Characterization and Functional Analysis of Adipose-Derived Stromal Vascular Fraction in Osteogenesis Imperfecta Pediatric Patients

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Abstract

Osteogenesis imperfecta (OI) is a heritable connective tissue disorder often associated with long- bone deformation and bone fragility that are a major impediment to daily activities. The standard of care includes bisphosphonates and surgical correction of bone deformities with osteotomy and intramedullary rodding. However, delayed union or non-union of osteotomy site is a common adverse event from this surgery, leading to pain, bending of the fixation rods, repeated fractures, and loss of ambulation. Therefore, alternative solutions are required. Bone grafts or bone-marrow-derived mesenchymal stem cells (BM-MSC) that have been studied for bone regeneration have their limitations. However, a new stem cell source, the autologous adipose tissue-derived stromal vascular fraction (aSVF) is acknowledged as an easily accessible source of a heterogeneous mix of non-expanded cells derived from enzymatically digested adipose tissue and has anti-inflammatory, angiogenic, immunomodulatory, and regenerative properties. The mesenchymal stem cells of the aSVF (ASCs) has multi-differentiation capacity as BM-MSCs. And its regenerative properties have been studied extensively in the past two decades; however, to our knowledge, the healing and regenerative potential of aSVF has not been explored in OI. Here, we describe for the first time, the isolation of SVF from OI patients' adipose tissue; and its cellular characterization and functional capacity as a new source for bone regeneration. In this study, we obtained 42 adipose tissue samples derived from pediatric patients either diagnosed with OI (38 donors; age range: 2–22 years; 19 males, 19 females); or non-OI (controls; n= 4; age range: 4–14 years; 1 male, 3 females) undergoing corrective bone surgeries at the Shriners Hospital for Children (Montreal, Canada). The overall mean weight of harvested adipose tissue was 6.70 g in the OI group and 7.48 g in controls. And the overall number of isolated viable cells

and nucleated cells was comparable between HC and OI (OI aSVF was 85.6±3.8 and 80.5±5.3% in the control group). Further, type of OI, gender, age, and anatomical site of harvest had no effect on overall number of isolated viable cells. Characterization of the SVF cellular populations revealed significantly fewer endothelial progenitor cells in OI than in controls. Other cell populations such as MSCs ($CD45^-$/$CD34^+$/$CD73^+$/$CD90^+$) were similar between OI and controls. The distribution of SVF cell populations was similar between OI types. Moreover, control and OI MSCs had similar capacity of differentiation into osteogenic, adipogenic, and chondrogenic lineages. These results strengthen the hypothesis that SVF from OI patients' adipose tissue has the potential of being a new source of MSCs for autologous stem cell therapy in OI.

Keywords: Adipose tissue; Sstem cells; stromal vascular fraction; bone; delayed union; non-union; osteogenesis imperfecta.

Résumé

L'ostéogenèse imparfaite (IO) est un trouble héréditaire du tissu conjonctif, souvent associé à une déformation des os longs et à la fragilité des os, qui constitue un obstacle majeur aux activités quotidiennes. Le traitement standard comprend les bisphosphonates et la correction chirurgicale des déformations osseuses avec ostéotomie et baguette intramédullaire. Cependant, l'union tardive ou la non-union du site d'ostéotomie est un événement indésirable fréquent de cette chirurgie, entraînant une douleur, une flexion des tiges de fixation, des fractures répétées et une perte de mobilité. Par conséquent, des solutions alternatives sont nécessaires. Les greffes osseuses ou les cellules souches mésenchymateuses dérivées de la moelle osseuse (BM-MSC) qui ont été étudiées pour la régénération osseuse ont leurs limites. Cependant, une nouvelle source de cellules souches, la fraction vasculaire stromale autologue provenant du tissu adipeux (aSVF), est reconnue comme une source facilement accessible d'un mélange hétérogène de cellules non développées dérivées de tissu adipeux digéré par voie enzymatique et possède des propriétés anti-inflammatoires, angiogéniques, propriétés immunomodulatrices et régénératrices. Les cellules souches mésenchymateuses de l'aSVF (ASC) ont une capacité de différenciation multiple en tant que BM-MSC. Et ses propriétés régénératrices ont été largement étudiées au cours des deux dernières décennies; Cependant, à notre connaissance, le potentiel de guérison et de régénération de l'AVSF n'a pas été exploré dans l'OI. Ici, nous décrivons pour la première fois l'isolement du SVF du tissu adipeux des patients atteints d'OI; et sa caractérisation cellulaire et sa capacité fonctionnelle en tant que nouvelle source de régénération osseuse. Dans cette étude, nous avons obtenu 42 échantillons de tissu adipeux provenant de patients pédiatriques ayant reçu un diagnostic d'OI (38 donneurs; groupe d'âge: 2 à 22 ans;

19 hommes, 19 femmes); ou non OI (témoins; n = 4; tranche d'âge: 4-14 ans; 1 homme, 3 femmes) subissant une chirurgie osseuse corrective à l'Hôpital Shriners pour enfants (Montréal, Canada). Le poids moyen global du tissu adipeux récolté était de 6,70 g dans le groupe IO et de 7,48 g chez les témoins. Et le nombre total de cellules viables isolées et de cellules nucléées était comparable entre HC et OI (OI aSVF était de 85,6 ± 3,8 et 80,5 ± 5,3% dans le groupe témoin). De plus, le type d'OI, le sexe, l'âge et le site de prélèvement anatomique n'avaient aucun effet sur le nombre total de cellules viables isolées. La caractérisation des populations cellulaires de SVF a révélé significativement moins de cellules progénitrices endothéliales chez les IO que chez les témoins. D'autres populations cellulaires telles que les MSC (CD45- / CD34 + / CD73 + / CD90 +) étaient similaires entre l'OI et les témoins. La distribution des populations de cellules SVF était similaire entre les types d'OI. De plus, les CSM témoins et OI avaient une capacité similaire de différenciation en lignées ostéogéniques, adipogènes et chondrogéniques. Ces résultats renforcent l'hypothèse selon laquelle les FSV provenant du tissu adipeux des patients atteints d'OI pourraient constituer une nouvelle source de CSM pour la thérapie par cellules souches autologues dans l'OI.

Mots-clés: tissu adipeux; Cellules souches; fraction vasculaire stromale; OS; union tardive; non-union; ostéogenèse imparfaite.

Frequently Used Abbreviations

aMSCs	Adipose-derived mesenchymal stem cells
ASCs	Adipose-derived stem cells
aSVF	Adipose tissue-derived stromal vascular fraction
CD	Cluster of differentiation
COL1A1/A2	Collagen type
DMEM: F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
EDTA	ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HC	Human control
HLA-DR	Human Leukocyte Antigen – DR isotype
IFATS	International Federation for Adipose Therapeutics and Science
ISCT	International Society for Cellular Therapy
MSCs	Mesenchymal stem cells
OI	Osteogenesis imperfecta
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SVF	Stromal vascular fraction

Contribution of Authors

Dena Bakhsh was the primary author of this thesis reviewed by Dr. Hadil Al-Jallad and with constant input from Dr. Reggie Hamdy. All protocols and experiments were designed and optimized by Dr. Hadil Al-Jallad. Isolation and of the adipose tissue-derived mesenchymal stem cells were performed by Dena Bakhsh. Characterization experiments of the adipose tissue-derived mesenchymal stem cells were performed by Mayumi Umebayashi and Dena Bakhsh. Flowcytometry analysis was performed by Dena Bakhsh with technical support from Marie-Helene Lacombe and Ekaterina Iourtchenko. Statistical analysis was performed by Damian Rauch. Dena Bakhsh and Mayumi Umebayashi, along with Josephine Tauer analyzed all the data presented in this thesis under the supervision of Dr. Hadil Al-Jallad with final input from Dr. Reggie Hamdy.

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Introduction

Discovery of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) represent a class of cells that could be isolated and expanded in culture while maintaining their in vitro capacity to be induced to form a variety of mesodermal phenotypes and tissues [1-8]. Mesenchymal stem cells have been largely investigated over the years for a broad array of clinical applications, hundreds of clinical trials using human MSCs has revealed their potential effectiveness and possibility to treat and cure several conditions [1, 9-13]. Stem cells were first described in the 19th century by Dr. Alexander Maximow as a unique type of cell present in bone marrow (BM) with a potential for self-renewal and differentiation into mature blood cells. This discovery marked the start of a new era in BM transplantation and lymphoproliferative disorders treatment. These cells are known today as the hematopoietic stem cells [14].

After that, non-hematopoietic stem cells were discovered by Dr. Alexander Friedenstein when he placed samples of whole bone marrow in plastic culture dishes and found a unique small number of spindle-shaped cells were adherent to plastic. These cells formed the foci of two to four cells that remained dormant for two to four days and then began to multiply rapidly. However, the most striking feature of the cells was that they had the ability to differentiate into colonies that resembled small deposits of bone or cartilage [15]. The role of those cells was investigated and explained further by multiple researchers [16-18]. These studies established that the isolated cells were multipotent and readily differentiated into osteoblasts, chondroblasts, adipocytes, and even myoblasts. Professor Arnold Caplan explained the role of these cells and proposed the term "mesenchymal stem cells" (MSCs) in the late nineties [7, 18]. In the

20th century, Professor Zuk discovered the presence of MSCs in adipose tissue obtained from elective liposuction procedures. Professor Zuk was able to characterize their multilineage differentiation into osteoblasts, adipocytes, chondroblasts, and myocytes [19].

Moreover, the discovery of MSCs in other areas in the human body has been achieved over the years. MSCs have been isolated from multiple sources including blood, bone marrow, muscle, lung, liver, pancreas, intestine, skin, synovial membrane, embryonic tissue, umbilical cord and placenta [20-26], and adipose/adipose tissues [19, 27]. Adipose tissue has been proven to be rich source of stromal vascular fraction (SVF), which is a collection of heterogeneous cells that give rise to a substantial number of multipotent adipose-derived stem cells (ASCs) [28].

ASCs are becoming the first choice for cell isolation due to the many advantages they have over other stem cell sources. These advantages include accessibility and high cell yields of MSCs, which can be obtained in substantially greater amounts compared with other tissue sources [28, 29]. Moreover, obtaining adipose tissue via liposuction which is a minimally invasive method, associated with a lower morbidity rate compared with bone marrow [30, 31]. It has been also shown that adipose-derived mesenchymal stem cells (aMSCs) are easier to culture, expand more rapidly, and a high percentage retain their stem cell phenotypes and mesenchymal pluripotency during long-term culture [32-34]. Additionally, the use of stem cells isolated from adipose tissue avoids the ethical concerns associated with embryonic stem cells [35, 36].

Over the last two decades, the biologic and clinical interest in aMSCs as risen dramatically as evidenced by the ever-increasing number of research groups studying these cells. The isolation, expansion, characterization, and referring terms of these cells have been reported differently by different groups, leading to confusion in the field. Thus, the International Federation for Adipose Therapeutics and Science (IFATS) adopted the term "adipose-derived stem cells" (ASCs) to identify the isolated, plastic-adherent, multipotent, MSC-like cell population. The IFATS proposed the establishment of minimal criteria for the identification of SVF-derived ASCs in a joint statement with the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT). This was done in an effort to solve the issue of uniform MSC characterization, facilitate the exchange of data among investigators, and make it easier to compare and contrast study outcomes. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, D73, and CD90, and lack expression of CD45, CD34, CD4 or CD11b, CD79a or CD19, and HLA-DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes, or chondroblasts *in vitro* [28, 37].

Moreover, in 2017 Professor Caplan recently urged the need to change the name of MSCs to 'Medicinal Signaling Cells' to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors that are immunomodulatory and trophic (regenerative) meaning that these cells make therapeutic drugs in situ that are medicinal. It is, indeed, the patient's own site-specific and tissue-specific resident stem cells that construct the new tissue as stimulated by the bioactive factors secreted by the exogenously supplied MSCs [38].

Stromal Vascular Fraction

Over the last decade, stem cell research has moved further into the focus of adipose-derived stromal vascular fraction (SVF) capacities and its applications in regenerative medicine [39-45]. Multiple clinical trials indicated the safety and beneficial effects of using SVF for a wide variety of conditions including, but not limited to, wound care, neurologic, cardiac, autoimmune, pulmonological and orthopedic conditions [46-62]. The SVF is defined as a collection of heterogeneous cell populations derived from adipose tissue after enzymatic dissociation and subsequent cell concentration by centrifugation that give rise to a substantial number of aMSCs differentiating along the adipocyte, chondrocyte, myocyte, neuronal, and osteoblast lineages [28, 41, 63]. It is thought that SVF regenerate tissue through a variety of mechanisms, along the presence of different types of cell including ASCs with multilineage differentiation potential. SVF shown Its ability to promote angiogenesis, through secretion of various growth factors such as vascular endothelial growth factor (VEGF) [64, 65], the presence of endothelial progenitor cells [66], supra adventitial cells and the supportive role of ASC with pericytic properties [67]. SVF also shown to display anti-inflammatory effects [68, 69] through the presence of cytokines [70], macrophages [71] and T cells.

Moreover, recent advances in the area of tissue regeneration have put SVF on a par and at times even above MSCs. Some studies showed that SVF treatment is significantly superior to aMSC treatment alone [72] although SVF is similar to aMSCs in its properties such as immunomodulation, anti-inflammatory, and angiogenesis. It is believed that SVF has more advantages over aMSCs for two fundamental reasons: (1) SVF is much more easily acquired and without the need for any cell separation or culturing conditions. Thus, the therapeutic cellular product is instantaneously obtained

and has minimal contact with reagents, making it comparatively safer and subject to less stringent regulatory criteria unlike aMSCs; and (2) the distinctive heterogeneous cellular composition of SVF may be responsible for a better therapeutic outcome as observed in comparative animal studies [72]. Nevertheless, it should be noted that although ASCs find utility in both allogeneic and autologous treatments, SVF, owing to the presence of various cell types known to cause immunological rejection, is suitable for autologous treatments only.

SVF heterogeneity depends on which site the adipose tissue is harvested from and the digestion protocol [28]. The cell populations of SVF consist not only of adipose mesenchymal stromal cells (15–30%), but also comprise many different cell subsets, such as mature endothelial cells (10–20%), progenitor cells (< 0.1%), pericytes (3–5%), monocytes/macrophages (5–15%), hematopoietic stem cells (25–45%), granulocytes (10–15%), and lymphocytes (10–15%) [28, 39, 72-75].

SVF can be isolated from adipose tissue that is either resected as intact tissue or aspirated using tumescent liposuction [28]. Most experimental studies isolate SVF by applying the protocol developed by Zuk et al. [19, 75]. The methods of SVF isolation fall into two categories: (1) an enzymatic method that combines washing and shaking with the use of proteolytic enzymes to assist in tissue dissociation [63, 76], and (2) mechanical methods such as washing, shaking, vibrating, or centrifuging in order to separate stromal cell populations from lipoaspirate samples [41, 72-74]. There are advantages and disadvantages to both methods. The enzymatic methods tend to be ideal for the clinical setting due to the significantly high yield of nucleated cells and its phenotypic cellular composition is superior to the mechanical method as it tends to isolate a higher frequency of stromal/stem cells and lower frequency of cells with

hematopoietic origin. On the other hand, mechanical methods offer the advantage of being cost-effective, less time-consuming, and eliminate the potential of triggering an allergic reaction to the collagenase or unwanted tissue degradation as in the enzymatic method if not adequately removed or neutralized during isolation [41].

Conversely, the adipose tissue-derived mesenchymal stromal/stem cell population adheres to plastic in culture, maintains its mesenchymal phenotype, and its multipotent toward the mesenchymal lineage differentiation *in vitro* into several mesenchymal lineages such as adipocytes, chondrocytes, osteoblasts, and cardiomyocytes [28, 63]. The aMSCs within freshly isolated SVF expresses CD73+, CD90+, and CD34+. Nevertheless, after culturing, cells go through a phenotypic transition as they proliferate and during which they down-regulate CD34 expression and up-regulate CD105 expression; afterwards, these cells are referred to as "mesenchymal stem cells" (aMSCs) [77].

Furthermore, previous literature shows great variation in the isolation, expansion, and phenotypical characterization of SVF cellular composition. As a result, it has become difficult to draw final conclusions on the cell composition of SVF. Thus, the International Federation for Adipose Therapeutics and Science (IFATS) recommended parameters for a basic characterization of both SVF cells and aMSCs in a joint statement with the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) [28]. Viability is recommended to be >70%. And the guidelines for SVF phenotyping indicated the use of a multicolor flow cytometric and cytological analysis by quantifying the co-expression of a viability marker to eliminate dead or apoptotic cells in combination with several negative and positive hematopoietic, non-hematopoietic, and endothelial markers. Primary stable

positive markers for stromal cells are CD13, CD29, CD44, CD73, CD90 (> 40%), and CD34 (> 20%). Primary negative markers for stromal cells are CD31 (< 20%) and CD45 (< 50%). Further, the proposed minimal criteria for SVF-derived aMSC identification are as follows. First, aMSCs must be plastic-adherent when maintained in standard culture conditions. Second, aMSCs must express CD105, D73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. Third, aMSCs must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [28, 37]. The multipotent differentiation potential of SVF-derived aMSCs must be confirmed both at cellular and molecular levels by multilineage differentiation and reverse transcription polymerase chain reaction (RT-PCR).

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a rare hereditary connective tissue disorder, in most patients it's usually caused either by a dominant mutation in one of the collagen type I coding genes, *COL1A1* or *COL1A2*; or by recessive mutations in genes, such as SERPINF1, CRTAP, WNT1, coding for proteins involved in collagen type I biosynthesis, function, transport, or secretion [78-82]. OI is estimated to affect about 1/13,500–15,000 live births [83-85]. OI patients are categorized based on clinical manifestations and inheritance to assess the prognosis and the effect of therapeutic interventions.

The most widely used classification of osteogenesis imperfecta is by Sillence et al. [86], and it distinguishes four clinical types. Rauch and Glorieux have further delineated three additional groups of patients with clinical diagnoses of the disorder but who present clearly distinct features [79, 87-89]. The clinical severity of OI ranges from very mild forms without fractures to severe bone deformity, scoliosis, and recurrent fractures or perinatal lethality. However, the hallmark of all types of OI is increased bone fragility, recurrent long-bone fractures, deformities. The severity of which increases in the order type I < types IV, V, VI, VII < type III < type II. OI clinical manifestations also include short stature and other extraskeletal manifestations which can be associated variably with the disorder and include blue sclera, dentinogenesis imperfecta, hyperlaxity of ligaments and skin, hearing impairment, and the presence of Wormian bones on skull radiographs [90-94].

Unfortunately, no cure exists yet for OI. Cell therapy approach for OI children was initially attempted in 1999 using systematic transplantation of allogeneic bone

marrow-derived mesenchymal stem cells in 3 patients with severe OI [95]. A small proportion of donor derived osteoblast cell was seen on bone biopsies. But, improved parameters of bone formation, increased total body bone minerals content and increased growth velocity about 7 months after the procedure. The same group reported in 2001 that cell transplantation resulted in improved the growth, mobility and reduced fracture incidence. However, the effect is transient and for this reason re-transplantation may be needed [96]. In 2005, pre- and post-natal transplantation of fetal allogenic MSCs was performed in 2 fetuses with severe OI and resulted in improved growth, mobility and fracture incidence for several months [97]. Thereafter in 2014, the study patients received a second post-natal transplantation where the positive effects of the cell therapy was reassumed without any toxicity or alloreactivity toward the donor MSCs [98]. In 2015, a review article summarized the limited experiences currently known in this field (too few patients) and indicated the need for proper studies to accurately determine whether MSC transplantation is of clinical benefit in the treatment of OI [99].

With such an uncorrectable disease-causing genetic defect, the overall goal of OI therapy is to optimize the patient's gross motor abilities and to achieve a level of independence. This is largely accomplished with a multidisciplinary therapeutic approach, by medical treatment with bisphosphonates to reduce bone resorption, induce bone mineralization and reduce the fracture incidence [100, 101]. Physical rehabilitation: and life-long orthopedic interventions such as osteotomies to correct bone deformities, which are the mainstay of treatment as the therapeutic efforts aim to get the most out of mobility and functional capabilities [82, 102-105]. Therefore, the treatment of long bone deformities has a serious impact on OI patients' quality of life.

If left untreated, it may have a serious impact on their ambulation and development [102, 106-109]. Currently, the best-known surgical management is to re-align the long-bone deformity by corrective osteotomies and intra-medullary rodding, specifically the Fassier–Duval rods [101, 108, 110]. This surgical technique usually yields satisfactory results with improvement in ambulation, daily activities, and quality of life [102, 108, 111, 112]. However, in OI, delayed healing and non-union of osteotomy sites, common complications after intramedullary rodding, which can lead to pain, bending of the intra-medullary rod, repeated fractures, loss of ambulation [113-118]. Additional surgical intervention may be required that often involves resection of tissue in the non-union gap, intramedullary fixation, and autogenous bone grafting. So far, fracture healing investigated in different mouse models of OI has revealed a delay in early fracture healing and delayed callus remodeling [119-122]. Improving fracture healing by treatment with either bisphosphonate [122]; antibody against bone-resorption differentiation factor 'RANKL' [120]; or antibody against bone-formation inhibitor 'sclerostin' [121] did not improve fracture healing in those OI mouse models.

In order to repair a fracture, mesenchymal stem cells (MSCs) are recruited to the site of injury and this leads to callus formation that is then replaced by new bone [123, 124]. MSC, present in several tissues including bone marrow (BM) and periosteum, are capable of differentiating into mesenchyme cell types such as osteoblasts, chondrocytes, and adipocytes [125]. In clinic, different strategies for example, local injection of MSCs or in combination with injectable scaffolds have been reported to deliver MSC to the injury site and to regenerate bone [126]. Currently, BM is the primary source of MSC, but BM aspirates alone are not consistent or sufficiently rich in MSCs. Further, aspiration of BM is a highly invasive procedure, and BM-MSCs

lose their ability to proliferate and differentiate with increasing risk of cell senescence [127]. However, to date, one clinical phase 2/3 trial evaluates bone regeneration capacity of *in vitro* expanded autologous BM-MSCs in combination with platelet lysate as treatment of non-unions in adult patients (#NCT02448849; https://www.clinicaltrials.gov/). As alternative to BM, it has been shown that adipose tissue also contains cells that express specific stem cell markers with similar differentiation capacity as BM-MSCs [75, 128]. The main advantage of using adipose-derived mesenchymal stem cells (ASCs) is the accessibility of such cells and simpler isolation procedure [129]. Further, one gram of fat has 1,000 times more stem cells than one gram of BM [126]. Additionally, ASCs were shown to have anti-inflammatory, angiogenic, immunomodulatory, and regenerative properties [130]. In addition, stem cell quality and proliferation capacity do not decline with patient age [19, 131]. Thus, stromal vascular fraction (SVF) of adipose tissue is a promising source of ASCs that can be used to treat non-unions in OI.

However, as OI is a systemic disorder affecting many connective tissues, it is presently unknown whether the cell populations of adipose tissue are altered in OI, and whether ASCs derived from OI patients have multi-differentiation capacity. Further, available protocols for human SVF isolation uses lipoaspirates [28, 75, 132-135]. It remains unclear if SVF and aMSCs isolated from resected adipose tissue of OI patients can be regenerative and has therapeutic potential as that of the healthy individuals. It's also unclear if there will be any variation between the different type of OI mutations *(COL1A1, COL1A2,* LRP5, SERPINF1, WNT1, IFITM5, CRTAP) ASCs in the in vitro, in vivo functional capacities and characteristics. We aim to fill this gap and start to address the challenging problem of delayed healing

and non-union in children with OI by obtaining adipose tissue samples from pediatric with OI and from controls that did not have OI during corrective bone surgery. After optimizing the isolation procedure. And characterizing the OI-derived SVF cell viability; quantify the number of stem cells in a given volume of SVF per gram of adipose tissue to determine the immunophenotypic identity of SVF cellular composition, and analyzed ASCs differentiation capacity into osteogenic, adipogenic, and chondrogenic lineages. We hypothesize that harvesting adipose tissue from patients with OI differ from age and sex is feasible and matched the controls in the SVF composition (frequency of cell subpopulations) and yield a sufficient number of aMSC in the SVF in which the isolated cells identity will be confirmed to be functional and osteogenic.

By defining the phenotypical and functional characteristics of OI adipose-derived SVF, we put the fundamental stone for our long-term clinical goal, which is to combine the powerful regenerative potential of freshly prepared adipose tissue autologous SVF —containing aMSCs— with a state-of the art osteoconductive scaffold (fluoride-coated magnesium) in a one–step surgical approach with intra-operative adipose tissue harvesting, cell isolation, cell seeding onto an osteoconductive scaffold, and subsequent implantation in the patient within a surgical time frame of 2 hours. This will be performed at the end of the initial surgery for the deformity correction, following the osteotomy and insertion of the intra-medullary rod. To use SVF for autologous stem cell transplantation within the same corrective bone surgery is uncertain because of the unknown parallel time frame for isolating ASC-containing SVF from OI patients. However, we strongly believe that this approach would accelerate the healing of the osteotomy site and prevent non-union. This accelerated and improved healing outcome would allow the child and family to have a normal lifestyle.

Materials and Methods

Study population

Samples were collected from patients in the age range of 2–22 years either diagnosed with OI or other disorders (human controls, HC). From August 2017 to November 2018, adipose tissue samples were collected from patients undergoing corrective Orthopaedic surgery at the Shriners Hospital for Children-Canada in Montreal. Ethical permission (McGill Research Ethics and Compliance Committee ID# A02-M15-11A) and informed consent from the patients or legal guards were obtained.

The recruiting of Control patients undergoing corrective bone surgeries at the Shriners Hospitals for Children in Canada (SHC) is challenging given that it is a tertiary center and a world leader in the care of patients with metabolic bone disease. And more than 500 patients with OI are treated and followed in the SHC. These patients have a tendency to develop deformities following the recurrent long bone fracture which accounts for the unbalanced recruited numbers of control patients in comparison with the OI patients.

Adipose-Derived Stromal Vascular Fraction Isolation

Isolation of the SVF was performed according to the optimized method by Tevlin et al. [134] with minor modifications.

Sample Collection

Signed informed consent was obtained from patients' parents/legal guardians before their enrolment in the study. The tissue samples were harvested by the principal investigator, Dr. Raggie Hamdy. Dissection and tissue collection steps were performed in strictly sterile conditions. Once collected, samples were labeled to ensure adequate traceability, picked up by one of the study team members, and transported to the stem cell research laboratory for further processing in compliance with Health Canada regulations.

Sample Preparation

Once received, harvested adipose tissue sample were weighted and incubated in fetal bovine serum (FBS)-free ice-cold culture medium (DMEM:F12 medium supplemented with 10% Qualified Fetal Bovine Serum (FBS) → gibco 12483-020, 1% antibiotic-antimycotic (AA) → gibco 15240-062, and 1% penicillin-streptomycin 10,000 units/mL, streptomycin 10,000 µg/mL (P/S) → gibco 15140-122) for 10 minutes at 4 °C to enhance the separation of adipose tissue from blood.

Adipose Stem Cell Medium Preparation (500 mL)

To prepare the stem cell medium, the following materials were added, mixed well, and kept at 4°C: DMEM:F12 medium (440 mL) (1:1) (X1) → (gibco 11330-032), 10% Qualified Fetal Bovine Serum (50 mL) (FBS → gibco 12483-020), 1% antibiotic-

antimycotic (5 mL) (100X) (AA)→ (gibco 15240-062), and 5 mL 1% penicillin-streptomycin (5 mL) 10,000 units/mL; streptomycin 10,000 µg/mL (P/S) → (gibco 15140-122).

Sample Processing

After 10 minutes in the ice-cold medium, the adipose tissue samples were minced manually into small 1x1 mm pieces using sterile surgical scissors until a homogeneous mixture was attained. If needed, ice cold medium was added to facilitate cutting. The mixture was then homogenized by using a 25 mL Sarstedt serological pipette (Thermo Fisher Scientific) until a milky solution was obtained. Moreover, the mixture was dispensed in a sterile 50 mL conical tube (Thermo Fisher Scientific).,

Collagenase Solution Preparation (10 mL)

The needed volume of digestion-collagenase buffer was calculated according to the sample weight and prepared freshly once ready after mincing the adipose tissue at a ratio of 1:2 (W/V) (gram of adipose tissue: digestion buffer) by dissolving the weighted 10% BSA powder, 0.3 g of BSA/g of adipose tissue (Bovine Serum Albumin powder → SIGMA A7030-50G) in the required volume of 37°C warmed Hank's balanced salt solution (HBSS) medium (HANK'S balanced salt solution → SIGMA H6648) in a 50 mL conical tube (Thermo Scientific). The solution was then vortexed using the mini vortex mixer, filtered using Corning 0.20 µm membrane filters (Sigma Aldrich), and kept in a 37°C water bath for 5 minutes. Added afterwards 2.2 mg/mL collagenase (0.024 g in 10 mL) and 65 µL of collagenase for every 3 mL of the solution/1 g of adipose tissue (collagenase NB 6, GMP grade → SERVA 17458.03).

Adipose Tissue Digestion with Collagenase Solution

The digestion buffer/collagenase solution was prepared as mentioned above and mixed with the homogenized samples in a ratio of 1:2 (weight per volume, W/V; gram of adipose tissue/buffer). The tube was then capped with parafilm, cleaned with alcohol, dried, incubated in the water bath at 37°C for 60 minutes (shorter times will be tested to facilitate future clinical application), with vigorous shaking for 60 seconds every 10 minutes. After digestion was completed, collagenase was neutralized by adding double the amount of collagenase solution of the complete culture medium (DMEM:F12 + 10% Fetal Bovine Serum, 1% P/S, 1% A/A) at a ratio of 1:2 (digestion buffer: complete medium) and mixed gently by pipetting up and down. Afterwards, the mixture was filtered into a new sterile 50 mL conical tube using 100 µm membrane filters (Falcon Cell Strainer, Fisher Scientific) and centrifuged at 700 g X 10 minutes (2.1 rpm) (0.7 rcf). The supernatant was removed by suction while ensuring that the cell pellet was not touched. Then, 5 mL of complete medium were added and mixed gently. A second centrifugation was done at 500 g X 5 minutes (1.8 rpm) (0.5 rcf) and the supernatant was removed by suction while ensuring the cell pellet was not touched. The cell pellet was collected as SVF. After the SVF was obtained, complete medium was added to the pellet and mixed. And the number of nucleated cells counted using trypan blue. Finally, SVF was either prepared for flow cytometric analysis or dispensed at 3000 cells/cm^2 in a sterile flask and incubated at 37°C in growth medium and plated for multilineage *in vitro* differentiation, RNA isolation, and RT-PCR. Figures 1 and 2 illustrate the cycle of sample processing and the schematic of the isolation of the Stromal Vascular Fraction (SVF) from adipose tissue.

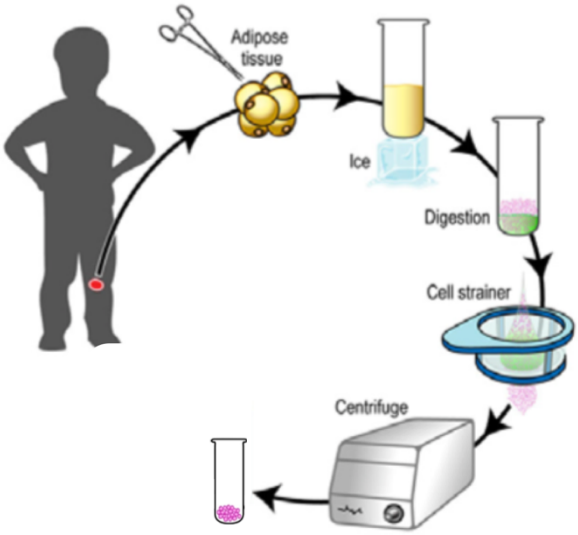

Figure 1. An illustration of the sample proccessing cycle.

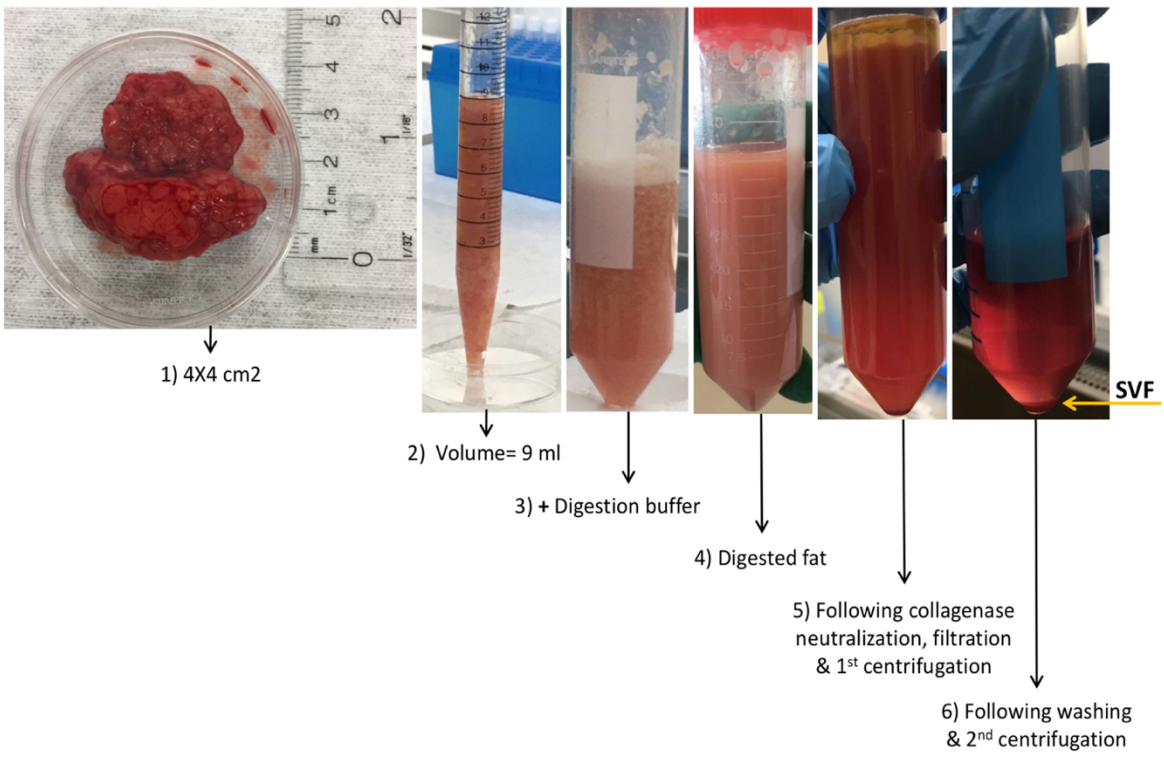

Figure 2. Schematic of the Isolation of the Stromal Vascular Fraction (SVF) from Adipose Tissue.

Cell Counting

A solution at a ratio 1:10 from the re-suspended pellet to Trypan Blue stain solution was prepared. The cells were counted with a hemocytometer. Viable cells appear transparent and reflect light while dead cells are permeable and take up the blue dye; as an alternative, an automatic cell counter might be used in future studies.

Stromal Vascular Fraction Characterization

Characterization of the SVF cell subpopulation by flow cytometry was carried out according to recommendations of the International Federation of Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). After counting, the cells were subjected to two different treatments.

First, the cells were mixed at a certain ratio with magnetic-activated cell sorting buffer to prepare the cells for identification and quantification, i.e., to determine the amounts of specific cell types present in the SVF by using flow cytometry. The isolated stromal cells were screened for CD31, CD45, CD34, CD90 and CD73 to confirm their identity. Mesenchymal Stem Cells must be positive for CD34, CD90, CD73 and negative for CD45 and CD31. Cells with low CD105 expression are more likely to form bone. Second, cells were re-suspended at a cell density of 3000 cells/cm^2 in a sterile flask and incubated at 37°C in growth medium and plated for multilineage *in vitro* differentiation, RNA isolation and RT-PCR.

Immunophenotyping by Fluorescence-Activated Cell-Sorting Fortessa Cell Analyzer

Once the freshly isolated SVF was obtained, it was prepared for identification and quantification to determine the amounts of specific cell types. The needed volume of cell suspension is calculated by dividing the number of cells needed by the number of cells counted. A total of 2 x10^6 nucleated cells per ml were used and divided by 14, which is the number of tubes required for each experiment. Nine compensations and five mixes used to avoid emission overlapping, fluorochrome combinations were distributed into separate independent measurements. The compensation tubes (single stains) included the following: unstained, CD34-APC, CD90-FITC, CD73-PE, CD31-APC-Cy7, CD146-PerCP-Cy5.5, CD45-PE-Cy7, Hoechst 33342, and Viability dye. The mixes tubes included the following: unstained (needed for every sample), Mix 1 (classic MSC markers), Mix 2 (activation marker), pericytes/endothelial progenitors, and M1/M2 (monocytes/macrophages).

The needed cell suspension was centrifuged at 0.6 rfc for 3 minutes. The supernatant was removed, and cells were re-suspended in 700 μL of ice cold magnetic activated cell-sorting (MACS) buffer. The MACS buffer solution was prepared in advance and kept at 4° C by mixing 450 mL phosphate buffered saline (PBS → gibco 1930-106), 2.5 g bovine serum albumin (BSA) using 1.0% BSA to make 0.5% BSA, and 50 mL using 0.1 M ethylenediaminetetraacetic acid (EDTA) to make 10 mM EDTA. Afterwards, cells were stained with Hoechst 33342 trihydrochloride for nucleated cells (Thermo Fisher Invitrogen) and the following panel of fluorochrome-conjugated antibodies were used to identify the targeted cells: CD14-PerCP-Cy5.5 (monocyte marker), CD45-PE-Cy7 (hematopoietic marker) from Thermo Fisher Invitrogen, CD3-PE (T cell marker), CD31-APC-Cy7 (endothelial marker), CD34-APC (MSC marker,

early), CD73-PE (MSC marker), CD90-FITC (MSC marker), CD105-PerCP-Cy5.5 (MSC marker, late), CD146-PerCP-Cy5.5 (pericyte marker), CD206-APC (M2 macrophage marker) from BD Pharmingen, and HLA-DR-APC (BD Pharmingen). Stained cells were then vortexed and incubated at 4˚C for 45 minutes in the dark. Then, 2 mL of MACS+ were added into each tube and centrifuged at 1350 rpm Sorvall H-1000B at 4˚C for 10 minutes and the supernatant discarded. Next, 200 µL of MACS+ were added to each tube.

Immediately before signal acquisition viability dye eFluor 506 and counting beads from BD Pharmingen were added for Mix1, Mix2, pericytes/endothelial progenitors, and monocytes/macrophages. Signals were determined using Fortessa cell analyzer, the multicolor FACS instrument (Figure 3). And analysis by Flow Jow software v.10.1. revealed the immunophenotype and frequency of cellular subpopulations present in the SVF samples. Tables 1 and 2 summarize the used fluorochrome-labeled antibodies and viability markers and the expression of these markers by the targeted cells.

Table 1. Surface markers expressed by the targeted cells within the SVF

What it Stains?	**Stains**
Nucleated cells	Hoechst 33342
Hematopoietic marker	CD45 PECy7
Monocyte marker	CD14 PerCPCy5.5
Macrophage marker	CD206 APC
Endothelial marker	CD31 APCCy7
Pericyte marker	CD146 PerCPCy5.5
MSC marker (early)	CD34 APC
MSC marker	CD73 PE
MSC marker	CD90 FITC
MSC marker (late)	CD105 PerCP Cy5.5
T cell marker	CD3 PE
Activation marker	HLA-DR APC
Counting beads	CountBright absolute counting beads
Dead cells	Fixable Viability Dye eFluor™ 506

Abbreviations: MSC, mesenchymal stem cells. CD; cluster of differentiation.

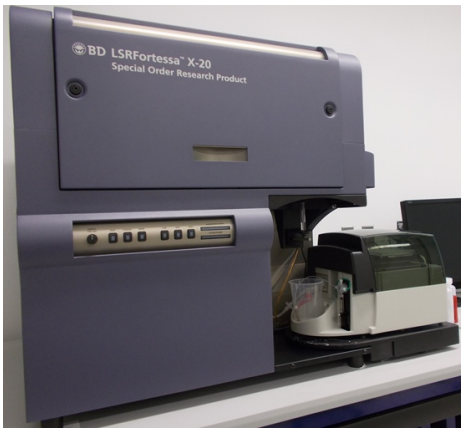

***Figure 3. BD LSR Fortessa X-20*.**

Table 2. Fluorochrome-labeled antibodies to the surface antigens of the targeted cells and viability markers used

Phenotype	CD45	CD3	CD14	CD206	CD31	CD34	CD146	CD73	CD90	CD105
Cells of Hematopoietic Origin	**+**	**+**	**+**	-	-	-	-	-	-	-
Macrophages	**+**	-	**+**	**+**	-	-	-	-	-	-
Monocytes	+	-	+	-	-	-	-	-	-	-
Pericytes	-	-	-	-	**-**	-	+	-	-	-
Endothelial	-	-	-	-	+	+	-	-	-	-
Supra adventitial cells	-	-	-	-	**-**	+	-	-	-	-
Early MSCs	-	-	-	-	-	+	-	+	+	-
MSCs	-	-	-	-	-	-	-	+	+	+

Abbreviations: CD, cluster of differentiation; MSC, mesenchymal stem cells.

Gaiting Strategy

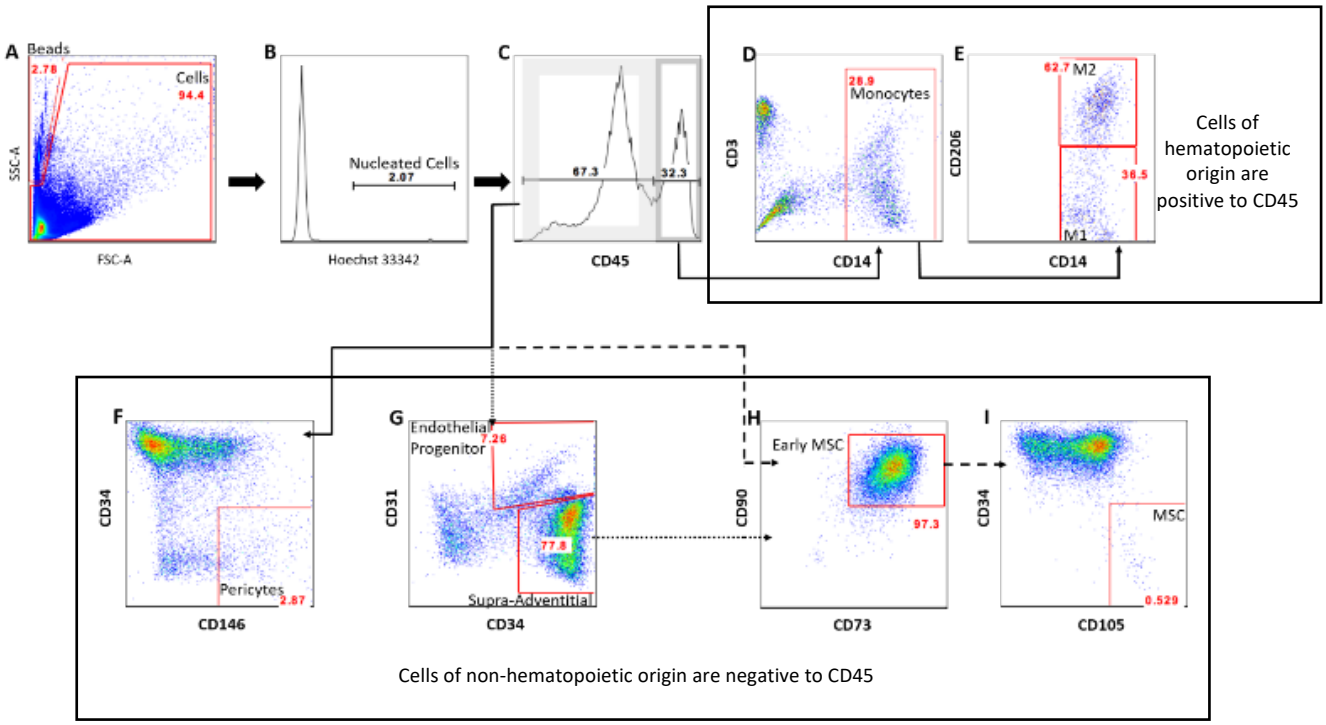

Figure 4. Gating strategy for the analysis of SVF cell populations by flow cytometry.

For our gating strategy for the analysis of SVF cell populations by flow cytometry (Figure 4). *The SVF cell* was stained with different CD markers and dead cells were excluded by using EBioscience fixable viability dye labeling.

(A) Forward and side scatterplot of viable cells (FSC-A axis; in Figure 94.4 % viable) and cell debris (SSC-A axis; in Figure 2.78%).

(B) Analysis of viable cells for live nucleated cells using Hoechst 33342^{+} dye (in Figure 2.07% live). *This step allowed us to identify the cell population and exclude cell debris and cell chunks. In this step, we used the back-gating method to identify our live nucleated cells and confirm our gating strategy. It allowed us to analyze the nucleated cells identified in a gate on dot plots with the live parameter.*

(C) CD45 marker was used to distinguish between non-hematopoietic (in Figure 67.3%) and hematopoietic cell populations (in Figure 32.3%).

(D) Hematopoietic population was further characterized by using CD14 and CD3 markers to separate monocytes/macrophage population (in Figure 28.9%),

(E) which was further characterized by using (E) CD14 and CD206 markers to individualize monocytes ($CD45^+/CD14^+/CD206^-$, in Figure 36.5%) and macrophages ($CD45^+/CD14^+/CD206^+$, in Figure 62.7%).

(F) Non-hematopoietic cell population was characterized by using CD146 marker to identify pericytes ($CD45^-/CD34^-/CD146^+$; 2.87%).

(G) and CD31 marker to separate cells from endothelial origin ($CD45^-$ $CD34^+$ $CD31^+$; 7.26%), and supra-adventitial stromal cells ($CD45^-$ $CD34^+$ $CD31^-$; 77.8%).

(H) Supra-adventitial stromal cells and non-hematopoietic cell population were pooled and further analyzed for the presence of early mesenchymal stem cells ($CD45^-/CD34^+/CD73^+/CD90^+$; in Figure 97.3%) using CD73 and CD90 markers.

(I) Applying the marker CD105, early mesenchymal stem cells were further separated for the presence of activated mesenchymal stem cells ($CD45^-/CD34^+/CD73^+/CD90^+/CD105^+$; in Figure 0.529%).

Multilineage Differentiation and Cell Culture

Directly after isolation, SVF for cell culture was seeded in T25 flasks and non-adherent cells were removed the next day. Cells were harvested by TrypLE Express Enzyme at 80% confluence then seeded at a density of 3000 cells/cm^2 in T75-culture flasks. The medium was replaced every 3 days using control medium (DMEM: F12 (1:1) (X1), 10% Qualified Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, penicillin 10,000 units/mL; streptomycin 10,000 µg/mL (P/S), 1% antibiotic-antimycotic (100X) (AA)).

Cells of passages 2 to 4 were used to examine the capacity of the aMSCs to differentiate along osteogenic, adipogenic, and chondrogenic lineages. Cells were seeded in duplicates at a density of 3000 cells/cm^2 in 6-well plates (Sarstedt Inc.) for histochemical analysis and 12-well plates (Sarstedt Inc.) for RNA extraction. At 80% confluence, culture medium was replaced by osteogenic, adipogenic, or chondrogenic maintenance medium (in the results referred as 'treated') or grown again in culture medium as control cells (in the results referred as 'non-treated'). For osteogenic and adipogenic differentiation, cells were exposed to induction media for 3 days, followed by maintenance media until the end of the experiment. For chondrogenic differentiation, cells were exposed to chondrogenic induction medium during the entire experiment, Media were replaced every 3 days for 21 days. Table 3 summarizes the supplements used for the differentiation medium.

Osteogenic Differentiation

The medium was replaced on the 80% confluent cultures with osteogenic induction medium for osteogenic differentiation. Osteogenic inductive medium is a control medium supplemented with 10 nM dexamethasone (Sigma BCBT5644), 5 mM B- β-glycerol phosphate (SLBV2601 Sigma), and 50 ug/ml Ascorbate-2-Phosphate (SLBN3833V Sigma). Control cultures where fed with control medium. The medium was changed every three days. Cells were exposed to induction media for 3 days, followed by maintenance media until the end of the experiment. On days 7, 14, and 21, cells were fixed in 70% ethanol at −20°C for 1 hour and then osteogenic differentiation was assessed by incubating the fixed cells in 40 mM (pH 7.0) Alizarin Red S stain (MKBS9114V Sigma Aldrich) for 10 minutes at room temperature, followed by washes with distilled water and PBS. Pictures were taken with LEICA DMRB microscope equipped with an Olympus DP70 digital camera, 10x/0.30 PL FLUOTAR objective or 40x/0.70 PL FLUOTAR objective, and the DP controller software. Visualization of mineral deposition into the formed collagenous extracellular matrix at the indicated time points confirmed osteogenic differentiation.

Adipogenic Differentiation

The medium was replaced on the 80% confluent cultures with adipogenic induction medium for adipogenic differentiation. Confluent cultures were incubated in adipogenic induction medium. Adipogenic medium is a control medium supplemented with 10 ug/ml Insulin (16634 Sigma), 1 uM Dexamethasone (Sigma BCBT5644), and 500 uM Isobutyl-Methylxanthine (IBMX) (I5879 Sigma), whereas the maintenance medium contained only 1 mg/mL insulin (16634 Sigma). Induction was performed by culturing cells for three days in induction medium and then in maintenance medium

while the control cultures where fed with control medium. The medium was changed every three days. Adipogenesis was assessed by Oil Red staining: cells fixed in 4% formalin at room temperature for 1 hour. And then incubated in fresh Oil Red stain (N1142 Invitrogen) for 15 minutes at room temperature. Pictures were taken with LEICA DMRB microscope equipped with an Olympus DP70 digital camera, 10x/0.30 PL FLUOTAR objective or 40x/0.70 PL FLUOTAR objective, and the DP controller software. Visualization of lipid droplet accumulation is indicative of adipogenic differentiation.

Chondrogenic Differentiation

The medium was replaced on the 80% confluent cultures with chondrogenic induction medium for chondrogenic differentiation. Chondrogenic medium is a control medium supplemented with 10 nM dexamethasone (Sigma BCBT5644), 50 ug/ml Ascorbate-2-Phosphate (SLBN3833V Sigma), 10 ng/ml TGF-β3 (MKBW3520V Sigma), 40 ug/ml Proline (SLBQ5606V Sigma), and 50 ug/ml ITS (SLBS7045 Sigma). Control cultures where fed with control medium. Cells were exposed to chondrogenic induction medium during the entire experiment, Media were replaced every 3 days for 21 days. On day 21, cells were fixed in 4% formalin for 1 hour at room temperature and then chondrogenic differentiation was assessed by incubating the fixed cells in Alcian blue stain for 120 minutes at room temperature. Pictures were taken with LEICA DMRB microscope equipped with an Olympus DP70 digital camera, 10x/0.30 PL FLUOTAR objective or 40x/0.70 PL FLUOTAR objective, and the DP controller software. Visualization of a red color is indicative of chondrogenic differentiation.

Table 3. A summary of the supplements used for the multilineage Cell Culture Media.

Media	**Composition**
Osteogenic induction media	DMEM: F12
	10% FBS
	1% penicillin-streptomycin
	1% antibiotic-antimycotic
	10 nM dexamethasone
	10 mM β-glycerol phosphate
	50 µg/mlascorbic-acid-2-phospahate
Osteogenic maintenance media	DMEM: F12
	10% FBS
	1% penicillin-streptomycin
	1% antibiotic-antimycotic
	10 nM dexamethasone
	5 mM β-glycerol phosphate
	50 µg/ml ascorbic-acid-2-phospahate
Adipogenic induction media	DMEM: F12
	10% FBS
	1% penicillin-streptomycin
	1% antibiotic-antimycotic
	10 µg/ml insulin
	1 µM dexamethasone
	500 µM 3-isobutyl-1-methylxanthine
Adipogenic maintenance media	DMEM: F12
	10% FBS
	1% penicillin-streptomycin
	1% antibiotic-antimycotic
	10 µg/ml insulin
Chondrogenic induction media	DMEM: F12
	10% FBS
	1% penicillin-streptomycin
	1% antibiotic-antimycotic
	10 nM dexamethasone
	50 µg/ml ascorbic-acid-2-phospahate
	10 ng/ml recombinant human TGF-β3
	40 µg/ml L-proline
	50 µg/ml ITS liquid media supplement

Abbreviations: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12); FBS, fetal bovine serum.

Quantification of Deposited Minerals

For quantification of deposited minerals, alizarin red stained cells were washed with deionized water and stain dissolved in 10% acetic acid (EMD Millipore). Absorbance was measured at 405 nm wavelength using a microplate reader (VICTOR Nivo, PerkinElmer). The amount of calcium deposits is directly proportional to the intensity of Alizarin red stain (red color).

RNA Extraction and Real-time qPCR Analyses

On days 7 and 21, total RNA was extracted by using TRIzol™ Reagent (15596018 Thermo Fisher Scientific) according to the manufacturer's protocol.
The purified RNA (500 ng/mL) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) in the presence of RNase inhibitor (Applied Biosystems) under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 10 minutes. Real-time PCR assessment of gene expression was performed with 25 ng of cDNA using an ABI 7500 Real-Time PCR Machine, and the 12.5 µL TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 µL human FAM labelled TaqMan® gene expression primers. (Applied Biosystems), and 6.25 µL Milli-Q water under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 15 seconds of denaturation at 95°C, and 1 minute of annealing and elongation at 60°C. TaqMan probes are runx2(Hs01047973-m1) and ALPL(Hs01029144-m1) for osteogenic differentiation, pparg (Hs01115513-m1), leptin(Ha00174877-m1), and LEPR(Hs00174497-m1) for adipogenic differentiation, sox9(Hs00165814-m1), ACAN(Hs00153936-m1) and col10(Hs00166657-m1) for chondrogenic differentiation, and *β-actin* (Hs01060665-g1) for a housekeeping gene (Applied Biosystems). Beta-actin was used as

endogenous control. Gene expression was analyzed according to the delta-delta Ct method. It represents fold-changes of differentiated samples (treated) to non-differentiated samples (non-treated).

Statistical Analyses

Unless stated otherwise, data presented here are shown as mean ± SEM (minimum - maximum). Data were evaluated for normal distribution using Shapiro-Wilk test. Non-normal distributed data were evaluated either using Mann -Whitney-Wilcoxon U-Test for 2 independent parameters such as HC vs OI, gender, and anatomical site, or using Kruskal-Wallis-H test for evaluation of statistical difference for >2 independent parameters like age-dependency, type of OI, and disease-causing collagen mutation. Normally distributed data were evaluated using independent t-test for 2 independent samples or one-way ANOVA for <2 independent samples. Calculations were performed using SPSS software (v 24.0; SPSS Inc). Statistical differences in gene expression between treated and non-treated samples were analyzed by paired t-test. Significant differences in genetic fold-change of OI versus HC were assessed by unpaired t-test. $P < 0.05$ was considered significant. The graphical illustration was generated by GraphPad Prism version 8.1.1 (GraphPad Software, San Diego, California, USA, www.graphpad.com).

Table 4. Key Resource

Reagent	Source	Catalog number
Chemicals and reagents		
TRIzol Reagent	Ambion	15596018
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	4368813
RNase Inhibitor	Applied Biosystems	N8080119
TaqMan Universal PCR Master Mix	Applied Biosystems	4364340
TrypLE Express Enzyme	Gibco	12604012
Bovine serum albumin (BSA)	Sigma-Aldrich	A7030-50G
EDTA	Fisher Chemical	S311-500
Trypan blue 0.4%	Gibco	T8154-100ML
Fetal bovine serum (FBS)	Gibco	12483020
Low glucose Dulbecco's Modified Eagle Medium Nutrient Mixture F-12(Ham) (DMEM:F12)	Gibco	11330032
Collagenase NB 6, GMP grade	SERVA	17458.03
HANK'S balanced salt solution (HBSS)	Sigma-Aldrich	H6648-1L
PBS pH7.4 (1x)	Gibco	10010023
Penicillin-streptomycin	Gibco	15140122
Antibiotic-antimycotic	Gibco	15240062
10% Buffered Formalin Acetate	Fisher Chemical	SF99-4
Ethanol	Greenfield Global	P016EAAN
Dexamethasone	Sigma-Aldrich	D4902-100MG
β-glycerol phosphate	Sigma-Aldrich	G9422-50G
Ascorbic-acid-2-phospahate	Sigma-Aldrich	A8960-5G
Insulin	Sigma-Aldrich	I2643-0MG
3-isobutyl-1-methylxanthine	Sigma-Aldrich	I5879-1G
Recombinant human transforming growth factor-beta 3 (TGF-β3)	Prospec	CYT-368
L-Proline	Sigma-Aldrich	P5607-25G
ITS liquid media supplement	Sigma-Aldrich	I3146-5ML
Acetic Acid	EMD	AX0079-2
Antibodies		
CD34-APC	BD Pharmingen Inc	555824
CD90-FITC	BD Pharmingen Inc	555595
CD73-PE	BD Pharmingen Inc	550257
CD31-APC-Cy7	BD Pharmingen Inc	563653
CD146-PerCP-Cy5.5	BD Pharmingen Inc	562134
CD45-PE-Cy7	BD Pharmingen Inc	560915
CD105-PerCP-Cy5.5	BD Pharmingen Inc	560819
CD14-PerCP-Cy5.5	BD Pharmingen Inc	562692
CD206-APC	BD Pharmingen Inc	561763

CD3-PE	BD Pharmingen Inc	555333
CountBright absolute counting beads	Thermo Fisher Invitrogen	C36950
Fixable Viability Stain eFluor506	Thermo-Fisher	65086614
Hoechst 33342 trihydrochloride	Thermo-Fisher	H3570
Staining solutions		
Alizarin Red S stain	Sigma-Aldrich	A5533-25G
Oil Red staining	Sigma-Aldrich	O0625-25G
Alcian blue staining	Sigma-Aldrich	A5268-10G
Primers		
Sox9	Applied Biosystems	Hs00165814-m1
OCN	Applied Biosystems	Hs01587814-g1
Runx2	Applied Biosystems	Hs01047973-m1
ALPL	Applied Biosystems	Hs01029144-m1
PPARg	Applied Biosystems	Hs01115513-m1
Leptin	Applied Biosystems	Ha00174877-m1
LEPR	Applied Biosystems	Hs00174497-m1
Sox7	Applied Biosystems	Hs00846731-s1
ACAN	Applied Biosystems	Hs00153936-m1
Col10	Applied Biosystems	Hs00166657-m1
β-actin	Applied Biosystems	Hs01060665-g1

Results

Characteristics of the Study Population:

Btween August 2017 and November 2018, adipose tissue samples were harvested and analyzed from 42 patients (age range: 2–22 years; 21 males, 21 females) during corrective Orthopaedic surgery at the Shriners Hospital for Children, Montreal, Quebec, Canada. Thirty-eight samples were harvested from patients diagnosed with OI (OI-I, n=1; OI-III, n=10; OI-IV, n=20; OI-V, n=2; OI-VI, n=2; OI-VII, n=3) (age range: 2–22 years; 19 males, 19 females) and four samples from HC (genu valgum, genu varum, acetabular dysplasia, fibular hemimelia with symmetrical amputation), (age range: 4–14 years; 1 male, 3 females). Demographics of Study Cohort shown in Table 5. Of the 42 samples, eleven were from OI patients with mutations mostly in the *COL1A1* gene, eighteen from patients with *COL1A2* gene mutation, nine from patients with recessive mutations (including LRP5, SERPINF1, WNT1, IFITM5, CRTAP), and four from control patients with other diagnoses. Twenty-two of the adipose tissue samples were harvested from the patients' thighs, fourteen from the patients' legs, and four from both sides.

	Sex	Age (years)	Diagnosis	Mutated gene	Nucleotide Change	Anatomical site of the harvested adipose tissue	Adipose tissue weight (g)	Total viable cell yield ($x10^6$)	Cells per gram of adipose tissue ($x10^4$)
1	F	10	HC	N/A	N/A	Tibia L	2.5	0.75	30
2	F	4	HC	N/A	N/A	Hip R	3.4	1.4	40
3	M	14	HC	N/A	N/A	Tibia R	5.2	5.0	96
4	F	12	HC	N/A	N/A	Tibia R	18.8	2.1	19
5	M	3	OI-IV	COL1A2	c.911G>T	Femur R	1.8	0.56	32
6	F	6	OI-IV	COL1A1	c.4325_4335delTGGCCCCCTTG	Tibia R	3.6	2.0	55
7	F	6	OI-IV	COL1A1	c.4325_4335delTGGCCCCCTTG	Femur R	7.7	6.0	78
8	F	5	OI-IV	COL1A1	C.2461G>A	Femur R	4.0	0.67	17
9	F	2	OI-IV	COL1A1	c.994G>A	Femur and Tibia L	2.6	3.3	125
10	F	6	OI-IV	COL1A1	c.4325_4335delTGGCCCCCTTG	Tibia L	5.8	2.6	45
11	M	14	OI-IV	COL1A1	c.3208-2_3213del	Femur and Tibia R	8.6	8.1	95
12	M	9	OI-IV	COL1A1	c.2461G>A	Femur R	15	9.0	58
13	F	5	OI-III	COL1A2	c.3277G>T	Tibia R	4.7	4.1	88
14	F	4	OI-VII	CRTAP	C.472-1021C>G	Femur R	8.0	0.94	12
15	M	3	OI-IV	COL1A2	C.911G>T	Femur R	3.4	2.7	80
16	M	13	OI-VI	SERPINF1	c.295C>T	Femur R	7.3	1.6	22
17	M	21	OI-IV	COL1A2	c.2008G>C	Tibia R	1.2	1.4	117
18	M	21	OI-IV	COL1A2	c.2008G>C	Femur R	2.3	1.5	66
19	M	22	OI-IV	COL1A2	c.2008G>C	Femur and Tibia L	4.4	2.7	62
20	F	3	OI-I	COL1A1	c.1792C>T	Femur R	6.6	2.3	35
21	F	2	OI-IV	WNT1	BENDING	Femur R	2.0	3.5	175
22	M	5	OI-III	COL1A2	c.1756G>A	Tibia R	2.6	1.7	67
23	M	5	OI-III	COL1A2	c.1756G>A	Femur R	6.2	1.9	32
24	M	10	OI-IV	COL1A1	c.2461G>A	Femur L	29	2.0	6.9
25	M	5	OI-III	COL1A2	c.1756G>A	Femur and Tibia L	6.4	2.5	42
26	M	9	OI-IV	COL1A2	c.982G>A	Femur R	4.4	1.3	28
27	M	6	OI-IV	COL1A2	c.1031_1033del	Tibia L	0.9	0.14	15
28	M	8	OI-III	COL1A2	c.2297G>A	Tibia R	8.4	1.8	21
29	M	5	OI-IV	COL1A2	c.2305G>T	Femur R	7.0	1.4	20
30	F	2	OI-IV	COL1A1	c.1093G>A	Femur R	5.5	1.4	25
31	F	8	OI-IV	COL1A1	c.3226G>A	Femur R	36	10	28
32	M	5	OI-VII	CRTAP	c.472-1021C>G	Tibia R	2.0	0.25	1.3
33	F	5	OI-VII	CRTAP	C.472-1021C>G	Femur R	13	1.4	13.8
34	M	10	OI-III	COL1A1	c.2596G>A	Tibia R and L	4.7	0.5	10
35	F	5	OI-III	COL1A2	c.1964G>A	Femur R	6.8	5.5	80.8
36	M	4	OI-IV	COL1A2	c.911G>T	Tibia L	2.6	4.9	10.8
37	M	2	OI-VI	SERPINF1	c.271_279dup	Femur L	6.6	0.31	7.4
38	F	16	OI-III	COL1A2	c.1378G>A	Femur R	4.5	3.4	7.6
39	F	16	OI-III	COL1A2	c.1378G>A	Femur R	4.5	3.7	8.2
40	F	16	OI-III	COL1A2	c.1378G>A	Femur R	4.5	2.5	5.5
41	F	14	OI-V	IFITM5	c.-14C>T	Tibia L	5.0	0.63	12.5
42	F	14	OI-V	IFITM5	c.-14C>T	Tibia L	5.0	0.75	15

Table 5. Demographics of Study Cohort

HC, human control; R, right side; L, left side.

Isolation and Cellular Outcome of The Stromal Vascular Fraction from Pediatric Adipose Tissue:

The overall mean weight of harvested adipose tissue was 6.70 g (SEM: 1.11 g) in the OI group and 7.48 g (SEM: 3.82 g) in controls. Within about 90 minutes, adipose tissue samples were processed to obtain SVF from HC and OI donors. The overall average number of nucleated cells per gram of adipose tissue was 426,000 cells per gram tissue (SEM: 6.43x10^4cells/g) in OI and 462,000 cells per gram tissue (SEM: 17.13 x10^4cells/g) in controls, Characteristics of isolated adipose tissue samples Table 6. Cell outcomes did not vary significantly with sex, age, type of OI, disease-causing collagen mutation, or anatomical site of harvest Table 7.

Table 6. Characteristics of isolated adipose tissue samples

	HC patients (N=4)	**OI patients (N=38)**	**P–value**
Adipose tissue weight (g)	7.48±3.82 (2.50–18.8)	6.70±1.11 (0.9–36.0)	0.88
Number of cells per gram of adipose tissue (x10^4)	46.25±17.13 (19.0–96.0)	42.62±6.43 (1.25–175)	0.49
Total viable cells yield (x10^6)	2.31±0.94 (0.75–5.0)	2.65±0.38 (0.14–10)	0.95

Data represent mean ± SEM (minimum - maximum). P values represent significance of the difference between HC and OI patients using Mann-Whitney-U-Test.

Table 7: Statistical Analysis of Harvested Adipose Tissue Weight and Isolated Number of Cells of OI and HC Patients

		Adipose tissue weight (g)		**Total viable cell yield (x10^6)**		**Number of cells per gram of adipose tissue (x10^4)**	
	N	**Median (25^{th}–75^{th} P)**	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**
HC vs OI							
All	42	4.85 (3.20–7.08)	0.88	1.94 (1.17–3.45)	0.95	29 (13.44–66.25)	0.49

HC	4	4.3 (2.73–15.4)		1.75 (0.91–4.28)		35 (21.75–82)	
all OI	38	4.85 (3.20–7.08)		1.94 (1.17–3.45)		28 (12.38–66.25)	
Male vs female							
All	42	4.85 (3.20–7.08)	0.49	1.94 (1.17–3.45)	0.30	29 (13.44–66.25)	0.85
Male	21	4.70 (2.45–7.15)		1.74 (0.91–2.70)		32 (12.90–66.50)	
Female	21	5 (3.8–7.25)		2.3 (1.16–3.60)		28 (13.13–66.50)	
Age–dependency							
All	42	4.85 (3.20–7.08)	0.12	1.94 (1.17–3.45)	0.64	29 (13.44–66.25)	0.10
Prepubertal (1–12 years)	31	5.5 (2.60–7.70)		1.88 (0.94–3.25)		30 (15–58)	
Pubertal (13–16 years)	8	5 (4.5–6.78)		2.95 (0.96–4.67)		13.75 (7.77–76.75)	
Post pubertal (<16 years)	3	2.3 (1.2–4.4)		1.5 (1.4–2.7)		66 (62–117)	
Type of OI							
All	38	4.85 (3.2–7.08)	0.69	1.94 (1.17–3.45)	0.10	28 (12.38–66.25)	0.09
OI–IV	20	4.2 (2.38–7.53)		2.32 (1.36–4.53)		50 (21.25–79.5)	
OI–III	10	4.7 (4.5–6.5)		2.48 (1.77–3.8)		26.5 (8.05–70.45)	
OI–I	1	NA		NA		NA	
OI–V	2	5 (3.75–5.66)		0.69 (0.47–2.47)		13.75 (9.38–13.16)	
OI–VI	2	6.95 (4.95–5.48)		0.96 (0.23–1.2)		14.72 (5.57–16.5)	
OI–VII	3	8 (2–13)		0.94 (0.25–1.38)		12 (1.25–13.75)	
Disease causing gene mutation							
All	38	4.85 (3.2–7.08)	0.06	1.94 (1.17–3.45)	0.17	28 (12.38–66.25)	0.28
COL1A1	11	5.84 (4.00–8.60)		2.3 (1.40–6.00)		35 (17–78)	
COL1A2	18	4.45 (2.53–6.25)		2.17 (1.39–3.5)		32 (13.95–70.25)	
Others [$]	9	6.6 (3.5–10.5)		0.94 (0.47–2.55)		13.75 (9.72–40)	
Anatomical side: left vs right							

All	42	4.85 (3.20–7.08)	0.80	1.94 (1.17–3.45)	0.22	29 (13.44–66.25)	0.18
Left	12	5 (2.6–6.55)		1.69 (0.66–2.68)		15 (11.23–44.25)	
Right	30	4.7 (3.4–7.4)		1.94 (1.39–3.80)		30 (18.5–78.5)	
Anatomical side: femur vs tibia							
All	36	5 (2.80–7.23)	0.07	1.83 (1.02–3.48)	0.38	26.5 (12.81–64)	0.65
Femur	22	6.4 (4.3–7.78)		1.94 (1.33–3.55)		26.5 (11.05–60)	
Tibia	14	4.15 (2.38–5.36)		1.76 (0.72–3.00)		25.5 (14.37–72.25)	

Data given in median (25^{th}–75^{th} percentiles). Shapiro-Wilk test indicated that all data collected for each parameter are not distributed normally. Therefore, parameters with 2 independent samples were evaluated using the Mann-Whitney-Wilcoxon U-Test and parameters with <2 independent samples were evaluated using Kruskal-Wallis-H Test. NA, not applicable, SERPINF1, IFITM5, Wnt1

Quantification of Cell Subpopulation of The Stromal Vascular Fraction:

FlowJo, software analysis of the acquired aSVF using the multicolor FACS instrument. Following the gating strategy illustrated previously in the method section, the analysis revealed the immunophenotype and frequency of cellular subpopulation present in the aSVF samples. Distribution analysis of SVF cell populations revealed similar number of viable cells in OI and HCs (Table 8). The overall average percentage of viable cells in OI aSVF was 85.6±3.8 and 80.5±5.3% in the control group (*P*-value: 0.30). The average frequency of each subcellular population (the proportion of cell type relative to the total number of viable nucleated cells) in the SVF of OI and compared to the controls is as follows: Early mesenchymal stem cells ($CD45/CD34^{+}/CD73^{+}/CD90^{+}$), were 35.6±4.9 % of the OI aSVF and 34.8±2.8 % of the HC (*P*-value: 0.95), and the activated mesenchymal stem cells ($CD45^{-}/CD73^{+}CD90^{+}CD34^{+}CD31^{-}$) represented about 0.04±0.02 % of the entire viable population of the OI aSVF and 0±0 % of the HC (*P*-value: 0.47). The endothelial progenitor cells (EPC = $CD45^{-}/CD34^{+}/CD31^{+}$) were 5.4±1.4 in OI aSVF and 12.6±4.9

% in the HC (*P*-value: 0.05). And pericytes ($CD45^-/CD34^-/CD146^+$) were about 3.7±1.4 % of the OI aSVF and 4.0±1.7 % of the HC (*P*-value: 0.32). supra-adventitial cells ($CD45^-/CD31^-/CD34^+/ CD146^-$) were 38.2±5.2 % of the OI aSVF and 41.8±5.0 % of the HC (*P*-value: 0.63). Monocytes ($CD45^+/CD14^+/CD206^-$) were 2.6±0.8 % of the OI aSVF and 3.6±2.2 % of the HC (*P*-value: 0.53), whereas macrophages ($CD45^+/CD14^+/CD206^+$) represented about 4.2±0.9 % of the OI aSVF and 2.2±0.3 % of the HC (P-value: 0.60). Hematopoietic cells ($CD45^+$) were 15.6±1.7 % of the OI aSVF and 17.4±2.5 % of the HC (*P*-value: 0.69). To summarize, the composition of aSVF subcellular population were similar between HC and OI patients except for the endothelial progenitor cells which were found to be significantly lower in OI than in HC samples (p=0.05), Quantified Cellular Subpopulations of the Stromal Vascular Fraction of Human Controls and OI Patients Figure 5.

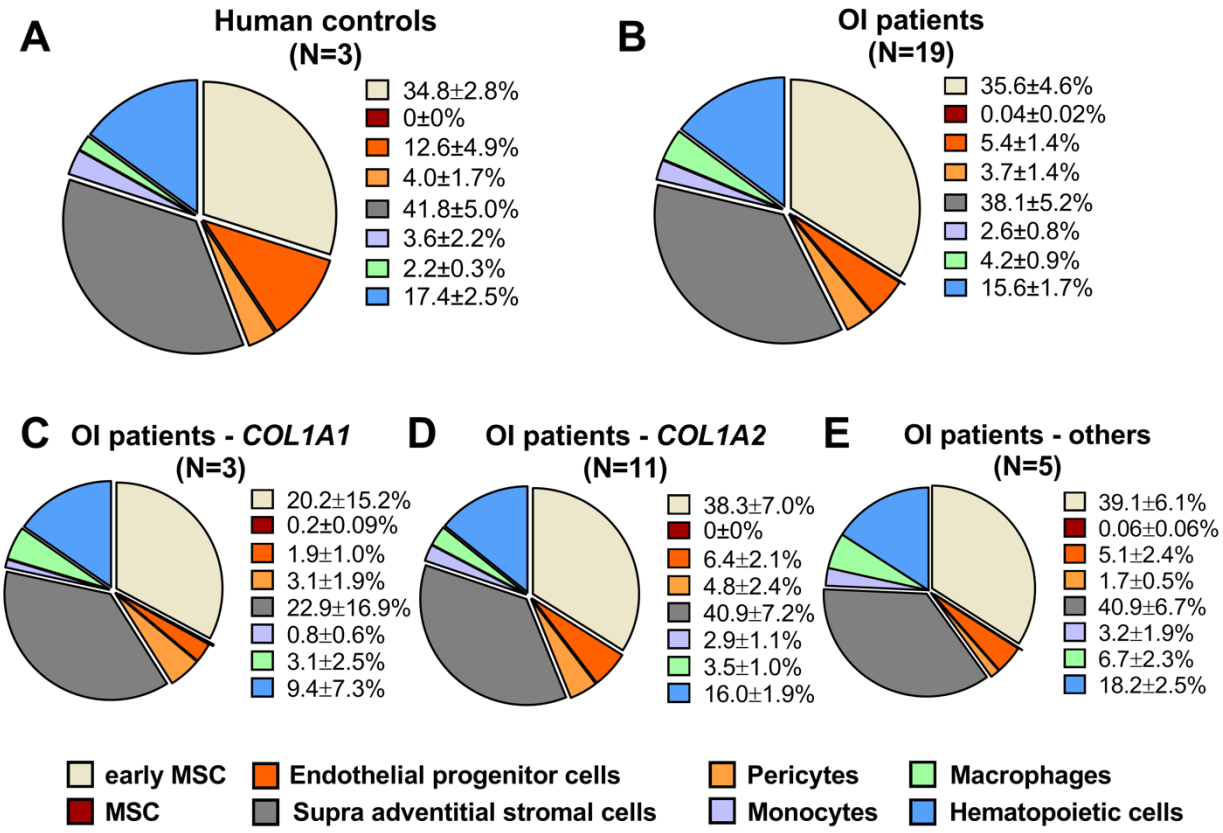

Figure 5: Quantified Cellular Subpopulations of the Stromal Vascular Fraction of Human Controls (A) and OI Patients (B).

Panel C, D, and E depicts cellular subpopulations of the stromal vascular fraction of OI patients according to genetic mutation. Others: OI patients with mutation in either SERPINF1, CRTAP, and WNT1. Values represent mean±SEM. MSC, mesenchymal stem cells.

Table 8. Composition of the Stromal Vascular Fraction Cell populations absolute frequencies descriptive statistics

	HC patients (N=3)	**OI patients (N=19)**	**P–value**
Viability	80.5±5.3% (71.8–90.2)	85.6±3.8 (44.3–97.6)	0.30
Early mesenchymal stem cells	34.8±2.8 % (30.0–39.9)	35.6±4.9 % (2.2–85.5)	0.95
Activated mesenchymal stem cells	0±0 % (0–0)	0.04±0.02 % (0–0.33)	0.47
Endothelial progenitors	12.6±4.9 % (4.4–21.4)	5.4±1.4 % (0.4–20.3)	0.05
Pericytes	4.0±1.7 % (1.2–6.9)	3.7±1.4 % (0.1–24.7)	0.32
Supra-adventitial stromal cells	41.8±5.0 % (32.7–49.9)	38.2±5.2 % (2.6–87.2)	0.63
Monocytes	3.6±2.2 % (0.3–7.9)	2.6±0.8 % (0.04–11.4)	0.53
Macrophages	2.2±0.3 % (1.7–2.8)	4.2±0.9 % (0.3–13.4)	0.60
Hematopoietic cells	17.4±2.5 % (12.8–21.5)	15.6±1.7 % (0.6–29.3)	0.69

Data represent mean±SEM (minimum - maximum). P values represent significance of the difference between HC and OI patients using Mann-Whitney-U-Test.

Statistical evaluation of the effect of age, sex, type of OI, disease-causing collagen mutation, and anatomical side of harvest on cell viability and cellular composition revealed i) significantly higher cell viability in SVF from OI type III donors (p=0.02); ii) lower number of endothelial progenitors in female donors (p=0.004); iii) higher amount of early ASCs in SVF from male donors (p=0.02) and in SVF from prepubertal (age 1–12 years) donors; iv) higher amount of supra-adventitial stromal cells in post pubertal donors (age < 16 years, p=0.02); and v) higher amount of pericytes in pre- and post-pubertal donors (p=0.02), **Table 9**. However, the anatomical site of harvest had no effect on cell viability and cellular composition, **Table 10.**

Table 9. Continuing Statistical Analysis of SVF Composition of OI and HC Patients

	N	**Activated mesenchymal stem cells** (CD45–/CD34+/CD73+/CD90+/CD105+)		**Progenitor endothelial cells** (CD45–CD34+CD31+)		**Pericytes** (CD45–/CD34–/CD146+)		**Monocytes** (CD45+/CD14+/CD206–)		**Macrophages** (CD45+/CD14+/CD206+)	
		Median (25^{th}–75^{th} P)	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**	**Median (25^{th}–75^{th} P)**	**P–value**
HC vs OI											
All	22	0 (0–0)	0.47	3.18 (1.62–11.11)	**0.05**	1.78 (0.70–4.17)	0.32	1.39 (0.27–3.67)	0.53	2.7 (1.17–4.71)	0.6
HC	3	0 (0–0)		11.97 (4.41–21.42)		3.97 (1.18–6.91)		2.64 (0.3–7.89)		2.17 (1.71–2.83)	
OI	19	0 (0–0)		3.00 (1.39–6.37)		1.63 (0.64–2.89)		1.07 (0.16–3.18)		3.24 (1.15–6.52)	
Female vs male											
All	22	0 (0–0)	0.1	3.18 (1.62–11.11)	**0.004**	1.78 (0.70–4.17)	0.74	1.39 (0.27–3.67)	0.06	2.7 (1.17–4.71)	0.29
Female	12	0 (0–0.12)		2.01 (1.31–3.60)		2.05 (0.30–6.18)		0.54 (0.10–2.49)		2.20 (0.79–5.92)	
Male	10	0 (0–0)		8.60 (3.24–19.52)		1.78 (0.75–3.26)		2.46 (0.52–7.16)		3.29 (1.57–5.94)	
Age–dependency											
All	22	0 (0–0)	0.32	3.18 (1.62–11.11)	0.34	1.78 (0.70–4.17)	**0.02**	1.39 (0.27–3.67)	0.07	2.7 (1.17–4.71)	0.17
Prepubertal (1–12 years)	13	0 (0–0.08)		3.304 (1.94–8.20)		2.89 (0.75–6.94)		1.07 (0.33–2.91)		2.84 (1.16–5.31)	
Pubertal (13–16 years)	6	0 (0–0)		2.01 (1.34–10.13)		0.34 (0.13–1.36)		0.46 (0.08–3.64)		2.83 (0.76–5.03)	

Post pubertal (<16 years)	3	0 (0–0)		10.82 (3.32–19.26)		2.57 (1.63–2.75)		6.92 (2.71–11.38)		3.85 (3.74–12.23)	
Type of OI											
All	19	0 (0–0)	0.51	3 (1.39–6.37)	0.51	1.63 (0.64–2.89)	0.15	1.07 (0.16–3.18)	0.36	3.24 (1.15–6.52)	0.36
OI–IV	9	0 (0–0.08)		3.32 (2.17–15.04)		2.75 (1.55–5.88)		2.06 (0.62–6.02)		3.74 (0.99–6.14)	
OI–III	6	0 (0–0)		2.41 (1.34–3.15)		0.44 (0.13–4.29)		0.25 (0.08–1.19)		2.39 (0.76–4.13)	
OI–VII	2	0.16 (0–0.24)		7.25 (0.47–10.40)		1.69 (0.56–1.98)		5.65 (0.37–8.10)		6.73 (2.43–7.66)	
Disease causing genetic mutation											
All	19	0 (0–0)	**0.02**	3.00 (1.39–6.37)	0.52	1.63 (0.64–2.89)	0.97	1.07 (0.16–3.18)	0.43	3.24 (1.15–6.52)	0.24
COL1A1	3	0.16 (0–0.33)		1.39 (0.38–3.78)		1.46 (0.72–6.97)		0.16 (0.06–2.06)		0.83 (0.31–8.17)	
COL1A2	11	0 (0–0)		3 (2.33–10.82)		1.63 (0.25–4.78)		1.07 (0.14–5.13)		2.56 (1.15–3.85)	
Others [$]	5	0 (0–0.16)		3.04 (1.16–10.11)		1.92 (0.59–2.76)		1.70 (0.63–6.51)		4.10 (2.31–11.82)	
Anatomical side: left vs right											
All	22	0 (0–0)	0.39	3.18 (1.62–11.11)	0.23	1.78 (0.70–4.17)	0.93	1.39 (0.27–3.67)	0.61	2.7 (1.17–4.71)	0.55
Left	4	0 (0–0)		11.40 (3.98–13.39)		1.75 (0.51–5.87)		1.70 (0.56–5.85)		3.04 (1.74–9.98)	
Right	18	0 (0–0)		3.02 (1.38–4.90)		1.78 (0.70–4.17)		1.39 (0.16–3.66)		2.40 (1.07–4.71)	
Anatomical side: femur vs tibia											
All	20	0 (0–0)	0.95	3.02 (1.46–10.57)	0.84	1.55 (0.66–4.31)	0.84	1.39 (0.21–3.06)	0.5	2.7 (1.15–4.04)	0.72
Femur	13	0 (0–0)		3 (1.37–10.12)		1.92 (0.5–3.83)		1.07 (0.15–3.67)		3.24 (0.99–6.14)	

Tibia	7	0 (0–0.16)	3.32 (1.70–11.97)	1.18 (0.64–6.92)	2.64 (0.53–3.18)	2.56 (1.38–3.85)

NA, not applicable; $, SERPINF1, IFITM5, Wnt1.
Analysis of normal distribution using Shapiro-Wilk test indicated that all data are not normally distributed. Therefore, data are given as the median (25^{th}–75^{th} percentiles) and were evaluated using Mann-Whitney-Wilcoxon U-Test for parameters with 2 independent samples and Kruskal-Wallis-H Test for parameters with <2 independent samples.

Table 10. Statistical analysis of SVF composition of OI and HC patients

	Cell viability			**N**	**Early mesenchymal stem cells** (CD45–/CD34+/CD73+/CD90+)		**Supra-adventitial stromal cells** (CD45– CD34+ CD31–)		**Hematopoietic lineage** (CD45+)	
	N	**Median (25^{th}–75^{th} P)**	**P–value**		**Mean (SD)**	**P–value**	**Mean (SD)**	**P–value**	**Mean (SD)**	**P–value**
HC vs OI										
All	19	90.18 (75.2–97.20)	0.3	22	35.52 (20.07)	0.95	38.59 (21.00)	0.79	15.82 (7.00)	0.69
HC	3	79.63 (71.78–90.18)		3	34.82 (4.92)		41.77 (8.63)		17.39 (4.36)	
OI	16	92.18 (76.94–97.20)		19	35.63 (21.61)		38.10 (22.46)		15.57 (7.40)	
Female vs male										
All	19	90.18 (75.2–97.20)	0.1	22	35.52 (20.07)	**0.02**	38.59 (21.00)	0.13	15.82 (7.00)	0.59
Female	9	92.13 (78.52–97.26)		12	26.74 (18.14)		29.39 (19.92)		16.58 (7.70)	
Male	10	82.85 (69.12–95.13)		10	46.06 (17.64)		49.64 (17.19)		14.91 (6.35)	
Age–dependency										
All	19	90.18 (75.2–97.20)	0.06	22	35.52 (20.07)	**0.01**	38.59 (21.00)	**0.02**	15.82 (7.00)	0.65

Prepubertal (1–12 years)	10	90.23 (78.52–95.13)		13	35.12 (16.11)		38.27 (17.54)		15.08 (8.22)	
Pubertal (13–16 years)	6	97.2 (79.56–97.46)		6	22.89 (14.40)		25.80 (15.75)		15.64 (1.95)	
Post pubertal (<16 years)	3	65.5 (44.3–83.55)		3	62.50 (24.10)		65.55 (24.15)		19.39 (8.79)	
Type of OI										
All	16	92.18 (76.94–97.2)	**0.02**	19	35.63 (21.61)	0.38	38.09 (22.46)	0.4	15.57 (7.40)	0.94
OI–IV	7	75.2 (65.5–85.95)		9	44.75 (26.38)		47.83 (27.37)		14.55 (9.05)	
OI–III	6	97.2 (96.04–97.31)		6	22.78 (12.52)		25.19 (13.35)		15.47 (5.86)	
OI–VII	1	NA		2	35.56 (0.53)		35.55 (5.29)		19.65 (10.27)	
Disease causing genetic mutation										
All	16	92.18 (76.94–97.2)	0.95	19	35.63 (21.61)	0.43	38.09 (22.46)	0.47	15.57 (7.40)	0.26
COL1A1	1	NA		3	20.18 (26.31)		22.96 (29.32)		9.43 (12.59)	
COL1A2	11	94.08 (70.33–97.2)		11	38.27 (23.28)		40.91 (23.74)		16.03 (6.25)	
Others	4	88.38 (76.94–96.83)		5	39.10 (13.71)		40.96 (15.07)		18.24 (5.61)	
Anatomical side: left vs right										
All	19	90.18 (75.2–97.20)	0.55	22	35.52 (20.07)	0.82	38.59 (21.00)	0.96	15.82 (7.00)	0.27
Left	4	89.08 (80.61–96.83)		4	33.34 (8.51)		38.05 (10.90)		19.36 (7.69)	
Right	15	90.18 (71.78–97.20)		18	36.00 (21.99)		38.71 (22.89)		15.03 (6.83)	
Anatomical side: tibia vs femur										

All	17	90.28 (73.49–97.2)	0.92	20	35.70 (21.06)	0.9	38.84 (22.04)	0.79	15.04 (6.62)	0.79
Tibia	6	86.85 (64.91–97.35)		7	36.54 (25.33)		40.67 (25.87)		14.49 (7.34)	
Femur	11	90.28 (75.2–97.2)		13	35.24 (19.50)		37.85 (20.77)		15.34 (6.50)	

NA, not applicable; $, SERPINF1, IFITM5, Wnt1.

Analysis of normal distribution using Shapiro-Wilk test indicated that cell viability data are not normally distributed while data collected for early MSC, supra-adventitial stromal cells, and hematopoietic lineage are normally distributed. Therefore, cell viability data are given as the median (25^{th}–75^{th} percentiles) and data for early mesenchymal stem cells, supra-adventitial stromal cells, and hematopoietic lineage are given as the mean (standard deviation). Cell viability data were evaluated using Mann-Whitney-Wilcoxon U-Test for parameters with 2 independent samples and Kruskal-Wallis-H Test for parameters with <2 independent samples. Early mesenchymal stem cells, supra-adventitial stromal cells, and hematopoietic lineage data were evaluated using independent t-test for 2 independent samples or one-way ANOVA for <2 independent samples.

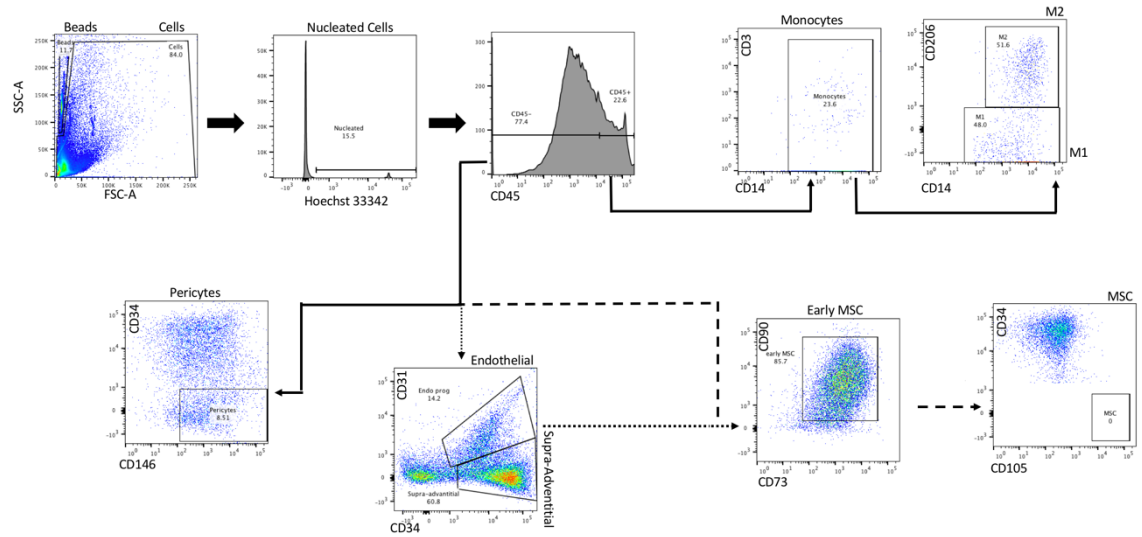

Figure 6A. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 1 HC.

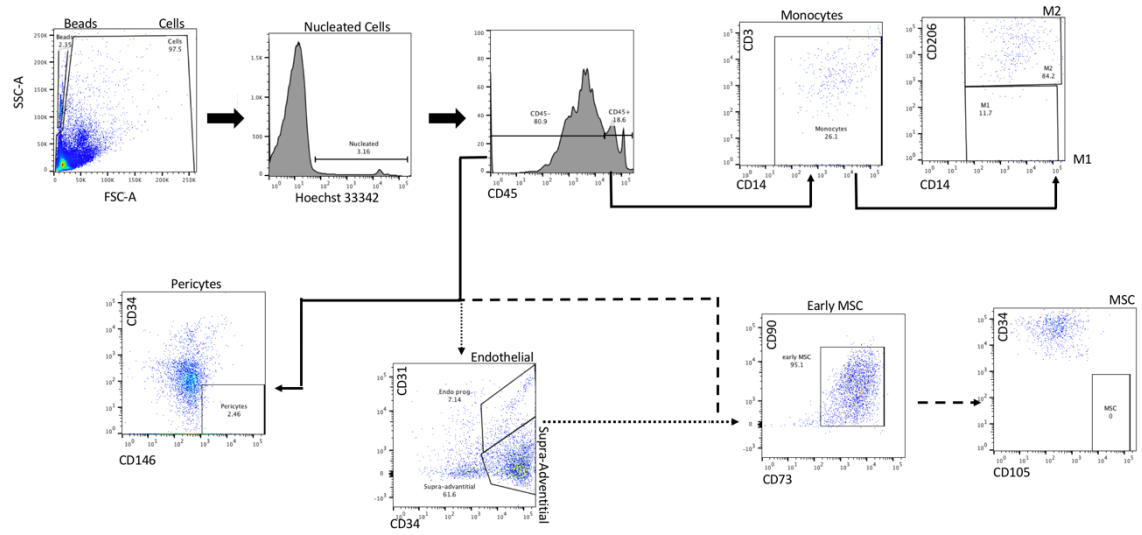

Figure 6B. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 2 HC.

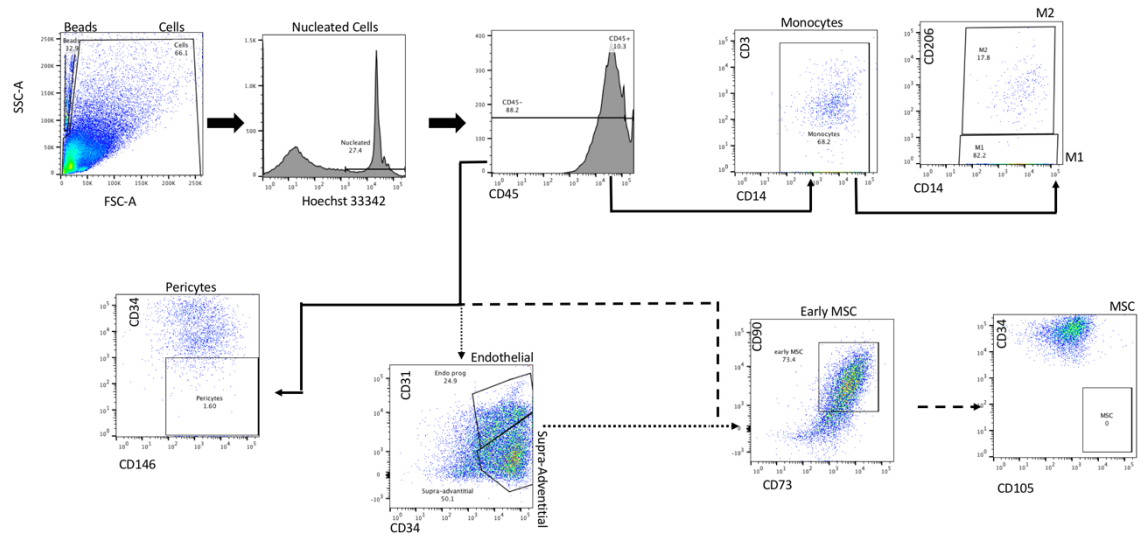

Figure 6C. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 3 HC.

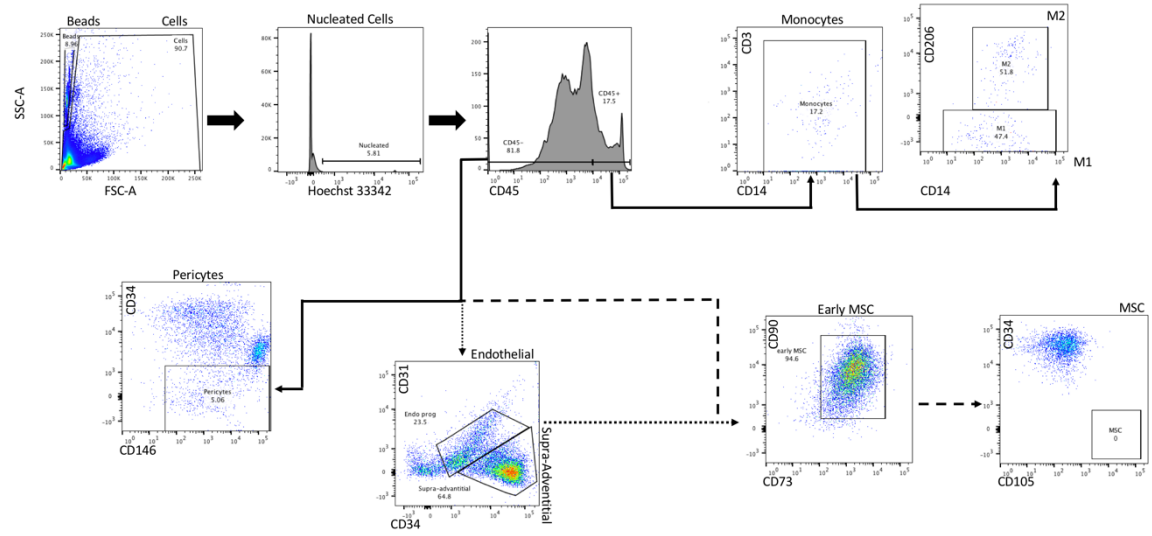

Figure 6D. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 5 OI-IV, COL1A2.

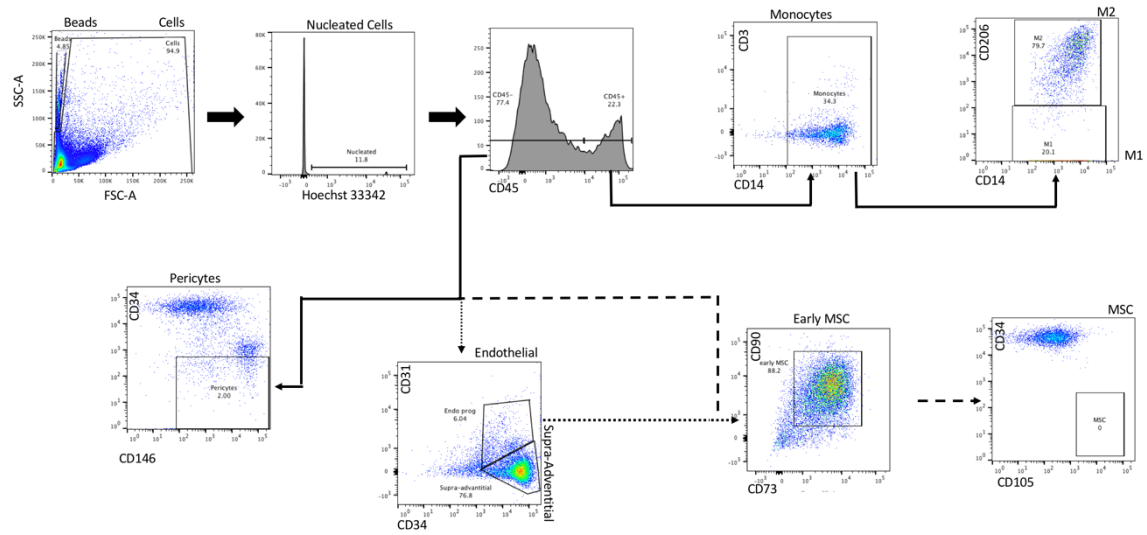

Figure 6E. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 8 OI-IV, COL1A1.

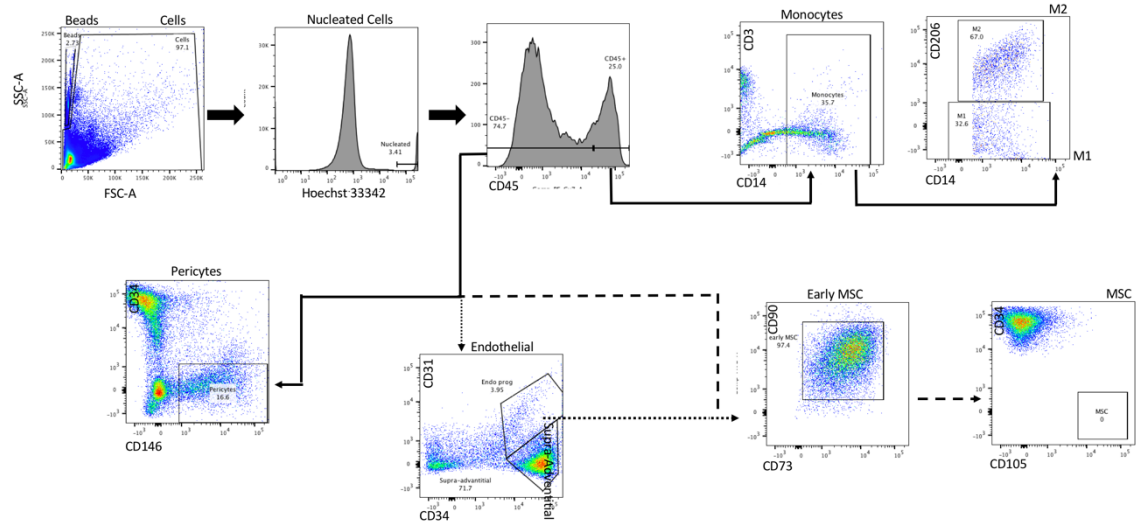

Figure 6F. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 13 OI-III, COL1A2.

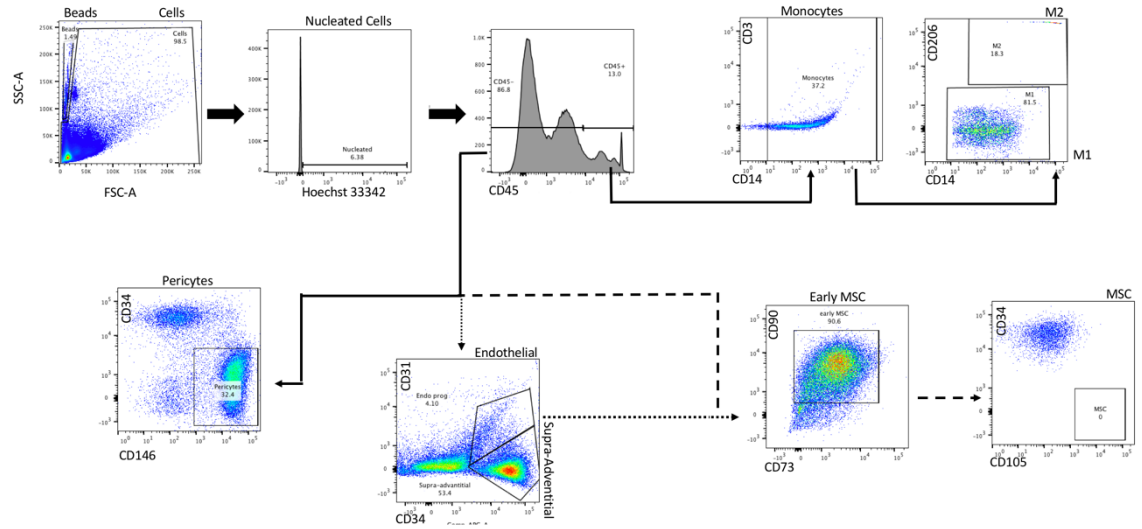

Figure 6G. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 15 OI-IV, COL1A2.

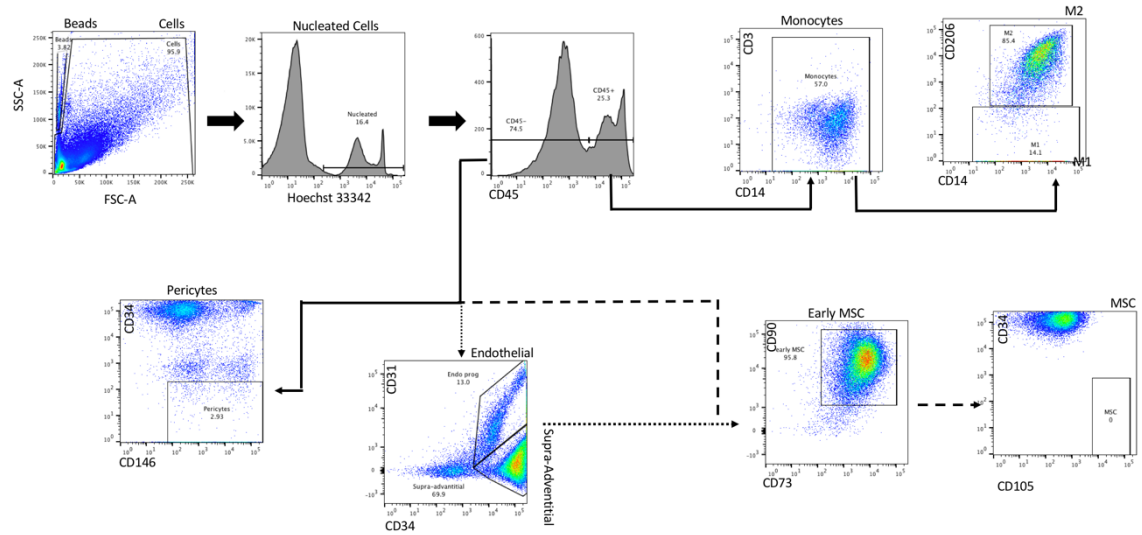

Figure 6H. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 16 OI-IV, SERPINF1.

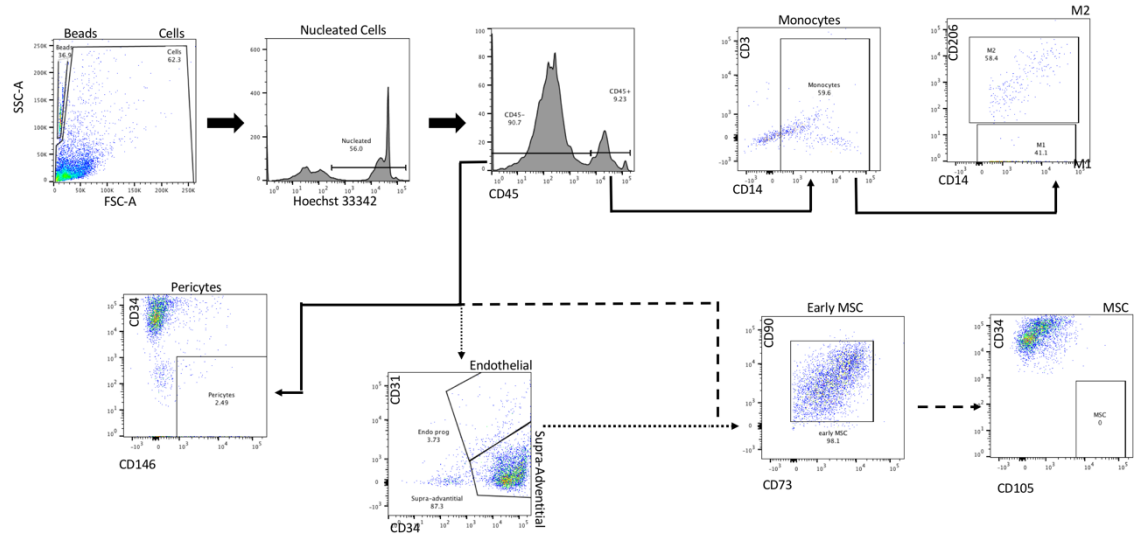

Figure 6I. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 17 OI-IV, COL1A2.

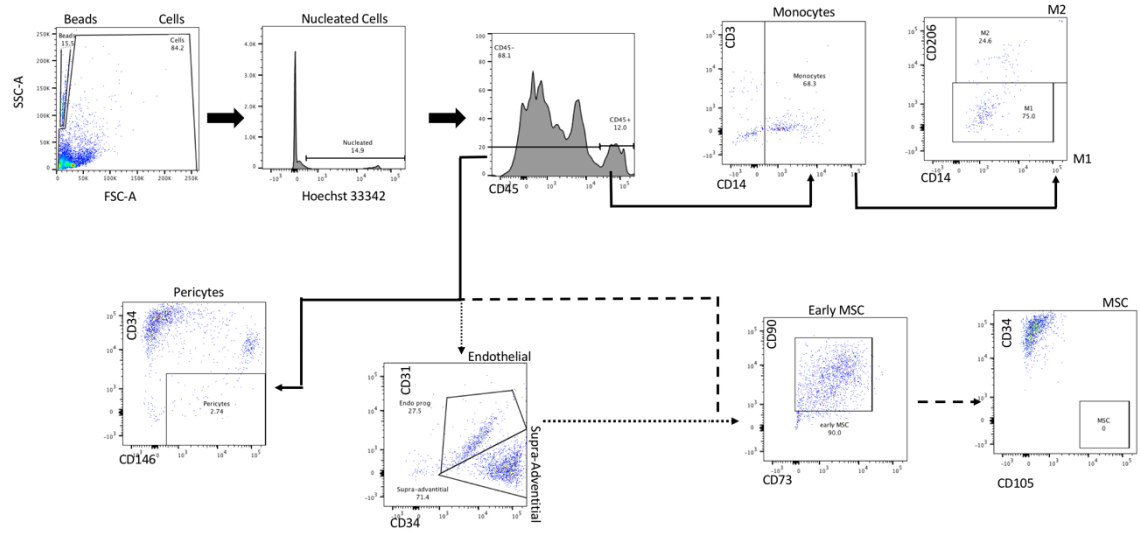

Figure 6J. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 18 OI-IV, COL1A2.

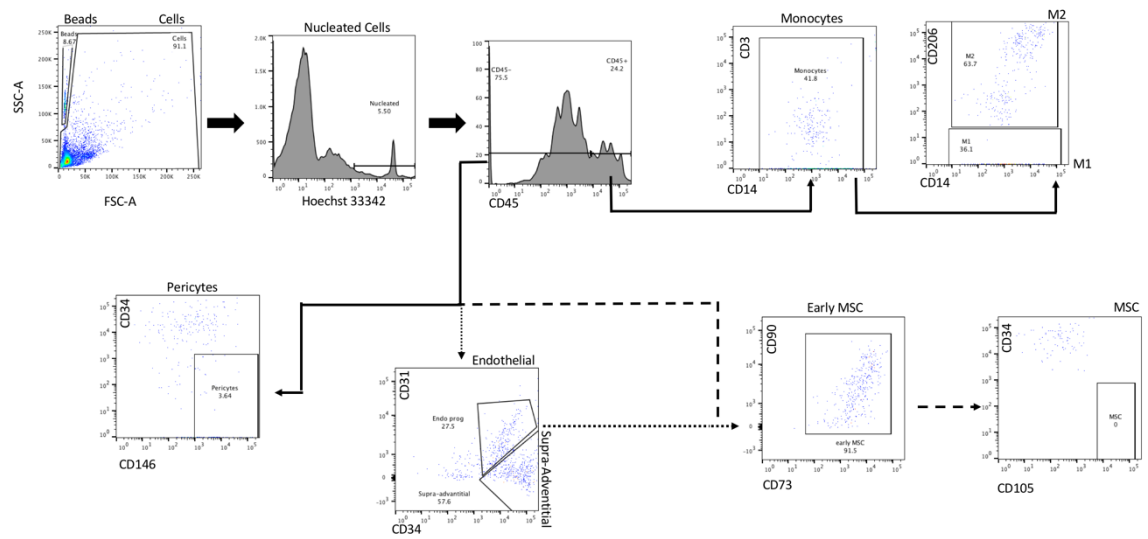

Figure 6K. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 19 OI-IV, COL1A2.

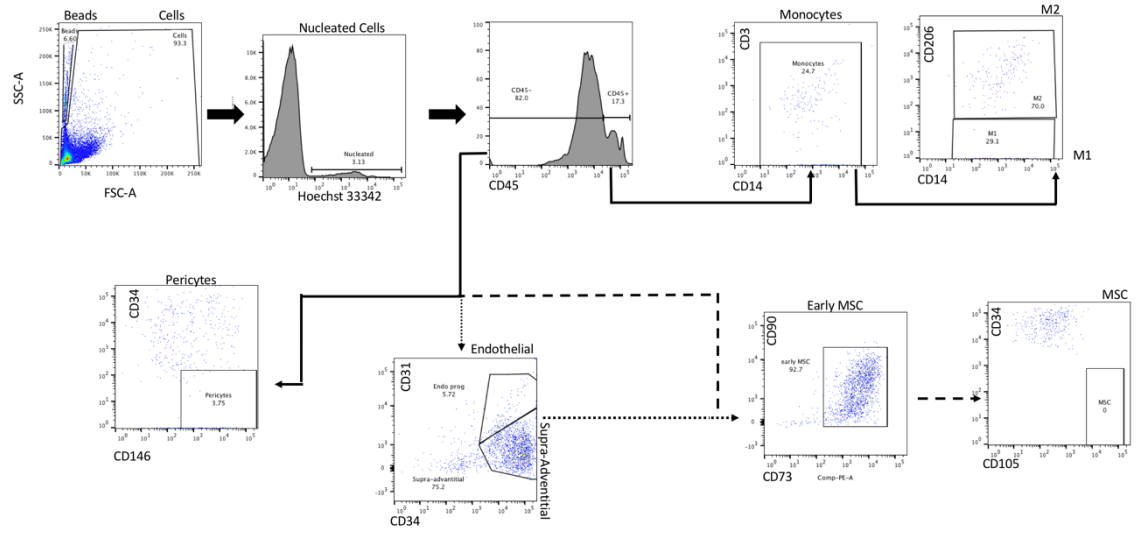

Figure 6L. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 21 OI-VII, WNT1.

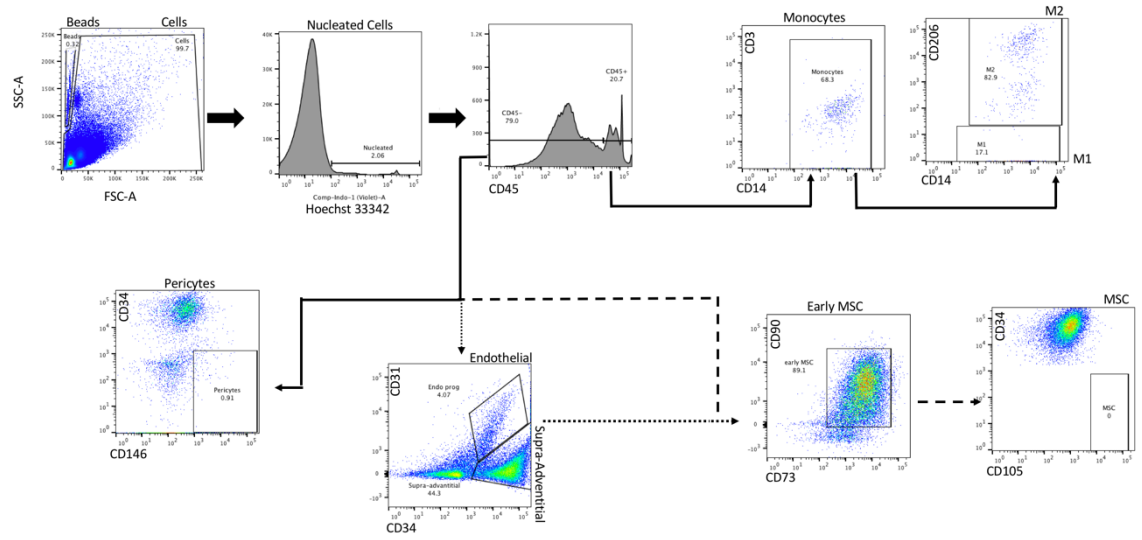

Figure 6M. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 22 OI-III, COL1A2.

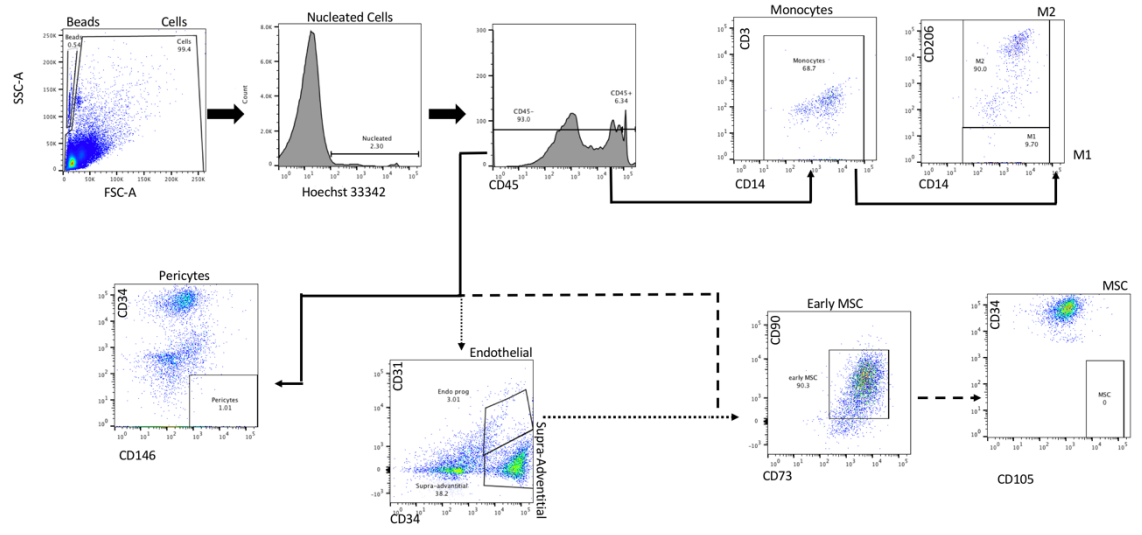

Figure 6N. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 23 OI-III, COL1A2.

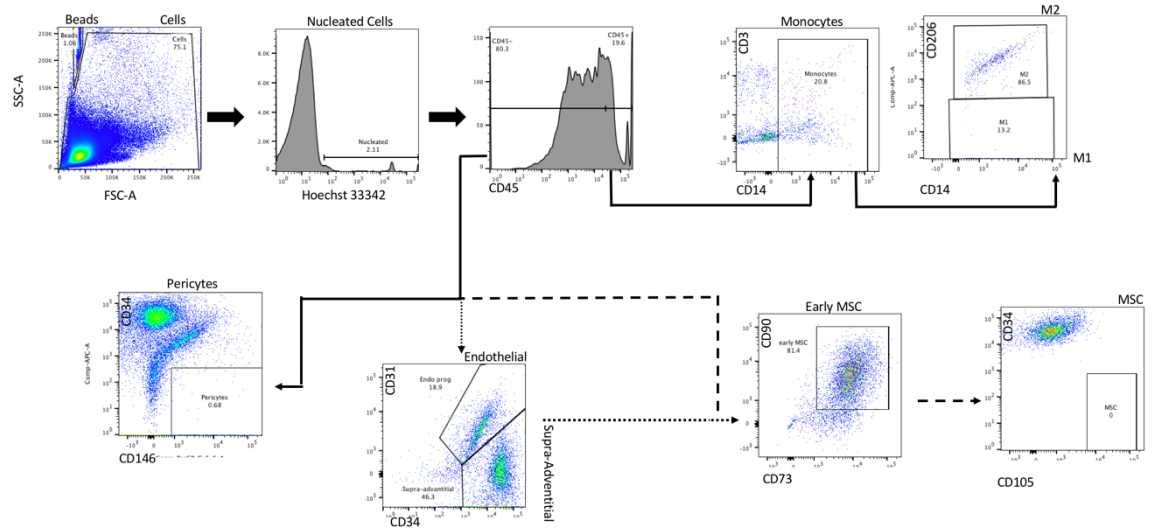

Figure 6O. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 37 OI-VI, SERPINF1.

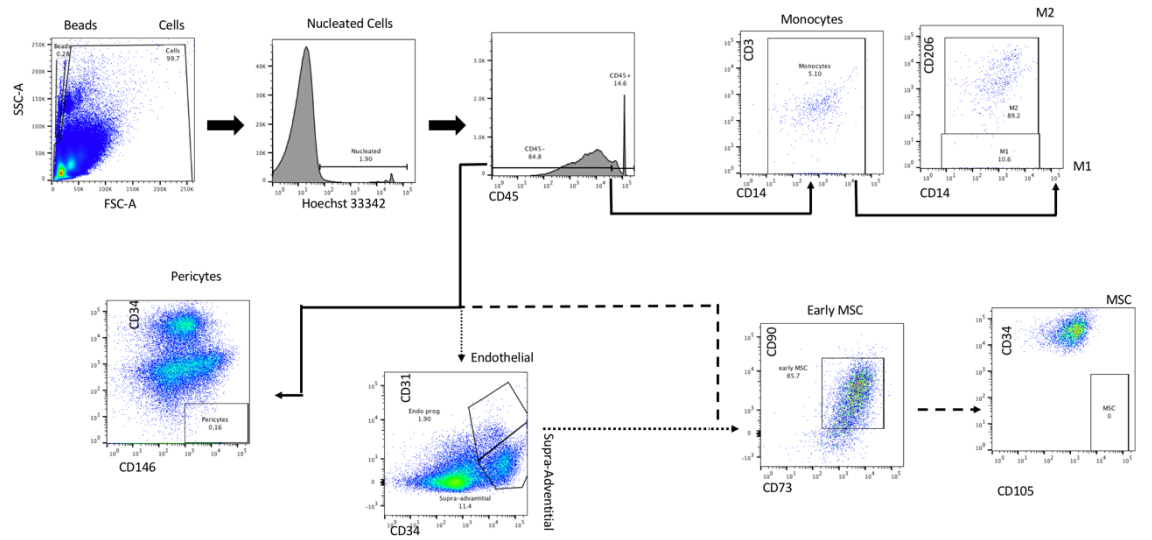

Figure 6P. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 38 OI-III, COL1A2.

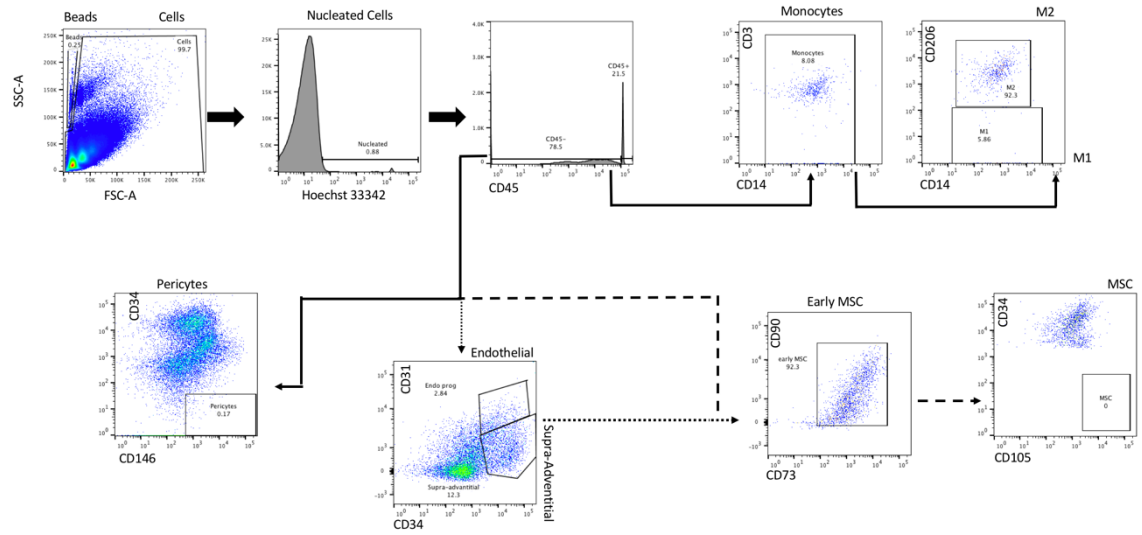

Figure 6Q. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 39 OI-III, COL1A2.

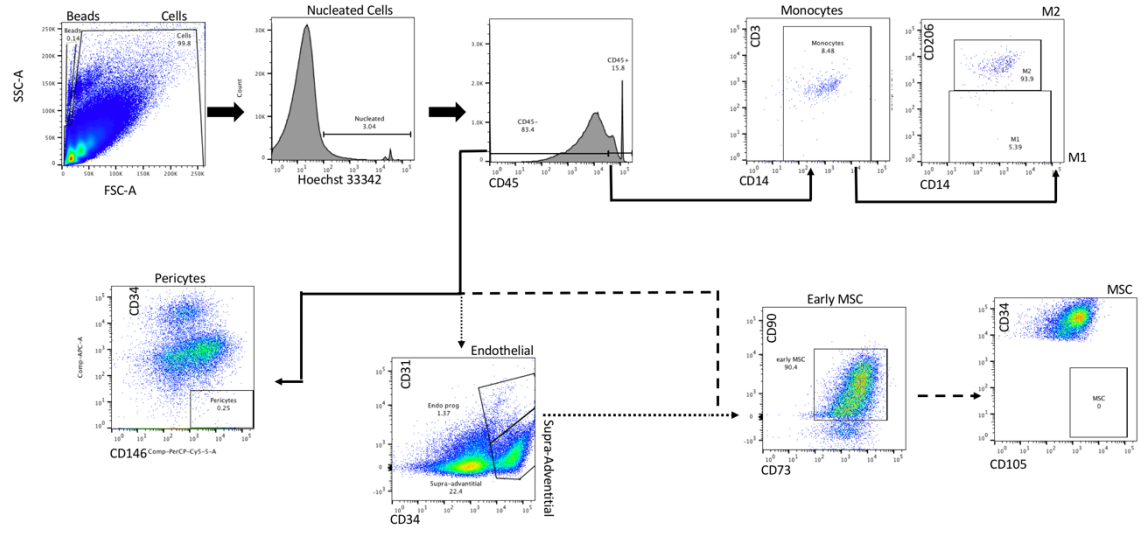

Figure 6R. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 40 OI-III, COL1A2.

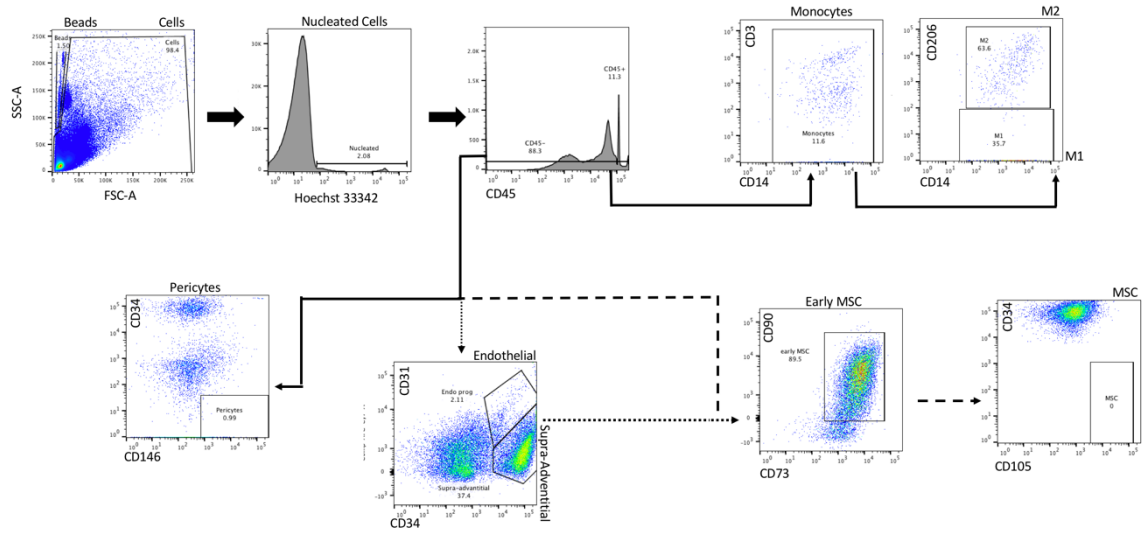

Figure 6S. Immunophenotypical characterization of stromal vascular fraction cell populations by flow cytometry for sample 41 OI-V, IFITM5.

HC- and OI- ASC Multilineage Differentiation Capacity:

When SVF cells were cultured under osteogenic conditions, expression of alkaline phosphatase, runt-related transcription factor 2 (runx2) and osteocalcin at day 7 and day 21 was higher than in non-treated samples. HC and OI samples had similar results (Figures 8A–8F). At the end of the 21-day induction period, Alizarin red staining of deposited minerals confirmed successful differentiation towards functional osteoblasts (Figure 7A–7C), (Figures 8H). Moreover, the histogram of the quantification of deposited minerals shows that the osteogenic induction of stem cells was successful, and mineral deposits were significantly higher in the differentiated cells than in the control group's untreated cultures (*P*-value: 0.003). Figure 8G shows Alizarin red quantification at day 21 in culture. When SVF cells were cultured under adipogenic conditions, there was significant upregulation of PPARy, LEPR, and leptin compared with similar results for HC and OI samples (Figure 7A–7C), (Figures 9 A-F). Consistent cell vacuolation was evident in the induced cells on day 21 of induction, oil droplet accumulation confirmed adipogenic differentiation (Figure 9 G). At day 21, under chondrogenic conditions cultured SVF cells showed significant upregulation of collagen 10, SRY-Box 9 (sox9) and aggrecan were observed with comparable results for HC and OI cells (Figure 10A –10F). Collagen blue staining confirmed chondrogenic differentiation (Figure 7A–7C), (Figure 10G). Evaluation of the influence of collagen mutation on multilineage differentiation capacity revealed similar differentiation competence towards osteogenic and adipogenic lineage of SVF cells with *COL1A1*, *COL1A2*, and WNT1 mutations. Regarding chondrogenic lineage, SVF cells harboring *COL1A1* mutation seemed to have a higher differentiation competence than mutations in other genes. However, given the limited number of samples this effect was not statistically significant.

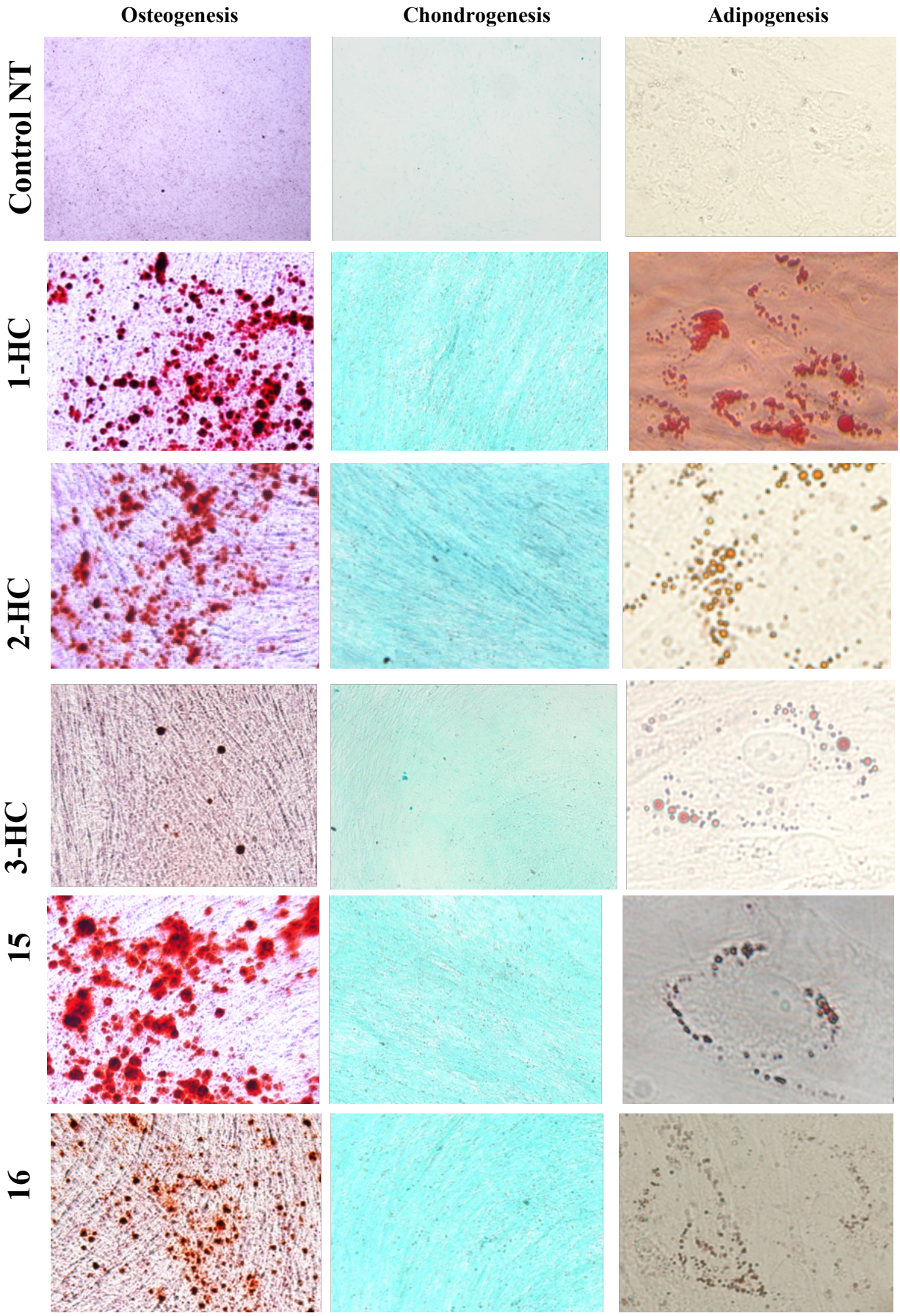

Figure 7A. Trilineage potency of osteogenesis imperfecta-mesenchymal cells and compared to human control.

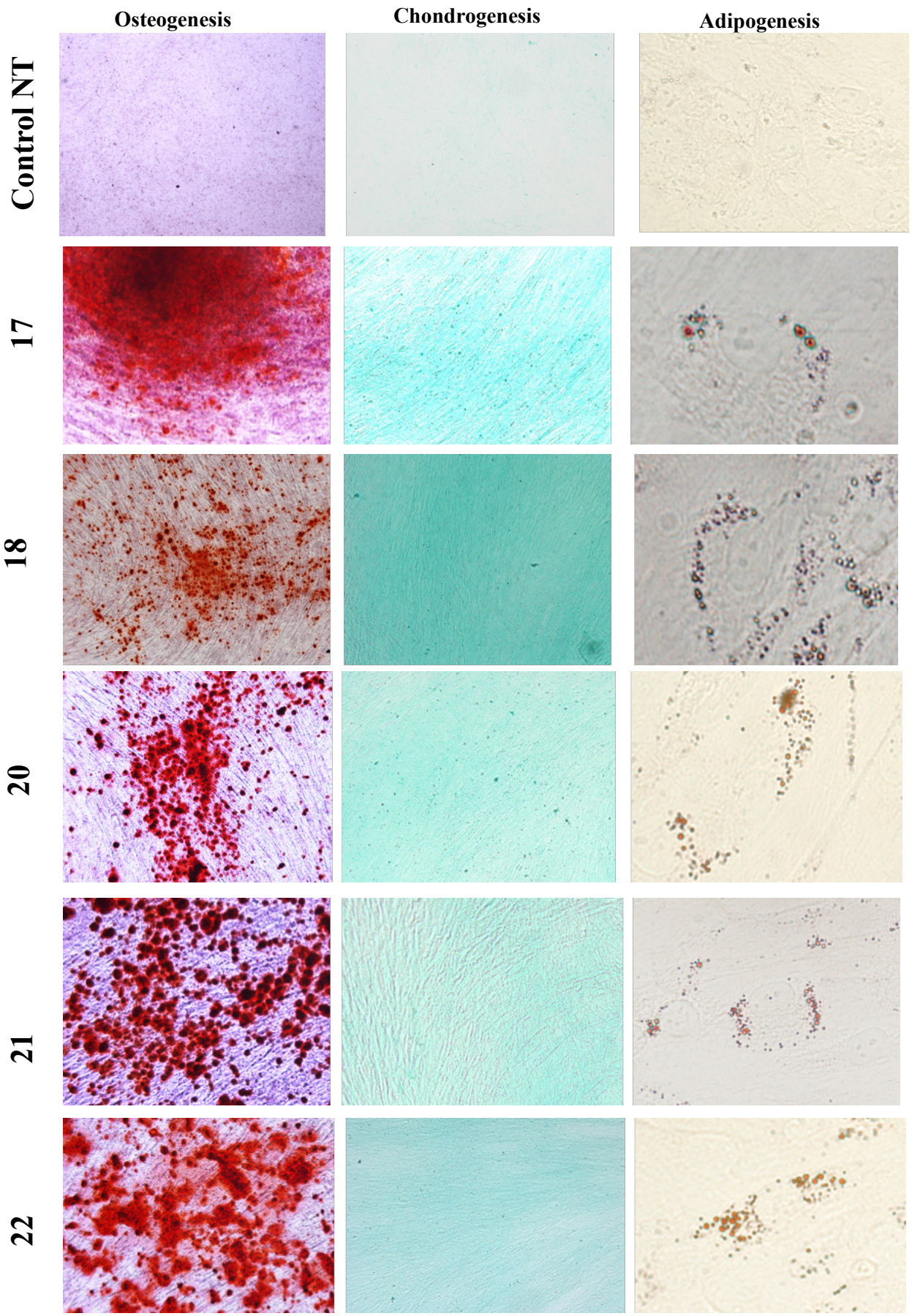

Figure 7B. Trilineage potency of stromal vascular fraction-mesenchymal stem cells for osteogenesis imperfecta and compared to human control.

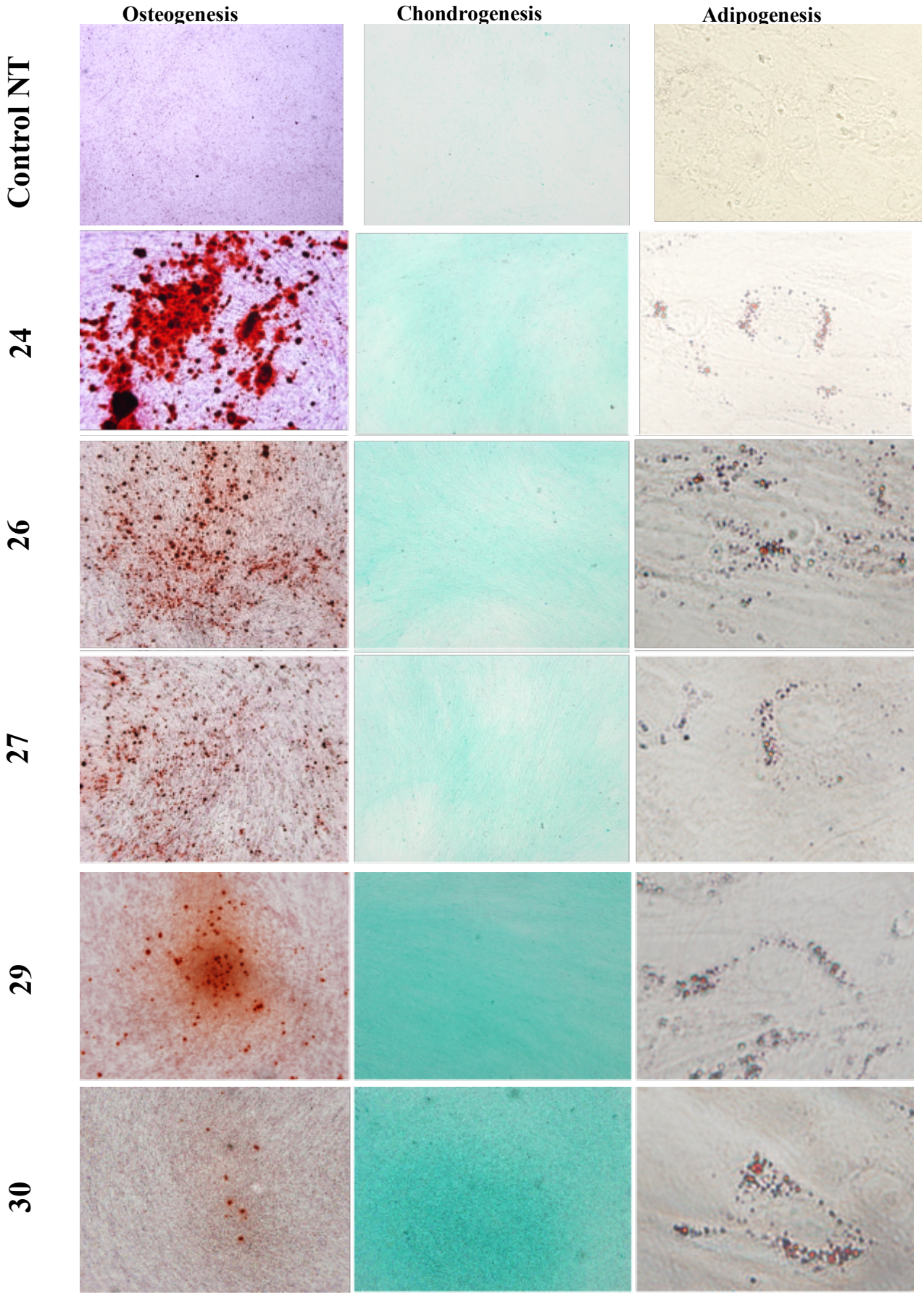

Figure 7C. Trilineage potency of osteogenesis imperfecta-mesenchymal cells and compared to human control.

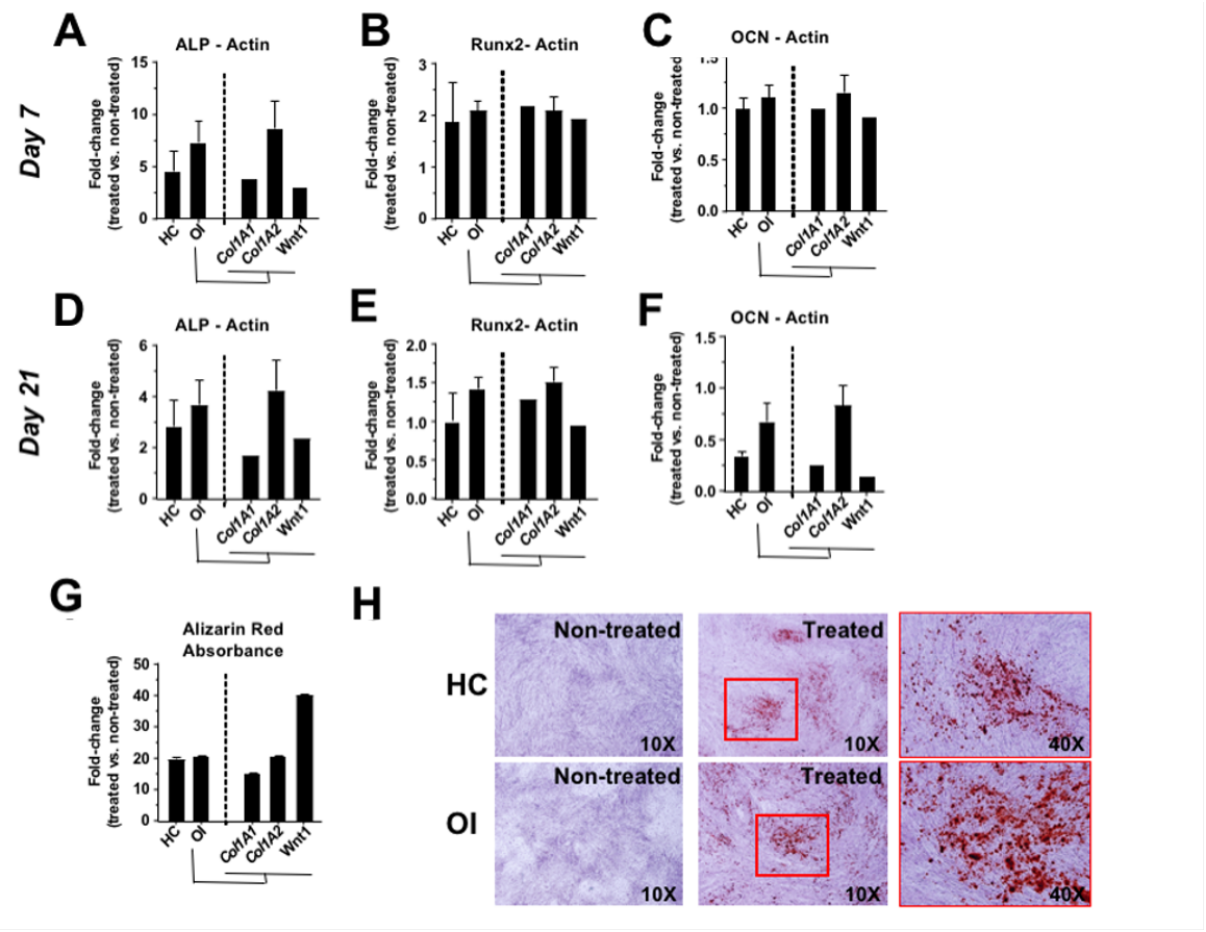

Figure 8. Osteogenic Gene Expression Profile of SVF Cells Obtained from Human Controls (HC) or OI Patients at Days 7 and 21 in Culture

(A–C) Fold-change of gene expression of treated versus non-treated cells at day 7 of culture.

(D–F) Fold-change of gene expression of treated versus non-treated cells at day 21 of culture.

(G) Quantification of alizarin red staining via absorbance measurements displayed as fold-change of treated versus non-treated samples.

(H) Representative images of alizarin red staining of treated and non-treated HC and OI samples. HC, n=2; OI, n= 9; OI-COL1A1, n=1; OI-COL1A2, n= 6; Wnt1, n=1. ALP, alkaline phosphatase; OCN, osteocalcin. Unpaired t-test of genetic fold-change of HC versus OI patients showed similar expression for all genes tested.

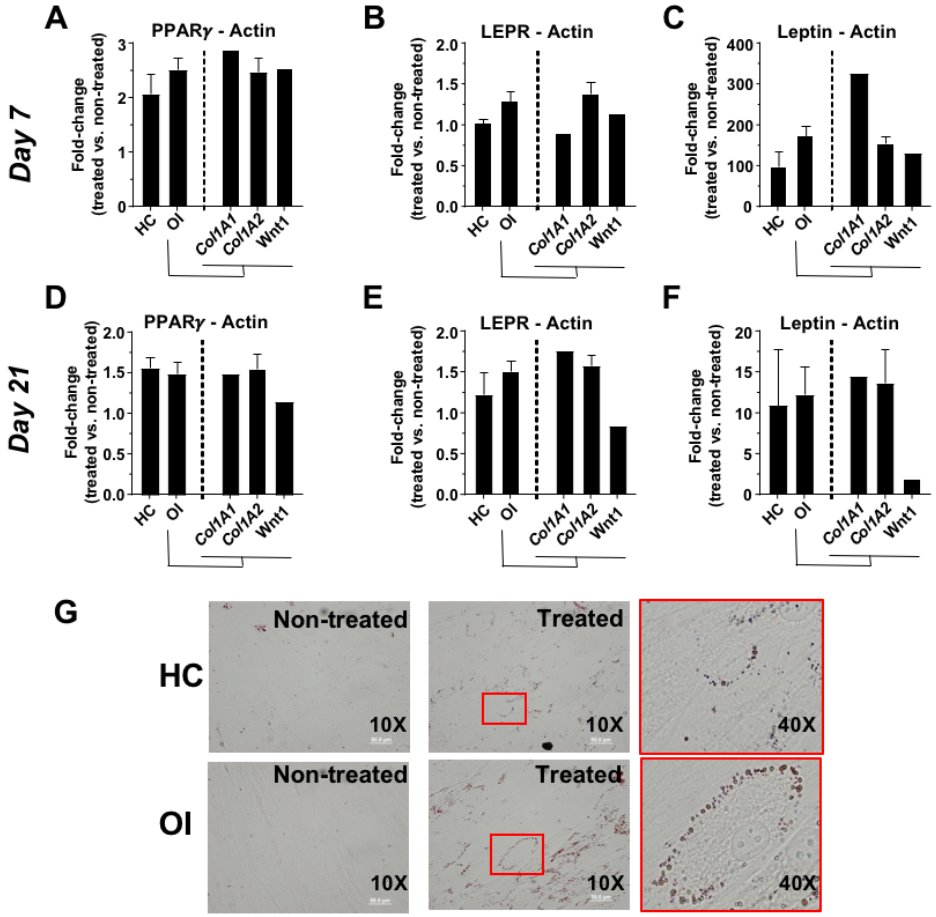

Figure 9. Adipogenic Gene Expression Profile of SVF Cells Obtained from Human Controls (HC) or OI Patients at Days 7 and 21 in Culture.

(A–C) Fold-change of gene expression of treated versus non-treated cells at day 7 of culture.

(D–F) Fold-change of gene expression of treated versus non-treated cells at day 21 of culture. (G) Representative images of oil red staining of treated and non-treated HC and OI samples. HC, n=2; OI, n= 9; OI-COL1A1, n=1; OI-COL1A2, n= 6; Wnt1, n=1. Unpaired t-test of genetic fold change of HC versus OI patients showed similar expression for all genes tested.

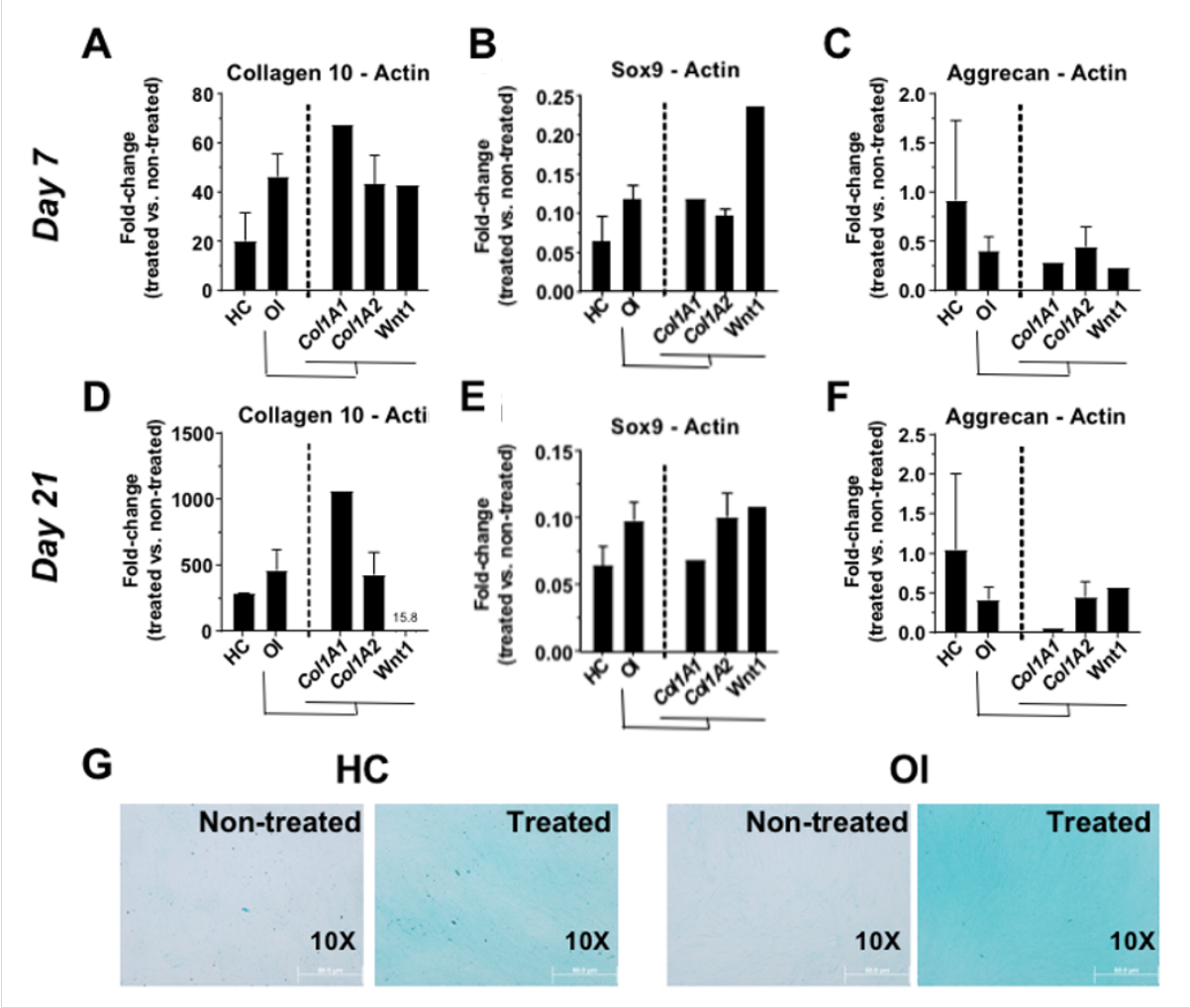

Figure 10. Chondrogenic Gene Expression Profile of SVF Cells Obtained from human Controls (HC) or OI Patients at Days 7 and 21 in Culture

(A–C) Fold-change of gene expression of treated versus non-treated cells at day 7 of culture.

(D–F) Fold-change of gene expression of treated versus non-treated cells at day 21 of culture. (G) Representative images of collagen blue staining of treated and non-treated HC and OI samples. HC, n=2; OI, n= 9; OI-COL1A1, n=1; OI-COL1A2, n= 6; Wnt1, n=1. Unpaired t-test of genetic fold-change of HC versus all OI patients showed similar expression for all genes tested.

Discussion

The future purpose of the in vitro characterization of OI derived SVF cellular composition is to locally use ASC-containing SVF for bone regeneration, nonunion prevention and improving of healing at the site of osteotomies in OI patients and to isolate and use SVF within the same surgery. To do so, we have optimized the methods for isolation and performed the characterization of the cellular composition SVF by using a multicolor flow cytometrical analysis (FACS) and in vitro assays following the basic recommendations from the IFATS and ISCT, established in 2013 [28]. And demonstrated SVF isolation from adipose tissue of pediatric patients with OI within about 90 minutes.

Existing literature in the field of tissue engineering and culler therapy have confirmed and described the excellent regenerative properties of SVF in multiple preclinical studies [5, 136-142] and clinical trials [41, 143-151]. These studies indicated the positive outcomes using SVF locally [46-61, 143, 152-154] and systematically [44, 154-161] for a wide variety of medical problems and diseases including its ability to facilitate bone regeneration [59, 62, 162, 163]. The count of nucleated cell and cellular viability are indicators of the efficiency of the isolation process and will be used to ensure accurate dosing in clinical applications. Yet, the number of viable SVF cells required for the treatment of a particular condition is still unknown due to; the insufficient data to establish a reliable dose versus effect relationship. Adding to that, the large variation in the obtained cell yield and viability of the isolated SVF which might be a result of the variability of donors as well as the site and method of adipose tissue harvesting [29, 164-167]. In general, because no

adverse effects are reported with the use of autologous SVF the largest number of cells isolated at the point of care without expansion in culture is typically used.

Evidence in regard adult adipose derived SVF isolation, characterization and differentiation potential has been growing substantially over the years [1, 28, 39, 63, 142, 168-173]. However, to date, information concerning pediatric stromal cells properties are still lacking. Few studies has reported the use of the conventional procedures of isolation and characterization validated for adults stromal cells [174]. The reported analysis of healthy pediatric derived adipose tissue SVF indicated that it contains large numbers of aMSC and that these could differentiate into trilineage cells [175]. Furthermore, the data of comparative studies led to the conclusion that pediatric aMSC are efficient and have similar in vitro properties as adult aMSC concerning extraction, in vitro expansion and immunosuppressive effect [176]. Nevertheless, there are no reports on the cellular composition of the SVF and the *in vitro* potency of aMSC derived from OI patients.

To our knowledge the only cell therapy approach applied in OI treatment is the allogeneic systematic transplantation of bone marrow stromal cells, or marrow-derived mesenchymal stem cells [100]. This approach tested whether the intravenously infused bone-marrow stromal cells find their way into the skeleton and differentiate into osteoblasts that start producing normal bone. The first study was undertaken in three toddlers (age range 13-32 months) with severe OI (type III) received with unmanipulated bone marrow transplantation from HLA- identical or single-antigen-mismatched siblings after they had received ablative conditioning therapy [95]. The average of the total nucleated infused cell dose is 5.8-6.5 x 10^8 cells per kg.

Transplanted patients showed small percentage of donor-derived osteoblast cells on bone biopsies (1.5–2.0% donor cells) but improved parameters of bone formation, increased total body bone mineral content, and increased growth [96].

The experience of performed pre- and post-natal transplantation of fetal allogeneic MSC for treatment of OI is limited to two published cases of two fetuses with severe OI (type III and IV). The patients were transplanted with cultured HLA-unmatched first trimester liver-derived MSC; 6.5 × 106/kg in gestational week 31 for the OI type III case and 30 × 106/kg in gestational week 31 for the OI type IV case [97]. The transplantation resulted in improved linear growth, mobility, and fracture incidence for several months. When the patient's condition began to deteriorate, the OI type III patient was re-transplanted with 2.8 × 106/kg same-donor cells at 8 years of age. And the OI type IV patient postnatal transplantation of 10 × 106/kg same donor MSC was performed at 1 year and 6 months of age [98]. The second, post-natal transplantation resumed these positive effects without any toxicity or alloreactivity toward the donor MSCs. Yet too few patients have been treated with this approach and the beneficial clinical effects of this approach were short-lived. And researchers conceded that although it holds great promise, more experience needs to be accumulated [99].

Thereby, using the local administration approach of autologous freshly isolated adipose tissue derived SVF in OI patients as it is the case for our long-term clinical goal would be of a tremendous clinical importance. As it may overcome the riddle associated with systematic administration of whether or not the cells will be able to localize the site of tissue injury and find their way into the skeleton and differentiate

into osteoblasts that start producing normal bone [177]. It also holds no ethical concerns allogeneic stem cells transplantation [178], avoids the lengthy and costly in vitro cell culture expansion [164]. And yield higher number of cells compared to bone marrow [30, 31]. As well as the better therapeutic effects it provides due to its distinctive heterogeneous cellular composition [69, 170, 179].

Our results of SVF obtained using manual, collagenase-based digestions of resected adipose tissue derived from pediatric OI patients are novel and original. In this study we have looked into the viability, the cell yield, the cellular composition, and the number of ASC within the SVF and its multilineage diffrentiation capacity. The number of isolated cells and cell viability was comparable between OI and HC and independent of age, gender, anatomical site of harvest, or genetic OI mutation.

The average cell yield of isolated SVF per gram of adipose tissue excised from the patient's lower limbs using manual enzymatic processing is 42.62±6.43 $x10^4$ cells per gram tissue among the OI derived SVF samples with 85.6±3.8 viability and 46.25±17.13 $x10^4$ cells per gram tissue among the controls with 80.5±5.3% viability with no statistical significance (P-value: 0.49, 0.30, cell yield, viability respectively). This result goes in line with the mean cell yield values obtained in other published report using excised chest subcutaneous adipose tissue manual collagenase based enzymatic method. The number of total nucleated cells isolated in that study was 7.28 x 10^5 ± 5.52 x 10^4/mL with 82.0 ± 3.45% cell viability [180]. Another study reported the isolation of approximately 2 $x10^6$ cells using manual enzymatic processing, from 0.5-cm^3 excised abdominal adipose tissue specimen. With cell viability ranged between 70 - 80% of the total recovered cell population [181]. However, no studies

found reporting the isolation of stromal cell in pediatric patient using excised adipose tissue from the lower limb.

Moreover, there was some variation in the numbers of cells obtained between the different donors. Which appears to be a controversial issue that highly depends on the characteristics of the donors involved in the study. Published reports showed that variables such as donor factors, age, gender, body mass index. The site of adipose tissue collection; abdomen, thigh, neck, back, breast, and knee and the harvesting surgical procedure: resection or aspiration. As well as the tissue processing techniques and isolation protocols may influence the yield, viability, and replication potential of the recovered SVF cells [29, 150, 164, 165, 182]. Therefore, the IFATS and ISCT recommendations indecates that cells should have a viability of >70 % for SVF [28]. Which makes the viability of our cell yield is in the higher range of what is reported in the literature [183, 184]. This is might be related to the elimination of the red blood cell (RBC) lysis step in our modified protocol; since our goal is to use the SVF in patients, our aim was to reduce the use of reagents as much as possible.

There are many ways to obtain stromal cells from adipose tissues, including the Automated or Semi-automated Mechanical or Enzymatic and the Manual Mechanical or Manual Enzymatic [129, 183, 185]. Among them, the enzymatic dissociation of lipoaspirates and resected adipose tissue is the most commonly used, efficient and reliable procedure [129, 183, 185]. Some studies report a higher yield when using an automated procedure, the higher yield of nucleated cells/ml of adipose tissue obtained by automated or semi-automated adipose-processing platforms is 7.19 x 105 and 83% cell viability. [165, 186-189]. Of these studies one compared the cell yield of enzymatic dissociation of abdominal lipoaspirate manually and automated.

The manual procedure yielded 1.6 x10^5 nucleated cells/ml of liposuction. While the automated, enzymatic procedure was able to isolate 2.6 x10^5 cells/ml of liposuction, with over 90 % viability was reported in both methods [187].

Published reports showed that enzymatic isolation performance is better compared to mechanical isolation [74]. Enzymatic methods for SVF isolation report significantly higher yields of nucleated cells/cc of lipoaspirate processed and it can be up to 1000 times more effective in SVF cell recovery than mechanical methods [183]. Reported yields of viable, nucleated SVF cells achieved using manual, collagenase-based digestions range from 100,000 nucleated cells/cc to 1,300,000 of lipoaspirate processed [183]. Whilst the cell yields using mechanical methods range from 10,000 nucleated cells/cc of lipoaspirate to 240,000 nucleated cells/cc of lipoaspirate. Adding to that, the composition of the cell populations recovered through greater frequency of progenitor cells and a substantially lower number of peripheral blood mononuclear cells compared to the simple centrifugation and other non-enzymatic methods [185, 190, 191]. This is because aMSCs are concentrated in the small and medium sized vascular structures of adipose tissue, and without enzymatic lysis of the collagen-based extracellular matrix many progenitor cells remain trapped within the vascular endothelium layers and connective tissue fragments [185].

Generally, to obtain adipose tissue, several harvesting techniques exist. It can typically be divided into "liquid" fat (e.g., liposuction procedures) and "solid" fat (e.g., resection of fat tissue). Not many direct comparisons exist in the literature. It is shown that liposuction material lacked large vessels and had significantly more dead cells than resected adipose tissue. The number of stromal cells was about 50% lower in the

liposuction compared to the resected tissue [169]. The quantity of ASCs isolated from dissected adipose tissue was comparable to yields achieved by liposuction and enzymatic ASC isolation (about 25%–30%) [133]. The cell yields per gram of resected adipose tissue during abdominoplasties have been reported to be always in the same range of 2.95 × 104 cells/g [192]. However, in most studies SVF isolation was obtained from adipose tissue via liposuction. The highest isolated numbers of stromal cells/g lipoaspirate with a viability that is more than 80% ranged between 0.5 - 1 million [165, 183, 184, 193]. Some studies reported that the rage of 0.308 x 106 - 0.5 x 106 /ml [168, 182, 194, 195]. Lower numbers were also reported, with a range of 0.148 x 106 - 0.287 x 106 /ml [187, 196, 197].

Therefore, among the studies tested the effect of liposuction and resection on the yield, viability and differentiation potential of adipose drives stromal cells. Some studies reported no significant difference in regard the quantities of nucleated cell yield isolated following adipose tissue harvested by tissue resection and the cell yield for processed lipoaspirate. Although, the amount of counted cells varied between the studies, one amounted to 1830.42 - 273.42 cells/ml tissue to resected tissue and amounted to 5050.87 - 1565.37 cells/ml to the lipoaspirate and one isolated 2.5 × 10^6 cells/ml with resection and 2.9 × 10^6 cells/ml lipoaspirate [184, 198]. Whereas the other study the average cell yield in both the resected and lipoaspirates is 0.5 x 10^6/ml [182]. It's showed in other studies that the cell yield obtained through liposuction is higher than the yield of resected adipose [199], significant difference with ($p<0.0001$) in the cell yield between the resected adipose tissue with 1.39 x110^6 and the yield obtained through aspiration is 2.56 x10^6 with no difference in the viability [200]. In the contrast, another studies showed that the resected adipose tissue significantly

associated with the numbers of total SVF cells and aMSCs yielded than lipoaspiration [29, 201]. Moreover, greater frequency of aMSCs in SVF harvested from excisional fat compared to those from lipoaspirates (*$p < 0.05$), with an average of aMSCs in processed lipoaspirates specimens was 42.4 %, and in resected adipose tissue was 55.8 %. However, the reduction of cell yield in lipoaspirates, did not result in an impaired cells ($p = 0.53$) [193].

To harvest ASC-containing SVF, traditionally harvested lipoaspirate is exposed to enzymatic dissociation followed by several centrifugation steps [28, 75, 132, 133, 135]. This relative time-consuming procedure could not be performed in parallel to a surgery. Therefore, we performed SVF isolation according to the optimized method for lipoaspirates described by Tevlin et al. [134] but applied minor modifications in order to use this method for resected adipose tissue. We incubated the dissected adipose tissue for about 10 minutes on ice to separate blood and fat and we incubated the mechanically homogenized adipose tissue for up to 60 minutes at 37ºC in digestion buffer using high collagenase I concentration. As described by Tevlin et al. a high concentration of collagenase I seems to be tolerated and does not influence isolated cell populations or multi-potency of isolated ASCs [134]. Still, with this protocol, we were able to isolate SVF with about 90% viable cells from OI and HC. This is comparable to established non-intraoperative isolation protocols and intraoperative isolation procedures [202]. Further, isolated ASCs presented the typical MSC markers.

Additionally, we found that the yield of ASCs was irrespective of anatomical harvest site as described previously [203, 204]. Several studies investigated whether

the anatomic site from which the adipose tissue was harvested affect the cell yield and viability of the isolated stromal vascular fraction. In one of the studies demonstrated that the yield of viable nucleated cells in the SVF of adipose tissue harvested from different tissue-harvesting sites was similar, the cell yield/g of adipose tissue harvested from the abdomen, hip and thigh region and mamma is $0.7x10^6$, $0.5x10^6$, $0.6x10^6$ respectively with no significant differences (P /0.3) [29]. Or a significant differences between the cell yield of adipose tissue harvested from abdominal site and the sub-abdominal site [182]. On the contrary, another study reported that the total yield of SVF cells counted among different donor areas, the abdomen revealed 2.97×10^6 cells, the waist 5.61×10^6, the inner thigh 6.34×10^6, the outer thigh 12.31×10^6 and the inner knee 7.96×10 cells. No significant differences were observed among regions of liposuction with regard to number of SVF cells, except for the outer thigh samples, which exhibited significantly higher SVF cell count compared to any other donor sites ($p<0.05$). [204]. And another harvest site influences the stem and progenitor cell content of abdominal of subcutaneous adipose tissue yielded,1.7-fold more than tissue from the thigh tissue (P <0.03). [196]. Moreover, one study looked into the effect of the collection site on SVF yield independent of the chosen harvesting technique, taking into account the effect of gender, age, and body mass index. It showed only gender seem not to influence the cell yield among the different site. And that in males the best collection site in terms of cell yield is the abdomen with respect to the back (P, 0.001) and to the knee (P < 0.02), whereas in females no significant differences were found between collection site and cellular yield (P, 0.086, 0.631, 0.091, 0.813) respectively thigh, back, breast, knee to the abdomen [200].

Aging is believed to negatively impact tissue repair and healing. Stem cell function generally declines with age. Several studies on aMSCs have reported alterations in the number, proliferation, and differentiation potential of MSCs with respect to donor age [36, 180, 205]. Yet only a limited number of studies looked into the impact of age and gender on the cell yield of adipose tissue derived SVF. The results of which are conflicting, some studies indicated that age has no influence on the cell yield [166, 188, 192, 195, 197, 200]. Others indicated a clear relationship between donor age and number of cells obtained from adipose tissue [165, 182].

It is reported that old aged donors (45-74y) had a significantly lower cell yield compared with the middle-aged donors (38-44y) but not the young donors (25-37y), with no statistical different seen by the influence of gender [182]. In one of the studies where all samples were obtained from females abdominal lipoaspirates) and the same isolation technique (mechanical enzymatic), concluded that there is a clear statistically significant decline in SVF cell yield with increasing age. The aged group (>60y) had the lowest cell yield, whereas the youngest group (<30y) had the highest cell yield [165]. Contrariwise, in other studies where samples were obtained from females thighs lipoaspirates and resected mammary tissue didn't demonstrate a significant correlation between the number of cells obtained per milliliter of lipoaspirate with the age of the donor [166, 188]. Moreover, several other studies suggested that there is no statistically significant correlation between cell yield with age or gender [195, 197, 200, 206, 207].

The knowledge in regards pediatric SVF cell yield per gram of adipose tissue in pediatrics is understudied. A study evaluated the effects of age on the quantity and quality of stem cells isolated from excised chest subcutaneous adipose tissue were

unable to demonstrate a significant difference between the different age-groups. Although there was a trend indicating that the SVF cell yield decreases with increasing donor age. The number of total nucleated cells was 7.28 x 10^5 with 82% cell viability in the child group, 6.32 x 10^5 /mL with 80% cell viability in the young adult group, and 5.53 x 10^5 /mL with 80% cell viability in the elderly group [180]. Other study reported significantly higher aMSC cell yield of donors aged 38 - 44 years compared with older donors ages > 45 years [182]. In contrast, report higher cellular aMSC yields for female donors > 45 years of age (0.61 x 106 ml) compared with female donors <35 years of age (0.27 x 106 ml) [208]. Conversely, all age groups exhibit similar osteogenic paracrine activity, and we posit that clinical applicability of adipose-derived mesenchymal stem cells is conserved despite age [167]. A study focused on the properties of pediatric aMSCs has shown that stem cells derived from pediatric abdominal fat following either enzymatic digestion or explant cultures can be significantly expanded and have comparable properties, making them a potential valuable source of stem cells for autologous cell-based reconstructive surgery in children [176].

The reported SVF compositions of adipose tissue are divided into two major subpopulations based on the expression of CD45, which is a hematopoietic cell marker: adipose derived (CD45-Ve) and blood derived (CD45+ve). [202] Adipose-derived cell populations can be divided into endothelial cells (CD31+Ve) and stromal cells (CD31-Ve) [170]. Nevertheless, the stromal cell population (CD45-Ve/CD31-Ve), including pericytes: (CD34+Ve/-Ve/CD146+Ve), ASCs: (CD34+Ve/CD90+Ve/CD105low), and supra-adventitial stromal cells: (CD34+Ve/CD146-Ve), are the most important cell types in regenerative therapies

because of their multilineage differentiation capacity [19, 170, 209]. Supra-adventitial stromal cells and pericytes are both identified as precursor cells of ASCs, although there remains some controversy [210-213]. Composition of isolated SVF from OI and HC was similar to each other and comparable to published data [28, 39, 72-75, 202]. However, endothelial progenitor cells as reported in the literature usually represent 10–20% of total nucleated cells within the SVF which is seen in our HC SVF (12.6±4.9%). Still, SVF from OI patients revealed a significantly lower percentage of endothelial progenitor cells (5.4±1.4%) suggesting impaired capacity of angiogenesis in OI, but this remains a question for future studies.

ASCs are the most studied and characterized cells from the SVF, and usually represents 15- 30% of the SVF [39]. Automated enzymatic methods of SVF isolation showed an increase in frequency and clonogenic fraction of the adipose-derived MSC [214]. However, other study showed that there was no difference in ASCs characteristics and function comparing manual versus automated procedure [215]. SVF obtained from OI and HC revealed similar numbers of ASCs and represented more than 30% of the total nucleated cells. This might be influenced by the use of the ice-cold media incubation step in our isolation procedure, which removes the majority of blood-derived cells and allows us to compare a more consistent population of stromal vascular cells released from adipose tissue.

Furthermore, our results showed that the percentage of macrophages ($CD45^+/CD14^+/CD206^+$) in the OI SVF were 4.2±0.9 % which is double the percentage of the control samples 2.2±0.3 %. Macrophages influence bone regeneration though their contributions in many key events from the initial inflammatory phase of healing

and early phases of fracture healing to complete tissue regeneration [216, 217]. The rule of macrophage in tissue repair was demonstrated in both humans [218] and animal models [219, 220]. There's also evidence supporting macrophage contribution to collagen deposition and mineralization in non-osseous tissues [221].

Regarding multilineage differentiation capacity, ASCs had similar osteogenic, adipogenesis, and chondrogenic differentiation between OI and HC. These are in line with published studies of human ASCs [19, 75, 222-225]. However, during our repeated multilineage differentiation experiments we noticed that although all samples were able to differentiate into the 3 cell lines, the rate of proliferation and differentiation capacity varies between the OI patients' samples. This variations in the in vitro properties can be due to the reported donor-to-donor variations [226, 227] that might be contributed by unidentified factors in the medical history and the physiological status of the donors. As MSCs properties also varies between samples obtained from the same donors at different times [228]. These variations can also be attributed to the genotype phenotype specifications and the site of the unique genetic mutation for each patient which counts for the variation in the type of extracellular matrix deposition. Future studies of the gene expression pattern to know which factors exactly are up regulated and which are down regulated in each mutation are needed.

Moreover, ASCs derived from OI patients showed significant Alcian blue staining of differentiated SVF, which confirms its capacity towards chondrogenic lineage diffrentiation histologically. Gene expression analysis also showed significant upregulation of collagen 10, SRY-Box 9 (sox9). Yet it shows downregulation of aggrecan expression. During chondrogenesis, Sox9 is required for mesenchymal cell

condensation and the expression of cartilage-specific extracellular matrix genes [229]. And type X collagen being used as a marker of late stage chondrocyte hypertrophy which facilitates endochondral ossification by regulating matrix mineralization and compartmentalizing matrix component [230]. Aggrecan is a core protein that is an integral part of the extracellular matrix in cartilaginous tissue which react with the Alcian blue stain[231]. This observed reduction in aggrecan expression might be associated with impaired induction of the extracellular matrix markers of chondrogenesis which could be secondary to the OI underlying collagen mutations. Moreover, in addition to growth factors, several environmental factors influence MSC chondrogenesis these include, oxygen tension, mechanical loading and hydrostatic pressure. Which might also played a part in this Aggrecan down-regulation [232]. However, definite conclusions can't be drawn given the limitations of our study domain, and small sample size. Future studies are needed.

Moreover, farther target specific genes therapy approaches may play great rule in overcoming any osteoconductive concerns in regards using autologous Osteogenesis Imperfecta Adipose-Derived Stromal Vascular Fraction and its use in bone generation and preventing non-union post operatively, which is one of the limitations of this study. Limitations of this study also include that we only investigated pediatric patients, did not compare multilineage differentiation of isolated ASCs to BM-MSCs, have not yet performed complex 3D functional in vitro assays and the in vivo testing in an animal model. In which it required to evaluate the SVF bone regeneration capacity in OI or human control patients.

It has been shown that ASC isolation and bone regeneration/wound healing of autologous transplanted ASCs in patients between 6 and 72 years of age were similar [206, 207]. It has also been demonstrated that compared to BM-MSCs, ASCs have a better resistance to cell senescence [32, 233] and are more effective in multilineage differentiation [32, 234-237] (Osteogenic potential of ASCs in combination with bioengineered scaffolds was proven in various animal models [238] with like-calvarial defect [239-242]; femoral head osteonecrosis [243]; femur defect [244]; distraction osteogenesis [245]; and spine fusion [246]. The potential of ASCs for bone regeneration has been investigated in case studies and small-size clinical trials with cranial defects, cranio-maxillofacial skeleton defect, or osteoarthritis [163, 247-249]. This facilitates the future approach of ASC-containing SVF for bone regeneration in OI. But future studies are needed to evaluate the optimal delivery system of SVF to the injury site perhaps by bioengineered scaffolds. It needs to be evaluated if human SVF from diseased patients have the capability of bone regeneration in non-union fracture animal models or mouse models of OI. Promising results were published recently that showed successful bone regeneration of undifferentiated synovial-fluid-MSCs of the temporomandibular joint of patients with temporomandibular dysfunctions on 3D polyetherketoneketone scaffold in a rabbit calvarial critical-sized defect [250].

Our study demonstrated the feasibility of isolating ASC-containing SVF from adipose tissue from pediatric OI patients and age-matched human controls within 90 minutes. We demonstrated that yields of isolated ASC from OI patients are comparable to the ASCs from human controls. And we verified that no alterations of the subcellular composition of the stromal vascular fraction of pediatric OI patients compared to human controls. Adipose tissue-derived stem cells of pediatric OI

patients and human controls express the same MSC surface markers and are capable of differentiation into chondrogenic, adipogenic, and osteogenic lineage. Most importantly, osteogenic differentiation potential was irrespective of an OI mutation. As a platform for therapeutic use, adipose derived stem cells can be used as a new source of MSCs for autologous stem cell therapy in OI.

Now that we have characterized the functional properties and the cellular composition SVF/ASC of culture expanded aMSC of OI pediatric donors in vitro. And in order to reach our long-term clinical goal, to use SVF for autologous stem cell transplantation within the deformity correction surgeries to accelerate the healing of the osteotomy site and prevent non-union. Our research group is working on identifying the optimal dose of stromal vascular fraction for subsequent preclinical and clinical studies and testing the safety and in vivo bone formation capacity of stromal vascular fraction (containing human ASCs) in an immunodeficient mouse model. Then after the pre-clinical experiments in animal models: To determine which scaffold will be used in patients. Bone healing will also be assessed using each scaffold in combination with the SVF/ASCs in two animal models: the mouse model of OI. Early phase I/II clinical study to determine the safety of using autologous adipose tissue SVF on a scaffold intended to promote healing of osteotomy sites in children with OI.

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