Mesenchymal stem cell isolation and characterization from ligamenta flava, interspinous ligaments and facet joints

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การศึกษาคุณสมบัติเซลล์ต้นกำเนิดชนิดมีเซนไคมอลจากเอ็นหุ้มไขสันหลัง เอ็นยึดระหว่างปล้องสันหลัง และข้อฟาเซต

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บาลเดอร์ คริสเตียนสัน : การศึกษาคุณสมบัติเซลล์ต้นกำเนิดชนิดมีเซนไคมอลจากเอ็นหุ้มไขสันหลัง เอ็นยึดระหว่างปล้องสันหลัง และข้อฟาเซต (Mesenchymal stem cell isolation and characterization from ligamenta flava, interspinous ligaments and facet joints) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. นพ. สิทธิศักดิ์ หรรษาเวก, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.พิเศษ นพ. วรวรรธน์ ลิ้มทองกุล, 119 หน้า.

การศึกษาเซลล์ต้นกำเนิด mesenchymal stem cells (MSCs) และ adult progenitor cells ได้รับความสนใจและเป็นที่นิยมอย่างมากในการประยุกต์ใช้ทางวิศวกรรมเนื้อเยื่อและทางการแพทย์ในโรคต่าง ๆ การหนาตัวและการเปลี่ยนสภาพเป็นกระดูกของชิ้นเอ็นและข้อต่อฟาเซ็ตบริเวณกระดูกสันหลังมีส่วนสำคัญต่อการเกิดโรคกระดูกสันหลังส่วนเอวเสื่อมที่มีช่องไขสันหลังตีบแคบ การศึกษาก่อนหน้าพบเซลล์ต้นกำเนิดอยู่ภายในชิ้นเอ็น ligamentum flavum และชิ้นเอ็น posterior spinal ligament ซึ่งคาดว่ามีส่วนเกี่ยวข้องกับการดำเนินโรคนี้ ดังนั้นวัตถุประสงค์ของการศึกษานี้ เพื่อตรวจสอบเซลล์ต้นกำเนิดในชิ้นเอ็น interspinous ligament และเนื้อเยื่อจากข้อต่อฟาเซ็ตบริเวณกระดูกสันหลังส่วนเอว โดยเปรียบเทียบกับเซลล์ต้นกำเนิดที่ได้จากชิ้นเอ็น ligamentum flavum และศึกษาตำแหน่งของเซลล์ต้นกำเนิดจากเนื้อเยื่อทั้ง 3 แห่ง โดยเก็บชิ้นเนื้อตัวอย่างที่ได้จากผู้ป่วยเข้ารับการผ่าตัดจำนวน 10 ราย วิเคราะห์ตัวบ่งชี้บนผิวเซลล์ (cell surface markers) จากเซลล์เพาะเลี้ยงทั้ง 3 ชนิด ได้แก่ CD29, CD34, CD44, CD45, CD73, CD90 และ CD105 ด้วยเทคนิค flow cytometry ศึกษาการเจริญของเซลล์ต้นกำเนิดทั้ง 3 สายพันธุ์ (tri-lineage potential cells) ในอาหารเลี้ยงเซลล์ที่มีการชักนำสภาวะ osteogenic, adipogenic และ chondrogenic induction นอกจากนี้ยังย้อมชิ้นเนื้อเพื่อศึกษาพยาธิสภาพของเซลล์ ผลการศึกษาพบ MSCs markers ในเซลล์ต้นกำเนิดจากชิ้นเอ็น interspinous ligament และข้อต่อฟาเซ็ตได้แก่ CD29, CD44, CD73, CD90 และ CD105 ส่วน haematopoietic markers CD34 และ CD45 ให้ผลการทดสอบเป็นลบ เซลล์ต้นกำเนิดจากทั้ง 3 แห่งสามารถถูกชักนำให้เปลี่ยนเป็นเซลล์กระดูก เซลล์กระดูกอ่อน และเซลล์ไขมันได้ ซึ่งยืนยันผลด้วยการย้อมพบแคลเซียม (Alizarin Red S stain) พอลิแซ็กคาไรด์ (Toluidine blue และ Alcian Blue stain) และไขมัน (Oil Red O stain) และพบการแสดงออกของยีนบ่งชี้การเปลี่ยนสภาพของเซลล์ (cell differentiation) ด้วยเทคนิค reverse transcription-polymerase chain reaction และยังพบว่าเซลล์ต้นกำเนิดมีการตอบสนองอย่างดีเยี่ยมต่อการชักนำในสภาวะ osteogenic induction ซึ่งสังเกตได้จากคอโลนีของเซลล์แต่ละชนิด การศึกษาทางพยาธิวิทยาพบเซลล์และหลอดเลือดจำนวนมากล้อมรอบบริเวณที่มีเส้นใยคอลลาเจนหนาแน่นจากเนื้อเยื่อทั้ง 3 แห่ง การศึกษานี้เป็นการศึกษาแรกที่ค้นพบเซลล์ต้นกำเนิดภายในชิ้นเอ็น interspinous ligament และเนื้อเยื่อข้อต่อฟาเซ็ตซึ่งมีตัวบ่งชี้จำเพาะต่อเซลล์ต้นกำเนิด มีความสามารถในการเปลี่ยนสภาพเซลล์ทั้ง 3 สายพันธุ์ และแสดงการตอบสนองต่อการชักนำให้มีการสร้างกระดูก กระดูกอ่อน และไขมัน ซึ่งชี้ให้เห็นว่าเซลล์ต้นกำเนิดจากเนื้อเยื่อทั้ง 3 แห่งบริเวณกระดูกสันหลังอาจมีส่วนเกี่ยวข้องโดยตรงต่อการสะสมแคลเซียมในชิ้นเนื้อเยื่อเหล่านี้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: MESENCHYMAL STEM CELL (MSC) / SPINAL LIGAMENTS / LIGAMENTA FLAVA (LF) / FACET JOINT (FJ) / INTERSPINOUS LIGAMENT (IL) / SURFACE MARKERS / GROWTH KINETICS

BALDUR KRISTJANSSON: Mesenchymal stem cell isolation and characterization from ligamenta flava, interspinous ligaments and facet joints. ADVISOR: PROF. SITTISAK HONSAWEK, M.D., Ph.D., CO-ADVISOR: ASSOC. PROF. WORAWAT LIMTHONGKUL, M.D., 119 pp.

Mesenchymal stem cells (MSCs) are among the most prominent adult progenitor cells and are interesting for their potential in tissue engineering applications as well as their involvement in various diseases. Hypertrophy and calcifications of spinal ligaments are progressive conditions were MSCs are believed to play a role and the existence of MSCs has been demonstrated within the ligamentum flavum (LF) and posterior longitudinal ligament. The aim of this study was to investigate whether these cells could also be found within the interspinous ligaments (ILs) and facet joints (FJs), if they were comparable to LF-derived MSCs and wherein these three tissues cells were localized. Samples were harvested from 10 patients undergoing spinal surgery and cells isolated using direct tissue explant technique. Surface antigen profiling for CD29, CD34, CD44, CD45, CD73, CD90 and CD105 was performed via flow cytometry and to evaluate tri-lineage potential cells were grown in osteogenic, adipogenic and chondrogenic induction medium for 21 days. Histological staining on tissue samples were performed to visualize wherein the tissue cells were localized. Flow cytometry analysis revealed both cell lines expressed the MSCs markers CD29, CD44, CD73, CD90 and CD105 and were negative for the haematopoietic markers CD34 and CD45. All cell lines were capable of osteogenic, adipogenic and chondrogenic differentiation confirmed with histochemical staining of calcium (Alizarin Red S stain), lipids (Oil Red O stain) and polysaccharides (Toluidine blue and Alcian Blue stain). RT-PCR gene expression analysis also confirmed the expression of differentiation related markers. Moreover, a very strong osteogenic response was observed from individual colonies subjected to induction media. Vascular cell rich areas surrounded the avascular dense collagenous areas in LF, FJs and ILs tissues. To the best of our knowledge, this study is the first to demonstrate that MSCs can be found within both the interspinous ligaments and facet joints. Our findings show that they are positive of MSCs markers, capable of tri-lineage differentiation and show a strong osteogenic response suggesting that they might be directly involved in the calcifications of soft tissues in the vertebral column.

Field of Study: Medical Science
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
°C	Degree Celsius
µg	Microgram
µL	Microliter
µM	Micromolar
3’	3’ untranslated region
5’	5’ untranslated region
ACAN	Aggrecan
ALDH	Aldehyde dehydrogenases
ALP	Alkaline phosphatase
AMSCs	Adipose-derived MSCs
ANOVA	Analysis of variance
APC	Allophycocyanin
BMP-2	Bone morphogenetic protein 2
BMSCs	Bone marrow-derived MSCs
Bp	Base pair
CA12	Carbonic anhydrases 12
CADH5	Cadherin-5
cAMP	Cyclic adenosine monophosphate
Cbfa1	Core-binding factor alpha (1)
CD	Cluster of differentiation
cDNA	Complementary DNA

CFU-F	Colony-forming unit fibroblast
Cm^2	Squared centimetres
COL10A1	Collagen-type X-alpha 1 chain
COL2A1	Collagen-type II-alpha 1 chain
Cy5	Cyanine5
dd	Distilled Deionized
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphates
DSLR	Digital single-lens reflex
EDTA	Ethylenediaminetetraacetic acid
F	Female
FBS	Foetal-bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FJs	Facet joints
FoxF1	Forkhead box F1
FSP-1	Fibroblast secretory protein-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMP	Good manufacturing practice
H&E	Haematoxylin and Eosin
HIF-α	Hypoxia inducible factor-alpha
HLA-DR	Human leukocyte antigen-DR
HLA-I	Human leukocyte antigen-I

HLA-II	Human leukocyte antigen-II
IgG1	Immunoglobulin G
IL-1RA	Interleukin-1 receptor antagonist
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-11	Interleukin-11
ILs	Interspinous ligaments
iPSCs	Induced pluripotent stem cells
IRB	Institutional Review Board
ISCT	International Society for Cellular Therapy
ITGA11	Integrin, alpha 11
ITS	Insulin–transferrin–selenium
KDR	Kinase insert domain receptor
KRT19	Keratin 19
LF	Ligamenta flava/Ligamentum flavum
LPL	Lipoprotein lipase
LSCS	Lumbar spinal canal stenosis
M	Male
M	Molar
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar

mm^3	Cubic millimetre
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
ng	Nanogram
nm	Nanometre
nM	Nanomolar
OCT4	Octamer-binding transcription factor 4
OLF	Ossification of the ligamenta flava
OPLL	Ossification of the posterior longitudinal ligament
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Population doublings
PE	Phycoerythrin
PGE2	Prostaglandin E2
pH	Potential of hydrogen
PICP	Procollagen type 1 carboxyl-terminal peptide synthesis
PPAR-γ2	Peroxisome proliferator-activated receptor gamma 2
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
RUNX2	Runt-related transcription factor 2
Sca-1	Stem cells antigen-1
SDF-1	Stromal cell-derived factor 1

Sox2	SRY (sex determining region Y)-box 2
Sox9	SRY (sex determining region Y)-box 9
SSEA4	Stage-specific embryonic antigen-4
STRO-1	Stromal precursor antigen-1
TAE	Tris-acetate-EDTA
TGF-β	Transforming growth factor-β
TGF-β1	Transforming growth factor-β1
TGF-β3	Transforming growth factor-β3
TSA	Trichostatine A
U	Unit
V	Volts
v/v	Volume/volume
vWf	von Willebrand factor
w/v	Weight/volume
α-MEM	Alpha-minimum essential medium
α-SMA	Alpha -smooth muscle actin

CHAPTER I

INTRODUCTION

1. Background

Mesenchymal stem cells (MSCs) were first described as precursor cells of bone and cartilage when isolated by Alexander Fridenstein in 1966 (1). Since then they have been found in numerous adult and foetal tissues and today they are considered multipotent progenitor cells with vast potential for tissue engineering applications. They are well known for their tri-lineage differentiation potentials being able to differentiate into osteoblasts, adipocytes and chondrocytes (2). Equally, epitope profiling can be used to identify them and a number of surface molecules have been associated with MSCs (3).

The International Society for Cellular Therapy (ISCT) has put forward a minimum criteria for defining human MSCs including: spindle-shaped morphology, plastic adherence and tri-lineage differentiation potentials (4). Their criteria also requires MSCs to display the surface antigens CD73, CD90 and CD105 while lacking the expression of the haematopoietic antigens CD11b or CD14, CD79 or CD19, CD34, CD45 and HLA-DR. Many of those surface molecules are also shared with pericytes, adventitial cells and some fibroblasts, suggesting a vascular origin of MSCs (5).

When it comes to tissue engineering applications, MSCs have many favourable characteristics such as trophic and migration properties as well as being immunosuppressive (6, 7). However, this has also caused concern about their potential to give rise to or support tumours or forming ectopic tissues if used in treatment. Notwithstanding, they have been used successfully in a number of human clinical trials and no complications involving tumour formation have been reported.

There are more reasons for studying MSCs in the human body than just for their potential in tissue engineering. MSCs have been reported to be involved in

numerous pathological conditions such as tumour metastasis, aortic valve calcification and myelofibrosis (8-10). Diseases can also affect the number of MSCs present in tissues either increasing or decreasing their number. Additionally, MSCs can lose some of their functions, stemness and show altered differentiation profiles in the presence of diseases. Among these diseases are the degenerative spinal conditions lumbar spinal canal stenosis (LSCS), ossification of the posterior longitudinal ligament (OPLL) and ossification of the ligamenta flava (OLF) where MSCs are suspected to play an important role in the diseases progression (11-13).

The human vertebral column is a complex structure composed of various individual components. It is a major weight bearing structure which is also involved in the movement of the body. Thus, the spinal ligaments are frequently under high mechanical stress and degenerative conditions affecting the joints and ligaments are common. LSCS, OPLL and OLF mainly affect the posterior longitudinal ligament and ligamenta flava (LF). Both are connective ligaments of mesodermal origin found the human vertebral column (14). In degenerative spinal conditions, they can become hypertrophied, compressing the spinal cord (15). Furthermore, ectopic calcification may also be present. Likewise, hypertrophy and calcification have also been reported in the facet joints (FJs) and interspinous ligaments (ILs) which are also connective tissues in the vertebral column (16-18). The FJs are synovial joints connecting the articular process between vertebras and the ILs are ligaments that pass between and connect adjacent vertebral spinous processes. The cause and pathogenesis of these diseases are poorly understood, but genetics, environmental factors and mechanical stress are all believed to be crucial factors (15, 19, 20). MSCs are also believed to play an important role due to their osteogenic potentials.

MSCs have previously been described in ligaments and joint tissues (21, 22). They have been isolated from multiple tissues within the knee joint and ligaments of the shoulder joint. It is however only recently that MSCs were first identified in spinal ligaments when they were described in LF and posterior longitudinal ligaments that

meet the minimum criteria of the ISCT (23, 24). Localization of LF- and posterior longitudinal ligament-derived MSCs has also been shown to be around micro vessels in the LF and posterior longitudinal ligament giving support to the theory of perivascular origin of MSCs (25). What is more, MSCs from ligaments of OPLL patients have been shown to possess superior osteogenic potentials without losing their multipotency, suggesting that MSCs might be directly involved in the ossification process (13). MSCs from LF have also been evinced to have alternative differentiation pathways, showing features of nucleus pulposus-like cells when co-cultured with healthy nucleus pulpous cells from intervertebral disc (26). Taken together, the aforementioned studies show that MSCs in spinal ligament are a worthy study subject for defining the microenvironment in the vertebrae column, their involvement in the progression of pathological conditions, and as a potential source for small scale tissue engineering applications. Accordingly, the objective of this study was to investigate whether MSCs could also be isolated and cultured from other structures of the human spinal ligament and if they shared similarities with the previously isolated LF- and posterior longitudinal ligament-derived MSCs.

2. Research questions

a. Can mesenchymal stem cells be isolated from the interspinous ligaments and facet joints?

b. Are they clonogenic and high proliferating?

c. Do they exhibit a differentiation profile similar to that of MSCs found in ligamenta flava?

3. Objectives

a. Isolation and characterization of mesenchymal stem cells from ligamenta flava, interspinous ligaments and facet joints.

b. Comparison of their colony forming efficiency, expandability, epitope profile and differentiation pathways.

c. Identification of which tissue is the best source of MSCs in terms of colony forming efficiency and growth rate.

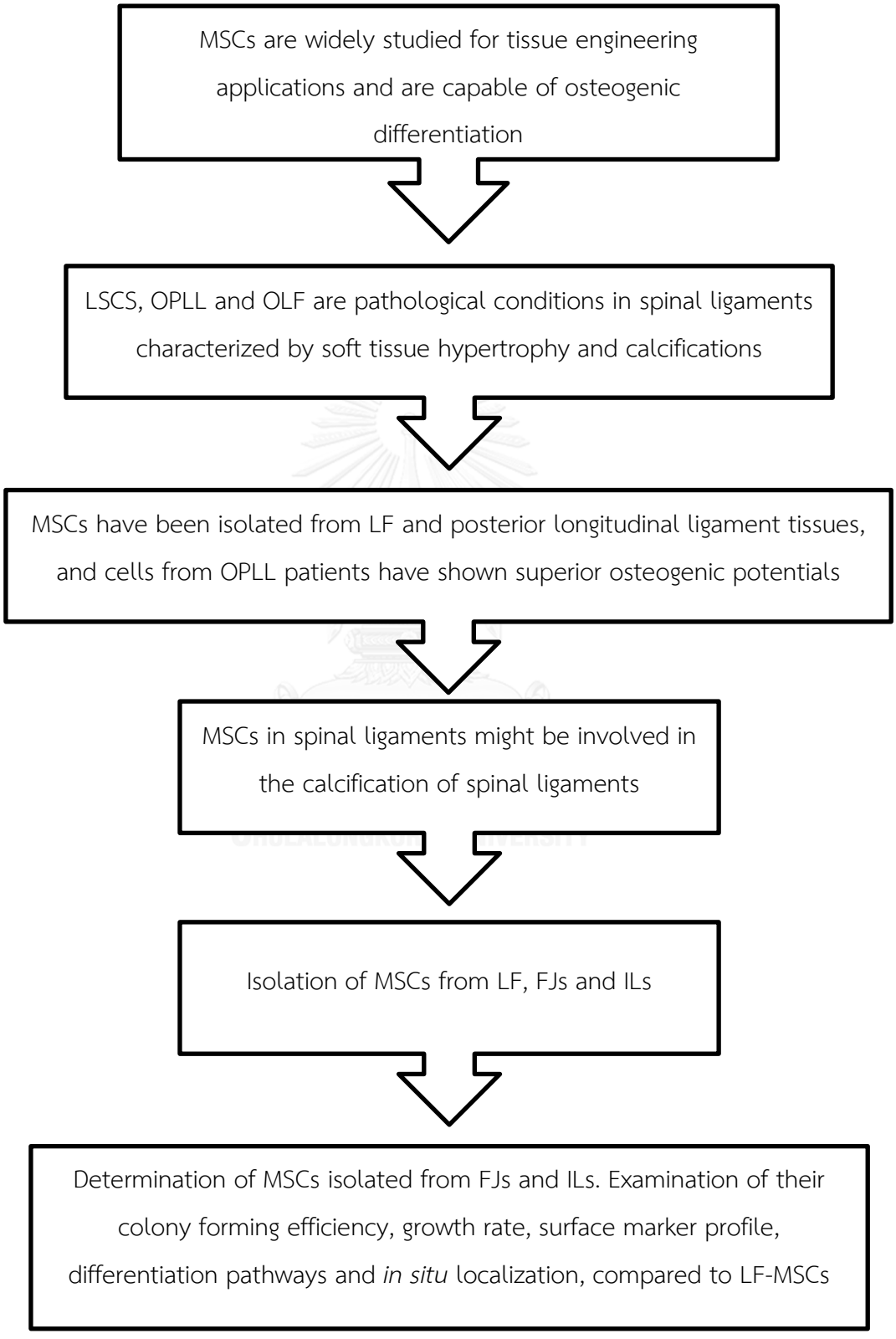
4. Hypothesis

Mesenchymal stem cells can be found in vascular areas of ligamenta flava, interspinous ligaments and facet joints. They are clonogenic, high proliferating, with a similar epitope profile of bone marrow MSCs, and exhibit osteogenic, chondrogenic and adipogenic potentials.

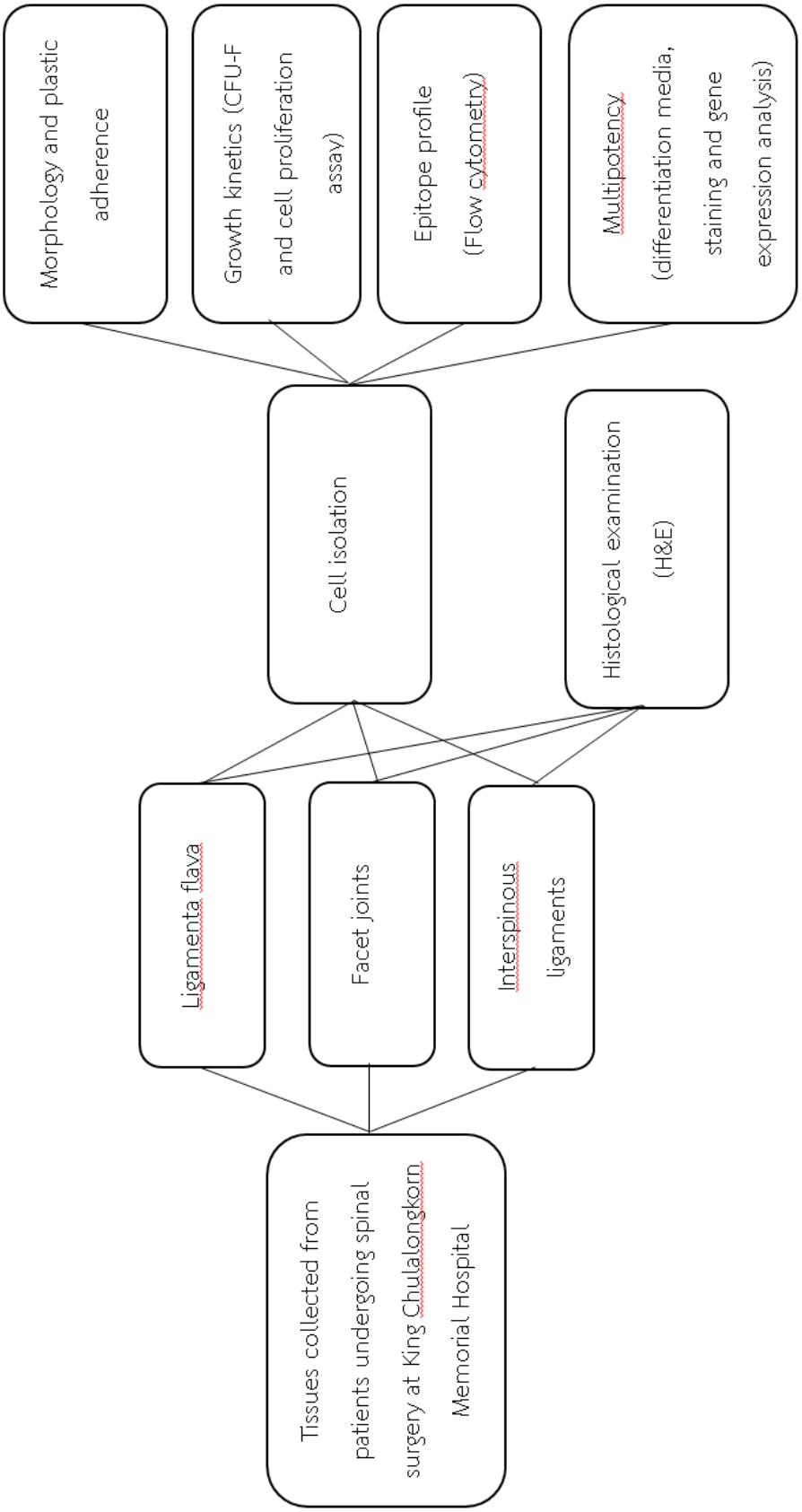
5. Key words

Mesenchymal stem cells (MSCs), Spinal ligaments, Ligamenta flava (LF), Facet joints (FJs), Interspinous ligaments (ILs), Differentiation, Surface markers, Growth kinetics

6. Conceptual framework



7. Experimental design



8. Expected benefits of the study

The benefit of this work is to identify new sources of MSCs that can help in investigation of the pathologic conditions such as LSCS, OPLL and OLF. Furthermore, if the cells show superior differentiation properties into a specific lineage or unique differentiation pathways, they might be interesting for tissue engineering applications.

9. Limitations

Since samples cannot be obtained from healthy individuals, they were harvested from patients undergoing spinal surgeries. Underlying condition might affect the cells harvested for example they might be more or less active, have altered differentiation profiles or be in greater or lesser numbers than those in healthy tissues.

10. Ethical considerations

The present study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (IRB No. 063/54). Written informed consent was obtained prior to patients' participation in the study. Following an informed consent, LF, FJs and ILs were harvested aseptically from discarded tissues of the lumbar spine, L1-S1 levels. This study was carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice.

CHAPTER II
LITERATURE REVIEW

Mesenchymal stem cells

Mesenchymal stromal cells or mesenchymal stem cells (MSCs) are multipotent progenitor cells. They were first described as fibroblast precursors within the bone marrow by Alexander Fridenstein in 1966 (1). Since then they have been shown to exhibit vast mesodermal differentiation potentials able to give rise to osteocytes, adipocytes, chondrocytes, myocytes and tenocytes (2). MSCs are involved in the maintenance and regeneration of connective tissues and are known to migrate to tissues as a result of injury or inflammation where they participate in the repair of the damage (6, 7). They are immunoprivileged cells with immunosuppressive properties by inhibiting the proliferation of CD4+ and CD+8 T-cells, B-cells and natural killer cells (27). MSCs also possess trophic properties and are known to secrete a number of cytokines including PGE2, GM-CSF, IL-1RA, IL-7, IL-8, IL-10 and IL-11, chemokines such as SDF-1 and growth factors (28-31). MSCs are adult stem cells that can be found in numerous post-natal organs and tissues, with some even suggesting that they can be found in all adult tissues (32). They reside predominantly within the bone marrow but other common harvest sources include adipose tissues, skeletal muscles, umbilical cord blood and Wharton's Jelly (33-35). Whilst being a well-studied and a widely used cell line bone marrow-derived MSCs (BMSCs) make up only a small fraction, estimated to be only 0.001% of the mononuclear cells found in the bone marrow (36).

MSC potency

Cell potency can be divided into a few categories (37). Totipotency is the ability of a single cell to give rise to a whole organism such as zygotes and spores (38). Pluripotency refers to cells that can differentiate into any of the three germ layers, endoderm, mesoderm and ectoderm. Pluripotent cells are for example

embryonic stem cells and induced pluripotent stem cells (iPSCs), which are chemically or genetically manipulated somatic cells that regain their differentiation potentials (38, 39). Multipotency is the ability of cells to give rise to a number of cell lines that are usually within the same germ layer, for example haematopoietic stem cells that can give rise to a number of blood cell types (40). Oligopotency is the function of cells to give rise to just a few cell types and unipotent cell just one type (37).

MSCs are in general considered multipotent meaning they can differentiate into multiple cell lines, most notably osteocytes, adipocytes, chondrocytes, myocytes and tenocytes but all of these cell lines are derived from the mesoderm during embryogenesis (41). MSCs have also been shown to differentiate into nerve cells and hepatocytes which are normally derived from the other two embryonic layers, the ectoderm and endoderm (42, 43). This phenomena is known as trans-differentiation and MSCs can be considered as partly pluripotent showing great plasticity (Figure 1.) (44, 45).

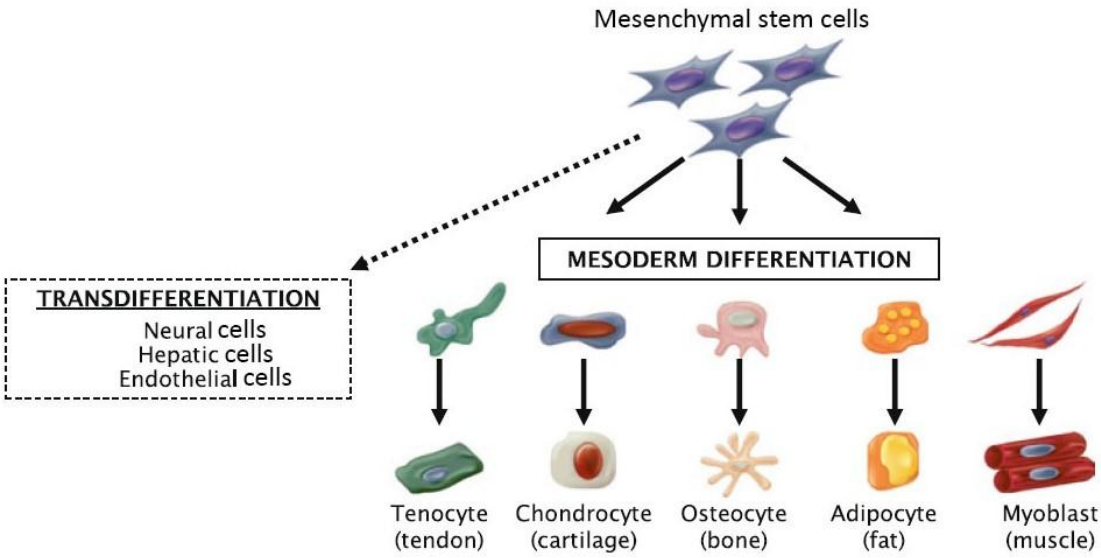


Figure 1. Differentiation potential of mesenchymal stem cells. Adapted from Vemuri et. al. 2011 (46).

Identifying MSCs

MSCs are an elusive niche of cells that share many characteristics with other mononuclear cells. Under normal culture conditions, MSCs display a fibroblast-like morphology, are adherent to plastic and can form colonies from single cells referred to as colony-forming fibroblast units (31), however, only a fraction of the population remains clonogenic. All MSCs are able to give rise to osteocytes, adipocytes and chondrocytes but some populations can give rise to other cell types such as BMSCs that are able to provide the stromal support system for haematopoietic stem cells (hence the name mesenchymal stromal cells) (47). However, not all MSCs fall under this definition such as a subpopulation of BMSCs and adipose-derived MSCs (AMSCs) that are non-adherent to plastic but still exhibit all the other properties of MSCs (48, 49).

Immunophenotypic by detecting cell surface proteins is an important and widely used tool for identifying MSCs. Currently over 363 surface proteins have been identified in both mouse and human cells and they are commonly referred to as cluster of differentiation (CD) markers (3). A number of CD markers have so far been

identified in MSCs most of whom are also in common with fibroblasts and pericytes. In 2006 the International Society for Cellular Therapy (ISCT) put forward a minimum criteria for defining human MSCs in order to standardise the field (4). Their criteria require MSCs to display the surface antigens CD73, CD90 and CD105 while lacking the expression of the haematopoietic antigens CD11b or CD14, CD79 or CD19, CD34, CD45 and HLA-DR (50). At present these markers are the most commonly used markers for identifying MSCs. CD73 is an ecto-5'-nucleotidase playing an important role in stromal interactions and cell migration (51, 52). CD105 (endoglin) is a TGF-β receptor believed to be involved in TGF-β signalling pathway and involved in chondrogenic differentiation (53). Less is known about CD90 (Thy1) functions but all three markers are found on MSCs from most sources. Notwithstanding, CD73, CD90 and CD105 are displayed by adventitial cells, pericytes, skin fibroblasts and umbilical vein endothelial cells making it harder to prove MSCs identity (5). This has led researchers to investigate other markers such as CD10, CD26, CD106, CD146 and ITGA11 do better discriminate pericytes and fibroblasts from MSCs (Table 1).

Table 1. Surface antigens on MSCs, fibroblasts and pericytes.

Surface antigen	**MSCs**	**Fibroblasts**	**Pericytes**	**References**
CD9	+	+		(54)
CD10	±	+	+	(34, 55)
CD13	+	+	+	(34, 55-58)
CD26	Variable	+		(55)
CD29	+	+	+	(54, 58-61)
CD44	+	+	+	(34, 54, 58-60, 62)
CD49a	+	+		(55)

Surface antigen	MSCs	Fibroblasts	Pericytes	References
CD49b	+	+		(55)
CD49d	-	+		(60)
CD49e	+	±	±	(57, 58, 63)
CD54	+	+	±	(55, 58, 60)
CD56	±	+	-	(34, 60)
CD71	+	+		(59)
CD73	+	+	+	(34, 54, 58, 59, 62)
CD90	+	+	+	(34, 54, 55, 58-60)
CD105	+	+	+	(34, 54, 55, 59, 60, 62)
CD106	+	-	-	(34, 54, 55, 60)
CD138	Variable	Variable		(55)
CD146	±	±	+	(34, 54-56, 58, 60, 64)
CD164	+	+	+	(34, 55)
CD166	+	+	+	(34, 54, 55, 58-60)
CD271	±	±	+	(56, 63)
ITGA11	+	-		(54)
CD11a	-	-		(55)
CD14	-	-	-	(55, 58, 59, 62)
CD16	-	-		(55)
CD31	-	-	-	(34, 58-60, 62, 63)

Surface antigen	MSCs	Fibroblasts	Pericytes	References
CD33	-	-	-	(55, 58)
CD34	-	-	-	(34, 55, 58-60)
CD40	-	+		(60)
CD43	-	-		(55)
CD45	-	-	-	(34, 54, 55, 58-60, 62)
CD80	-	-		(60)
CD86	-	-		(55, 60)
CD117	-	-		(55, 60, 63)
CD133	-	-	-	(34, 59, 60)
CD184	-	-		(55)
CD85k	-	-		(55)
HLA-DR	-	-	-	(34, 55, 58)
Sca-1	-	±		(56, 63, 65)
SSEA4	+		+	(34, 56, 63, 66)
STRO-1	±	±	±	(36, 58, 60, 63)
ALDH	±	±		(60)
HLA-I	+	+	+	(34, 58, 60)
HLA-II	-	-	-	(58, 60)
vWf	-	-	-	(34, 60)
KDR	-	-	-	(58)

Surface antigen	MSCs	Fibroblasts	Pericytes	References
GLYPA	-	-	-	(58)
AC 133	-	-	-	(58)
FSP-1	-		±	(58)
CADH5	-	-	-	(58)

Variability in surface antigen profile exists within MSC populations due to difference between individuals and tissue sources. This has made it increasingly difficult to distinguish between cell populations. Some have suggested a perivascular origin of MSCs and identified adventitial cells and pericytes as precursors of MSCs (34). Adventitial cells are non-pigmented cells mainly found around larger vessels and are not associated with endothelial as pericytes are (67, 68). Pericytes are progenitor cells that encircle endothelial cells in capillaries and microvessles and share many features with MSCs (69). They are highly myogenic and have also been shown to be capable osteogenic, chondrogenic and adipogenic differentiation. This has made it very difficult to distinguish between these two cell lines and but CD146 and NG2 have been identified as potentials markers since they are highly expressed by pericytes *in vivo* and *ex vivo* (34). Fibroblasts are the other cell type highly similar to MSCs sharing many key features with both MSCs and pericytes (58). However, many fibroblast cell lines are terminally differentiated with low clonogenic potentials and low or no osteogenic, chondrogenic and adipogenic potentials. In a study from 2011, Alt et al. found that commercially available human AMSCs, human skin-derived fibroblasts and embryonic lung fibroblasts all shared a similar surface marker profile (62). Furthermore, AMSCs and skin-derived fibroblasts were highly clonogenic and could differentiate into osteocytes, chondrocytes and adiposities, whilst embryonic lung fibroblasts lacked that function. In another study from 2013 Brohem et al.

compared bone marrow, skin and adipose-derived MSCs to commercially available MSCs and fibroblasts (70). Likewise, they found that all shared a similar epitope and gene expression profiles. However, in their study the fibroblasts were only capable of osteogenic differentiation but adipogenic and chondrogenic potentials were very low compared to MSCs. This demonstrates how hard it can be to differentiate between those two cell types and often the difference between them is unclear.

Mesenchymal stem cells in tissue engineering

The properties of MSCs can be used for therapeutic applications to treat a number of conditions such as autoimmune diseases, diabetes mellitus, multiple sclerosis and osteoarthritis (71-73). Most commonly the patients' own MSCs are isolated, expanded and used as an autologous cell line in treatment. This eliminates the risks of host rejection, disease transmissions as well as ethical concerns. The use of allogenic cells is also a possibility although the risk of rejections and disease transmission is higher; currently there are a number of trials investigating the effects of allogenic MSCs. Before MSCs can be used in treatment, they are usually expanded *in vitro* first to create a sufficient numbers to work with. However, unlike embryonic stem cells, MSCs do not show unlimited self-renewal capacity and cannot be maintained and expanded indefinitely *in vitro*, raising the questions if they can truly be considered as stem cells. They can be maintained and expanded to confluence for 5-15 passages before losing the ability of self-renewal and differentiation. When used in treatment they are usually grown to confluence for 2-5 passages, before being applied. Further passaging results in loss of function in addition to mutations and possible tumour genetic effects. Although transplantations into immunodeficient animals have shown no evidence of tumour formation and recent studies have shown that unlike MSCs from many mammals human MSCs do not undergo spontaneous transformation when cultured *in vivo*, the risk should not been underestimated (74, 75). In 2011 Suzuki et al. showed that MSCs can support tumour growth by supporting the growth of the tumour stroma (76). They observed a

significant increase in tumour growth when co-injecting mice with tumour cells and MSCs. However, their results were also controversial since they observed no increased tumour growth when using a different MSC lineage. This suggests that MSCs could indirectly contribute to tumour formation and the *in vivo* effects of introducing MSCs into the body have not yet been fully investigated.

A number of considerations have to be taken into account when selecting a source of MSCs for tissue engineering. Harvesting the cells should result in minimum morbidity to the patients or donors and collection should not result in tissue defects. The source should yield a sufficient amount of fully functional MSCs that can be expanded easily in culture and introduced to the target site without causing host rejection or adverse effects. Therefore, obtaining cells for tissue engineering can be a major technical issue. Hitherto, the most common sources of MSCs have been autologous BMSCs and recently the focus has also shifted towards AMSCs. BMSCs can be collected easily without causing tissue defects by drilling into the bone and aspirating the bone marrow (77). Major harvest sites include the iliac crest, tibia and femur, all of which can yield a plethora of bone marrow, from which MSCs can by isolated from and expanded. Adipose tissues are also considered a good source of MSCs and it has been estimated that up to 1,000 times more MSCs can be obtained from each gram of adipose tissue compared to bone marrow making it a very potent source (78). Likewise, BMSCs harvesting AMSCs is relatively simple wherein fat-pads are major harvest sites. Before cultivated cells are applied in treatment, they are usually confirmed as MSCs by immunophenotyping. If this is not done there is no way of knowing if the cells truly are MSCs or simply a niche of unipotent cells that were able to proliferate under the given culture conditions. Although autologous cells have been favoured by researchers, a number of clinical trials focusing on the use of allogenic cells are now listed on clinicaltrials.gov (79). Using allogenic cells from just a few donors might help in standardising treatment since MSCs seem to vary from person to person. Furthermore, age greatly affects the proliferation and

differentiation ability of MSCs. Nevertheless the disadvantages of allogenic cells, host rejection, disease transmission and tumour formation should not be disregarded.

MSCs are an advanced medical therapy and as such they should comply with the good manufacturing practice (GMP) guidelines for medicinal products. However, a therapy that utilises living cells cannot be standardised to the same extent as chemically synthesised medicine and the GMP guidelines for medicinal products is not yet fully capable of dealing with cell based therapies. GMP guidelines vary between countries and regions; in the USA the Food and Drug Administration (FDA) provides the guidelines, whilst in Europe the European Medicines Agency provides them. The FDA has defined two categories of human cell products; the ‘’minimally manipulated’’ category and the ‘’more than minimally manipulated’’ category in which MSCs fall into (80). GMP production requires a clearly defined and well documented manufacturing process, which requires validation at every step. For cell based products that includes routine checks of cell isolations, and cultures for any infectious agents, unwanted elements or cross-contamination of other mononuclear cells, as well as keeping an intense record of the cells origin and donors (81). Although no lab-produced stem cell therapy has been GMP-approved for commercial production in Europe or the USA, a number of clinical trials have been approved and conducted under GMP guidelines.

Despite MSCs being a promising tool for tissue engineering and playing an important role in supplying recovery cells, they also contribute to pathological conditions such as tumour metastasis, aortic valve calcification and myelofibrosis (8-10).

Mesenchymal stem cells in joints and ligaments

Joints are made out of tissue mainly originating from the mesoderm and unsurprisingly MSCs can be found in both synovial and solid joints as well as the ligaments of the mammalian body. In the human synovial joint they were first

described by De Bari et al. when they successfully isolated MSCs from the knee joint synovial membrane in 2001 (21). They have also been isolated and characterised in the meniscus, ligament, fat pad and cartilage of the synovial knee joint suggesting that MSCs play an important role in the maintenance and function of these tissues (82-85). These cells are similar to MSCs from other sources, capable of self-renewal and tri-lineage differentiation, but MSCs from the synovial fluid have been shown to have greater clonogenecity and chondrogenic capacity than those from bone marrow. Furthermore, they show clonal heterogeneity with individual clonal populations exhibiting variable proliferation and differentiation potentials (86). They might therefore seem like an obvious choice for cartilage repair and trials using rabbit models have shown promising results (87). However, difficulties in extraction and limited studies of synovial-derived MSCs have favoured other sources of cells and heretofore only one clinical trial has been performed in humans (88).

Whilst MSCs are so widely distributed within the synovial joint, their function has not been fully elucidated. It is likely that they play an important role in providing an opulent reservoir of repair cells that can be activated for growth, repair and remodelling. Another function might be to reduce inflammation by suppressing the activity of T-cells (79). MSCs can be found in cartilage, albeit they seem to lack the ability for functional repair just like chondrocytes, as it is well known that cartilage fails to regenerate following injury. Whereas MSCs are precursors of chondroblasts, which are immature chondrocytes, they might also serve other purposes such as replenishing the surface zone with proteoglycan lubricant to minimise friction within the joint (89).

A significantly greater number of MSCs can be recovered from the effected joints of osteoarthritis or rheumatoid arthritis patients as well as those of ligament injury compared with that from healthy joints. Furthermore, the number of MSCs also increases with the severity of the disease suggesting they might originate in the degrading synovium (90). In 2002, Murph et al. showed that MSCs from patients with

end-stage osteoarthritis had reduced *in vitro* proliferation and differentiation potentials. They compared MSCs from patients who underwent total knee arthroplasty surgery and compared them with samples from matched healthy individuals. They observed a significantly reduced yield and proliferation activity with cells having reduced chondrogenic and adipogenic activity and increased osteogenic potentials (91). Similar results were produced for retro patellar fat pad-derived MSCs from elderly osteoarthritis patients showing that age and osteoarthritic condition had significantly reduced the differentiation capacity and expression of stemness genes (92). More strikingly it has been evinced that synovial fluid from donors with osteoarthritis or rheumatoid arthritis inhibits the chondrogenic differentiation of MSCs from healthy donors (93). Factors secreted by the synovial membrane of osteoarthritis patients also show similar results. It has been observed that these functional deficiencies can be improved with supplementation of the medium with growth factors (94). Taken together, this suggests degenerative diseases can alter the function of MSCs and that the cells might also be directly involved in the progression of the conditions.

The human vertebral column

The human vertebral column is a complex structure consisting of bones, fibrous tissues, joints, ligaments and muscles. It is made up of approximately 33 vertebrae and forms the spinal canal which houses the spinal cord. These vertebrae are divided into 5 groups depending on morphology and location. At the top of the column there are 7 cervical vertebrae between the thorax and the skull, they are small in size and have a foramen in each transverse process. The middle consists of 12 thoracic vertebrae which are connected to rib bones via synovial joints. Inferior to the thoracic vertebrae is the lumbar region which has 5 lumbar vertebrae. These vertebrae are the largest of the vertebrae and form the skeletal support for the posterior abdominal wall. At the bottom of the vertebral column are the sacral and coccygeal vertebrae. The sacral vertebrae are 5 in number and are fused into a single

bone called the sacrum, the coccygeal vertebrae vary in number but are usually four and are also fused into a single bone called the coccyx (14) (Figure 2.).

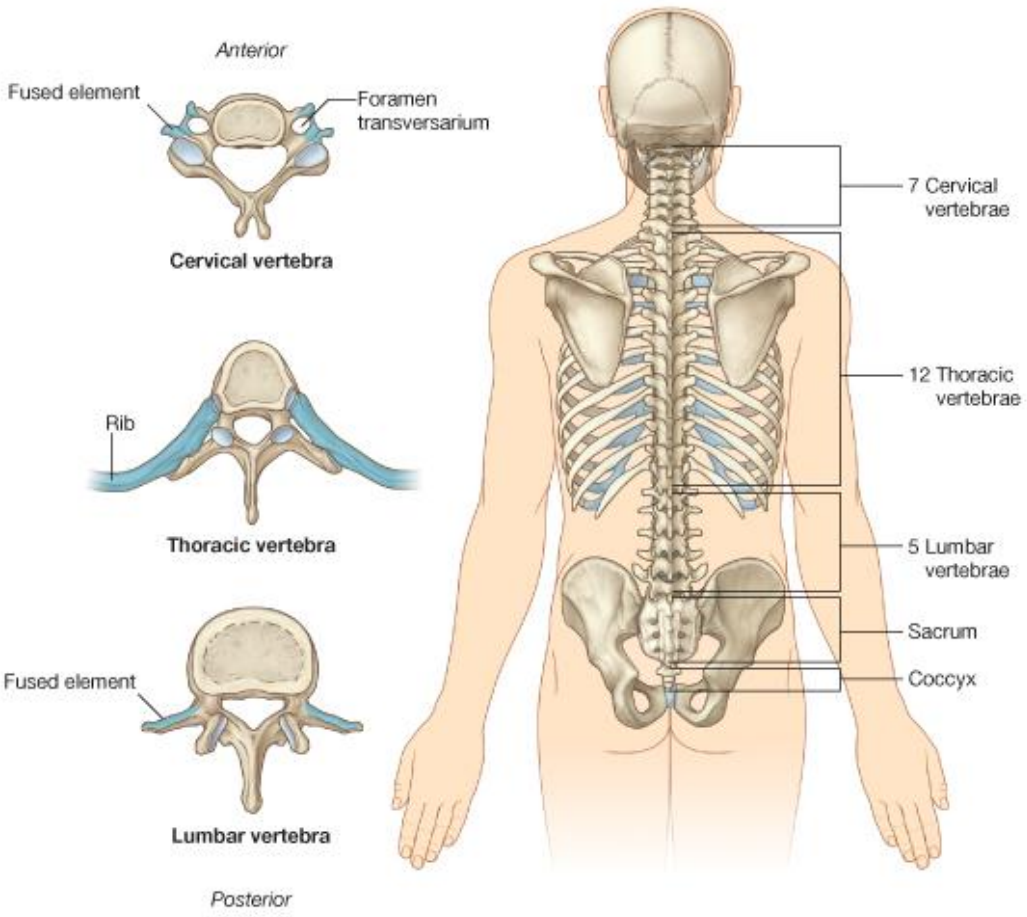


Figure 2. The human vertebral column. Adapted from Gray's Anatomy for Students, 2004 (95).

The 24 articulating vertebrae are held together by a number of ligaments, joints and muscles. There are two types a joints found between vertebrae. A solid joint connects adjacent vertebrae with symphyses which is made of hyaline cartilage and connects to intervertebral discs which are made of collagen, fibrocartilage and

gelatinous substance which is able to absorb compression forces between vertebrae. Two synovial joints know as facet joints (FJs) or zygapophysial joints connect the superior articular process of one vertebra and the inferior articular process of the vertebra directly above it (Figure 3.). Their function is to guide and limit the movement of the spinal motion segment and in the lumbar spine they are curved and adjacent processes interlock, protecting the motion segment from anterior shear forces, excessive rotation and flexion. Ligaments in the vertebrate column also play an important role. The posterior longitudinal ligament and anterior longitudinal ligament extent along most of the vertebral column with the posterior longitudinal ligament inside the spinal canal. The ligament consists of smooth, shining, longitudinal fibers that are denser than those that can be found in the anterior longitudinal ligament that runs down the anterior surface of the spine. The function of these two ligaments is to prevent hyper flexion of the vertebral column (14, 96). Each spinal ligament has two ligamenta flava (LF; singular, ligamentum flavum) which are yellow looking ligaments that connect the laminae of adjacent vertebrae (Figure 4.). They are relatively thin and broad but thinnest in the neck region and thickest in the lumbar region and consist predominantly of elastin which makes them yellow. Their function is to preserve the upright posture and resuming it after flexion (14, 97). Ligamentum nuchae is attached to the skull and runs down to the last cervical vertebrae where it meets the supraspinous ligament which connects the tips of the vertebral spinous processes from the last cervical vertebrae all the way to the sacrum. The interspinous ligaments also known as interspinal ligaments (ILs) are white ligaments that pass between and connect adjacent vertebral spinous processes (Figure 5.). They are thin and membranous and meet with the LF in the front and the supraspinal ligament behind. They are thickest in the lumbar region but in the neck region they are so small and undeveloped that they are often considered part of the nuchal ligament. The function of the ILs is to limit the flexion of the spine (14).

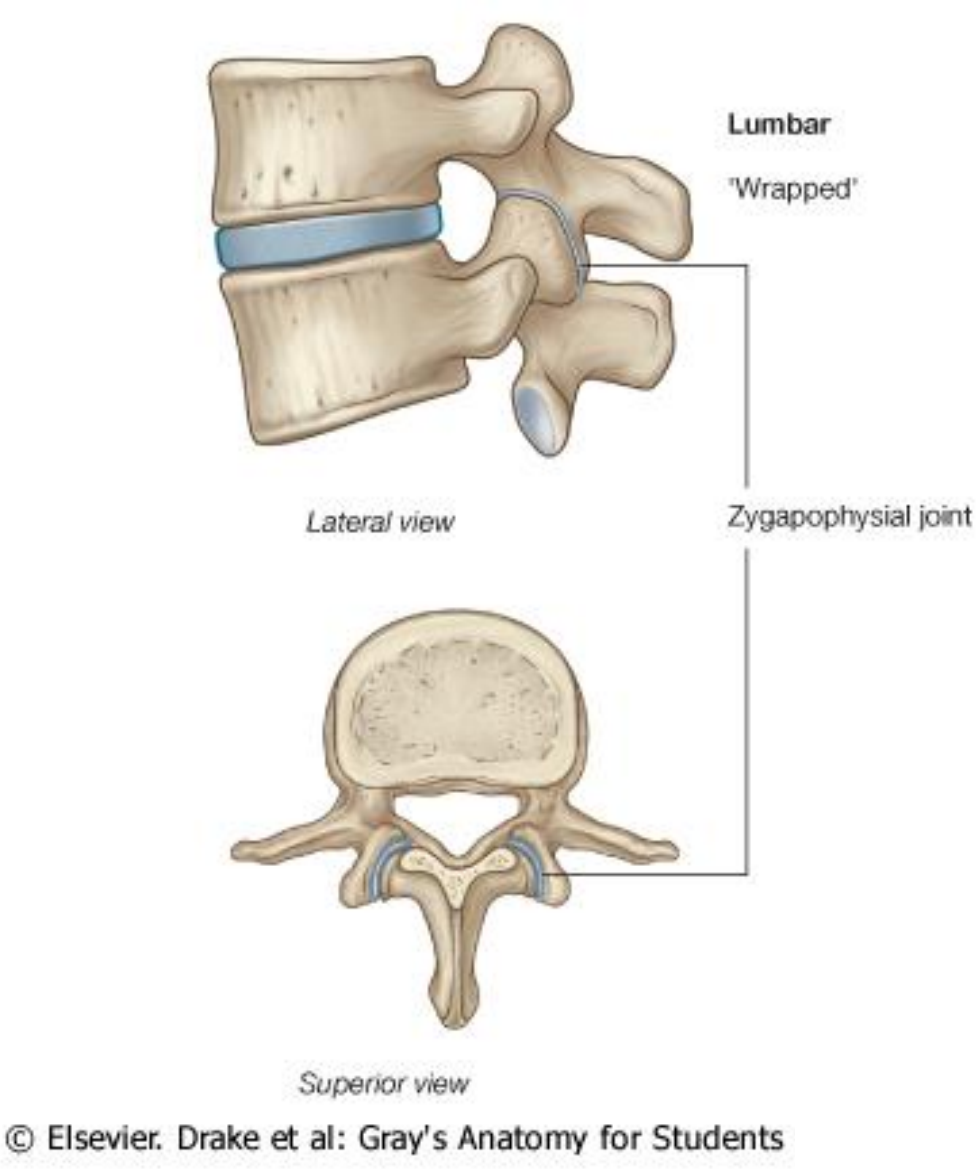


Figure 3. Facet joints (Zygapophysial joints) in the lumbar region of the human vertebral column. Adapted from Gray's Anatomy for Students, 2004 (95).

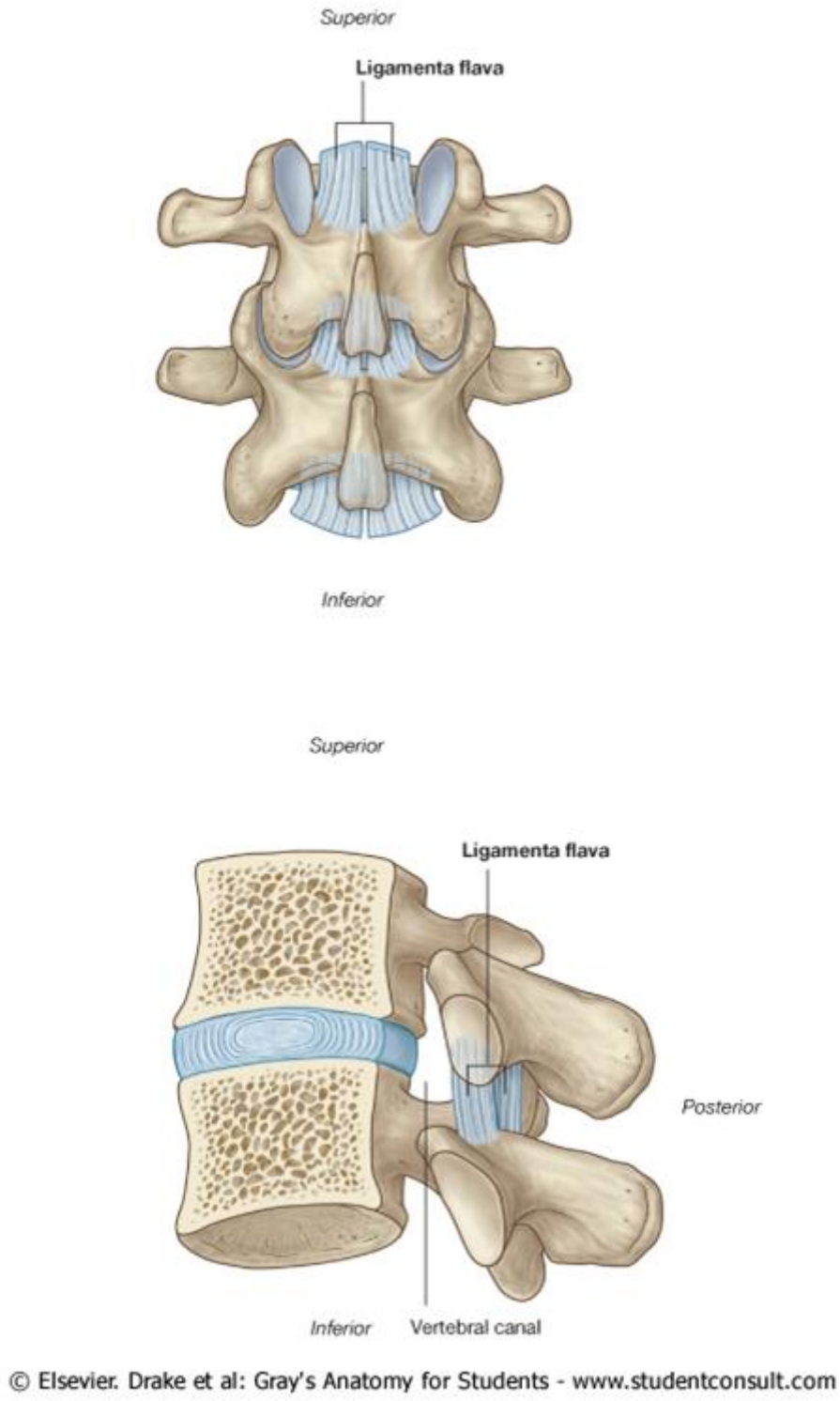


Figure 4. Ligamenta flava in the human vertebral column. Adapted from Gray's Anatomy for Students, 2004 (95).

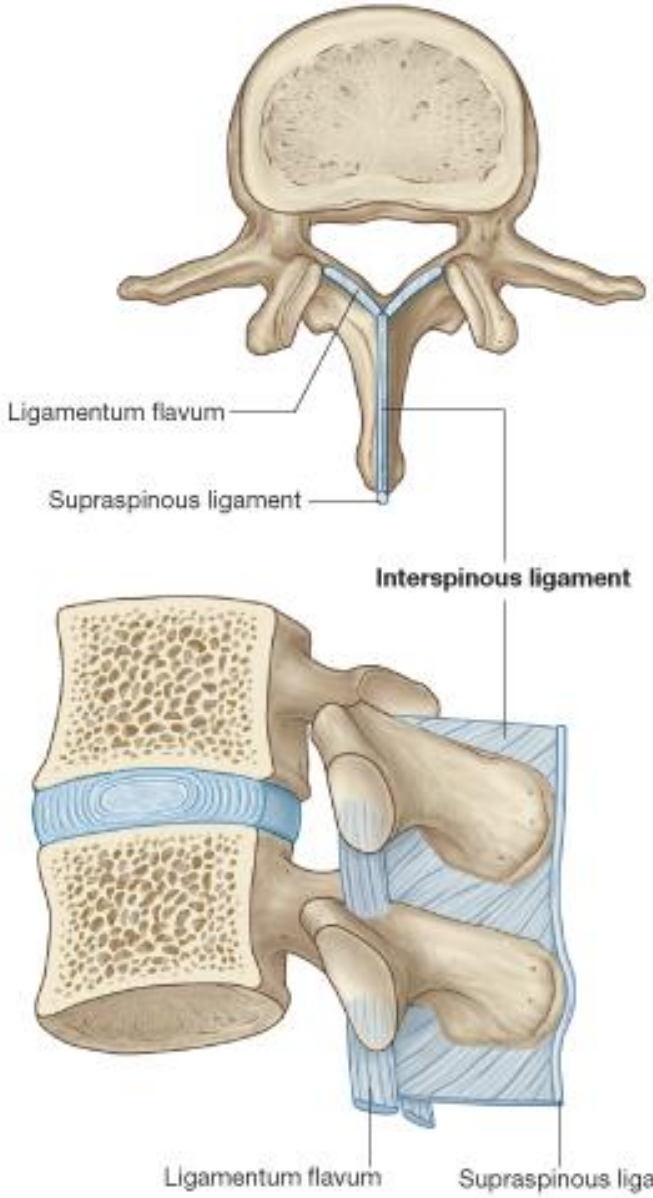


Figure 5. Interspinous ligaments in the human vertebral column. Adapted from Gray's Anatomy for Students, 2004 (95).

The vertebral column is a major weight bearing structure which is also involved in the movement of the body. Consequently, the spinal ligaments are under a lot of mechanical stress and degenerative conditions affecting the joints and ligaments are not uncommon. Since the vertebral column houses the spinal cord, then any changes in the spinal canal can be a major source of pain and cause serious and irreversible damage to the spinal cord. The intervertebral disc, posterior longitudinal ligament, LF, FJs and ILs are all part of the spinal canal and number of diseases can cause hypertrophy or ectopic calcifications in these structures.

Lumbar spinal canal stenosis, ossification of the ligamentum flavum and ossification of the posterior longitudinal ligament

Lumbar spinal canal stenosis (LSCS) is a narrowing of the spinal canal caused by hypertrophy of the LF and sometimes in the FJs as well. Spinal canal stenosis mainly affects the lumbar region and may result in the compression of the spinal cord and nerves of the lumbar vertebrae. LSCS usually occurs in elderly patients and spinal degeneration as the result of ageing and mechanical stress have been suggested as the cause of the condition, albeit the pathogenesis remains undetermined. The LF is mainly composed of elastin and collagen fibres and with age the elastin-to-collagen ratio decreases and the LF loses its elasticity and becomes stiffer. This may cause the LF to hypertrophy sometimes results in LSCS. Although, LSCS is mainly characterized by the hypertrophy of the LF, calcification and ectopic bone formations may also be present (98-102). The reason for the loss of elastin-to-collagen ration and calcifications in LSCS remains unknown but genetics and mechanical stress have been associated with it (20, 103). Furthermore, this condition can also affect the FJs where hypertrophy and calcifications have also been reported in LSCS patients (16).

Ossification of the ligamentum flavum (OLF) is an ossification process that mainly occurring in elderly Japanese people although this disease has also been reported in Korean, Chinese and European people (104-109). It is similar to LSCS in a

way as the LF becomes calcified and compress the spinal cord. OLF can occur anywhere in the vertebral column and has been reported as the cause of thoracic myelopathy (110). Genetics, diet and extrinsic causes have been suggested as causes of OLF (111, 112). However, pathogenesis of the condition has not been fully elucidated yet.

Ossification of the interspinous ligament is a very rare condition and only a few case reports describing the condition exist (17, 18, 100).

Ossification of the posterior longitudinal ligament (OPLL) is a disease of progressive ectopic calcification of the posterior longitudinal ligament (113). Like OLF this condition mainly affects the Japanese population but has also been reported in other Asian and Caucasian population (114, 115). OPLL is characterized by the calcification and bone formation that mainly occurs in the cervical and thoracic segments of the posterior longitudinal ligament. It causes narrowing of the spinal canal and may result in compressive myelopathy and radiculopathy (113). The condition has been extensively studied and linked to various epidemiological, genetic, metabolic and mechanical factors (116, 117). However, the pathologies of the disease are not fully understood but it is believed that stem cells play an important part in the progression of the condition (11-13).

The causes and pathogenesis of all the aforementioned conditions remain poorly understood. Notwithstanding, ossification and fibrocartilage formation play an important role in the progression of them all. MSCs are capable of both osteogenic and chondrogenic potentials and might be involved ossification and fibrocartilage formation. Even so there have been few studies on their involvement and impact in degenerative spinal conditions.

Mesenchymal stem cells in spinal tissues

MSCs in spinal tissues were first isolated by Chen et al. in 2011 (23). They hypothesized that MSCs could be isolated from the human LF and might play an important role in the pathogenesis of LF hypertrophy. They were successful in isolating and culturing cells from LF of six patients who underwent spine surgery. The LF samples were minced down and digested with type 1 collagenase and the resulting nucleated cells plated and cultured. They examining the morphology, surface marker profile and differentiation potentials of the isolated cells. The cells isolated in this study were spindle shaped and showed a fibroblast-like morphology. They were positive for the MSC markers CD29, CD44, CD73, CD105 and CD166 but negative for the haematopoietic markers CD34, CD45 and CD133. This is the same morphology and a similar surface marker profile as reported from BMSCs (4). The cells were also able to differentiate into osteocytes, chondrocytes and adipocytes confirmed by Alizarin Red S, Oil Red O, Alcian blue and collagen type III staining, and gene expression analysis of the osteogenic markers *Runx2* and *Osteopontin*, the adipogenic markers *PPAR-γ2* and *LPL* and the chondrogenic markers *COL2A1* and *Aggrecan*. Together these data confirm the existence of MSCs in the ligamentum flavum. In addition, they checked the MSCs in response to transforming growth factor beta (TGF-β1) and trichostatine A (TSA). They demonstrated that administration of TGF-β1 stimulated cell proliferation and the expression of genes linked with osteogenic and myogenic differentiation; however, TSA blocked this effect.

With OLF and OPLL being prevalent diseases in Japan, the Japanese scientists have also been interested in the role of MSCs in spinal conditions. In 2011, Asari et al. isolated MSCs from the LF and posterior longitudinal ligament (24). Spinal ligament samples were harvested during surgery from eight patients, four suffering from LSCS and 4 from OPLL. They isolated cells from 6 ligamenta flava samples and 2 posterior longitudinal ligament samples. They performed colony forming unit-fibroblast (CFU-F) assay, cell proliferation assay at both low and a high seeding

densities, flow cytometry, cell differentiation assay, gene expression analysis and immunohistochemical staining of LF and posterior longitudinal ligament with CD34, CD45 and CD90. The results showed that colony-forming efficiency, expansion potential and proliferation ability of the isolated cells was no less than that of BMSCs that are commonly used. The cells were positive for the classical MSCs markers CD73, CD90 and CD105 while being negative or low for CD11b, CD19, CD34, CD45 and HLA-DR. Furthermore, the cells showed osteogenic, adipogenic and chondrogenic potentials when grown in differentiating medium. This was confirmed by immunohistochemical staining with Alizarin Red S, Oil Red O and Toluidine blue. In addition, real-time PCR analysis showed that the expression of osteogenic genes: *BMP2, Runx2* and *ALP*, adipogenic genes: *PPAR-γ2* and *LPL*, chondrogenic genes: *Sox9, COL2A1* and *COL10A1* were upregulated during differentiation. Tissue staining of the LF and posterior longitudinal ligaments showed that cells in the tissues stained positive for the MSCs marker CD90 and negative for the haematopoietic markers CD34 and CD45. This meets the definition of MSCs put forward by the ISCT and they were able to confirm their isolated cells as MSCs with self-renewal capacity similar to those of bone marrow, synovium adipose tissue and knee muscle derived MSCs. Although the cells in this study were derived both from LF and posterior longitudinal ligament, they did not perform any separated analysis of the cell populations.

In a follow-up study from the Japanese team, they compared the localization of MSCs in ossified human spinal ligaments to that of non-ossified tissues (25). They visualized where in the tissues the cells were with immunohistochemical *in situ* staining using antibodies for MSC markers CD73, CD90 and CD105, endothelial marker CD31 and pericyte marker α-SMA and chondrocyte maker S100. Following a double immunofluorescence staining, the tissue sections were examined by confocal laser scanning microscope. Their results showed that MSCs were concentrated around blood vessels in both ossified and non-ossified samples and a greater number

existed in ossified samples and they suggested there was a close relationship between neovascularization on the accumulation of MSCs during damage ligament repair. They also found that MSCs in the collagenous matrix of the non-ossified samples were sparse but in the ossified samples MSCS situated amongst the irregular arrangement and fragmented collagenous fibers. This suggests that MSCs migrate from certain areas to micro-injured ligament tissue sites to restore damaged ligamentous tissues. In December 2013, the Japanese team submitted yet another paper on their findings. They were able to show that MSCs from patients with ossified spinal ligaments had superior osteogenic potentials (13). They quantified the osteogenic potential by measuring the absorbance of bound Alizarin Red S by first extracting it and then measuring it at 570 nm in a spectrophotometer. This showed a significant difference between the cells from ossified samples and the controls. Furthermore, there was a significant difference in alkaline phosphatase activity. They also investigated chondrogenic and adipogenic potentials using similar techniques but found no significant difference between groups. These findings further support that MSCs are involved in the ossification of the spinal ligament and that they have an altered differentiation profile showing superior osteogenic potentials without losing the ability to differentiate into adipocytes or chondrocytes.

MSCs have also been isolated from the bone marrow of the vertebrae. In 2013 Barbanti et al. isolated and compared cells from the iliac crest bone marrow, vertebrae bone marrow, colon mucosa and dental pulp (118). They investigated the growth kinetics, immunophenotype and differentiation potentials of the cells. Their results showed that vertebrae bone marrow derived cells could be maintained in culture for a longer period of time than cells from the other sources. They grew at the same rate as iliac crest derived cells but could be maintained in culture twice as long and accumulated over 20 population doublings. Nonetheless, they did not accumulate as many population doublings as the rapidly growing colon mucosa and dental pulp derived cells. The surface marker profile of the cell populations were

similar being positive for the MSCs markers CD44, CD90, CD105 and CD146, whilst negative for the haematopoietic markers CD34 and CD45. Interestingly, vertebrae derived cells showed greater plasticity than both colon mucosa and dental pulp derived cells and generated osteocytes, adipocytes and chondrocytes with a greater efficiency. They concluded that vertebrae bone marrow derived cells was the best source of cells in term of expansion and differentiation and might be applied in clinical application for spinal fusion in spine surgery.

The potential use of spinal ligament MSCs for tissue engineering application has also drawn the attention of scientist. In 2015, Han et al. studied the differentiation of human LF-derived cells towards intervertebral nucleus pulposus-like cells in a co-culture system (26). First they isolated MSCs from the LF and compared them to BMSCs. They isolated the cells in a similar way as in previous studies and examined the expression of the stem cell genes *Nanog, Sox2* and *OCT4* (usually associated with pluripotent embryonic stem cells and iPSCs (39)), surface marker profile and differentiation potentials. The expression of *Nanog* and *Sox2* was similar between cells from LF and BMSCs, but interestingly the expression of *OCT4* was significantly higher in LF cells. The cell population was positive for CD29, CD44, CD73, CD90 and CD105 and negative for STRO-1, CD34, CD45 and CD133. In agreement with previous reports they grew MSCs in osteogenic medium for 21 days and confirmed osteogenesis with both Alizarin Red S staining and gene expression analysis. However, for adipogenic differentiation they subjected cells to a 96-hour treatment cycle with both induction media and control media that was repeated 5 times. Likewise, adipogenesis was confirmed with Oil Red O staining and gene expression analysis. They also employed a different approach for chondrogenic differentiation where they used a micromass-culture to generate cell rich droplets and grew them in multi-well plates for 21 days. Alcian blue staining and gene expression analysis confirmed the chondrogenic potentials of their cell population and thus the cells multipotency. For the co-culture model, they grew LF cells in

direct contact with nucleus pulpous cells through 1-μm-pore-size, high-pore-density wells to promote differentiation. The cells were co-cultured for 14 days under two different oxygen settings, hypoxia (2% O_2) and normoxia (20% O_2). Cell differentiation was evaluated by morphology, presence of CD24, and real-time and Western-blotting analysis of KRT19, CA12, COL2A1, HIF-**α**, FoxF1, ACAN and Sox9. They observed a change in morphology with LF cells becoming more round shaped when co-cultured. A significant increase in levels of CD24+ was also observed in cells grown in co-culture compared to control cells, confirmed both with flow cytometry analysis and staining. Real-time and Western-blot analysis also showed significant difference between co-cultured and control cells for all genes tested. Furthermore, the expression of *KRT19, CA12, COL2A1, HIF-**α**, ACAN* and *Sox9* was significantly increased in cells grown in hypoxia compared to cells from normoxia cultures. This might stem from the fact that the environment of the intervertebral disc is hypoxic and growing cells in hypoxia might better mimic their natural environment as well as reducing oxidative stress (119, 120). In summary, they concluded that MSCs could be isolated from the LF that showed high similarity with BMSCs but where not identical. Additionally, the cells showed nucleus pulposus-like properties when co-cultured with nucleus pulposus cells especially when cultured under hypoxia.

CHAPTER III

METHODOLOGY

Materials, equipment and reagents

Materials

- 100 mm cell culture dish (Eppendorf, Hamburg, Germany)
- 6-well plates (Thermo Fisher Scientifics, Waltham, MA, USA)
- Disposable gloves (Proglove, Thailand)
- Disposable pipette: 25 mL
- Glass Pipette: 1 mL, 5 mL, 10 mL (Witeg, Germany)
- Glass slide, SuperFrost® Plus (Thermo Scientific, Germany)
- Microcentrifuge Tube: 0.2 mL, 0.5 mL, 1.5 mL (Bio-Rad, USA)
- Needle, 18G, 20G, 22G, Sterile (Nipro, Thailand)
- Parafilm (American National Can, USA)
- Petri Dish (Sterilin, UK)
- Pipette Tip: 10 µL, 200 µL, 1000 µL (AxyGen, USA)
- Polypropylene Conical Tube, Sterile: 15 mL, 50 mL (Elkay, USA)
- Single use syringe, sterile (Nipro, Japan)
- Surgical blade, stainless steel no. 23 (Feather, Japan)
- T175 culture flask (TPP, Trasadingen, Switzerland)
- T25 cell culture flask (TPP, Trasadingen, Switzerland)
- T75 culture flask (Nunc, Roskilde, Denmark)

Equipment and programs

- Accujet® pro Pipette Controller (BrandTech Scientific, Inc., USA)
- Autoclave (Hydroclave Harvey, USA)
- Balance (Sartorius, Germany)

- Cell culture incubator; Forma Series II water jacked CO_2 incubator (Thermo Scientific, Germany)
- Centrifuge, Refrigerated Centrifuge (Eppendorf, USA)
- Centrifuge, Micro-centrifuge High Speed (Eppendorf, USA)
- Centrifuge, Kokusan H-103N Series (Kokusan, Japan)
- Class II biological safety cabinet: Model BH-120 (GelmanSciences, Australia)
- Combs (Bio-Rad, Hercules, USA)
- Electrophoresis Chamber Set (Bio-Rad, USA)
- BD FACSCaliburTM (BD Bioscience, San Jose, CA, USA)
- FlowJo (FLOWJO LCC, Ashland, OR, USA)
- Forceps
- Freezer -80°C. (Forma Scientific, USA)
- Gel Doc 1000 (Bio-Rad, USA)
- GraphPad Prims 5 (GraphPad Software, Inc., La Jolla, CA, USA)
- IMB SPSS Statistics 22 (IMB, Armonk, NY, USA)
- Microscope (40x, 100x, 200x, 400x) with camera (Nikon, Japan)
- Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA)
- Nanodrop® ND-1000 Spectrophotometer (Scientific, USA)
- Thermal cycler PCR machine, Mastercycler personal (Eppendorf, Hamburg, Germany)
- pH Meter (Eutech Cybernataics)
- Pipette 10 µL, 200 µL, 1000 µL (Gilson, France)
- Power Supply Model 250 (Bio-Rad, USA)
- Reagents Bottle 100 mL, 250 mL, 500 mL, 1000 mL (Duran, USA)
- Refrigerator (Sanyo, Japan)
- Scalpel

- Stirring-Magnetic Bar
- Test Tube Racks
- Vortex Mixer (Scientific Industry, USA)
- Water Purification Equipment (Water Pro Ps, Labconco USA)
- Water Bath, Memmert WB45 (Memment, Germany)

Reagents

- Sample collection
    - PBS
- Cell isolation and culture
    - 70% Ethanol
    - Distilled water
    - Foetal bovine serum (Hyclone® Laboratories Inc., USA)
    - Penicillin/streptomycin (Hyclone® Laboratories Inc.)
    - Sodium Bicarbonate (UCS, Thailand)
    - α-MEM (Hyclone® Laboratories Inc., USA)
- Colony-forming unit fibroblast assay (CFU-F)
    - Crystal Violet (Carlo Erba, Italy)
- Flow cytometry
    - APC anti-human CD45 (BioLegend, USA)
    - APC anti-human CD73 (BioLegend, USA)
    - FITC anti-human CD34 (BioLegend, USA)
    - FITC anti-human CD90 (BioLegend, USA)
    - Paraformaldehyde (Sigma-Aldrich, USA)
    - PE anti-human CD105 (BioLegend, USA)
    - PE anti-mouse/human CD44 (BioLegend, USA)
    - PE/Cy5 anti-human CD29 (BioLegend, USA)
    - Sodium azide (NaN3) (Sigma-Aldrich, USA)

- Cell Differentiation
    - 100% Ethanol
    - 3-isobutyl-1-methylxanthine (MP Biomedicals, USA)
    - Alizarin Red S (Sigma-Aldrich, USA)
    - Ammonia
    - Ascorbate-2 phosphate (Sigma-Aldrich, USA)
    - Dexamethasone (Sigma-Aldrich, USA)
    - Indomethacin
    - Insulin
    - Insulin–transferrin–selenium (ITS supplement) (MP Biomedicals, USA)
    - Isopropanol
    - Neutral buffered formaldehyde (Sigma-Aldrich, USA)
    - Oil Red O (Fluka, Germany)
    - Toluidine blue (Merck, USA)
    - Transforming growth factor-β3 (ProSpec, Rehovot, Israel)
    - β-glycerophosphate (Sigma-Aldrich, USA)
- RNA isolation
    - RNeasy Mini Kit (QIAGEN, USA)
        - Buffer RLT
        - Buffer RW1
        - Buffer RPE
        - RNase-free water

- Reverse transcription-polymerase chain reaction (RT-PCR)
    - TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems, USA)
        - 10x Taqman RT buffer
        - 25 mM MgCl2
        - 100 mM dNTP
        - Random hexamer
        - RNase inhibitor
- Polymerase chain reaction (PCR)
    - 1× TAE Buffer (Bio Basic Inc, Thailand)
    - 10 µM Primer Forward (Bio Basic Inc, Thailand)
    - 10 µM Primer Reverse (Bio Basic Inc, Thailand)
    - 100 bp DNA ladder
    - Agarose Molecular Grade (Sigma, USA)
    - Ethidium Bromide (Sigma, USA)
    - Loading dye
    - PerfectTaq Plus MasterMix Kit (5 PRIME, Hilden, Germany)
        - Mastermix
- Haematoxylin and eosin staining of spinal ligaments
    - Haematoxylin and Eosin (C.V. Laboratories, Thailand)

Sample collection

The present study was approved by the Institutional Review Board (IRB) on Human Research of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained prior to patients' participation in the study. LF, FJs and ILs were harvested aseptically from 10 patients, 7 females and 3 males. The patients aged ranged from 50-84 years with a mean age of 64.2 ±11.5 years (Table 2). The samples were collected from the lumbar and sacrum spine, L1-S1 levels of

patients undergoing surgery suffering from LSCS, spondylolisthesis, degenerative scoliosis and herniated nucleus pulposus. LF, FJs, ILs were collected *en bloc* from discarded tissues during spinal surgery. They were washed and stored in phosphate buffered saline (PBS) at 4°C until cell isolation.

Table 2. Characteristics of patients in the current study

Patient no.	**Age**	**Sex**	**Diagnosis**	**Spinal ligament collected**
1	52	F	Spondylolisthesis	LF, FJ,IL
2	59	M	LSCS	LF, FJ,IL
3	60	F	Spondylolisthesis	LF, FJ,IL
4	74	M	LSCS	LF, FJ,IL
5	80	F	Degenerative scoliosis	LF, FJ,IL
6	64	F	Degenerative scoliosis and claudication	LF, FJ,IL
7	84	F	Degenerative scoliosis with LSCS	LF, FJ,IL
8	50	M	Herniated nucleus pulposus and LSCS	LF, FJ,IL
9	58	F	Spondylolisthesis	LF, FJ,IL
10	61	F	Degenerative scoliosis	LF, FJ,IL

Cell isolation

Cells were isolated via direct tissue explant. LF, FJs and ILs tissue samples were washed with sterile PBS to remove blood, and placed on a petri dish. If present, bone fragments, debris and calcified areas were carefully removed. Subsequently the samples were cut into small pieces (1-2 mm^3) using a scalpel and forceps. Approximately, 20-40 pieces from each sample were placed into T25 cell culture flask (TPP, Trasadingen, Switzerland) using sterile syringe and needle. Small drops of culture medium, α-minimum essential medium (α-MEM; Hyclone® Laboratories Inc., South Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone® Laboratories inc.) and 200 U/mL penicillin/streptomycin (Hyclone® Laboratories Inc.) were placed at each piece to allow adhesion and cell expansion. Samples were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture medium changed twice a week using a sterile syringe and needle or a sterile glass pipet. After cells emerged (passage 0) culture medium per flask was increased to 1-3 mL and when cells reached 70-80% confluence, tissue pieces were carefully detached and removed with a needle before cells were harvested with 0.25% Trypsin EDTA (Hyclone® Laboratories Inc.) and incubated for 5 minutes at 37°C and the trypsin neutralised with 3 mL of culture medium. The cell suspension was centrifuged at 1,500 rpm for 10 minutes, counted using haemocytometer and cells passed into T75 culture flask (Nunc, Roskilde, Denmark) for further culturing. Flasks showing no cell outgrowth after 30 days incubation were discarded.

Colony-forming unit fibroblast assay (CFU-F)

To evaluate colony-forming efficiency colony-forming unit fibroblast (CFU-F) assay was performed on passage 0 cells. Cells from passage 0 were diluted in culture medium and cells seeded at the density of 10, 100 and 1,000 cells per well in 6-well plates (Thermo Fisher Scientifics, Waltham, MA, USA) with 2 mL of culture medium in each well. Plates were incubated for 14 days at 37°C in a humidified atmosphere of

95% air and 5% CO_2 and culture medium changed two times per week. Following incubation period, plates were washed with PBS and stained with 0.5% Crystal Violet (Carlo Erba, Italy) in methanol for 10 minutes at room temperature. Stained plates were washed with distilled water until all excess dye was removed and visible colonies of 5 mm or greater in diameter counted. This experiment was performed in triplicates on cells from all tissues of all patients enrolled.

Cell proliferation assay

Proliferation capacity of isolated cell was measured by passing cells counting population doublings (PDLs) until cells entered senescence. Cells from passage 0 were counted and diluted in culture medium and seeded at the density of 50 cells/cm^2 in T25 culture flasks with 5 mL of culture medium. The flasks were incubated for 14 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture medium changed two times per week. After 14 days incubation time cells were harvested with 0.25% Trypsin EDTA, centrifuged at 1,500 rpm for 10 minutes and counted in haemocytometer and reseeded at 50 cells/cm^2. This process was repeated until the cells stopped proliferating and entered senescence state. PDLs were calculated using the following formula were X = initial PDL, I = cell inoculum (number of cells plated in the flask), Y = final cell yield (number of cells at the end of the growth period) (46).

Equation 1. Equation for calculating population doublings.

$$PDL = X + 3.322\ (\log(Y) - \log(I))$$

Growth curve was established by plotting PDL against days in culture. This experiment was performed on cells from all tissues of all patients enrolled

Flow cytometry

Cell-surface expression profile was analysed using flow cytometry. Approximately 1.5 x 10^6 cells were needed for each flow cytometry run. In order to gain sufficient numbers of cell to process for flow cytometry, cells at passage 2-3 were seeded in T175 culture flask and incubated until confluent. Cells were harvested with 0.25% Trypsin EDTA and transferred to 15 mL tubes and centrifuged at 1,500 rpm for 10 minutes. Pellets were suspended and washed with 15 mL of cell staining buffer consisting of PBS with 2% FBS. The suspensions were centrifuged at 1,500 rpm for 5 minutes and supernatant discarded, this was repeated twice. After that the pellets were resuspended in cell staining buffer and cells counted with haemocytometer. Afterwards, 500,000 cells for each CD marker/CD marker set or reference run were transferred to a new 15 mL tube. Each tube was washed with 15 mL of cell staining buffer containing sodium azide (NaN_3), centrifuged at 1,500 rpm for 5 minutes and the supernatant discarded. Subsequently, the pellet was resuspended in 100 µL of cell staining buffer containing sodium azide and conjugated fluorescent, purified primary antibodies added at predetermined optimum concentrations (5-20 µL). The reaction was incubated on ice for 30 minutes, away from any light source. The total of 7 surface markers were analysed, the MSCs markers CD29 (Intergrin beta-1), CD44, CD73 (5'-nucleotidase), CD90 (Thy-1) and CD105 (Endoglin) along with the haematopoietic markers CD34 and CD45 (Protein tyrosine phosphatase receptor type C). The CD markers were divided into two sets depending on their fluorochrome, set A contained CD29, CD44, CD45 and CD90 whilst set B was composed of CD34, CD73 and CD105 (Table 3). Following staining cells were washed twice with 15 mL cell staining buffer containing sodium azide before being suspended in 100 µL cell staining buffer with 0.5% (v/v) paraformaldehyde and transferred to FACS tubes. Tubes were ran in BD FACSCaliburTM (BD Bioscience, San Jose, CA, USA) cell sorter and flow cytometry output analysed with FlowJo (FLOWJO LCC, Ashland, OR, USA).

Table 3. Primary antibodies used for flow cytometry analysis.

Primary antibody	**Fluorochrome**	**Clone**	**Isotype**	**Target Cell type**	**Usage per reaction**
Anti-human CD29	PE/Cy5	TS2/16	Mouse IgG1, k	MSCs	10 µL
Anti-human CD34	FITC	4H11	Mouse IgG1, k	Haematopoietic stem cells	10 µL
Anti-mouse/human CD44	PE	IM7	Rat IgG2b, k	MSCs	10 µL
Anti-human CD45	APC	HI30	Mouse IgG1, k	Haematopoietic stem cells	10 µL
Anti-human CD73	APC	AD2	Mouse IgG1, k	MSCs	5 µL
Anti-human CD90	FITC	5E10	Mouse IgG1, k	MSCs	10 µL
Anti-human CD105	PE	43A3	Mouse IgG1, k	MSCs	20 µL

Cell differentiation

The *in vitro* differentiation potentials of LF-, IL- and FJ-derived cells were examined using the classical tri-linage differentiation assay for MSCs in which cells are subjected to osteogenic, adipogenic and chondrogenic medium. Differentiation potentials were evaluated by histochemical staining of differentiation specific components and gene expression.

Osteogenesis

For osteogenic differentiation cells were grown in three settings, for detecting calcium deposits cells were grown in 6-well plates at both high and low cell density, for gene expression analysis cells were grown in T75 culture flasks and for evaluating the osteogenic potentials of individual cells they were first allowed to form colonies before being subjected to osteogenic medium.

Calcium accumulation

Cells at passage 1-3 were suspended with 0.25% Trypsin EDTA. Cells were counted using haemocytometer and seeded at two densities, for low density 1,000 cells were seeded per well in 6-well plates, 3 wells for induction and 3 wells for control per cell line and for high density approximately 150,000 cells per well were seeded in the same manner. After 24 hours incubation, the culture medium was replaced in induction wells and osteogenesis initiated by osteogenic medium. Osteogenic medium consisted of culture medium supplemented with 10 nM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich) and 50 µg/mL ascorbate-2 phosphate (Sigma-Aldrich). Plates were incubated for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture- and osteogenic medium changed twice a week.

Extracellular calcium accumulation and a build-up of an extracellular matrix is a part of osteogenesis. After 21 days of culturing cells in osteogenic medium, both osteogenic- and control cultures were stained with 0.2% (w/v) Alizarin Red S which stains calcium deposits red. Medium was aspirated and wells washed 3 times with PBS and once with distilled water. Cells were fixed with 100% ethanol for 15 minutes. Alizarin Red S solution was prepared by dissolving 0.2 g of Alizarin Red S in distilled water and pH adjusted to 6.4 with ammonia. Following fixing cells were stained with 0.2% Alizarin Red S solution for 40 minutes at room temperature. After staining the wells were washed with distilled water until all excess dyed was washed away. Staining was observed under light microscope and photos taken at 100x magnification.

Gene expression

To isolated sufficient amounts of mRNA for gene expression analysis, cells were grown in T75 culture flask. Cells at passage 1-3 were grown to 70-80% confluence before being subjected to osteogenic medium, controls were grown in normal culture medium. The flasks were incubated for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture- and osteogenic medium changed two times per week.

Osteogenic potentials of individual colonies

To evaluate the osteogenic potentials of individual colonies 100 passage 1 cells were seeded in 100 mm culture dishes. The culture dishes were incubated with 10 mL of culture medium for 14 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and medium changed two times per week. Subsequently, the culture medium was switched out for osteogenic medium and the cultures grown for additional 21 days. Following osteogenic induction, the dishes were fixed and stained with 0.2% Alizarin Red S as in the previously described process. After staining dishes were photographed at 1x magnification using a Canon DSLR camera. Additionally,

cells were afterwards stained with 0.5% Crystal Violet in the same way as colonies in the CFU-F assay, producing a double staining of Alizarin Red S and Crystal Violet on the colonies. In addition, double stained colony dishes were photographed at 1x magnification.

Adipogenesis

For adipogenic differentiation, cells were grown in three settings. Cells were grown in 6-well plates at both high and low cell density for detecting lipid droplets. For gene expression analysis, cells were grown in T75 culture flasks. For evaluating the adipogenic potentials of individual cells, they were first allowed to form colonies before being subjected to adipogenic medium.

Lipid accumulation

Cells at passage 1-3 were suspended with 0.25% Trypsin EDTA. Cells were counted using haemocytometer and seeded at two densities. For low density 1,000 cells were seeded per well in 6-well plates, 3 wells for induction and 3 wells for control per cell line. For high density approximately 170,000 cells per well were seeded in the same manner. After 24 hours incubation, the culture medium was replaced in induction wells and adipogenesis initiated by adipogenic medium. Adipogenic medium consisted of culture medium supplemented with 100 nM dexamethasone, 50 µg/mL indomethacin, 0.45 mM 3-isobutyl-1-methylxanthine (MP Biomedicals, Santa Ana, CA, USA) and 10 µg/mL insulin. Plates were incubated for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture- and osteogenic medium changed two times per week.

Extracellular as well as intracellular lipid accumulation is part of adipogenesis. After 21 days of culturing cells in adipogenic medium, both adipogenic- and control cultures were stained with 0.3% (w/v) Oil Red O which stains intracellular and extracellular lipid droplets. Medium was aspirated and wells washed 2 times with PBS. Cells were fixed with 4% neutral buffered formaldehyde for 1 hour at 4°C. A

0.3% Oil Red O working solution was obtained by dissolving 0.5 g of Oil Red O in 100 mL of isopropanol before mixing with 67 mL of distilled water. Following fixing cells were stained with 0.3% Oil Red O working solution for 15 minutes at room temperature. After staining the wells were washed with distilled water until all excess dyed was washed away. Staining was observed under light microscope and photographs taken at 100x magnification.

Gene expression

To isolate sufficient amounts of mRNA for gene expression analysis, cells were grown in T75 culture flask. Cells at passage 1-3 were grown to 70-80% confluence before being subjected to adipogenic medium, controls were grown in normal culture medium. The flasks were incubated for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and culture- and adipogenic medium changed two times per week.

Adipogenic potentials of individual colonies

To evaluate the adipogenic potentials of individual colonies 100 cells from passage 1 were seeded in 100 mm culture dishes. The culture dishes were incubated with 10 mL of culture medium for 14 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and medium changed two times per week. Subsequently, the culture medium was switched out for adipogenic medium and the cultures grown for additional 21 days. Following adipogenic induction the dishes were fixed and stained with 0.3% Oil Red O as in the previously described process. After staining dishes were photographed at 1x magnification using a Canon DSLR camera. Additionally, cells were afterwards stained with 0.5% Crystal Violet in the same way as colonies in the CFU-F assay, producing a double staining of Oil Red O and Crystal Violet on the colonies. In the same manner double stained colony dishes were photographed at 1x magnification.

Chondrogenesis

Chondrogenic differentiation was done in a pellet culture both for histological staining and gene expression analysis. Cells from passage 2-3 were suspended with 0.25% Trypsin EDTA, counted using haemocytometer and approximately 1 x 10^6 cells transferred to sterile 15 mL polypropylene culture tubes. Cells were washed with culture medium and tubes centrifuged at 1,500 rpm for 10 minutes to create a solid visible pellet in the bottom of the tube. After 24 hours incubation, the culture tubes were centrifuged at 1,500 rpm for 2 minutes and the medium carefully removed without disturbing the cell pellet. Medium was replaces with culture medium with only 1% FBS for control cultures and chondrogenic medium for induction cultures. Chondrogenic medium consisted of culture medium with only 1% FBS, supplemented with 10 nM dexamethasone, 10 ng/mL transforming growth factor-β3 (ProSpec, Rehovot, Israel), and 6.25 µg/ml insulin–transferrin–selenium (ITS supplement). Tubes were incubated for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Medium was replaced two times per week after centrifuging the tubes at 350 rpm for 3 minutes.

When growing pellet cells in chondrogenic medium, cells synthesize extracellular cartilage. After culturing cells in a pellet with chondrogenic or culture medium for 21 days, pellets were stained with Alcian blue (Sigma-Aldrich, USA) which stains glycosaminoglycans in cartilage and Toluidine blue (Merck, USA) which stains nucleic acid and polysaccharides. Cell pellets were fixed in 10% formaldehyde for 24 hours before being dried out in ethanol and embedded in paraffin. The pellets were cut into 4-5 µM sections and stained with Alcian blue and Toluidine blue. The sections were observed for cartilage formation and photographed at 100x magnification.

Gene expression analysis

RNA isolation

On day 21 of differentiation induction, the total RNA was isolated from osteogenic-, adipogenic cultures and chondrogenic pellets as well as controls grown for each induction. For osteogenic and adipogenic cultures, cells were harvested with 0.25% Trypsin EDTA and transferred to 15 mL polypropylene tubes and centrifuged at 1,500 rpm for 10 minutes. The supernatant was discarded and the cell pellet transferred to 1.5 mL Eppendorf tubes and used for RNA isolation. For chondrogenic pellets, the medium was carefully aspirated and pellet carefully placed in 1.5 mL Eppendorf tubes. RNA was isolated using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) following the protocol provided by the manufacturer. Briefly, cells were ruptured by suspending the pellets in 350 µL Buffer RLT and vortexing the lysate. The lysate was homogenized by passing it through a 20-gauge needle using a sterile syringe. Following, 350 µL of 70% ethanol were added to the lysate and mixed by pipetting. The lysate was transferred to RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded and 700 µL of Buffer RW1 added and centrifuged for 15 seconds at 10,000 rpm. Again the flow through was discarded and 500 µL of Buffer RPE added and centrifuged for 15 seconds at 10.000 rpm, this step was repeated twice. Finally the RNA was eluted by adding 30 µL RNase free water to the column. RNA concentrations were measured by Nanodrop.

Reverse transcription-polymerase chain reaction (RT-PCR)

To determine the gene expression of osteogenic, adipogenic and chondrogenic markers, cDNA was synthesized from the isolated mRNA using reverse transcription-polymerase chain reaction (RT-PCT). RT-PCR was performed using TaqMan® Reverse Transcription Reagents Kit (Applied Biosystems, Branchburg, New Jersey, USA) using the reaction listed in Table 4. RNA concentration for each reaction was 50 µg.

Table 4. RT-PCR reaction compounds

Reagents	**Volume (µL) per reaction**
10x Taqman RT buffer	2
25 mM MgCl2	4.4
100 mM dNTP	0.4
Random hexamer	1
RNase inhibitor	0.4
Multiscribe reverse transcriptase	0.5
Template total mRNA + RNase free water	11.3
Total	20

The reaction tubes were placed in a PCR machine and the conditions listed in Table 5. used for cDNA synthesis

Table 5. RT-PCR reaction conditions

Step	**Temperature (°C)**	**Time (minutes)**
Hold incubation	25°C	10
Reverse transcription	48°C	30
Inactivation	95°C	5
Hold	4°C	∞

Polymerase chain reaction (PCR)

Following cDNA synthesis, a regular polymerase chain reaction (PCR) was used to amplify selected genes for induction cultures and controls. Two genes were selected for each induction and *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* used as a reference gene. The genes were as follows: for osteogenic induction *Runt-related transcription factor 2* (*Runx2*) and *Osteopontin*, for adipogenic induction *Peroxisome proliferator-activated receptor gamma 2* (*PPAR-γ2*) and *Lipoprotein lipase* (*LPL*) and for chondrogenic induction *SRY (sex determining region Y)-box 9 (Sox9)* and *Aggrecan*. The primers, product length and annealing temperatures for each gene are listed in Table 6.

Table 6. Primers of genes used for gene expression analysis

Gene	Forward primer	Reverse primer	Base pairs	Annealing temperature (°C)
Runx2	5‘TATGGCACTTCT TCAGGATCC‘3	5‘GCGTCAACACCA TCATTCTGG‘3	422	54
Osteopontin	5‘TGAAACGAGTCA GCTGGATG‘3	5‘TGAAATTCATGG CTGTGGAA‘3	162	58
PPAR-γ2	5‘GCTGTTATGGGT GAAACTCTG‘3	5‘ATAAGGTGGAGA TGCAGGCTC‘3	351	54
LPL	5‘GAGATTTCTCTG TATGGCACC‘3	5‘CTGCAAATGAGA CACTTTCTC‘3	277	50
Sox9	5‘ATCTGAAGAAGG AGAGCGAG‘3	5‘TCAGAAGTCTCC AGAGCTTG‘3	264	54
Aggrecan	5‘TGAGGAGGGCT GGAACAAGTACC‘3	5‘GGAGGTGGTAAT TGCAGGGAACA‘3	350	56
GAPDH	5’GTGAAGGTCGGA GTCAACGG’3	5’TCAATGAAGGGG TCATTGATGG’3	107	56

PCR was performed using PerfectTaq Plus MasterMix Kit (5 PRIME, Hilden, Germany) using the reaction listed in Table 7.

Table 7. PCR reaction compounds

Reagents	**Volume (µL) per reaction**
Mastermix	6.25
Forward primer	0.25
Reverse primer	0.25
ddH2O	2.75
Template cDNA	3
Total	12.5

The reaction tubes were placed in a PCR machine and the conditions listed in Table 8. used for gene amplification.

Table 8. PCR reaction conditions

Step	**Temperature (°C)**	**Time (minutes)**	**No. of cycles**
Initial denaturing	94°C	3	1
Denaturing	94°C	0.5	35
Annealing	50-58°C	0.5	
Extension	72°C	1	
Final extension	72°C	10	1
Hold	20°C	∞	1

The resulting PCR products were electrophoresed on 2% agarose gel (Research Organics, St. Cleveland, OH, USA) at 100 V for 30 minutes, stained with ethidium bromide (Sigma-Aldrich) and photographed promptly in Gel DocTM XR+ (Bio-Rad, Hercules, CA, USA).

Haematoxylin and eosin staining of spinal ligaments

To determine the tissue composition and cell localization of spinal ligaments LF, FJs and ILs samples were stained with Haematoxylin and Eosin (H&E). FJs and ILs samples of 2 patients were washed with PBS and placed on petri dishes. If present, bone fragments, debris and calcified areas were carefully removed. Subsequently the samples were cut into small pieces (5-6 mm^3). The samples were fixed in 10% formaldehyde for 24 hours before being dehydrated through a series of ethanol washes and cut into 5-µM sections, and stained with H&E (C.V. Laboratories, Bangkok, Thailand).

Statistical analysis

Statistical analysis were performed on patients' age, and cell colony forming efficiency and growth rates. Mean and standard deviation were calculated using Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA) and IBM SPSS Statistics 22 (IMB, Armonk, NY, USA). One-way-ANOVA was used to analyse any significance between the groups in the CFU-F and cell proliferation assay, with $P<0.05$ as significant difference. All graphs and diagrams CFU-F and cell proliferation assay were created using GraphPad Prims 5 (GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER IV
RESULTS

Cell isolation

Cells were successfully isolated from LF, FJs and ILs of all 10 patients involved in this study (Table 2.). Cell isolation was performed via direct tissue explants as explain in the methodology chapter. Following 3-7 days from tissue explanting, a homogenous population of spindle-shaped and plastic-adherent cells started creeping and could be visible around the periphery of the tissue pieces (Figure 6). These cells quickly covered the area around the tissue pieces from where they expanded to the rest of the tissue culture flask. The cells evinced a high proliferation rate usually reaching 70-80% confluence in 10-15 days after the emerging of the first cells (Figure 7.). The cells isolated at this stage were defined as passage 0 cells. After reaching confluence the cells were reseeded as passage 1 cells.

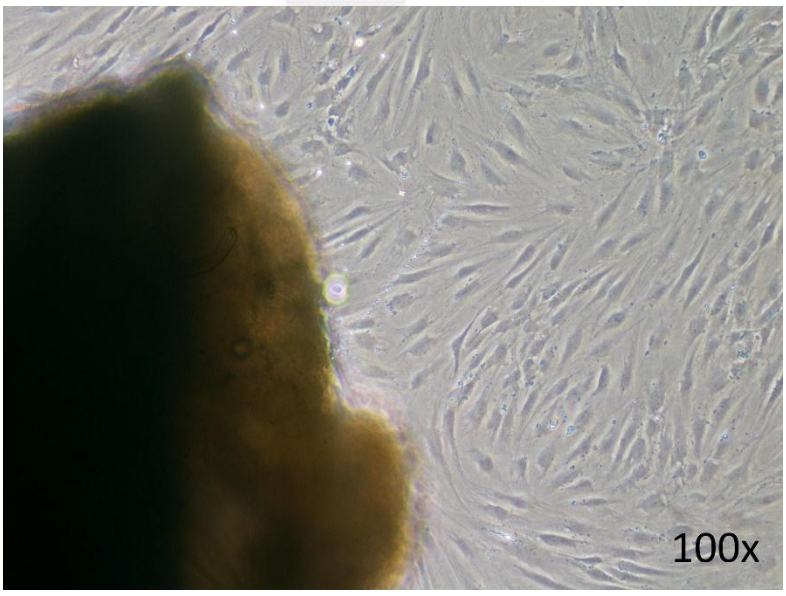


Figure 6. Cell emerging and creeping around a piece of ligamentum flavum. 100x original magnification.

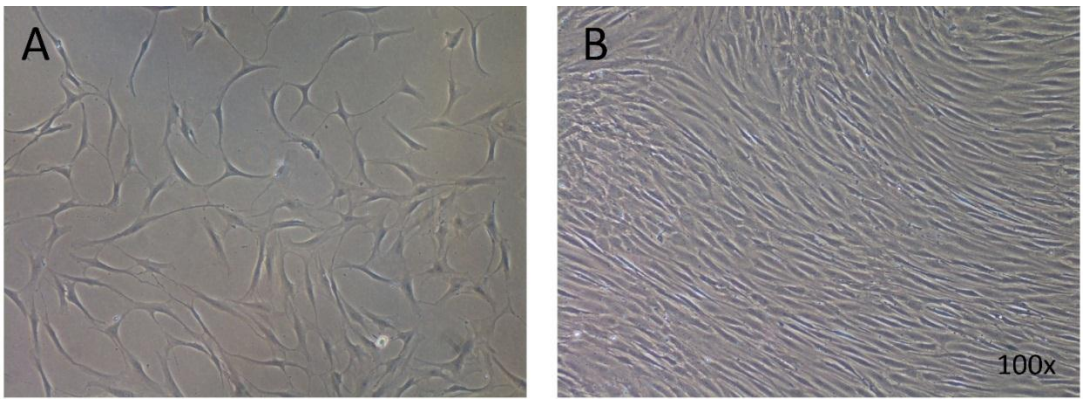


Figure 7. Ligamentum flavum derived cells at a low density (A) and at confluence (B).

Colony forming efficiency

The colony forming efficiency of each cell line was tested using CFU-F assay in 6-well plates. When 1,000 cells were seeded in a single well they formed a plethora of colonies which turned into an uncountable monolayer. When 100 cells were seeded per well, they gave rise to a number of colonies which came in contact with each other making counting nearly impossible. Seeding only 10 cells per well still yielded colony forming cells which gave rise to easily distinguishable colonies making counting easy (Figure 8A). Hence, 10 cells per well was the density at which cells were seeded and counted to measure the colony forming efficiency of each cell line from each patient. All cells in each individual colony were derived from a single clonogenic cell. They were plastic adherent, spindle shaped and stained bright purple with crystal violet making counting of colonies easier (Figure 8B).

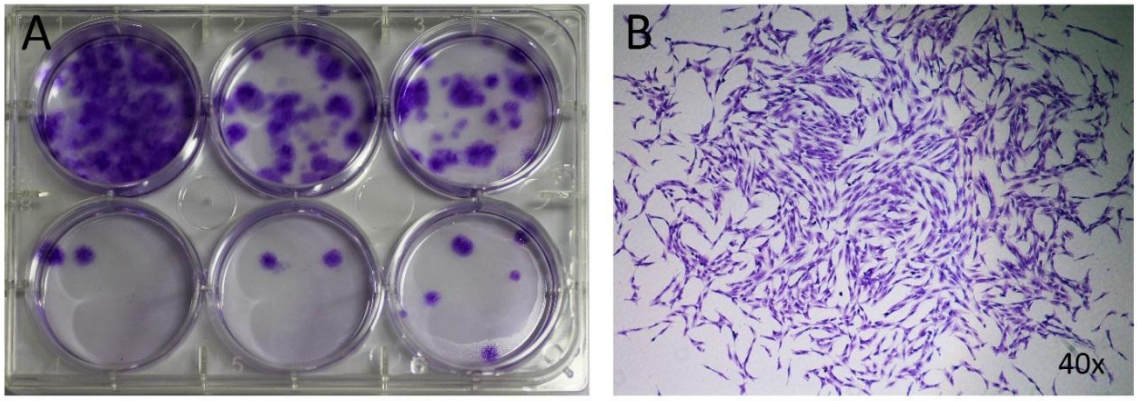


Figure 8. A 6-well plate with cell seeding at different densities; in the top left corner 1,000 cells, in the top middle and right corner 100 cells and that the bottom wells 10 cells per well (A). A single colony of IL derived cells stained with Crystal Violet. 40x magnification (B).

Colony forming efficiency varied greatly between individuals as well as source of tissue from which cells were isolated. For LF derived cells it ranged from 6.7% - 83.3%, for FJ derived cells 6.7% - 80.0% and for IL derived cells 16.7% - 73.3% respectively (Table 9.).

Table 9. Colony forming efficiency of each cell line from individual patient

Patient no.	LF colony forming efficiency (%)	FJ colony forming efficiency (%)	IL colony forming efficiency (%)
1	50.0%	16.7%	63.3%
2	13.3%	60.0%	26.7%
3	63.3%	73.3%	73.3%
4	83.3%	80.0%	50.0%
5	26.7%	43.3%	10.0%
6	63.3%	66.7%	46.7%
7	53.3%	63.3%	40.0%
8	6.7%	10.0%	16.7%
9	33.3%	6.7%	26.7%
10	6.7%	6.7%	53.3%

The mean colony forming efficiency of LF-, FJ- and IL-derived cells was similar. For LF-derived cells it was 40.0% (± 26.7%), for FJ-derived cells 42.7% (± 29.8%) and for IL-derived cells 40.7% (± 20.5%), respectively (Table 10.) (Figure 9.). There was no significant difference in colony forming efficiency between cell lines when analysed by one-way ANOVA.

Table 10. Mean colony forming efficiency of LF-, FJ- and IL-derived cells

Tissue	**Colony forming efficiency**
Ligamentum flavum	40.0% (± 26.7%)
Facet joints	42.7% (± 29.8%)
Interspinous ligaments	40.7% (± 20.5%)

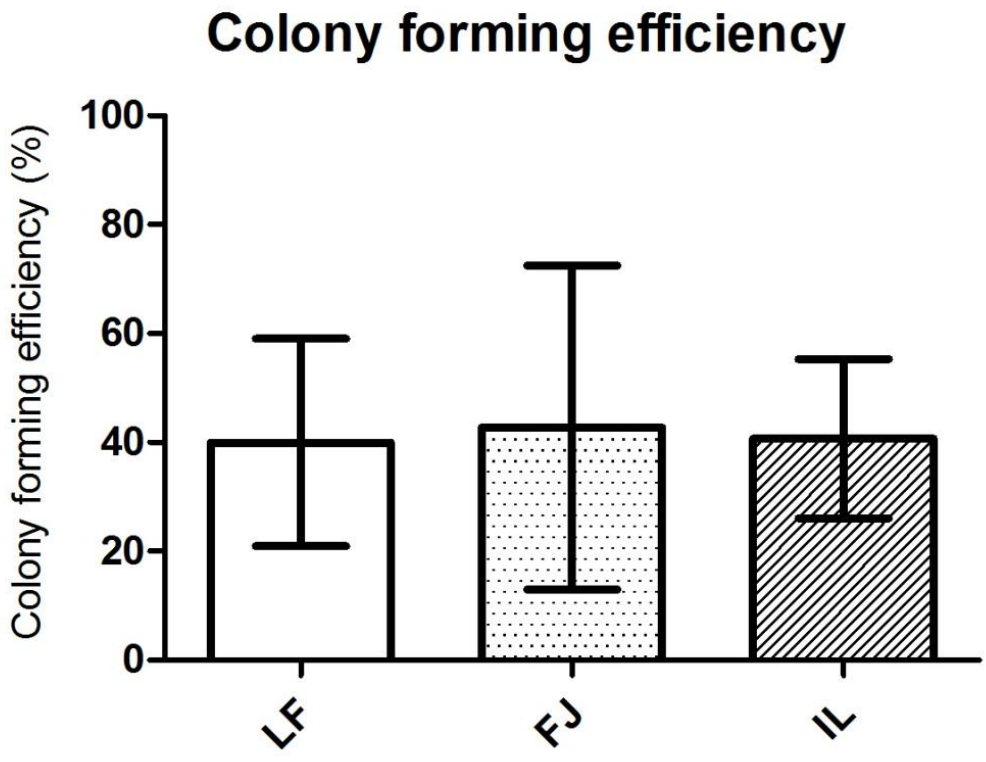


Figure 9. Mean colony forming efficiency of LF-, FJ- and IL-derived cells. The bars show standard deviation.

Cell proliferation

Cell proliferation was measured by seeding cells at a low density and calculating PDLs. Cell proliferation and PDLs varied between patients (Figure 10). In some cases cell lines could only be maintained in culture for 28 days, whilst other cell lines could be maintained and expanded for up to 70 days. Accumulated PDL between cell lines and individual patients ranged from 9.2 – 36.4, after which cells entered senescence state and could not be proliferated any further. PDL for LF-derived cells were between 15.5 – 34.9, for FJ-derived cells 11.2 – 36.4 and for IL-derived cells 9.2 – 34.3, respectively. Mean PDLs for each cell line at each time point were calculated and plotted together and separately (Figure 11.). It showed a similar rapid growth curve for all cell lines.

In addition, mean PDLs were calculated using the accumulated PDL of each patient regardless of how long cells were maintained in culture. For LF-derived cell mean PDL was 23.8 (±5.6), for FJ-derived cells 25.6 (±7.4) and for IL-derived cells 24.0 (±7.6), respectively (Figure 12). There was no significant difference between accumulated PDLs or mean PDLs at any individual time point between cell lines when analysed by one-way ANOVA.

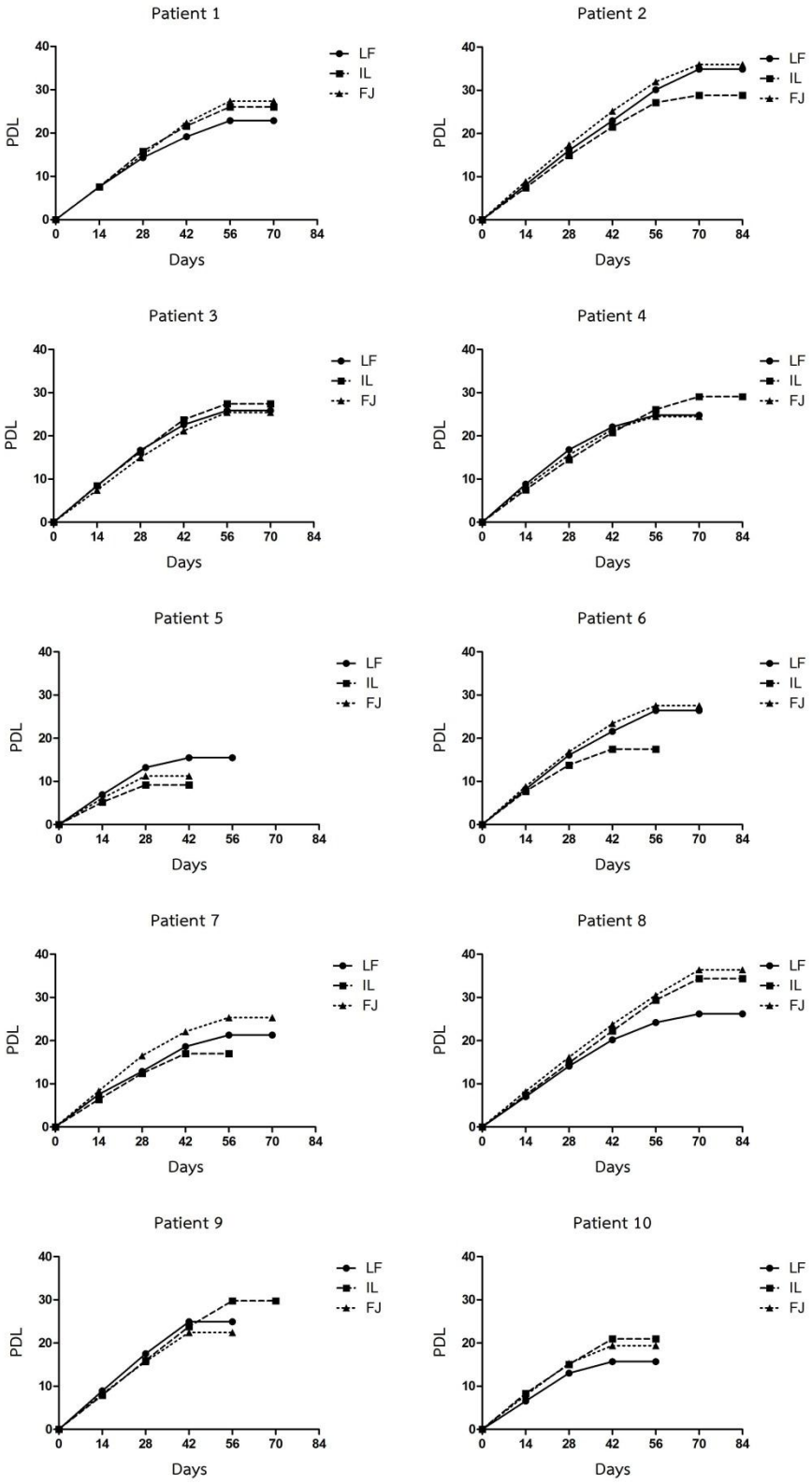


Figure 10. Growth curves of LF-, FJ- and IL-derived cells for individual patients. The bars show standard deviation

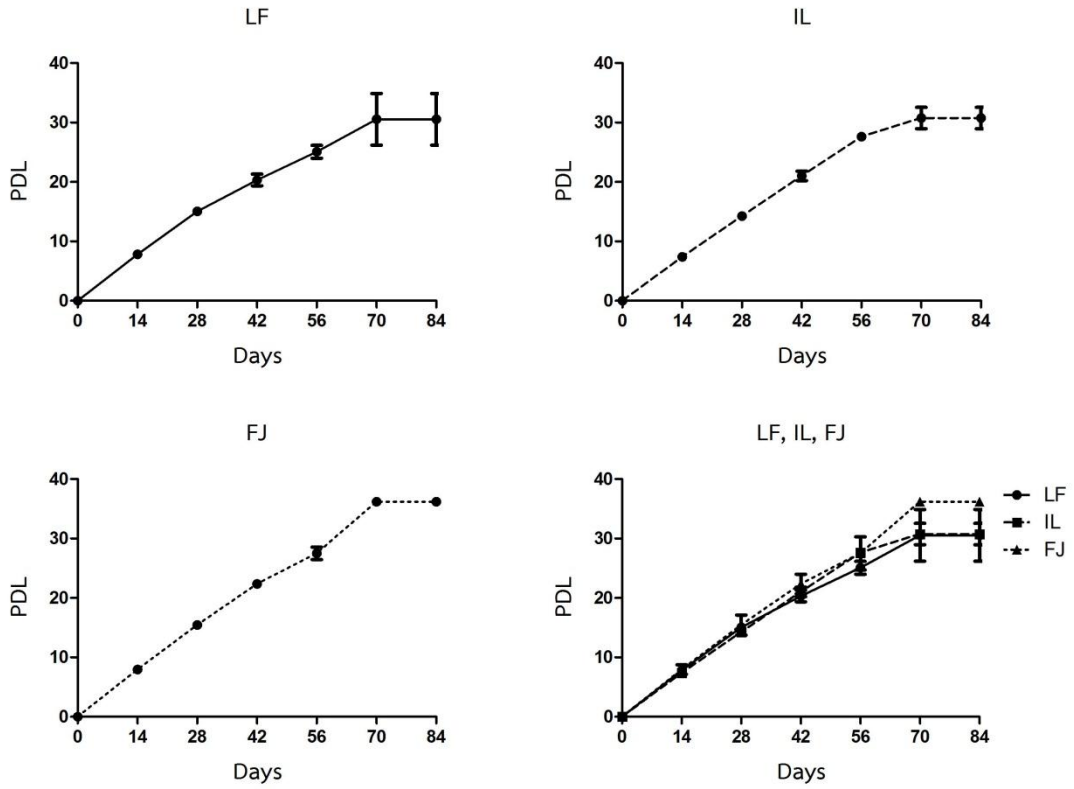


Figure 11. Growth curves with mean PDLs per time point from all patients. The bars show standard deviation

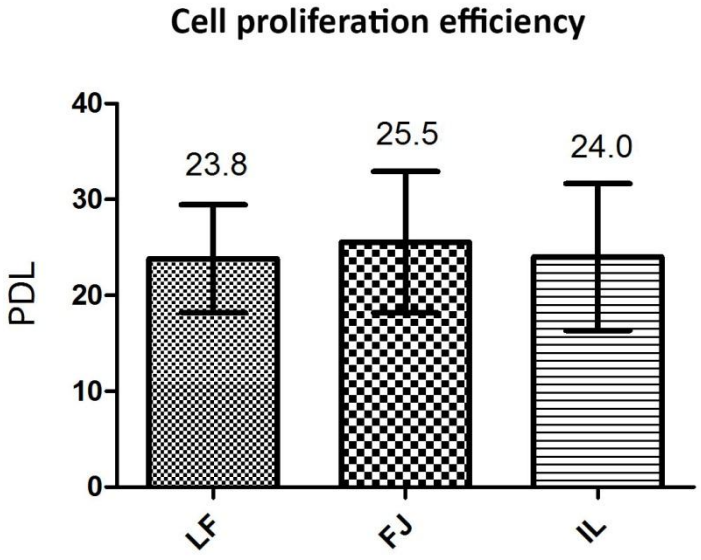


Figure 12. Accumulated PDL for LF-, FJ- and IL-derived cells. The bars show standard deviation.

Cell surface marker profile

Flow cytometry was used to determine the expression of selected MSCs surface markers and haematopoietic markers. The MSCs markers included in this study were CD29, CD44, CD73, CD90 and CD105. All isolated cell lines were fully or partly positive for all MSCs markers tested. LF-derived cells were 63.7% positive for CD29, 96.6% positive for CD44, 63.1% positive for CD73, 89.6% positive for CD90 and 86.4% positive for CD105. FJ-derived cells were 50.6% positive for CD29, 98.4% positive for CD44, 59.8% positive for CD73, 97.1% positive for CD90 and 75.7% positive for CD105. IL-derived cells were 61.7% positive for CD29, 98.6% positive for CD44, 59.0% positive for CD73, 96.1% positive for CD90 and 56.4% positive for CD105. The haematopoietic markers in this study were CD34 and CD45. These two markers were almost absent in all cell isolated cell lines. In LF-derived cells CD34 was 0.3% positive and CD45 was 1.4% positive. For FJ-derived cells CD34 was 0.2% positive and CD45 was 0.9% positive. For IL-derived cells CD34 was 0.3% positive and CD45 was 1.0% positive (Figure 13.).

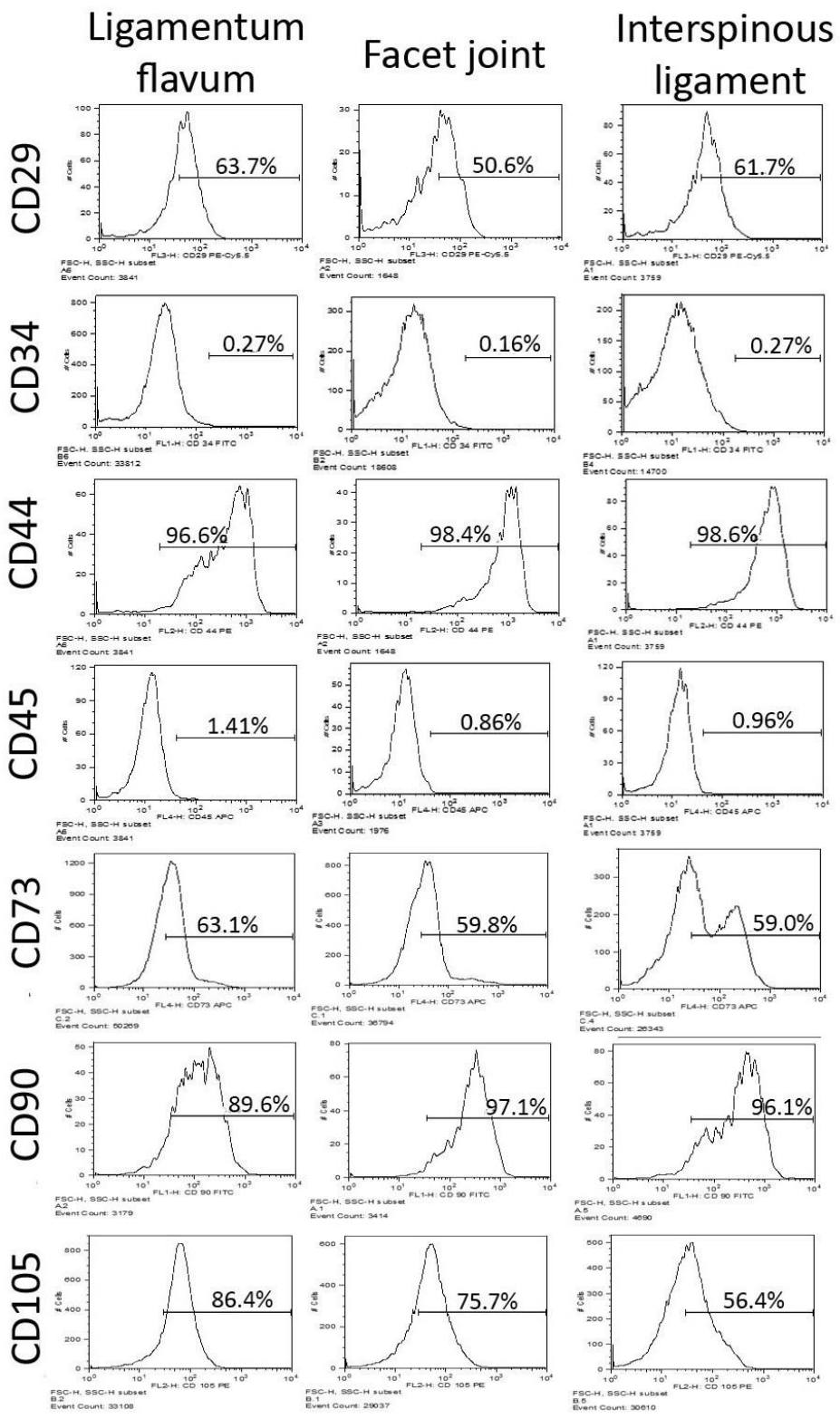


Figure 13. FACS histograms of CD29, CD34, CD44, CD45, CD73, CD90 and CD105 expression in LF-, FJ- and IL-derived cells.

Cell differentiation

All cell lines were exposed to osteogenic-, adipogenic- and chondrogenic medium. Staining and gene expression were used to evaluate differentiation potentials.

Osteogenic differentiation

Osteogenic differentiation was performed at a high and low density as well as investigating the osteogenic potentials of individual colonies and gene expression of osteogenic markers.

Osteogenic differentiation of cell at high density

Cells were seeded at a high density in 6-well plates and introduced to osteogenic medium 24 hours later. After 7 days of initial seeding a slight change in morphology became visible. The cells become more round shaped and denser. After 14 days a mineralized matrix started to be visible on the top of the cells. At day 21 the matrix was very dense and it could be seen with bare eyes, whereas the cells were barely visible under the microscope. When stained with Alizarin Red S the induction wells became bright red whereas the control did not stain at all (Figure 14.). The stained confirms the accumulation of calcium deposits in the induction culture.

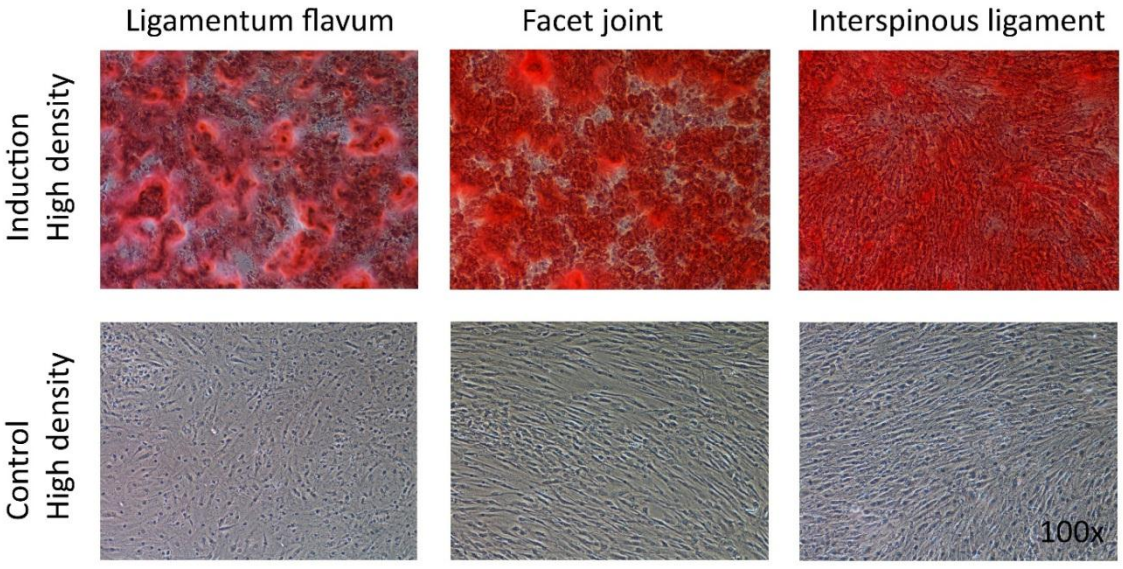


Figure 14. Osteogenic potentials of LF-, FJ- and IL-derived cells at high density. Alizarin Red S staining. 100x original magnification.

Osteogenic differentiation at low density

Cells were also seeded at a low density in 6-well plates and cultured in the same manner as the cells seeded at high density. A morphology change was also visible at low density although not as robust as in the cells at high density. After 14 days mineralized areas became visible, however, there was no matrix as detected at high density. After 21 days of incubation in osteogenic medium the cells were stained with Alizarin Red S. Calcium rich areas stained bright red showing that calcium accumulation was also possible when cells were seeded at a low density. No stained was observed in control culture (Figure 15.).

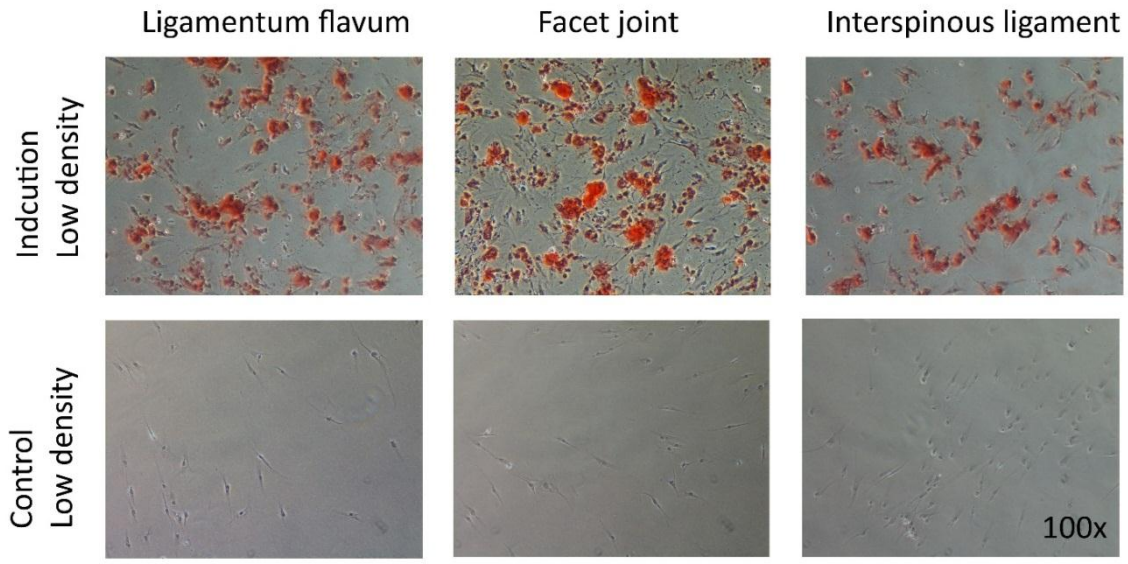


Figure 15. Osteogenic potentials of LF-, FJ- and IL-derived cells at low density. Alizarin Red S staining. 100x original magnification.

Osteogenic potentials of individual colonies

To evaluate the osteogenic potentials of individual colonies, single cells were first allowed to form colonies for 14 days before being switched to osteogenic medium for 21 days. After 14 days of seeding 100 cells in 100 mm culture dishes, a number of colonies could be seen when view under the microscope. After 7 days of switching to osteogenic medium, the cells showed similar morphology as cells seeded at a high density and at 14 days a mineralized matrix was also observed. After growing the plates for 21 days in osteogenic medium the plates were stained with Alizarin Red S. The colonies become bright red confirming the presence of calcium. When stained again with Crystal Violet, the bright red colonies became dark purple and no additional colonies were detected (Figure 16.).

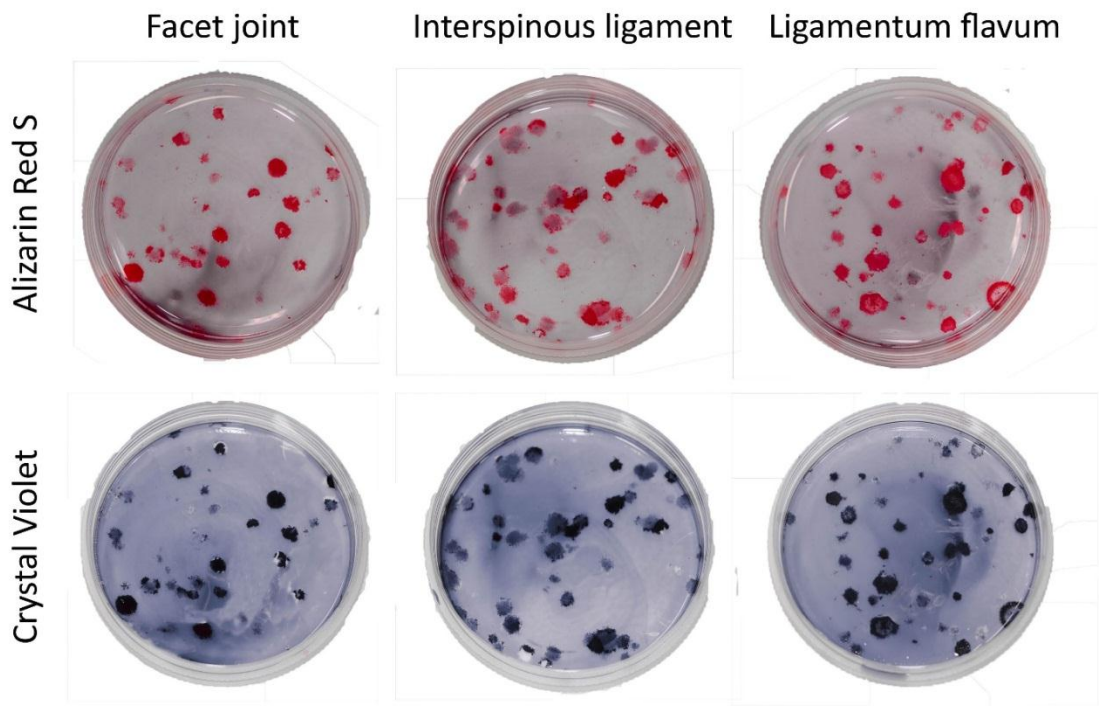


Figure 16. Osteogenic potentials of individual colonies from LF-, FJ- and IL-derived cells. The top half shows calcium staining with Alizarin Red S and the bottom half DNA staining with Crystal Violet.

Gene expression analysis of osteogenic markers

Total RNA was isolated from cells grown in T75 culture flask in osteogenic medium. RT-PCR analysis of the osteogenesis master regulator gene *Runx2* showed that its expression was elevated in the induction cultures (I) compared to the controls (C) where it was also detected as previously reported (121). Nonetheless, *Runx2* expression in LF-derived control cells was very faint. *Osteopontin* was highly expressed in the induction culture and a very faint expression was detected in the controls as well. The expression of the housekeeping gene *GAPDH* was observed in all cultures (Figure 22.).

Adipogenic differentiation

Like osteogenic differentiation, adipogenic differentiation was performed at a high and low density as well as determining the adipogenic potentials of individual cells and gene expression of adipogenic markers.

Adipogenic differentiation at high density

Cells were seeded at a high density in 6-well plates and introduced to adipogenic medium 24 hours later. After 7 days of initial seeding a change in morphology and culture composition. The cells became elongated and flat, and the cell density of the culture was decreased. This continued throughout the culture period. At day 21 the cultures were stained with Oil Red O and a faint red colour covered the wells. When viewed under the microscope, a red staining of both intracellular and extracellular lipid droplets was visible showing the accumulation of lipids. In the control cultures, only a very weak staining of intracellular lipid components was observed (Figure 17.).

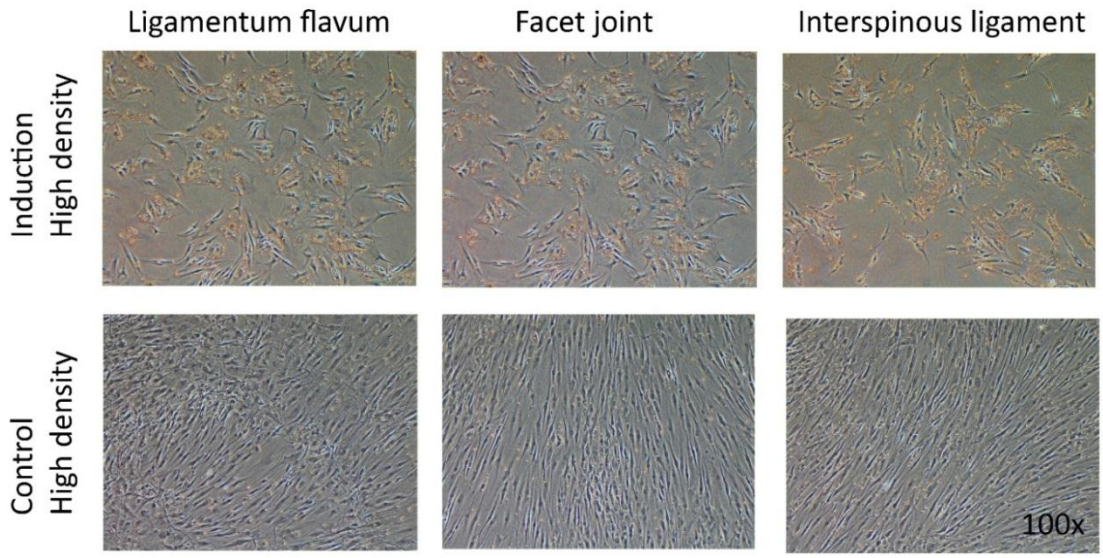


Figure 17. Adipogenic potentials of LF-, FJ- and IL-derived cells at high density. Oil Red O staining. 100x original magnification.

Adipogenic differentiation at low density

When cells were seeded at a low density, a similar change in morphology was evident as in cells seeded at high density. At day 21, the cultures were stained with Oil Red O. A bright red staining of intracellular and extracellular lipids was likewise detected as in cells seeded at high density. Albeit, the accumulation of extracellular lipids seemed even higher than in cells seeded at high density. In the control cultures, only a very weak staining of intracellular lipid components was observed (Figure 18.).

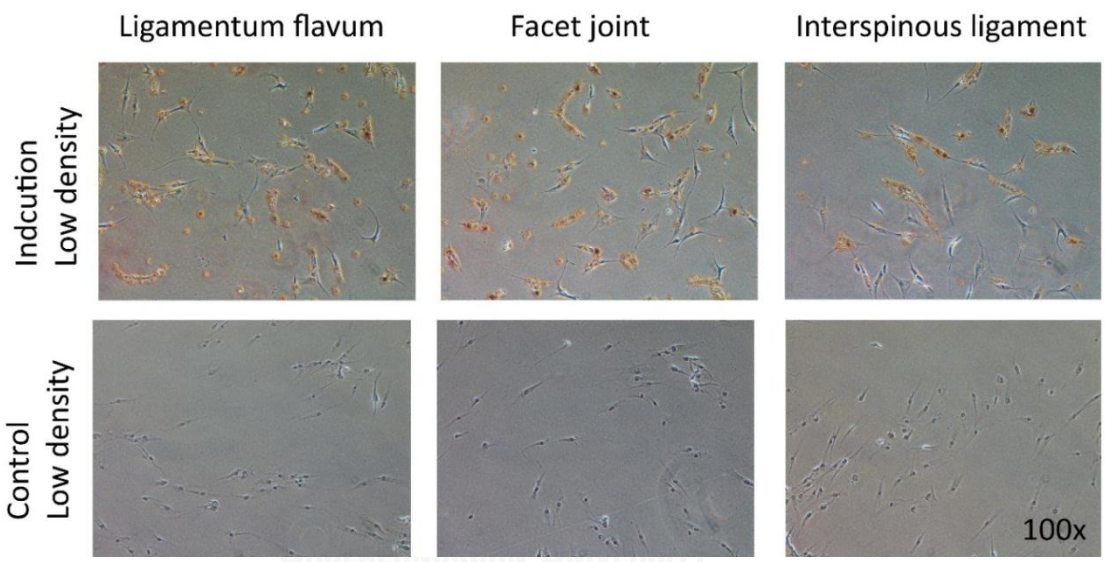


Figure 18. Adipogenic potentials of LF-, FJ- and IL-derived cells at low density. Oil Red O staining. 100x original magnification.

Adipogenic potentials of individual colonies

To evaluate the adipogenic potentials of individual colonies, single cells were first allowed to form colonies for 14 days before being switched to adipogenic medium for 21 days. After 14 days of seeding 100 cells in 100 mm culture dishes, a number of colonies could be seen when view under the microscope. After 7 days of switching to adipogenic medium, the cells showed similar morphology as cells seeded at a high and low density and also evinced a decreased density of cells. After growing the plates for 21 days in adipogenic medium, the plates were stained with Oil Red O. A faint red staining was observed on the colonies and when viewed under the microscope intracellular and extracellular lipid droplets were stained in the same way as cell grown at high density. When stained again with Crystal violet a number of cells became visible (Figure 19.).

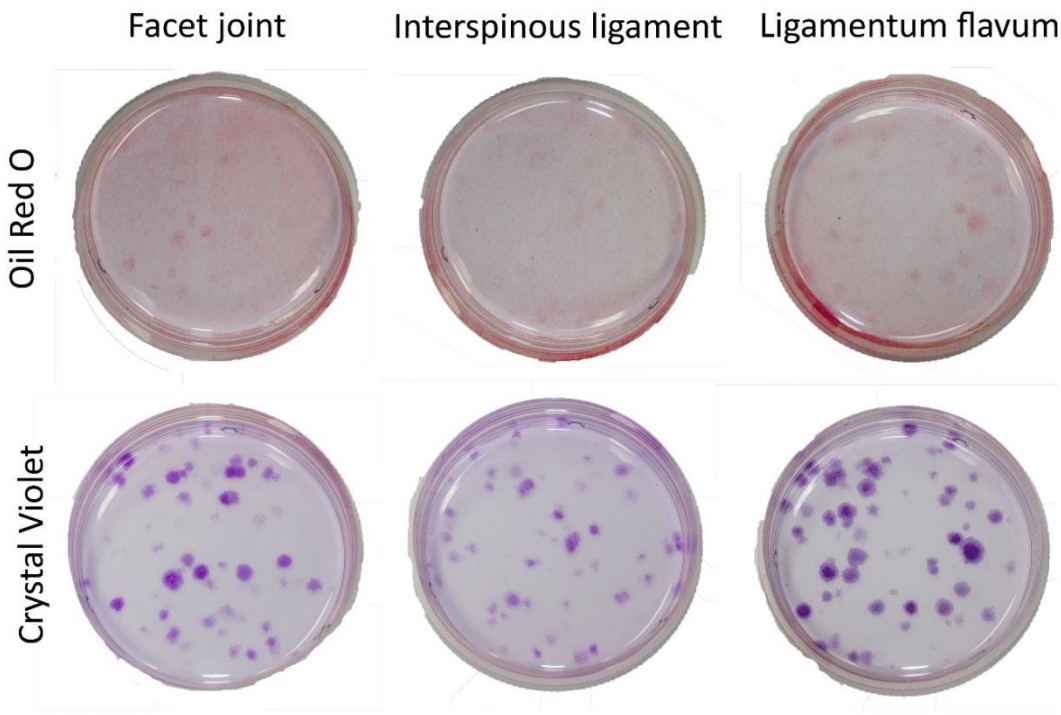


Figure 19. Adipogenic potentials of individual colonies from LF-, FJ- and IL-derived cells. The top half shows calcium staining with Oil Red O and the bottom half DNA staining with Crystal Violet.

Gene expression analysis of adipogenic markers

Total RNA was isolated from cells grown in T75 culture flask in adipogenic medium. RT-PCR analysis of the adipogenesis master regulator gene *PPAR-γ2* showed that it was highly expressed in the induction cultures of all cells lines and barely visible in the control cultures (122). The expression of *LPL* was also detected in the induction cultures but not in the controls. The expression of the housekeeping gene *GAPDH* was detected in all cultures (Figure 22.).

Chondrogenic differentiation

Unlike osteogenic and adipogenic differentiation, chondrogenic differentiation was done in a pellet culture. Cells were grown in a pellet at high density for both staining at gene expression analysis of chondrogenic markers. Cells grown in pellets could not be viewed under the microscope.

Pellets that were grown in chondrogenic medium became denser and easier to handle than cells grown in culture medium who easily dispersed when handled. The pellets were cut into thin sections and stained with Toluidine blue and Alcian Blue. Toluidine blue staining showed a vast build-up of extracellular matrix in the induction pellets. The extracellular matrix stained blue and the cells which were either embedded in the matrix or on the outskirts of the pellet stained dark blue. More extracellular matrix was present in pellets from FJ- and IL-derived cells and they formed a round dense pellet. Little to no extracellular matrix was observed in control pellets (Figure 20.). Control pellets were composed of cells which stained dark blue like, cells in induction pellets. Staining with Alcian blue produced the same results (Figure 21.)

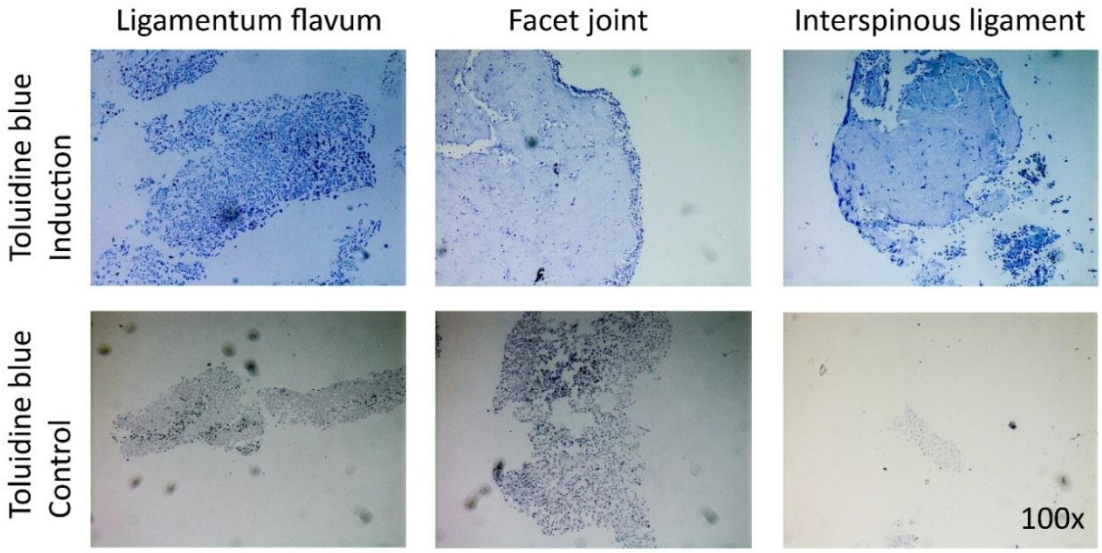


Figure 20. Toluidine blue staining of pellets grown in chondrogenic induction media (top) and control media (bottom). 100x original magnification.

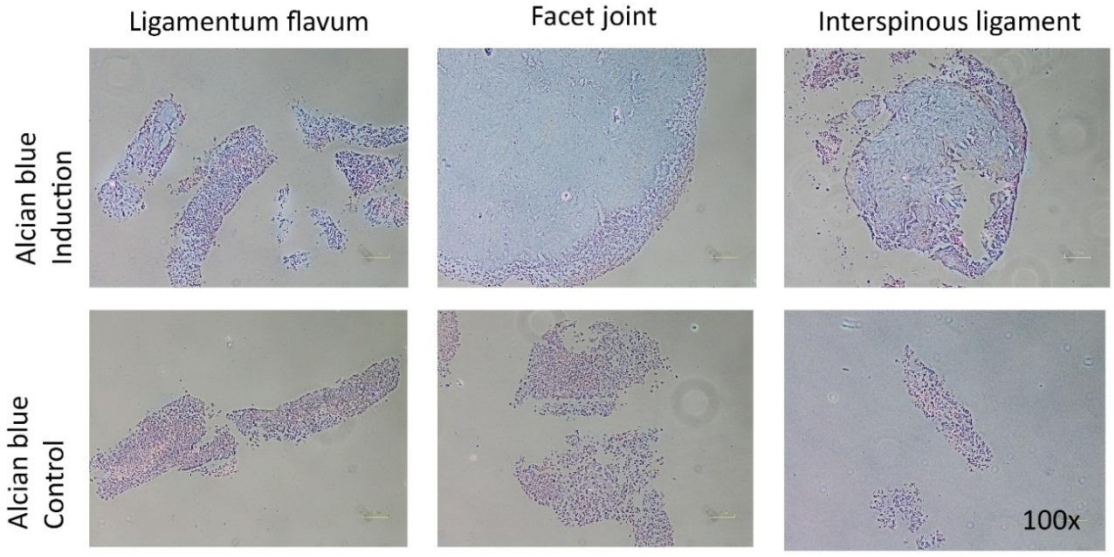


Figure 21. Alcian blue staining of pellets grown in chondrogenic induction media (top) and control media (bottom). 100x original magnification.

Gene expression analysis of chondrogenic markers

Total RNA was isolated from pellets cultured in chondrogenic and regular culture medium. RT-PCR analysis of the chondrogenesis master regulator gene *Sox9* showed that it was highly expressed in the induction cultures of all cells lines and barely visible in the control cultures. The expression of the regulator gene *Aggrecan* was also detected in the induction cultures but not in the controls. The expression of the housekeeping gene *GAPDH* was detected in all cultures (Figure 22.).

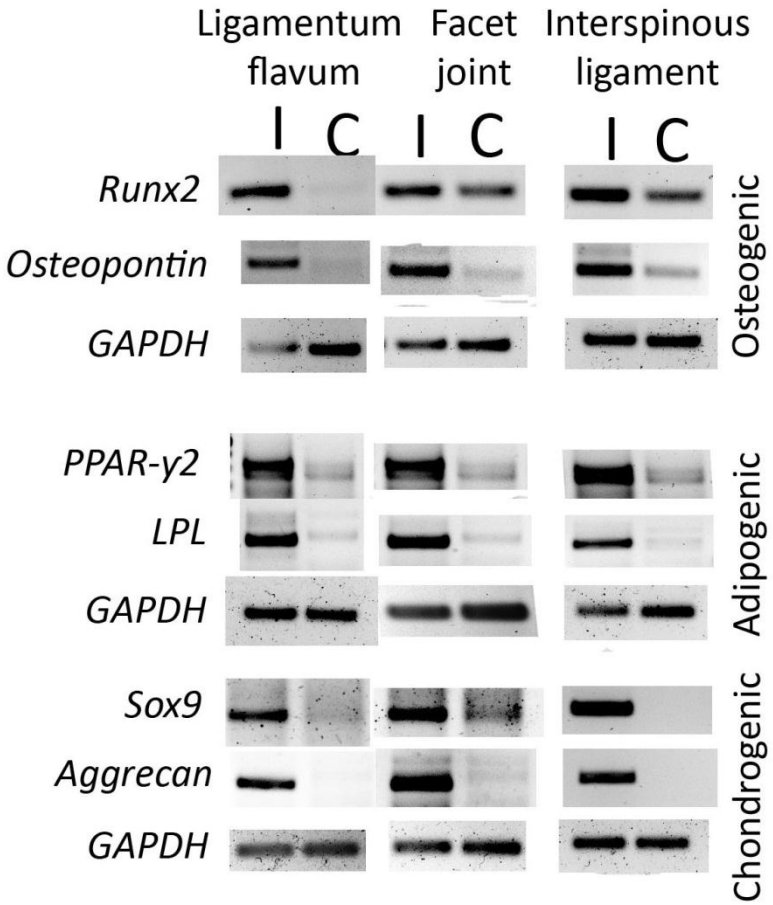


Figure 22. Gene expression analysis of osteogenic markers *Runx2* and *Osteopontin*, adipogenic markers *PPAR-**y**2* and *LPL* and chondrogenic markers *Sox9* and *Aggrecan*. Lanes "I" represent induction and "C" controls. The housekeeping gene *GAPDH* was used as internal control.

Tissue composition and localization of cells in LF, FJs and ILs tissues

LF, FJ, and IL tissues were stained with H&E stain. H&E staining showed that the tissues were mainly composed of dense avascular collagenous tissue areas that were surrounded by less dense, vascular, cell rich areas. Red blood cells and endothelial cells were visible in the cell-rich zones within the vascular areas. The cell-rich areas were less dense than the inner tissue allowing greater cell mobility. Vast collagenous areas with only a few or no cells were also observed in all tissues stained. These avascular dense areas made up for most of the stained tissue in all tissues (Figure 23.).

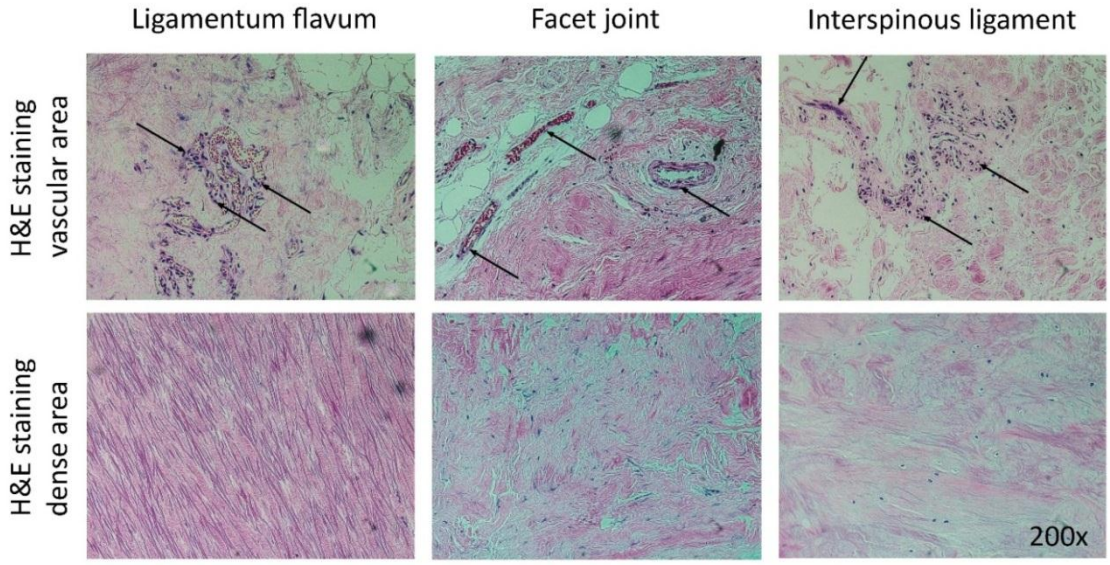


Figure 23. H&E staining of ligamentum flavum, facet joint and interspinous ligament tissues. The tissues consisted of both vascular cell rich areas and dense fibrous areas. 200x original magnification. The black arrows are pointing at endothelial cells in cell rich areas.

CHAPTER V
DISCUSSION AND CONCLUSION

Discussion

It is becoming apparent that progenitor cells of various kinds can be found throughout the adult human body. MSCs have been gaining increased interest both for their therapeutic potentials in tissue engineering and also their involvement in the development of various degenerative conditions. Hence, MSCs have been isolated from a number of novel sources over the last few years. The present study provides a strong evidence for the existence of MSCs within the LF, FJs and ILs. MSCs have been previously reported in the LF but to the best of our knowledge this is the first time they have been isolated from the FJs and ILs. In this study, plastic-adherent cells could be isolated from the LF, FJs and ILs of patients suffering from various conditions. They showed high proliferation rates and expressed the known specific surface markers of MSCs CD29, CD44, CD73, CD90 and CD105. Furthermore, they were negative for the haematopoietic markers CD34 and CD45. When subjected to induction medium, cells were able to differentiate into osteocytes, adipocytes and chondrocytes. However, this is not the first time cells from spinal ligament tissues have drawn the attention of scientists. With a high prevalence of OLF and OPLL in the Japanese population, the Japanese Ministry of Public Health and Welfare has launched and funded a number of studies on the aetiology of these conditions since 1975 (114). Amid these studies are studies that describe the isolation and characteristics of cells from human spinal ligaments.

In 1993, Ishida and Kawai isolated and described the characteristics of cultured cells from the posterior longitudinal ligament of OPLL patients and healthy controls (123). They reported that cells from control subjects did not display any osteoblastic properties but cells from OPLL patients showed various phenotypic characteristics of osteoblasts; osteoblast morphology, *in vitro* calcification, high

alkaline phosphatase activity, and elevated cAMP levels. Furthermore, they investigated the effects of bone-seeking hormones on DNA synthesis, cAMP level and alkaline phosphatase activity on their cells. They reported that proliferation and differentiation of the cells were controlled by various types of bone-seeking hormones (124). In 1997, Kon et al. investigated the effects of bone morphogenetic protein-2 (BMP-2) on the differentiation of cultured posterior longitudinal ligaments cells (125). They reported an increased alkaline phosphatase activity, DNA synthesis and procollagen type 1 carboxyl-terminal peptide synthesis (PICP) in cells from OPLL patients, whereas they only observed increased DNA synthesis and in one case PICP synthesis in cell from healthy controls. They concluded that BMP-2 stimulated osteoblast differentiation of cells from OPLL patients. In 1998, Goto et al. investigated the distribution of insulin-like growth factor I in the posterior longitudinal ligament and its effect on cultured cells (126). They observed an increased alkaline phosphatase activity, DNA synthesis and PICP synthesis was significantly higher in cells from OPLL patients than in cells from healthy controls. Mechanical stress in the vertebrae column might also play a major role in the development of OPLL. In 2001, Tanno et al. studied the effects of uni-axial cyclic stress on cells from OPLL patients and healthy controls (127). They observed a significant increase in mRNA expression of osteogenic related genes in cells from OPLL patients compared to healthy controls. An up regulation of prostaglandin I_2, bone morphogenetic proteins, and Cbfa1 has also been reported after subjecting posterior longitudinal ligament cells to uni-axial cyclic stress (128-130). Collectively, these studies suggested that cells found within the posterior longitudinal ligament were involved in the ossification of the ligament. Additionally, cells from OPLL patients responded differently compared to cells from healthy controls when exposed to mechanical stress and various growth factors, evincing strong osteogenic response. Notwithstanding, none of these studies investigated the cells in a wider spectrum and therefore did not define them as MSCs

although reporting multiple MSCs characteristics such as fibroblast like morphology, plastic adherence and osteogenic potentials.

LF-derived cells have also been isolated and studied in regard to LSCS and OLF. In 2000, Specchia et al. isolated and characterized the osteogenic and chondrogenic features of cells from LF tissue of LSCS patients and healthy controls (131). They found that cells from LSCS patients expressed high levels of alkaline phosphatase and produced a collagen rich matrix that mineralized upon stimulation with β-glycerophosphate and ascorbic acid (which are among the core chemicals used in the osteogenic differentiation in the present study). The addition of parathyroid hormone also increased the cAMP levels in the cultures suggesting strong osteoblastic features of cells from LSCS patients. Additionally, they stained cells for chondrogenic proteins, showing that the cells also displayed some chondrogenic features. They did not observe these osteogenic and chondrogenic features in cells from control subjects. In 2004, Murata et al. investigated the effects of dexamethasone on healthy LF derived cells (132). They reported that supplementation with dexamethasone resulted in morphological changes in cells, and at high concentration suppressed growth. In addition, they observed an increased osteogenic activity. Dexamethasone was included in all differentiation mediums used in this study. In a study from 2006, Ikeda et al. compared gene expression during osteogenesis of cells from OPLL patients, OLF patients, healthy controls and commercially available MSCs (133). They observed an upregulation of 24 genes during osteogenesis and a strong osteogenic response in both cells from OPLL and OLF patients. Although they did not identify their cells as MSCs they compared them with a commercially available MSCs and observed similarities in morphology and process of osteogenesis.

The cells found in LF and the posterior longitudinal ligament were first described as MSCs in 2011, when Chen et al. reported the isolation of MSCs from the ligamentum flavum and their response to TGF-β (23). And in 2012, Asari et al.

isolated MSCs from the ligamentum flavum and posterior longitudinal ligaments (24). Like in the previous studies, the emphasis was on the ligamentum flavum and the posterior longitudinal ligaments due to these structures often being hypertrophied and ossified in LSCS and OPLL patients. These were the first studies that explored the proliferation and differentiation potentials, and surface marker profiles of cells from spinal ligament tissues, and found similarities with other established MSCs cell lines.

In the present study, cells were isolated from the LF, FJs and ILs using direct tissue explant technique. Tissue pieces were placed directly in culture flasks and cells allowed to emerge from the tissue pieces. This method is both simple and cost-effective but involves a long lag face compared to other conventional methods. When working with BMSCs or AMSCs cells can be easily harvested directly from the nucleated cell populations obtained from these tissues. However, when cells are embedded in tissues, this can be a more challenging task. In previous reports, cells were isolated by digesting the tissue pieces with collagenases and passing it through a nylon cell strain (23, 24, 82, 134). Our method might be less specific but it recruits all plastic adherent cells within the tissues regardless of collagenase digestion and size. Nonetheless, our cell lines consisted on a homogeneous population of plastic-adherent cells with spindle shaped fibroblast like morphology. This method might not be suitable for clinical applications as a 95% purity of cell culture is recommended by the ISCT (4). Regardless, studies using directly isolated, unexpanded and unpurified AMSCs has given good results in osteoarthritis patients (135). In recent years, the use of unexpanded cells has been gaining interest. Culturally expanding cells is both expensive and time consuming. Additionally, it ages the cells and extended culturing might increase the risk of tumour formation and decreases the cells differentiation potentials (136-138). Consequently, sources rich in MSCs such as adipose tissue have been suggested for various tissue engineering applications. Adipose tissue is abundant, easy to access, results in minimum

morbidity and patients are usually pleased to lose fat mass. AMSCs are frequently isolated by centrifuging liposuction fat mass which contains an array of cells; AMSCs, endothelial and mural cells, smooth muscle cells, pericytes, fibroblasts, and circulating cells (139). A number of enriching methods have been suggested and tested to further purify MSCs from adipose tissue and bone marrow. Cells can be sorted by flow cytometer for STRO-1+, CD146+ and CD271+ or through the use of binding columns (140-142). With LF, FJs and ILs being relatively small and inactive structures, the number of cells isolated can be expected to be somewhat low. In this study the initial number of cells that were visible creeping around the tissue samples seemed fairly low and it took up to 2 weeks to observe them proliferate at full capacity during passage 0. Therefore, they might not be suitable as a source for cells for major clinical applications that require a large number of cells such as cartilage repair. Albeit, they might be useful for small local tissue engineering procedures such as spinal fusion or degenerative disk repair as suggested by Han et al. (26). In this study all tissue samples were obtained from discarded tissues that result from spinal surgery. In a clinical perspective, autologous cells could be isolated and expanded from the discarded tissues following a surgery and applied during follow up treatment if applicable. Even though the number of cells from LF, FJs and ILs is low, they show a high clonogenic and proliferation potentials.

Clonogenecity is the ability of a cell to give rise to other cells by forming colonies from a single cell units. This is an important feature of MSCs as they are considered progenitor cells. However, clonogenecity between MSCs varies greatly depend on source and culture methods. There are 3 conventional CFU-F methods for measuring cell colony forming efficiency. The most precise method is done by seeding 1 cell at a time in the wells of a 96-well plates using flow cytometer and counting colonies formed after 14 days of incubation. The other two methods (both used in this study) are based on manually diluting cells to either 100 cells per 100 mm culture dish or 10 cells per well in 6-well plates (46). This is less accurate than

using a flow cytometer to manually gate 1 cell at a time and might influence the results presented here. In this study, CFU-F assay using 6-well plates was used to measure the clonogenetic potentials of cells. The average colony forming efficiency ranged from 40.0% (± 26.7%) – 42.7% (± 29.8%) with no significant difference between cell lines. However, the clonogenecity between individuals varied greatly ranging from 6.7 – 83.3%. This is higher than reported by Asari et al. in 2011 when they isolated and cultured cells from LF and posterior longitudinal ligament tissues (24). They reported 8.8% (± 6.8%) colony forming efficiency making no distinguishing between cell sources. In their study they seeded 100 cells on a 100 mm culture plate and grew for 14 days before counting colonies. In 2013, Harada et al. reported 25.2% colony forming efficiency of cells from posterior longitudinal ligament of healthy subjects and 27.2% of cells from OPLL patients using single cell seeding method in 96-well plates (13). This differs from the method used here as 10 cells were plated per well in 6-well plates and it might partly explain the difference as diluting becomes increasingly difficult at lower levels. Nevertheless, when osteogenic and adipogenic potentials of individual colonies were measured by seeding 100 cells in 100mm culture dishes, crystal violet staining revealed multiple colonies showing that colony forming efficiency was still greater than reported by Asari et al. when using the same culture technique. It has been shown that colony forming efficiency can be improved by bead enrichment of cells. In 2003, Shi and Gronthos revealed that colony forming efficiency could be improved 6-fold by enriching dental pulp MSCs for STRO-1+ and 7-fold when tested for CD146+, reaching up to 96% colony forming efficiency compared to that of the unfractioned pulp cell population (143).

Another distinct feature of MSCs, especially for tissue engineering, is their high proliferation rate, multiplying their number over a short period of time. Proliferation can be measured both at a high- and low-seeding density by counting PDL over time. In this study, PDL were counted after seeding cells a low-density for up to 5 passages before cells entered senescence state. Average accumulated PDLs were 23.83 (±5.62)

for LF-derived cells, 25.55 (±7.36) for FJ-derived cells and 24.00 (±7.64) for IL-derived cells. A similar trend was observed for individual patients and cell lines as in the CFU-F assay with PDL ranging from 9.20 – 36.39. This is a high proliferation capacity comparable or greater to that of other conventional sources of MSCs such as BMSCs, AMSCs and synovial MSCs (144). In contrary, this is less than reported by Asari et al. when they seeded LF and posterior longitudinal ligament cells at the same density (24). They reported a similar growth rate but were able to maintain their cells in culture for a greater length of time reaching passage 7 before cells entered senescence state, whereas they entered senescence at passage 5 as reported here. Asari et al. also investigated the proliferation rate at high density by seeding cells at 5000 cells/cm^2 and observed over 10 PDL during 80 days culture period.

The results from the CFU-F assay and cell proliferation assay are no surprise since cells vary greatly between individuals. Age, genetic predisposal, disease and environmental factors all contribute to the proficiency of cells within our bodies and it applies to MSCs as well (134). This is one of the challenges in tissue engineering since making a standard treatment with predictable results is immensely difficult when using autologous cells. Thus, allogenic cells have been suggested as an alternative source. Due to the destructive nature of various disease this might prove beneficial by using healthy, unaffected MSCs from young donors. Moreover, it eliminates the harvest and cultivation of autologous MSCs, causing less discomfort to the patients and treatment can start contiguously. Whilst a paucity of research exists regarding allogenic MSCs and their potential as a treatment is still being investigated, they have been shown to be successful for the treatment of osteoarthritis (145). Additionally, xeno-free culturing methods utilizing human platelet lysate for growth support instead of the commonly used FBS are gaining ground. The clinical advantages of xeno-free conditions is the absence of secondary effects potentially cause by FBS vectors in the culture and as human platelet lysate is GMP approved it can be directly applied in clinical therapy without extensive testing (146-148). In

2015, Li et al. compared BMSCs and AMSCs grown under xeno-free conditions as a potential culture method for cell therapy (149). They reported over 10% colony forming efficiency for BMSCs and over 12% for AMSCs. The cells proliferated for up to 5 passages which is the same as reported here, but reached only 13.7 ± 0.5 PDL for BMSCs and 17.2 ± 0.3 PDL for AMSCs. In addition, both cell lines expressed common MSCs markers and were capable of tri-lineage differentiation. BMSCs grown under xeno-free condition have also been used in treatment when Centeno et al. treated 227 patients suffering from cartilage defects in peripheral joints or intervertebral discs using BMSCs grown in autologous platelet lysate. They, reported only three stem-cell related complications all of which were minor and easily remedied (148, 150, 151).

Immunophenotyping showed all cell lines to be positive for the established MSC markers CD29, CD44, CD73, CD90 and CD105. However, positivity varied both between markers and cell lines. CD73, CD90 and CD105 are considered to be the golden standard markers as they are the core markers recognized by the ISCT for defining MSCs (4). Whilst, the positivity for CD90 and CD44 was very high being around 90% and up, the positivity for CD29, CD73 and CD105 varied more, ranging from 50.6% - 86.4%. Dissimilarities in epitope profiles are a common occurrence in the vast populations of MSCs isolated from multiple tissues that serve diverse functions. Although CD73, CD90 and CD105 positivity are among the minimum criteria for defining MSCs as put forward by the ISCT, Utsunomiya et al. reported sources of fully functional MSCs with a very low positivity for CD105 (22). In their study they isolated cells from the synovium, bursa, tendon and enthesis of shoulder tissues involved in rotator cuff tears. The cells were colony forming, evinced high proliferation rates and where capable of tri-lineage differentiation. When they investigated the epitope profile, they observed a high positivity for CD44 and CD90 as reported here, but surprisingly the positivity for CD105 was as low as 0.1% in the case of enthesis-derived cells. This was also true for other MSCs markers such as CD147 and CD166 used in their study. The tendon and enthesis tissues used in their study

share some similarities as the tissues used here being collagenous connective tissues under high mechanical stress. Nevertheless, cell lines presented here were positive for CD105 meeting the minimum criteria put forward by the ISCT. Moreover, all cell lines were negative for the haematopoietic markers CD34 and CD45, suggesting that there was little to no contamination of haematopoietic stem cells in the culture. Haematopoietic contamination is a concern as they are often accompanied by MSCs (152). Both are progenitor cells found predominantly within the bone marrow but serve dissimilar functions.

Although, epitope profile of MSCs varies greatly the tri-lineage differentiation is an important attribute shared by all defined MSCs. Osteogenic differentiation is an important tissue engineering feature of MSCs also shared by pericytes, adventitial cells and some sources of fibroblasts (34, 67, 153, 154). In this study all cell lines were highly osteogenic when subjected to osteogenic media, but there was no osteogenic activity observed as reported in cells from posterior longitudinal ligament of OPLL patients when grown under normal culture conditions (13, 123). The matrix build-up became so dense and strong making trypsin cell detachment difficult when cells were extracting for RNA isolation. Osteogenic differentiation was successful at both low- and high-seeding densities and the matrix was rich in calcium, the major building component of bones as revealed by Alizarin Red S staining. The expression of *Runx2* was also elevated when cells were grown in osteogenic medium. A base expression of *Runx2* has been reported before in MSCs but surprisingly the results in this study showed the base expression to by higher in cells from FJs and ILs compared to LF-derived cells (121). *Osteopontin* was also highly expressed in induction cultures and a slight expression of *Osteopontin* was also observed in the control cultures further supporting the hypothesis that cells in native conditions are highly osteogenic. Moreover, a very strong osteogenic response was evident from individual colonies and no additional colonies were observed after the second staining with Crystal Violet showing that all clonogenic cells were also osteogenic.

This is a stronger osteogenic response from clonogenic cells than reported by Utsunomiya et al. in 2013 when they investigated the osteogenic potentials of shoulder derived MSCs in a similar fashion (22). Nonetheless, supporting the results presented here, Harada et al. reported a significant increase in osteogenic potentials of cells from OPLL patients compared with healthy controls (13).

This robust osteogenic response is a strong indicator that MSCs might be directly involved in the calcification of spinal ligaments in LSCS, OLF and OPLL patients. Although, calcification is more commonly reported in LF and posterior longitudinal ligaments compared to FJs and ILs, the cells from FJs and ILs still showed a high osteogenic response. This suggests that the conditions might be systematic, not only affecting the ligaments involved but also the surrounding structure and that FJs and ILs might be contributing factors in LSCS, OLF and OPLL. Notwithstanding, no quantitative experiments of osteogenic potentials were performed in this study.

Although, adipogenic differentiation has a very limited use for tissue engineering application, it is still considered a major differentiation pathway of MSCs. All cell lines tested showed adipogenic potentials at both a high- and a low-seeding density. Even so, a fairly low staining was observed when stained with Oil Red O. Intracellular as well as extracellular lipid droplets stained and were visible under the microscope but barely detectable with the naked eye. The lipid droplets appeared larger when cells were seeded at low density than when seeded at high density. This is conflicting to what has been reported before, where it was suggested that high density of cells resulted in a stronger osteogenic response (46). The low amount of staining observed might partly be due to the loss of cell density, differentiation medium used and staining method. However, gene expression analysis showed a strong expression of the adipogenic markers *PPAR-γ2* and *LPL* compared to cells grown in normal culture medium showing that the cells were undeniably responding to the induction medium. The adipogenic potentials of individual colonies were not

as strong as the osteogenic potentials. After Oil Red O staining some colonies stained red and were visible, but Crystal Violet staining revealed a plethora of other colonies not stained by Oil Red O. This suggests that adipogenic potentials of clonogenic cells are not very strong, especially when compared to osteogenic response. However, when viewed under the microscope both intracellular and extracellular lipid droplets stained by Oil Red O could be observed, whereas only small intracellular droplets were seen in control cultures. This is consistent with Utsunomiya et al. findings that shoulder derived MSCs showed lower adipogenic potentials at an individual level than osteogenic potentials, especially in cells from tendon and enthesis which are highly similar to LF, FJs and ILs (22). A partial explanation for this low adipogenic response observed here and by Utsunomiya et al. might be the nature of the tissues involved. All are high in collagen proteins which are common components in bone and cartilage, which might affect the cells residing within theoretically favouring osteogenic and chondrogenic differentiation pathways.

Despite tissue engineering applications rarely focusing on the restoration of adipose tissue, AMSCs have become popular source of cells for multiple procedures as they can be harvested in great number requiring minimal manipulation before being applied in treatment. They have been used successfully for cartilage restoration in numerous clinical trials (155-157).

Chondrogenic differentiation is the third key differentiation pathway of MSCs noted by the ISCT and a commonly studied one. All cell lines in this study were able to undergo chondrogenic differentiation when grown in a pellet culture in chondrogenic induction medium. When the pellets were harvested, there was a notable difference in the pellets grown in chondrogenic medium and the pellets grown in normal culture medium. Pellets grown in chondrogenic medium were dense and easily held together due to the extracellular matrix formed during chondrogenesis holding them together. Whereas the pellets grown in normal culture medium contained little to no extracellular matrix and easily fell apart when

handled. This extracellular matrix was visible when sections were stained with Toluidine blue and Alcian blue. More extracellular build-up was observed in pellets of FJs and ILs compared to that of LF-derived cells. Gene expression analysis of *Sox9* and *Aggrecan* further confirmed the chondrogenic potentials of all cell lines. The results presented here are similar to the once previously reported by Utsunomiya et al. when they studied shoulder derived MSCs (22). They grew their pellets in a similar way and Toluidine blue staining showed a build-up of extracellular matrix with cells visible on the edges of the pellet. In addition, they performed collagen type II staining which further confirmed the collagenous nature of the extracellular matrix. Chen et al. also reported similar staining of LF-derived MSCs when they stained pellets with Alcian blue and collagen type II (23). As described here staining of cartilage components and gene expression analysis were used to confirm chondrogenic potentials. Moreover, pellet diameter and wet-weight can also be measured as done by Harada et al. when they compared MSCs from OPLL and healthy controls and showed that the chondrogenic potentials of MSCs from OPLL patients were not reduced (13). Pellet culturing is a challenging task and initiating and measuring the chondrogenic response can be complicated. Nonetheless, this study confirmed that LF-, FJ- and IL- derived cells were all capable of chondrogenic differentiation.

Cartilage regeneration is a challenging task in the field of tissue engineering and orthopaedics, especially for patients with degenerative joint arthritis conditions. Clinical trials using MSCs have shown promising results for numerous arthritis condition where MSCs can by directly injected into the affected joint providing a minimally invasive treatment option. Better results have been produced with a high numbers of MSCs injected although development of the treatment is still on-going (158). Hitherto, researchers have favoured BMSCs and AMSCs for these applications, but in 2015 Ichiro Sekiya et al. reported their success on using synovial derived MSCs for the first time in a human clinical trial (88). Previous *in vitro* and *in vivo* studies had

shown the superior chondrogenic potentials of synovial derived MSCs and they hypothesized that they could also be used for the treatment of cartilage defects *in vivo* (144, 159). Unlike BMSCs and AMSCs, synovial MSCs are local cells in joints and therefore do not need to adapt to the microenvironment when applied in treatment theoretically giving them an advantage over other sources of MSCs. Equally, MSCs derived from spinal ligament tissues might possess advantage for tissue engineering applications focusing on the connective tissues of the vertebral column as well as surrounding structures.

Albeit, the localization of MSCs *in vivo* still remains a subject of great controversy. A number of studies have suggested a vascular origin of MSCs both from pericytes and adventitial cells (34, 67, 160). Vascular cell rich areas as well as avascular dense areas were observed in tissues stained in this study when stained with H&E. Endothelial cells were visible and it is possible that pericytes were present as well and that they might be the source of the cells isolated in this study. Chen et al. showed that LF-derived MSCs highly expressed the protein *α-smooth muscle actin (α-SMA) in vitro* (23, 26). A high expression of *α-SMA* has also been reported in pericytes and endothelial cells but not adventitial cells which suggests that LF-derived cells might have a perivascular origin (34, 67, 161). Moreover, Asari et al. reported a positive CD90 *in situ* staining of LF and posterior longitudinal ligaments (24). Their results revealed the presence of CD90 in cells around micro vessels and an absence of the haematopoietic markers CD34, found in adventitial cells and CD45. The isolated cells were also highly CD90 positive, but CD90 is shared by MSCs, pericytes, adventitial cells and a number of fibroblasts (Table 1.). Adding to the debate of MSCs origin is the fact that MSCs have been shown to migrate to tissues under stress, for example from inflammation and injury (6, 7). This might be a factor in numerous MSC studies including this one as cells are often isolated from tissue under stress. Hence, the origin and localization of MSCs still remains disputed.

There are several noteworthy limitations to the present study. Cells were isolated from all tissues 10 patients providing a weak support for any statistical analysis performed. This was due to the cost and time of MSCs primary isolation, proliferation assays, immunophenotyping and tri-lineage differentiation. Nonetheless, isolation of cells from 5-15 study subjects is a common occurrence when identifying new sources of MSCs. Furthermore, all patients enrolled in this study were above 50 years of age. Age has been shown to reduce the proliferation and osteogenic potentials of BMSCs and AMSCs (162). Even so we report high proliferation rates, colony forming efficiency and osteogenic potentials of the isolated cells. Antithetically, it has been shown that phenotypic and tri-lineage potentials, of MSCs are not altered by age and all cells isolated in this study were capable of tri-lineage differentiation (163). In addition, all patients involved were suffering from either LSCS, spondylolisthesis or degenerative scoliosis, causing increased stress to the spinal ligaments, hence, effecting the tissues studied. As aforementioned, inflammation and injury can affect the number and characteristics of cells as MSCs are capable of migration upon paracrine signalling. However, studies from LF and posterior longitudinal ligament have identified MSCs in healthy subjects with similar properties as MSCs from patients suffering from degenerative spinal conditions (13, 23, 24). At last there were no quantitative experiments on tri-lineage capacity performed.

This study confirms the existence of MSCs within the LF, FJ and IL. They all show strong osteogenic potentials suggesting that they might be involved in calcification processes of the ligaments. However, the osteogenic potentials have not been quantified. Future studies could focus on the osteogenic potentials of the cells from these three sources by comparing cells from healthy subjects to cells from patients suffering from LSCS. This could identify if cells from LSCS patients show superior osteogenic potentials, possibly having an altered cell profile. This could help to better identify if the effects are systematic not only affecting cells within the LF but also the FJs and ILs. In addition, it would be interesting to grow cells under

hypoxia, reducing oxidative stress and determine if they behave in the same way as this condition might better simulate their native environment. Furthermore, *in situ* staining of the MSCs and pericytes surface makers CD73, CD90, CD105, CD146 and **α**-SMA could better identify the origin and localization of MSCs within the LF, FJ and IL tissues. This would better clarify if MSCs were of pericyte origin as CD146 and **α**-SMA are highly expressed in those cells. Differentiation potentials of spinal MSCs towards specialized cell types such as tenocytes and nucleus pulposus cells commonly found in spinal ligaments and intervertebral disc should also be investigated. If they are found to possess those differentiation potentials they might provide a superior source of local cells that might be applicable in small tissue engineering applications in the vertebral column.

Conclusion

In summary, this study has shown that cells can be isolated from LF, FJ and IL tissues and that they can be classified as MSCs meeting the standard criteria put forward by the ISCT as follows:

(1) Spindle shaped morphology and plastic adherence

(2) Clonogenic and high proliferating

(3) Positive for a number of surface markers associated with MSCs and negative for markers associated with haematopoietic stem cells

(4) Multipotent differentiation potentials

This is the first time MSCs have been identified within the FJs and ILs. Here we demonstrate that they are highly similar to the previously identified LF-derived MSCs. They are highly clonogenic, capable of forming colonies even when seeded at low density. Immunophenotyping revealed that cell sources were positive for the common MSCs surface markers CD29, CD44, CD73, CD90 and CD105, whilst negative for the haematopoietic markers CD34 and CD45. Tri-lineage differentiation revealed

all cells sources to be highly osteogenic suggesting that they might be involved in the calcification process of spinal ligaments. In addition, they also showed good chondrogenic and adipogenic potentials although no large lipid droplets were observed in induction cultures. The origin of the cells described here is still unknown but vascular cell rich areas were observed, suggesting that they might be of perivascular origin. We conclude that MSCs within LF, FJs and ILs might be involved in degenerative spinal conditions as well as having a potential for small scale tissue engineering applications. Additional studies are required to better understand their localization and involved in disease progression as well as potentials for tissue engineering. MSCs in spinal tissues merit further investigation.

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APPENDIX

STATISTICAL ANALYSIS OUTPUT

1. Age

MEANS

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
VAR00001	10	100,0%	0	0,0%	10	100,0%

Report

VAR00001

Mean	N	Std. Deviation
64,2000	10	11,45814

2. CFU-F assay

MEANS

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
LF	10	100,0%	0	0,0%	10	100,0%
FJ	10	100,0%	0	0,0%	10	100,0%
IL	10	100,0%	0	0,0%	10	100,0%

Report

	LF	FJ	IL
Mean	3,9996	4,2663	4,0663
N	10	10	10
Std. Deviation	2,66679	2,97657	2,04763

One way ANOVA
Post Hoc Tests

Multiple Comparisons

Dependent Variable: SET1

	(I) FJ	(J) FJ	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1,00	2,00	-,26670	1,15944	,971	-3,1414	2,6080
		3,00	-,06670	1,15944	,998	-2,9414	2,8080
	2,00	1,00	,26670	1,15944	,971	-2,6080	3,1414
		3,00	,20000	1,15944	,984	-2,6747	3,0747
	3,00	1,00	,06670	1,15944	,998	-2,8080	2,9414
		2,00	-,20000	1,15944	,984	-3,0747	2,6747
Bonferroni	1,00	2,00	-,26670	1,15944	1,000	-3,2261	2,6927
		3,00	-,06670	1,15944	1,000	-3,0261	2,8927
	2,00	1,00	,26670	1,15944	1,000	-2,6927	3,2261
		3,00	,20000	1,15944	1,000	-2,7594	3,1594
	3,00	1,00	,06670	1,15944	1,000	-2,8927	3,0261
		2,00	-,20000	1,15944	1,000	-3,1594	2,7594

Homogeneous Subsets

LF

	FJ	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	1,00	10	3,9996
	3,00	10	4,0663
	2,00	10	4,2663
	Sig.		,971

2. Population doublings

MEANS

Case Processing Summary

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
LF	10	33,3%	20	66,7%	30	100,0%
IL	10	33,3%	20	66,7%	30	100,0%
FJ	10	33,3%	20	66,7%	30	100,0%

Report

	LF	IL	FJ
Mean	23,8310	24,0000	25,5470
N	10	10	10
Std. Deviation	5,62099	7,63922	7,35456

One way ANOVA
Post Hoc Tests

Multiple Comparisons

Dependent Variable: VAR00001

						95% Confidence Interval
	(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound
Tukey HSD	1,00	2,00	-,16900	3,09885	,998	-7,8523
		3,00	-1,71600	3,09885	,845	-9,3993
	2,00	1,00	,16900	3,09885	,998	-7,5143
		3,00	-1,54700	3,09885	,872	-9,2303
	3,00	1,00	1,71600	3,09885	,845	-5,9673
		2,00	1,54700	3,09885	,872	-6,1363
Bonferroni	1,00	2,00	-,16900	3,09885	1,000	-8,0787
		3,00	-1,71600	3,09885	1,000	-9,6257
	2,00	1,00	,16900	3,09885	1,000	-7,7407
		3,00	-1,54700	3,09885	1,000	-9,4567
	3,00	1,00	1,71600	3,09885	1,000	-6,1937
		2,00	1,54700	3,09885	1,000	-6,3627

Homogeneous Subsets

VAR00001

	VAR00002	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	1,00	10	23,8310
	2,00	10	24,0000
	3,00	10	25,5470
	Sig.		,845

α-MEM PREPERATION

1. Measure and add 890 mL ddH_2O to a 2 L glass container

2. Weigh 10.2 g α-MEM powder and 2.2 g sodium bicarbonate

3. Add α-MEM powder and sodium bicarbonate to the water and stir for 15-30 minutes

4. Bring the container to a sterilized flow hood

5. Add 10 ml Penicillin-Streptomycin antibiotic (1%)

6. Add 100 ml FBS (for 10% FBS)

7. Stir using a glass rod

8. Filter liquid medium using sterilized filter system (0.22 μm)

9. Store at 4°C

VITA

Mister Baldur Kristjánsson was born on the 5th of January 1989 in Reykjavík, Iceland. He is the son of the psychiatric nurse Guðrún Inga Tryggvadóttir and cement truck driver Kristján Sigurðusson. He has one son with his girlfriend Namfon Mayoe, named Egill Mayoe, born on the 5th of February 2015 in Chiang Rai, Thailand. He graduated with a Bachelor degree in biology with First Class honours from the University of Iceland in 2012 and started his Master degree at Chulalongkorn University in 2013. He was selected as an oral presenter for the 35th Annual Meeting of the Royal College of Orthopaedic Surgeons of Thailand conference where he presented the work of Thakoon Thitiset on periosteum derived-MSCs and novel scaffold composites for bone tissue engineering.

Moreover, parts of his graduate studies were presented at the 2015 Orthopaedic Research Society´s Annual meeting in Las Vegas, Nevada, USA in March/April 2015. Additionally, he received awards at the 2015 Graduate Research Competition held in Chulalongkorn Univeristy, where he presented his work in April 2015.

In his free time, he enjoys spending time with his family, travelling, hiking, photography and web-browsing.

Publications

Kristjánsson B, Limthongkul W, Yingsakmongkol W, Jirathanathornnukul N, Honsawek S. Isolation and characterization of human mesenchymal stem cells from facet joints and interspinous ligaments. Spine 2015 Submitted.

Kristjánsson B, Honsawek S. Current perspectives in mesenchymal stem cell therapies for osteoarthritis. Stem Cells International 2014 Dec 194318

Kristjánsson B, Honsawek S. Current trends of stem cell-based approaches for knee osteoarthritis. OA Tissue Engineering 2013 Jun 01;1(1):7.

Kristjánsson B, Mabey T, Yuktanandana P, Parkpian V, Honsawek S. Mesenchymal Stem Cells for Regeneration of Cartilage Lesions: Focus on knee osteoarthritis. The Thai Journal of Orthopaedic Surgery. 2013: 37 No.2-4: 67-78.

Conference contributions

2015 Princess Maha Chakri Sirindhorn Congress. Oral presentation: Mesenchymal stem cells in human spinal ligaments. Nonthaburi, THAILAND

2015 Joint Conference In Medical Science 2015. Poster presentation: Mesenchymal stem cell isolation and characterization from ligamenta flava, interspinous ligaments and facet joints. Bangkok, THAILAND.

2015 Orthopaedic Research Society 2015 Annual meeting. Poster presentation: Comparison of Human Mesenchymal Stem Cells Derived from Various Spinal Tissues: Superiority of Facet Joint and Interspinous Ligament. Las Vegas, NV, USA.

2013 The 35th Annual Meeting of the Royal College of Orthopaedic Surgeons of Thailand (RCOST). Oral presentation and poster: Isolation of Mesenchymal Stem Cells from Human Periosteum and Biomaterial Scaffolds for Bone Tissue Engineering, Pattaya, THAILAND.