ISOLATION AND CHARACTERIZATION OF HUMAN GINGIVAL FIBROBLASTS

By

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Abstract

Background*:* Gingival fibroblasts (GFs) that exhibit characteristics similar to adult stem cells are also known as gingival mesenchymal stem cells (GMSCs). These cells can be isolated using either tissue explants or enzymatic digestion, however it is unknown whether the isolation method influences the GMSCs potential for differentiation. In addition, no specific mesenchymal stem cell (MSC) marker has been reported to identify and distinguish GMSCs from GFs. Recently, the cell surface molecule known as cluster of differentiation (CD) 146 has been identified as a potential MSC cell surface marker.

Objective: To investigate the differentiation potential of GMSCs based on CD146 expression.

Hypothesis: CD146 is the MSC surface marker that identifies GMSCs among a population of GFs regardless of the isolation method employed.

Materials and methods: GFs were isolated by two techniques: tissue explants or enzymatic digestion. GFs were cultured and expanded in a medium containing fibroblast growth factor 2 (FGF-2), and magnetically sorted according to CD146 expression. Four experimental groups were collected: $CD146^{low}$ and $CD146^{high}$ from explant-derived isolation and $CD146^{low}$ and $CD146^{high}$ from digestion-derived isolation. Each group of cells was expanded and then tested for stem cell markers using flow cytometry before it was subjected to osteogenic and chondrogenic differentiation. Multilineage differentiation outcome was tested after 21 days using histology, immunofluorescence, real-time quantitative PCR (qPCR), and glycosaminoglycan (GAG) to DNA ratio (GAG/DNA) assays.

Results: As confirmation of osteogenic differentiation, alizarin red staining was positive for all groups with no significant difference between osteogenic gene expressions. The

absence of Safranin O staining accompanied by low GAG production negates chondrogenic differentiation. This lack of chondrogenesis was further confirmed by immunofluorescence assay, which indicated no deposition of collagen type II in the extracellular matrix (ECM) of GFs aggregates.

Conclusion: CD146 is not the specific stem cell surface marker to identify and enrich the GMSC population. Its expression did not enhance the osteogenic potential differentiation potential of sorted GFs regardless of the implemented isolation method.

Preface

This thesis is an original work by Samira Diar-Bakirly. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Effect of Gingival Fibroblasts and Ultrasound on Tooth Root Resorption in Humans,” No. Pro00056111, October 28, 2014 (Approval Renewal Date).

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Table of Contents

List of abbreviations

GFs: gingival fibroblasts

GMSCs: gingival mesenchymal stem cells

FBS: fetal bovine serum

P1: passage 1

CFU-F: colony forming unit-fibroblast

IL10: interleukin 10

CD: cluster of differentiation or cluster of designation

qPCR: quantitative polymerase chain reaction

DSPP: dentine sialophosphoprotein

SHED: stem cells from human exfoliated deciduous teeth

DPSC: dental pulp stem cells

PDLSC: periodontal ligament stem cell

GAG: glycosaminoglycan

ECM: extracellular matrix

PDGF: platelet-derived growth factor

TGF-ß: transforming growth factor ß

VEGF: vascular endothelial growth factor

RUNX2: runt-related transcription factor2

ALP: alkaline phosphatase

BMP: bone morphogenetic protein

IGF I and II: insulin growth factor I and II

HLA-DR: human leukocyte antigen-DR

mAb-FITC: monoclonal antibodies conjugated to fluorescein isothiocyanate

mAb-PE: monoclonal antibodies conjugated to phycoerythrin

FACS buffer: fluorescence-activated cell sorting buffer

COLI: collagen type 1

COLII: collagen type 2

HPRT1: hypoxanthine phosphoribosyltransferase 1

RPL13: ribosomal protein L13a

YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

List of Tables

List of Figures

Chapter 1 Literature Review

1.1 Anatomy and Physiology of the Gingiva

The gingiva, both anatomically and functionally, is a unique structure that is firmly attached to the alveolar bone surrounding the teeth by well-developed collagenous bundles[1]. The two main tissue components of the gingiva are the gingival epithelium and the underlying connective tissue, called the lamina propria.

The gingival epithelium is relatively thick, apart from a small zone of the free gingiva. The epithelial surface of the gingiva is tough and resistant to abrasion and tightly bound to the underlying lamina propria (**Figure 1**). The epithelial cells maintain their structural integrity through a process of continuous mitotic cell divisions in the basal cell layer. These cells migrate to the surface and replace those that have been shed. Thus, the cells of the gingival epithelial cells can be recognized as both, progenitors (providing new cells) and maturing cells that form the protective surface, the keratinized oral mucosa [1].

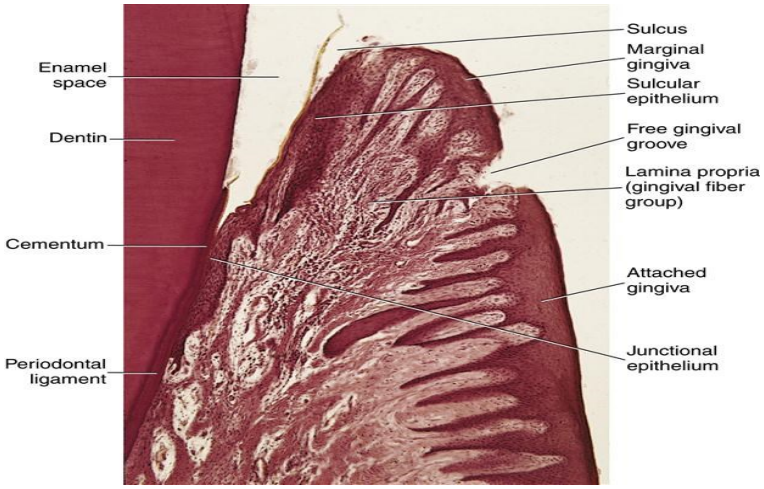

Figure 1. Photomicrograph of gingival and dentogingival tissue. The lamina propria underlies the epithelium and is continuous with the periodontal ligament, which is adjacent to the hard tissues of the tooth.

The connective tissue of the gingiva consists of collagen fibers embedded in the extracellular matrix, blood and lymphatic vessels, nerves, and a heterogeneous group of cells including fibroblasts, immune cells, and endothelial cells[1]. The predominant cells of the gingival connective tissue are the gingival fibroblasts (GFs), however, the existence of various subpopulations of GFs has been reported[2-4]. Although these subpopulations are phenotypically different, they share fibroblast-like structures and require identical growth conditions[2]. In healthy gingival tissue, GFs exhibit low mitotic activity as well as low biosynthetic activity of extracellular matrix (ECM) components[5, 6]. When activated, as in response to an injury, GFs increase the production of ECM components.

GFs secrete the majority of the components of the ECM including collagen fibers, proteoglycans, glycoproteins, cytokines, and growth factors[7]. The collagen matrix of the gingival connective tissue is well organized into fiber bundles, including type I and type III collagen as the primary fibers occupying nearly 60% of the extracellular space; and type IV and type VII occurring as part of the basal lamina[8]. GFs are able to adhere to these fibers, forming a 3D network that becomes embedded within the ECM[7].

Heterogeneous protein-carbohydrate complexes composed of proteoglycans and glycoproteins maintain the integrity of the gingival tissue by interacting with the components of the ECM and contributing to cell adhesion and signaling[9]. The proteoglycans consist of a protein (polypeptide) core to which glycosaminoglycans (hexose sugars) are attached. Dermatan sulphate, chondroitin sulphate (sulphated), and hyaluronan (non-sulphated) are the primary glycosaminoglycans (GAGs) forming the proteoglycans decorin, versican, syndecan and biglycan within the gingival ECM [9].

Growth factors, including fibroblast growth factor 2 (FGF-2)[10]. exert their effect on cells after binding to specific cell receptors during certain conditions; binding of these growth factors to the proteoglycans within the ECM constitute the reservoir of active molecules that can be used when needed[1]. A more detailed discussion of FGF-2 can be found in Section 1.4.

As a result of the unique properties of GFs, there are several aspects that distinguish the gingival ECM from the ECM generated by skin fibroblasts. GFs produce larger dermatan sulphate proteoglycans and higher levels of hyaluronan compared to dermal fibroblasts[11]. In addition, the glycoproteins have a polypeptide chain to which only few simple hexoses are attached and the microfibrillar component of the gingival ECM includes fibulin 5 and fibrillin-1 and -2. Furthermore, proteins such as periostin, osteopontin and type V collagen are not highly expressed in the gingiva[12]. Taken together, these differences between GFs and dermal fibroblasts in the production of the ECM lends the gingiva its distinctive characteristics, including as faster healing after injury in the absence of scar formation[12,13].

1.1.1 Tissue Repair of the Gingiva

Given their anatomical location in the oral cavity, gingival cells are in direct and continuous contact with a complex cellular milieu comprised of bacterial products and immune cells[3]. Thus, it is unsurprising that at every instance of damage to oral tissues, bacteria immediately load the injury site[3]. The inflammatory reaction induced by injury and bacterial invasion to the site of injury triggers an inflammatory response with a unique cytokine response from the GFs. Studies revealed that during gingival healing, GFs exhibit

many fetal fibroblast-like properties including their migration properties and production of migrating stimulating factor [6, 13].

GFs rapidly respond to bacterial products by synthesizing hyaluronic acid, a GAG found in the ECM that is known to increase cell motility[5, 6]. Once the ECM is disturbed by injury, the migration, proliferation, and contraction abilities of GFs will increase as a response to the large number of chemokine and growth factors secreted into the wounded area by the degranulated platelets after injury. These growth factors include platelet-derived growth factor (PDGF), transforming growth factor ß (TGF-ß), insulin-like growth factors I and II (IGF-I and II), FGF-2, and epidermal growth factor (**Figure 2**)[5]. The activated GFs will then release chemokines such as interleukin 8 (IL-8) that will increase the number of immune cells in the wounded area and aid in its repair [5].

Over the course of scarless oral wound healing, inflammatory phase is followed by development of granulation tissue, which contains several types of cells including fibroblasts, macrophages, and endothelial cells, as well as a dense capillary network. The epithelial layer is also re-established, and the ECM undergoes restoration. The further migration and differentiation of fibroblasts and regenerative stem cells allows for the development and remodeling of the ECM as it progresses towards the center of the wound. Continual differentiation of the fibroblasts results in contraction and closure of the wound[14].

Taken together, the scarless wound healing and regenerative capacity of the gingiva elicited researchers to identify the stem cell population residing within the gingiva with the ability to self-renew.

1.2 Stem Cell Biology

Stem cells are defined as cells that have the potential for unlimited or prolonged self-renewal, as well as the ability to give rise to at least one type of mature, differentiated cells. Self-renewal is achieved by the ability to divide asymmetrically: one cell remains a daughter self-renewing stem cell, and the other cell replicates and differentiates into a mature cell type[15]. Stem cells can be divided into two types: embryonic stem cells and adult stem cells.

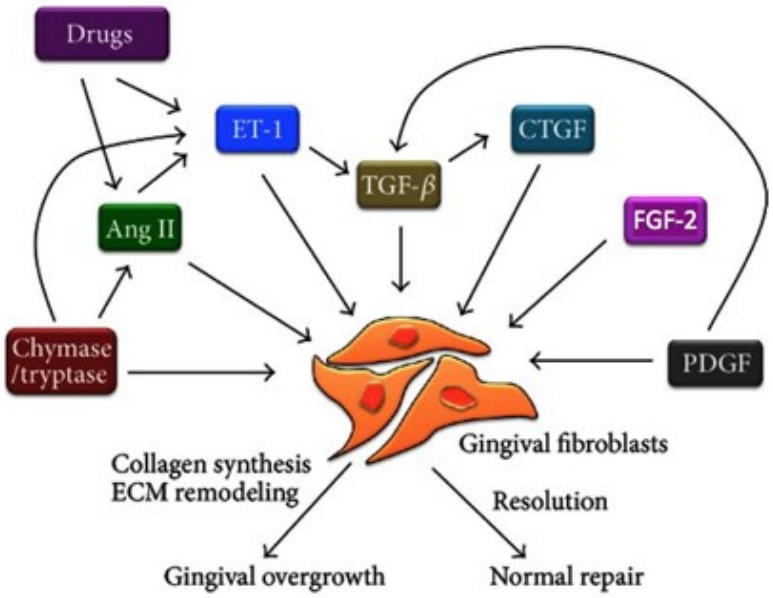

Figure 2. Wound healing in the gingiva. Fibroblast activation and regeneration are promoted by the following: Ang II: angiotensin II; CTGF: connective tissue growth factor; ET: endothelin; FGF-2: fibroblast growth factor-2; PDGF: platelet-derived growth factor; TGF: transforming growth factor; ECM: extracellular matrix.

After fertilization, the cells of the zygote are totipotent, which means they are able to give rise to all cell types in the body as well as the placenta. Embryonic stem cells are derived from the blastocyst, which forms 5 days after fertilization. These cells are

pluripotent and have the ability to produce all three germ layers and consequently all cell types in the body[15].

In contrast, adult stem cells are rare cells that do not expand limitlessly and have a specific differentiation potential. Stem cells in adult tissues are in an inactive quiescent state and they maintain their long-term homeostasis through the self-renewal process where they divide into undifferentiated cells[10]. Adult stem cells, as the name implies, are present in various adult tissues. It is believed that adult stem cells are responsible for replacing the cells of certain tissues whenever these cells are destroyed by disease or injury[10].

Stem cells can be further categorized based on characteristics acquired upon activation. Transit-amplifying cells (TACs) are produced from stem cells and have the capacity to divide quickly several times, before progressing to progenitors that will terminally differentiate to a specific tissue type[16]. It is thought that TACs are able to provide feedback to stem cells in order to generate further progenitor cells for regeneration processes in response to injury[16].

Progenitor cells are derived from stem cells and have the capacity to differentiate into one specific type of cell after several rounds of cell division (**Figure 3**)[15]. A specialized, and well-known type of adult stem cell progenitor is the mesenchymal stem cell (MSCs).

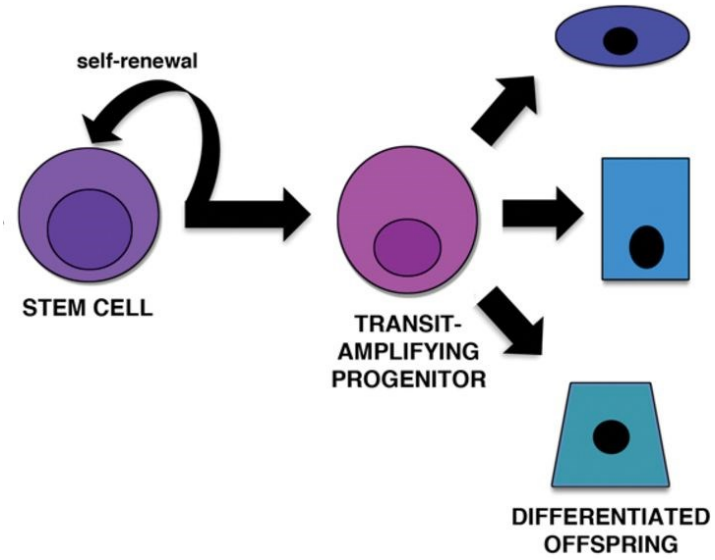

Figure 3. Identification and progression of stem cells. Stem cells are able to self-renew and also produce more differentiated progenitors. These progenitors are highly proliferative and able to differentiate into various cells types.

1.2.1 Mesenchymal Stem Cell

Mesenchymal stem cells are defined as non-hematopoietic stromal cells that were first identified from mononuclear cells of bone marrow[17]. Friedenstein *et al*. noted that these fibroblast-shaped cells proliferate to form round shaped colonies that could differentiate into an osteogenic lineage[17, 18]. They also reported that specific cells formed osteogenic nodules, and cells with high osteogenic potential also exhibited high mitotic activity [18]. Later, these fibroblast-shaped cells were determined to be mesenchymal stem cells that can differentiate into multiple mesenchymal lineages including osteogenic, chondrogenic, and adipogenic lineages (i.e., multipotent cells)[19]. MSCs have also been called stromal stem cells in reference to their location in the stroma of the tissue [20].

Existing studies reported the presence of MSCs in various tissues such as placental tissue, umbilical cord blood, perivascular cells, dental pulp tissues, the synovial membrane, adipose tissue, compact bone, periosteum, and fetal tissues (**Figure 4**)[20, 21]. MSCs have also been extracted from many tissues in the body, including those believed to be post-mitotic like the heart and kidneys. MSCs derived from different anatomical sites display differences in their mitotic ability, multipotency, and self-renewal capacities[20]. These features are thought to be due to regulatory cues from cells within the local tissue microenvironment – this is often referred to as the stem cell niche [22].

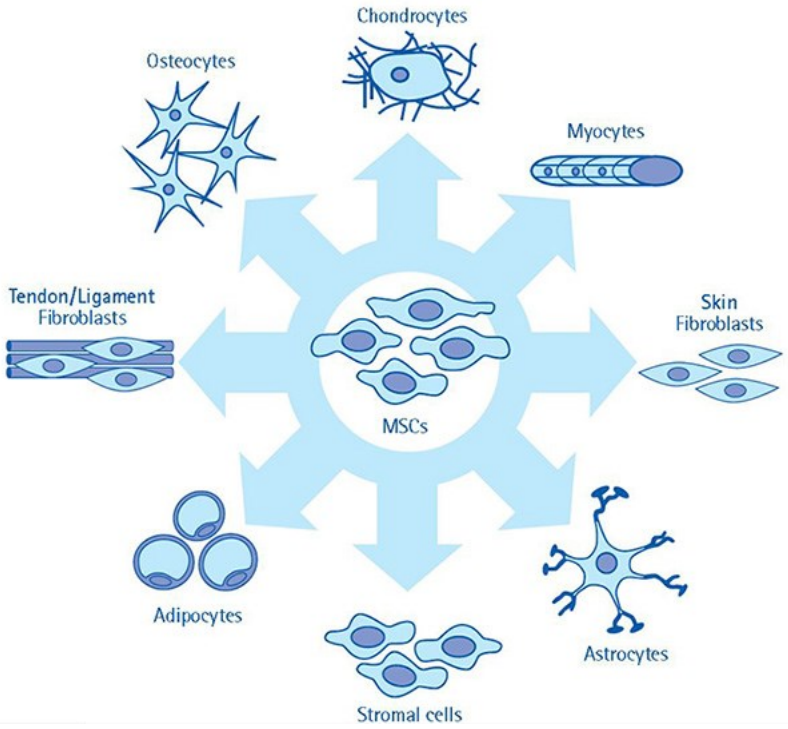

Figure 4. Differentiation potential of MSCs. Mesenchymal stem cells have been isolated from a variety of tissues and have the capacity to differentiate into a variety of cells types, which is especially attractive for regenerative medicine. Adapted from https://www.sigmaaldrich.com/technical-documents/articles/biology/cell-culture/mesenchymal-stem-cell-faq.html

1.2.2 Stem Cell Niche

The specific microenvironment that supports the development and function of the stem cells in a tissue is referred to as the *niche*. The niche embeds the signals generated from the surrounding blood vessels, supportive cells, and the ECM[10]. These signals are essential to regulate stem cell self-renewal, proliferation, survival, and cell death[10]. In addition, spatial relationships and adhesion between stem cells and the supporting stromal cells and/or ECM promotes asymmetric cell divisions, migration, and survival[23].

The idea of the stem cell niche is such that the determinate factor of differentiation and eventual cell fate of the daughter stem cell is the microenvironment in which the cells reside. Understanding the niche and how it is maintained is important to the replication ability and the differentiation process of stem cells *in vitro*. For example, hematopoietic stem cells receive signals from differentiated macrophages in the bone marrow that prevent further mobilization to the bloodstream [24, 25]. In addition, stem cells located in the crypt of the intestinal lining receive signals from differentiated Paneth cells to induce self-renewal[26]. It has also been shown that stem cells in the hair follicle receive cues from keratin 6-positive cells to inhibit further activation when the regenerative process is stable [27]. Signals from polar cells within the ovary of Drosophila have also been described to guide the function of follicular stem cells [28]. Taken together, there is ample data to support the existence of a stem cell niche, a microenvironment that can provide instructions to stem cells to initiate or signal the end of a regenerative process.

1.3 Dental Stem Cells

Craniofacial development is a complex process in which stem cells with varying developmental origins are involved. The teeth alone have at least two embryonic origins:

ectoderm-derived oral epithelium that forms dental enamel and a neural crest that leads to the development of the remaining dental structures (i.e., pulp, dentine, and cementum)[29].

Several types of stems cells have been derived from the various structures within the teeth (**Figure 5**). Teeth are composed of enamel, dentine, and soft dental pulp tissue and are connected to the alveolar bone by the periodontal ligament (PDL)[1]. Stem cells isolated from dental pulp are known as dental pulp stem cells (DPSCs) and were the first adult stem cells identified from dental tissues[30]. Stem cells from human exfoliated deciduous teeth (SHED) were isolated from the pulp tissues and are osteoconductive when implanted *in vivo*[31]. Periodontal ligament stem cells (PDLSC) isolated from the periodontal connective tissue have the potential to regenerate bone, cementum, and PDL-like tissues and could thus be used for regenerative purposes[32 33]. Stem cells from the apical papilla (SCAP) exhibited greater population doubling and superior regeneration and migration capabilities compared to PDLSCs[34]. Dental follicle stem cells (DFPCs) have been isolated from the mesenchymal condensation surrounding the tooth germ during its development[35]. All of the above dental-derived MSCs and differentiated into multiple phenotypes (i.e., osteogenic, chondrogenic, adipogenic, and neural lineages)[36, 37].

1.3.1 Stem Cells in the Gingiva

Stem cells are verifiably present in various dental tissues. However, extracting a primary tooth to collect the dental follicle and devitalising or extracting an adult tooth to collect the pulp or periodontal tissue are examples of dental tissue collection procedures that will compromise the function and vitality of dental structures[38]. Thus, gingival tissue represents an ideal source of tissue biopsies and gingival fibroblasts (GFs) due to its accessibility and significantly reduced donor site morbidity compared to other dental

tissues[38-40]. The literature offers overwhelming evidence to support the hypothesis that a group of cells within the GF population of cells from the gingival tissue possess mesenchymal stem cells (MSC) properties – and are thus called gingival mesenchymal stem cells (GMSCs) (**Figure 5**)[41].

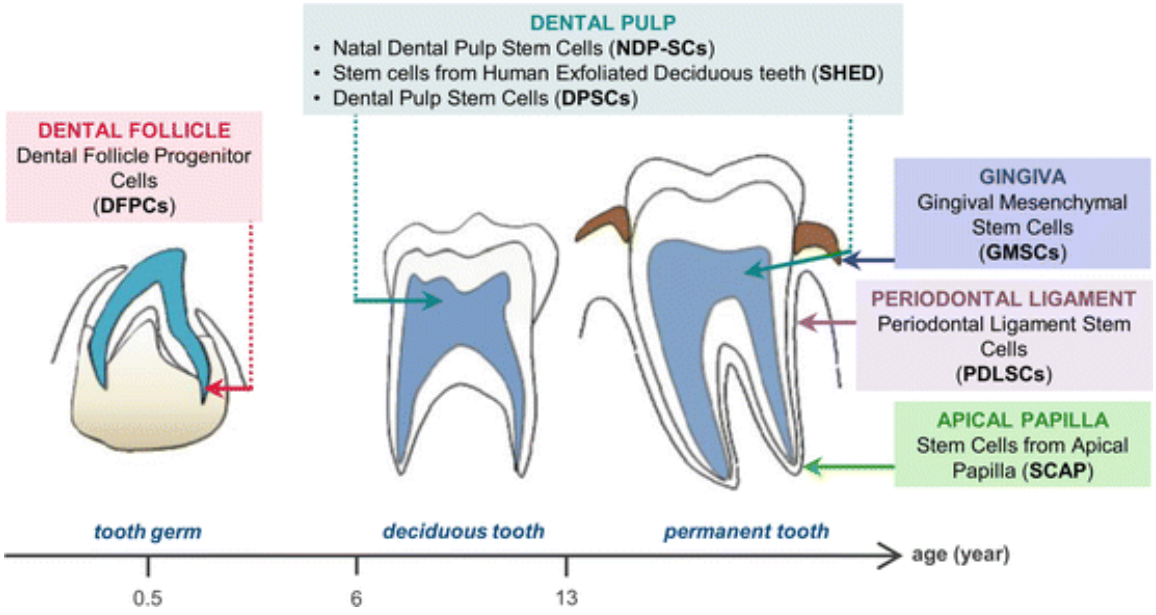

Figure 5. Dental-related stem cells and their sources.

Whether sorted (enriched) or unsorted, several studies have demonstrated that these GMSCs are able to differentiate into more than one lineage *in vitro* including osteogenic, chondrogenic, and adipogenic[2, 36, 42-44]. The majority of GMSCs are derived from cranial neural crest cells, however, mesoderm-derived GMSCs also exist within the GMSC population. When their differentiation potential was compared, the neural crest-derived GMSCs displayed a higher differentiation potential to neurogenic and chondrogenic lineages as well as superior immuno-modulatory effects than those derived from a mesodermal source[36, 45].

1.4 Role of FGF Signaling in MSCs

Given the ability of GMSCs for osteogenic differentiation, and the fact that FGFs are expressed during all stages of tooth development in the dental epithelium and mesenchyme (**Figure 6**)[46], it is an important consideration for the optimization of experimental conditions in order to fully elucidate the therapeutic effects of GMSCs.

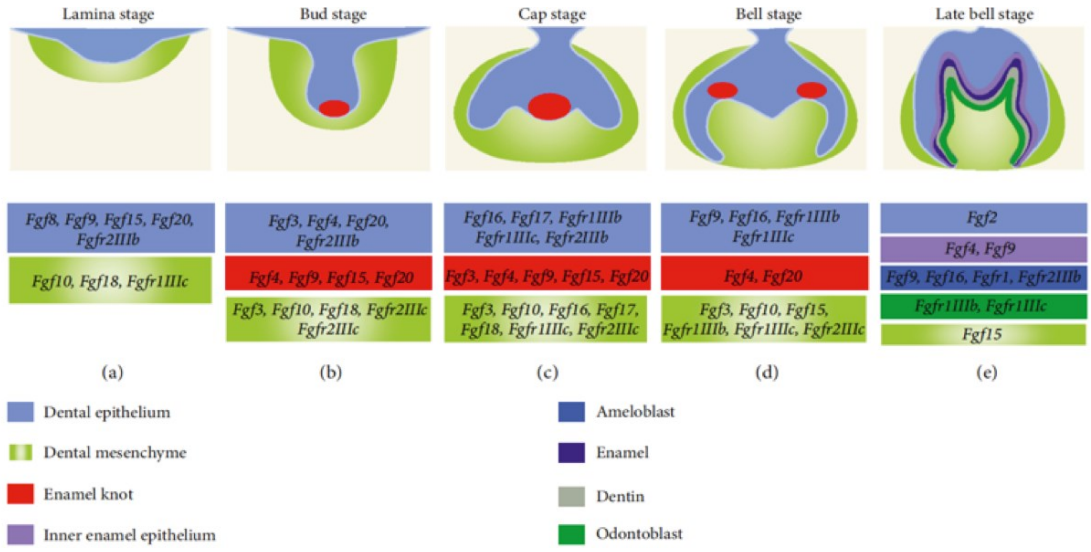

Figure 6. Expression of FGF family members in the developing tooth.

FGFs were discovered in 1974 in the form of a protein that strongly induces proliferation in fibroblasts isolated from cow pituitary glands[47]. Initially, two proteins, basic and acidic, were identified as FGF isoforms. Since then, seven total subfamilies have been classified with 22 identified genes. Most FGFs bind to a tyrosine kinase-type receptor that is activated as a result of receptor pairing and autophosphorylation[48]. Activation of these receptors will elicit a series of signaling pathways that play an important role in tissue repair and regeneration[49]. FGFs have been shown to induce angiogenic activity and proliferation in undifferentiated mesenchymal cells and lead to new blood vessel

formation, induce wound repair, play a role in skeletal muscle development, initiate hematopoiesis, and heal bone fractures[50].

FGF-2 is expressed specifically within the differentiating osteoblasts of the developing alveolar bone surrounding the developing tooth, where it stimulates chondrocytes, osteoblast proliferation, and increased production of collagen type I[111]. In the context of dental tissue regeneration, *in vivo* studies have examined the effect of FGF-2 on periodontal regeneration, and a large multi-center, randomized clinical study was performed in humans[51, 52]. In addition, in studies on dogs and primates to assess root resorption following a fault injury, FGF-2 promoted significant regeneration of the periodontium through an increase in dentin, cementum, alveolar bone deposition, and periodontal attachment level[53-56]. More specifically, FGF-2 significantly improved tooth root resorption in a model of tooth auto-transplantation compared to controls [56].

FGF-2 expression during the healing process implies several potential benefits of including FGF-2 treatment with GMSCs therapies to augment regenerative effects. Van Gastel *et al*. has demonstrated that exogenous treatment with FGF-2 during expansion of bone-forming progenitor cells is necessary and required for the cells to maintain their ability to form bone. This data provides evidence that FGF-2 can prime cells to enhance their regenerative potential, as well as limits both the need for differentiation of cells *in vitro* and the use of exogenous growth factors *in vivo*[57].

The use of FGF-2 *in vitro* during culture and differentiation of different dental MSCs has not been widely implemented and requires further study[58]. Whether its use during culture is necessary to increase cellular proliferation or to enhance differentiation also requires further investigation and is of interest for the study of GMSCs in this thesis.

1.5 Methods to Identify Stem cells

The heterogeneity of stem cell populations requires analysis of the ability to self-renew and the differentiation potential of the cells. Selective growth methods have been used to isolate stem cells, including enrichment of the stem cell populations as opposed to pure isolation processes, which can be limiting due to available tissue. Physical separation or *in situ* analysis of individual cells define the host of single-cell, or clonal, analyses available. Methods include lineage tracking by recombination, introduction of a unique clonal marker in the cell, single-cell transplantation, and *in vitro* or *in vivo* imaging of single cells (**Figure 7**)[59]. Each method has advantages and disadvantages including the unknown effects of the methods on cell behavior when they are not in their natural environment.

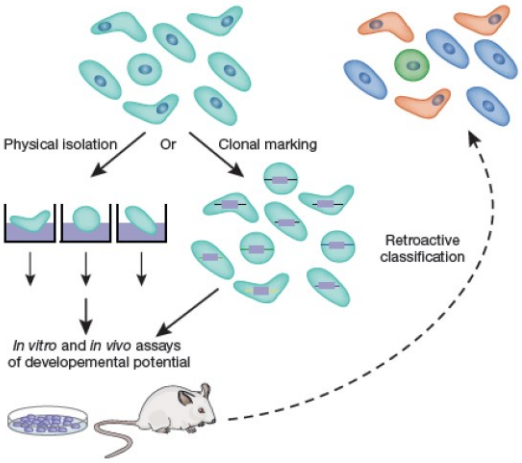

Figure 7. Clonal assays to identify stem cells. Determining the identity of heterogenous cell populations can be performed using physical isolation or genetic labeling in order to track the cells, and then assaying for various properties using ***in vitro*** or ***in vivo*** assays.

Separation and identification of cells can also be performed using centrifugation or sorting methods. Since centrifugation or density-based separation does not provide concrete evidence of the cell type, two of the most widely used methods apply affinity-

based techniques: fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS)[60]. FACS has been shown to provide a more pure population (>95%), while MACS has a higher throughput with a substantial purity (~75%). Both methods employ antibodies that are specific to the stem cell markers used to identify these unique populations.

1.5.1 Mapping the Stem Cell Hierarchy Using Surface Markers

As mentioned above, stem cells can be identified and characterized by the expression of various cell surface proteins. These surface proteins, which can also act as receptors or ligands, are formed during cell development and maturation[14]. For the purpose of understanding the physiology and phenotype of stem cells, there are numerous clusters of differentiation (CD) or clusters of designation that have been defined [61]. There is no single CD marker that uniquely distinguishes MSCs from among other fibroblast populations[3,61,62]. The majority of the identified stem cell markers are not universal, and ongoing research seeks to identify the marker or set of markers that can be used to identify MSCs in different tissues[62].

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT, 2006) defined the MSC population of cells as having at least 95% of the MSC surface markers CD73, CD90, and CD105, and less than 2% of the hematopoietic antigens CD34, CD45, CD19, CD14, and HLA-DR[15]. This percentage of surface markers meets one of the three criteria needed to identify these cells based on the *in vitro* properties of the cultured cells[63]. The other two criteria include the ability to adhere to plastic and the capacity for tri-lineage differentiation into osteogenic, chondrogenic, and

adipogenic lineages[63]. While some markers positively identify an MSC population, the hematopoietic stem cell markers CD45 and CD34 are rarely expressed in human MSCs[64].

When compared to bone marrow-derived mesenchymal stem cells (BMSCs), GMSCs maintain their stem cell surface markers (CD44, CD90, CD105, CD73, and CD29) **(Figure 8)**[65] after passage 13; and at passage 18, they still exhibit 85% of their marker expression[66]. Additionally, GMSCs possess higher proliferation rates and an increased cell yield within a shorter period of time than BMSCs[66]. Furthermore, GMSCs are able to maintain a telomerase activity as well as stable morphology and phenotype with a normal karyotype in extended-time cultures and at higher passages[66]. When compared to dermal stem cells and other types of dental stem cells from periodontal tissues, GMSCs reflected previously proven results indicating higher proliferative and colony-forming ability but maintained medial values in terms of osteogenic differentiation potential when compared to dermal and PDLSCs[67].

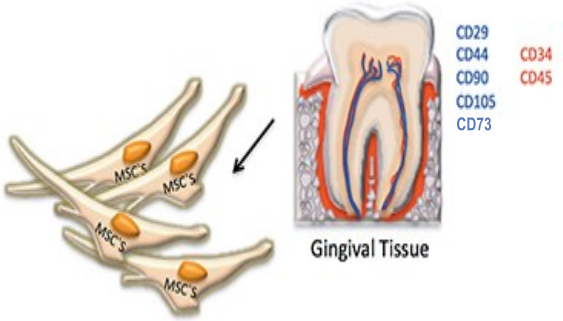

Figure 8. Phenotype of GMSC. Positively expressed markers are shown in blue, negative markers are shown in red

Other proposed MSC markers include the positive expression of the cell surface receptor endoglin, CD105, which in addition to its role in enhancing angiogenesis and neovascularization, has been linked to MSC osteogenic differentiation[17]. CD90 is also known as T lymphocyte differentiation antigen (Thy-1), and is a membrane glycoprotein

expressed in more than one type of cell, osteoblast-like cells, and as a late osteoblast precursor[68]. STRO-1 is another surface marker protein believed to indicate an enriched MSC population of cells[62, 69]. However, STRO-1 is not universally expressed in all reported MSC types, and its utility as the sole marker for MSC has yet to be verified[62]. Several studies have sorted MSCs based on a pre-selected set of applicable surface markers including CD56, CD271, STRO-1, and CD146[70-74]. However, CD271 is not universally expressed in all MSC types and is thus eliminated from consideration as the sole MSC marker[62]. Notably, CD146 has been reportedly found in almost every type of MSC and has since emerged as the marker of choice for identifying MSCs[62].

1.5.2 CD146

CD146 has been identified as a melanoma cell adhesion molecule that is primarily expressed at the intercellular junction and cell-matrix adhesion sites[75]. It has also been identified as a ligand, and, more recently, as a surface receptor that plays a role in transducing signals across the cell membrane, thus affecting cellular motility and invasion[76].

CD146 is believed to play an integral role in multiple functions related to cell proliferation, development, signal transduction, and angiogenesis[75-77]. It has also been linked to cancer metastases, immune response, and cell migration[75-77]. Additionally, CD146 has been identified as a pericyte cell marker, MSC marker, an endothelial progenitor cell marker, and an osteoblastic marker and has been reportedly expressed during embryonic tissue development[75-77]. While some groups report that CD146 expression has no effect on the differentiation potential of MSCs[78, 79], others have shown that CD146 exerts a variety of effects on stem cells, including an increase of differentiation

potential towards more than one lineage or a decrease in the overall differentiation potential of the cell[77, 80].

These CD146^{high} and CD146^{low} populations have been described for MSC cultures that have consistent expression of CD90, CD105, and CD73, combined with the lack of the hematopoietic cell markers CD45 and CD34 (**Figure 7**)[77, 78, 80, 81]. However, variability in the expression of the hematopoietic markers CD34 and CD45 in enriched CD146 cultures has been reported as well[70]. Moreover, CD146 expression is variable and has been linked to the location of the tissue: High CD146 expression was reported to be largely present in sub-endothelial sinusoidal cells, whereas low CD146 expression was reported in bone lining cells[78]. Therefore, the ability of CD146 to be a determinant marker distinguishing GFs from GMSCs remains to be elucidated.

CD146 has also been recently identified as a marker for pericytes which are perivascular cells located within the basement layer of capillaries and around the vessel wall[82]. Generally, pericytes are involved in blood flow regulation, vasoconstriction, and angiogenesis. More recently, pericytes have been described to contain multipotent populations of cells with stem cell characteristics[83]. Notably, pericytes express the MSC markers CD90, CD73, CD44, and CD105 which are widely employed as MSC markers. Expression of these MSC markers indicates that their proposed multipotency and stem cell characteristics do not develop after *in vitro* culture, rather, it suggests that perivascular sites may be another niche for MSCs and that MSCs are derived from perivascular cells[83, 84].

1.6 Potential Clinical Applications

GMSCs are known to enhance angiogenesis, polarize macrophages toward the regenerative M2-type, decrease local and systemic inflammation, and increase anti-

inflammatory markers[3,53]. Therefore, the use of MSCs for therapeutic purposes is extremely attractive in the development of novel and safe treatments for a variety of diseases and pathological conditions[85-88].

1.6.1 Therapeutic and Tissue Regeneration

GMSCs have been used to repair skin wounds and to treat patients with rheumatoid arthritis and other immune diseases[85]. In addition, they reduced the severity of colonic inflammation both clinically and histopathologically, contributed to the regeneration of tendons and bone, induced tumor cell necrosis and apoptosis, and healed mucositis in the murine tongue by regenerating the damaged epithelial layer[39,86,87].

Dentally, the use of GMSCs has been shown to promote the regeneration of various dental tissues, including the ability to repair periodontal defects in a porcine model[88]. In this study, the cells were loaded into two types of scaffolds before implantation: an inorganic porous matrix of deproteinised bovine cancellous bone scaffold and an organic collagen scaffold[88]. Both scaffolds demonstrated similar regeneration of the periodontium, and both were favored over the unloaded scaffold controls. In a separate study, GMSCs were loaded into an IL-1 short-term releasing hyaluronic acid hydrogel synthetic extracellular matrix[89]. The results showed that periodontitis could typically damage tissues within a relatively short time, however groups treated with GMSCs have displayed increased regeneration of bone, cementum/cementum like material and periodontal tissue[89].

Clinically, the use of GMSCs from gingival biopsies has been shown to increase the width of the gingiva when used over a non-woven matrix of benzyl ester derived from hyaluronic acid[90]. Furthermore, maxillary gingival recessions treated with autologous

fibroblast cell cultures isolated from palatal gingival tissues in a collagen matrix placed under a coronally advanced flap demonstrated beneficial effects[91]. Researchers are currently testing various scaffold materials for xenografts with type I and type III collagen and allografts from the fascia of the thigh with human GMSCs that might aid in periodontal regeneration for use in future clinical treatments[92-94].

1.6.2 Immunomodulation

Generally speaking, MSCs are non-immunogenic and possess immunomodulatory capabilities[95,96]. Immunomodulatory actions do not induce a systemic immune response in the body[12]. Instead, they control the immune system by inducing, amplifying, or attenuating the existing immune pathways[39,97,98]. The inhibition or alteration of the function of either innate or adaptive immune cells, such as inhibiting the pro-inflammatory action and recruitment of T-cells, is an example of immunomodulatory actions[39,98]. The abundance of recent evidence suggesting that GMSCs possess immunomodulatory properties is of great therapeutic interest[3,6,13,95,99,100]. It has been shown that GMSCs modulate the recruitment of macrophages, mast cells, and neutrophils to injury sites, resulting in less infiltration compared to skin injuries[53]. Concurrent with this recruitment, GMSCs repolarize inflammatory M1-macrophages to anti-inflammatory M2-reparative cells[3].

GFs isolated and cultured from healthy and inflamed gingival tissues exhibit comparable colony forming unit fibroblasts (CFU-F), universally possess tri-lineage differentiation, and express MSC-associated markers. One study reported that Population from both healthy and inflamed gingival tissue display a similar capacity to undergo osteogenic, adipogenic, and chondrogenic differentiation, both *in vitro* and *in vivo*[101]. In a

comparison between hyperplastic gingival tissue, healthy gingival tissue cells, and bone marrow stem cells (BMSCs), all shared an immunomodulatory feature with their bone marrow counterpart when placed in a proper immune-activated environment[43].

1.7 Limitations

Fibroblasts and MSCs cultures possess a very similar spindle configuration of cells[20]. Fibroblasts are the predominant cells in connective tissues, and they maintain the structural framework of tissues by secreting both the extracellular matrix[7]. It was previously thought that fibroblasts possessed a uniform cell type regardless of specific tissue function. However, this assumption has been challenged, and studies have proven phenotypic heterogeneity in fibroblastic cultures derived from different tissue sources and within clonal MSC populations[4,7,20]. Fibroblast heterogeneity was demonstrated in cell surface antigen expression, collagen production, morphology, proliferation rate, differentiated reaction to inflammatory cytokines, and wound healing [4,7].

The epithelial-mesenchymal transition process suggests that fibroblasts originate from organ epithelia where the epithelial cells break away from the surrounding cells to develop into mesenchymal fibroblasts[102]. Other studies reported that hematopoietic stem cells differentiate and give rise to fibroblasts [102]. while yet others describe pericytes and bone marrow mesenchymal stem cells as sources of fibroblast populations[103]. Determining the origin of fibroblasts will help to isolate the fibroblasts that develop MSC properties that are necessary for obtaining a purified or enriched MSC culture that may be used for future cell-based therapies[20].

GMSCs hold properties such as self-renewal, multipotent differentiation, expression of MSC associated cell markers, and immuno-modulatory and anti-

inflammatory properties[95,104]. The difficulty in identifying and isolating these GMSCs from GFs lies in distinguishing between them. This confusion is due to their incredibly similar morphology, stem cell marker expression, and the ability of both GFs and GMSCs to adhere to plastic treated plates when cultured *in vitro* [3,95,105].

With specific regard to GMSCs, seeding them after isolation by any method allows for the identification of MSCs within gingival fibroblast-derived cultures. The fibroblasts will then begin to multiply and form discrete colonies (CFU-F) which may grow from a single cell or an aggregation of multiple cells[17]. Upon expansion, these colonies demonstrate self-renewal properties, and are considered to be rich in MSCs[3]. It is vitally important to reach a consensus regarding the ideal method of isolating these cells and to develop a consistent protocol for their *in vitro* purification and enrichment to ensure their use in future clinical settings where a specific lineage is required.

Taken together, the combined attributes of GFs, including their accessibility, self-repair, immunomodulatory effects, and potential for tissue regeneration, may lead to their use in the development of future tissue engineering and cellular therapeutic modalities. The vast body of evidence accumulated through *in vitro* and *in vivo* studies supports the use of GFs and GMSCs for the purpose of tissue regeneration[106]. However, optimization of the most feasible isolation, expansion, identification, differentiation protocols, and improving the clinical handling of these cells remain crucial for the success of randomized clinical trials in proving the regenerative power of GMSCs clinically[96].

1.8 Current Gaps in Knowledge

Given the complexity of the various factors that are involved in developing cellular therapeutic models, in order to ensure consistent results, it is necessary to: 1) develop a

consistent protocol for cellular isolation; 2) identify a standard set of stem cell marker(s); and 3) identify the growth factors that could potentially play a role in MSC function[107]. Although these elements are extensively discussed in the literature, they have yet to be adequately investigated and understood.

The identification of surface markers and their definitive utility in isolating pure MSC populations are of great value. There is a significant demand for further experimentation with additional surface markers and different MSC populations to identify the most reliable MSC marker for use in an *in vitro* setting that can also be recommended for future *in vivo* studies.

1.9 Study Aim and Hypothesis

This study was designed to investigate the role of CD146 in distinguishing GMSCs from the isolated GFs population based on two different isolation methods. We *hypothesize* that CD146 is the surface marker that distinguishes GMSCs from within a GF population regardless of the isolation method employed.

Chapter 2 Materials and Methods

2.1 Isolation and expansion of human gingival fibroblasts

The gingival tissue collection procedure began after receiving approval from the University of Alberta Health Research Ethics Board (Pro00001454). Gingival tissues were collected from six adolescent patients undergoing extractions for orthodontic purposes from healthy interdental papilla at the University of Alberta Dental Clinic (detailed characteristics are reported in **Table 1**). All patients read and signed a consent form prior to the collection of tissues.

Patient	Gender	Age (years-months)	Tissue Weight (g)
JO 104	**Male**	**17-08**	**0.21**
KE 62	**Male**	**14-0**	**0.18**
RO 76	**Male**	**13-06**	**0.21**
SI 121	**Female**	**16-04**	**0.19**
LU 36	**Female**	**18-0**	**0.18**
SA 178	**Female**	**18-0**	**0.20**

Table 1. Participant Demographics (n=6)

Tissues were weighed and immediately stored in a sterile saline solution for one to four hours before processing. Cells were extracted from the gingival tissues of each patient using the two most common established methods of cellular extraction reported in the

literature [3]. Gingival tissues were washed thoroughly -10 times consecutively- using a phosphate buffer saline (PBS) solution to dilute the oral bacterial flora of the gingival tissue. Following the PBS wash, the tissues were weighed and cut into small pieces of between 1 and 2 mm^2 using a No.10 surgical blade (Sigma-Aldrich®, Missouri, US)

Each sample was divided into two equal portions, and one portion was used for each of the two isolation methods.

Method 1: Enzymatic digestion. This technique entails incubating the tissue in a collagenase I (2mg/mL; Worthington Biochemical, Lakewood, NJ, USA) solution for one hour at 37°C in a 5% CO_2 incubator. The tissues were then filtered through a 100µm-nylon mesh filter (Falcon, BD Bioscience, NJ, USA) and centrifuged for 10 minutes at 1500 rpm. The cells were then re-suspended in a fresh medium and plated at a density of 10^5 cell/cm^2 in 25 cm^2 tissue culture flasks (Falcon® Tissue Culture Flasks, Sterile, Corning®, Corning, New York). After 48 hours, the medium was replaced. The medium used for culture and expansion consisted of standard alpha minimum essential medium Eagle (αMEM) supplemented with 10% v/v FBS, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U/mL penicillin, 100 µg/mL streptomycin (all from Sigma-Aldrich®, Missouri, US) with the addition of 5 ng/mL of FGF-2 (Neuromics, MN, US, Catalog#: PR80001). The medium was changed every 2 to 3 days. Once the flask was confluent (after approximately one week of culture), the cells were passaged. At passage 1 (P1), the cells were trypsinized (0.05% w/v Trypsin/EDTA, Invitrogen), counted, and magnetically sorted (**Figure 9**). At the conclusion of the magnetic sorting procedure, four groups of cells were obtained and then expanded to passage 2 (P2) and passage 3 (P3). The number of cells at P3 was sufficient to set up the experiment.

Method 2: Tissue explants. The gingival tissue was cut into small pieces and plated over 25 cm^2 tissue culture flasks and incubated for 48 hours, undisturbed, at 37°C in a humidified incubator with 5% CO_2. After 48 hours, the medium was exchanged for a fresh medium. As with the enzymatic group, the cells at P1 were magnetically sorted and expanded to P2 and P3, where the numbers of cells were sufficient to set up the experiment. A schematic diagram of the full experimental procedure is illustrated in **Figure 9**.

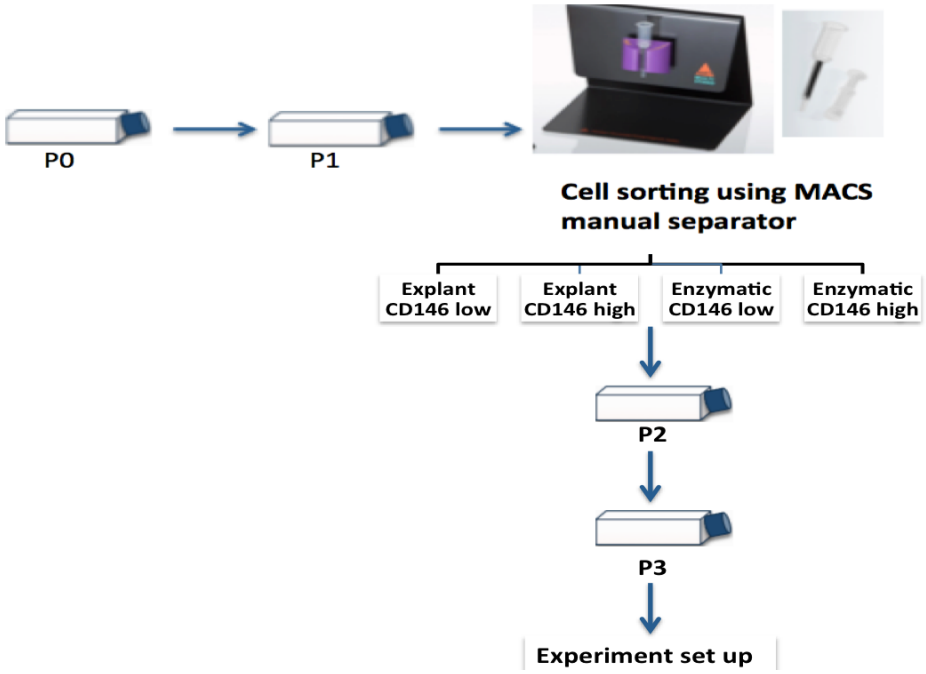

Figure 9. Graphic representation of cell expansion. Four groups are obtained at the end of the magnetic sorting procedure: Explant $CD146^{low}$, Explant $CD146^{high}$, Enzymatic $CD146^{low}$, and Enzymatic $CD146^{high}$. Each of the four groups was then expanded to passage 2 and passage 3 where the number of cells was sufficient to set up the experiment.

At P1, the cells of both groups (explant and enzymatic digestion) were trypsinized and counted. A CD146 microbead kit (Mitenyi Biotec Cat. no: 130-093-596) was used to magnetically sort the GFs based on their CD146 surface marker expression. Following the manufacturer's instructions, 10^7 cells were incubated in a fragment crystallisable receptor (FcR) blocking reagent, followed by CD146 microbeads for 15 minutes in a refrigerator.

Incubation with FcR blocking reagent increases the specificity of microbeads to the cells, thus increasing the purity of the cells after magnetic separation. Following incubation, the cells were washed, centrifuged, and re-suspended in a prepared buffer solution with pH=7.2 containing PBS 0.5% w/v bovine serum albumin and 2mM Ethylenediaminetetraacetic acid (EDTA) pursuant to the manufacturer's protocol.

The suspension of cells with the magnetic beads was passed through a magnetically activated cell sorting (MACS) LS column (Mitenyi Biotec LS Columns #130-042-401) that was attached to a highly magnetic board. Each step of the process was performed under a Class II Type A2 biological safety cabinet. The first elution contained cells that we characterized as: *$CD146^{low}$ expressors*. The cells with no CD146 antigen remained unresponsive to the CD146 microbeads, and, thus, did not adhere to the MACS LS tube wall. The MACS LS tube was then removed from the magnetic board, the buffer solution was passed through the MACS LS magnetic tube, and a plunger was used to drive the solution with the attached cells out from within (**Figure 9**). The suspension of cells obtained at this stage was expected to be rich with CD146 surface marker and were therefore characterized as: *$CD146^{high}$ expressors*. Flow cytometric analysis was conducted to determine the percentage of CD146 expression immediately following separation in order to confirm the expression of CD146 in both groups.

At the conclusion of the magnetic separation phase, four groups of cells were plated at the same concentration used throughout the entire experiment: 10^5 cells/cm^2 in T-75 tissue culture flasks. The experimental groups were described as follows: *enzymatic $CD146^{high}$*, *enzymatic $CD146^{low}$*, *explant $CD146^{high}$*, *explant $CD146^{low}$*. The growth medium was exchanged every 2 to 3 days. Once the cells were confluent, they were

trypsinized (0.05% w/v Trypsin/EDTA, Invitrogen), counted, and divided into different groups to set up the experiment.

2.2 Phenotypic analysis by flow cytometry

To characterize the population of cells used to set up the experiment, 25 x 10^4 cells were washed with a prepared ice-cold FACS buffer (PBS, 0.5% v/v FBS and 0.1% w/v sodium azide) then incubated with fluorochrome primary monoclonal antibodies conjugated to fluorescein isothiocyanate (mAb-FITC) or to phycoerythrin (mAb-PE) for 45 minutes. The antibodies with conjugated fluorescence, in this case, were CD146-PE, CD90-FITC, CD105-FITC, CD73-FITC, CD34-FITC, and CD45-FITC. The cells were then washed and fixed with 2% v/v paraformaldehyde for 15 minutes, washed again, and suspended in 1 ml FACS buffer. Isotype-matched controls were incubated with FITC and PE-conjugated mIgG1. 10^4 cells were acquired using a Fortessa SORP flow cytometer. The results of the flow were analysed using the FlowJo software application (FlowJo, LLC., Oregon, US).

2.3 Osteogenic and chondrogenic differentiation

Osteogenic and chondrogenic media were prepared and used to differentiate the gingival fibroblasts into osteogenic and chondrogenic lineages. For osteogenic induction, $10^5/mm^2$ cells were cultured in a monolayer in every well of 6-well plates. Three of the wells were used for staining, while the other three were used for gene analysis. Osteogenic medium used was composed of Dulbecco′s Modified Eagle′s Medium (DMEM), 100 U/mL penicillin, 100 µg/mL streptomycin, 10% v/v FBS, 0.1 mM ascorbic acid, 10mM ß-glycerophosphate, 10nM Dexamethasone (all medium component products are produced by Sigma-Aldrich®, Missouri, US). Ascorbic acid stimulated the synthesis of collagen type

I, ß-glycerophosphate was added to promote calcium phosphate deposition, and dexamethasone stimulated osteogenesis by increasing alkaline phosphatase activity. The medium was changed every 3 to 4 days for a period of 21 days. Following this period, alizarin red staining was used to determine whether the cells differentiated into osteogenic lineage and deposited any calcium. Alizarin red stain is a dye used to detect calcium deposits by binding to calcium through a chelation process to form red alizarin-calcium complexes. Cells from each of the wells were collected for further gene expression analysis by quantitative real-time polymerase chain reaction (qPCR).

For chondrogenic induction, 25 x 10^4 cells were counted and centrifuged in 1.5ml tubes, the pelleted cells were incubated at 37°C with 5% CO_2 in a chondrogenic differentiation medium composed of DMEM, 365µg/ml ascorbic acid 2-phosphate, 10 nM dexamethasone, 125µg/ml human serum albumin (all from Sigma-Aldrich®, Missouri, US), 10ng/ml TGFß3 solution (ProSpec, New Jersey, USA, Catalogue #: cyt-11), 40µg/ml L-proline, and ITS+ Universal Culture Supplement Premix (Corning Discovery Labware II, California, US, catalogue #CACB354352). The cells within the pellet aggregated to form a spherical shape that did not adhere to the walls of the tube. The medium was changed every 3 to 4 days over a period of 21 days. Following this period, the cellular aggregates were collected for assays to confirm chondrogenesis qualitatively through safranin O and alcian blue histologic staining. In addition, the GAG content of every cellular aggregate was spectrophotometrically measured. The same spherical aggregates were used to examine the collagen type formed in the ECM, whether type I or type II by performing an immunofluorescence assay.

2.3.1 Osteogenic assays

2.3.1.1 Alizarin red staining

The cells from the four experimental groups were cultured in a monolayer as described in the osteogenic differentiation assay. After a period of 21 days, the wells were washed with distilled water twice and fixed with 10% w/v formalin neutral buffer (Anachemia Canada Inc., Quebec, CAN) for 15 minutes at room temperature. Alizarin red staining was used to stain the cultured wells for 10 minutes. The wells were washed again on a shaker for 15 minutes. Finally, microscopy images were captured and used to qualitatively assess the alizarin stained mineralized nodules using a light microscope.

2.3.1.2 Gene analysis after osteogenic differentiation

Genes that are related to osteogenic differentiation and dentinogenesis were compared across the four experimental groups and included: 1) runt-related transcription factor 2 (*RUNX2*) gene; *RUNX2* is essential for osteoblast differentiation; 2) alkaline phosphatase liver/bone/kidney type (*ALPL*) gene; ALPL is a metalloenzyme expressed during osteogenesis[8,121,122]; 3) osteocalcin (*OCN*) gene; OCN which constitutes the majority of noncollagenous bone matrix proteins, is considered a late osteogenic marker, and has recently been found to play a regulatory role for transcription factors during mineralization[108]; 4) collagen type IA1 (*COLIA1*) gene; collagen type I is the most abundant organic component of bone ECM, is believed to play an important role in enhancing osteogenesis through MSC integrin –collagen type I binding[109]; and 5) dentin sialophosphoprotein (DSPP) gene, DSPP is abundant in odontoblast cells and plays an important role in mineralization, DSPP gene was evaluated as the marker gene for odontogenic differentiation[110]. The primers sequences are detailed in **Table 2**.

Gene	Forward	Reverse
Osteocalcin (*OCN*)	AATCCGGACTGTGACGAGTTG	CCTAGACCGGGCCGTAGAAG
Alkaline Phosphatase (A*LPL*)	CCTGGCAGGGCTCACACT	AAACAGGAGAGTCGCTTCAGAGA
Runt related transcription factor 2 (*RUNX2/CBFA1*)	GGAGTGGACGAGGCAAGAGTTT	AGCTTCTGTCTGTGCCTTCTGG
Collagen type I alpha 1 chain	GCCTCGGAGGAAACTTTGC	TCCGGTTGATTTCTCATCATAGC
Dentin sialophosphoprotein (DSPP)	TGGGCAAAGGCAATGTCAA	TGGCCAGGTCCTTCTATGTTG

Table 2. Primers sequences for genes of interest

Trizol (Life Technologies) was used to extract the RNA from the monolayer osteogenic cultures. The RNA concentrations were measured using a NanoDrop-2000C Spectrophotometer (Thermo Fisher Scientific, Delaware, US). cDNA was then synthesized following the reverse transcription reaction by using 1µg of Oligo dT (Promega, Wisconsin, US). Dilutions of 1:10 were prepared from the samples to be used in real-time PCR (qPCR). A 10µl real-time reaction mixture was prepared by adding 3 µl of cDNA, 1 µl each of forward and reverse primers and 5 µl of Takyon™ No Rox Probe MasterMix dTTP Blue (Eurogentec North America, Inc., California, US). The dilutions were then suspended in the 96-well block of the CFX real-time PCR detection system. Hypoxanthine phosphoribosyltransferase 1(HPRT1), Ribosomal Protein L13a (RPL13), and Tyrosine 3-Monooxygenase/Tryptophan 5- Monooxygenase Activation Protein Zeta (YWHAZ) were used as internal controls in each run. These three latter reference genes were used for the

accurate quantification of data. Fluorescence data was obtained and plotted against cycle number and then analyzed using CFX connect software.

To determine the relative expression of the genes of interest, we used three reference genes as normalizers. After determining C_T values, the difference between the reference and target gene C_T values is calculated. The relative expression of the target gene is then determined by using the $2^{-\Delta C_T}$ formula to compare and evaluate the osteogenic gene expression between groups.

2.3.2 Chondrogenic assays

2.3.2.1 Safranin O staining

After 21 days of chondrogenic differentiation, the pellets were fixed in 10% w/v neutral buffered formalin overnight at 4°C. To preserve the cells and increase hydrophobicity, the pellets were dehydrated before being embedded in wax blocks by immersing them in incrementally higher concentrations of ethanol. The pellets were then embedded in paraffin and cut into sections that were 5µm thick. To detect whether the sulphated proteoglycans matrix formed within the pellets, the mounted sections of the pellets were stained with 0.01% w/v safranin-O and counterstained with 0.02% w/v fast green (Sigma-Aldrich®, Missouri, US). Safranin O staining was used to histologically assess the chondrogenic differentiation of the gingival fibroblasts. Safranin O, a cationic stain basic dye, stains the acidic proteoglycans with an orange-red color. Fast green is a sulphate group containing acidic substrate, which binds strongly to the amino group on protein and stains the non-collagen sites.

2.3.2.2 Alcian blue staining

Alcian blue stain detects all polysaccharides, including any proteoglycan deposits. Here, the alcian blue stain was prepared in distilled water using alcian blue powder with pH=1. The pellet sections were then washed in UltraClear™ (Avantor Performance Materials, Inc., Pennsylvania, US), an isoparaffin-based clearing agent that can be used as a more environmentally friendly xylene replacement during tissue embedding, deparaffinization, and staining processes. The pellets sections were then washed in varying concentrations of ethanol (70-100% v/v) and held under running tap water for 5 minutes, drained, counterstained for 1 minute with a prepared neutral red stain by dissolving 1 g of neutral red in 100 ml of distilled water. Afterward, 0.1 ml of glacial acetic acid was added, mixed, and filtered. The slides were rapidly dehydrated in absolute 100% ethanol. The microscopic images were then assessed qualitatively.

2.3.2.3 Biochemical analysis for gingival fibroblast chondrogenesis

After 21 days of chondrogenic induction using the chondrogenic medium, the pellets were washed with PBS. Next, 250µl Protease K (1 mg/mL in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A; all from Sigma-Aldrich®, Missouri, US) was used overnight at 56°C to digest the pellet. The GAG content was measured spectrophotometrically after using 1,9-dimethylmethylene blue, and chondroitin sulphate (Sigma-Aldrich®, Missouri, US) was used as a standard. The DNA content was determined using the CyQuant cell proliferation assay kit (Invitrogen, Ontario, CAN) with supplied bacteriophage λ DNA as standard.

2.4 Immunofluorescent staining for collagen type I and collagen type II

5µm sections of pellet slices were deparaffinized after being dipped in UltraClear™ solution followed by ethanol and washed with distilled water. Due to the formation of

methylene bridges during fixation of the pellets, the slides were incubated 30 minutes at room temperature in an antigen retrieval enzyme, protease XXV (AP-9006-005, Thermo Scientific, Massachusetts, US), in order to unmask the antigen sites and allow the antibodies to bind. To increase the specificity of the antibodies, the slides were incubated in hyaluronidase (H6254, Sigma-Aldrich®, Missouri, US) for half an hour at 37ºC. The pellet slices were then incubated in bovine serum albumin (BSA) 5% w/v to reduce non-specific binding of the antibodies. After BSA incubation, the pellet slices were incubated in primary antibodies: rabbit anti-collagen I (CL50111AP-1, Cedarlane, Ontario, CAN), mouse anti-collagen II (II-II6B3, Developmental Studies Hybridoma Bank, Iowa, US) using a 1:200 dilution at 4°C overnight. For the purpose of fluorescent detection, the preceding step was followed by incubation with a fluorochrome-conjugated secondary antibody1:200 dilution for both, goat anti-rabbit IgG (H&L Alexa Fluor 594, Abcam, UK) for collagen type I and goat anti-mouse IgG (H&L Alexa Fluor 488, Abcam, UK) for collagen type II.

The sections were then stained with DAPI (4′, 6-diamidino-2-phenylindole, Cedarlane), to stain the cell nuclei, and mounted with a 1:1 glycerol-PBS solution. Immunofluorescent images were visualized by an Eclipse Ti-S microscope (Nikon Canada, Ontario, CAN).

2.5 Statistical analysis

The data presented in the graphs represent the average and standard deviation in each group for the six donors. Statistical analysis was performed using SPSS (Version 23; IBM Canada, Ltd., Ontario, CAN) and Excel 2016 (Microsoft, Washington, US). A normality test to assess the distribution of the data was performed using the Shapiro-Wilk

test. A repeated two-way ANOVA was used to determine whether there was any interaction between the isolation method and sorting groups followed by further assessment of the primary effect of the isolation method and CD146 expression. Statistical significance was considered when $p < 0.05$.

Chapter 3 Results

3.1 Magnetic separation using CD146 magnetic beads

At the conclusion of P1, magnetic separation was performed on the expanded gingival fibroblast cellular cultures, which were obtained either by enzymatic digestion or the tissue explant technique. The objective was to compare the two differentiation potentials of these populations: one that is enriched, with high expression of CD146 and one that is low in CD146 expression. After the cellular populations were sorted, flow cytometry was performed on a sample of the cells to confirm that the sorting process was successful. **Figure 10** confirms that CD146 was highly expressed in the enriched groups, and there was low expression of CD146 among the low expression groups.

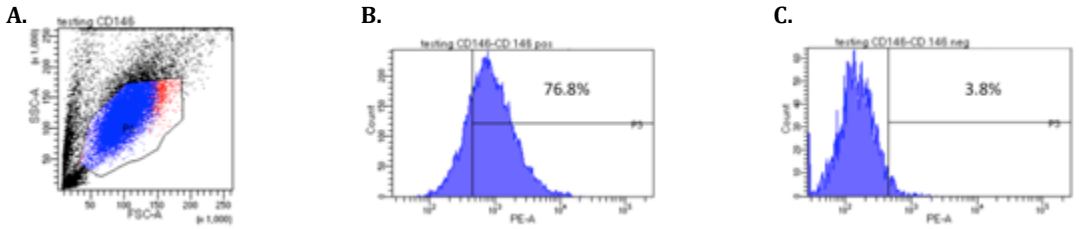

Figure 10. CD146 expression immediately following magnetic sorting. (A) Forward and side scatter of the GF population. (B) CD146 was expressed in 76.8% of the GF population of $CD146^{high}$ cells. (C) CD146 was expressed in 3.8% of the GF population of $CD146^{low}$ cells.

3.2 Phenotypic analysis by flow cytometry

Flow cytometry was performed following the culture and expansion of the four experimental groups of cells collected from each patient (N=6) at P3 and before conducting osteogenic and chondrogenic experiments. The expression of CD146 and stem

cell markers CD90, CD105, and CD73, and the negative expression of the hematopoietic surface markers CD45 and CD34 were all assessed.

All four experimental groups expressed the positive MSC markers CD90, CD105, and CD73. None of these groups expressed CD45 or CD34. No statistical significance was detected across all groups for all CD markers.

All patients exhibited low expression of CD146 in the $CD146^{low}$ groups compared to the $CD146^{high}$ groups whether the enzymatic or explant method was used. One patient exhibited a higher expression of CD146 than expected in $CD146^{low}$ group. A possible explanation for this phenomenon could be a fault in the magnetic cell sorting that allowed $CD146^{high}$ cells to be passed into the $CD146^{low}$ cultures.

However, not all patients maintained the high expression of CD146 in the $CD146^{high}$ explant or enzymatic groups as initially anticipated. **Figure 11** represents the average percentage of CD marker expression in all patients (N=6) along with the standard deviation. Flow cytometry data, for all CD markers assessed in each individual patient sample is presented in the appendix (**Figure A1a** to **A1f**).

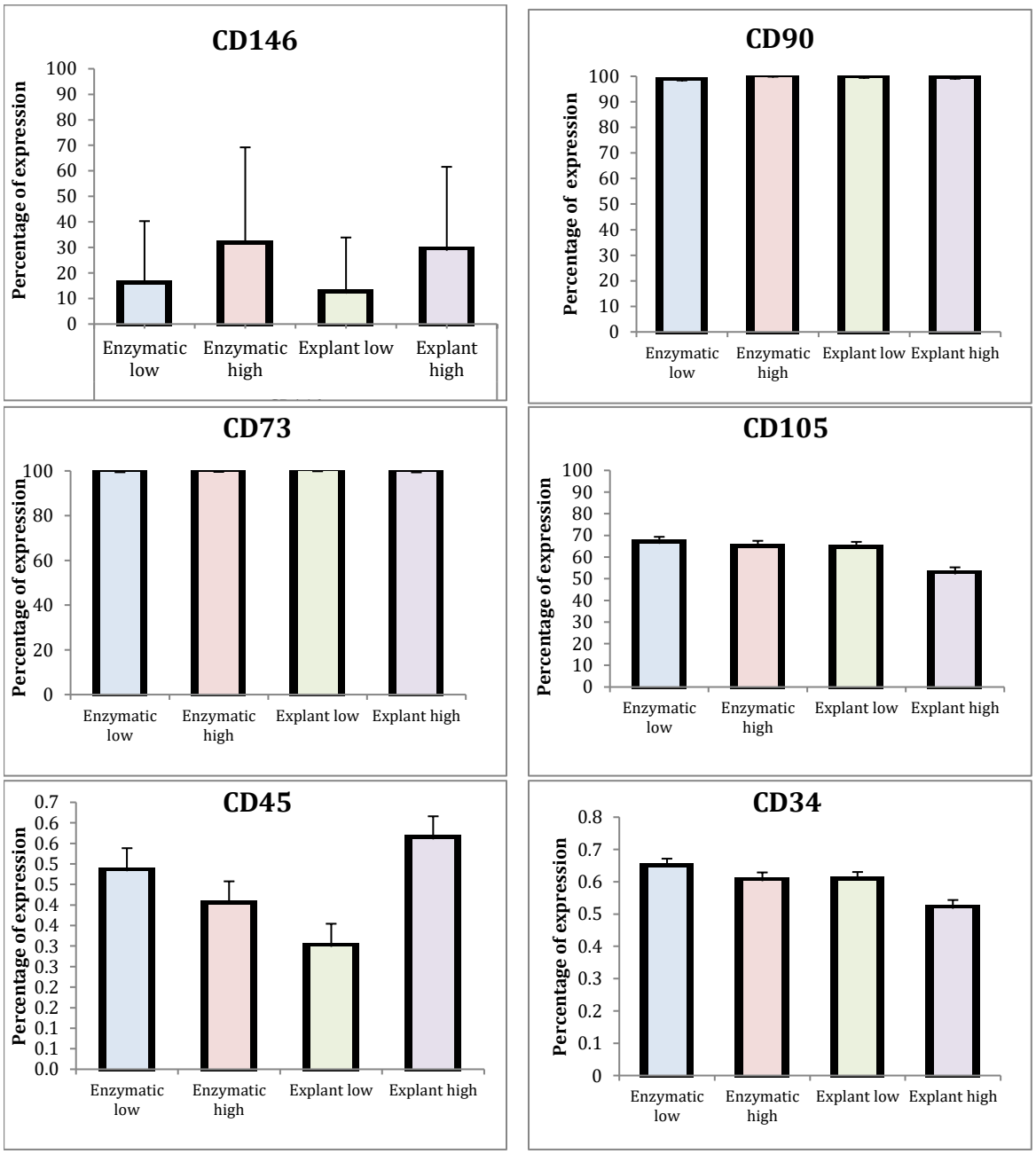

Figure 11. Flow cytometry of CD surface markers. The bars represent the average expression of surface markers: CD146, CD90, CD70, CD105, CD34, and CD45 in all four experimental groups. Values are expressed as the average ± standard deviation. No significant difference was observed ($P>0.05$).

3.3 Osteogenic assays

3.3.1 Alizarin red staining

Osteogenic differentiation was attempted using the differentiation medium described above to investigate the osteogenic differentiation potential of the gingival fibroblasts. The alizarin red stain is used to detect any calcium nodules deposited after osteogenic differentiation. All patient samples exhibited alizarin red staining in all groups of the 6-well monolayer cultures (**Figure 12**).

3.3.2 Gene analysis following osteogenic differentiation

The alizarin red stain indicates the osteogenic differentiation of GFs. The fold-change of relative osteogenic gene expression levels was compared to further investigate whether there were any differences between the capacities of the four groups of cells to osteogenically differentiate. The dentin sialophosphoprotein (*DSPP*) gene was evaluated to investigate whether the production of the dentin protein regulating gene during osteogenic differentiation is upregulated[110]. No significant differences were observed in any of the experimental groups from all patient samples in terms of the expression of the *RUNX2, OCN*, *OPN*, and *DSSP* genes (**Figure 13**).

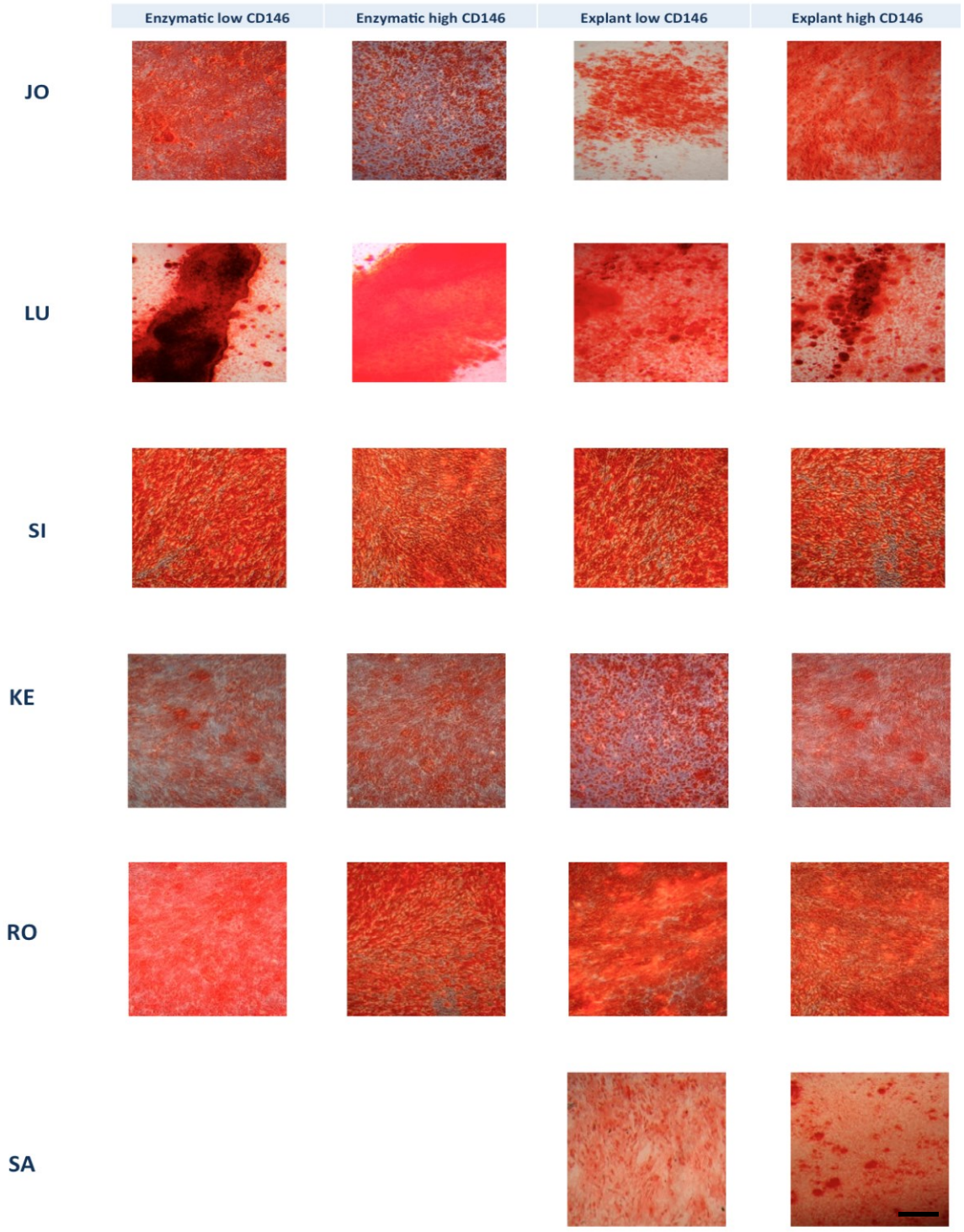

Figure 12. Osteogenic differentiation of GMSCs. All experimental groups from each patient: JO, LU, SI, KE, RO, and SA. Mineralization nodules were detected with alizarin red staining after 21 days of osteogenic differentiation. Scale bar (black) 100 µm.

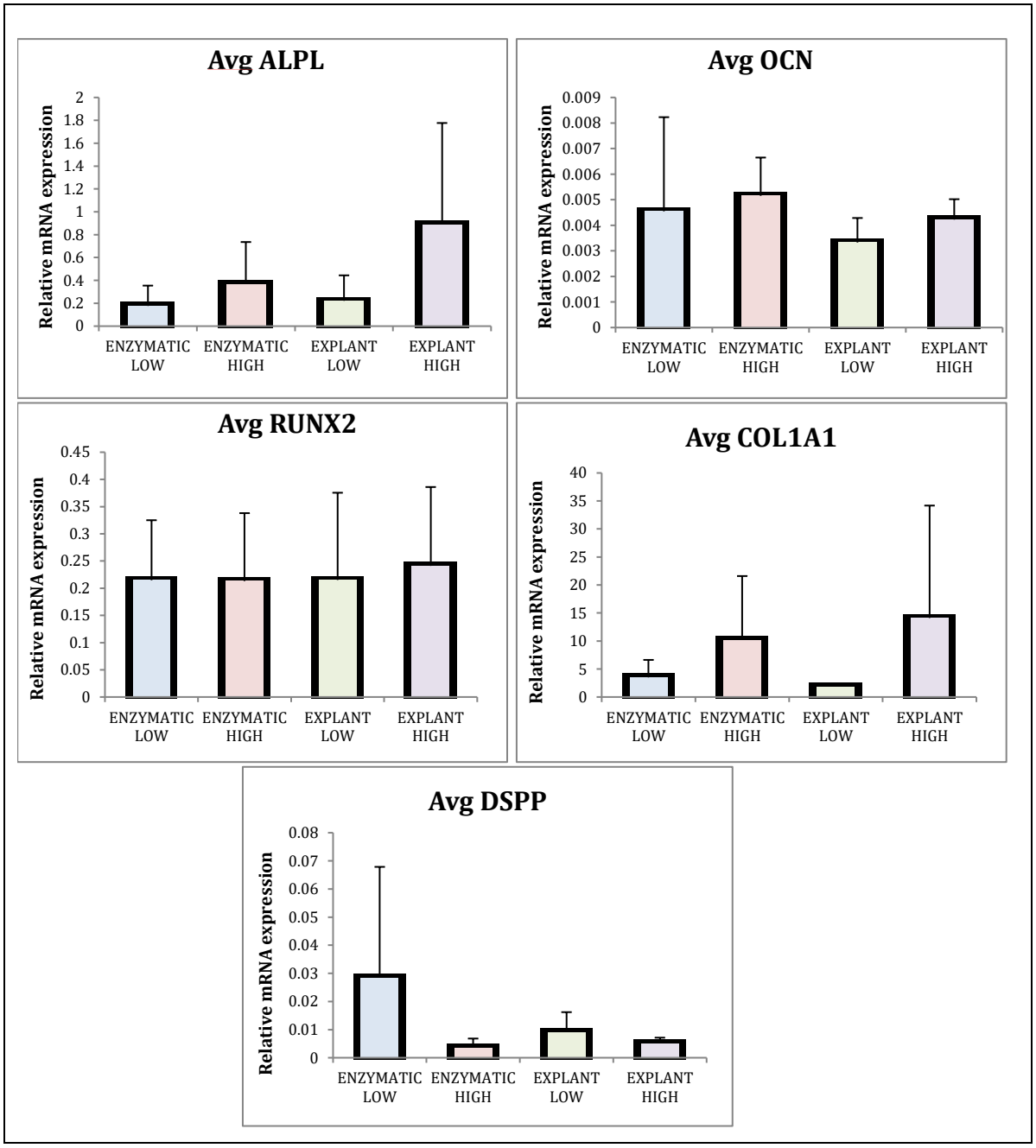

Figure 13. Relative osteogenic gene expression. Average value and standard deviation for all patients (N=6). None of the genes [alkaline phosphastase (*ALPL*), osteocalcin (OCN), runt related transcription factor 2 (*RUNX2*), type I collagen (*COL1A1*), and dentine sialophosphoprotein (*DSPP*)] displayed significant differences in the relative gene expression across the four groups (P>0.05).

3.4 Chondrogenic assays

3.4.1 Safranin O staining

Safranin O was used to stain pellet slices following chondrogenic differentiation to detect sulphated proteoglycans. The safranin O staining of the pellet slices from all patients across all four groups showed no significant difference across all experiment (**Figure 14**). Patient SA Explant group of cells was contaminated and omitted from the figure.

3.4.2 Alcian blue staining

Alcian blue was used to stain the pellet slices from all patients across all four groups to detect all proteoglycans deposited during chondrogenic differentiation. Figure 6 shows the results of the alcian blue staining of the pellets. Patient SA Explant group of cells was contaminated and omitted from the figure.

3.4.3 Biochemical analysis for gingival fibroblast chondrogenesis

After 21 days in chondrogenic media, the pellets were analyzed to detect their specific glycosaminoglycan (GAG) content. Figure 7 shows that the GAG content was very low for all groups. However, there was no significant difference between the four groups or all patients. Similarly, when the DNA content was normalized against the DNA content of the cells from every pellet, no significant difference between the groups was found.

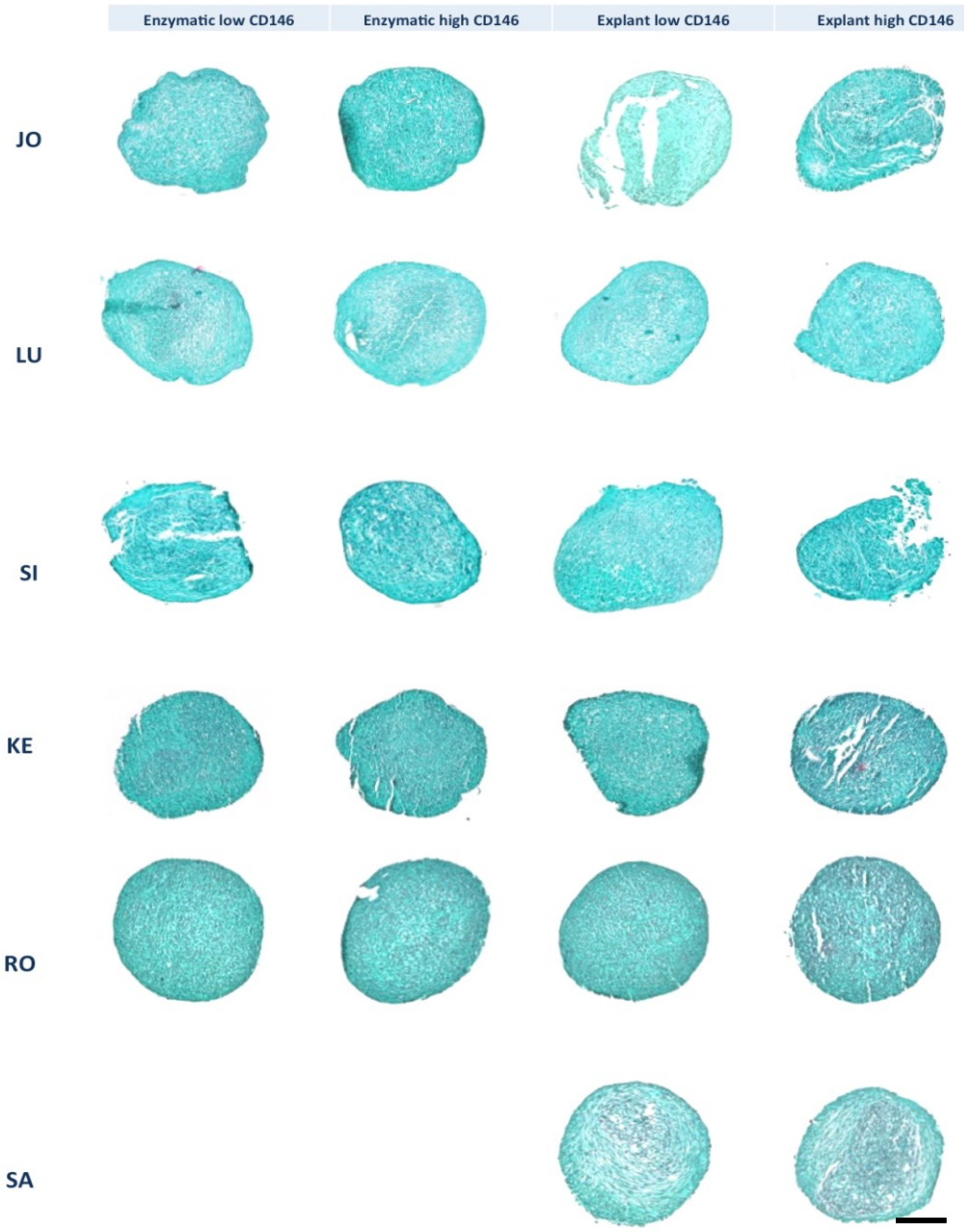

Figure 14. Chondrogenic differentiation of GMSCs. All experimental groups from each patient: JO, LU, SI, KE, RO, and SA. None of the pellet slices exhibit the pink safranin O stain that should indicate the sulphated proteoglycans in the pellets after chondrogenesis. **Scale bar (black) 100 µm.**

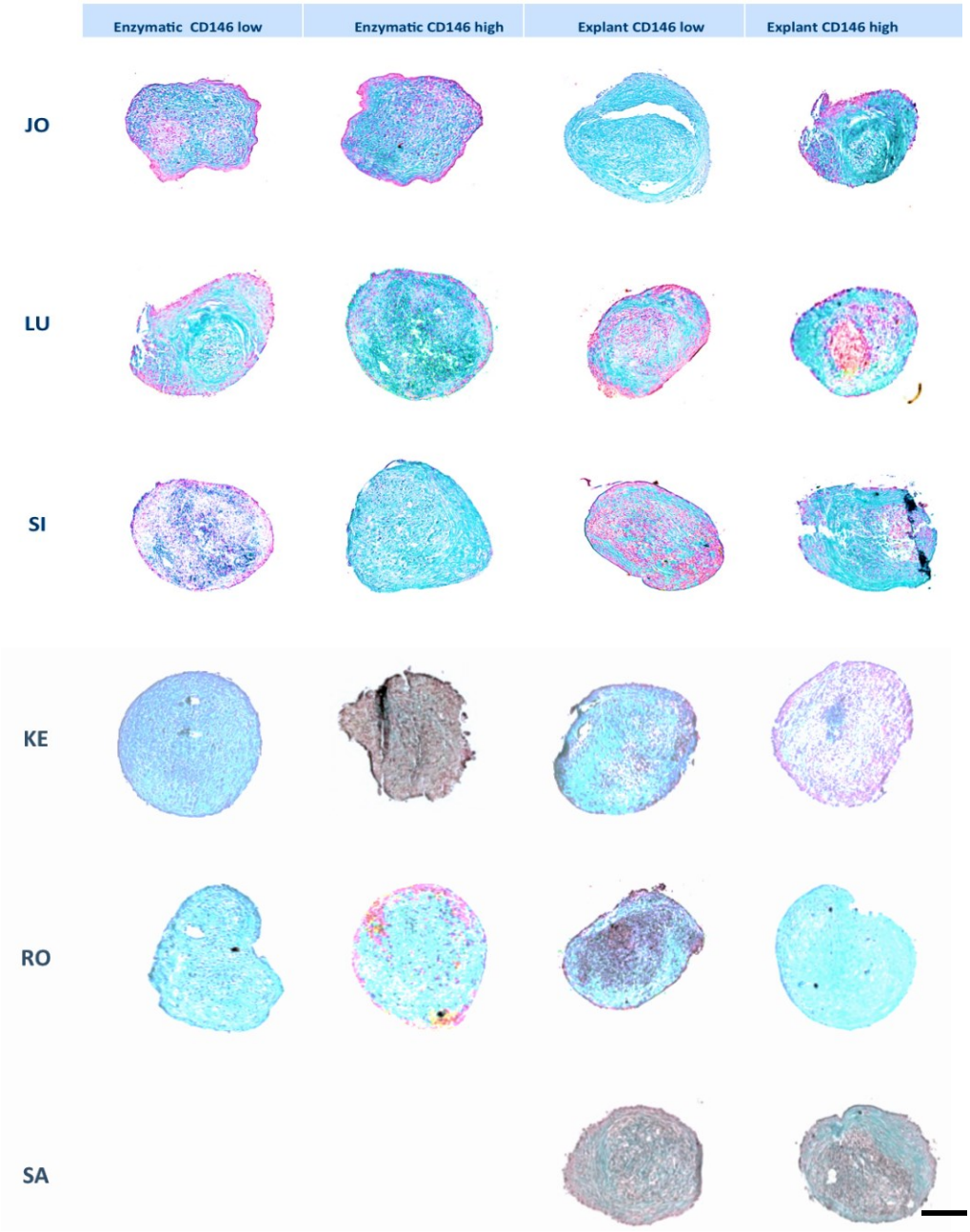

Figure 15. Chondrogenic differentiation of GMSCs. All experimental groups from each patient: JO, LU, SI, KE, RO, and SA. All samples from cell pellets stained blue, indicating proteoglycan formation within the pellets following chondrogenic differentiation. **Scale bar (black) 100 µm.**

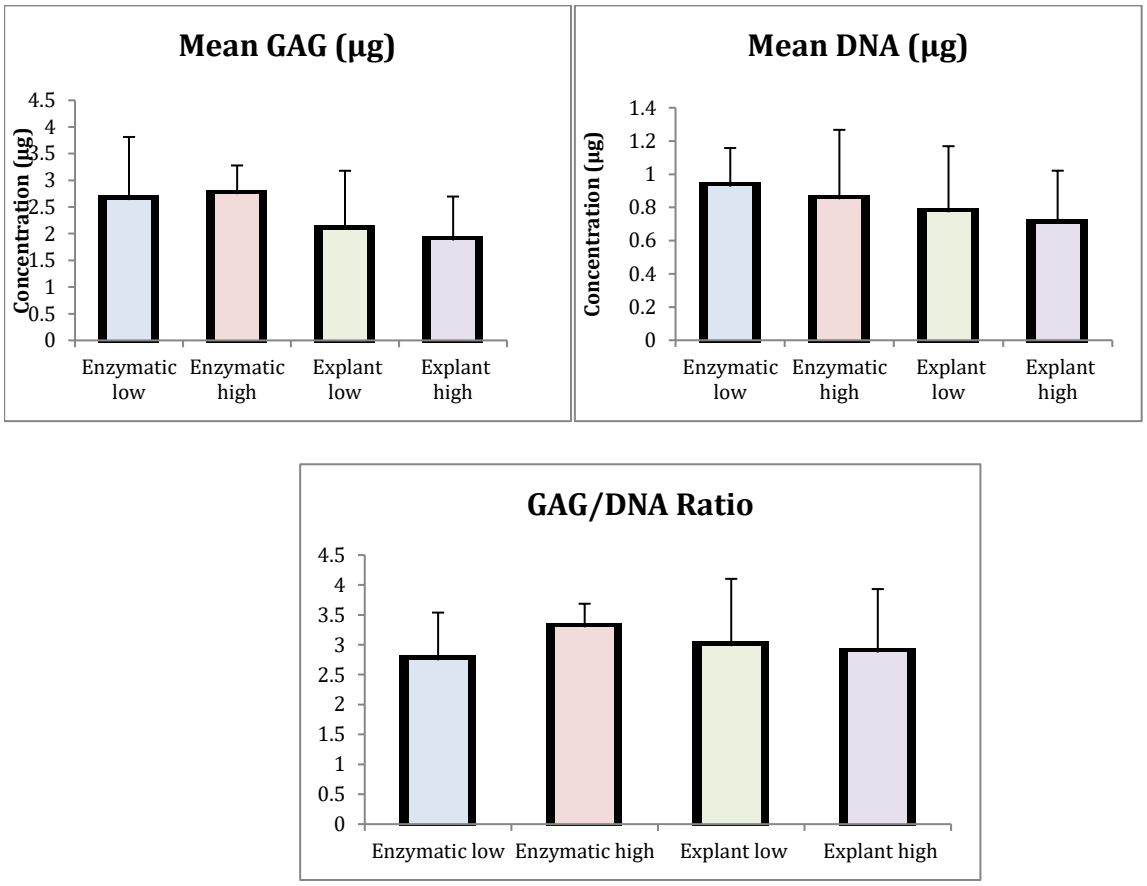

Figure 16. GAG, DNA, and GAG/DNA ratios. Pelleted cells from all patients (N=6) in all experimental groups were analyzed by RT-PCR. Values expressed as mean ± standard deviation. no significant difference was observed (P>0.05).

3.5 Immunofluorescent staining for collagen type I and collagen type II

To identify the cells within the pellet, we used blue immunofluorescent stain 4′,6-diamidino-2-phenylindole (DAPI), which binds strongly to the adenine-thymine rich region of DNA, the nucleus. The ECM components collagen type I and collagen type II were stained using a primary antibody to collagen type I and collagen type II. Collagen type I (red) was detected in each experimental group from all patients (**Figure 17**).

Qualitatively, the cell nuclei stained by DAPI were uniformly distributed within the pellet. Collagen type I was produced throughout the entirety of each pellet of each patient, and no outstanding variations were observed among the four groups. Collagen type II was not detected in the extracellular matrix of the pellets, a result that aligns with the low GAG content, negating chondrogenic differentiation potential that was presumed in the pelleted gingival fibroblasts.

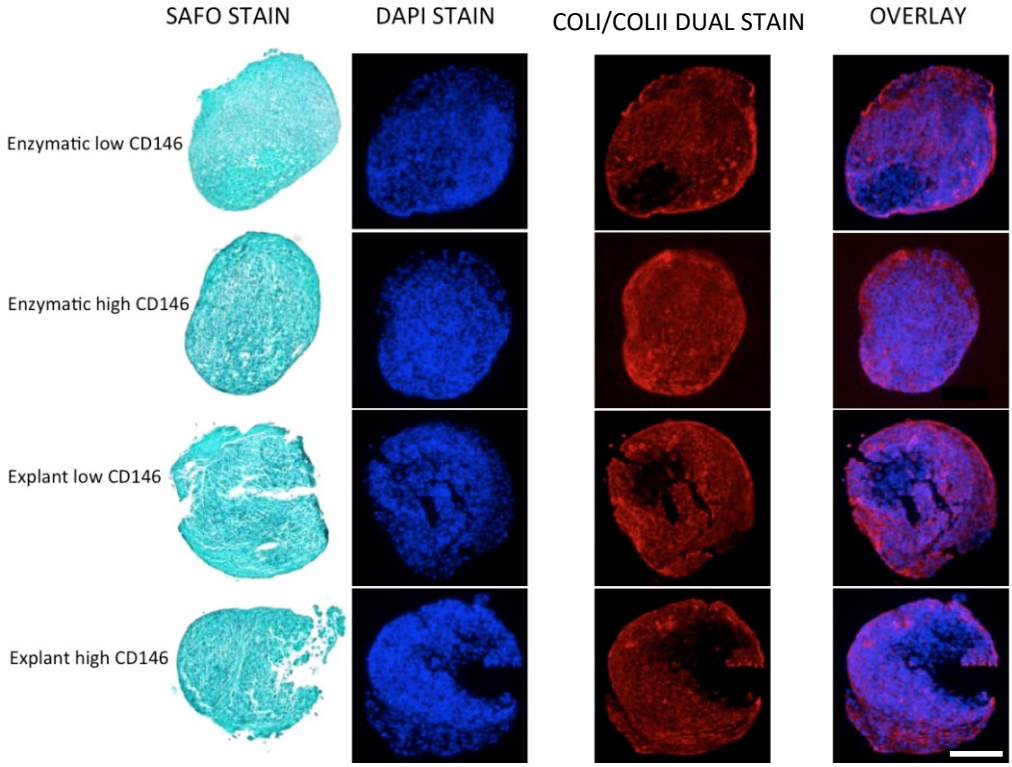

Figure 17. SAFO staining to detect chondrogenesis. Representative images from one patient. The nuclei of GFs in the pellets were counterstained with DAPI (blue). GF pellets were fluorescently labelled with antibodies for collagen type I (red) and type II (green). Dual stain of collagen type I and type II shows only DAPI nuclei stain and red stain of collagen type I; no collagen type II was detected. **Scale bar (white) 100 µm.**

Chapter 4 Discussion

The use of MSC surface markers in the studies described in the literature, despite its long history, is an imprecise process that may lead to the contamination of cultures with cells other than MSCs, including fibroblasts and other supportive cells [111].

The experiment described in this thesis utilized gingival tissue to isolate and expand GFs, specifically due to its ease of access and the promising multilineage potential of these cells [3, 33, 36, 96, 98, 112]. More importantly, we attempted to identify GMSCs within GF cultures. Since MSCs are considered heterogeneous, containing subpopulations within the same isolated culture, the identification of the specific GMSC marker(s) is essential for tissue engineering treatments, not only for dental tissue regeneration, but potentially other organ systems [77].

MSC Surface Marker Identification

CD146 has been identified as a unique MSC marker [70, 77-80, 113]. $CD146^{low}$ and $CD146^{high}$ cultures express similar levels of MSC-positive marker expression, with the expected negative expression of hematopoietic markers (CD34 and CD45), regardless of the isolation method. Moreover, the loss of CD146 expression in three of our six samples of the $CD146^{high}$ expression groups was similar to previously published work, which showed a loss of CD146 expression from P1 to P6 [80]. The results of this thesis ran contrary to other research showing that CD146 maintained high expression after passage 6 [77]. This could be attributed to the different tissue sources used: bone marrow Vs. gingival interdental tissue. It has also been reported that CD146 expression was inconsistent in the cells that were studied and greatly affected by the anatomical site from which the tissues

were derived [78, 79]. The range of studies that used different tissue sources to isolate the MSCs and the variability in the literature regarding CD146 expression and how it is affected by culture prevents solid conclusions regarding the role and function of CD146.

Osteogenic and Chondrogenic Differentiation

Our results revealed that the osteogenic differentiation potential of both $CD146^{high}$ and $CD146^{low}$ cultures was similar when assessed phenotypically following alizarin red staining. Quantitative analysis bore similar results: neither CD146 expression nor the isolation methods had any effect on *ALPL, OCN, COLIA1, or DSPP* gene expression. Studies using MSCs of a dental origin have reported contradictory results with regard to the differentiation potential between $CD146^{low}$ and $CD146^{high}$ cultures. Several studies have demonstrated increased differentiation potential of MSCs for $CD146^{high}$ cultures as opposed to $CD146^{low}$ cultures[113-115]. In addition, Sorrentino *et al*. reported an increased differentiation potential and stem cell marker expression among $CD146^{high}$ cells[81]. However, this study used bone marrow-derived cells and made no comparison to $CD146^{low}$ cells[81].

Two additional studies using BMSCs reported a similar differentiation potential for both $CD146^{low}$ and $CD146^{high}$ cultures. Both studies obtained their cells from fresh bone marrow aspirate and healthy donors, and both used basic MSC expansion media without the addition of growth factors[78,79]. Furthermore, a recent study reported no difference in osteogenic gene expression between $CD146^{high}$ and $CD146^{low}$ cultures[77]. However, it is important to note that publications attempting to replicate this data were unable to observe similar findings[77]. Both high and low groups laid bone, but the $CD146^{low}$ cultures differentiated and laid significantly more bone compared to the

$CD146^{high}$ cultures, which formed more bone marrow. The authors linked their finding to the heterogeneity of the MSC populations - either mature MSCs (laid more bone) or immature MSCs (laid more marrow)[77]. In another study, GMSCs were sorted by El-Sayed *et al*. according to STRO-1 and CD146 expression and concluded that $CD146^{low}$ cells possessed superior osteogenic differentiation potential[70]. Another study supported the superior osteogenic potential has been reported in $CD146^{low}$ cultures despite a difference in CD146 expression after passaging[80]. In summary, it remains unclear whether the expression level of CD146 has any substantial effect on the ability of MSCs to differentiate.

We attempted to differentiate the GFs into two lineages: osteogenic and chondrogenic. Our results indicate a high osteogenic differentiation potential in all four groups of isolated cells. However, the quantitative assessment of GAG production was not statistically different among all groups and did not support chondrogenic differentiation.

In addition, our pellets have shown similar qualitative results following safranin O staining and none of the pellets displayed positive stains for sulphated glycosaminoglycans. By contrast, when using alcian blue, which stains polysaccharides including the GAGs, all pellets stained positive [116]. Despite the fact that the safranin O stain specifically detects sulphated GAGs, the alcian blue could have picked all the anionic molecules in the tissue formed. That is why alcian blue is not a specific staining to use. An error in the pH adjustment could have happened which led to the positive staining of the pellets.

For further identification of the ECM collagenous component produced in the cellular aggregates, an immunofluorescence assay was performed. Immuno-fluorescent results have shown high and equal production of collagen type I in all pellets; no collagen type II production was detected. After compiling the results of the chondrogenic ECM components in our pellet models, following chondrogenic differentiation, we might assume that the sorted GFs did not undergo chondrogenic differentiation. In the context of our data, the isolated and sorted GFs may be considered to be osteoprogenitor cells rather than mesenchymal stem cells that possess multilineage differentiation potential.

Effects of FGF-2 on GFs differentiation potential

In this thesis, we used FGF-2 to culture and expand the GFs that were collected from human tissue. FGF-2 is known for its mitogenic effects on MSCs, enhancing MSC proliferation while maintaining MSC multipotency[52,53]. The increased expression of VEGF, an angiogenesis marker, has also served as evidence of the ability of FGF-2 to promote regeneration[117]. The mitogenic effects of FGF-2, along with its angiogenic capability, are well established and are frequently examined to gain a better understanding of the mechanism of action that leads to enhanced tissue regeneration[117-119].

The multipotent differentiation of MSCs cultured in FGF-2 supplemented media has been confirmed[58,120], with the demonstration of osteogenic differentiation and increased expression of OCN in cultures receiving FGF-2 treatment[121,122]. However, the versatility of FGF-2 is evidenced by the varying regulatory mechanisms within different cell types. In periodontal ligament PDL MSCs, FGF-2 inhibits OCN, ALPL, and COLI; whereas increased expression of these genes is observed in murine bone cellular lines[123]. With regard to the gingiva, differential expression of FGF-2 receptors has been reported in the

gingival epithelium, GFs, and PDL cell[124], Specifically, FGF-2 receptors are abundant in GFs and PDLs but not in gingival epithelial cells[124], These results reveal a selective mechanism of FGF-2 depending on the cell type – potentially due to the niche in which it is functioning[122].

Few studies have experimented with the effects of FGF-2 on sorted dental MSCs based on a set of pre-selected MSC surface markers[51,58]. PDL MSCs have been sorted using a combination of STRO1/CD146 surface markers[58]. The addition of FGF-2 to this sorted population resulted in several observations: FGF-2 stimulated an increase in the number of cells expressing both STRO1 and CD146; the overall number of cells in FGF-2 cultures increased 15-fold compared to cells without FGF-2; and the sorted cells maintained their osteogenic and adipogenic differentiation potential[51]. To our knowledge, there has yet to be a study that examines the multipotent differentiation of GFs cultured in FGF-2 media based on the CD146 selected surface marker. Our study demonstrated consistent osteogenic differentiation of GFs between all groups when cultured in media containing FGF-2.

Assessing Methodological Differences in GFs Isolation

As mentioned earlier, extractions of dental MSCs from different anatomical locations have been performed using either tissue explants or enzymatic digestion. Here, we tested whether the isolation method had any effect on the expression of MSC markers or on CD146 expression and compared the osteogenic/chondrogenic differentiation potential. We report that, regardless of the isolation and culture method, the expression of MSC markers, including CD146, and the differentiation potential remained unchanged in our isolated populations. To our knowledge, this is the first study that has considered the

effects of isolation methods on MSC marker expression and differentiation potential of GFs derived from gingival tissue. The importance of this experiment relies in the fact that a consistent protocol will be required to advance tissue-engineering models for therapeutic or regenerative purposes.

Conclusion

We attempted to identify and isolate GMSCs from GF populations using the two most common methods: the explant and enzymatic digestion methods. We then attempted to enrich the isolated cells by magnetically sorting them based on their CD146 expression.

Neither of the isolation methods we used had any significant effect on the expression of MSC markers nor the differentiation potential of these cells. Thus, either isolation method could be used to isolate GF cells. CD146 expression did not affect the differentiation potential of the enriched group of cells. Based on the results of our study, we cannot conclude that CD146 is a specific MSC surface marker to isolate or enrich the MSC population from among GFs. Adding FGF-2 growth factor to the culture media during cell expansion might have led to greater consistency in the results seen across all the groups tested. Our results cannot be conclusively adopted due to the lack of a group of non-FGF-2 media for comparison. However, the addition of FGF-2 growth factor to the culture medium might represent a viable step during the expansion of the GFs.

Limitations

Our study was not without limitations. It is possible that statistical significance was not achieved due to the relatively small sample size. Furthermore, the inclusion of experimental control groups that did not receive FGF-2 treatment was lacking. Furthermore, we used magnetic sorting by passing the cells with magnetic beads only

once in the extraction tube. Passing the cells twice in the tube or using flow assisted cell-sorting machines, which yield more accurate and purer populations of cells, may have presented superior methods for sorting cells. It is also possible that the isolation methods could be optimized, which would be the subject of future studies.

Future Studies

The identification of a specific surface cell marker(s) that identifies GMSCs is necessary for the development of a consistent stem cell isolation protocol. By extension, developing a consistent protocol will increase the reliability of tissue engineering models. The objective of this study was to identify the GMSC population that could be employed in tissue engineering or cellular therapy protocols for dental tissue regeneration. There is previous data to support, and great potential for additional data gained by ongoing and future research to be utilized for dental clinical application in four broad categories: direct pulp capping, pulp revitalization, pulp tissue engineering, and periodontal ligament regeneration.

Based on our results, and the limitations discussed, there are several recommendations for future studies.

1) Increasing the number of samples analyzed. Using a power caclculation and based on the results of previously published studies regarding the number of cells recovered and the purity of the population, we estimate that with 10 samples for each experimental condition, we will be sufficiently powered (>90%) with high confidence levels to detect association of specific cell surface markers at a significance level of of $P < 0.05$ in the range found in other studies.

2) Continue to assess the various combinations of MSC markers in GFs populations in order to reliably identify and isolate the optimal GMSC population for future translational animal and clinical studies and novel therapeutic applications.
3) Continue to assess the effects of FGF-2 on the characteristics of GMSCs, and include untreated control groups for each patient sample.
4) Continue to assess the differential results based on modified isolation methods.
5) Additional studies could also be significantly powered to understand any potential difference in number of cells recovered, surface marker expression, or differentiation potential based on:
    a. Gender
    b. Age – Since age-associated changes have been observed in the regenerative capacity of stem cells, comparing recovery and potential of cells from young (adolescent), adult, or aged patients should be performed.
    c. Race
    d. Health status of the individual – comparing recovery and potential of cells from healthy or diseased gingiva
6) Optimize identification of cells by obtaining antibodies for all surface markers conjugated to different fluorophores that can be analyzed in one sample by flow cytometry (requires a flow cytometer for analysis or sorting with sufficient laser compatibility).

7) Evaluate the potential for cryopreservation and storage of isolated GMSCs while maintaining their therapeutic potential.
8) Once the specific GMSC population has been identified and defined, the therapeutic potential of the cells should be evaluated using *in vitro* assays and *in vivo* models.

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Appendix

Table A1. Summary of published studies using GFs

Author	Cellular population	Method of isolation	Medium used for expansion	MSC markers	
				High expression	Low expression
1. Diomede, et al. (2018)[125]	Anatomical site not reported	Explant: Tissues ground then washed several times with PBS, subsequently cultured using TheraPEAK™MSCGM-CD™ 37°C in 5 % CO_2.	Cells were cultured in MSCGM-CD™serum-free medium then seeded in a commercial polylactide (PLA) 3D printed scaffold treated with conditioned medium obtained from GMSCs, collected at passage 2.	Oct3/4 Sox-2 SSEA-4 CD29 CD44 CD73 CD90 CD105	CD34 CD14 CD45

2. Hyunjin, et al. (2018)[126]	Obtained during periodontal treatments.	De-epithelialized, minced into 1 to 2mm fragments and digested in 0.2-mm filtered MEM with 2 mg/mL collagenase IV incubated in a humidified incubator at 37°C.	10% FB 4 mM L-glutamine 100 U/mL penicillin 100 mg/mL streptomycin Osteogenic medium with valproic acid (VPA)	Not reported	Not reported
3. Gugliandolo, et al. (2017)[127]	Not reported	Explant: Tissues were de-epithelialized and washed with PBS, 37 °C in 5 % CO_2.	DMEM high-glucose medium 10% FBS humidified atmosphere, 5% CO_2.	CD29 CD44 CD73 CD90 CD105 OCT3/4 SSEA4 SOX2	CD14 CD34 CD45
	In vitro	Polylactic acid polymer filaments, 3D printed.			
4. Vadim, et al. (2017)[128]	Biopsies of the alveolar mucosa of the retromolar area and attached gingiva from the distal mandibular molar level	αMEM supplemented with 10% FBS. 20 mg/mL gentamicin, 0.05% solution of collagenase type II Incubated for 12 hours at 37°C.	DMEM/F12 20% FBS 20 m g/mL gentamicin	CD73 CD90 CD105	CD324, cytokeratins (14, 15, 16, 19) CD31 CD34 CD45

5. Subbara-yan, et al. (2017)[129]	Unspecified	Enzymatic + overnight plating: Overnight at 4°C with dispase 2mg/ml. Tissues were minced into 1 to 3mm^2 fragments Collagenase IV 4 mg/ml at 37°C for 2 hours. [Adopted from Zhang (2009)]	αMEM 10% FBS 100U/mL penicillin 100 mg/mL streptomycin 2mM l-glutamine 100mM nonessential amino acid 550 mM 2-mercaptoethanol 37°C in a humidified tissue culture incubator with 5% CO_2. MSCs were cultured as a suspension culture in ultra-low attachment dishes to obtain 3D spheroid formation. [Adopted from Zhang (2012)]	CD45 CD34 CD73 CD90 STRO-1 VIMENTIN OCT-4A NANOG SOX-2 SSEA-4 TRA-1-60 TRA-1-81	CD45 CD34 CD11b CD19 HLA-DR
6. Subbaray an, et al. (2017)[130]	Crown lengthening procedure or operculectomy	Explant:	αMEM 10% FBS	CD73 CD90 CD105	CD45 CD34 CD11b

		Tissue minced and suspended in the selected medium, left undisturbed at 37°C in a humidified incubator with 5% CO_2.		CD44 STRO1 OCT-4, NANOG, SOX-2 SSEA-4 TRA1-60 TRA 1–81	CD19 HLA-DR
7. Ansari, et al. (2017)[131]	Tissues obtained from patients undergoing third molar extractions	Not reported	MSC culture media	CD73 CD105 CD146	CD34 CD45
	In vitro	Alginate hydrogel with hyaluronic acid.			
8. Santamaría, et al. (2017)[132]	After prescribed periodontal surgery in the palatal maxilla.	Enzymatic: Collagenase I 3mg/ml, dispase II 4 mg/ml, 1 hour 37°C in 5 % CO_2.	DMEM: F12 supplemented 10% FCS 100 U/mL penicillin 100 µg/mL streptomycin 50 µg/ml gentamycin 2 mM L- glutamine	CD 73 CD 90 CD 105	CD 34 CD 45 HLA-DR CD11b CD19
9. Rao, et al. (2016)[133]	Crown-lengthening procedure or operculectomy.	Enzymatic: Cut into 1 to 3mm^2 pieces and digested at	αMEM 10%FBS 100 U/mL penicillin	CD90 CD105 CD73 Oct-4	CD45 CD34 CD11b CD19

		37°C for 2 hours in a sterile medium containing 1mg/ml collagenase type II with gentle agitation applied.	100 µg/mL streptomycin 100 µg/mL amphotericin B 2 mM L-glutamine cultured at 37 °C with 5% CO_2.	NANOG SOX-2 SSEA-4 TRA-1-60 TRA-1-81	HLA-DR
	In vitro	Cells incapsulated in 3D hydrogel (Indian-Del No. 1413del2013).			
10. Ha (2016)[134]	Gingival derived stem cells In vitro	Enzymatic: De-epithelialized and minced into 1 to 2 mm^2 fragments digested in 0.2 ml filtered αMEM containing dispase (1 mg/mL and collagenase IV) 2 mg/ml at 37°C for 30 minutes. After discarding the initially digested cell suspension, the tissues were digested in the same solution for 90 minutes at 37°C. The cell suspension was filtered with a 70 µm cell strainer. [Adopted from Jin et al. (2015)] Cells were incubated with Tacrolimus-loaded poly (lactic-co-glycolic acid) microspheres.	αMEM 15% FBS 100 U/mL penicillin 100 lg/mL streptomycin 200 mM L-glutamine 10 mM ascorbic acid 2 phosphate [Adopted from Jin et al. (2015)]	Not reported	Not reported

11. Van Pham, et al. (2016)[135]		Explant: Tissue minced and suspended in the medium and left undisturbed at 37°C in a humidified incubator with 5% CO_2.	DMEM/F12 10% FBS 1% antibiotic antimycotic solution	CD13 CD44 CD73 CD90 CD105	CD14 CD34 CD45 HLA-DR
12. Ansari, et al. (2016)[136]	GMSCs – not specified In vitro & in vivo	Only enzymatic: 2 mg/ml collagenase type I 4 mg/ml dispase II 1 hour at 37°C	αMEM 15% FBS 2 mM L – glutamine 100 nM Dex 100 mMascorbic acid 100 mM ascorbic acid 2 mM sodium pyruvate 100 µg/ml streptomycin	CD73, CD105, and CD146 ≈ 70%	CD34 CD45
13. El-Sayed, et al. (2015)[70]	From the third molar region with free gingival soft tissue collars	Explant: Rinsed several times with Eagle's minimum essential medium alpha modification supplemented with 100 U/ml penicillin 100 mg/ml 21 streptomycin and 1% amphotericin.	αMEM 15% fetal calf serum 400 mmol/ml L-glutamine 100 U/ml 21 penicillin 100 mg/ml 21 streptomycin 1% amphotericin	CD73 CD90 CD105 MUC18 STRO-1 CD146 ≈ 8.4–92.7%	CD14 CD34 CD45

	In vitro	Placed into dry culture flasks for 30 minutes to allow them to adhere to the bottoms of the flasks. Cells were then left to grow in the basic media at 5% carbon dioxide at 37C.			
14. Jin, et al. (2015)[137]	Tissues collected after clinical crown lengthening procedures	Enzymatic: De-epithelialized and minced into 1 to 2 mm^2 fragments digested in 0.2 ml filtered αMEM containing dispase (1 mg/mL and collagenase IV) 2 mg/mL at 37°C for 30 minutes. After discarding the first digested cell suspension, the tissues were digested in the same solution for 90 minutes at 37°C. The cell suspension was filtered with a 70 μm cell strainer.	αMEM 15% FBS 100 U/ml penicillin 100 lg/ml streptomycin 200 mM L-glutamine 10 mM ascorbic acid 2 phosphate	CD44 CD73 CD90 CD105 SSEA-4 STRO-1 CD146, CD166 CD271	CD14 CD19 CD34 CD45
15. Wu, et al. (2014)[138]	After crown lengthening procedures or distal wedge periodontal surgeries In vitro	At 37°C and 5% CO_2	αMEM 10% FBS 1%penicillin streptomycin (P/S)	CD90 CD105 CD73 STRO-1	Not reported
16. XU, et al. (2014)[139]	Anatomical site not identified	Enzymatic:	αMEM medium 15% FBS	CD73 CD105	CD144 CD31

	In vitro & in vivo	Washed several times with PBS. Incubated in 2mg/ml dispase at 40°C overnight. Connective tissue is then minced and digested in 2mg/ml collagenase (type not reported) for 4 minutes at 37°C.		CD29 CD44 Stro-1	HLA-DR CD34 CD45
17. Gao, et al. (2014)[140]	Gingival tissue after 3rd molar extraction In vitro & in vivo	Enzymatic + CFU-F Overnight at 40°C with 2mg/ml dispase to separate the epithelial and spinous layers. The tissue was minced into fragments and digested with 4 mg/mL collagenase IV at 37°C for 2 hours followed by colony forming units.	αMEM supplemented with 10% FBS	STRO-1 CD29 CD90 CD105 CD146 8.5%	CD34 CD45
18. El-Bialy, et al. (2014)[141]	Gingival tissue around the premolar extraction site following ortho treatment In vitro	Explant: Cut into small pieces, isolated on glass slides, placed in a culture plate at 37°C in a humidified atmosphere of 5% CO_2.	DMEM 10% FBS 100 U/mL penicillin 100 l g/mL streptomycin	CD73 CD90 CD105	CD11b CD34 CD45 CD31
19. Gay, et al. (2014)[71]	Human gingival tissue –	Enzymatic: 3 mg/ml collagenase type I	DMEM with 10% FBS and 1% Pen-strep at 37°C	CD105, CD29,	Not reported

	unspecified anatomical site In vitro	4 mg/ml of dispase		SSEA4 OCT4 STRO-1 OCT4 NANOG	
20. Moshaverinia, et al. (2014)[142]	Human gingival tissue – unspecified anatomical site	Enzymatic: Minced and digested in collagenase IV solution at 37°C for 2 hours. Cell suspension filtered through a 70mm cell strainer. Incubated at 37°C in 5% CO_2.	αMEM 15% FBS 100 mM ascorbic acid 2-phosphate 2mM glutamine 100U/ml penicillin, 100 mg/ml streptomycin 550 mM 2-mercaptoethanol	CD166 CD146 ≈70%	CD34
	In vitro & In vivo	Cells encapsulated in RGD alginate capsules.			
21. Moshaverinia, et al. (2014)[87]		Enzymatic: Minced and digested in collagenase IV solution at 37°C for 2 hours. Cell suspension filtered through a 70mm cell strainer. Incubated at 37°C in 5% CO_2.	αMEM 15% FBS 100 mM ascorbic acid 2-phosphate 2mM glutamine 100U/ml penicillin, 100 mg/ml streptomycin 550 mM 2-mercaptoethanol	CD105 CD146	CD34
	In vitro & In vivo	RGD-coupled alginate			

22. Li, et al. (2013)[143]	Gingival tissue after gingivectomy (healthy and inflamed) In vitro & in vivo	Enzymatic and Explant: 0.4% dispase at 37°C for 30 min. gingival epithelium stripped off, type 1 collagenase 0.66mg/ml for 40min	DMEM 10% FBS 0.292 mg/ml of glutamine 100 U/ml penicillin 100 mg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.	CD29 CD90 CD44 CD105 less expressed Stro-1 CD146 ≈ 8-11%	CD34 CD45
23. Hao Yang et al. (2013)[144]	GMSCs – from the third molar cervical ridge In vitro & In vivo	Enzymatic followed by Explant: 0.1% dispase 0.2% collagenase IV 30 minutes at 37°C Tissue incubated until cells grew out.	α-MEM 10% FBS 0.292 mg/mL glutamine 100 units/mL penicillin streptomycin	STRO-1 CD90 CD105 CD29 CD146 55.2 %	CD31 CD45
24. Hsu, et al. (2012)[145]	GMSCs – unspecified site, subpopulation expressing high (Oct4, Nanog) & (Slug and Sox10) In vitro	Explant: Gingival tissue cut into pieces and explanted into 60mm tissue culture polystyrene dishes.	α-MEM 10% FBS 100 mg/ml streptomycin 100 U/ml penicillin 50 mg/ml gentamicin	CD29 CD44 CD73 CD90 CD105 CD106 &STRO1 CD146 ≈61-91%	CD31 CD34 CD45

25. Ge, et al. (2012)[146]	Collected from patients undergoing crown lengthening surgery	Enzymatic + CFUF: Collagenase type 1 (3 mg/ml; Dispase II neutral protease) 4 mg/ml for 2 hours at 37°C and filtered through 70-µm cell strainer + CFUF selection	α-MEM 20% FBS 2 mM l-glutamine 100 mM l-ascorbate-2 phosphate 1 mM sodium pyruvate 50 U/mL penicillin G 50 µg/mL streptomycin 2.5 µg/mL amphotericin B 37°C with 5% CO_2.	CD44 CD73 CD90 CD105 CD166	CD14 CD34 CD45
26. Tang, et al. (2011)[147]	Gingival tissue following gingivectomy (healthy and inflamed) In vitro & In vivo	Enzymatic: 0.4% dispase at 37°C for 30 minutes followed by physically stripping the gingival epithelium. The remaining propria tissues were digested with type I collagenase (0.66 mg/ml) for 50 minutes. Single cell suspensions.	DMEM 10% FBS 0.292 mg/mL glutamine 100 U/mL penicillin G 100 mg/mL streptomycin	CD29 CD44 CD90 CD105 Stro-1 CD146 ≈ 85-93%	CD34 CD45

27. Wang, et al. (2011)[148]	The gingival tissues were discarded tissues following conventional dental procedures In vitro	Enzymatic: Removal of epithelial layer Gingiva tissue was minced and incubated in a mixture of 0.1% dispase and 0.2% collagenase IV for 15 minutes at 37°C. The initially digested cell suspension was discarded and incubated in 0.2% trypsin for 5, 10, and 15 minutes at 37°C. All cell fractions were collected.	αMEM 10% FBS 100U/mL penicillin 100 mg/mL streptomycin in at 37°C in 5% CO_2.	CD29 CD105 STRO-1 CD90	CD34 CD45
	In vivo	Cellmatrix type I-A kit (Cellmatrix_ Type 1-A)			
28. Keren, et al. (2010)[149]	Oral mucosa – gingival and periosteal tissue	Explants only: αMEM 12% FCS 2 mM glutamine Antibiotics (penicillin [100 U/ml] Streptomycin [0.1 mg/ml] Fungisone [0.25 μg/ml]).	low-glucose Dulbecco's modified Eagle's medium (LGDMEM) 10% FBS	CD29 CD73 CD90 CD105, and CD166 Stro1 and CD106 human leukocyte antigen-DR (HLA-ABC) CD146 ≈ 16% SSEA4,	CD34 CD45 human leukocyte antigen-DR (HLA-DR) SSEA3 SSEA1 Tra1-60, Tra1-81 CD117

	In vitro			Oct4, and Sox2	
	In vivo (Formed a teratoma)	Fibrin membranes			
29. Mitrano, et al. (2010)[42]	Gingival tissue was taken from the maxillary tuberosity.	Explanted method only: Deepithelialized with a scalpel, #15, leaving only the connective tissue. The explants were placed in tissue culture dishes.	α-MEM, 10% FBS and 1% penicillin, streptomycin, and amphotericin.	CD90 CD105 CD73 CD44 CD13	CD34 CD38 CD45 CD54
30. Tomar, et al. (2010)[66]	Gingival tissue – unspecified location	Enzymatic method only: 0.1% collagenase 0.2% dispase for 15 minutes at 37°C The first cell fraction was discarded. Tissues were further incubated in enzyme solution for 5, 10, and 15 minutes and all cell fractions were	α-MEM 10% FBS	CD44 CD29 CD73 CD90 CD105	CD34, CD14 CD45

		pooled.			
31. Fournier, et al. (2010)[2]	All gingival samples were obtained from buccal marginal tissues	Explant cultures + CFU-F: Primary explant cultures were established. The CFU-F were then transferred and cultivated.	DMEM 20% fetal calf serum (FCS), 100 mg penicillin ml 100 mg/ml streptomycin 2 ng/ml amphotericin B	CD29 CD44 CD73 CD90 CD105 STRO-1 CD271 CD146 ≈ 3-17%	CD34 CD45 CD117 CD200 HLA-DR
32. Moshaverinia, et al. (2012)[150]	3rd molar site following tooth extraction	Enzymatic: Minced and digested in collagenase IV solution at 37°C for 2 hours. Cell suspension filtered through a 70-mm cell strainer. Then incubated at 37^0C in 5% CO_2.	αMEM 15% FCS 100 mM ascorbic acid 2-phosphate 2mM glutamine 100U/mL penicillin, 100 mg/mL streptomycin 550 mM 2-mercaptoethanol	CD73 CD146	CD34
	In vitro In vivo	Alginate microcapsules			
33. Tomasello, et al. (2017)[151]	Oral surgery procedures for tooth extraction.	Enzymatic: 5 mg/ml collagenase G 2 mg/ml collagenase H in a 4:1 ratio for 4 hours at 37°C under agitation.	StemLine Mesenchymal Stem Cell Expansion Medium with 0.5 µg/mL gentamicin	Stro-1 CD146 CD29 SSEA4 CD146 ≈16.5-75.2%	CD 34 CD 45 HLA-DR

			0.25 µg/mL levofluoxacin 0.10 µg/mL vancomicin 0.25 µg/mL fluconazole 5% FBS and incubated at 37°C in 5% CO_2.		
34. Zhang Q. et al (2009)[39]	The gingival tissues were obtained as remnant or discarded tissues following routine dental procedures In vitro	Enzymatic + overnight plating: overnight at 4°C with dispase 2 mg/ml tissues were minced into 1- to 3-mm^2 fragments collagenase IV 4 mg/ml at 37°C for 2 h	α-MEM 10% FBS 100 U/mL penicillin 100 µg/mL streptomycin 2 mM L-glutamine 100 mM nonessential amino acid 550 µM 2-ME 37°C tissue culture incubator with 5% CO_2 and 95% O2.	Oct-4 CD29 CD90 CD73 Less expression CD105 SSEA-4 Stro-1 CD146 7.1%	CD45
	In vivo	hydroxyapatite/tricalcium phosphate ceramic powder			

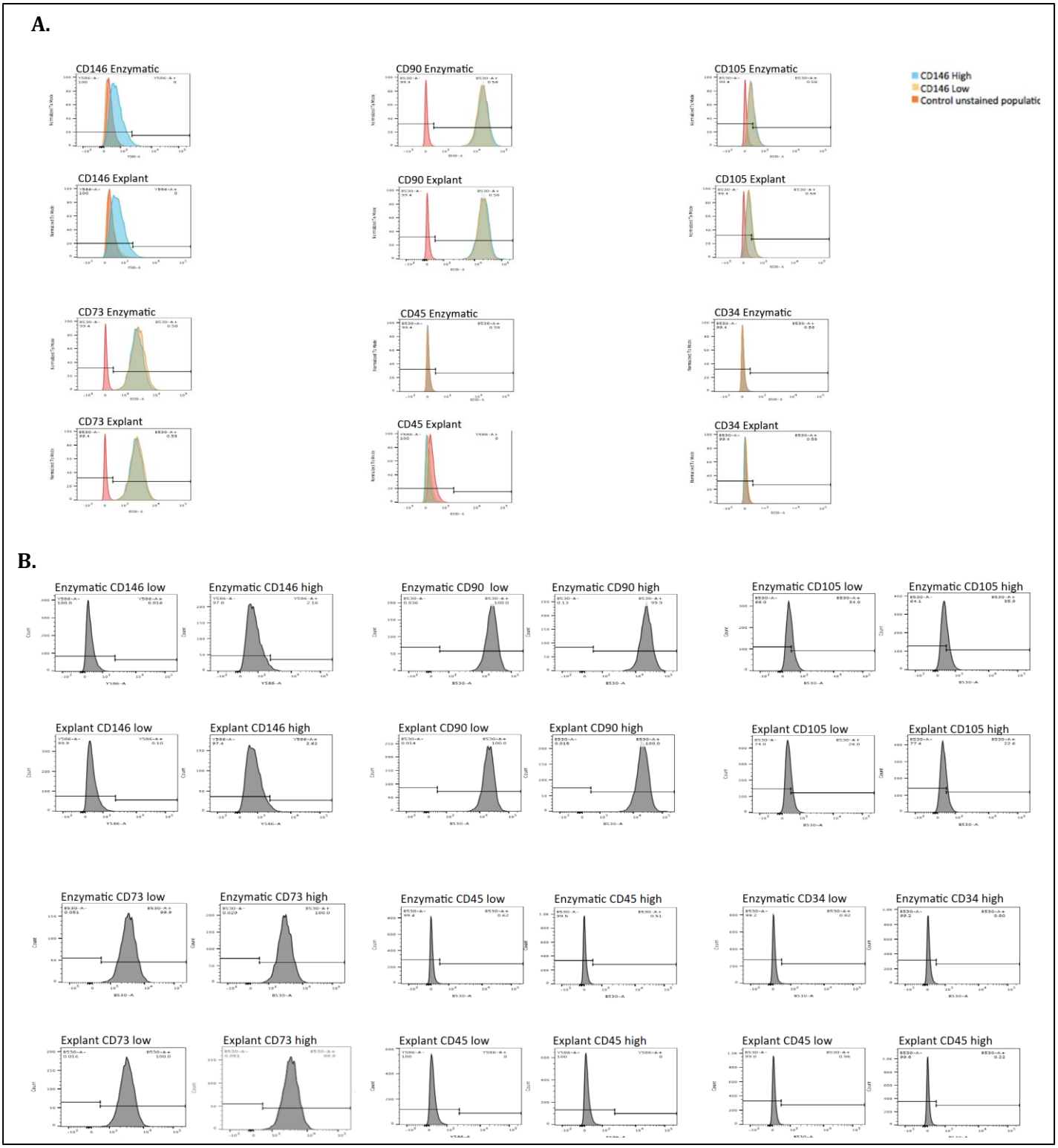


Figure A4.*a* Patient JO: A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, CD146^{high}/CD146^{low}. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.

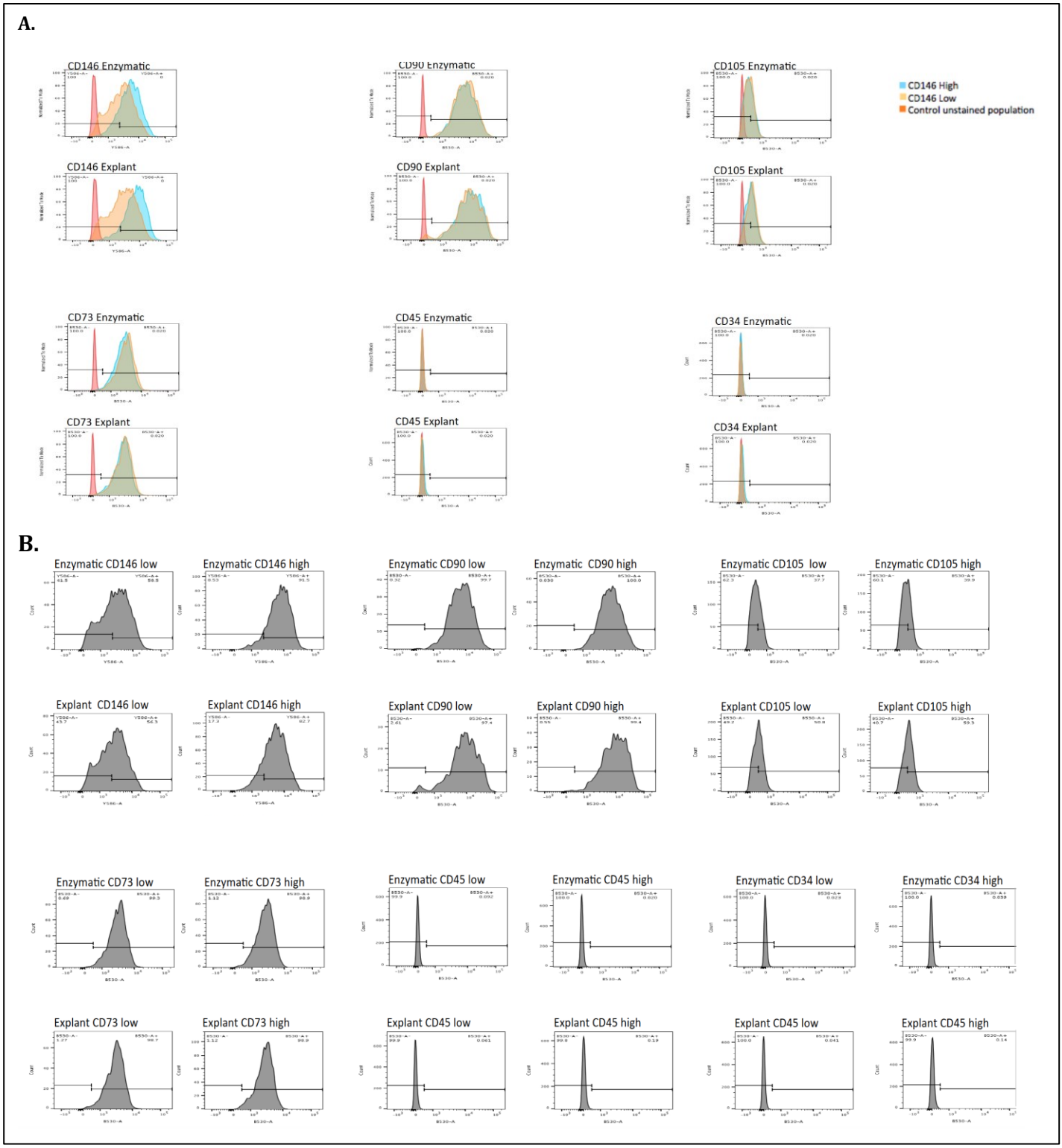
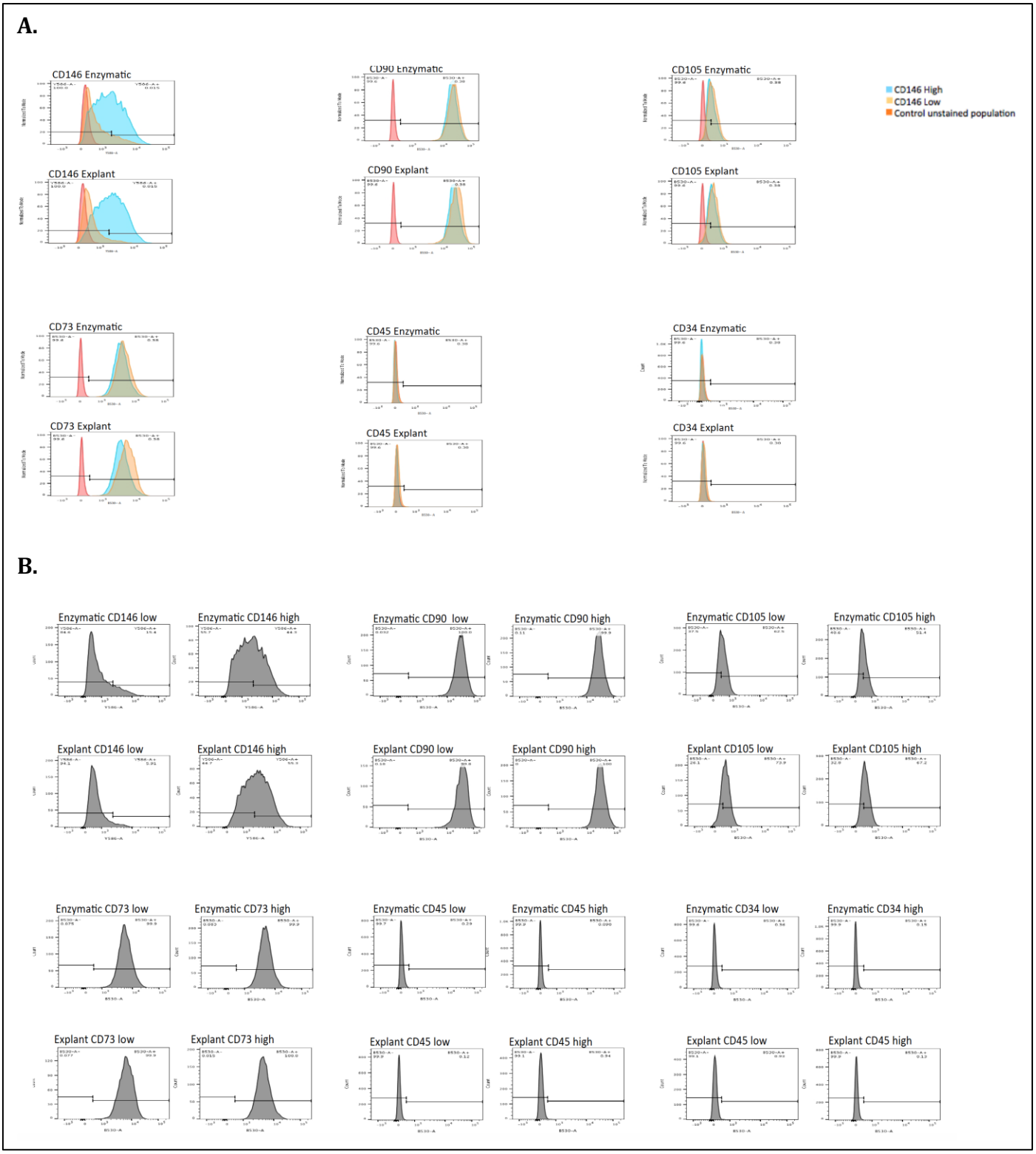


Figure A4.*b* Patient LU: A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, $CD146^{high}/CD146^{low}$. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.



Figu4.*c* Patient SI: A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, $CD146^{high}/CD146^{low}$. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.

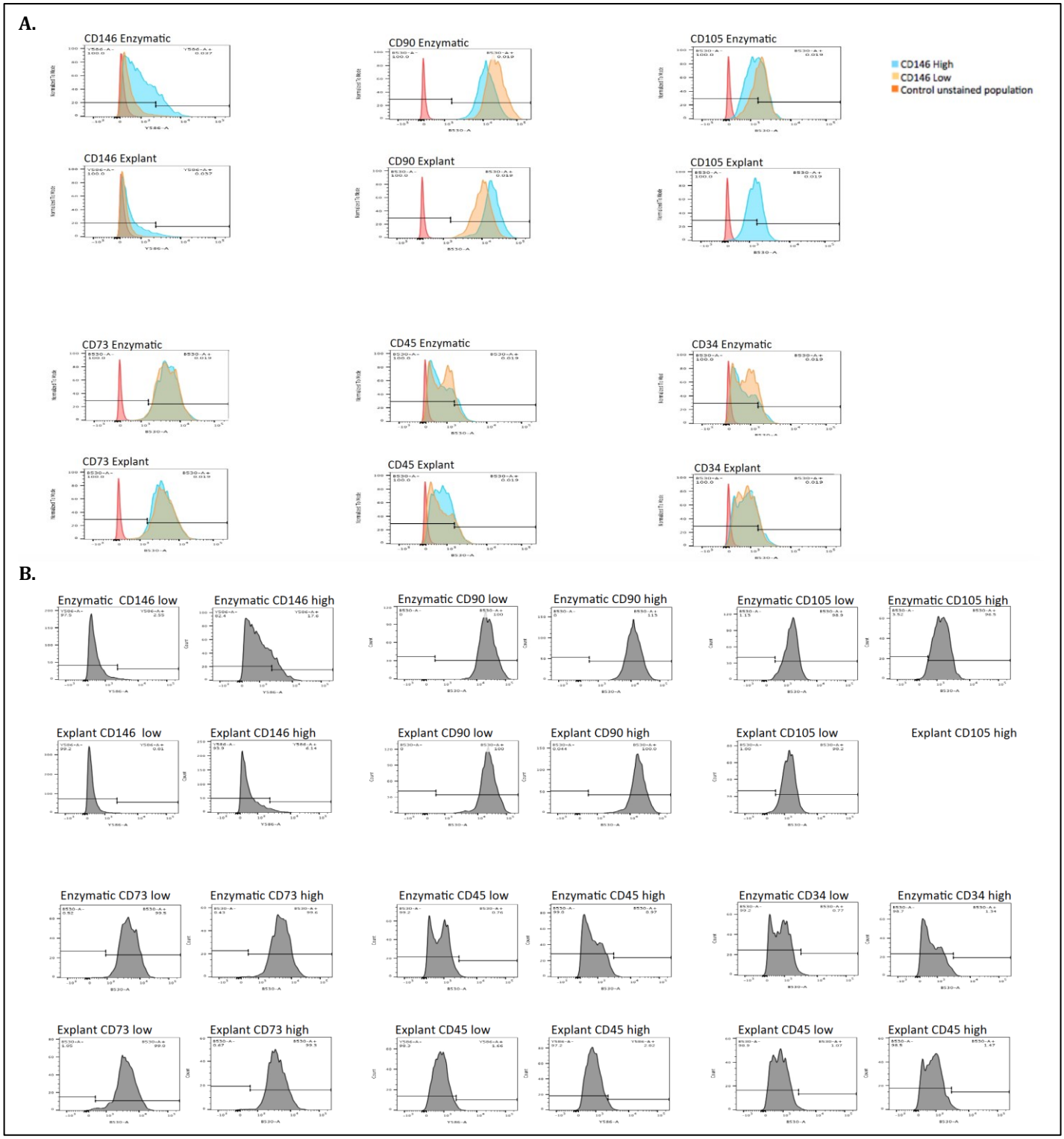


Figure A4*d* Patient KE: A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, CD146^{high}/CD146^{low}. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.

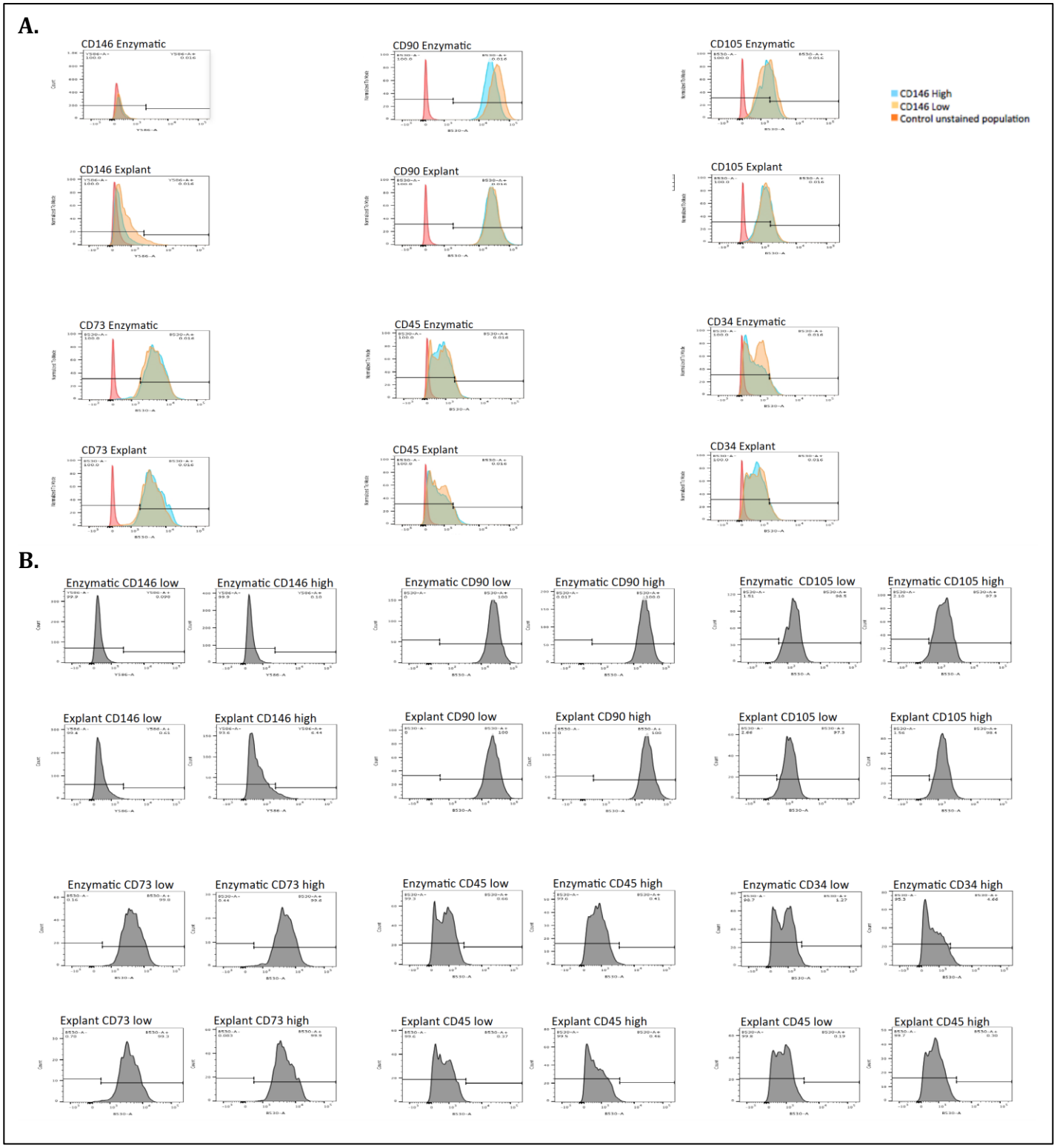


Figure A4.*e* Patient RO: A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, $CD146^{high}/CD146^{low}$. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.

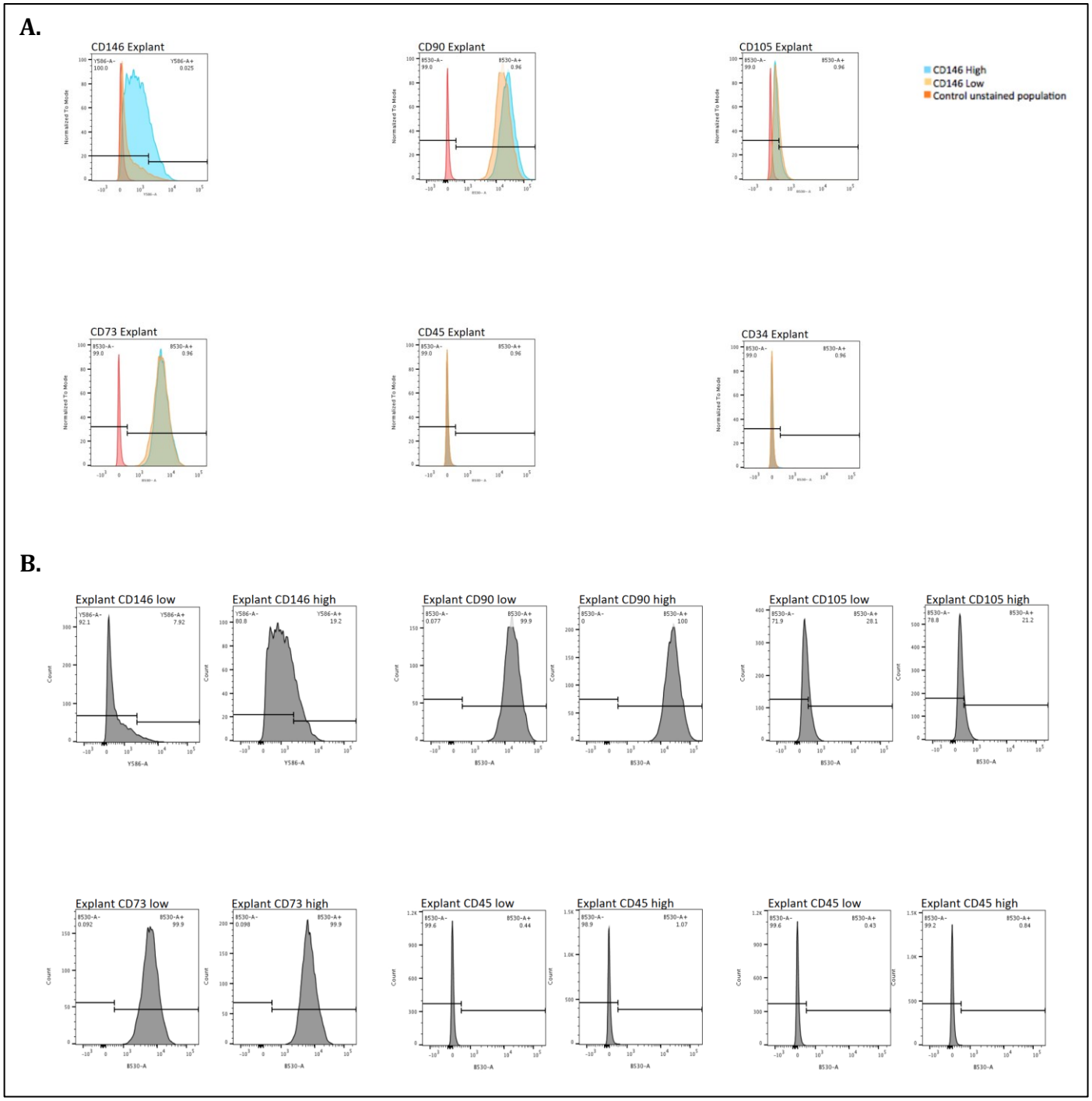


Figure A4.*f* Patient SA. A) Flow cytometry histogram with overlay of CD marker expression in Enzymatic/Explant, CD146^{high}/CD146^{low}. B) Individual analysis of CD146, CD90, CD105, CD73, CD45, and CD34.

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