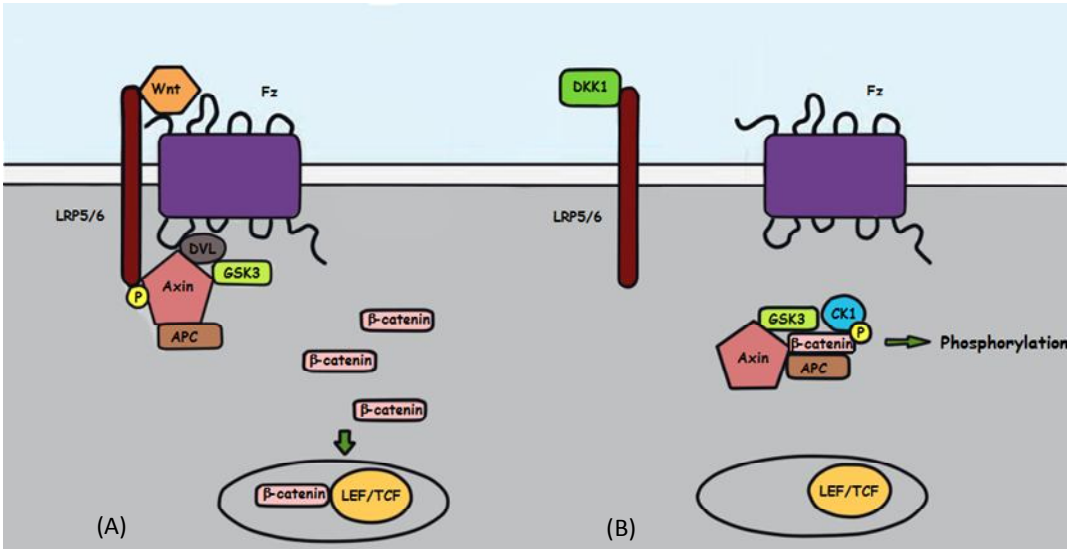

Figure 2.4 Wnt canonical pathway (A) and its inhibition (B).

2.6.2.2 Wnt non-canonical pathways

The second category of Wnt signaling pathways is referred to as the non-canonical pathway. This type of pathway has not been as extensively characterized, and may involve multiple receptors on the cell surface. To date, there are two well summarized mechanisms that have been identified in Wnt non-canonical signaling: the Dishevelled (DVL) dependent pathway and the Ca^{2+} dependent pathway [22, 128-130] (Figure 2.5).

Dishevelled-dependent pathway

In the non-canonical Wnt signaling pathway, Fz binds with Wnt proteins in the absence of LRP5/6 [128]. The Wnt/Fz complex triggers DVL to initiate the DVL-dependent pathway, which is also referred to as the planar cell polarity (PCP) pathway. In *Drosophila*, PCP signaling is pivotal for establishing cell polarity in the orientation of wing hairs, the organization of multifaceted eye structures, the orientation of hair cells in the inner ear, and the orientation of bristles and other asymmetric polar structures [131].

Activation of the PCP pathway leads to intracellular-localized activation of the Rho-family GTPases (RHOA) and JNK pathways. JNK is also known as stress activated MAP kinase. In the Wnt DVL-dependent non-canonical pathway, JNK is one of the downstream signaling pathways shown in Figure 2.5. This specific pathway can be activated through exposure to physical stresses and inflammatory signals, and may be responsible for stress-induced apoptosis [132]. It also plays an important role in epithelial planar polarity during embryonic development [133]. A critical role of

JNK appears to be in the regulation of activator protein-1 (AP-1) transcription activity, which is involved in cell polarity during gastrulation of chondrogenesis [134, 135]. Inhibition of JNK activity using the small-molecule inhibitor SP600125, or RNA-interference-mediated knockdown of JNK2, strongly enhances chondrogenesis in terms of sGAG production and chondrogenic marker gene expression [136]. Compressive force promotes Sox9, type II collagen and aggrecan expression and results in chondrogenesis in mouse embryonic limb bud mesenchymal cells [137]. The JNK pathway is also closely associated with osteogenic differentiation [119, 138]. Studies have shown that alendronate, a bisphosphonate drug, promotes osteogenesis of bone marrow MSCs via the JNK signaling pathway [138].

RHOA is another downstream signaling pathway of DVL activation (Figure 2.5) that mediates cell contraction, local adhesion, and cell motility, which are all considered to be important in cell differentiation [139]. Studies have shown that RHOA may be involved in cytoskeletal reorganization during differentiation [140] and may influence cell fate in a density-dependent manner [141]. The inhibition of the RHOA pathway may result in a decrease in the transcript levels of collagen II and aggrecan, as well as reduced activity of Sox9-responsive reporter genes. Sox5 and 6 transcriptional levels also decrease upon inhibition of RHOA. [142, 143]

Ca^{2+}-dependent pathway

Activation of the Wnt Ca^{2+}-dependent pathway occurs through the interaction of Wnts with Ror2 (may also include the co-receptor Fz). This interaction causes an increase in intracellular calcium, which subsequently activates the

calcium/calmodulin-dependent protein kinase II (CAMKII) and protein kinase C (PKC). CAMKII mediates serine/threonine protein kinase (NLK) and phosphorylates the TCF/LEF transcriptional factors to inhibit the Wnt canonical pathway. The downstream elements of PKC are less well studied, but the effects may be associated with cell adhesion and/or migration during gastrulation [144].

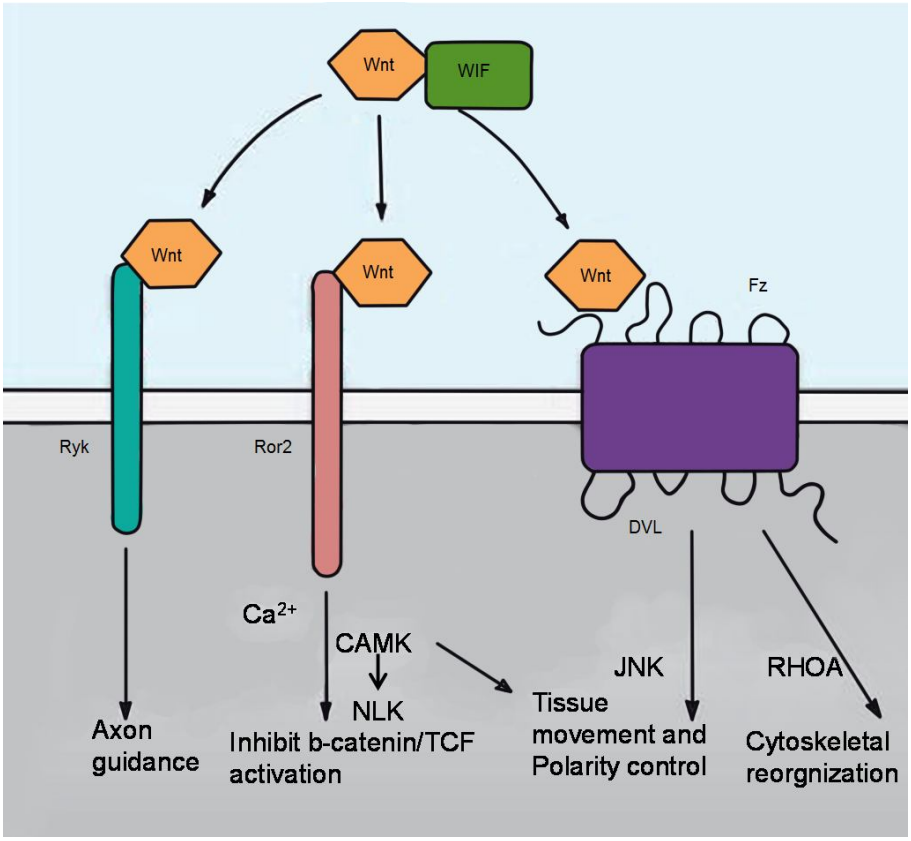

Figure 2.5 Wnt non-canonical pathway and its inhibitor.

Wnt proteins have also been shown to interact with the receptor tyrosine kinase (Ryk), mediating axon guidance on the contralateral side of the corpus callosum [145].

2.6.3 Wnt Inhibitors:

Known antagonists of Wnt signaling include the Dickkopf proteins (DKK1-4, and Soggy (DKKL-1)) [146], secreted Frizzled-related proteins (sFRP 1-5) [147], and Wnt Inhibitory Factor-1 (WIF-1) [148]. Two different inhibition mechanisms have been identified.

Recombinant DKK and DKKL bind with LRP5/6 (Figure 2.4B) and the transmembrane protein Kremen 1/2, which leads to LRP5/6 membrane depletion. In addition, Wnt proteins can also bind with LRP5/6 alone to prevent the formation of the LRP5/6 - Fz complex, inhibiting the Wnt canonical pathway. Alternatively, recombinant WIF and sFRP can act as free ligands in the extracellular space. Free Wnt proteins in the medium are captured by these ligands, which therefore limit the availability of Wnt proteins to bind to cell surface receptors, serving to inhibit both the canonical and non-canonical pathways. [22, 128, 144, 149, 150]

2.6.4 The Role of Wnt Proteins in MSC Self-Regulation and Differentiation:

There is evidence, primarily from mouse ESC research, that Wnt signaling components are involved in ESC self regulation. The activation of Wnt signaling promotes the undifferentiated phenotype of mouse ESCs by genetically eliminating the function of the negative regulator APC [151]. Wnt signaling maintains ESC pluripotency; however, contradicting results have also been shown. In Pereira *et al.*'s work, the Wnt-controlled transcription factor, TCF3, repressed self renewal activity

through the nanog gene [152]. Further, Oct-4, another gene associated with self renewal, is associated with increased β-catenin transcriptional activity [153].

The Wnt signaling pathways play critical roles during lineage specification and differentiation of ASCs both *in vitro* and *in vivo*. Figure 2.6 summarizes some of the key interactions (both positive and negative) associated with the Wnt pathways that have been identified to date that play a role in mediating MSC differentiation.

Wnt canonical pathway

The Wnt canonical pathway directly regulates the expression of Sox9 in osteo-chondro progenitor cells. While the Wnt canonical pathway is active, Sox9 expression is reduced, and genetic transcription of Runx2 is favored, thereby committing ASCs towards the osteogenic fate. Alternatively, when the Wnt canonical pathway is blocked, the Runx2 gene is suppressed, and Sox9 expression occurs at high levels, pushing the cells towards non-hypotrophic chondrogenesis. The canonical pathway also promotes the initiation of chondrogenesis via crosstalk with the TGF-signaling pathways [21].

Similar to the osteogeneic /chondrogenic relationship, Wnt proteins can also act as a switch between adipogenesis and osteogenesis. Recombinant GSK3β blocks adipogenic differentiation, indicating that excessive β-catenin in the nucleus inhibits adipogenic initiation. Overall, Wnt signaling suppresses adipogenesis by blocking the induction of PPARγ and CEBP/α. Conversely, the disruption of exogenous and endogenous Wnt signaling can result in spontaneous adipogenic differentiation in cultured MSCs [154].

Wnt signaling is also associated with myogenesis during embryonic development, as well as postnatal muscle regeneration. Canonical Wnt signaling can control the balance between myogenesis and adipogenesis. Through Wnt inhibition, adipogenic gene expression is upregulated in favor of the myogenic differentiation process. [22]

Non-canonical pathways

The effect of the non-canonical pathways in stem cell differentiation has been less well characterized, as it involves multiple receptors and downstream signaling pathways. However, every lineage-specific differentiation process involves morphological changes, cell migration (associated with tissue polarity control) and changes in cell metabolism. These activities involve cytoskeletal reorganization, including actin microtubule reconstruction in the cytoplasm, which may require signaling through the non-canonical Wnt pathways.

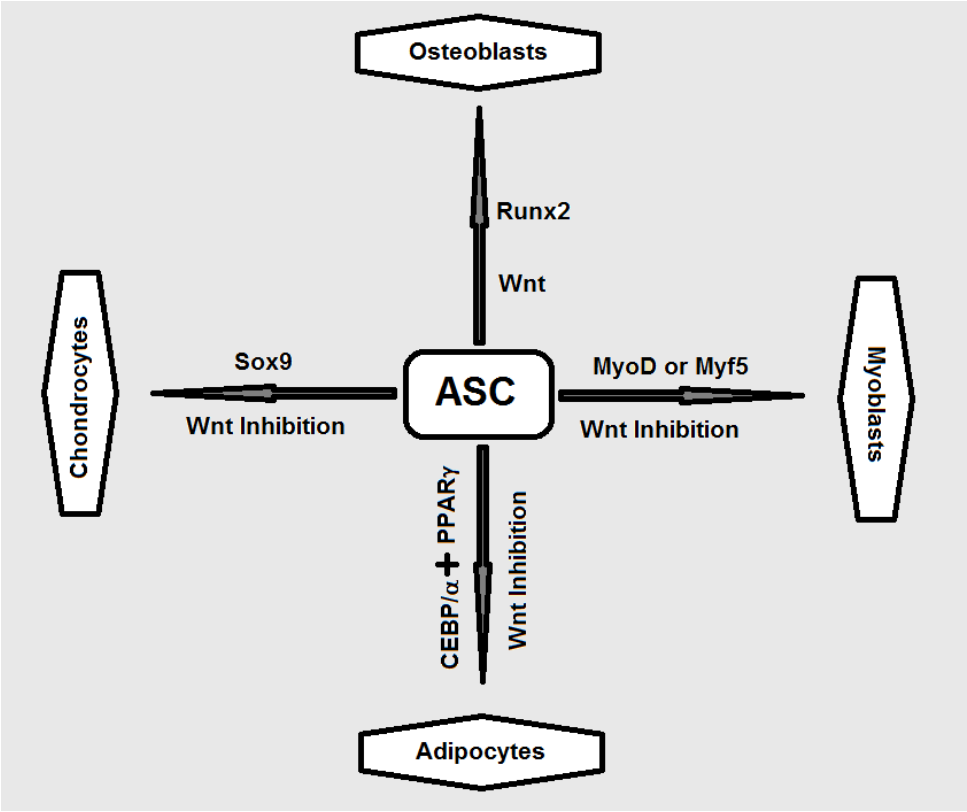

Figure 2.6 Wnt Signaling pathway and its inhibition with differentiation

Because signal transduction is often initiated at the level of extracellular ligands, different Wnt proteins have distinct affinity to the cell-surface receptors which have been discussed previously [155]. In general, the Wnt 5a class (Wnt 5a, 4, and 11) tends to participate in the Wnt/Ca^{2+} and Wnt/ JNK pathways, whereas the Wnt 1 class (Wnt 1, 3a, 8 and 8b) favors the canonical Wnt/ β-catenin pathway [156]. Therefore, each Wnt protein may trigger different cellular responses dictated by different co-receptors. Wnt 5a is necessary to maintain osteogenic potential of MSCs. Further, inhibition of WNT 5a and 10b signaling promotes adipogenesis of human ASCs [157]. Wnt 3a, which is able to influence cell motility through RHOA activation, is specifically regulated by disheveled-2 (DVL2) [158]. In one study, Wnt 2 was shown

to specifically target Fz 9 in 293T cells, activating T-cell factor (TCF)-dependent transcription [159]. Wnt 5a and Wnt 11 signaling through distinct non-canonical Wnt pathways have opposing effects on type II collagen expression by chondrocytes through the JNK and PKC pathways respectively [160]. In spite of these interesting results, more studies are needed to understand the impact of the Wnt pathways in a general and broader scope.

2.7 Cell-cell Interactions in Co-culture Environments

2.7.1 Overview

Most tissues consist of more than one cell type, and each cell type is responsible for different functions in tissue growth, homeostasis, repair and regeneration after injury [161]. In stable tissues, MSCs are normally quiescent, and are distributed amongst the mature cell populations. In response to cell-cell and cell-ECM signaling, the MSCs can be stimulated to proliferate and replenish the mature cells through differentiation [161, 162]. The interactions between MSCs and mature cells in normal tissues influence both proliferation and differentiation. These interactions can occur through cell–cell/cell-ECM direct contact, autocrine/paracrine signaling, and/or endocrine signaling via the blood stream [162]. For example, the development, remodeling and repair of bone are tightly controlled to coordinate the proliferation, differentiation, migration and apoptosis of many cell types, including osteoblasts,

osteoclasts, HSCs and MSCs [163]. Similar situations exist in normal homeostasis in other mesenchymal-derived tissues and organs.

Co-culture is a technique that has been used to study the dynamic interactions between mature cells and MSCs. Through mimicking the cellular microenvironment in normal tissues, co-culture provides simplified and controllable conditions *in vitro* that can be used to model and understand the more complex *in vivo* regenerative processes [164].

In co-culture experiments, various cell-cell communication mechanisms can occur. For example, cells can communicate through gap junction formation or direct cell contact, juxtacrine signaling and/or paracrine signaling. Gap junction formation, which requires cell-cell contact, directly connects the cytoplasm of two cells, allowing for various small molecules and ions to pass freely. Ca^{2+} and ATP are two molecules that are known to pass through gap junctions between adjacent cells [165]. Juxtacrine signaling between neighboring cells is typically transmitted via oligosaccharide, lipid or protein receptors on the cell membrane [166]. These signaling pathways may affect either the emitting cell (i.e. autocrine signaling) or the immediately adjacent cells, and frequently require physical contact between the two cells involved. Paracrine signaling depends on the secretion of soluble signaling factors, which are released into the extracellular space and interact with neighboring cells. Although close proximity is sufficient, cell-cell direct contact is not required via this mechanism. [18]

2.7.2 Direct Co-culture

In direct co-culture experiments (Figure 2.7), cells can communicate through all three of the mentioned signaling pathways. Direct cell-cell contact can often provide better efficiency in exchanging factors between the cellular compartments; however, the mixture of two cell types within the direct co-culture experiment can create problems for later assessment in terms of cell separation and the differentiation of the observed effects. For example, Tsuchiya *et al* used direct co-culture of bMSCs with passage 2 (P2) chondrocytes to observe the chondrogenic differentiation of mixed cell pellets. Elevated FGF-2, TGF-β, IGF-1 and BMP-2 levels were detected in the cultures and supported differentiation, however, the investigators were not able to identify which cell population(s) was secreting each factor [88]. Thus, while direct co-culture is easy to conduct, the available assessment methods have limitations and it is difficult to separate the complex cell-cell/cell-ECM effects.

2.7.3 Indirect Co-culture

For most co-culture systems in ASC differentiation, indirect cultures (Figure 2.7) allow for a more clear elucidation of the effects, and in particular, of those processes mediated by cell-secreted paracrine factors. Indirect co-cultures are commonly facilitated by transwell inserts, which have a permeable membrane at the bottom to separate the cell populations [154]. The two cell types of interest can be separated by this membrane, but are cultured at a close distance of 1-2 mm. The membrane allows signaling molecules, such as proteins, growth factors, and hormones, to exchange

freely in the medium and to facilitate cellular communication. The transwell system has a limited influence on paracrine signaling, but prevents gap junction and direct cell-cell effects by eliminating the contacting surfaces.

Several studies have used this type of system to observe the interactions between bMSCs and osteoblasts [154, 163]. These studies have shown that osteoblasts are capable of inducing bMSCs to commit to osteogenesis. Similarly, in a study of the effect of co-culture between primary and passaged chondrocytes, indirect co-culture restored the chondrogenic activity that is typically lost upon serial passaging [167]. There are very few studies that have investigated the effect of co-culture between adipocytes and MSCs, potentially due to the inconvenience of handling mature adipocytes. To circumvent this problem, Stacey et al. provided an innovative way of co-culturing more committed adipogenic progenitors with ASCs, and assessed them by intracellular adipogenic protein accumulation [168]. To date, no correlation was found between the specific cellular interactions and the developmental stage of the cells. While cell–cell contact in some co-culture experiments seems to be crucial for cellular interactions, in other experiments co-culture in the same medium without cell–cell contact is sufficient as well [169-171].

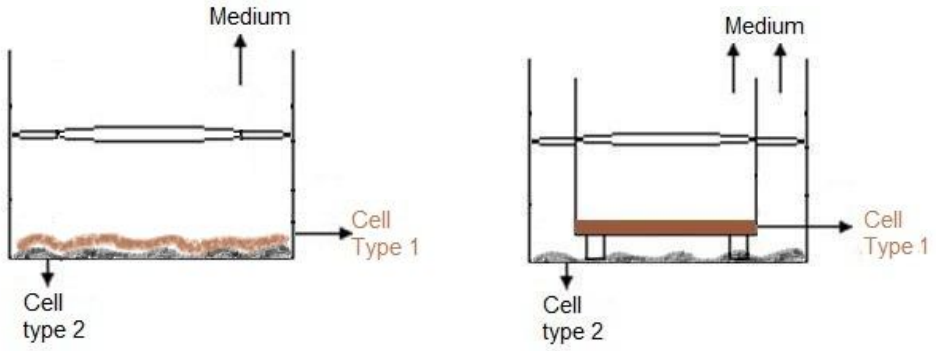

Figure 2.7 Direct (Left) and Indirect (Right) co-cultures.

Chapter 3:

The Characterization of Bovine Adipose-Derived Stem Cell Proliferation and Differentiation in Conventional Culture

* Large portions of this chapter have been submitted for publication in *Cells Tissues Organs* (Y. Zhao, S.D. Waldman, L.E. Flynn. The Effect of Serial Passaging on the Proliferation and Differentiation of Bovine Adipose-derived Stem Cells. Submitted to Cells Tissues Organs Dec. 2010.). All experimental work was conducted by Y. Zhao.

3.1 Introduction

Recent research has demonstrated that adipose-derived stem cells (ASCs) are capable of long-term self-renewal in culture, while maintaining multi-potency in several species, such as: human, mouse and rat [11, 12, 14, 63]. Several papers have also probed alternative animal models for *in vitro* and *in vivo* experimental purposes [62, 63, 66], however, the presence of a multipotent stem cell population within bovine adipose tissue has not yet been thoroughly investigated. As discussed in Chapter 2, there are significant differences observed between ASCs isolated from different species, as well as from various adipose tissue depots within the same organism [71]. Further, no studies to date have investigated the use of ASCs from a structural adipose tissue source, which primarily functions in mechanical cushioning,

rather than in metabolism and maintenance of the energy balance.

For most cell biology and tissue-engineering applications, the cells of interest first need to be expanded *in vitro* in order to obtain sufficient quantities of purified cells for cell culture studies and/or implantation models, with most studies using stem cells between passages 1 - 5. Therefore, it is important for ASCs to maintain their stem cell capacity to differentiate while undergoing extensive *in vitro* expansion. Bone marrow-derived mesenchymal stem cells (bMSCs) have been shown to maintain osteogenic differentiation capacity for 10-15 passages, but adipogenic potential decreases rapidly with increasing time in culture [50, 172]. While Zuk et al. [173] showed that sub-populations of human ASCs obtained from lipoaspirates proliferate well for up to 15 passages, adipogenic, chondrogenic and osteogenic differentiation was assessed only at the first passage. As an extension of this work, Wall et al. [37] found that only a fraction of human ASCs retain their ability to differentiate along the adipogenic and osteogenic lineages through 10 passages, using defined differentiation media containing cocktails of stimulatory factors. Overall, it remains unclear whether ASCs retain a satisfactory level of differentiation after extensive *in vitro* culturing.

These studies raise the issues of whether there is a threshold for maintained ASC proliferation ability and multi-potent differentiation capacity, as well as when this threshold might occur. As an initial step in the research project, a thorough understanding of the bovine ASC response within a conventional culture environment was conducted to confirm the presence of a multipotent cell population, and to ensure that optimized conditions could be utilized in the co-culture studies discussed in the

next chapter. As such, the objectives of this chapter were to isolate and characterize the ASC population from the bovine interdigital fat pad, and to investigate the influence of the conventional *in vitro* cell culture environment on the proliferation ability and multi-lineage differentiation potential of the cells after serial passaging. The growth factor stimulation differentiation studies also helped to establish a fundamental validation to demonstrate the feasibility of the co-culture study (Chapter 4). Similarly, this study also provided a baseline for comparison between the traditional and co-culture environments.

3.2 Materials and Methods

3.2.1 Materials:

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and were used as received.

3.2.2 Bovine ASC Isolation and Culture:

Bovine adipose-derived stem cells (bASCs) were extracted under sterile conditions from the interdigital fat pad found in the hoof, from tissues obtained from a commercial slaughterhouse (calves 12 – 18 months in age). Cell isolation methods were based on human ASC extraction protocols described by Flynn et al. [174]. The fat pads were minced and digested under agitation (120 rpm) at 37 ºC for 1 h in Kreb's Ringer buffer containing 2% collagenase type VIII, 3 mM glucose, 25 mM

HEPES, and 20 mg/mL bovine serum albumin (BSA). After digestion, the overall mixture was filtered through 250 μm stainless steel mesh to get bASC-enriched cell fraction and the undigested tissue fragments were discarded. The bASC-enriched cell fraction was pelleted by centrifugation at 1200 x g for 5 min. Red blood cells were lysed using erythrocyte lysing buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid (EDTA)) under gentle agitation for 10 min at room temperature. The bASCs were then re-suspended in growth medium (1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient mixture (DMEM:Ham's F-12), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin) [175] and filtered through a 100 μm nylon filter. The cells were then washed 3 times in sterile cation-free phosphate buffered saline (PBS), and re-suspended in growth medium. Isolated bASCs were plated in 75 cm^2 flasks (Corning T-75, 30,000 cells/cm^2) and incubated at 37 ºC with 5% CO_2. The growth medium was changed 24 h after isolation and every 2 – 3 days thereafter. For passaging, cells at 90% confluence were trypsin-released (0.25% trypsin/0.1% EDTA, Gibco, Burlington, Canada), washed, counted and re-plated in new flasks at a density of 30,000 cells/cm^2.

3.2.3 bASC Proliferation:

bASCs (n=3, N=5) from passage 1 to 5 were seeded in 12-well plates at a density of 2,000 cells/cm^2, and 3 wells of each passage were tryspin-released and counted daily using a hemocytometer with trypan blue viability staining, for up to 8 days after

seeding. The gene expression patterns of the stem cell markers, CD90, CD34 and Sca-1 (ATXN-1), were also analyzed by end point reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm the presence of stem cells within the culture expanded cells under proliferation conditions, as described in detail in section 3.2.5.

3.2.4 bASC Differentiation and Preliminary Characterization:

Passage 2 (P2) and passage 5 (P5) bASCs, from the same donor source were differentiated towards the adipogenic, chondrogenic or osteogenic lineages, with characterization carried out at 14 days after the induction of differentiation. Triplicate negative control samples (i.e. non-induced) cultured in growth medium (DMEM:Ham's F-12, 10% FBS, 1% pen-strep), were included in every trial.

3.2.4.1 Adipogenic Differentiation:

bASCs were plated at a density of 50,000 cells/cm^2 and cultured in growth medium for 48 h. The cells were then cultured in adipogenic induction medium (0.5mM isobutylmethlxanthine (IBMX), 0.25 μM dexamethasone (DEX), 2.5 μg/mL insulin, 5 μM troglitazone and 1% penicillin- streptomycin in DMEM:Ham's F-12) for 48 hrs and then transferred into adipogenic differentiation medium (5% FBS, 2.5 μg/mL insulin, 5 μM troglitazone and 1% penicillin-streptomycin in DMEM:Ham's F-12) [67] for the remainder of the culture period, with medium exchanges 3 times per week. Adipogenesis was assayed in terms of glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity (n=3, N=3) as well as oil red O staining of intracellular lipid accumulation (n=3, N=3).

GPDH activity:

14 days after the adipogenic induction, triplicate wells from induced and control cultures (P2 and P5) were rinsed with PBS. GPDH enzyme activity was then determined using a GPDH measurement kit (Cat #: KT-010, Kamiya Biomedical, Seattle, WA, USA). To extract the total intracellular protein content, the cells were disrupted by sonication with 3, 5-s bursts in 2 mL of extraction buffer (supplied with the kit) with intermittent cooling on ice. The samples were then centrifuged (13,000 x g for 10 min at 4 ºC) and the supernatants were immediately assayed according to the manufacturer's instructions within 2 hours. Data was normalized to the total intracellular protein concentration measured using a Bio-Rad protein assay (Cat #: 500-0002, Bio-Rad Laboratories, Inc. CA, USA) with an albumin standard. One unit of GPDH activity was defined as the amount required to consume 1 μmol of NADH in 1 minute.

Oil red O staining:

Intracellular lipid accumulation was assessed by oil red O staining 14 days after adipogenic induction. Cultures were rinsed thoroughly with PBS and fixed for 30 min in 10% neutral buffered formalin. Oil red O stock solution (3 g/L in isopropanol) was prepared according to the protocol of Flynn *et al.* (2007). Briefly, 1 mL of oil red O working solution (stock solution diluted 3:2 in deionized water) was applied to each well for 5 min, followed by extensive rinsing with distilled water (dH_2O) and counterstaining with hematoxylin for 2 min.

3.2.4.2 Chondrogenic Differentiation:

Three-dimensional (3-D) pellet cultures of bASCs (2 x 10^6 cells/pellet) were obtained by centrifugation (300 x g for 10 min), and then transferred into 24-well plates coated with 2% agarose (type VII) and initially cultured in growth medium. After 48 h, the pellets were transferred into chondrogenic medium (10 ng/mL transforming growth factor-beta 1 (TGF-β1), 50 μg/mL ascobate-2-phosphate (A2P), 6.25 μg/mL bovine insulin, 100 nM DEX in growth medium) [63] with medium exchanges 3 times per week. Chondrogenesis was analyzed using toluidine blue staining of glycosaminoglycans (GAG) (n=3, N=3), immunohistochemical (IHC) localization of collagen types I and II (n=3, N=3), as well as quantitative analysis of sulphated GAG (sGAG) content, using the dimethylene blue (DMMB) assay (n=3, N=2), and hydroxyproline content, as an estimation of total collagen (n=3, N=2).

Toluidine blue staining:

For histology and IHC analysis, 14 days after induction, the pellets were fixed overnight in 4% paraformaldehyde, paraffin-embedded and sectioned (5 μm sections) (John L. Dacosta, Department of Pathology and Molecular Medicine). Sections were incubated overnight at 65 °C, de-paraffinized, and rehydrated in dH_2O prior to toluidine blue staining (0.1% for 2-3 min).

IHC staining for Collagen I and II:

After de-paraffinization, sections were incubated in 0.5 U/mL chondroitinase and 0.5 U/mL hyaluronidase, each for 30 min, to facilitate antigen availability. Endogenous peroxidase activity was neutralized with 0.3% hydrogen peroxide for 30

min, followed by overnight incubation with the 1° antibody (either 1:200 dilution of mouse polyclonal anti-Collagen II or 1:10 dilution of anti-rabbit collagen I (Biolynx, Brockville, ON, Canada)) at 4 °C. Detection was facilitated using the Vectastain ABC Kit (mouse IgG for collagen II and rabbit IgG for collagen I), (Vector Labs, CA, USA) with DAB as the substrate and counterstained using methyl green [176]. Controls without primary antibodies were included to detect non-specific staining.

Sulphated GAG and Collagen Content:

After 14 days of culture in chondrogenic medium, triplicate bASC pellets (n=3, N=2) were washed with PBS and digested in papain solution (40 mg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol (DTT)) for 72 h at 65 ºC. As described, triplicate negative control pellets (n=3, N=2) cultured in proliferation medium were analyzed using identical methods. The papain digest aliquots were stored at -20 ºC and assayed separately for the sGAG [177], hydroxyproline and DNA contents [178]. sGAG content was assessed according to the established DMMB assay from Farndale *et al*. [179]. Collagen content was estimated from the determination of the hydroxyproline content, using the standard assumption that hydroxyproline accounts for 10% of the total collagen mass in cartilage [180]. In brief, aliquots of the papain digest were hydrolyzed in 6 N HCl at 110 ºC for 18 h and the hydroxyproline content was determined using the chloramine-T/Ehrlich's reagent assay [181]. For data normalization purposes, the DNA content was determined from aliquots of the papain digest using the Hoechst dye 33258 assay.

3.2.4.3 Osteogenic differentiation:

bASCs were plated at a density of 20,000 cells/cm^2 and cultured in growth medium for 48 h. The cells were then transferred to osteogenic medium (50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 nM DEX in growth medium) [12] for 14 days, with medium changes 3 times per week. Osteogenic differentiation was measured using the alkaline phosphatase (ALP) activity assay (n=3, N=3), as well as von Kossa staining of calcium deposition (n=3, N=3).

Alkaline phosphatase activity:

Triplicate wells from both induced and control cultures (P2 and P5) were rinsed with PBS. Total intracellular protein was collected, using the methods described in the GPDH section. The ALP activity was measured using the p-nitrophenol phosphate liquid substrate system [182-184]. Briefly, 100 μL of liquid substrate was added into a well of a 96-well plate containing 100 μL of the extracted intracellular cell supernatant from centrifugation or p-nitrophenol standards. The plates were incubated at 37 °C for 15 min and quenched with 100 μL of 1 M NaOH per well. The absorbance was assayed using a microplate reader (405 nm wavelength) and the results were normalized to the total intracellular protein content, measured using the Bio-Rad protein assay. One unit of ALP activity was defined as the amount of enzyme required to catalyze the liberation of 1 μmol of p-nitrophenol per minute.

Von Kossa staining:

14 days after seeding, cultures were rinsed thoroughly with PBS and fixed for 30 min in 10% neutral buffered formalin. After rinsing with dH_2O, cultures were stained

with silver nitrate (1% in dH_2O), incubated under UV light for 1 h, followed by incubation in sodium thiosulphate (5% in dH_2O) for 5 min. The cultures were then counterstained with hematoxylin for 2 min.

3.2.5 Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis:

In addition to the assessment of the stem cell markers, lineage specific gene expression was assessed in all three investigated lineages at 14 days after induction (n=3, N=3). Total RNA was extracted from the cells (induced and control samples from both P2 and P5) using TRIzol® Reagent (Invitrogen Canada Ltd., Burlington, ON, Canada) according to the manufacturer's instructions. As positive controls, RNA was also extracted from mature adipocytes, chondrocytes and osteoblasts. The total RNA concentration and purity was determined using a NanoDrop spectrophotometer, and the 260/280 ratio generally ranged between 1.9 and 2.1. cDNA was synthesized from 1 μg of total RNA using random primers (Invitrogen) and SuperScript™ II Reverse Transcriptase (RT) (Invitrogen). The reverse transcription was carried out in a 20 μL reaction volume containing first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.09 OD_{260} units of random primers (Invitrogen), 0.5 mM of each dNTP (Invitrogen) and 200 units of SuperScript™ II RT. Gene-specific primers were designed using Primer3 software and are listed in Table 3.1. GAPDH and 18S were included as the housekeeping genes [185]. The melting temperature for all primers was 60 °C.

Each PCR reaction was conducted in a 50 μL reaction volume with 2.5 μL of

diluted cDNA (containing 50 ng of input RNA), 1X Taq buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 250 nM forward primer, 250 nM reverse primer, 250 nM of each dNTP, 2.5 mM $MgCl_2$ and 0.375 units of recombinant Taq DNA Polymerase (Fermentas International Inc., Burlington, ON, Canada). PCR was conducted for different cycles (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) in Table 3.1 using a Bio-Rad C1000 thermal cycler. Expression was analyzed following 5% agarose gel electrophoresis by ethidium bromide staining (G:box Chemi HR16, Syngene, Cambridge, UK). Minus-RT and no template controls were included in every run. The presence of each specific product was determined based on the size of the amplicon (Figure A 1 in appendix).

Table 3.1 Bovine-specific primer pairs used for RT-PCR

Markers	Gene	Primer sequence (5'~3')	Amplicon size	Cycles
Chondrocytes	Collagen II	Forward:CTCAAGTCCCTCAACAACCAG Reverse: TTGGGGTCGATCCAGTAGTC	134	40
	Aggrecan	Forward: CAGTCACACCTGAGCAGCAT Reverse: CCTTCGATGGTCTTGTCGTT	104	40
	Sox9	Forward: AGAAGGACCACCCGGACTAC Reverse: CGTTCTTCACCGACTTCCTC	57	40
Osteoblast	Collagen I	Forward: ATGGAACTCCAGGTCAAACG Reverse: CACCAACACGTCCTCTCTCA	60	40
	Osteocalcin	Forward: CATGGGAGCTGGGAGAGTAA Reverse: AAGGGGCAAATGGATTAAG	113	40
	RUNX2	Forward: CAGATGACTCCTCCGTCCAT Reverse: ACTGAGAGTGGAAGGCCAGA	147	40
Adipocytes	C/EBPα	Forward: ATCGACATCAGCGCCTACAT Reverse: CGGGTAGTCAAAGTCGTTGC	138	40
	PPARγ	Forward: CAGTGTCTGCAAGGACCTCA Reverse: GATGTCAAAGGCATGGGAGT	128	40
	LPL	Forward: TGCTGGTATTGCAGGAAGTC Reverse: AAAATCCGCATCATCAGGAG	124	40
Stem Cell Markers	CD90	Forward: CACCATGAACCCTACCATCG Reverse: TGTCAGGCTGGTCACCTTCT	82	35
	CD34	Forward: CAGCATCTGCCTGAAGCTAA	103	35

		Reverse: CTCGGCCTGTTTCTTCTGAC		
	Sca-1 (ATXN1)	Forward: CAGCACTGACCTGAAGATC Reverse: TACCAAAACTTCCACGCTGA	127	40
Housekeeping	GAPDH	Forward: CACCCTCAAGATTGTCAGCA Reverse: GTCTTCTGGGTGGCAGTGAT	135	40
	18S	Forward: ATGGCCGTTCTTAGTTGGTG Reverse: GAACGCCACTTGTCCCTCTA	136	40

3.2.6 Statistical Analysis:

Unless otherwise stated, all numerical data is presented as Mean ± Standard Error. For each experiment, the number of samples per group (n), plus the number of times each experiment was repeated with cells from a different donor (N) are indicated. Within an individual trial, all groups were seeded with bASCs isolated from one donor to eliminate the potential for donor-to-donor variability. The effect of passage number was assessed using a one-way ANOVA with the Tukey's post-hoc test, performed using Origin Pro8 statistical software. A *p*-value of less than 0.05 was considered to be statistically significant.

3.3 Results

3.3.1 Cellular Isolation:

Fat dissected from the bovine interdigital region (Figure 3.1 A) was light yellow in color, with little or no visible vascularization (Figure 3.1 B). Any white fibrous tissue associated with the pads was removed before digestion. After seeding, the isolated cells appeared to be small and flat, with several extending processes (Figure 3.1 C). When the cells approached confluence, they became more spindle-like and

elongated, closely packing together (Figure 3.1 D).

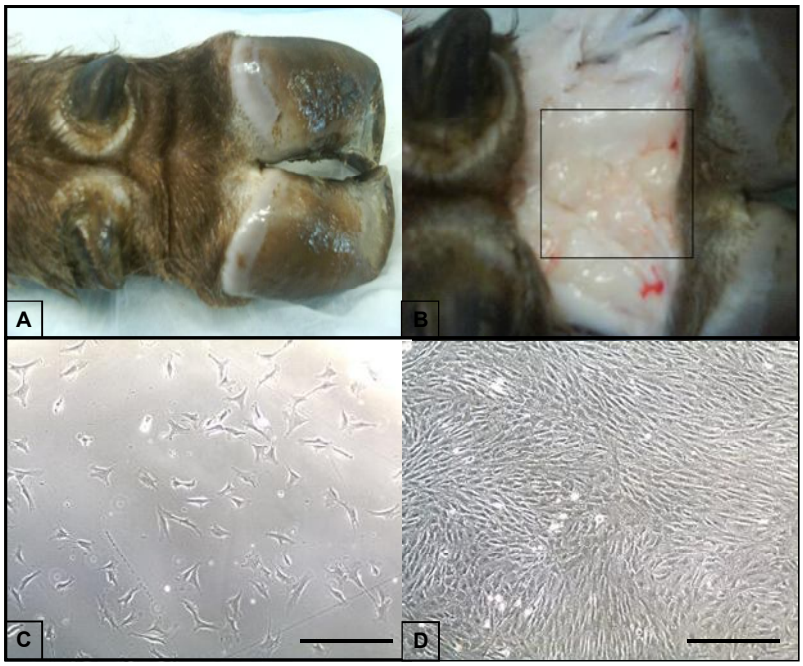

Figure 3.1 **(A)** Cow hoof interdigital region, **(B)** Exposed bovine interdigital fat pad. Representative cell morphology at **(C)** 24 h after seeding, and **(D)** confluence, observed by optical microscopy. Scale bars = 100 μm.

3.3.2 Proliferation Study:

bASCs at each of the 5 passages were examined for changes in growth kinetics through an 8-day proliferation study (Figure 3.2). Only passage 1 and 2 cells reached a plateau after day 7, whereas the other passages (P3 through P5) appeared to proliferate through the 8-day culture period.

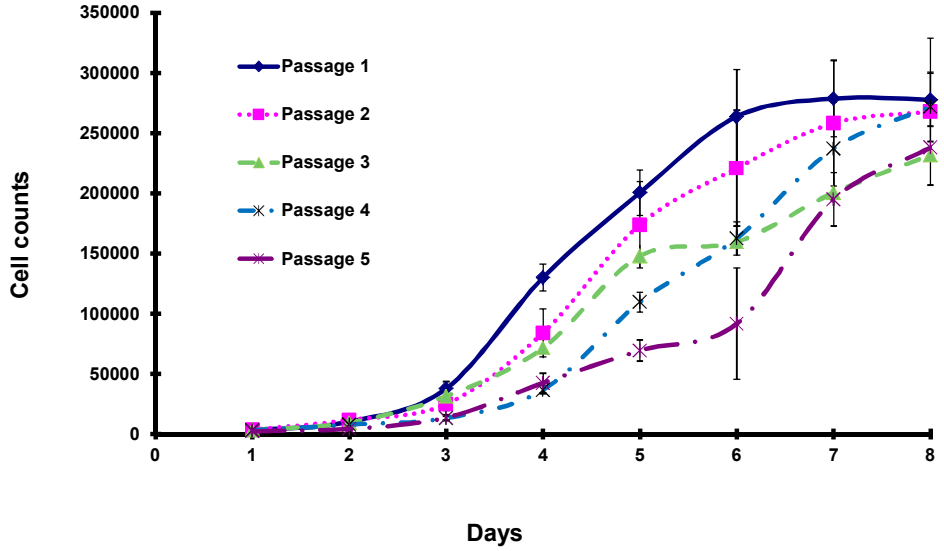

Figure 3.2 Growth kinetics of bASCs. Hemocytometer cell counts represent the average number of viable cells in triplicate samples for each passage. N=5 donors, with 3 replicates per donor. Error bars represent the standard deviation.

Although total cell yields from each passage were not statistically different at the end of the culture period, the growth rate declined with increasing passage number (Table 3.2). The doubling time of cells from passages 1- 4 was the same, ranging from 16 – 19 h, which significantly increased ($p < 0.05$) at passage 5 to approximately 22 h. As a significant difference in the doubling time was only observed at P5, the differentiation studies were conducted using P2 (most commonly used to study differentiation) and P5 cells.

Table 3.2 Doubling times for passage 1 – 5 bASC. Error represents the standard deviation (n=3, N=5)

	P1	P2	P3	P4	P5
Mean (hr)	**16.44**	**17.39**	**17.71**	**18.66**	**22.06**
Error	**0.33**	**0.26**	**0.14**	**0.11**	**0.32**

Examination of stem cell markers showed that primary isolated bASCs (P0 cells)

had the strongest expression for all three genes investigated (CD90, CD 34 and Sca-1). P2 cells showed a slight reduction in CD90, but no observable decay in CD34 or Sca-1. P5 cells gave the lowest expression of all three markers compared to both P0 and P2 cells (Figure 3.3).

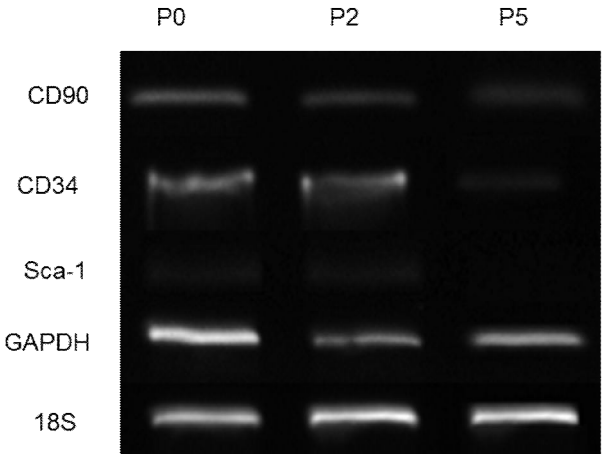

Figure 3.3 Stem cell gene expression (CD90, CD34, and Sca-1) in P0, P2 and P5. GAPDH and 18S are included as internal housekeeping genes.

3.3.3 Adipogenic Differentiation:

Cells from P2 and P5 were analyzed for levels of GPDH enzyme activity, intracellular lipid accumulation and mRNA expression of adipogenic markers to determine the ability of passaged bASCs to undergo adipogenesis (Figures 3.4 - 3.6). bASCs undergoing intracellular lipid accumulation tended to be found in clusters, while the remainder of the cells were unchanged. Approximately 40 - 50% of the cells differentiated, depending on the donor and passage number. GPDH assay results (Figure 3.4) indicated that both P2 and P5 induced cultures had significantly upregulated GPDH enzymatic activity relative to the control cultures in proliferation

medium. However, P5 cells had approximately 50% of the GPDH activity of the P2 cells.

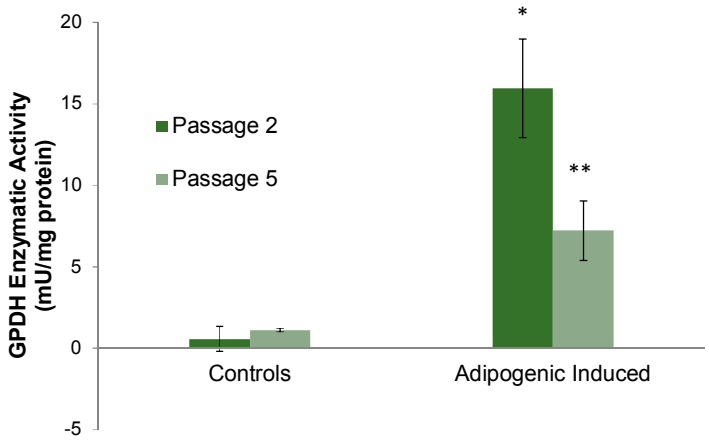

Figure 3.4 Adipogenic differentiation of bovine ASCs assessed by GPDH enzyme activity. Both P2 and P5 showed upregulation in induced cultures, with higher activity in P2. * was statistically higher than **; * and ** were significantly higher than both controls.

The reduced adipogenesis at P5 was confirmed by Oil Red O staining (Figures 3.5 A - D), which showed lower levels of intracellular lipid accumulation and a less mature phenotype in the P5 cells. More specifically, differentiated P2 cells had a more unilocular morphology, with large singular lipid droplets in the cytoplasm. In contrast, P5 induced cells contained multiple, small lipid droplets located around the nucleus. Both P2 and P5 control cultures in proliferation medium stained negative for intracellular lipid.

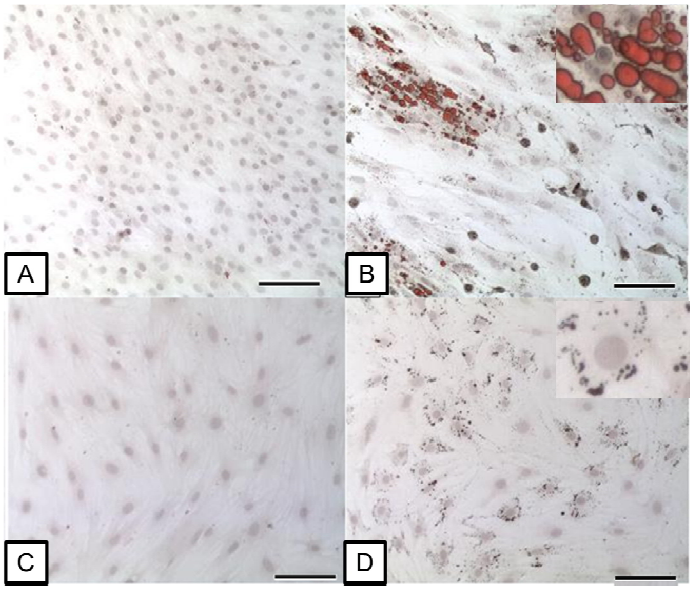

Figure 3.5 Oil red O staining of bASCs in **(A)** P2 control culture, **(B)** P2 induced culture, **(C)** P5 control culture, and **(D)** P5 induced culture. Scale bars = 100 μm. Boxed regions from (B) and (D) are shown in detail below to visualize intracellular lipid accumulation at higher magnification (Original magnification 200X). Scale bars = 25 μm.

The CCAAT/enhancer binding protein-α (C/EBPα), peroxisome proliferator activated receptor-gamma (PPARγ) and lipoprotein lipase (LPL) genes were all expressed in P2 induced cultures as well as mature bovine adipocyte samples (positive control) (Figure 3.6). In the P5 induced cultures, C/EBPα and LPL genes were expressed at lower levels (compared to P2 cells) and PPARγ gene expression was undetected.

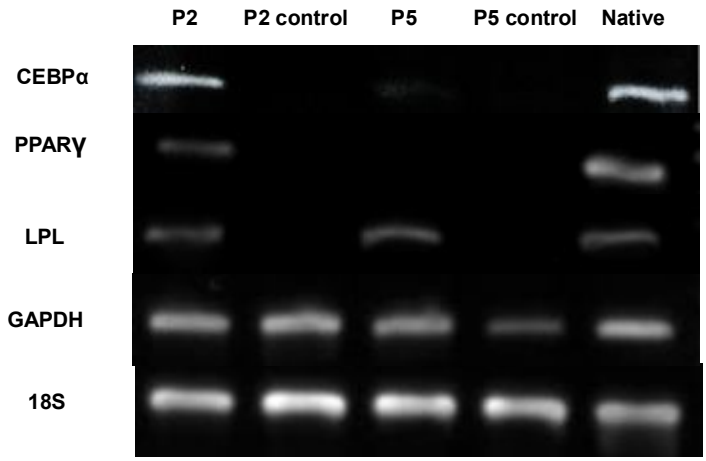

Figure 3.6 Adipogenic gene expression (C/EBPα, PPARγ, and LPL) in P2 and P5, and in freshly-isolated bovine mature adipocytes (Native; positive control). GAPDH and 18S are included as internal housekeeping genes.

3.3.4 Chondrogenic Differentiation:

Pellet cultures created from P2 and P5 cells were analyzed for GAG accumulation, synthesis of collagen I and II and chondrogenic gene expression analysis to measure chondrogenesis. In all pellets, the cells maintained a packed and rounded morphology for the 2-week culture period. Control pellets from both P2 and P5 cells displayed limited GAG staining (Figure 3.7 A and C), whereas both induced pellets (P2 and P5) had more intense staining, with P2 induced cultures showing greater GAG accumulation as compared to P5 induced cultures (Figure 3.7 B and D).

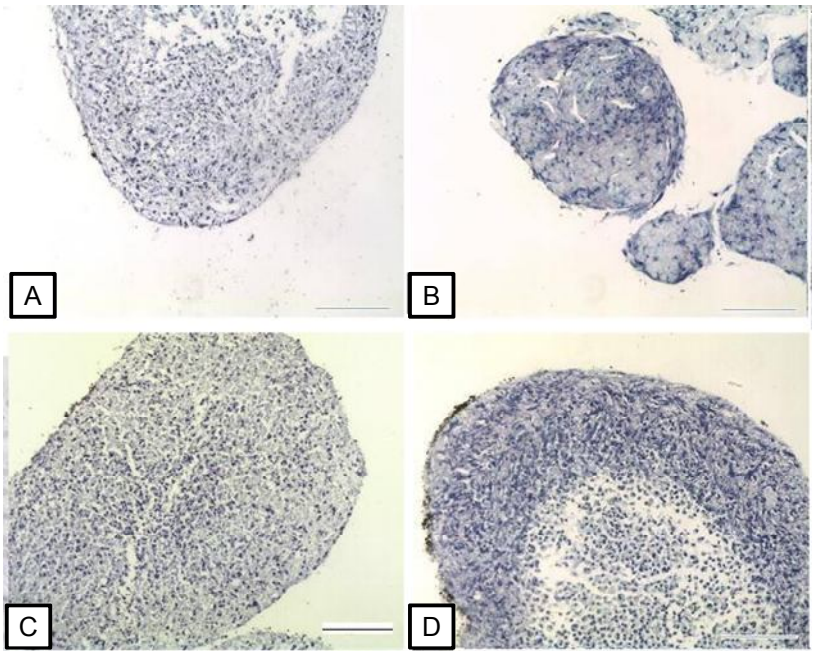

Figure 3.7 Chondrogenic differentiation of bovine ASCs assessed by toluidine blue staining of GAG in **(A)** P2 control pellets, **(B)** P2 induced pellets, **(C)** P5 control pellets, and **(D)** P5 induced pellets. Scale bars = 100 μm.

P2 and P5 induced pellets stained positive for collagen type II (Figure 3.8 B and D), with the P2 induced pellets displaying more intense staining than P5 induced pellets. All control pellets displayed no obvious staining for collagen II (Figure 3.8 A and C).

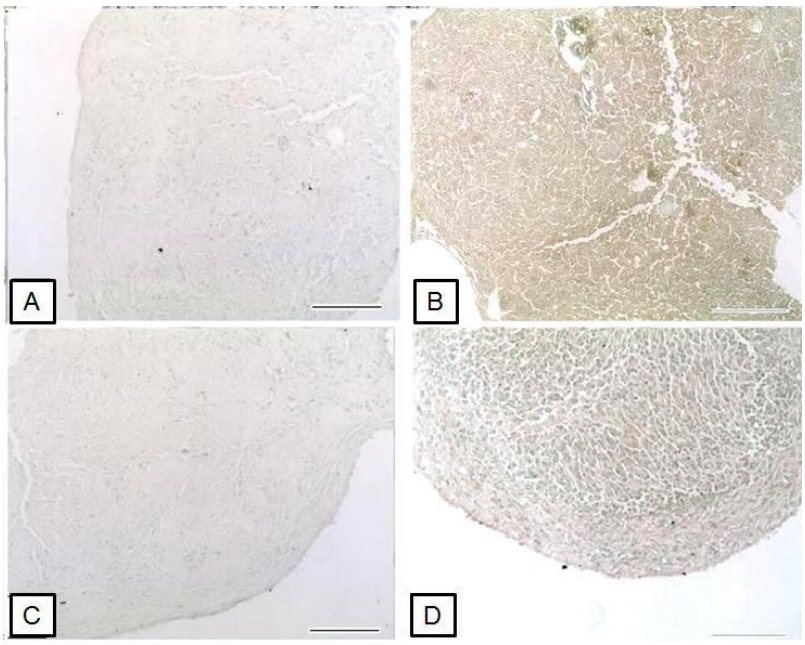

Figure 3.8 Collagen II IHC staining in **(A)** P2 control pellets, **(B)** P2 induced pellets, **(C)** P5 control pellets, and **(D)** P5 induced pellets. The pellets are also counterstained with methyl green. Scale bars = 100 μm.

In contrast, collagen I was detected in both the control and induced pellets from both passages (Figure 3.9 A - D); however, P5 pellets displayed more intense staining than P2 for both the control and induced samples.

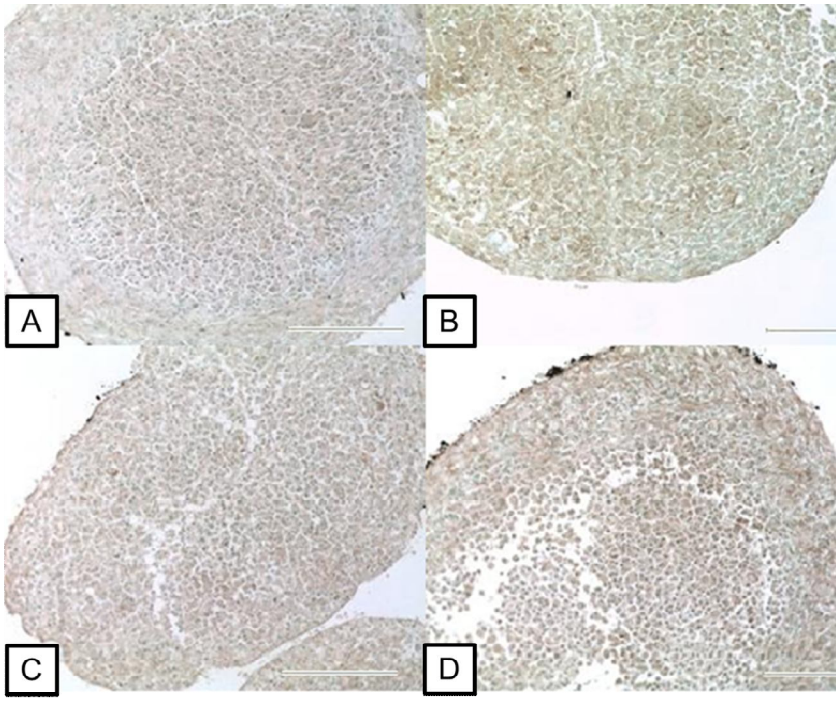

Figure 3.9 Collagen I IHC staining in **(A)** P2 control pellets, **(B)** P2 induced pellets, **(C)** P5 control pellets, and **(D)** P5 induced pellets. Scale bars = 100 μm.

In terms of gene expression (Figure 3.10), P2 induced pellets expressed collagen II, aggrecan (AGC) and SOX9, whereas P5 induced pellets had lower expression of AGC and SOX9, and no collagen II gene expression.

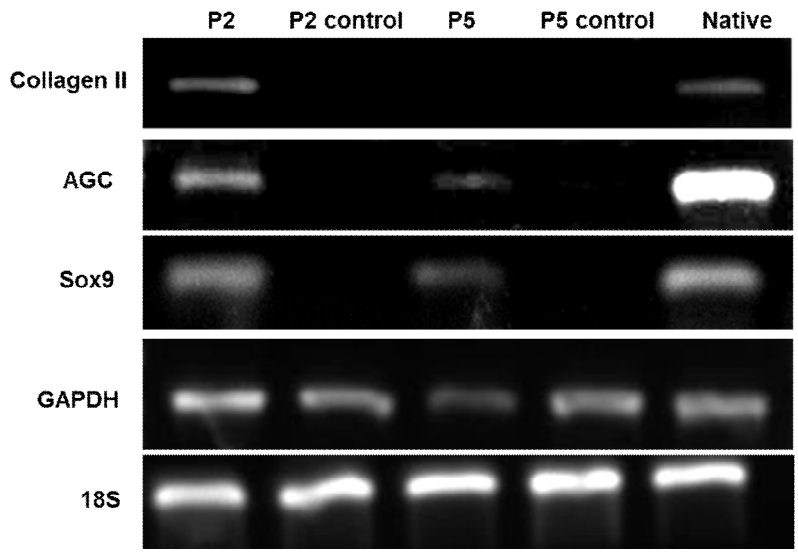

Figure 3.10 Chondrogenic gene expression (collagen II, aggrecan and SOX9) compared between P2, P5, and freshly-isolated bovine chondrocytes (Native; positive control). GAPDH and 18S are included as internal housekeeping genes.

Supporting the previous data, the DMMB assay indicated that there was statistically higher sGAG content in the P2 induced pellets than in the P5 induced pellets (Figure 3.11). Further, based on the hydroxyproline content measurements, the total collagen content in the P2 induced cultures was statistically greater than all other samples (Figure 3.11).

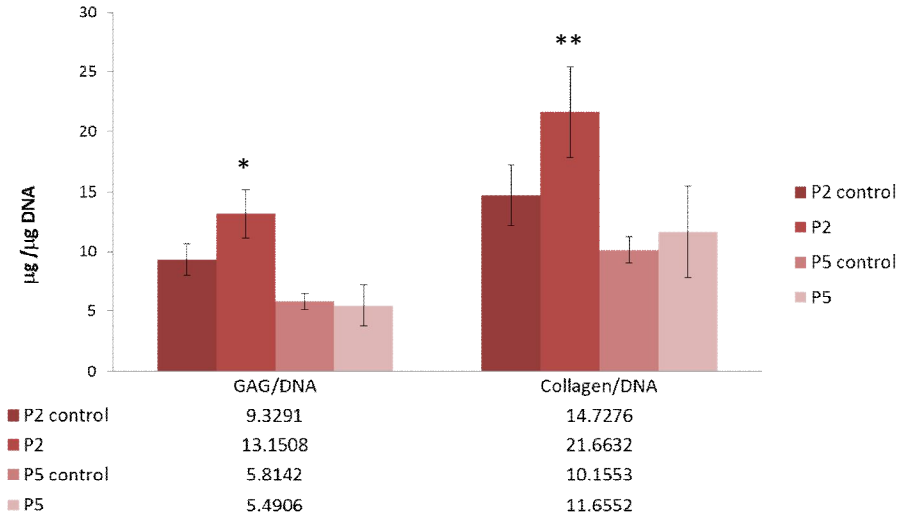

Figure 3.11 Total collagen content and sulphated GAG content, measured using the DMMB assay, in the chondrogenic testing of pellet-cultured bASCs (n=3, N=2). The hydroxyproline assay was used as an estimation of the total collagen content. All data was normalized to the total DNA content in each sample. Control samples were non-induced and cultured in proliferation medium. Error bars represent the standard deviation. * and ** were significantly higher than others.

An ALP assay was carried out on the P2 and P5 induced pellets, to assess the potential of osteogenic differentiation under the chondrogenic culturing conditions. Interestingly, the P2 induced samples had an average activity of 12 ± 2 U/mg protein and P5 induced samples had an average activity of 13 ± 1 U/mg protein, which was similar to the ALP activity levels measured in the osteogenic induced cultures (as shown in Figure 3.12).

2.3.5 Osteogenic Differentiation:

Cells from P2 and P5 were analyzed for levels of ALP enzyme activity, calcium deposition and osteogenic gene expression using RT-PCR to determine the osteogenic potential of the passaged bASCs. After 14 days, the induced bASCs became more cubical in morphology and had secreted extensive extracellular matrix. After

prolonged culture (~15 – 18 days), the cell sheets would detach from the plates, forming matrix-rich nodules. ALP activity (Figure 3.12) was upregulated in both the P2 and P5 induced cultures, with P5 induced cells having higher ALP activity than P2 induced cells.

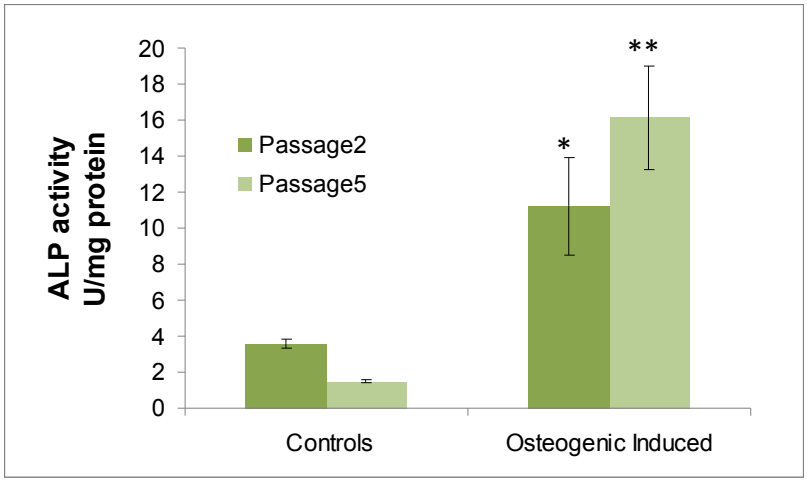

Figure 3.12 Osteogenic differentiation of bovine ASCs assessed by ALP enzyme activity showed significant upregulation in the induced cultures, with higher activity at P5. * was statistically higher than **; * and ** were significantly higher than both controls.

While calcium deposition (observed by von Kossa staining) was undetectable in the control cultures (P2 and P5) (Figure 3.13 A and C), positive staining for calcium deposition of similar intensity was observed for both induced cultures (P2 and P5) (Figure 3.13 B and D).

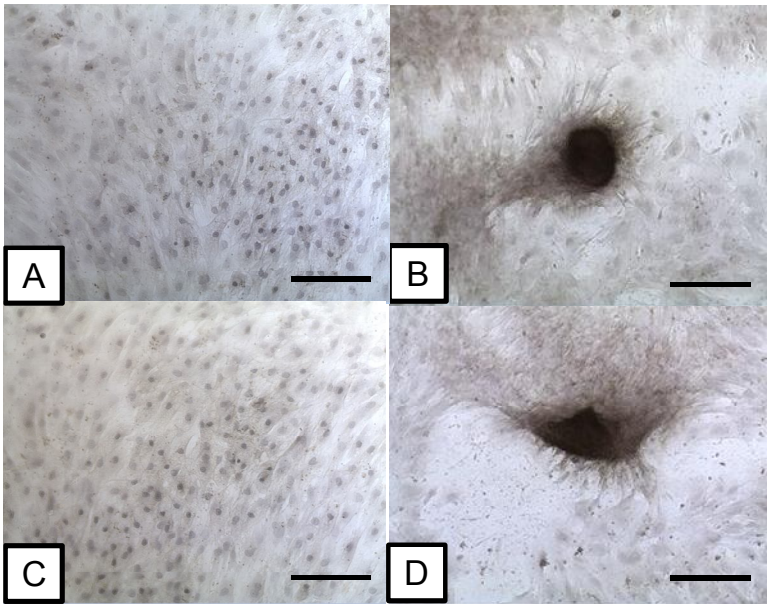

Figure 3.13 Osteogenic differentiation of bovine ASCs assessed by von Kossa staining for mineralization of **(A)** P2 control culture, **(B)** P2 induced culture, **(C)** P5 control culture, and **(D)** P5 induced culture. Scale bars = 25 μm.

Gene expression results were similar, without a detectable difference in the expression of RUNX2, osteocalcin (OCN) and collagen I, between the P2 and P5 induced cultures (Figure 3.14).

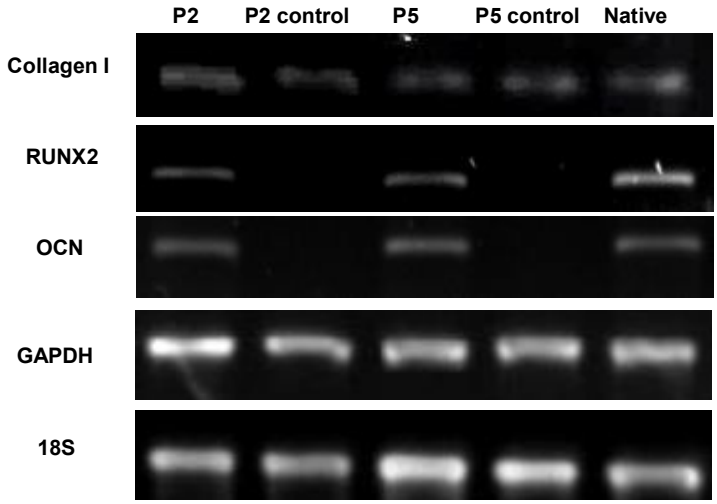

Figure 3.14 Osteogenic gene expression (collagen I, RUNX2, and osteocalcin) in P2 and P5 samples, as well as in mature bovine osteoblasts (Native; positive control). GAPDH and 18S are included as internal housekeeping genes.

3.4 Discussion

In this chapter, conventional growth factor stimulation was used to demonstrate that ASCs isolated from bovine interdigital fat, a structural adipose source, are capable of long term *in vitro* expansion and multi-lineage differentiation up to passage 5. While bovine preadipocytes have been identified previously [67, 68], no published studies to date have investigated the multi-potent stem cell population from bovine adipose tissue. The identification and characterization of this cell population is of interest in terms of developing a better understanding of ASCs in general, including the influence of species variability, as well as for applications in tissue-engineering models based on bovine sources [178, 186, 187].

In order to characterize the proliferation ability and multi-potent differentiation capacity of these cells, we first examined the growth kinetics of bASCs from passage 1 to passage 5. While there was no significant difference in the population doubling time between passages 1 through 4 (16 - 19 h), P5 cells displayed a statistically longer doubling time (22 h). This result is consistent with the study by Wall *et al.* [37] who found that human ASC doubling times were significantly longer at passage 6 and later. In addition to the slower growth, changes in response to chemical stimuli were also observed after passage 4.

Gene expression analysis of stem cell markers on freshly isolated (P0), P2 and P5 bASCs also showed a significant decay at P5, as compared to P0 and P2. These findings are consistent with Mclntosh's work on the profile of ASC surface protein

expression with serial passaging [23]. Hence, using traditional cell expansion methods, it is likely that there is a threshold for ASC growth *in vitro*, after which the proliferative ability declines with further passaging. This result has significant implications in terms of the application of these cells in tissue-engineering strategies. The observed increase in doubling time and decay in stem cell gene expression may be indicators of specific changes in the bASC population, including a reduction in stem cell potency.

A loss of multi-lineage differentiation potential with sub-culturing has been observed for other MSC sources [50, 188, 189]. In terms of models, Caplan [55] introduced the concept of multipotent MSCs, and Aubin [56] postulated that these MSCs give rise to more restricted tri- or bi-potential progenitors before ultimately becoming limited, monopotential precursors. Sarugaser *et al*. [57] utilized clonal self-renewal and differentiation analyses to postulate that expanding MSCs become more restricted self-renewing progenitors by gradually losing their differentiation potential until they become fibroblasts. In these previous studies, adipogenic potential declines first, followed by chondrogenic potential. The osteogenic lineage is preserved the longest in culture, before the proliferating cells become fibroblastic. For bone marrow-derived MSCs, this trend is supported by the work of DiGirolamo *et al*. [50], who found a decay in the adipogenic differentiation potential with passaging, combined with calcium deposition in the adipogenic cultures after passage 8. However, the effects of serial passaging on the multi-potent differentiation potential of ASCs have not been thoroughly characterized.

To better understand the effects of expansion on bASC differentiation, we compared the extent of differentiation towards the adipogenic, chondrogenic and osteogenic lineages between P2 and P5, based on the proliferation data and the common use of early passage cells in tissue-engineering applications. P2 was selected as a relevant control, as it is commonly used in differentiation studies in order to minimize the time in culture, while still obtaining a sufficiently sized cell population for experimental purposes [190]. In the present study, induced P2 bASCs had comparable levels of differentiation towards all three lineages investigated as what has been reported for ASCs from other species [11, 12, 14, 63, 93].

In terms of adipogenic differentiation, both GPDH enzyme activity and the presence of intracellular lipids indicated that there was a significantly higher level of differentiation in the P2 cells as compared to the P5 cells. This result was confirmed by C/EBPα and PPARγ gene expression analysis — two early markers of adipogenesis [191]. Both genes are required for lipogenesis, and the forced expression of either gene can induce adipogenesis in non-adipogenic cells [192-194]. While the P5 cells showed minimal expression of C/EBPα, PPARγ expression was absent in these cultures, indicative of a less-differentiated state. Similarly, Wall *et al*. [37] found decreases in PPARγ gene expression in human adipogenic cultures with extended serial passaging. LPL, a downstream gene of C/EBPα [195], was expressed in both the P2 and P5 cells at similar levels, indicating that the bASCs at both passages were responsive to the adipogenic stimuli by expressing this lipase, which is required for triglyceride uptake during lipogenesis [196].

The chondrogenic differentiation capacities of the bASCs were similar to the results observed with adipogenesis, in which there was a higher level of differentiation in the P2 cells. While the extent of collagen II staining was somewhat higher in the P2 induced cultures, GAG staining gave a better understanding of the differentiation state. The presence of GAG was more pronounced in the P2 induced cultures (compared to the induced P5 cultures) indicating an enhanced chondrogenesis in the early passage cells. This result was confirmed by quantification of sGAG content in the pellets by the DMMB assay, which clearly showed a statistically higher concentration in the P2 induced pellets. Similarly, the quantitative analysis of hydroxyproline content indicated that the P2 induced pellets contained a statistically higher total collagen content. Gene expression results confirmed these trends, as lower expression of SOX9 (an early chondrogenic marker [98, 197, 198]) and AGC (and no collagen II expression) were found in the P5 induced pellets. Interestingly, collagen I was detected in all of the induced cultures. The presence of collagen I may indicate the cultures were not fully differentiated towards a chondrogenic phenotype. It is well recognized that there is a close relationship between chondrogenesis and osteogenesis both *in vitro* and *in vivo* [129]. Thus, it is possible that the bASCs were driven towards an unstable chondrogenic state that could ultimately lead to osteogenesis in the cultures. This phenomenon is supported by the ALP data, which showed high levels of enzyme activity in the induced chondrogenic pellets.

In terms of the osteogenic potential of the bASCs, a different trend was observed. No detectable differences between P2 and P5 induced cultures were observed in

relation to all of the markers investigated, with the exception of ALP activity. Higher ALP activity levels were found in the P5 induced cultures, indicating a trend towards enhanced osteogenesis with serial passaging. This result is consistent with previous work on human bone marrow-derived MSCs, which demonstrated that osteogenesis tends to dominate at later passages, at the expense of both the adipogenic and chondrogenic potentials [36, 37]. In contrast, extensive passaging of porcine bone marrow-derived MSCs resulted in a loss in osteochondrogenic potential while favoring adipogenesis [66].

The variation in these studies emphasizes that it is critical to understand the differences in differentiation potential between cell donor sources, both in terms of the species, as well as the specific tissue depot from which the cells originate. The influences of the cell culture micro-environment are well recognized as an important factor in mediating cellular behavior. In particular, the growth substrate stiffness has been shown to influence stem cell specification [199]. Traditional plate culturing methods using 2-D tissue culture polystyrene provide a high-stiffness substrate, which is more similar to bone matrix, rather than that of fat or cartilage. Thus, the results combined with these findings, may suggest that long-term *in vitro* expansion using traditional cell culture substrates favors the osteogenic lineage.

To date, the growth kinetics and multi-potency of bASCs have not been well studied. In fact, for many species, the changes in the extent of multi-lineage differentiation of culture-expanded ASCs, even at early passages, have not been well characterized. For this investigation, the interdigital fat pad was selected on the basis

of convenience of acquisition and the assurance of sterility. Most ASC studies have been carried out using cells isolated from the subcutaneous abdominal, hip or breast regions. The literature results indicate that there is great variability in the ASCs isolated from the various tissue depots, which respond differently to various hormonal, chemical or mechanical stimuli [15]. As there is increasing evidence that MSCs reside in a perivascular niche [200, 201], the different degrees of vascularization of the adipose tissue sources may influence the characteristics of the isolated stem cell populations. In particular, the limited apparent vascularity of the interdigital fat pad may have played a role in the results obtained in this study. Overall, fat tissues from different locations behave differently in terms of growth kinetics and differentiation potential [190]. However, based on our results, the ASCs from bovine interdigital fat are capable of long-term *in vitro* expansion and multi-lineage differentiation potential similar to stem cells isolated from other sites on other species.

The success of utilizing stem cells in tissue-engineering applications is highly dependent on maintaining a satisfactory level of differentiation potential after extensive *in vitro* expansion. This chapter demonstrates that there are changes in the proliferation ability and multi-potency of bASCs upon cell expansion. While the P2 and P5 bASCs were capable of undergoing multi-lineage differentiation (adipogenic, chondrogenic and osteogenic), it was shown that adipogenesis and chondrogenesis were favored at lower passage, and osteogenesis was maintained, or potentially improved, upon increased passaging. Based on these findings, future studies should utilize bASC P2 cells as an effective cell source to study how differentiation can be

induced or enhanced during co-culture with freshly isolated mature cells, such as: adipocytes, chondrocytes and osteoblasts.

Chapter 4:

Multi-lineage Differentiation of Bovine Adipose-Derived Stem Cells in Co-culture Environments

4.1 Introduction

Exogenous growth factors are routinely used as stimulators for stem cell proliferation and differentiation. Both insulin-like growth factor (IGF) and fibroblast growth factor (FGF) have been shown to enhance mesenchymal stem cell (MSC) growth rate, while the transforming growth factor (TGF) superfamily and bone morphogenic proteins (BMPs) are highly effective at inducing differentiation towards musculoskeletal lineages, including: chondrogenesis, osteogenesis, and myognesis [12, 90, 202, 203]. Within the body, these mitogenic and differentiation factors are secreted by terminally-differentiated (i.e. mature) cells. For instance, adipocytes are highly active endocrine cells that secrete growth factors, cytokines, hormone-like molecules that can mediate the adipogenic differentiation of neighboring adipose-derived stem cells (ASCs) [204, 205], such as FGF-2 [206], adiponectin and insulin [207]. Chondrocytes also use autocrine and paracrine signaling, such as through the TGF-β superfamily and IGF, which promote differentiation [208]. Therefore, mature cells could be used in theory as a source of exogenous induction factors to enhance or enable stem cell differentiation within a co-culture environment.

Co-culturing of cells is a commonly-used method to study the dynamic interactions between different cells. *In vitro* co-culture of embryonic stem cells (ESCs) and somatic cells has successfully identified specific signaling molecules exchanged between these two cell types, which function in normal embryonic development [18]. Co-culturing of human bone marrow-derived MSCs (bMSCs) with osteoblasts from the same donor has also shown promise at enhancing bMSC differentiation along the osteogenic lineage [154, 163]. ASCs have also been investigated in direct co-culture (i.e. mixed cultures) with chondrocytes, which may induce chondrogenic differentiation of the ASCs [209]. However, as a relatively recently-identified cell source, ASCs have not yet been thoroughly investigated in co-culture environments with mature populations. The advantage of using co-culture with the ASCs is that it may be possible to more closely simulate the natural micro-environment in which MSCs are situated in the body, to promote more normal cellular organization and behavior. It is possible that co-culture may be used as a means to reduce or eliminate the dependence on costly inductive differentiation factors and hormones, which could be associated with undesirable systemic effects if applied *in vivo*. Co-culture may also hold the potential to promote a more homogenous cell response, as growth factor induction is typically only effective at inducing the differentiation of sensitize sub-populations of cultured MSCs, as observed in the histological staining experiments in the previous chapter.

As discussed in Chapter 2, there are two types of co-culture that are commonly implemented. Direct co-culture involves mixing two or more cell types together, and

facilitates communication through cell-cell contact. In contrast, indirect co-culture eliminates physical contact between the different populations, and therefore depends on paracrine signaling to facilitate crosstalk. [18] In this study, indirect co-culture was the focus, as it facilitates assessment on the individual cell populations, without the need for complex cell-separation techniques. Further, the results of previous studies with bMSCs and serially-cultured chondrocytes have indicated that cell-secreted paracrine factors are most crucial for mediating cell differentiation in co-culture [88].

Wnt proteins have been suggested to play an essential role in cell renewal and differentiation in both ESCs and MSCs. More specifically, signaling through the Wnt pathways can trigger the expression of early differentiation markers in cultured stem cells in a similar fashion to that of growth factors [21, 22]. Although the exact signaling mechanisms have not yet been precisely identified, there is evidence that both Wnt signaling pathways (i.e. canonical and non-canonical) may be involved. For example, through the β-catenin dependent canonical signaling pathways, Wnt 10b has been shown to block adipogenesis while promoting osteogenesis, and Wnt 3a is also commonly used to enhance osteogenesis [126, 130, 134, 144]. The activation of the Wnt non-canonical pathways can also influence cell fate, including through cytoskeletal reorganization via RhoA kinase (RHOA) and via c-Jun N-terminal kinase (JNK) during gastrulation [131, 140, 149, 210].

Since mature cells are known to self-regulate through autocrine signaling, it is of interest to investigate whether these factors can also direct ASC lineage commitment and differentiation (adipogenic, chondrogenic, osteogenic). Thus, this study will

present the assessment of differentiation induced by co-culturing bovine ASCs (bASCs) with mature bovine adipocytes, chondrocytes and osteoblasts in terms of characteristic enzymatic activity, extracellular matrix (ECM) accumulation and gene expression of key transcriptional factors. As Wnt signaling plays an important role in stem cell differentiation, it was also of interest to probe the specific influence of these pathways on the differentiation process in the co-culture environments.

While Chapter 3 focused on the use of conventional growth factors to induce differentiation, this Chapter demonstrated the feasibility of inducing differentiation through co-culturing ASCs with mature cells in the absence of key exogenous factors. This study also sought to determine if Wnt signaling pathways played a role in co-culture induced ASC differentiation towards each lineage. Overall, this is the first study to investigate ASC multilineage differentiation through co-culturing with mature cells of three different lineages (adipogenesis, chondrogenesis and osteogenesis).

4.2 Materials and Methods

4.2.1 Materials:

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and were used as received.

4.2.2 Cell isolation and Co-culture:

For co-culture experiments of all three lineages, bASCs in co-culture (referred to as co-culture) and bASCs cultured alone (ASC-alone) were cultured in parallel in defined co-culture medium. Triplicate samples were included in every assessment.

4.2.2.1 Bovine ASC Isolation and Culture:

bASCs were extracted under sterile conditions from the interdigital fat pad in the hoof, from tissues obtained from a commercial slaughterhouse. Cell isolation and culture methods were based on human ASC extraction protocols described by Flynn *et al.* [174], and are described in detail in section 3.2.2. Passage 2 bASCs were used for all co-culture experiments.

4.2.2.2 Adipocyte Isolation and Adipogenic Co-culture:

a) Adipocyte Isolation

The fat pads harvested from the bovine interdigital area were minced and digested under agitation (120 rpm) at 37 ºC for 1 hour in Kreb's Ringer buffer containing 2% collagenase type VIII, 3 mM glucose, 25 mM HEPES, and 20 mg/mL bovine serum albumin (BSA). The mixture was filtered through a 100 μm nylon mesh and then centrifuged at 300 x g for 10 min. The adipocytes present in the layer on top of the supernatant were then transferred into a new 50 mL conical tube using a sterile spoonula. The extracted adipocytes were then gently washed 2 times with 20 mL of

growth medium (DMEM:Ham's F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep)) followed by centrifugation at 300 x g for 10 min. Finally, the adipocytes were transferred into a 35 mm Petri dish overnight before seeding. For each adipocyte suspension obtained from 5 mL of minced adipose tissue, 5 mL of growth medium was added to the culture. The cultured adipocytes were evenly suspended, floating on top of the medium, and were used for co-culture 12 – 24 h after isolation.

b) Adipogenic Co-culture

To prepare the co-cultures the freshly isolated adipocytes (as described in the previous section) were gently mixed, and then the adipocyte cell suspension was transferred (0.5 mL/insert) into pre-sterilized transwell standing inserts (Cat # PICM01250, Millicell, MA, USA) using a 5 mL serological pipette. The inserts were placed in 24-well cell culture plates, which had been seeded (48 h prior) with passage 2 bASCs at a density of 50,000 cells/cm^2. Both co-culture and ASC-alone wells were supplied with a total of 1.5 mL of growth medium per well, and the medium was changed 3 times per week. One week after the initiation of the co-culture, the adipocytes were discarded by aspiration and replaced with fresh adipocyte isolates, to avoid the potential for confounding effects due to adipocyte de-differentiation. The transwell configuration for the adipogenic co-culture is illustrated in Figure 4.2 A.

The level of adipogenesis was assayed on the bASCs in both co-culture and ASC-alone configurations using the glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity assay (n=3, N=3) as well as Oil Red O staining for intracellular lipid

accumulation (n=3, N=3). All assays were conducted 14 days after the initiation of co-culture, using the methods described in Chapter 3 (Section 3.2.4.1).

GPDH enzymatic assay:

Triplicate wells of bASC in co-culture and bASC-alone were measured using the GPDH assay, with a GPDH measurement kit (Cat #: KT-010, Kamiya Biomedical, Seattle, WA, USA). The data was normalized to the total intracellular protein concentration in each sample, measured using the Bio-Rad protein assay (Cat #: 500-0002, Bio-Rad Laboratories, Inc. CA, USA) with an albumin standard. One unit of GPDH activity was defined as the amount required to consume 1 μmol of NADH in 1 minute.

Oil red O staining:

For Oil red O staining, 1 mL of Oil red O working solution [174] was applied to each well for 5 min, followed by extensive rinsing with distilled water (dH_2O) and counterstaining with hematoxylin for 2 min.

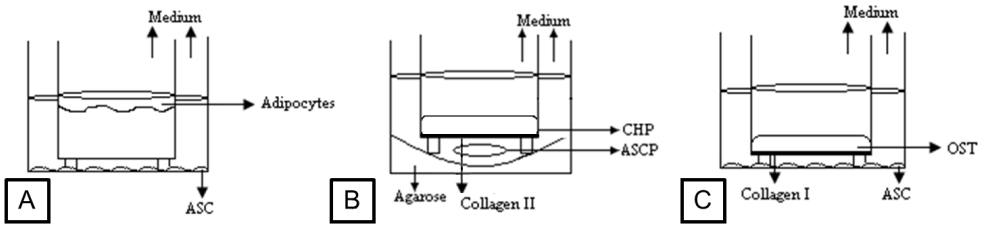

Figure 4.1 Transwell setup for the (A) Adipogenic (B) Chondrogenic and (C) Osteogenic co-cultures. CHP – chondrocyte pellet, ASCP – ASC pellet, OST – osteoblasts.

4.2.2.3 Chondrocyte Isolation and Chondrogenic Co-culture:

a) Chondrocyte Isolation

Primary articular chondrocytes were isolated from the metacarpalephalangeal joint of 12- to 18-month-old calves by sequential enzymatic digestion, as described by Boyle *et al*. [186] Chondrocytes were obtained from 2 - 3 animals and pooled together in order to have a sufficient number of cells for each trial. Briefly, cartilage pieces were removed surgically and digested in protease solution (P5147) (0.5% w/v in Ham's F-12 (SH3001002, Utah, HyClone)) for 2 hours. Then, samples were washed with Ham's F12 media 2 times and followed by 0.15% w/v collagenase A (Roche) digestion for 18 hours. The mixture were then filtered, washed and centrifuged to obtain chondrocytes pellets. Prior to co-culture seeding, the chondrocytes were maintained in growth medium. The isolated primary chondrocytes were freshly prepared and seeded for each co-culture experiment right after isolation.

b) Chondrogenic Co-culture

For the co-culture experiments, three-dimensional (3-D) pellet cultures of bASCs (2×10^6 cells/pellet; ASCP) and chondrocytes (2×10^6 cells/insert; CHP) were prepared. Passage 2 bASC pellets were obtained by centrifugation (300 x g for 10 min) in 15 mL conical tubes, and then cultured in 24-well cell culture plates coated with 2% agarose (type VII, in DMEM:Ham's F-12), to minimize cell attachment and spreading. To prepare the CHP, primary chondrocytes (freshly isolated) were seeded on collagen II (C9301)-coated transwell inserts at density of 2×10^6 cells/insert in 0.5 mL of growth medium. The chondrocyte layer naturally condensed at the bottom of the insert by gravity settling. Both seeded bASCP and CHP were maintained in growth medium for 48 h to promote stable cell pellet formation. The bASCP and CHP

transwell inserts were then combined in the agarose-coated 24-well cell culture plate, as shown in Figure 4.2 B. The growth medium was removed by aspiration, and each well was supplied with 2 mL of chondrogenic co-culture medium (50 μg/mL ascorbate-2-phosphate (A2P), 6.25 μg/mL insulin, 100 nM dexamethasone (DEX) in growth medium), which was changed 3 times per week. For the ASC-alone control cultures, the bASCs were maintained in the pellet culture configuration (ASCP) in chondrogenic co-culture medium, as described.

The level of chondrogenesis was analyzed on the bASCs in both co-culture and ASC-alone cultures using toluidine blue staining of glycosaminoglycan (GAG) (n=3, N=3) and fluorescence immunohistochemical (FIHC) localization of collagen types I and II. Quantitative analysis of sulphated GAG, by the dimethylene blue (DMMB) assay, and collagen content, estimated by the hydroxyproline assay, were also utilized as measures of chondrogenic differentiation. All assays were conducted at 14 days after the initiation of co-culture.

Toluidine Blue Staining:

For toluidine blue staining, triplicate (n=3) bASC pellets from both the co-culture and ASC-alone configurations were placed in 5.4% sucrose solution (in phosphate buffered saline (PBS)) overnight, followed by immersion into O.C.T. freezing medium (Tissue-Tek®) and snap freezing in liquid nitrogen. All frozen samples were sectioned by Judy Pang in the department of Anatomy and Cell Biology, Queen's University for histological and FIHC staining. For toluidine blue staining, the slides were stained with 0.1% toluidine blue O (Sigma, T3260) solution for 2 - 3 minutes.

FIHC staining for Collagen I and II:

For FIHC staining, triplicate sections of each co-culture and ASC-alone sample were incubated in 0.5 U/mL chondroitinase (Sigma, C2905) and 0.5 U/mL keratanase (Sigma, K2876), each for 30 min, to facilitate antigen availability. The sections were then blocked with serum for 30 min, and then incubated overnight with the 1° antibody (either 1:200 dilution of mouse polyclonal anti-collagen II (Iowa Hybridoma Bank, Iowa City, IA) or 1:10 dilution of anti-rabbit collagen I (Fitzgerald 70-XR90, Boston MA)) at 4°C. Detection was facilitated using the collagen II anti-mouse vector F1-2000 (fluorescein) for collagen II and the collagen I anti-rabbit vector T1-1000 (Texas Red) for collagen I (Biolynx). 100 µl of DAPI (D9564) solution at a concentration of 100 nM was used as the counterstain to detect cell nuclei. Controls without primary antibodies were included to detect non-specific staining.

Sulphated GAG and Collagen Content:

The sulphated GAG and collagen content were measured after 14 days in culture in the chondrogenic co-culture medium. Triplicate bASC samples (n=3) in co-culture and ASC-alone were washed with PBS and digested in papain solution (40 mg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol (DTT)) for 72 h at 65 ºC. The papain digest aliquots were stored at -20 ºC and assayed separately for the sulphated GAG [177] hydroxyproline and DNA contents [178], as described in Chapter 3 (Section 3.2.4.2).

4.2.2.4 Osteoblast Isolation and Osteogenic Co-culture:

a) Qsteoblast Isolation

Primary osteoblasts were isolated from the cancellous bone of the sacral vertebrae of 12- to 18-month-old calves, obtained from a commercial slaughterhouse, based on adaptations to established protocols [211]. For decontamination purposes, the bovine tails were first washed with tap water and soaked in 70% ethanol for 90 min. The overlying muscle was removed using surgical blades (Figure 4.1). In order to access the cancellous bone, a sterilized electrical saw blade was used to cut off the compact bone, to create a surface for cancellous bone harvesting. A sterile curette was use to extract the cancellous bone chips, which were transferred into separate 50 mL conical tubes.

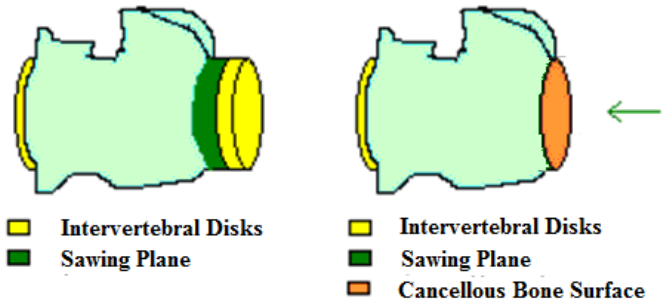

Figure 4.2 Illustration for bone chip harvesting.

The bone chips obtained from 3 – 4 vertebrae were harvested and pooled together, and washed in DMEM:Ham's F12 media under agitation (120 rpm) at 37 ºC for 5 min, followed by enzymatic digestion in 0.25% trypsin/0.1% EDTA (Gibco, Burlington, Canada) for 5 min. The bone chips in each tube were then further digested with 25 mL of 0.5 % collagenase A (in DMEM:Ham's F12 media) at 37 ºC for 30 min, and then

washed twice with DMEM:Ham's F12 medium. Bone chips from each tube were then transferred into 75 cm^2 tissue culture flasks (T-75, Corning) with 20 mL of growth medium (DMEM:Ham's F12, 10% FBS, 1% pen-strep). The medium was changed 3 times per week, and the isolated osteoblasts would reach 80% confluence within 1 - 2 weeks.

b) Osteogenic Co-culture:

To prepare for co-culturing, passage 2 bASCs were plated in 24-well plates at a density of 20,000 cells/cm^2 and cultured in growth medium. Similarly, the isolated osteoblasts were seeded onto collagen I-coated (Sigma, C3867) transwell inserts at a density of 20,000 cells/cm^2 and cultured in growth medium. After 48h, the osteoblast-seeded inserts were placed in the bASC-seeded 24-well plates in osteogenic co-culture medium (50 μM A2P, 10 mM β-glycerophosphate, 100 nM DEX in growth medium) [12]. The medium was changed 3 times per week. Osteogenic differentiation was measured in the bASCs using the alkaline phosphatase (ALP) activity assay (n=3, N=3) as well as von Kossa staining for calcium deposition (n=3, N=3), at 14 days after the initiation of co-culture.

Alkaline Phosphatase Activity:

Triplicate bASC samples from the co-culture and ASC-alone configurations were used in the assay, using the methods described in Chapter 3 (Section 3.2.4.3). In brief, the p-nitrophenol phosphate liquid substrate system (Sigma, N7653) was used [182-184], and the results were normalized to the total intracellular protein content, measured using the Bio-Rad protein assay with an albumin standard. One unit of ALP

activity was defined as the amount of enzyme required to catalyze the liberation of 1 μmol of p-nitrophenol per minute.

Von Kossa Staining:

Triplicate samples of the bASCs in co-culture and ASC-alone were processed, as described in Chapter 3, Section 3.2.4.3

4.2.4 Wnt Inhibitor Study

To probe the role of the Wnt pathways in co-culture mediated bASC differentiation, recombinant human Wnt inhibitory factor-1 (WIF-1) and DKK-1 (R&D) were used as Wnt inhibition factors, and were added to samples of each of the 3 lineages, in the appropriate co-culture medium, at 200 ng/mL [212] and 20 ng/mL [163] respectively (Table 4.1). The co-culture without inhibition is referred to as "Co-None", co-culture with WIF-1 is "Co-WIF", co-culture with DKK-1 is "Co-DKK", while the bASC-alone without inhibition is "AL-None". All quantitative lineage specific assessments (i.e. GPDH, sGAG and collagen content, ALP) in section 4.2.3 were performed using identical methods for all samples, as listed in Table 4.1.

Table 4.1 Concentration of Recombinant Wnt inhibitors in co-culture environment

Adipogenic	Co-culture	ASC-alone
No inhibitor	None	None
W/DKK-1	20ng/ml	N/A
W/WIF-1	200ng/ml	N/A
Chondrogenic	**Co-culture**	**ASC-alone**
No inhibitor	None	None
W/DKK-1	20ng/ml	N/A
W/WIF-1	200ng/ml	N/A
Osteogenic	**Co-culture**	**ASC-alone**
No inhibitor	None	None
W/DKK-1	20ng/ml	N/A
W/WIF-1	200ng/ml	N/A

4.2.5 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis:

Lineage specific gene expression was assessed in all three investigated lineages at 14 days after the initiation of co-culture (n=3, N=3), using the methods described in Chapter 3, Section 3.2.5. In brief, total RNA was extracted from the cells (co-culture and ASC-alone samples, both with and without the Wnt inhibitory factors) using TRIzol® Reagent (Invitrogen Canada Ltd., Burlington, ON, Canada). cDNA was synthesized from 1 μg of total RNA using random primers (Invitrogen) and SuperScript™ II Reverse Transcriptase (RT) (Invitrogen), in a 20 μL reaction volume. The gene-specific primers for the PCR reactions are listed in Table 4.2, with GAPDH and 18S as the housekeeping genes [185]. Each PCR reaction was conducted in a 50 μL reaction volume with 2.5 μL of diluted cDNA (containing 50 ng of input RNA), with the number of cycles depending on the gene of interest (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) using a Bio-Rad C1000 thermal cycler, as presented in Table 4.2. Gene expression was analyzed following 5% agarose gel electrophoresis by

ethidium bromide staining (G:box Chemi HR16, Syngene, Cambridge, UK). Minus-RT and no template controls were included in every run.

Table 4.2 Bovine-specific primer pairs used for RT-PCR

Markers	Gene	Primer sequence (5'~3')	Amplicon size	Cycles
Adipogenic	C/EBPα	Forward: ATCGACATCAGCGCCTACAT Reverse: CGGGTAGTCAAAGTCGTTGC	138	40
	PPARγ	Forward:CAGTGTCTGCAAGGACCTCA Reverse: GATGTCAAAGGCATGGGAGT	128	40
	Lipoprotein Lipase (LPL)	Forward: TGCTGGTATTGCAGGAAGTC Reverse: AAAATCCGCATCATCAGGAG	124	40
Chondrogenic	Collagen II	Forward:CTCAAGTCCCTCAACAACCAG Reverse: TTGGGGTCGATCCAGTAGTC	134	40
	Aggrecan (AGC)	Forward: CAGTCACACCTGAGCAGCAT Reverse: CCTTCGATGGTCTTGTCGTT	104	40
	Sox9	Forward: AGAAGGACCACCCGGACTAC Reverse: CGTTCTTCACCGACTTCCTC	57	40
Osteogenic	Collagen I	Forward: ATGGAACTCCAGGTCAAACG Reverse: CACCAACACGTCCTCTCTCA	60	40
	Osteocalcin (OCN)	Forward: CATGGGAGCTGGGAGAGTAA Reverse: AAGGGGCAAATGGATTAAG	113	40
	RUNX2	Forward:CAGATGACTCCTCCGTCCAT Reverse: ACTGAGAGTGGAAGGCCAGA	147	40
Housekeeping	GAPDH	Forward: CACCCTCAAGATTGTCAGCA Reverse: GTCTTCTGGGTGGCAGTGAT	135	40
	18S	Forward: ATGGCCGTTCTTAGTTGGTG Reverse: GAACGCCACTTGTCCCTCTA	136	40
Wnt signaling pathway related genes	β-catenin	Forward: GGATGTGGATACCACCCAAG Reverse: CCCTCATCTAGCTCTCAGG	153	35
	RHOA	Forward: AAGGACCAGTTCCCAGAGGT Reverse: TTCCGGAGTCCATTTTTCTG	228	35
	JNK1	Forward: CAGTTGGTGGAGGCCTTTTA Reverse: TCCCATAGGCATGGAGTAGC	174	35

4.2.6 Statistical Analysis:

Unless otherwise stated, all numerical data is presented as the Mean ± Standard Error. For each experiment, the number of samples per group (n), plus the number of times each experiment was repeated with cells from a different donor (N) are indicated. Within an individual trial, all groups were seeded with bASCs isolated from one donor, to eliminate the potential for donor-to-donor variability. The significance of the quantitative data was assessed where appropriate using a one-way ANOVA with the Tukey's post-hoc test, performed using Origin Pro8 statistical software, with ($p < 0.05$).

4.3 Results

4.3.1 Cellular Isolation:

Adipocytes from the interdigital adipose tissue pad were easily isolated in the floating layer on top of the medium after enzymatic digestion and centrifugal separation. Small amounts of extracellular lipid, from cell lysis during digestion, were transferred with the adipocytes, but gentle technique was used to minimize the amount free oil.

For the osteoblast isolation, the digested bone chips in the flasks were maintained in static culture so that the osteoblasts from the inner porous structures migrated out of the bone matrix. Using the developed methods, as the osteoblasts proliferated, they expanded over the surface of the flasks. Osteoblasts in the expansion culture had a cubical morphology once a high cell density was achieved. During the extraction,

residual lipids, blood cells and debris from the bone marrow were eliminated at the initial medium change.

4.3.2 Adipogenic Co-culture:

Samples from the adipogenic co-cultures and ASC-alone cultures were analyzed for levels of GPDH enzyme activity, intracellular lipid accumulation and mRNA expression of adipogenic markers to determine the feasibility of co-culturing with mature adipocytes to induce bASC adipogenesis (Figures 4.3 - 4.5) in the absence of exogenous differentiation factors. bASC GPDH enzymatic activity showed an upregulation during co-culture in growth medium, with the average level approximately 3 times higher than that observed for the ASC-alone.

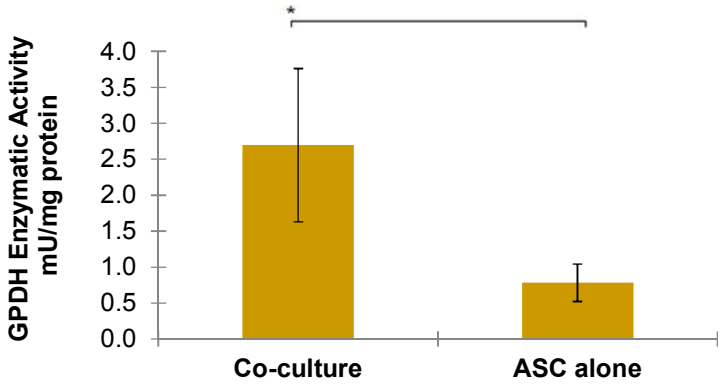

Figure 4.3 Adipogenic differentiation of bASCs in co-culture assessed by GPDH enzyme activity (n=3, N=2). bASCs showed an upregulation in GPDH activity in the co-culture environment, as compared to bASCs cultured alone. * indicates statistical significance ($p<0.05$).

While the bASC-alone samples showed no detectable intracellular lipid accumulation (Figure 4.4 A) after Oil red O staining, the bASCs in co-culture demonstrated extensive positive staining (Figure 4.4 B) all over the culture plates.

Almost 100% of the co-cultured bASCs contained multiple intracellular lipid droplets, as compared to the only 40 – 50% of cells that responded on average to adipogenic growth factor stimulation (Chapter 3). In co-culture, the droplets were small and less easy to distinguish; however, they were closely packed around the nucleus of the cell.

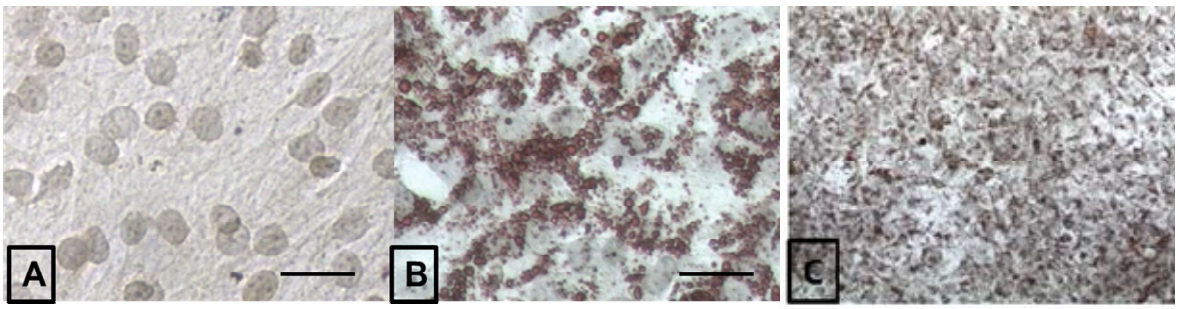

Figure 4.4 Oil red O staining of bASCs in **(A)** ASC-alone culture, and
(B) Co-culture with mature adipocytes. Scale bars = 10 μm.
(C) Differentiated ASC distribution at lower magnification, 200X original magnification.

The C/EBPα, PPARγ and lipoprotein lipase (LPL) adipogenic genes were all expressed in the bASCs in co-culture, but remained undetected in the ASC-alone cultures (Figure 4.5).

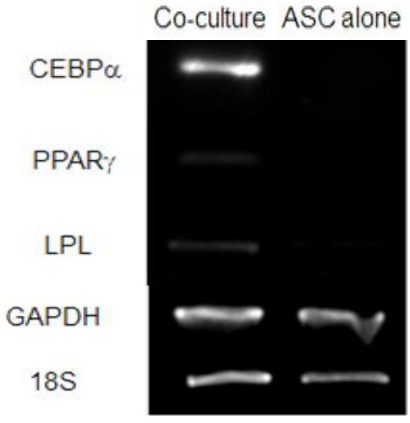

Figure 4.5 Adipogenic gene expression (C/EBPα, PPARγ, and LPL) in co-culture and ASC-alone cultures. GAPDH and 18S are included as internal housekeeping genes.

4.3.3 Chondrogenic Co-Culture:

bASC pellets in co-culture and ASC-alone cultures were analyzed for GAG accumulation, collagen I and II protein expression, as well as chondrogenic gene expression, to detect co-culture-induced chondrogenesis. In all of the pellets, the cells maintained a packed and rounded morphology for the 2-week culture period. The pellets in co-culture had a glassier appearance as compared to the ASC-alone pellets. There were no macroscopic size changes in the pellets by visual observation. The sections from the ASC-alone pellets displayed more limited GAG staining (Figure 4.6 A), whereas co-culture induced pellets had slightly more intense staining (Figure 4.6 B), potentially indicative of higher GAG content.

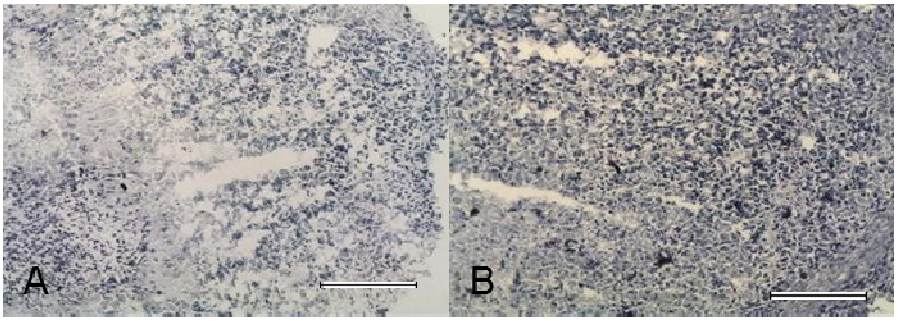

Figure 4.6 Chondrogenic co-culture differentiation of bovine ASCs assessed by toluidine blue staining of GAG in **(A) b**ASC-alone pellets, **(B)** bASC pellets in co-culture with chondrocytes. Scale bars = 100μm.

In the bASC co-culture pellets, collagen type II (Figure 4.7) was expressed (visualized as a bright green color by FIHC), with more intense staining in the exterior region of the pellets. DAPI-stained cell nuclei (blue) demonstrated cell distribution throughout the entire pellet, but there was also more intense staining in the exterior regions (Figure 4.7 A). In contrast, the ASC-alone culture had no

detectable staining for collagen II, but there was positive staining with DAPI (Figure 4.7 C & D). In contrast, collagen I staining with texas red was detected in both the co-culture and ASC-alone culture configurations, with no observable differences between the two groups (Figure 4.8). DAPI staining indicated cell distribution throughout the pellets.

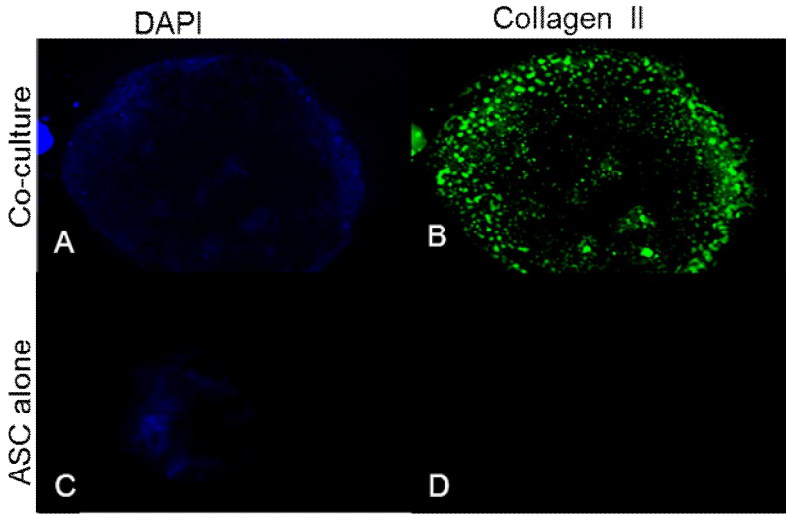

Figure 4.7 FIHC staining in bASC co-culture pellets, of **(A)** cell nuclei by DAPI, **(B)** Collagen type II; FIHC staining of ASC-alone pellets, of **(C)** DAPI, **(D)** Collagen type II.

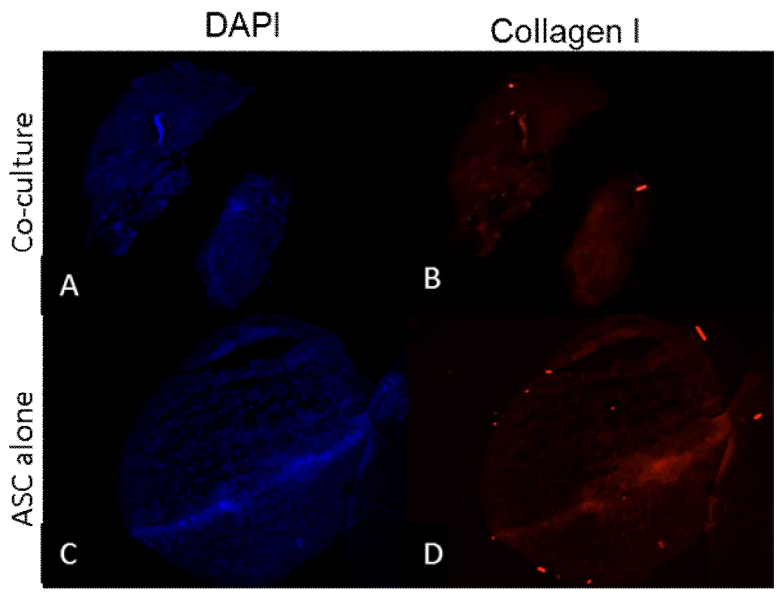

Figure 4.8 FIHC staining in bASC co-culture pellets, of **(A)** DAPI, and **(B)** Collagen type I; FIHC staining of bASC-alone pellets, of **(C)** DAPI, and **(D)** Collagen type I.

The DMMB assay (Figure 4.9) indicated that there was a statistically higher sGAG content in the co-culture bASC pellets than the ASC-alone pellets. Further, the higher mean hydroxyproline content measured in the bASC co-culture pellets suggested a higher total collagen content, as compared to the ASC-alone pellets. (Figure 4.10). Overall, sGAG and total collagen in the bASC pellets in co-culture were approximately twice as much as in the ASC-alone samples.

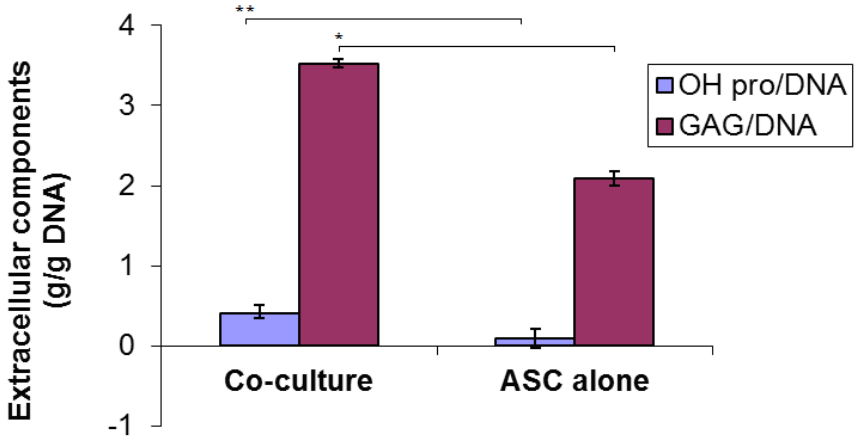

Figure 4.9 Mean hydroxyproline content and sulphated GAG content (measured using the DMMB assay) in the chondrogenic testing of pellet-cultured bASCs (n=3, N=2). All data was normalized to the total DNA content in each sample. Error bars represent the standard deviation. * and ** indicates statistical significance ($p<0.05$).

In terms of chondrogenic gene expression (Figure 4.10), the bASCs in co-culture expressed collagen II, aggrecan (AGC) and low levels of SOX9, while the ASC-alone pellets had no detectable expression of all three chondrogenic markers

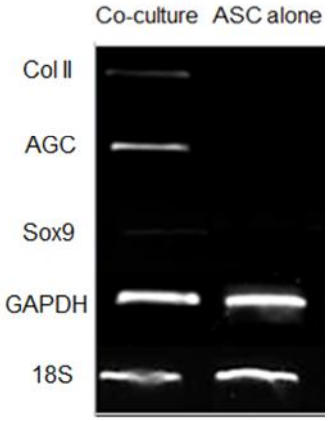

Figure 4.10 Chondrogenic gene expression (collagen II, aggrecan and SOX9) compared between bASC pellets in co-culture and ASC-alone culture. GAPDH and 18S are included as internal housekeeping genes.

4.3.4 Osteogenic Co-Culture:

bASCs from co-culture with osteoblasts and ASC-alone were analyzed for levels of ALP enzyme activity, calcium deposition, and osteogenic gene expression using RT-PCR to determine the feasibility of co-culture enhanced differentiation. After 14 days, white tissue aggregates could be readily visualized by eye within the bASC layers in both the co-culture and ASC-alone cultures. The ALP activity (Figure 4.11) of the bASCs in co-culture was approximately twice as high as it was in the ASC-alone cultures.

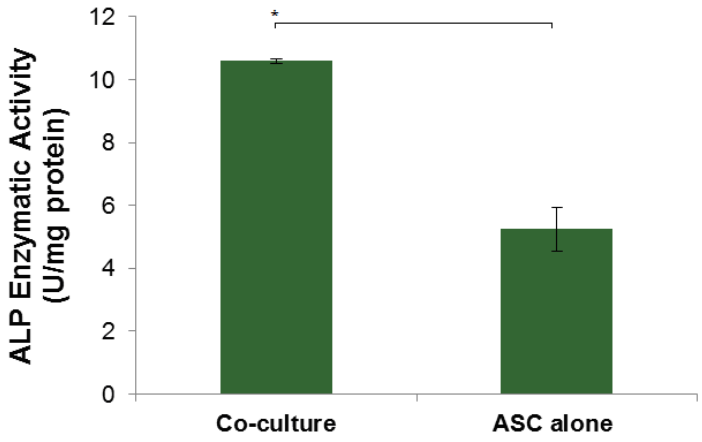

Figure 4.11 Co-culture enhanced osteogenic differentiation of bovine ASCs assessed by ALP enzyme activity. (n=3, N=2), * indicates statistical significance ($p<0.05$).

While calcium deposition demonstrated obvious staining in both the bASCs in co-culture and ASC-alone cultures, it was difficult to determine whether one configuration showed more extensive matrix mineralization. In general, the bASCs aggregated together to form defined bone nodules in both cultures (Figure 4.12).

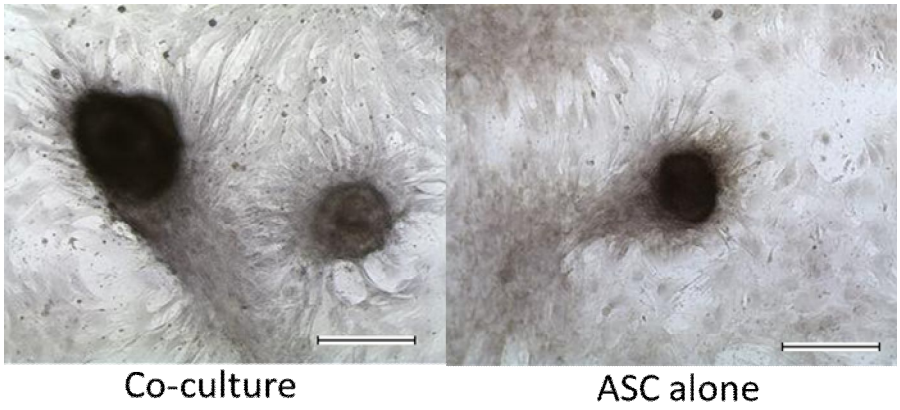

Figure 4.12 Osteogenic differentiation of bASCs assessed by von Kossa staining for mineralization of **(A)** co-culture and **(B)** ASC-alone culture. Scale bars = 25 μm.

The osteogenic gene expression results were slightly more inconsistent, as compared to the results for the other lineages. Collagen type I, the primary organic structure in bone, was upregulated in co-culture. Runx2 in the co-culture samples had a similar, or potentially slightly lower, intensity as compared to the ASC-alone cultures. Interestingly, OCN was significantly lower in co-culture than in the ASC-alone cultures (Figure 4.13).

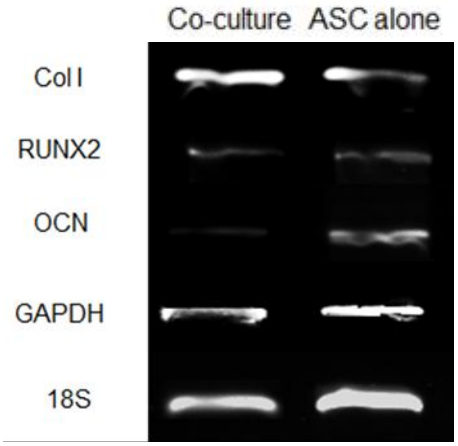

Figure 4.13 Osteogenic gene expression (collagen I, RUNX2, and osteocalcin) in co-culture and ASC-alone samples, GAPDH and 18S are included as internal housekeeping genes.

4.3.5 The Inhibiton of Wnt Signaling Pathways

4.3.5.1 Adipogenic Co-Culture

bASCs from the Wnt-inhibited and non-inhibited control cultures were tested for GPDH enzymatic activity and gene expression of the key adipogenic markers (C/EBPα, PPARγ, and LPL), as well as the Wnt-signaling pathway related markers (β-catenin, JNK1 and RHOA), by RT-PCR (Figure 4.14). According to the results, all of the co-cultures had a higher level of enzymatic activity than the ASC-alone cultures. Co-WIF was not significantly different from the Co-None group, but the Co-DKK group showed an almost 3-fold increase in GPDH activity relative to the Co-None group and a 9-fold increase relative to the ASC-alone culture group (Figure 4.14 A).

The gene expression analysis of all three adipogenic markers showed more intense bands in the Co-WIF and Co-DKK groups in comparison to the Co-None group. LPL expression was highest in the Co-DKK group, which supported the GPDH enzymatic assay results. Gene expression analysis of β-catenin showed a similar intensity in all co-culture samples, which was higher than the bASC-alone samples. JNK1 expression was similar between the ASC-alone and Co-None groups, but elevated in both the Co-WIF and Co-DKK groups, with the highest levels detected in the Co-DKK samples. RHOA was upregulated in all of the co-cultures, relative to the ASC-alone controls, and was highest in the Co-WIF group.

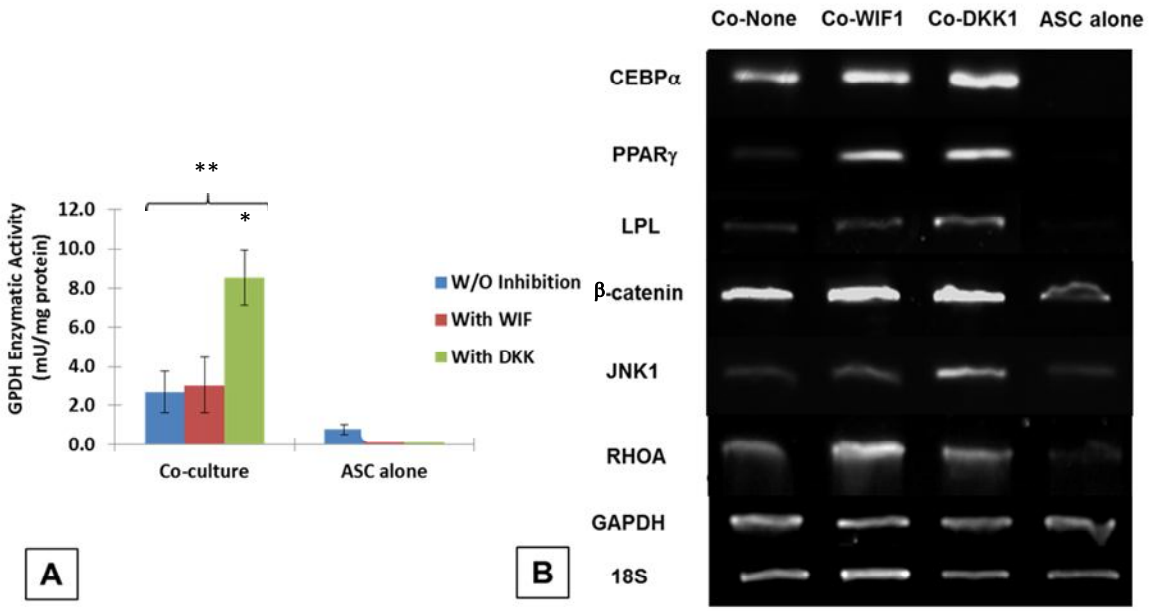

Figure 4.14 The effect of recombinant Wnt inhibitors on co-culture induced adipogenic differentiation (A). Co-culture with or without Wnt inhibitors (WIF-1 or DKK1). The GPDH enzyme activity was upregulated significantly in all co-cultures, with the highest levels in the DKK1 inhibitor group (* and ** represent statistically significant). (B) Adipogenic gene expression (C/EBPα, PPARγ, and LPL); Wnt signaling pathway related gene expression (β-catenin, JNK1 and RHOA) in bASCs in co-culture with mature adipocytes and ASC-alone culture. GAPDH and 18S are included as internal housekeeping genes.

4.3.5.1 Chondrogenic co-culture

The bASC pellets from the Wnt-inhibited and non-inhibited cultures were analyzed for sGAG and total collagen content, as well as gene expression analysis of the chondrogenic markers (Collagen type II, AGC, Sox9) and Wnt signaling pathway-related markers (β-catenin, JNK1 and RHOA), to determine how the Wnt pathways influenced the observed co-culture-induced chondrogenic differentiation of the bASCs.

A statistically higher hydroxyproline content was measured in the bASC co-culture pellets, consistent with the previous results that suggested they contained a

higher total collagen content relative to the ASC-alone pellets (Figure 4.15 A). Although there appeared to be decline in the hydroxyproline content with the addition of the Wnt inhibitors (i.e. Co-WIF and Co-DKK groups), the levels were not significantly lower than in the Co-None group. The DMMB assay (Figure 4.15 B) indicated that there was a statistically higher sGAG content in all of the co-culture induced pellets, with the highest levels measured in the Co-WIF group, although there was no statistical difference between the three co-culture conditions.

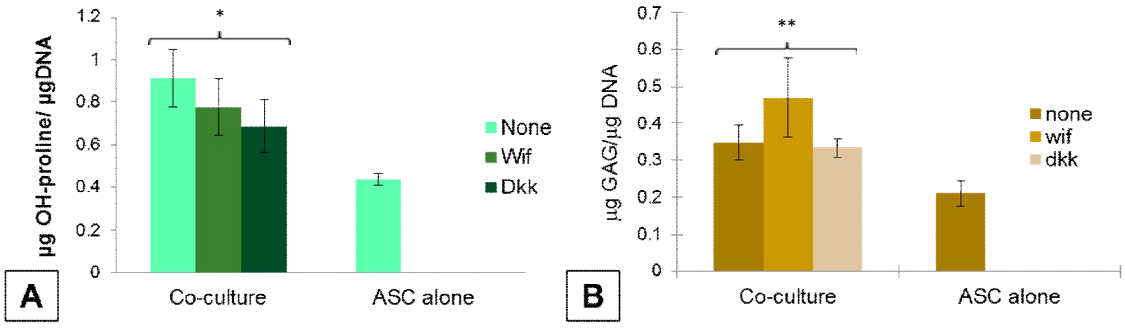

Figure 4.15 **(A)** Hydroxyproline content and **(B)** sulphated GAG content, in the bASC pellets (n=3, N=2) in the Wnt signaling pathway inhibition study. All data was normalized to the total DNA content in each sample. Error bars represent the standard deviation. * and ** Indicates statistical significance between co-cultures and ASC-alone cultures ($p < 0.05$).

In terms of the chondrogenic gene expression studies, collagen II showed increasing intensity in the co-culture groups, with the levels increasing in the order of the Co-None, Co-WIF and Co-DKK groups. The levels of intensity of AGC gene expression demonstrated no detectable differences among three co-culture conditions. Sox9 expression was slightly higher in Co-DKK, as compared to the Co-None group, although the expression was generally very low in all of the groups. All of the bASC co-cultures demonstrated downregulated expression of β-catenin relative to the ASC-alone group, with Co-DKK having the lowest levels of expression. Out of the

co-culture conditions, both β-catenin and JNK1 expression were highest in the Co-WIF group, with similar JNK1 expression in all of the other cultures. RHOA was downregulated in the Co-DKK and Co-WIF groups.

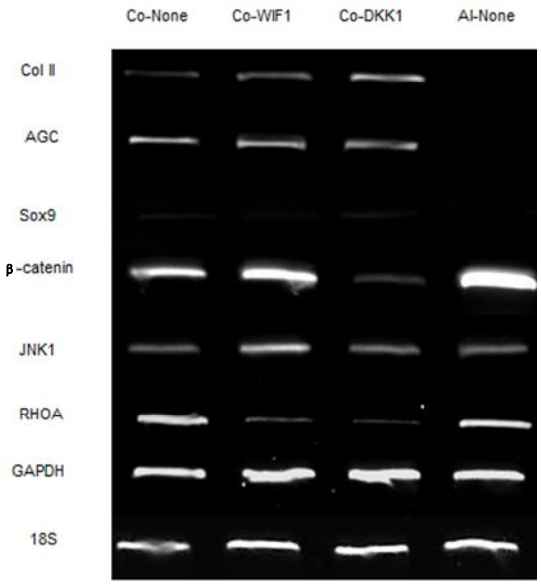

Figure 4.16 Chondrogenic gene expression (Collagen II, aggrecan, and Sox9); Wnt signaling pathway-related gene expression (β-catenin, JNK1 and RHOA) in chondrocyte-induced bASC co-cultures, both with and without the Wnt inhibitory factors, and ASC-alone cultures (AL-None). GAPDH and 18S are included as internal housekeeping genes.

4.3.5.1 Osteogenic Co-culture

bASCs from the Wnt-inhibited and non-inhibited cultures were analyzed in terms of ALP enzyme activity, as well as gene expression of osteogenic markers (ColI, Runx2, and OCN) and Wnt signaling pathway-related markers (β-catenin, JNK1 and RHOA) using RT-PCR, to probe the role of the Wnt pathways in osteogenic differentiation of bASCs in co-culture with mature osteoblasts.

All bASCs in co-culture demonstrated higher ALP enzymatic activity than the ASC-alone cultures, with the Co-DKK group showing a statistically lower level of

ALP enzymatic activity (Figure 4.17 A) than the other co-culture conditions. In terms of the osteogenic gene expression (Figure 4.17 B), OCN showed an increase in the Co-DKK co-culture condition and ASC-alone cultures, while Runx2 gene expression was decreased in the Co-DKK group. No detectable differences in collagen I expression were observed between any of the groups.

In terms of the Wnt signaling pathway-related genes, β-catenin showed the lowest levels of expression in the Co-None group, with similar levels detected in all of the other samples (Figure 4.17 B). The expression levels of JNK1 were similar in all of the samples, with the exception of the Co-DKK group, which was somewhat lower. The expression of RHOA was also similar in all of the culture conditions.

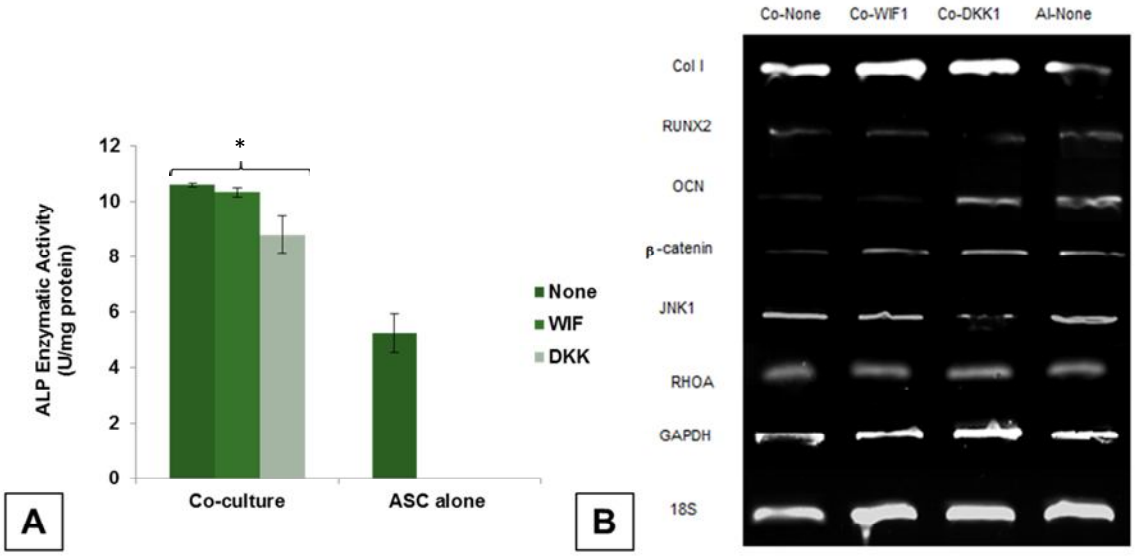

Figure 4.17 The effect of recombinant Wnt inhibitors on co-culture enhanced osteogenic differentiation of bASCs. **(A)** Co-culture with or without Wnt inhibitors upregulated ALP enzymatic activity. **(B)** Osteogenic gene expression (Collagen I, Runx2, and osteocalcin); Wnt signaling pathway related gene expression (β-catenin, JNK1 and RHOA) in osteoblast-enhanced co-culture of bASCs and ASC-alone cultures. GAPDH and 18S are included as internal housekeeping genes. * indicates all co-cultures had statistically higher ALP levels than ASC-alone cultures ($p < 0.05$).

4.4 Discussion

In this study, it was demonstrated that bASCs in co-culture with allogenic mature cells (adipocytes, chondrocytes, osteoblasts) displayed lineage-specific markers of differentiation in the absence of exogenous growth factor stimulation. Methods were established to conduct indirect co-culturing with the bASCs using transwell systems with freshly isolated adipocytes, chondrocytes and osteoblasts respectively. Passage 2 bASCs were selected based on the results from Chapter 3, as they demonstrated maintained multipotency, while enabling *in vitro* expansion to obtain sufficient cell populations for the experimental purposes.

The medium used in the adipogenic co-cultures was simple growth medium, which contained 10% FBS, and excluded all of the hormones and cytokines from the adipogenic differentiation medium used in Chapter 3. For adipogenesis, GPDH enzymatic activity was approximately 3-fold higher in co-culture, as compared to the ASC-alone cultures. The Oil red O staining results were very interesting, demonstrating that almost every single bASC in the co-culture system had responded and had begun to accumulate intracellular lipid. While the size of the intracellular lipid droplets were much smaller than those observed in the medium induction experiments in Chapter 3, the more homogenous cell response is a desirable characteristic for tissue-engineering applications.

These results could suggest that the bASCs may have been uniformly "sensitized" to differentiate by the co-culture conditions, by secreted paracrine factors from the mature adipocytes that mediated the receptor expression profile of the bASCs to drive

differentiation. In contrast, the use of inductive medium may have functioned differently, by stimulating differentiation within a select sub-population of the bASCs (i.e. the 40 – 50 % of cells that responded), which had the appropriate receptors for the stimulating factors included in the medium. It is possible that with extended time in culture that a more mature phenotype would have been observed in the bASCs in co-culture with mature adipocytes. The adipogenic gene expression analyses confirmed the results that the co-culture was successfully stimulating the adipogenic differentiation of the bASCs. The low expression of LPL in co-culture, relative to results reported in the literature [12, 63] and in Chapter 3 for growth factor stimulation, may be a further indication of the less mature phenotype of the co-culture induced bASCs. The positive results are supported by the work of Stacey *et al*, involving the co-culture of human ASCs and adipocytes in adipogenic differentiation medium, which showed that the level of adipogenic cytokines, such as leptin, glycerol and adiponectin, were significantly upregulated through co-culturing [168].

For the chondrogenic co-culture studies, the medium used was chondrogenic differentiation medium without TGF-β, which is the key differentiation factor for chondrogenesis [90, 91]. This medium was selected to minimize the effects of exogenous growth factor stimulation, while still providing a stable environment for the mature chondrocytes. The toluidine staining showed a slightly higher intensity of staining in co-culture, as compared to the ASC-alone cultures, although the results were not conclusive. However, the FIHC staining to localize collagen I and II in the pellets showed that collagen II was only present in the bASC pellets in co-culture,

with more intense staining in the exterior regions. These results may suggest that there was inductive signaling occuring at the surface of the pellets. However, the lower intensity of DAPI staining in the central regions also indicated that there was a lower cell density at the core, which may have contributed to the lower levels of collagen II expression. In contrast, collagen I was expressed throughout all of the pellets, potentially indicating that the bASCs were undergoing osteo-chondrogenesis, as discussed in Chapter 3 [129]. The collagen type I may have also been secreted by undifferentiated or partially-differentiated ASCs, which still possessed stem cell properties. Both sGAG and total collagen content were statistically higher in the bASC pellets in co-culture than in the ASC-alone cultures. These results, combined with the expression of the chondrogenic gene markers, suggest that the mature chondrocytes stimulated bASC differentiation towards chondrogenesis, without exogenous TGF-β. This phenomenon is supported by the recent work of Lee and Im, which confirmed that signals from mature chondrocytes can effectively promote the chondrogenic differentiation of human ASCs during *in vitro* pellet culture [213].

In terms of the osteogenic co-culture, the medium used was osteogenic medium, as used in Chapter 3 for the ASC osteogenic differentiation. This medium selection was based on preliminary ALP enzyme studies that indicated that all of the supplements (50 μM A2P, 10 mM β-glycerophosphate, 100 nM DEX) [117] were required by the osteoblasts to maintain their phenotype. However, the results still indicated that the ALP enzymatic activity was 2-fold upregulated in bASCs in co-culture, relative to the conventional osteogenic-induced bASC. The bASC

co-culture and ASC-alone cultures were both positively stained with von Kossa, verifying calcium deposition in the matrix of a similar intensity. The gene expression results of the osteogenic markers were more difficult to interpret. Collagen I was more highly expressed in co-culture, consistent with the protein expression data, and the Runx2 expression levels were somewhat similar. Interestingly, the expression of osteocalcin (OCN), a relatively late-stage marker [125], was much lower in bASCs in co-culture, as compared to the ASC-alone culture. These results might suggest that the bASCs in co-culture were in an earlier stage of differentiation as compared to the ASC-alone in osteogenic medium, but that the co-culture was promoting a stronger differentiation response. Combining the results of all of the assays, it is possible that the osteoblasts were able to enhance the osteogenic differentiation response of the bASCs in co-culture.

Overall, the results indicated that bASC differentiation towards all three lineages was successfully induced or enhanced by co-culture with mature cells. While this is the first study to investigate all three lineages in co-culture with ASCs, the results are supported by other studies that have shown a positive response in MSCs in co-culture specific with mature populations. For example, Gan and Kandel found that passaged, de-differentiated bovine chondrocytes were able to regain the chondrogenic phenotype through co-culture with primary chondrocytes [167]. The results of Wang *et al.* and Zhou *et al.* both suggested that murine osteoblasts can produce paracrine factors that promote osteogenesis in bone marrow-derived MSCs [154, 163].

However, there have been some contradictory results reported in the literature. In

a study by Janke *et al.* [214], mature human adipocytes inhibited the *in vitro* differentiation of human ASCs via the angiotensin type I receptor. Additionally, Hendriks *et al.* postulated that paracrine signaling did not play a role in regulating the terminal differentiation of chondrogenic progenitor cells in co-culture [18]. The variations observed in these studies may be due to differences in the specific culture conditions and cell sources implemented, but emphasize that it is crucial to understand which factors promoted the positive responses observed in the co-culture models developed in this work.

Towards a similar goal, Wang *et al.* and Zhou *et al.* conducted experiments that concluded that the osteoblastic control of the fate of murine bone marrow-derived MSCs in indirect co-culture with osteoblasts involved the Wnt signaling pathways [154, 163]. In addition, it has been established that specific Wnt proteins can inhibit adipogenesis [22] in cultured MSCs, and can promote chondrocyte hypertrophy [21, 144, 150]. As such, it was decided that it would be interesting to probe the roles of the Wnt signaling pathways in the co-culture environments defined in this study. As such, a series of experiments was conducted using two different Wnt inhibitors (WIF-1 and DKK1) to probe the role of the canonical and non-canonical Wnt signaling pathways in mediating the interactions between the bASCs and the mature cells (adipocytes, chondrocytes, osteoblasts). Although the role of Wnt proteins has previously been investigated in osteogenic co-cultures of murine bone marrow-derived MSCs and osteoblasts, using a Wnt 3a-enriched medium [154, 163], this is the first study to investigate the Wnt pathways in multilineage co-culture with ASCs from any species.

A better understanding of these pathways, as well as the positive responses observed in co-culture, might help to elucidate ASC physiology and function, and contribute to optimized strategies incorporating these unique regenerative cells in a broad range of tissue-engineering applications.

The Wnt inhibitory factors DKK1 and WIF-1 were added separately to co-culture samples of all 3 lineages (adipogenic, chondrogenic, osteogenic). In general, as discussed in Chapter 2, DKK1 inhibits the Wnt canonical pathway only. In contrast, WIF-1 knocks down both the canonical and non-canonical signaling pathways by binding with free Wnt proteins within the extracellular space, preventing interactions with the receptors on the surface of the cells [126, 127, 144]. Theoretically, two inhibtors should have similar responses in terms of the inhibition of canonical pathway. In signaling through the canonical pathway, β-catenin accumulates and translocates into nucleus to trigger the expression of differentiation-related genes. However, this intra-nucleus activity may be interrupted by non-canonical pathway (Ca^{2+} dependent pathway in chapter 2.6.2.2). As downstream elements in the other non-canonical pathways, JNK1 is related to physical stimulation, cell migration and polarity control, and JNK1 and RHOA are both involved in cytoskeletal reorganization [132-134, 140].

For adipogenesis, GPDH enzymatic activity was highest in the Co-DKK culture, where the Wnt canonical pathway was inhibited. In contrast, the Co-WIF samples had GPDH levels that were similar to the Co-None samples, indicating that the non-canonical pathway had a negative effect on adipogenesis. Similarly, the gene

expression analysis of the later-stage adipogenic marker LPL indicated that the highest levels were detected in the Co-DKK group, supporting the enzyme activity data. However, CEBPα and PPARγ were both expressed at a similar level of intensity in both of the inhibitor groups. CEBPα and PPARγ are early adipogenic markers, which self-propagate in a positive feed back loop during adipogenic differentiation, and that are responsible for inducing the expression of other adipogenic markers [79, 85]. Therefore, the data may suggest that the Wnt non-canonical pathway possibly interfered with the later stages of adipogenesis. In general, when the Wnt canonical pathway was blocked, adipogenesis in co-culture was significantly upregulated, which agrees with the inhibitory effects of Wnt described in the literature [22, 129, 154].

For Wnt-related gene expression, β-catenin was upregulated in bASCS in all of the co-cultures as compared to the ASC-alone group, which could suggest a potential role in mediating co-culture-induced adipogenic differentiation. However, β-catenin was also possibly involved in cadherin mediated cell-cell adhesion [215] and other cell activities, which might not be related to the Wnt signaling pathway. The elevation of JNK1 expression in the Co-DKK group suggested that when the canonical pathway was inhibited, signaling through this specific non-canonical pathway was upregulated, potentially by increasing the amount of available Wnt within the system while reducing binding events. The increased expression of RHOA in co-culture also suggests a potential role in mediating adipogenesis. However, the highest levels in the Co-WIF group were unexpected, as a similar differentiation response was observed in this group as compared to the Co-None group.

In general, the levels of protein expression for each of the elements might be of interest for future studies, as there could be post-transcriptional changes that could impact the activity levels of each specific pathway/protein, without affecting gene expression. Moreover, assessing the expression at additional time points would further elucidate the response, as peaks in gene or protein expression might occur at different time points in the different co-culture groups. As such, it is important not to over-interpret the results of this preliminary investigation into the Wnt pathways.

For the chondrogenic co-cultures, the decreased hydroxyproline levels observed with Wnt inhibition were not statistically significant. However, there was an interesting peak in sGAG content when both the canonical and non-canonical Wnt signaling pathways were blocked through WIF-1 inhibition. Further, collagen II gene expression was upregulated in both the Co-WIF and Co-DKK groups relative to the Co-None group, and Sox9 expression was also slightly higher in the Co-DKK group. Based on the literature, the Wnt canonical pathway represses chondrogenesis. Hence, it was expected that blocking this pathway should augment chondrogenesis [21]. While there were no statistically significant changes in the ECM with the addition of either of the factors, the gene expression data in the current study does support that chondrogenesis was upregulated with inhibition of the Wnt pathways.

In terms of Wnt-related gene expression, β-catenin expression was lower in the Co-DKK culture group than in all of the other groups, including the ASC-alone pellets, consistent with inhibition of the canonical pathway [163]. In addition to the Wnt canonical pathway, β-catenin also plays a role in mediating cell-cell adhesion via

the cadherin-β-catenin complex [215], which may also impact chondrogenesis [216]. Studies have indicated that β-catenin expression is high in pre-chondrogenic MSCs, but low in differentiated chondrocytes [217]. This trend is consistent with the observed results that the highest levels of β-catenin expression were detected in the ASC-alone group, which had a less differentiated state based on the chondrogenic gene expression studies. Potentially through changes in the JNK-mediated pathways, chondrocytes are sensitive to pressure and physical stimulation [131, 132, 218]. As such, the slightly higher expression of JNK in the Co-WIF group, relative to the other conditions, might be related to cellular changes associated with chondrogenesis. The reduction in RHOA expression in the WIF-1 inhibited cultures was consistent with the inhibition of the Wnt non-canonical pathway [219]. The downregulation of RHOA in the bASCs in both the Co-WIF and Co-DKK groups relative to the Co-None group was consistent with the literature reports that reduced RHOA expression is associated with enhanced chondrogenesis in MSCs and de-differentiated chondrocytes [142, 143].

In osteogenic co-culture, the bASCs in all of the co-culture groups had higher ALP enzymatic activity than the ASC-alone group, but the results indicated that the Wnt inhibitory factors had a limited effect on osteogenic differentiation over the time course in the current study. However, there was significant reduction in ALP activity in the Co-DKK group, as compared to the Co-None group. This result suggests that the repression of the canonical pathway reduces the level of osteogenesis, which is consistent with the literature findings that the inhibition of the canonical pathway

promotes adipogenesis, while limiting osteogenic differentiation of cultured MSCs [21, 126, 127, 144, 154]. However, the osteogenic gene expression results were more inconsistent, potentially confounded by the required use of the osteogenic induction medium. Although the expression of Runx2 was in concordance with the ALP enzymatic results, OCN gene expression was highest in the Co-DKK and ASC-alone groups at the 14-day time point, which was unexpected. Similarly, no clear trends in the Wnt-related gene expression patterns were detected in the experimental conditions studied. The slightly higher levels of β-catenin expression in the Co-None condition were consistent with enhanced osteogenesis and the literature reports that the osteogenic differentiation of MSCs is promoted through enhancements in this signaling pathway [220].

Overall, the results of the studies indicate that Wnt signaling does play a role in regulating bASC differentiation in the three co-culture systems investigated in the current body of work. As the bASCs did not demonstrate differentiation in the ASC-alone culture conditions, it is most likely that important signaling factors were secreted by the mature cells. Another possibility is that the paracrine signaling from the mature cells interacted with the bASCs and induced the secretion of endogenous Wnt proteins, which functioned in autocrine signaling. The paracrine factors may have also "sensitized" the bASCs, changing their cell surface receptor profile, and thereby mediating the observed differentiation response in culture. As discussed, this possibility is supported by the highly uniform response observed in the intracellular lipid accumulation within the bASCs in the adipogenic co-culture system. While the

results are interesting, further studies are needed to explore these possibilities in greater detail to better understand the response.

The success of utilizing stem cells in tissue-engineering applications not only depends on the quality of the cell source, but also on the method of differentiation. It is important to develop a thorough understanding of ASC differentiation both *in vitro* and *in vivo*, so that the cells can safely be applied in cell-based therapies with a negligible risk of tumorigenesis, and a high probability of regaining normal functionality. Wnt proteins have been commonly studied in the field of oncology, as the pathways are known to play essential roles in mediating cellular proliferation and differentiation [128]. In tissue engineering, an understanding of Wnt proteins will likely be important for safely and effectively applying MSCs in clinical therapies.

Chapter 5

General Discussion

The research in this thesis included the identification and characterization of bovine adipose-derived stem cells (bASCs), isolated from the interdigital fat pad in the hoof, in terms of *in vitro* proliferation and multi-lineage (adipogenic, chondrogenic, osteogenic) differentiation. The analysis of this new potential cell source represents a significant contribution to understanding ASC behavior in culture, and this work is useful for the development of mesenchymal stem cell (MSC) models based on the bovine system. The study indicated that a threshold existed for bASC degeneration during extensive *in vitro* expansion on two-dimensional tissue culture polystyrene (TCPS), which could possibly apply to other ASC sources. This work highlights the importance of complete cell characterization, as stem cell sources must maintain their proliferation and differentiation properties in order to be effectively and safely applied in cell-based therapies.

The research also demonstrated the limitations of using inductive media to stimulate ASC differentiation. These media formulations typically include costly growth factors and cytokines, at physiological concentrations or higher, which could have undesirable side effects when applied *in vivo*. Further, the response is typically very heterogeneous, with only selected sub-populations of sensitive cells responding to the inductive cues and successfully differentiating. The results clearly showed that

the fraction of cells that would differentiate along the adipogenic and chondrogenic pathways using standard culture methods declined with increasing time in culture and serial passaging.

This project was the first to develop co-culture models to study paracrine-mediated interactions between mature cells and bASCs across 3 lineages (adipogenesis, chondrogenesis and osteogenesis). The overall goal was to mimic the native tissue environment and the regenerative processes that naturally occur within the body, as MSCs proliferate and selectively differentiate during normal tissue homeostasis and wound healing. The research investigated the potential for mature cell populations (adipocytes, chondrocytes, osteoblasts) to induce or enhance the differentiation of bASCs through secreted soluble factors. Interestingly, all 3 of the developed co-culture systems effectively stimulated the expression of lineage-specific gene and protein markers in the bASC populations. In the adipogenic and chondrogenic cultures, differentiation was induced in the absence of key stimulatory factors that are normally required in the culture medium to observe a positive response. The results suggested that Wnt proteins and the associated canonical and non-canonical signaling pathways played important roles in mediating co-culture-stimulated bASC differentiation in all three lineages.

As bovine ASCs had never been characterized thoroughly before in terms of both proliferation and multipotent differentiation, this research also contributes to developing a better understanding of the common characteristics of ASCs in general, independent of species variability. The selected site of cell harvest was innovative

relative to previous ASC-based studies, as this was the first investigation of structural adipose tissue (i.e. not subcutaneous or visceral) as an ASC source. The study revealed that the interdigital bASCs maintained their doubling time until passage 4, which is an earlier passage to observe changes in proliferation as to what has been reported for ASCs from adipose tissue depots [12, 37, 66]. This result may suggest that ASCs isolated from structural adipose tissue are distinct, degrading and senescing more readily in culture. The adipose tissue in this fat pad is macroscopically more fibrous and stiff, with fewer visible blood vessels, as compared to human abdominal or breast adipose tissue [190]. As ASCs have been localized to a perivascular niche within many tissues, and are recognized as a highly active cell type that responds to endocrine signaling [205], the lower degree of apparent vascularity may have contributed to the unique properties of the cells from this new source. A better understanding of structural adipose tissues and ASCs is also important for the development of strategies in orthopedic soft tissue regeneration, such as in the augmentation of the fat pads in the feet of elderly patients.

Experiments with primary cell populations commonly require repeated trials with multiple donors to reduce donor differences [187] and statistical variation, in order to detect meaningful trends in the data. As a cell source, bASCs are generally abundant and accessible from tissues that routinely discarded at slaughterhouses, and are therefore a good cell source for *in vitro* studies in terms of availability. Further, the use of bovine cells in this study made it possible to develop the 3 co-culturing systems, which required healthy mature adipocytes, chondrocytes and osteoblasts. While

human adipose tissue is generally available as surgical waste, the ability to obtain human chondrocytes and osteoblasts from healthy donors for this type of research is very limited. In general, the use of the bovine large animal model made it possible to easily collect and culture all of the cell types of interest from discarded tissues and even from the same animal.

However, due to the fact that the bovine animal model is less well developed experimentally, as compared to the murine, rat, rabbit, and human models, there were some challenges associated with primer design, as well as the lack of confirmed antibody cross-reactivity, which limited the available assessment methods for bASC characterization. However, despite these restrictions, the data was still convincing that multipotent bASCs could be isolated from the interdigital fat pad, and were a good choice of cell source for the co-culture studies in this thesis.

The characterization of the bASCs from the interdigital fat pad provided a solid basis of understanding of this particular cell source. As discussed the cells maintained their stem cell properties for a shorter period of time using standard culture methods, as compared to other known ASCs [37, 50]. However, the concept of an *in vitro* "degeneration threshold" may also be applicable to other stem cell sources. While embryonic stem cells (ESCs) can theoretically self-renew infinitely, it remains to be determined how many replications specific MSC populations can undergo before starting losing their stem cell capacity to proliferate and differentiate. It is probable that every specific MSC source (i.e. depot, species, and individual) probably has a somewhat different response. As cell-based tissue engineering strategies may require

hundreds of millions of stem cells for a single application, it is possible that the primary stem cells from a single harvesting procedure may not be sufficient without undergoing extensive *in vitro* expansion. Therefore, it is critical to perform experiments to confirm the stem cell quality after expansion, and to develop optimized methods for both ASC proliferation and lineage-specific differentiation.

Since indirect co-culture is a novel tool that can be used to investigate the interactions between cells via paracrine signaling, and it allows for the individual assessment of each cell population without complex cell sorting [18], it is a powerful tool to study the interactions between precursors and mature cells. While growth factor cocktails could be used to induce the multilineage differentiation of bASCs, co-culture with mature cells of each lineage could also be used to induce or enhance the response, including in a medium without these growth factors.

Of the three co-culture systems studied, the adipogenic study showed the most surprising results. While the co-culture GPDH enzymatic activity level (Figure 4.3) was only approximately 20% of the level observed in the medium-induced samples (Figure 3.4) (both were higher than the controls, which were at a similar level), the differences in the Oil red O staining were very interesting. As compared to the 40 - 50% differentiation response in inductive medium, mature adipocytes induced lipid accumulation in almost every single bASC in co-culture. These results suggested that there was effective signaling with soluble factors secreted by the adipocytes that changed the bASC population, potentially altering the cell surface receptor profile and making the cells more uniformly responsive to differentiation factors. The system

may be mimicking the natural balance between ASCs and mature adipocytes, as found in adipose tissue in the body. As the lipid droplets in the bASCs in co-culture were small and multilocular, the data suggested that the cells were at an earlier differentiation stage as compared to the medium-induced cultures. The lower level of GPDH enzymatic in co-culture is consistent with the differential staining response. Overall, adipogenic co-cultures effectively induced early-stage differentiation, with a more consistent cell response, potentially replicating the natural processes involved in fat formation in the body more closely.

In the chondrogenesis studies, immunohistochemistry (IHC) (Figure 3.8 – 9) and fluorescence IHC (FIHC) (Figure 4.7 – 8) were used to localize collagen I and II in pellet cultures of the bASCs in co-culture with mature chondrocytes. Experimentally, the FIHC staining provided a more conclusive visualization of the gradient changes in the pellet sections, especially at low magnification. The bASC in both the co-culture and medium-induced cultures stained positively for collagen I throughout the whole pellets. These results suggested that the chondrogenic phenotype of the bASC may have been unstable, tending towards a more osteo-chondrogenic condition. In general, there were lower levels of glycosaminoglycan (GAG) and collagen II staining in the co-cultures (Figure 4.9), as compared to the growth factor (GF)-induced cultures (Figure 3.11). In conclusion, the mature chondrocytes were able to induce chondrogenic differentiation of bASCs in co-culture without TGF-β, but there was generally a lower level of cartilage-specific extracellular matrix (ECM) production, as

compared to the GF-induced cultures, potentially indicative of a less mature or stable chondrogenic phenotype.

The osteogenic co-culture demonstrated an enhancement in osteogenesis in the bASCs influenced by the mature osteoblasts in traditional osteogenic medium. As concluded in Chapter 3, the alkaline phosphatase (ALP) enzymatic and osteogenic gene expression comparisons suggested that the bASCs in the co-culture were in an earlier, but stronger, differentiation stage, with a similar intensity in the von Kossa staining of matrix mineralization.

To date, this thesis was the first to demonstrate the tri-lineage differentiation ASCs in transwell co-culture environments with mature cells. The study successfully demonstrated the feasibility of mature cell induced or enhanced differentiation via paracrine signaling. Comparison of the mature cell induced co-culture differentiation and GF-stimulated differentiation yields interesting observations. It seems to be common in the 3 lineages that the mature cells induced an early stage of differentiation, which was generally more uniformly potent than the early differentiation responses observed in the GF-simulated cultures. This data suggested that there were complex interactions in the co-cultures involving soluble factors, which may have induced a more “tissue-like” differentiation response in the ASCs. The temporal aspect of co-culturing would be interesting to probe in more depth, including an assessment of changes in the differentiation response in the bASCs with extended time in culture.

In the Wnt inhibition study, the adipogenic co-cultures responded very well to inhibition with DKK1, as expected [22]. Further, combining the findings from the adipogenic and osteogenic co-cultures, the Wnt canonical pathway was shown to have opposite influences on the two lineages, which is in agreement with the literature [22, 130]. At the gene-expression level, chondrogenesis was also somewhat upregulated by blocking the canonical pathway. These findings validated the positive effects of DKK as an inhibitor of the Wnt canonical pathway. However, based on the observed results, it was unclear whether a sufficient concentration of Wnt inhibitory factor-1 (WIF-1) was added to the co-cultures to effectively block the Wnt signaling networks. In the adipogenic co-cultures, WIF-1 showed similar results to DKK-1 on a genetic level, but not at the protein level, and the results were similar in the chondrogenesis experiments. In the osteogenic studies, the Wnt inhibitor results were more inconclusive. However, this may have been potentially due to confounding effects associated with the required use of osteogenic induction medium as the basis for all of the osteogenic cultures.

The co-culture experiments successfully proved that soluble differentiation factors were transported through the transwell membrane and stimulated bASCs differentiation. However, the specific factors involved, which may change over time, remain unresolved. While the results suggested that cell-secreted Wnts played an important role in the co-cultures, it was not within the scope of the current project to determine where these Wnt proteins came from, or which specific types of Wnt were involved in each system. One possibility is that the Wnt proteins were secreted by the

mature cells. However, it is possible that other cytokines or growth factors secreted by the mature cells triggered endogenous Wnt production in the bASCs, which may have acted in an autocrine fashion [163]. Another area worth investigating in future work would be whether the cell-surface receptor profile on the bASCs changed under co-culture conditions, making the cells more responsive to inductive stimuli in the media.

Chapter 6

Conclusions and Future Recommendations

6.1 Summary and Conclusions

Cell-based therapies hold great promise for tissue regeneration. Stem cells, especially adipose-derived stem cells (ASCs), have shown great potential in many tissue-engineering applications [9]. ASCs may be stimulated to differentiate in culture into specific lineages, such as fat, cartilage or bone, before they are applied, in order to promote specific tissue regeneration. Ideally, this differentiation process should be as close as possible to the natural mesenchymal stem cell (MSC)-based regeneration that occurs in native tissues [221]. Evidence suggests that within the body, mature cells have a high degree of interaction with local MSCs, which can help regulate the stem cell behavior. In this context, studying co-culture between mature populations and ASCs is of interest in understanding the mechanisms and soluble factors involved in mediating differentiation.

This study first demonstrated a new cell source, ASCs from the bovine interdigital fat pad, in terms of *in vitro* proliferation potential, as well as multi-lineage differentiation potency. Doubling time calculations and gene expression analysis of stem cell markers indicated that the proliferation potential of the cultured bASCs decayed at passage 5 (P5). The multi-lineage differentiation potentials were compared between passage 2 (P2) and P5, with the bASCs in P5 presenting significantly lower

levels of adipogenesis and chondrogenesis. Osteogenic potential, on the other hand, was maintained or even improved with serial passaging. The results indicated that there may have been a degeneration in the overall stem cell properties in the P5 cells. The study also suggested that there may be a threshold for ASC degeneration with serial passaging, including for other ASC sources as well. Therefore, each cell source must be thoroughly characterized before being applied in cell-based therapies. The characterization of the bASCs isolated from interdigital fat indicated that P2 cells were a suitable multipotent cell source for the co-culture experimentation.

In order to study the interactions between the mature cells and the bASCs, proper co-cultures (adipogenic, chondrogenic, and osteogenic) with defined culture medium were designed. Within the co-culture systems, the bASCs were successfully stimulated to differentiate along the 3 lineages, similar to the growth factor (GF)-induced differentiation characterized in the first phase. This phase of the study demonstrated the general feasibility of mature cell-induced or -enhanced bASC differentiation by soluble cell-secreted factors. More specifically, the results suggested that bASC differentiation was stimulated through signaling with paracrine secreted by the mature cells. When compared to GF-stimulated differentiation, the bASCs in co-culture presented an earlier, but potentially stronger, level of differentiation. This trend may have suggested that the soluble factors from the mature cells provided a more natural stimulation of tissue regeneration.

Among the cell-secreted factors, Wnt proteins, and the associated canonical and non-canonical signaling pathways, were indicated to play important roles in mediating

differentiation along all three lineages in co-culture. As confirmed in the literature, the canonical pathway is upregulated during osteogenesis, but inhibited during adipogenesis [22, 130]. The inhibition of chondrogenesis through the canonical pathway was suggested on a genetic level. Additional studies are needed to further elucidate the specific roles of each of the Wnt pathways in co-culture induced differentiation, including in terms of specific intracellular protein levels.

In conclusion, bASCs from the interdigital fat pad generally displayed similar properties to what has been reported for ASCs isolated from different fat depots in other species [11, 12, 14, 32]. Co-culture between mature cells and bASCs is a useful tool to probe the signaling pathways involved in differentiation and natural tissue regeneration. In addition, Wnt proteins and Wnt inhibitory factors may be useful in many ways to tune the differentiation of MSCs, and more specifically, ASCs. To date, only limited work has been done investigating the signaling pathways involved in cellular communication between mature cells and stem cells. This work represents the first study presenting ASC differentiation along 3 different lineages within a co-culture system, and may help to provide a better understanding of these important signaling networks.

6.2 Contributions

Specific contributions of this thesis to the field are listed as follows:

- Successfully isolated bASCs from the bovine interdigital fat pad
    - Characterized the proliferation potential of the bASCs in terms of growth kinetics and stem cell marker gene expression

    - Demonstrated the existence of a threshold for degeneration of the proliferation potential
    - Demonstrated the multi-lineage differentiation capacity of bASCs (adipogenesis, chondrogenesis, and osteogenesis)
- Explored the differentiation potential trends of the 3 lineages with serial passaging
    - Confirmed that the adipogenic and chondrogenic differentiation potentials decayed at the same time as the proliferation potential
    - Discovered that the differentiation potential towards osteogenesis was maintained, or even enhanced, with serial passaging.
- Designed 3 indirect co-culture systems for bASCs with mature bovine cells (adipocytes, chondrocytes, osteoblasts), with modified mediums.
- Successfully differentiated bASCs (adipogenesis, chondrogenesis and osteogenesis) in the designed co-culture systems.
- Probed the role of the Wnt canonical and non-canonical signaling pathways in multilineage co-culture, including Wnt/β-catenin, Wnt/JNK1 and Wnt/RHOA signaling.

6.3 Future Work and Recommendations

The results in this thesis indicate that early-passage bASCs from the interdigital fat pad are a satisfactory cell source for tissue-engineering studies. However, additional characterization studies could be conducted to more fully investigate the stem cell properties of the bASCs.

Further characterization of the bASCs

- Use other characterization methods to evaluate the bASCs in depth
    - Compare the expression of the stem cell markers on the differentiated cells with undifferentiated cells using real time RT-PCR, to evaluate the changes in the stem cell profile during culture and differentiation.

If appropriate antibodies were available, this could also be done by flow cytometery to compare the changes in the stem cell related surface marker profiles.

  - Conduct colony forming unit differentiation studies on the bASCs to identify sub-populations of tri-potent, bi-potent and uni-potential progenitors. This will help to confirm the identity of the bASCs as MSCs.
  - Examine the osteo-chondrogenic differentiation (i.e. the formation of an unstable phenotype within the chondrogenic cultures) through immunohistochemical staining or gene expression analysis of collagen X, alkaline phosphatase (ALP), and osteocalcin (OCN). These factors are the hypotrophic markers in chondrogenesis.

- Differentiate the bASCs towards other lineages (i.e myogenesis, cardiomyogenesis, epitheliogenesis, and neurogenesis) to further explore the possibility of this cell source.
- Compare the proliferation and differentiation potentials of bASCs from interdigital fat with bASCs from other adipose tissue depots. This might help to understand the depot-specific differences, and the potential insensitivity of structural fat to changes associated with hormones and body metabolism.

Micro-environment refinement

The cell culture micro-environment of the bASCs significantly impacts cell fate determination. Physical properties of the substrate, such as surface chemistry, porosity and stiffness, should be optimized for each lineage.

- Explore the effect of substrate stiffness on bASC proliferation and differentiation. Based on similar work with human bone marrow-derived MSCs, it would be interesting to probe alternative growth substrates, each designed to mimic a specific tissue stiffness, to determine if it is possible to maintain the adipogenic and chondrogenic potentials by altering the mechanical properties of the cellular microenvironment during the proliferation phase [141].

Co-culture

- Investigate co-culturing bASCs with other mature populations, such as muscle cells, liver cells, epithelial cells and other possible candidates, in non-inductive medium. The study of this interaction might help to explore the possible signaling pathways involved in lineage-specific differentiation
- Incorporate the co-culture with appropriate tissue-engineered scaffolding materials, designed to mimic lineage-specific ECM.

Wnt proteins and the Wnt signaling pathways

- Further investigate the Wnt signaling pathways, including Wnt/CAMKII, as well as additional genes associated with the pathways, such as serine/threonine protein kinase (NLK), to probe how the expression profile changes in the presence of the Wnt inhibition factors.
- Quantify the intracellular protein levels of β-catenin, JNK, RHOA, and CAMKII to assess the influences of the Wnt signaling pathways. Western blot or Elisa could be used in this case if the antibodies are available.
- Investigate the cell-surface receptor profile on the bASCs and mature populations, including receptors associated with the Wnt signaling pathways, such as Fz 1-10, LRP5/6, Ryk, ROR2, Kremen 1 and 2.
- The concentration of WIF used in the thesis might not be optimized. Repeat the experiment using higher concentrations (300, 400, 500 ng/mL) of WIF to optimize the working concentration. The experiment could also use an alternative inhibitor, such as sFRP-1 and 3, to replace WIF-1. However, optimization of concentration is still necessary.
- Refine the medium formulations with recombinant Wnt proteins or Wnt inhibitory factors and use them for lineage-specific bASC differentiation without mature cells present. Optimize the concentration of Wnt proteins and Wnt inhibitory factors in each medium.
- There are multiple Wnt proteins, and each Wnt protein has unique affinities for different receptors on the cell surface [222]. Therefore, it would be interesting to look at which Wnt proteins were involved in each signaling

pathway. This could be probed by using different recombinant Wnt proteins and quantifying the downstream intracellular proteins of each signaling cascade.

- Study other pathways and cytokines involved in the designed co-cultures, which may promote differentiation. i.e. growth factor/hormone related pathways.

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Appendix

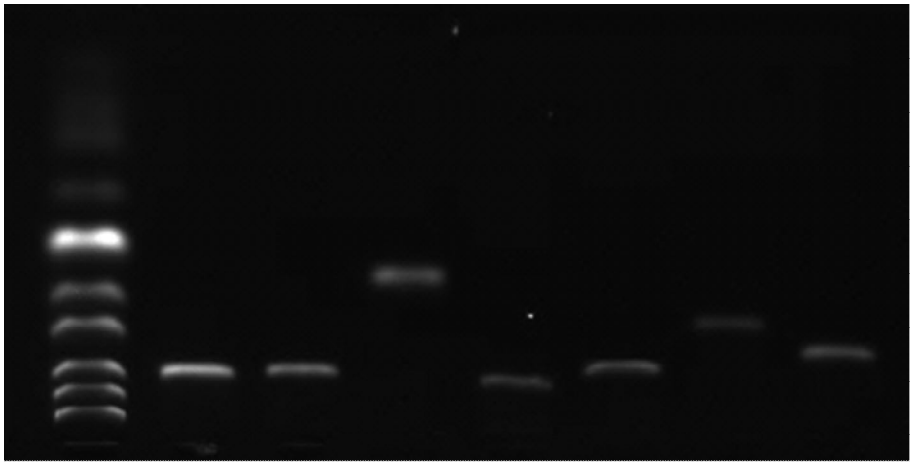

Figure A 1 Sample gene expression analysis (GAPDH, 18S, Collagen I, Runx2, osteocalcin, CD90 and CD34) on 5% agarose gels with an ultra low range DNA Ladder (Invitrogen). The ladder size ranges from 0 bp to 300 bp, with highest intensive band at 50 bp. Second row indicates sizes of the products

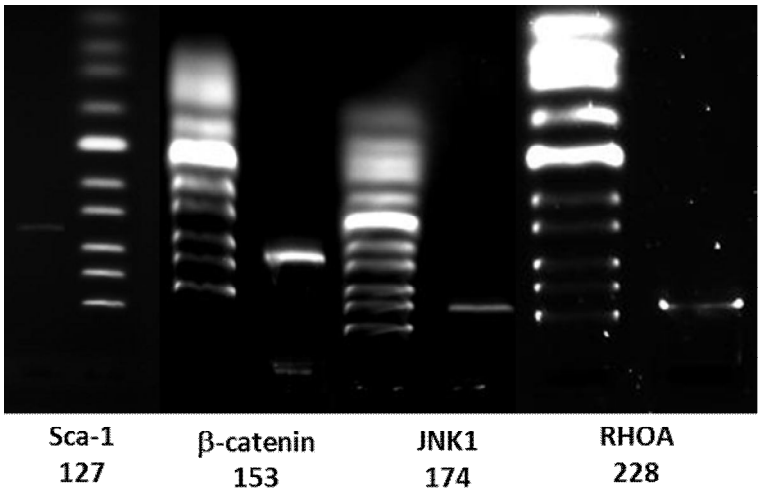

Figure A 2 Sample gene expression analysis (Sca-1, -catenin, JNK1 and RHOA) on 5% agarose gels with an ultra low range DNA Ladder (Invitrogen). The ladder size ranges from 10 bp to 300 bp, with highest intensive band at 50 bp. Second row indicates sizes of the products

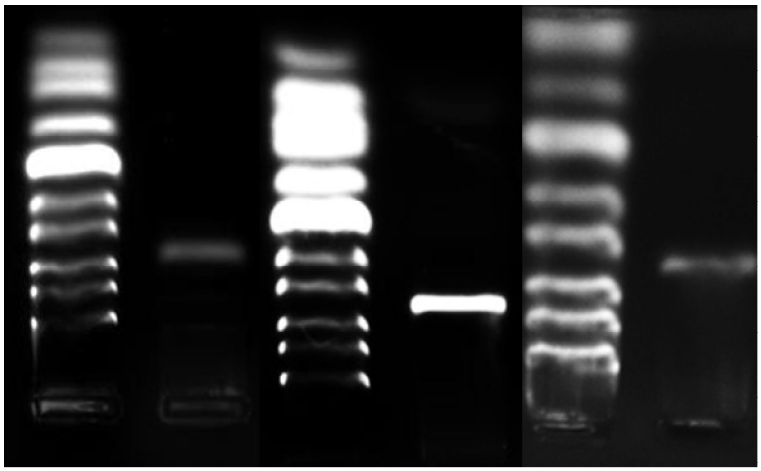

Figure A 3 Sample gene expression analysis (CEBPα, PPARγ and LPL) on 5% agarose gels with an ultra low range DNA Ladder (Invitrogen). The ladder size ranges from 10 bp to 300 bp, with highest intensive band at 50 bp. Second row indicates sizes of the products

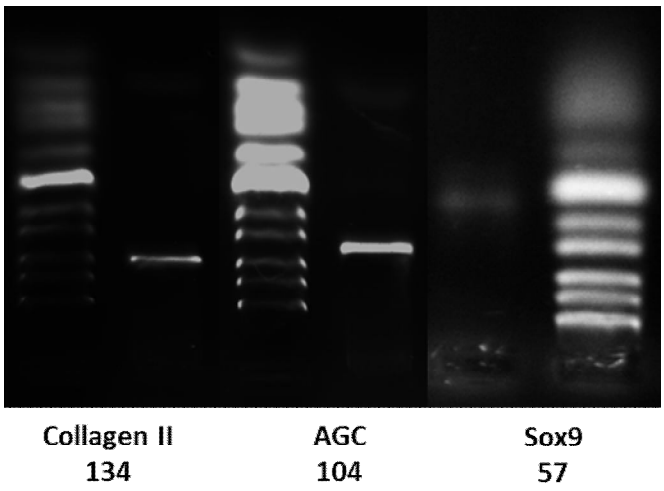

Figure A 4 Sample gene expression (Collagen II, AGC and Sox9) on analysis on 5% agarose gels with an ultra low range DNA Ladder (Invitrogen). The ladder size ranges from 10 bp to 300 bp, with highest intensive band at 50 bp. Second row indicates sizes of the products

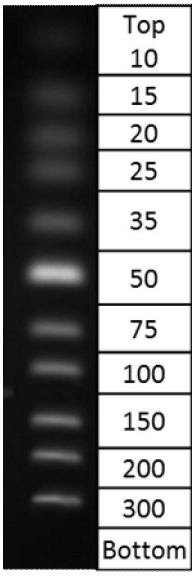

Figure A 5 The ultra low range DNA ladder, bands are labled with corresponding size from 10 bp to 300 bp, with highest intensive band at 50 bp.