

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR



FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO



Potential of miR-195 for Mesenchymal Stem/Stromal Cells- based Osteoregenerative Therapies

Mafalda de Montenegro Cortez

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Orientação: Dra. Maria Inês Almeida

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Abstract

Bone fractures are a common injury, with an estimated 5% to 10% of all human bone fractures experiencing impaired or delayed healing, progressing into non-union bone fractures. Non-unions can be caused by several factors, including: infection, excessive fracture dimensions and severity, extensive damage to surrounding soft tissues, and other co-morbidities that hinder bone regeneration. Patients with non-unions typically undergo invasive therapies, which are painful, long and costly. In extreme cases, limb amputation may be necessary. Therefore, there is a clinical need for new approaches and therapies to promote bone regeneration and facilitate fracture healing in non-union fractures, ultimately enhancing the care and quality of life for these patients.

Mesenchymal stem/stromal cells (MSC) have the ability to facilitate bone regeneration due to their osteogenic potential, immunomodulatory capacity and paracrine effect on angiogenesis. Several studies and clinical trials are currently being developed, leveraging the bone-healing potential of MSC to develop therapies for various musculoskeletal disorders, such as osteoarthritis, osteoporosis and non-union bone fractures. Genetic manipulation of MSC by targeting microRNAs (miRNA) can be used to modulate MSC behaviour and gene expression profiles. Consequently, transfecting MSC with miRNA-mimic or antagonist oligonucleotides can be used for directing MSC towards osteogenic differentiation and enhancing their potential for bone healing. Specifically, anti-miR-195 has been previously identified as having osteogenic and proliferative abilities in human bone-marrow derived MSC.

The aim of this thesis is to identify and validate miR-195 targets associated with bone extracellular matrix (ECM), MSC osteogenesis and proliferation. This involves conducting bulk RNA-sequencing (bRNA-seq) on human primary MSC transfected with an miR-195 antagonist sequence (anti-miR-195). The analysis of the sequencing data revealed 674 differentially expressed genes (DEGs) as potential targets of anti-miR-195, with 309 of them located in protein-coding regions. Gene ontology (GO) analysis revealed 145 genes linked to bone ECM, MSC proliferation, and angiogenesis, of which 19 were present in at least 2 categories and/or displayed an absolute fold change higher than 2. Seven of these genes were selected for further validation through quantitative real-time polymerase chain reaction (qRT-PCR). Out of those, Cyclin B1 (CCNB1), Nicotinamide Phosphoribosyltransferase (NAMPT) and Integrin Binding Sialoprotein

were shown to be upregulated when miR-195 levels were decreased in MSC, validating the bRNA-seq results at transcript level.

Additionally, immunofluorescence assays were conducted to evaluate CCNB1, NAMPT and IBSP at protein level at day 2 and 7 post-transfection with anti-miR-195. CCNB1 was not detected in the tested experimental conditions. NAMPT protein levels showed an increase at day 7 post-transfection under osteogenic conditions, in 2 out of 3 MSC donors, while no differences were found for IBSP protein levels at either day 2 and day 7 post-transfection.

To better mimic the features of a 3D environment, an alginate 3D hydrogel was optimized to support the MSC *in vitro* cultures. For this, different densities of cells and concentrations of alginate were tested. A cell density of 2.5 million cells/mL in 2% alginate hydrogels rendered the best survival rate at day 7 and MSC spreading at day 14. Assessment of NAMPT expression within the hydrogels at day 2 and 7, showed that NAMPT protein levels were higher at the later than at the earlier time-point.

Finally, since no targets could be validated with statistical significance at protein level and to expand the panel of genes to be analysed, the bRNA-seq data was reassessed by performing an untargeted analysis of the results, extending the list of potential miR-195 target candidates beyond the categories of the previous GO analysis. Out of the 30 genes that are up- or down-regulated in all donors with individual fpkm values higher than 0.01 and individual absolute fold change (FC) values greater than 2 for each donor, the six with the highest FC were selected for future validation.

In conclusion, bRNA-seq is a valuable tool to evaluate miRNA direct and indirect targets. This high-throughput analysis revealed anti-miR-195 delivery into MSC regulates osteogenesis- and proliferation-related genes. Although anti-miR-195 target candidates have been shown to be differently expressed at the transcript levels, this was not translated to the protein level. Therefore, future studies should expand the number of genes to be analysed and address their levels in 2D and 3D cultures of MSC, which have been optimized.

Keywords: microRNAs, NAMPT, alginate hydrogel, 3D models, non-union bone fractures, MSC-based therapies

Resumo

As fracturas ósseas são uma lesão comum. Estima-se que 5% a 10% de todas as fracturas ósseas em humanos resultem em não-consolidações ósseas. Estas podem ser provocadas por infeção, dimensão e severidade da lesão, dano extenso nos tecidos adjacentes à fractura e presença de comorbidades que limitam a capacidade regenerativa do osso. O tratamento de não-consolidações ósseas é invasivo, longo, doloroso e dispendioso. Em casos extremos, pode ser necessária amputação parcial. Assim, existe uma necessidade clínica de desenvolver novas abordagens e terapias para promover a regeneração óssea em fracturas de não-consolidação, para melhorar o tratamento e a qualidade de vida desses doentes.

As células mesenquimais estaminais do estroma (do inglês “*Mesenchymal stem/stromal cells*”, MSC) têm a capacidade de facilitar a regeneração óssea devido ao seu potencial osteogénico, capacidade imunomoduladora e efeito parácrino na angiogénese. Vários estudos e ensaios clínicos estão atualmente a ser desenvolvidos para desenvolver terapias baseadas em MSC para tratar doenças músculo-esqueléticas, como não-consolidações ósseas, osteoartrite e osteoporose. A manipulação genética das MSC por alteração dos níveis de microRNAs (miRNAs), pode ser utilizada para dirigir o comportamento e perfil de expressão das MSC. Consequentemente, a transfecção de MSC com oligonucleótidos que mimetizam ou antagonizam miRNAs pode ser utilizada para dirigir as MSC para osteogénese, promovendo o seu potencial regenerativo no osso. Em particular, o microRNA-195 (miR-195) tem efeito anti-osteogénico e anti-proliferativo em MSC humanas primárias.

O objetivo desta tese é identificar e validar alvos do miR-195 associados à osteogénese e proliferação. Para isso, MSC humanas foram transfectadas com um antagonista de miR-195 (anti-miR-195) ou com um oligonucleótido controlo (do inglês “*Scrambled*”, SCR) e o RNA foi isolado para análise por sequenciação de RNA (do inglês “*RNA-sequencing*”, RNA-seq).

A análise dos dados do RNA-seq revelou 674 potenciais alvos do miR-195 que são diferencialmente expressos após silenciamento do miR-195. Destes, 309 codificam proteínas. Análise da ontologia genética (do inglês “*gene ontology*”, GO) destes genes, mostrou que 145 estão associados à matriz extracelular (do inglês “*extracellular matrix*”, ECM), proliferação celular e angiogénese. Destes, 19 estão presentes em pelo menos 2 categorias e/ou apresentam uma expressão relativa igual ou superior a 2. Destes 19, 7

foram selecionados para validação por reação quantitativa em cadeia da polimerase em tempo real (do inglês “quantitative Real-Time Polymerase Chain Reaction”, qRT-PCR). Os resultados mostram que a ciclina B1 (CCNB1), a Visfatina (NAMPT) e a sialoproteína óssea (IBSP) estão aumentadas a nível transcricional após silenciamento do miR-195 em MSC, validando os resultados do RNA-seq.

Adicionalmente, foram realizados ensaios de imunofluorescência para avaliar a expressão de CCNB1, NAMPT e IBSP após 2 e 7 dias da transfecção com o anti-miR-195. O CCNB1 não foi detectado nas MSC nas condições experimentais testadas. Verificou-se um aumento da proteína NAMPT, 7 dias após transfecção com estímulo osteogénico na maioria dos doadores. Contudo, não se verificaram alterações nos níveis de IBSP nas condições testadas.

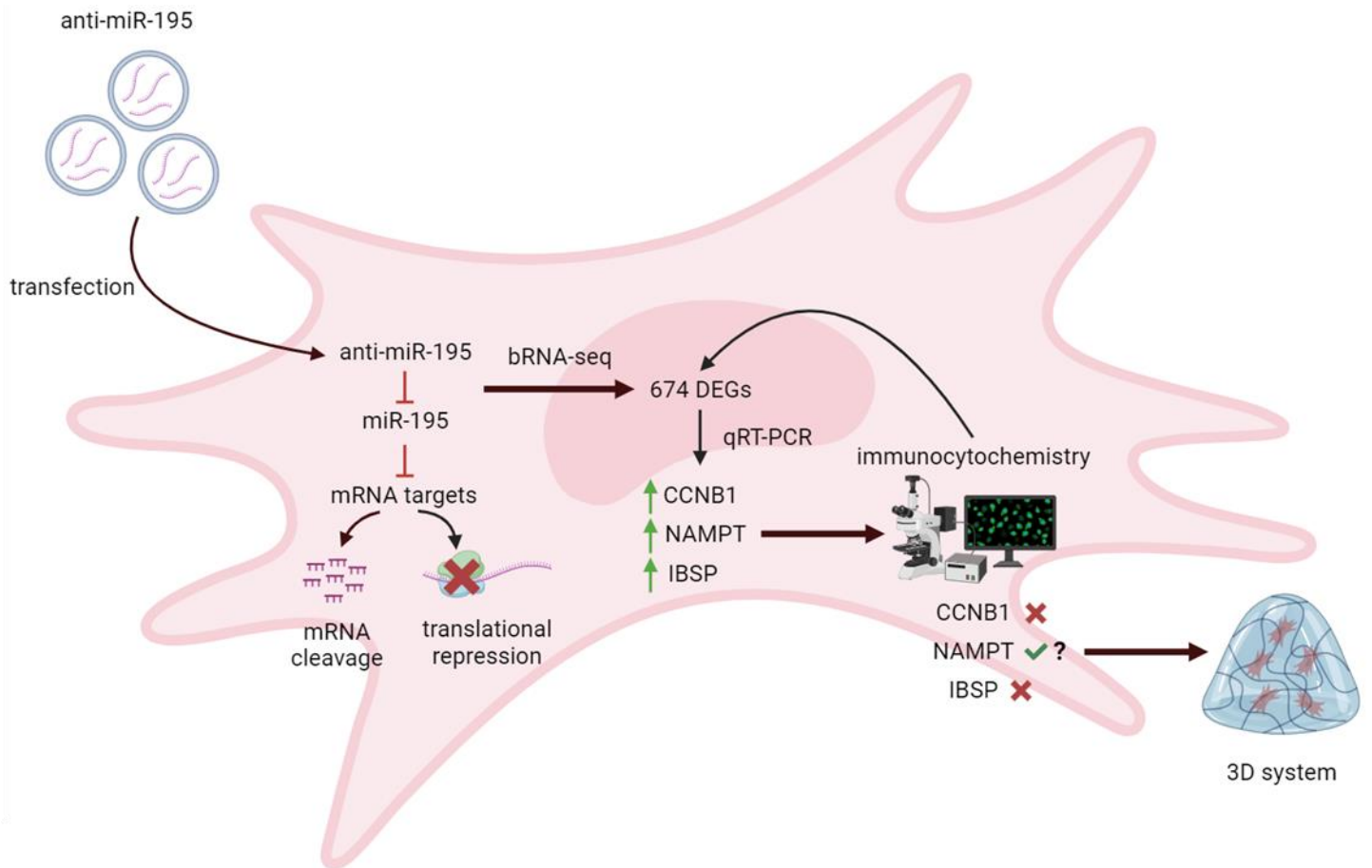
Para mimetizar melhor as características de um ambiente 3D, um hidrogel de alginato foi otimizado para culturas *in vitro* de MSC. Para tal, foram testadas densidades celulares e percentagens de alginato diferentes. As MSC nos géis com a densidade celular de 2,5 milhões de células/mL e a percentagem de alginato de 2% apresentaram a melhor taxa de sobrevivência celular ao dia 7 e a maior extensão celular ao dia 14. A avaliação dos níveis de NAMPT nestes hidrogéis determinou que existe mais NAMPT ao dia 7 que ao dia 2.

Finalmente, uma vez que não foi possível validar o NAMPT e o IBSP a nível proteico, foi realizada uma análise não dirigida dos dados do RNA-seq para expandir a lista de alvos potenciais do miR-195. Nesta análise identificaram-se 30 genes que estão sobre- ou sob-expressos em todos os doadores, apresentando valores de fpkm superiores a 0,01 e de expressão relativa absoluta igual ou superior a 2 em todos os doadores. Destes 30, os 6 com maior expressão relativa foram selecionados para validação futura.

Em conclusão, a sequenciação de RNA é uma ferramenta valiosa para identificar alvos diretos e indiretos de miRNAs. Esta análise revelou que a transfecção de MSCs com anti-miR-195 regula genes relacionados com a proliferação e a osteogénese das MSC. Apesar de ter sido possível validar alvos do miR-195 a nível transcricional, não foi possível observar os mesmos resultados a nível proteico. Assim, no futuro devem realizar-se estudos para expandir a lista de proteínas a testar e avaliar a sua expressão em ambiente 2D e 3D.

Palavras-chave: microRNAs, NAMPT, hidrogel de alginato, modelo 3D, não-consolidações ósseas, terapias celulares com MSC

Graphical abstract



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List of Abbreviations

A

- AGO – argonaute protein
- anti-miRNA – microRNA antagonist
- anti-miR-195 – microRNA 195 antagonist
- ALP – alkaline phosphatase
- AR – aspect ratio
- AT-MSC – adipose tissue derived mesenchymal stem/stromal cell
- AUD – Australian dollar

B

- BAMBI – BMP And Activin Membrane Bound Inhibitor
- BM-MSC – bone-marrow derived mesenchymal stem/stromal cell
- BMP – bone morphogenetic protein
- bRNA-seq – bulk RNA sequencing
- BSP – bone sialoprotein (same as IBSP)

C

- CCNB1 – cyclin B1
- circRNA – circular RNA
- CLEC3B – Tetranectin
- COL1a1 – collagen type Ia1

D

- DEG – differentially expressed gene
- DGE – differential gene expression
- DGCR8 – DiGeorge syndrome critical region in gene 8
- DMEM – dulbecco's modified eagle medium
- dsRNA – double stranded RNA

E

- ECM – extracellular matrix
- eRNA – enhancer RNA
- EUR – euro

F

- FBS – fetal bovine serum
- FC – fold change
- FGF – fibroblast growth factor
- FNDC5 – Fibronectin Type III Domain Containing 5 (same as Irisin)
- fpkm – fragments per kilobase of transcript per million mapped reads
- FZD - frizzled

G

- GBP – Great Britain pound
- GO – gene ontology

- gRNA – guide RNA

H

- hAT-MSC – human adipose tissue derived mesenchymal stem/stromal cell

I

- IBSP – integrin binding sialoprotein (same as BSP)
- IL-1 – interleukin 1
- IL-4 – interleukin 4
- IL-6 – interleukin 6
- lincRNA – long intergenic non-coding RNA
- lncRNA – long non-coding RNA
-

M

- MCSF – macrophage colony-stimulating factor
- miR-195 – microRNA 195
- miRISC – microRNA-induced silencing complex
- miRNA – microRNA
- mRNA – messenger RNA
- MSC – mesenchymal stem/stromal cell

N

- NAMPT – Nicotinamide phosphoribosyltransferase
- NCID – notch intracellular domain
- ncRNA – non-coding RNA
- NSAIDs – non-steroidal anti-inflammatory drugs
- NUSS – non-union scoring system

O

- OCN – osteocalcin
- OPN – osteopontin
- optiMEM – minimum essential media
- OSX – osterix

P

- PDLCs – periodontal ligament cells
- PFA – paraformaldehyde
- piRNA – piwi-interacting RNA
- PPAR- γ – peroxisome proliferator-activated receptor gamma
- pre-miRNA – micro RNA precursor
- pri-miRNA – primary micro RNA

Q

- qRT-PCR – quantitative real-time polymerase chain reaction

R

- RANKL – receptor activator of nuclear factor kappa-b ligand
- RISC – RNA-induced silencing complex
- RNA pol II – RNA polymerase II
- rRNA – ribosomal RNA

S

- SCR – scrambled control
- siRNA – small interfering RNA
- snRNA – small nuclear RNA
- snoRNA- small nucleolus RNA
- SOST - sclerostin

T

- TGF- β – transforming growth factor beta
- TGM2 – transglutaminase 2
- TNF- α – tumour necrosis factor alpha
- tRNA – transfer RNA

U

- UC-MSC – umbilical cord derived mesenchymal stem/stromal cell
- USD – United States dollar

V

- VEGF – vascular endothelial growth factor

I. Introduction

1. Incidence and socioeconomic impact of non-union bone fractures

Bone fractures are common injuries that often follow traumatic events (falls, accidents) or can be caused by repetitive strain or diseases, such as osteoporosis and bone tumours [1]. Their incidence is expected to double by 2050 [1], [2]. Generally, it is estimated that around 5-10% of bone-fractures have impaired healing and will evolve into non-union fractures [2], [3]. However, this incidence varies depending on the fracture site and study methodology. For example, Nicholson et al. reported in 2020 an incidence of 2% of all bone fractures, that could rise up to 20% for diaphyseal fractures[4].

In Spain, Padilla-Eguiluz et al. analysed the incidence of long bone (femur, tibia and humerus) non-unions over a period of 17 years and reported that 4% of all long bone fractures would evolve into non-unions [5]. They estimated an yearly prevalence of 4.5 non-union cases per 100 000 persons [5]. Meanwhile, in Scotland, a study determined that 1.9% to 9% of bone fractures would evolve into non-unions, depending on the fracture site and age group [6]. In Germany, between 2010 and 2015 up to 14 800 non-union cases were reported yearly and in the USA in 2017, Pajarinen et al. reported a yearly incidence of 100 000 non-union bone fractures in the USA alone [7],[8]. Overall, non-unions affect a considerable population worldwide.

Non-union bone fractures are an important contributing factor to the years lived with disability and are associated with increased pain, extended treatment time and costs, as well as decreased quality of life, thus representing an additional burden for patients

[9], [10]. They are difficult and expensive to treat [11]. For example, in the UK in 2007, the “best-case scenario” treatment cost of non-union long bone fractures per fracture was estimated to be between 15 556 GBP and 17 200 GBP (would approximately correspond to 10 896 and 12 040 EUR respectively in 2007) [12]. Moreover, in 2014 the treatment costs were greater with Hak et al. reporting a cost of 29 209 GBP (approximately corresponding to 23 534 EUR in 2014) [11]. A driving factor for the increased costs is the prolonged hospital stays and readmissions. In fact, in Australia, Ekegren et al. reported a 8.1% 2-year readmission rate for bone fracture patients, the majority (83.9%) of which due to non-union complications and associated a total cost of 4.9 million AUD (would approximately correspond to 2.45 million EUR in 2008) to these readmissions [10]. Another considerable factor in the global economic burden of non-union bone fractures are the indirect costs associated with morbidity, disability, absence of work and loss of productivity, which are difficult to estimate [10], [13], [14]. In the USA, median cost of total care of tibia shaft non-union bone fractures per patient, including cost with inpatient care, was estimated to be 32 770.4 USD in 2013 (approximately corresponding to 43 514 EUR), almost three times as much as for patients without non-union complications [9]. These patients were also more likely to be prescribed strong opioid pain treatments during longer periods of time [9]. The high treatment costs, consisting of direct and indirect costs, the last ones associated with morbidity, disability and absence of work, highlight the need for the development of novel therapies for the treatment of patients suffering from non-union bone fractures.

2. Bone composition and structure

2.1. Architecture and structure of long bones

The bones can be divided into flat and long bones. Long bones, such as the femur and tibia, consist of the diaphysis, the long tubular and hollow “tube” that connects the proximal and distal extremities and is filled with yellow bone marrow, and the epiphysis, which are the wider extremities of the bone and are filled with spongy bone [15]. Within spongy bone, the red bone marrow can be found intertwined in the trabeculae of the spongy bone [16]. There, stem cells, including MSCs, can be found, whereas yellow bone marrow is composed mainly of fat [16]. The interface between epiphysis and diaphysis is the metaphysis. At the ends of the metaphysis is the epiphyseal plate, where growth occurs [15]. In adults, when the growth has stopped, the epiphyseal plate is replaced by the epiphyseal line. The outer lining of the bone is called the periosteum, whereas the inner lining that lines the medullary cavity is the endosteum [15]. Osteoprogenitor cells, osteoblasts and osteoclasts can be found in both linings and bone remodelling occurs here [17]. Additionally, nerve fibres, blood and lymphatic vessels can be found in the periosteum. Flat bones, such as the cranial and facial bones, are composed by two layers of compact bone separated by spongy bone [15]. Spongy or cancellous bone is composed of a matrix of trabeculae covered by endosteum. It confers structural support to the bone in a lightweight manner, while compact bone is very hard and confers mechanical resistance to the bone. As previously mentioned, the spaces between trabeculae are filled with red bone marrow, where MSCs and hematopoietic stem cells (HSCs) are located [15]. In contrast to spongy bone, compact or cortical bone is a highly organized tissue composed of osteons or haversian systems, which are made of concentric lamellae of organic and mineralized bone ECM around the central canal or haversian canal, lined with endosteum. Blood and lymphatic vessels and nerve fibres run through the central canal and perforating or Volkmann’s canals to reach the endosteum and periosteum. Between neighbouring lamellae, lacunae with osteocytes can be found [15].

Figure 1 summarizes the composition, structure and architecture of long bones.

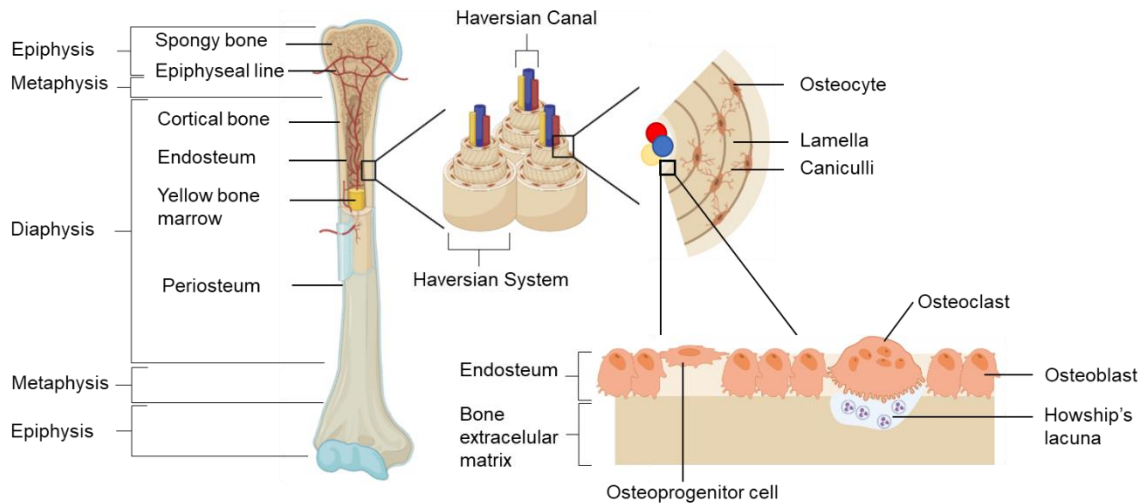


Figure 1. Structure and organization of long bones.

2.2. Bone cells

Bone tissue is defined by the National Cancer Institute NIH as the tissue that makes up bone and is composed of a strong and compact outer tissue (compact cortical bone) and a cancellous tissue (spongy bone) that contains the bone marrow [18]. At the cellular level, bone is composed of osteoblasts, osteocytes and osteoclasts. At the matrix level it is possible to distinguish between organic (25%) and mineralized (70%) matrix [19].

Regarding the cellular component of bones, it consists mainly of osteoblasts, osteocytes and osteoclasts. Osteoblasts are bone-forming cells that secrete osteoid, which is unmineralized freshly produced matrix, composed primarily of collagen and proteoglycans. Osteoid mineralizes when the secreted exosomes carrying phosphatases, calcium ions and phospholipids become saturated and burst, releasing the phosphate and calcium ions into the extracellular matrix (ECM), originating mineralized ECM, that contains hydroxyapatite [19]. Some osteoblasts become entrapped in the ECM they have produced, transforming into osteocytes. This happens to around 5-20% of mature osteoblasts, which undergo osteocytogenesis and turn into preosteocytes [20]. Then, preosteocytes or osteoid osteocytes can become mature osteocytes when the osteoid mineralizes[20]. Osteocytes are smaller than osteoblasts, have higher nucleus to cytoplasm ratio and establish a three-dimensional intercellular communication network (lacuno-canalicular system) with each other and with bone lining cells through cytoplasmatic extensions [19], [21]. These cells correspond to approximately 90% of the

cellular composition of the bone and were previously thought to be inactive cells [19]. However, today it is known that they have multiple functions, such as participating in the synthesis and mineralization of osteoid matrix and in the regulation of plasmatic calcium levels [21]. Furthermore, it is predicted that osteocytes orchestrate and regulate the recruitment of osteoclasts and osteoprogenitor cells, therefore regulating the bone remodelling and bone homeostasis [21]. Osteocyte-mediated MSC recruitment and regulation of bone formation may occur through osteopontin (OPN) secretion and regulation of the Wnt/ β -catenin pathway, whereas orchestration of bone resorption arises through Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) secretion, which supports osteoclastogenesis [22]–[24].

On the other side of the coin are the osteoclasts, which are the cells responsible for the resorption of old or damaged bone. These cells are derived from the monocytic hematopoietic lineage through osteoclastogenesis. They are large, multinucleated and apically polarized cells that resorb bone in Howship's lacunae by secreting protonic species and several enzymes (Figure 1), such as proteases. The protons released to the lacunae reduce the pH in the resorption pit, leading to the dissolution of the mineralized matrix. Afterwards, the degradation of the proteins of the organic matrix through the secreted enzymes occurs [19]. The equilibrium between bone formation, performed by the osteoblasts, and bone resorption, conducted by the osteoclasts, makes up the bone remodelling process. The balance of bone remodelling is crucial to mobilize calcium, phosphates and growth factors towards the bloodstream, contributing to the mineral homeostasis, and to replace old and/or injured/damaged bone with new healthy bone tissue [17]. The dysregulation of the bone remodelling equilibrium is observed in several diseases, such as osteoporosis and osteopetrosis (Figure 2).

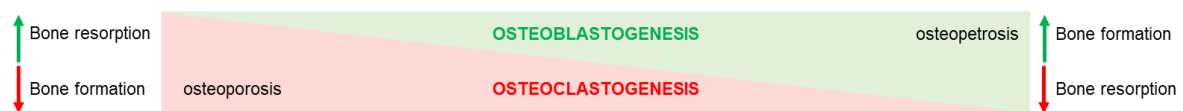


Figure 2. Bone remodelling equilibrium.

3. Bone fractures and non-union bone fractures: definition and treatment

3.1. Bone fractures and non-unions

Bone fractures are common injuries often caused by traumatic events (falls, accidents) or by repetitive strain or diseases, such as osteoporosis and bone tumours [1]. Fractures caused by repetitive strain to the bone are classified as stress fractures and are commonly associated with athletes. Meanwhile, fragility fractures occur typically in patients whose bone structure, composition and architecture has been compromised, for example, due to osteoporosis [13]. Bone fractures affect all age groups globally, having a significant impact on people's lives and representing a substantial economic burden [25]. Patients are often unable to work, while undergoing treatment, have impaired quality of life and experience health loss due to morbidity and disability associated with this type of injury [25].

Some bone fractures exhibit impaired healing, which can lead to non-union fractures. They are currently defined as “the absence of bone bridging and the presence of a fracture line after nine months from acute fracture, with absence of progressive signs of bone healing over three consecutive months” [5]. Previous definitions varied and, therefore, bone fractures were frequently considered non-unions if consolidation did not occur within six months [5]. There are different types of non-unions, including septic non-unions, pseudoarthrosis, hypertrophic non-union, atrophic non-union and oligotrophic non-union [7]. As the name suggests, septic non-unions are caused by the presence of an infection that hinders healing. While in hypertrophic non-unions there are viable bone ends, in pseudoarthrosis non-unions non-viable bone ends are present, that complicate the treatment process [7]. Atrophic non-unions suffer from poor vascularization of the bone ends, and oligotrophic non-unions possess intermediate characteristics of the previously mentioned non-union types [7]. Non-unions can be caused by high energy-traumas, surgeries, such as tumour resections and prosthetic revision surgeries, and diseases, including osteoporosis and infections, that cause bone defects with impaired healing greater than the critical size bone defect [3]. Risk factors include both patient dependent (smoking, advanced age, Non-Steroidal Anti-Inflammatory Drugs – NSAIDs – use, nutritional deficiency, medical comorbidities,

among others) and patient independent factors (such as fracture pattern, site and displacement, bone loss and soft tissue damage severity) [11].

Bone fracture healing is a complex well-orchestrated, multifactorial, multicellular and multistage process, which involves an anabolic phase and a catabolic phase that can be divided into four distinct overlapping stages: (1) hematoma formation, (2) inflammatory response, (3) callus formation and mineralization and (4) remodelling (Figure 3) [1], [7], [13]. During the anabolic phase the tissue volume expands and recruitment and differentiation of stem cells occurs [1]. This phase starts with the formation of a hematoma originated from the rupture of blood vessels and immune cell extravasation, which respond to the injury by eliciting an immediate inflammatory response that involves cytokine secretion and creation of a pro-inflammatory environment. Consequently, this pro-inflammatory environment promotes an adaptative immune response and the recruitment of mesenchymal stem/stromal cells (MSC). The recruited MSC differentiate into chondroblasts that secrete cartilage ECM and differentiate into chondrocytes, thus forming a cartilaginous callus [3]. This soft callus mineralizes transforming into a hard callus. At this point the catabolic phase starts. This phase is characterized by a volume decrease due to the resorption of the cartilaginous callus and its replacement with of woven bone [1]. The progression from anabolic phase to catabolic phase is also characterized by a switch from a pro-inflammatory environment to an anti-inflammatory environment, facilitating vascularization [1], [7]. Finally, bone remodelling occurs, and the immature woven bone is replaced by mature bone. In some cases, when the fracture is highly stable and compressed direct ossification occurs. In these cases, there is no intermediary soft callus formation and bone is regenerated directly by osteogenesis. Namely, MSCs undergo osteogenesis and differentiate into osteoblasts that secrete bone ECM and differentiate into osteocytes, when they become entrapped in the ECM they produced [3], [13].

During the distinct phases of fracture healing different signalling molecules are differentially expressed. For example the pro-inflammatory molecules Interleukin 1 (IL-1), Interleukin-6 (IL-6) and Tumour Necrosis Factor alpha (TNF- α) are upregulated in the inflammatory phase, whereas Transforming growth factor beta (TGF- β) and Bone Morphogenetic protein 2 (BMP-2) expression increases during soft callus formation [13]. During the replacement of cartilage with bone, BMP-3, BMP-4 and BMP-7 levels rise, as they promote the recruitment of osteoprogenitor cells. RANKL and Macrophage Colony-Stimulating Factor (MCSF) are also present in this phase [13]. RANKL and

MCSF play important roles in the differentiation and maturation of osteoclasts, thus being relevant for the bone resorption and remodelling process [26], [27].

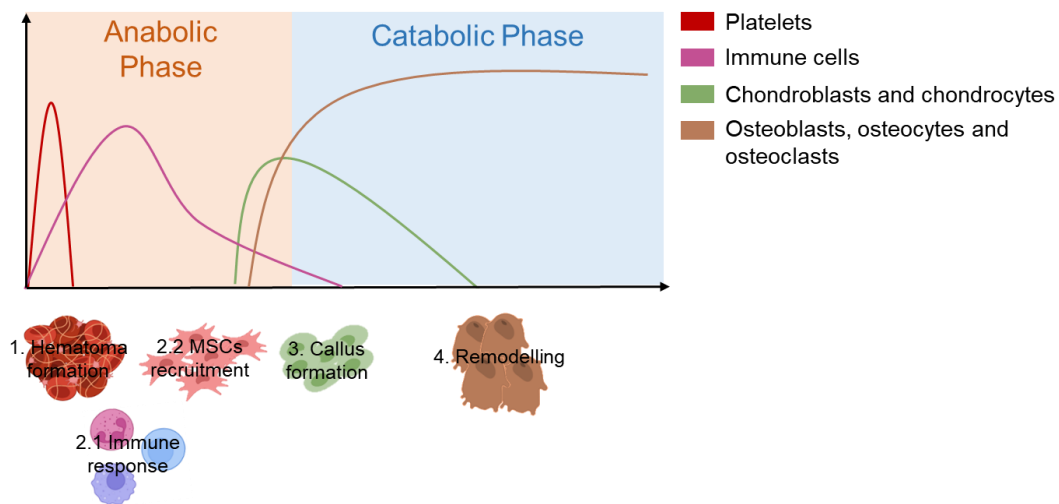


Figure 3. Fracture healing is a multistage and complex multicellular process.

3.2. Non-unions: a problem with insufficient solutions

To evaluate non-unions and provide treatment guidelines, the non-union scoring system (NUSS) has been proposed by Gaddi et al. [28]. NUSS relies on radiographic evaluation of the injury, history of the fracture, bone quality and degree of soft tissue damage to provide a treatment guideline. It also considers the individual patient constitution and clinical interventions performed up to the current point [7]. Currently, non-union bone fractures often involve multiple surgical procedures starting with fracture debridement, which involves the revitalization of the bone edges and removal of the soft callus that was formed during the initial stages of fracture healing and was unable to be remodelled into a hard bony callus. Traditionally, following site debridement, non-unions have been treated by internal fixation methods, such as internal plate fixation or intramedullary nailing, to avoid any movement at the fracture site [12]. These approaches involve the use of bioinert biomaterials, which could possibly evoke an inflammatory response and a foreign body response. These internal fixation methods are considered standard treatment for non-unions and they aim to stabilize and reduce the fracture to allow bridging to occur [7]. Treatment with antibiotics, which may be included in bone cement, can be used to combat infections in septic non-unions [7]. Autologous bone grafts can be used to fill the bone defect thus stimulating biologically the bone healing process

[7]. They are presently the gold standard to treat non-unions, but there is limited availability of graft tissue and this approach requires a bone harvesting surgery, increasing the risk of surgical complications, including infection and donor site morbidity [3], [12]. Spongy donor bone in autografts is typically collected from the iliac bone, and it aims to promote osteogenesis. Additionally, allogenic bone grafts from cadavers are also available to treat these fractures. However, they are rarely used and can result in graft-versus-host disease. Both auto- and allografts are acellular and serve as a scaffold and guide for osteogenesis and bone formation to occur. In the NUSS system, bone grafts are proposed for cases with higher score, and ultimately for the highest score amputation can be the final option [7]. External fixation methods, such as the Ilizarov circular frames, are also available and are used in cases where there is significant bone loss and soft tissue damage and segment transport is required (Figure 4) [7], [12]. If the fracture gap is too large, the surgeon may choose to either amputate a portion of the bone and use a prosthesis, for example in the cases of non-unions in the head of the femur. Alternatively, the surgeon may opt to fuse the fracture ends together, therefore shortening the limb. The surgeries required for the treatment of non-unions are complex and even in successful cases, the recovery time is extended and often painful [29].

Additionally, fracture healing can also be stimulated by non-invasive methods such as electrical stimulation and low-intensity pulsed ultrasound, which can be used supplementary to invasive approaches [9], [12]. Osteoinductive factors, such as bone morphogenetic proteins and platelet derived growth factors can also be used during fixation revision surgeries to biologically stimulate bone regeneration in non-unions as well as bone marrow aspirates, allografted bone, demineralized bone extracellular matrix [7], [9]. However, treatment with BMPs is controversial. Several studies have reported BMP-2 and BMP-7 to impact positively the healing of non-union bone fractures [30]–[32]. However, Woo et al. reported adverse effects of BMP-2 use in non-spinal bone applications, including infections, pseudarthrosis and inflammation [33]. BMP-7 has also been associated with high costs, safety concerns and reasonable failure rates [34]. Other possible adverse effects of treatment with BMPs include ectopic bone formation and vertebral osteolysis [34]. Therefore, BMP-7 was withdrawn from the market and BMP-2 use is now restricted [34]. In the case of pseudoarthrosis non-unions, removal of the non-viable bone ends is required and muscle flaps are available to treat soft tissue damage [7].



Figure 4. Scheme of a Ilizarov circular frame (Zamani et al., R. Soc. Interface (2009)).

Currently, research is being conducted to identify other BMPs that could positively impact fracture healing and Sreekumar et al. proposed BMP-9 as a possible substitute for BMP-7 in 2017 [34]. Vascular endothelial growth factor (VEGF) shows promise in bone fracture healing, due to its pro-angiogenic role, as well as platelet-rich plasma injections [35], [36]. Immunomodulation is also being investigated to treat impaired bone healing as well as mesenchymal stem/stromal cells-based approaches, due to their immunomodulatory and osteogenic properties [3], [7].

4. Mesenchymal stem/stromal cell

4.1. Mesenchymal Stem/Stromal cells: Promising Agents for Fracture Healing

Due to their ability to undergo osteogenesis, immunomodulatory potential and paracrine effect on angiogenesis, MSC-based approaches have been designed and studied to promote fracture healing and bone regeneration [37]. Mesenchymal stem/stromal cells are multipotent stem cells able to self-renew and to differentiate into multiple different cell lineages, including cartilage, adipose tissue, muscle and bone [3], [37], [38]. They make up less than 0.01% of the total cellular population of human bone marrow and can be isolated from bone-marrow tissue by density gradient centrifugation [39]. They have fibroblast-like morphology and are adherent [39]. Following minimal criteria defined by the International Society for Cellular Therapy [40], the MSC should:

- be adherent to plastic in standard culture conditions;
- be positive for CD73-, CD90- and CD105;
- be negative for CD45, CD34, CD19, CD14, CD11b, CD79 α and HLA-DR [3];
- be able to undergo osteogenesis, adipogenesis and chondrogenesis and differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*, respectively.

Another compelling feature that enhances the attractiveness of MSCs for clinical applications is their ease of acquisition from various sources, such as bone marrow, adipose tissue, umbilical cord, placental tissue, among others [3]. Interestingly, studies comparing MSC from different sources have been conducted and Kargozar et al. found that bone-marrow derived MSC (BM-MSC) have higher osteogenesis capacity than umbilical cord derived MSC (UC-MSC) [41]. However, UC-MSC were found to have greater angiogenesis promoting capacity [41]. Therefore, different MSC sources should be considered depending on the aim of the study. MSC-based approaches could be especially relevant for the treatment of non-union bone fractures, since MSC differentiate into osteoprogenitor cells and promote the formation of new bone as well as the repair of damaged bone. In fact, a study reported that human BM-MSC efficiently induced fracture healing in non-unions [42]. The behaviour and cell fate of MSC can be influenced *in vitro* and *in vivo* by endogenous growth factors, cytokines, cross-talk with the ECM, external mechanical stimuli, and epigenetic factors, such as microRNAs (miRNAs)[43].

MSC-based therapies often involve the delivery of the cells to the desired target location. The cells may be delivered to the defect site through a scaffold, such as a bioactive glass and gelatine construct, or a delivery-system, for example a collagen type I sponge [41], [44]. But they can also be transplanted locally without a carrier or supporting scaffold, as Shoji and colleagues did with human adipose tissue derived MSC (hAT-MSC) [45]. Shoji's et al. work assessed the fracture healing capacity of hAT-MSC versus fibroblasts and confirmed that, not only did the hAT-MSC promote fracture healing more efficiently than the control group, they also induced higher expression of BMP-2 and VEGF in the tissues surrounding the fracture. They also reported that the hAT-MSC underwent osteogenesis at the fracture site [45]. However, the use of scaffolds and delivery-systems allows for the simultaneous local delivery of the cells as well as other factors to stimulate bone regeneration, decreasing the risks of off target effects, such as ectopic bone formation. The scaffolds should be designed to promote MSC survival, proliferation and osteogenic differentiation, therefore increasing the efficiency of MSC-based treatments. Regarding the use of scaffolds for local MSC-delivery in bone engineering, hydrogels are being increasingly studied due to their enhanced biocompatibility and swelling properties [46].

Genetic manipulation of the MSC is also available and enables the commitment to a specific lineage and improvement of MSC features before implantation [3]. Popular targets of genetic manipulation are proteins traditionally associated with osteogenesis and bone formation, such as Osterix (Osx) and BMP-2. Actually, a decrease in Osx levels in mice results in impaired osteogenesis and bone formation, therefore Osx overexpression in BM-MSC should enhance osteogenic differentiation and promote bone fracture healing, which was what Tu et al. observed when they introduced Osx-overexpressing BM-MSC in calvarial bone defects in mice [44]. In fact, they determined that the group treated with Osx-overexpressing MSC formed five times more bone than the control group [44]. Another study transduced rabbit BM-MSC with either BMP-2 or VEGF and co-implanted the cells into critical-sized femoral fractures in rabbits and observed that by week 4 all rabbits had healed defects, thus concluding that BMP-2 and VEGF overexpressing BM-MSC accelerated the bone regeneration process [47].

More recently, the engineering of MSC using non-coding RNAs (ncRNAs) has been explored, with a particular focus on microRNAs (miRNAs), which are a class of small ncRNAs, typically consisting of 18-25 nucleotides long, that regulate gene expression by either blocking translation or promoting the degradation of specific

messenger RNA (mRNA) targets [3], [37]. MiRNAs are involved in the regulation of multiple physiological processes, including MSC migration, lineage commitment, immunomodulation abilities and proliferation. Their levels can be easily modulated through transfection with either miRNAs mimic or antagonists [3]. Freitas et al. conducted a review of miRNAs that have the potential to enhance fracture healing, such as miR-218, miR-222, miR-214, miR-135 and miR-21 [3]. Other osteogenesis-influencing miRNAs will be presented in the section 5.3.

4.1. Osteogenesis and Osteoblastogenesis: Intervening Factors and Pathways

4.1.1. From MSC to osteoblast

Fracture healing occurs either by direct intra-membranous ossification or indirect endochondral ossification. On one hand, the first bone healing process involves the direct differentiation of MSC into osteoblasts and bone ECM deposition and occurs in metaphysis fractures which present minimal fracture gap and are rigidly fixed [8]. On the other hand, endochondral ossification occurs in larger fractures, fractures that have lesser mechanical stability and/or are located in the bone diaphysis [8]. It involves the overlapping phases of inflammation, soft and hard callus formation, and bone remodelling, which have been previously described in section 1.1. In both types of ossification, osteoblastogenesis and osteogenesis play key roles.

Osteogenesis is the process of bone formation through differentiation of MSC into osteoprogenitor cells and lastly into osteoblasts, which secrete osteoid that mineralizes and forms the calcified bone matrix (Figure 5). It can be divided into different phases: early osteogenesis with recruitment and expansion of MSC, the second phase, involving the differentiation into pre-osteoblasts and ECM production, and late osteogenesis with ECM mineralization and osteoblast maturation [43]. Osteogenesis can be identified by the expression of osteogenic markers, such as type I collagen, bone sialoprotein (BSP or IBSP), osteocalcin (OCN), and osteopontin (OPN) [39]. The MSC to osteoblast transition is referred to as osteoblastogenesis[48]. During osteoblastogenesis, uncommitted MSC differentiate firstly into preosteoblasts, that become immature osteoblasts and finally mature osteoblasts (Figure 4). Osteoblastogenesis can be induced in vitro with ascorbic acid, β -glycerophosphate and dexamethasone [39]. Several key players are involved in these processes, being Runx2 a central regulator and integrator of several signalling pathways during osteoblastogenesis and osteogenesis [39].

Runx2 or Cbaf1 is a transcription factor, belonging to the Runx family, composed of Runx1, Runx2 and Runx3 [49]. Not only does Runx2 promote MSC differentiation into the osteoblastic lineage, but it also maintains the proliferation of preosteoblasts, by interacting positively with the Fibroblast Growth Factor (FGF) signalling [49]. It is also associated with the expression of bone ECM proteins. Knockout mice for Cbfa1 or Runx2 present impaired ossification and lack of osteogenesis- and osteoblast-related genes such as OPN and OCN[50]. They also presented decreased expression of bone extracellular matrix proteins, such as collagen type I, OPN and IBSP [51].

The expression of collagen type I, OPN, IBSP and other relevant osteogenesis-associated genes fluctuates, depending on the differentiation stage of the cells. Uncommitted MSCs present weak expression of collagen type Ia1 (COLIa1) and during differentiation into preosteoblasts, COLIa1 expression increases. While immature osteoblasts strongly express OPN and IBSP, mature osteoblasts present upregulated expression of COLIa1 and OCN. When they differentiate into ECM entrapped mature osteocytes, sclerostin (SOST) expression increases, which is a marker for osteocytes [49] (Figure 5).

Table 1 summarizes the main factors involved in the different stages of osteoblastogenesis and osteogenesis.

Table 1. Factors involved in osteogenesis.

Transition	Factors
MSC → Preosteoblast	Runx2 and Ihh [49]
Preosteoblast → Immature osteoblast	Runx2, β -catenin, and Dlx3/5/6 [48], [49]
Immature osteoblast → Mature osteoblast	Runx2, osterix, NFAT, and β -catenin [48], [49]
Mature osteoblast → Osteocyte	Osteopontin, osteonectin, and osteocalcin [48]

The regulation of osteoblastogenesis and osteogenesis is wide and complex and there are multiple influencing factors, signalling pathways and microenvironmental cues that have been associated with osteoblast differentiation and osteogenesis. In this section the most important signalling pathways and the effect of inflammation on MSC osteogenic differentiation will be explored.

4.1.2. Signalling pathways involved in osteogenesis and osteoblastogenesis

Several signalling pathways are involved during osteoblastogenesis and osteogenesis, including BMP-, Wnt-, Notch-, Hedgehog- and FGF- signalling pathways.

Starting with the BMP-signalling: BMP binds to BMP receptors, which can be divided into type I (BMPRI) and II (BMPRII) serin-threonine receptors. BMP binding to BMPRII leads to the recruitment of BMPRI and an activated ligand-receptor complex is formed, leading to the activation of intracellular Smad proteins through phosphorylation [39]. These proteins translocate to the nucleus, where they act as transcription factors for the Runx2 gene, which has already been previously presented and is intensively involved in several stages of osteoblastogenesis and osteogenesis [39], [49], [50]. In fact, Lee et al. and Wei et al. both concluded that BMP-2 is a promoter of osteogenesis [48], [52].

Wnt signalling involves either canonical or non-canonical Wnts. In canonical Wnt-signaling, Wnts bind to Frizzled (FZD), a transmembrane receptor, and to LRP5/6 co-receptor, leading to the intracellular accumulation of β -catenin, which translocates to the nucleus and interacts with transcription factors to promote the transcription of target genes, including Runx2, similarly to the BMP-signalling pathway [39]. Additionally, Wnt/ β -catenin activation inhibits the transcription of adipogenic transcription factors, such as the peroxisome proliferator-activated receptor- γ (PPAR- γ), which blocks the expression of Runx2 and its activity as a transactivator, thus inhibiting OCN expression [53]. Canonical Wnt-signalling activation also leads to the increased expression of Dlx5, a homeobox protein, and Osterix, which are both genes associated with bone lineage commitment [53].

The Notch intracellular signalling pathway is highly conserved and regulates stem cell proliferation and differentiation, both in embryonic and adult tissue, participating in stem cell commitment and fate decisions in the development and regeneration of skeletal tissue [54]. Notch itself is a transmembrane receptor present in the cellular membrane of several cell types, including MSCs. It has an intracellular domain (NCID – Notch intracellular domain) that is cleaved when the extracellular domain of Notch binds to membrane-bound ligands, namely Jagged or Delta, present in the membrane of neighbouring cells. The NCID then enters the cell's nucleus and activates the transcription factor CBF1 and binds to co-activators, inducing the transcription of target genes such as Hes and Hey [39]. In humans, Notch 1 to 4 interact with Jagged1 or Jagged2, or Delta-like 1, 3, or 4 ligands, causing the transcription of the genes *HES1*, *HES5*, and *HES7* and

HEY1, *HEY2*, and *HEYL*. *In vitro* studies with MC3T3 osteoblast cell line, have concluded that Notch signalling positively influences osteoblastic differentiation [55]. However, these observations are controversial, and the effects of Notch signalling activation probably depends on the cell type and time-point of differentiation. A study using ST-2 cells, a cell line of mice bone marrow stromal cells that has the ability to differentiate into osteoblasts, concluded that Notch 1 overexpression suppressed osteoblastogenesis by inhibiting Wnt/ β -catenin signalling [56]. Interestingly, Dishowitz et al. observed that proteins involved in Notch signalling were upregulated during bone regeneration in *in vivo* tibial fracture and calvarial defect injury models [57]. Particularly, Notch2 and Jagged 1 were upregulated in MSCs during bone regeneration [57]. Therefore, the way and extent Notch signalling influences osteoblastogenesis remains unclear.

Hedgehog signalling plays a key role during embryonic skeletal development and has been implicated in osteoblastogenesis through Runx-2 [58], [59]. There are three hedgehog homologs, and particularly Indian hedgehog (Ihh) has been long known to be essential for skeletal development and osteoblastogenesis. Already in 1999, St-Jacques et al. established that *Ihh* ^{-/-} mice had no mature osteoblasts in their long bones [60]. In 2005, Hu et al. confirmed these observations and reported that *Ihh*-null mice presented reduced Runx2, Osx, type I collagen and alkaline phosphatase (ALP) expression [61]. However, *Ihh* signalling inhibition at preosteoblastic stage no longer affected osteoblastogenesis [49]. Therefore, it seems that *Ihh* signalling is necessary for the commitment of MSC towards osteogenic lineage and differentiation into preosteoblasts, but no longer required for further differentiation into mature osteoblasts (Figure 5).

Finally, fibroblast growth factors (FGF) are a family of growth factors involved in the proliferation and differentiation of several different cell types, including MSC and preosteoblasts [39]. Specifically, FGF-2 has been associated with early stages of differentiation and lineage commitment of osteogenesis [39].

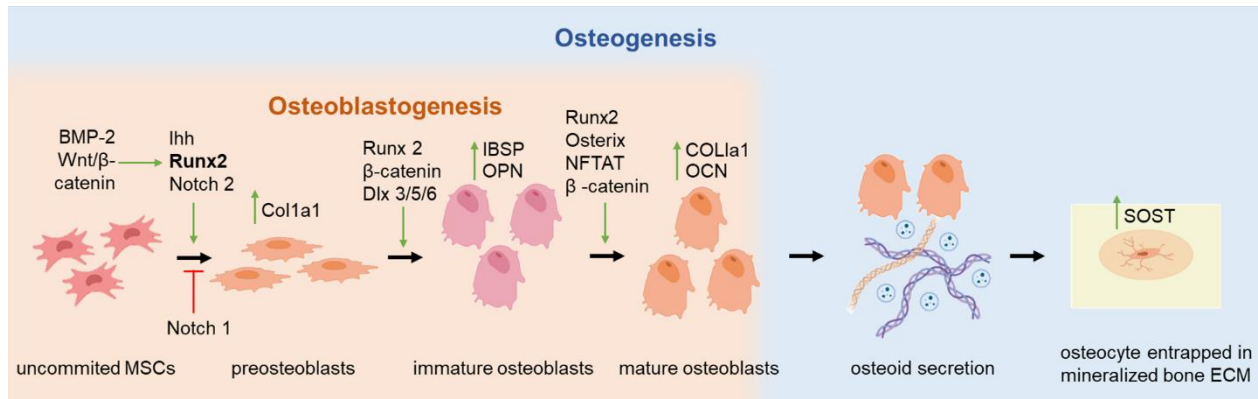


Figure 5. Osteogenesis and osteoblastogenesis.

4.2. The influence of inflammation on osteoblastogenesis

Osteoblastogenesis and osteogenesis of MSC during fracture healing are also influenced by the microenvironment at the injury site. As previously mentioned, after injury, an inflammatory response starts and immune cells extravasate from the blood vessels towards the fracture site and create a pro-inflammatory environment. These immune cells secrete cytokines, including TNF- α and Interleukin-6 IL-6, which promote osteoblastic differentiation of MSCs [48]. In the pro-inflammatory environment, M1 pro-inflammatory macrophages can be found. These cells promote early and middle stages of osteogenesis [8]. Then a switch to an anti-inflammatory environment occurs and M2 macrophages become more abundant. In vitro co-culture experiments with MC3T3 osteoblast cell line and M1 and M2 macrophages, concluded that an initial M1-dominant pro-inflammatory environment followed by prolonged co-culture with M2 macrophages enhanced bone formation [62]. Interleukin-4 (IL-4), which is secreted by M2 macrophages, seems to further promote osteogenesis of MC3T3 cells [8]. In conclusion, the inflammatory environment at the fracture site seems to be relevant for the bone regeneration process and the switch from an initial pro-inflammatory state to an anti-inflammatory state is necessary for the progression of the different bone healing phases.

5. Non-coding Transcripts: Epigenetic Regulators of Cell Differentiation and Tissue Regeneration

5.1. Definition and Importance

The majority of the human genome does not codify for any proteins and, instead, gives rise to non-coding RNAs. In fact, in the human genome only about 2% of the genes are protein-coding and up until the late 90s and early 2000s the rest of the genome was thought to be “evolutionary junk” [63], [64]. The protein-coding genes are transcribed into messenger RNAs (mRNAs) which are translated into amino-acid sequences, that will later be folded and acquire the structure of a protein, with the help of transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs). Up until recently, the functions of the rest of the non-protein-coding genome were unknown. However, today we know that these non-coding sequences have various important functions. Non-coding RNAs (ncRNAs) can be classified as either housekeeping or regulatory ncRNAs [65]. Housekeeping ncRNAs participate in central cellular functions and include the tRNAs, rRNAs, small nuclear RNAs (snRNAs), which participate in RNA splicing, and small nucleolus RNAs (snoRNAs), that are responsible for RNA modifications [65]. The regulatory ncRNAs modulate and regulate gene expression and can be categorized according to their size: small or short ncRNAs with less than 200 nucleotides (nt) and long ncRNAs (lncRNAs), which have more than 200 nt [64], [65]. NcRNAs can form complex structures, interact with other RNAs, proteins or DNA and orchestrate multiple cellular processes, such as growth, development, and organ function, being involved in cell proliferation, migration, differentiation, survival and gene expression. These transcripts have gained importance due to their roles in both physiological and pathological processes such as cancer, where they can act as biomarkers, targets or therapeutic agents [64], [65].

Long non-coding RNAs represent the majority of the ncRNAs and can reach lengths of several thousand nucleotides [65]. LncRNAs can be classified according to their genomic location and relative position to protein-coding genes (Table 2) [64]. They are a developing field of research and not as extensively studied as some classes of small ncRNAs, such as microRNAs (miRNAs).

Table 2. Classes of long non-coding RNAs according to genomic location.

Class of lncRNA	Characteristic
Sense lncRNA	Overlap at least one exon of a coding gene
Antisense lncRNA	Have partial or complete complementarity to transcripts on the opposite strand
Intronic lncRNA	Are transcribed from an intron of a gene
Bidirectional lncRNA	Share promoters with protein-coding genes, but are transcribed in the opposite direction
Long intergenic ncRNA (lincRNAs)	Are found between two protein-coding genes, but are transcribed independently from these genes
Enhancer lncRNA (eRNAs)	Originate from enhancer regions of protein-coding genes
Circular lncRNA (circRNA)	Are produced from splicing events of protein-coding genes and their ends are covalently bound to each other, forming closed loops

Small or short ncRNAs can have up to 200nt length, as previously mentioned. This class of ncRNAs includes several subclasses, the most notorious one being microRNAs (miRNAs). Other subclasses are the small interfering RNAs (siRNAs), guide RNAs (gRNAs), small nucleolar RNAs (snoRNAs) and piwi-interacting RNAs (piRNAs) [65], [66]. Table 3 summarizes the aforementioned subclasses of small ncRNAs.

Table 3. Classes of short non-coding transcripts.

Class of short ncRNA	Length (nt)	Function
gRNA	60-80	Edits site selection during RNA editing.
siRNAs	19-23	Interfere with gene expression and have complementary regions to protein-coding genes.
snoRNAs	60-300	Participate in the modification of ribosomal RNAs.
piRNAs	26-30	Participate in the silencing of some DNA-elements and in germline DNA integrity maintenance. Interact with piwi proteins.

MicroRNAs (miRNAs) are the most well-known and studied class of small ncRNAs. They are around 22nt long and regulate gene expression by binding specifically to complementary sequences in mRNAs and either blocking their translation or promoting mRNA degradation [64], [65]. This class of ncRNAs will be explored more extensively in the following section.

5.2. MicroRNAs: biogenesis pathway and mechanism

MicroRNAs belong to the regulatory RNAs and their length varies between 18 to 25 nt. They represent 1% to 5% of the human genome and predictably regulate more than 60% of all protein-coding genes [43], [67]. As previously mentioned, they regulate gene expression at the posttranscriptional level. They are conserved among species and present cell-type and tissue-specific expression, which facilitates their study and enhances their appeal as biomarkers and therapy targets [68]. They were firstly identified in *C.elegans* in the late 90s and early 00s with the identification of miRNA lin-4 and let-7 in 1996 by Lee et al. and in 2000 by Reinhart and colleagues, respectively [69], [70]. The let-7 gene was also found in the same year in other species, including humans and *Drosophila*, hinting toward the conserved characteristic of miRNAs [71]. Since then, miRNAs have been described in several species and physiological and pathological processes, including in MSC differentiation and osteogenesis.

MiRNAs genes are usually found in clusters within the genome mostly in intergenic regions, and few in intronic regions [64][71]. In animals, their biogenesis pathway comprises of four different phases: transcription, nuclear maturation, export and cytoplasmatic processing (Figure 6) [64]. During transcription, primary miRNAs (pri-miRNA) are formed by RNA polymerase II (RNA pol II), and in some cases, RNA polymerase III. These pri-miRNAs are approximately 70 nucleotides long, have a hairpin structure and present 5' capping and 3' poly(A) tail [64], [68]. After transcription, nuclear maturation of the pri-miRNA occurs, consisting of recognition of the 70nt pri-miRNA is by the DGCR8 (DiGeorge syndrome critical region in gene 8) protein in humans, which contains two double-stranded RNA (dsRNA)-binding domains [72]. The DGCR8 co-factor associates with the endonuclease Drosha, a class II RNase III enzyme, forming the DROSHA/DGCR8 complex, called the microprocessor [73]. The microprocessor cleaves the 3' and 5' ends of the pri-miRNA, originating a miRNA precursor (pre-miRNA) hairpin of 60nt [64], [71], [72]. Then, the pre-miRNA gets exported to the cytoplasm through the Exportin 5 - RanGTP complex. In the cytoplasm, the endonuclease DICER cleaves the looped end of the hairpin, forming an RNA duplex of 22nt composed of the mature miRNA strand and the passenger miRNA strand (miRNA*) [71]. The mature miRNA strand is loaded onto Argonaute 1 to 4 (Ago 1 - 4) proteins, forming the effector complex, also known as the RNA-induced silencing complex (RISC) or the miRNA-induced silencing complex (miRISC), whereas the miRNA* strand is exposed and is degraded [71].

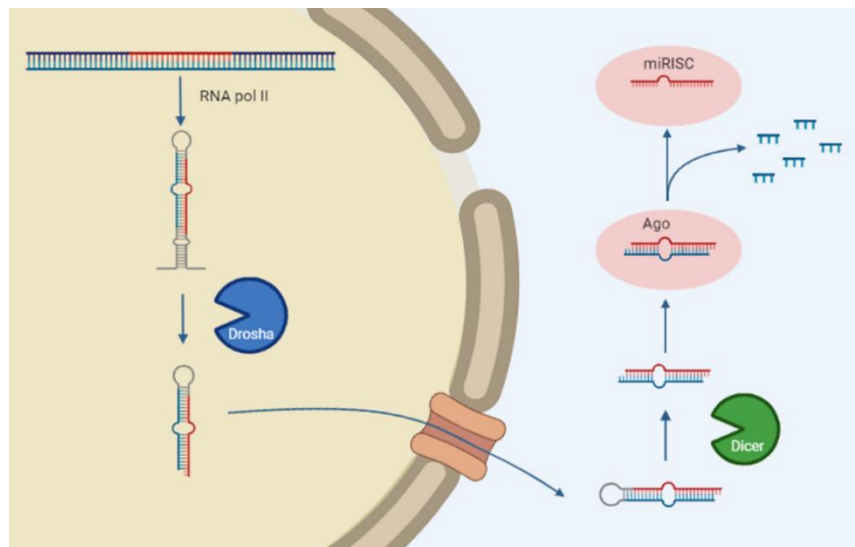


Figure 6. MicroRNA biogenesis pathway.

As previously mentioned, miRNAs regulate negatively gene expression at the posttranscriptional level. They direct the miRISC complex towards specific mRNA targets and perform either translational repression or promote mRNA cleavage, by binding specifically to sequences found mostly in the 3'-untranslated region (3'-UTR) of target mRNAs, therefore decreasing final protein expression [66], [71]. MiRNAs can also bind to complementary sequences found in the 5' UTR and coding sequences of target mRNAs [67]. When the complementarity between miRNA and mRNA is total, the mRNA target undergoes degradation. Otherwise, translational repression occurs, and protein levels decrease but are not eliminated [74]. However, miRNAs can impact positively the expression of indirect targets by decreasing the expression of direct targets, that block the expression of the indirect targets (Figure 7) [66]. One single miRNA can have multiple mRNA targets, having the ability to affect multiple pathways and cellular mechanisms simultaneously [43].

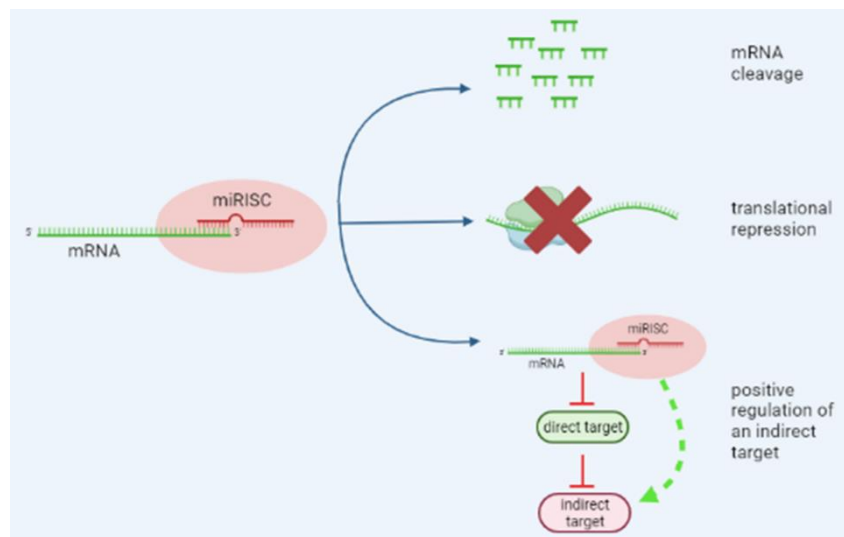


Figure 7. MicroRNA mechanisms of action.

Considering the nomenclature of microRNAs, some aspects need to be briefly clarified. Different microRNAs are identified by the abbreviation “miR” followed by a number, which is given sequentially based on the order of discovery [75]. When referring to the genomic loci, microRNAs names should be written in italic, whereas when referring to the mature microRNA, names aren’t written in italic and the strand of origin can be indicated by adding either 5p or 3p to the name, e.g. *miR-195*, miR-195-5p and miR-195-3p [76]. Mature microRNAs originated from different strands of the precursor can have different mRNA targets. Similar microRNAs that are evolutionarily related can be distinguished by a letter following the number, such as for example *miR-29a* and *miR-*

29b [75], [76]. Human microRNAs can be identified by the prefix “hsa”, e.g. has-miR-195 [76]. Identical mature microRNAs that originate from different loci are distinguished by adding another number to the name, for example *miR512-1* and *miR-512-2* [75], [76].

5.3. microRNAs involved in determining the cell fate of MSCs

MicroRNAs are epigenetic factors that can be employed to modulate the behaviour, proliferation, and commitment of MSCs towards osteogenesis. In this context, we will introduce some of the miRNAs that have already been documented *in vitro* and/or *in vivo* as influencers of osteogenesis and bone regeneration. For additional osteogenesis-related miRNAs, you can refer to the reviews by Mazziotta et al, Han et al and Freitas et al. [3], [43], [77].

MiR-21 has been reported to improve osteogenesis and bone fracture healing in a rat bone fracture model and showed to promote BM-MSCs migration, osteogenesis *in vitro* and new bone formation *in vivo* [78], [79]. Moreover, miR-21 has been shown to ameliorate apoptosis in BM-MSCs [80]. MiR-26a has also been described as having the ability to improve osteogenesis of rat BM-MSCs [81]. MiR-126 is an exosomal miRNA present in exosomes derived from MSCs cultured under hypoxic conditions and has been reported as promoting bone fracture healing as well [82]. Another osteogenic-promoting miRNAs is miR-29b [83].

MicroRNAs can also have a negative impact on osteogenesis, such as miR-16-2, miR-148a, miR-195, miR-381, miR-765 and miR-889 [37], [84]–[88]. Interestingly, miR-26a, which is pro-osteogenic in BM-MSCs from rats and mice is anti-osteogenic in mice adipose-derived MSCs (AD-MSCs) [89]. MiR-195 has been previously studied in the group and has been found to inhibit osteogenesis and MSC proliferation [37]. Additionally, it has also been described as decreasing angiogenesis by targeting VEGF [37]. Therefore, a suppression of miR-195 levels should have the opposite effect and promote osteogenesis, MSC proliferation and angiogenesis.

Table 4 summarizes the aforementioned osteogenesis-impacting miRNAs and introduces some new miRNAs.

Table 4. Osteogenesis-impacting microRNAs.

miRNA	Effect on osteogenesis	Cell line	In vivo studies
miR-16-2	-	hBM-MSCs [87]	×
miR-21	+	rBM-MSCs [78] rBM-MSCs [79]	✓ [78], [79]
miR-26a	+	rBM-MSCs [81], mBM-MSCs [89]	✓ [89]
miR-26a	-	mAD-MSCs [89]	✓ [89]
miR-29a	+	mBM-MSCs [90]	✓ [90]
miR-29b	+	hAD-MSCs [83], MC3T3 [91]	×
miR-126	+	hUC-MSCs [82]	✓ [82]
miR-148	-	rBM-MSCs [88]	✓ [88]
miR-195	-	hBM-MSCs [37]	×
miR-195-5p	-	human Periodontal Ligament Cells (PDLs) [92]	✓ [92]
miR-381	-	hBM-MSCs [84]	×
miR-765	-	hBM-MSCs [85]	×
miR-889	-	hBM-MSCs [86]	×

It is important to emphasize that only a small fraction of osteogenesis-affecting miRNAs was presented here to portray how osteogenesis is tightly regulated by several microRNAs and that many more microRNAs are involved in the regulation of osteogenesis.

5.4. MiR-195: a player in osteogenesis of MSCs

Previously, several miRNAs that influence the osteogenesis and the osteoregenerative potential of MSC were presented, including miR-195. As previously mentioned, this microRNA inhibits the osteogenesis and proliferation of MSC. Additionally, miR-195 interacts with VEGF, thus having a paracrine effect on angiogenesis [37]. So, miR-195 affects bone regeneration in three ways: through regulation of MSC osteogenesis and proliferation and through paracrine regulation of angiogenesis. Therefore, in this section the focus will be on miR-195.

A negative correlation between miR-195-5p levels and osteogenic differentiation of human primary PDLs has been established [92]. Overexpression of miR-195 in MC3T3-E1 cells inhibited osteoblast differentiation [93]. Additionally, there is extensive evidence in several cancers that miR-195 has a negative effect on cellular proliferation [94]. For example, in lung cancer miR-195-5p is downregulated and is thought to inhibit cell proliferation [95]. In colorectal cancer, miR-195-5p has been demonstrated to decrease the proliferation of colorectal cancer cells, downregulating the levels of cyclin B1 (CCNB1) by targeting directly FGF2 and regulating the canonical Wnt/ β -catenin pathway [96]. Interestingly, as previously stated, the canonical Wnt/ β -catenin pathway and FGF signalling are involved in osteoblastogenesis of MSCs. In osteosarcoma, *miR-195* decreases cellular proliferation, migration and invasion as well and miR-195 inhibition might act as tumour promoter [97]. Finally, miR-195 has been previously studied in the group and has been found to inhibit osteogenesis and MSC proliferation [37]. Additionally, it has also been described as decreasing angiogenesis by targeting VEGF [37]. Therefore, a suppression of miR-195 levels should have the opposite effect and promote osteogenesis, MSC proliferation and angiogenesis.

In conclusion, there is extensive evidence suggesting that *miR-195* is an important regulator of osteogenesis, osteoblastogenesis and cellular proliferation. This enhances the therapeutic potential of this microRNA. However, miR-195 protein targets involved in the regulation of MSC differentiation and proliferation remain to be identified.

II. Aim and Rational

This thesis aims to identify miR-195 targets involved in MSC behaviour at the transcriptional level and to validate the most relevant targets at the protein level in 2D and 3D *in vitro* cultures.

Firstly, the problem of bone fractures, including non-union fractures, was thoroughly investigated, and its socioeconomic impact was outlined. This was undertaken to deepen the understanding of non-union bone fractures and the currently available treatments. To develop a therapeutic approach for bone injuries, it is necessary to comprehend the biology, composition and architecture of bone, as well as the biological and cellular processes intrinsic to bone healing and regeneration. Given that MSCs play a pivotal role in bone fracture healing, it is pertinent to provide a definition of MSCs and explain their utility in the development of therapies that promote bone regeneration. As osteogenesis is key in bone formation, healing and regeneration, it is relevant to elaborate on these processes. Finally, the definition, biogenesis and mechanisms of miRNAs were investigated, and specific examples of miRNAs impacting osteogenesis were presented.

Secondly, the experimental work was divided into 4 main tasks: 1) Identification and selection of potential targets for anti-miR-195; 2) Validation of these targets through quantitative real-time polymerase chain reaction (qRT-PCR); 3) Assessment of the differential expression of selected targets at a protein level; and 4) Optimization of a cell-supportive 3D microenvironment aimed at facilitating future validation of the identified targets within a 3D context.

To fulfil the first objective of identifying miR-195 targets at the transcriptomic level, a bulk RNA-sequencing (bRNA-seq) was performed. Subsequently, gene ontology and gene enrichment analysis were performed, yielding a list of the most relevant targets for further validation. This validation was initially carried out at a transcript level with qRT-PCR. Furthermore, we assessed target expression at a protein level through immunofluorescence assays conducted on 2D cellular monolayers. Additionally, to simulate a 3D environment, we optimized MSC cultures in alginate hydrogels. Different cellular densities and alginate concentrations were experimentally tested and cell viability, survival and morphology were assessed. Additionally, we validated the immunofluorescence protocol within the hydrogels. Lastly, an untargeted analysis of the bRNA-seq results was performed to identify targets beyond the gene ontology categories.

III. Materials and Methods

1. Cell culture

Human bone-marrow derived MSC were previously isolated from bone tissue collected from 5 patients under 37 years of age undergoing anterior cruciate ligament rupture repair surgery at Centro Hospitalar Universitário São João (Porto, Portugal) and from 1 patient over 60 years that suffered fragility fractures and received surgical treatment at Centro Hospitalar Universitário São João (Porto, Portugal). All donors provided an informed consent for the collection of tissue and its use for research. The MSC were previously categorized in the group accordingly to the previously mentioned minimal criteria defined by the International Society for Cellular Therapy [40]. Additionally, commercially available MSC from 1 further donor were obtained (Lonza).

The cells were cultured on tissue-culture treated T₇₅ flasks with low-glucose (1g/L) Dulbecco's Modified Eagle Medium (DMEM – Corning) supplemented with 10% FBS (ThermoFisher Scientific) and 1% Pen/Strep (Labclinics). Media was changed twice a week.

Osteogenic stimulus was induced using culture media (in DMEM low glucose with 10% FBS for cell differentiation without Pen/Strep) supplemented with ascorbic acid (1:100 dilution), β -glycerophosphate (1:100 dilution) and dexamethasone (1:100 dilution).

2. Bulk RNA sequencing

RNA was extracted from MSC obtained from 3 donors that had been transfected with either negative inhibitor control (scrambled sequence - SCR) (Ambion) or with miR-195 antagonist (anti-miR-195) oligonucleotides (Ambion). Bulk RNA sequencing (bRNA-seq) was performed by Novogene using the Illumina NovaSeq platform (Human mRNA-Seq 6Gb). Briefly, the methodology encompassed a poly(A) selection to enrich the mRNAs, followed by PCR amplification and sequencing. Low-quality reads were excluded from the final results. The sequences were aligned to the human reference genome, the reads were quantified and fold change (FC) and p-value were calculated.

Differentially expressed genes (DEG) were only considered significantly differential when $p\text{-value} < 0.05$.

3. Gene ontology analysis

Bioinformatic analysis tools such as DAVID (<https://david.ncifcrf.gov/tools.jsp>) and PANTHER (<http://pantherdb.org/>) were employed for Gene Ontology (GO) analysis.

Categories spanning cellular component, molecular function, biological process, KEGG pathways and tissue expression were explored and the ones related to extracellular matrix, cell cycle and proliferation, angiogenesis and expression in the bone marrow were selected for a more detailed analysis.

Genes falling within these categories, which were either present in two or more categories and/or had a $|FC| > 2$, were selected for a list of potential targets. This list was then reviewed based on existing published literature that describes their roles and functions in the survival, proliferation and differentiation of MSC.

4. Transfection

Cells were detached from the cell culture flasks using trypsin (Sigma, T4799-100G) and counted using trypan blue (Sigma-Aldrich) and a Neubauer Chamber. Then, the MSC were plated up to 70% confluence in 6-well plates (VWR - Avantor) for RNA extraction and 24-well plates (VWR - Avantor) for immunocytochemistry (0.2 million cells/well for the 6-well plates and 0.04 million cells/well for the 24-well plates). After cell seeding, the cells were left to adhere for 24h with MSC growth medium and then the medium was replaced with either negative inhibitor control (scrambled sequence - SCR)

or miR-195 antagonist (anti-miR-195) transfection medium. Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions[98]. Briefly, SCR or anti-miR-195 sequences were diluted with in minimum essential media (OptiMEM – Thermo Fisher Scientific) in the proportion of 1:100 and 1:125 for 6-well plates and 24-well plates respectively. A solution of Lipofectamine 2000 and OptiMEM (1:25) was prepared and added in a 1.1 proportion to the SCR and anti-miR-195 mixes, which were left to incubate at room temperature (RT) for 15 minutes. Finally, SCR or anti-miR-195 transfection mix was added to the wells and the volume was completed up to 1mL or 0.4mL with low glucose DMEM supplemented with 10% FBS for 6-well plates or 24-well plates, respectively. Cells were left to incubate for 6h at 37°C and 5% CO₂ with the transfection reagent and then media was changed to complete cell culture media.

5. RNA extraction

RNA was isolated using TRIzol reagent (Invitrogen) 48h after transfection with either SCR or anti-miR-195 sequences, following the company's guidelines[99]. The MSC monolayer was disrupted using TRIzol (1 mL/well) and the lysed material was collected. Chloroform (Thermo Fisher Scientific) was added to the cell lysates (200µL chloroform per 1mL of TRIzol), mixed vigorously, incubated for 5 minutes at RT and centrifuged for 15 minutes, at 12 000g and 4°C to separate the organic and inorganic phases. The upper aqueous phase, which contains the RNA, was transferred to clean microcentrifuge tubes (Thermo Fisher Scientific) and isopropanol (ACROS Organics) was added, mixed vigorously, incubated at RT for 10 minutes and the centrifuged at 12 000g and 4°C during 10 minutes to form a RNA pellet. The supernatant was rejected, and the pellet was washed with 70% ethanol and centrifuged a third time (12 000g, 5 minutes 4°C). The supernatant was rejected, and remaining ethanol was left to evaporate at RT. Finally, the RNA was resuspended in DEPC-treated water (Ambion).

The RNA concentration was quantified using NanoDrop One (Thermo Fisher Scientific). The contamination with proteins or solvents was assessed by quantification of A260/A280 and A260/A230 absorbance ratios, respectively.

Gel electrophoresis was performed to assess the integrity of the RNA. Briefly, RNA was denatured for 2 min at 72°C, mixed with loading buffer and loaded in a 1%

agarose gel. The electrophoresis was performed at 100V for 20 minutes. Photos were taken at 10 minutes and 20 minutes using GelDoc XR⁺ Imaging System (BioRad).

All procedures were performed using RNase- and DNase-free material.

6. DNA digestion and Complementary DNA

DNA digestion was performed on 1500ng of extracted RNA using the TURBO DNA-free kit (Life Technologies) by adding DNase mix (DNase and 10x Turbo DNase Buffer – Invitrogen, both) to the RNA solution during 30 minutes at 37°C and then inactivating the enzyme with the inactivation solution (Invitrogen). After centrifuging, a DNA pellet is visible. The supernatant, which contains the DNA-free RNA, was collected and stored at -80 until further use.

Complementary DNA (cDNA) was synthesised from the extracted RNA pellet through reverse transcription using oligonucleotides (dNTPs from Invitrogen, Life Technologies), Random Hexamers (Invitrogen), Superscript III reverse transcriptase (Invitrogen), 5x First Strand Buffer (Invitrogen), DTTs (Invitrogen) and the T100 Thermal Cycler (Bio-Rad) according to the manufacturer's instructions [100]. Briefly, a priming mix was prepared with oligonucleotides (2µL oligonucleotides per 25µL RNA sample) and random hexamers (2µL random hexamers per 25µL RNA sample). This mix was added to the RNA solution and heated to 65°C for 5 minutes. Then a reverse transcription mix, containing the Superscript III reverse transcriptase (1µL Superscript III per 25µL RNA sample), DTTs (2µL DTTs per 25µL RNA sample) and enzyme buffer (8µL buffer per 25µL RNA sample) was added and the solutions were heated up to 25°C during 10 minutes, 50°C during 1hour and 70°C during 15 minutes.

7. mRNA amplification and quantification

Extracted RNA from 5 donors was diluted to a concentration of 30ng/µL and cDNA was prepared by adding 13µL of Mastermix to 2µL of RNA. The Mastermix was prepared with 10x RT Buffer (Thermo Fischer Scientific), dNTP Mix 100mM (Thermo Fischer Scientific), Multi Scribe reverse Transcriptase (Thermo Fischer Scientific), RNase Inhibitor (Thermo Fischer Scientific) and reverse transcription probes for the target miR-195 and the reference RNA U6: RT hsa-miR-195 probe (Thermo Fischer Scientific) and RT U6 snRNA (Thermo Fischer Scientific). The RNA and Mastermix were heated up to 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes using

the T100 Thermal Cycler (Bio-Rad). Then, the cDNA was diluted with 50 μ L DEPC water.

To perform the quantitative PCR, reaction mixes were prepared for miR-195 and U6 with Sso Advanced Universal Probes Supermix (BioRad) and the respective TaqMan probe: either TM hsa-miR-195 (Thermo Fischer Scientific) or TM U6 snRNA (Thermo Fischer Scientific). The reaction mixes and cDNA were added to a low profile 96-well PCR plate, and PCR was performed using the CFX96 Real-Time PCR Detection System (BioRad) as follows: 10 min at 95°C, 40 cycles of 15 seconds at 95°C and 1 min at 60°C.

8. mRNA expression levels assessment by qRT-PCR

The selected targets from the RNA-seq results were validated by qRT-PCR using CFX96 Real-Time PCR Detection System (BioRad) using iTaq Universal SYBR Green Supermix (BioRad), primers and cDNA synthesised from 7 donors (5 healthy donors, 1 commercial donor and 1 fragility donor). Forward (Fw) and reverse (Rv) primer sequences are shown in Table 5. The quantification cycle (C_q) method was used according to the MIQE guidelines[101]. Briefly, a priming mix was prepared for each gene with SYBR Green, DEPC treated water and the corresponding forward and reverse primers. To a 96-well PCR plate (BioRad) was added 6 μ L of priming mix and 2 μ L of prepared cDNA. Duplicates were performed for each condition, SCR or anti-miR-195, and target gene. The qRT-PCR was performed as follows: 3 min at 95°C, 40 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

Table 5. Primer sequences.

Target	Sequence
<i>BAMBI</i>	Fw: TGCACGATGTTCTCTCTCCT
	Rv: GAAGTCAGCTCCTGCACCTT
<i>IBSP</i>	Fw: CAGAAGGCACCACAGAGACC
	Rv: CCAAAGGTGGGGAAGTGGT
<i>CCNB1</i>	Fw: GACCTGTGTCAGGCTTTCTCTG
	Rv: GGTATTTTGGTCTGACTGCTTGC
<i>CLEC3B</i>	Fw: AGGAGCTCAAGAGCCGTCTG
	Rv: CTCGTGGAAGGTCTTCGTCT
<i>FND5</i>	Fw: GGCCTCCAAGAACAAGATGAG
	Rv: GCAGAAGAGGGCAATGACAC
<i>NAMPT</i>	Fw: AGGGTTACAAGTTGCTGCCACC
	Rv: CTCCACCAGAACCGAAGGCAAT
<i>TGM2</i>	Fw: TGTGGCACCAAGTACCTGCTCA
	Rv: GCACCTTGATGAGGTTGGACTC
<i>B-ACTIN</i>	Fw: TGGCACCCAGCACAATGAA
	Rv: CTAAGTCATAGTCCGCCTAGAAGCA

9. Immunocytochemistry validation of candidate targets

The antibodies against CCNB1, NAMPT and IBSP were tested. Anti-CCNB1 (Santa Cruz Biotechnology, sc-245) and anti-NAMPT (Santa Cruz Biotechnology, sc-393444) were both tested on the hepatic HeLa cell line and on human BM-MSCs from a healthy donor. Anti-IBSP (IBSP polyclonal antibody, Invitrogen, PA5-114915) immunohistochemistry was performed on a bone slide and on human BM-MSC from a healthy donor as well. The CCNB1 and NAMPT ICC staining were performed with the primary antibody dilution of 1:100 and with Alexa Fluor 594 goat anti-mouse secondary antibody (Invitrogen, A11005) (1:1000 dilution). IBSP staining on the bone slice was performed using anti-IBSP primary antibody with 1:100 dilution and with the goat anti-rabbit Alexa Fluor 647 secondary antibody (Invitrogen, A21245) (1:1000). Additionally, IBSP staining on an MSC cell monolayer was tested with the primary antibody concentration of 1:200 and with goat anti-rabbit AF488 secondary antibody (1:1000, Jackson Immuno Research Labs, 111-545-144). Nuclei were stained using DAPI nuclear stain (Invitrogen, LTID3571) at a dilution of 1:1000. Images were obtained with the

LeicaDMI6000 widefield fluorescence microscope. Table 6 summarizes the combinations of antibodies tested and the respective dilutions.

Table 6. Testing combinations of antibodies

Sample	Target protein	Primary antibody	Secondary antibody
HeLa cells	CCNB1	anti-CCNB1 (1:100)	anti-mouse AF594 (1:1000)
	NAMPT	anti-NAMPT (1:100)	anti-mouse AF594 (1:1000)
Bone slice	IBSP	anti-IBSP (1:100)	anti-rabbit AF647 (1:1000)
MSC	CCNB1	anti-CCNB1 (1:100)	anti-mouse AF594 (1:1000)
	NAMPT	anti-NAMPT (1:100)	anti-mouse AF594 (1:1000)
	IBSP	anti-IBSP (1:200)	anti-rabbit AF488 (1:1000)

Then, MSC from 2 healthy donors 1 and fragility fracture donor at different passages were plated at confluence in 24-well plates (0.04 million cells/well) and transfected the day after plating. After 48h, the cell monolayer was fixed, using PFA 4% in PBS. The cellular membranes were permeabilized with 0.1% Triton X-100 in PBS 1x and the cells were incubated at room temperature (RT) with blocking buffer (5% BSA, 0.1% Triton X-100 in PBS 1x). Afterwards, cells were left overnight at 4°C with the respective primary antibody solution in BB, either anti-BSP (1:200 dilution) or anti-NAMPT (1:100 dilution). The negative control was incubated overnight with BB. The morning after, cells were incubated with secondary antibody solution (goat anti-rabbit AF488 for IBSP and goat anti-mouse AF594 for NAMPT) (1:1000 dilution in BB) for 1h at RT and with DAPI (1:1000 dilution in 0.1% Triton X-100 in PBS 1x) for 5 minutes at RT. Intermediate washing steps were performed on the shaker for 3minutes at a time using PBS 1x.

Alternatively, cells were left in the incubator for 7 days after transfection with either basal media or osteogenic media. Then, cells were fixed and immunocytochemistry staining was performed as described above.

The stained wells were scanned using Operetta CLS at 20x amplification and the staining area was quantified in the negative control, SCR-transfected well and anti-miR-195-transfected well for all 3 donors with the Harmony High-Content Imaging and Analysis Software. To quantify the staining area, an intensity threshold was applied to select the stained area and the background staining present in the negative control was subtracted from the staining area measured for both transfection conditions. Additionally, to attenuate the effect of different cellular densities in the wells on the staining quantification, the staining area was normalized by the number of nuclei present in each well.

Only changes greater than 20% were considered as an up- or down-regulation of protein expression.

10. Alginate cell-laden gels construction

Oxidized alginate hydrogels functionalized with RGD were constructed. Regarding the structure of alginate: it is composed of blocks of consecutive guluronate residues (GGGGGG – polyG blocks), consecutive mannuronate residues (MMMMMM – polyM blocks) and blocks of alternating guluronate and mannuronate residues (GMGMGM) [102]. These blocks form linear chains and alginate gelation occurs by introduction of divalent cations, such as Ca^{2+} . The calcium ions bind to glucuronate residues, establishing an ionic bridge between polyG blocks from neighbouring chains, forming a structure called the egg-box model (Figure 17) [103], [104].

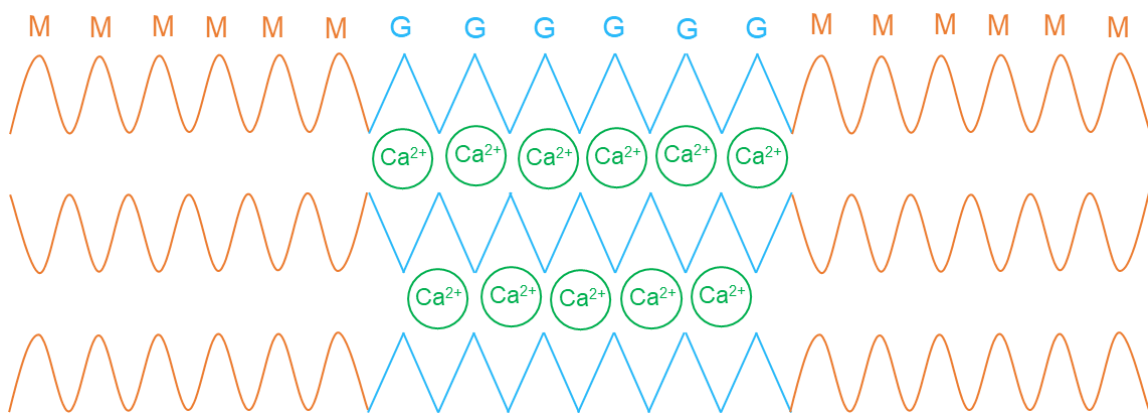


Figure 8. Egg-box model of alginate gelation (adapted from *Immobilization of planktonic algal spores by inkjet printing*, 2019, *Scientific Reports* [105]).

Hydrogel discs with the final concentration of 2.0% or 1.5% alginate (PRONOVA LVM) and a cellular density of either 5 million cells per mL, 3.78 million cells per mL or

2.5 million cells per mL were prepared using calcium carbonate (CaCO_3) and GDL (glucono- δ -lactone – Sigma-Aldrich). Briefly, CaCO_3 was added to the alginate and once GDL was added, the acidification of the pH, lead to the release of Ca^{2+} -ions from the CaCO_3 , which promotes the formation of ionic bounds between alginate chains, thus causing alginate gelification. How the internal gelification process occurs is described in greater detail ahead in chapter IV in section 6. The cellular suspension was quickly introduced and 10 μL drops were pipetted onto a hydrophobic Teflon surface, which was covered with a second Teflon plaque, separated by 500 μm spacers. The discs were left to polymerize for 20 minutes and were then transferred to a 24 well plate with MSC growth media.

10.1. Cell survival and viability assessment

Resazurin assay was performed on the gels at the time-points of 24h, 72h and 7days. Briefly, a solution of 20% resazurin (Sigma-Aldrich) (0.1 mg/mL in HBSS – Thermo Fisher Scientific) in MSC media was prepared and the gels were incubated with 500 μL of resazurin solution during 2h. Wells without gels were also incubated with resazurin during 2h as a blank. Then, 100 μL of the supernatant was transferred to a 96-well fluorescence plate and the fluorescence was read at 530/590nm using the Synergy Mx. The average blank value, which accounts for the fluorescence of the media, was subtracted. The working principle behind this assay is that resazurin (dark blue) is reduced by cell enzymes into resofurin (pink), which is fluorescent at 530 nm excitation wavelength[106]. The fluorescent intensity emitted at 590nm is proportional to the amount of metabolic active cells present in the well, therefore enabling an indirect estimation of cell viability. Higher fluorescence emission corresponds to more metabolically active cells.

Additionally, Live/Dead staining was performed at day 2 and 7 using calcein and ethidium homodimer. Shortly, calcein dye (1:500) and ethidium homodimer (1:800) were diluted in MSC media and 250 μL of each solution was added to the gels and left to incubate at 37°C and 5% CO_2 for 30 minutes. The calcein dye marks the live cells in green, whereas the ethidium homodimer binds to DNA and marks dead cells with compromised membrane integrity, since it cannot cross naturally uncompromised cell membranes[107]. The Leica SP5 Confocal microscope was used to visualize the staining and take photographs. The number of green/live cells was counted manually as well as

the number of red/dead cells and the cell survival percentage was calculated (Equation 1).

$$\%viable\ cells = \frac{nr\ of\ live\ cells}{nr\ of\ live\ cells + nr\ of\ death\ cells} \times 100$$

Equation 1. Percentage of viable cells in the Live/Dead assay.

10.2. Morphology evaluation of the cells

Phalloidin and DAPI staining were performed on the discs fixed at day 2, 7 and 14. Briefly, the hydrogels were fixed using PFA 4% in HBSS (Alfagene), incubated for 10 minutes at RT with NH₄Cl (50mM) and cell membrane permeabilization was achieved using a solution of 0.2% Triton X-100 in HBSS. Blocking was performed for 1h at RT with the blocking buffer of 1% BSA in 0.2% Triton X-100 in HBSS. Then, the discs were incubated with the phalloidin staining solution (1:100 dilution, Invitrogen) for 20 minutes at RT and, subsequently, with DAPI solution (1:1000) for 5 minutes at RT. Intermediate washing steps were performed with HBSS for 5 minutes at RT on the shaker. Visualization was achieved with the Leica Sp5 Confocal microscope and the cell morphology was assessed using the shape descriptors plugin in the ImageJ Fiji Software. The area, aspect ratio, roundness, compactness and form factor of the cells were measured and particles with a form factor greater than 1 and area smaller than 50 px² were excluded, since they were too small to be MSCs[108], [109].

The aspect ratio (AR) is the ratio between the cells major and minor axis (Equation 2, Figure 19) [110]. A bigger AR implicates a smaller roundness and indicates that the cell has spread further

$$AR = \frac{major\ axis}{minor\ axis}$$

Equation 2: Aspect ratio formula.

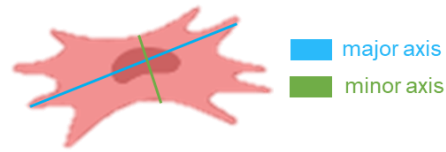


Figure 9. Representation of the cellular major and minor axis (Adapted from Martinez et al., Polymers, 2021).

10.3. NAMPT immunofluorescence assay

Similarly to the immunocytochemistry protocol, after fixing the hydrogels in 4% PFA in HBSS for 20 minutes at RT, a washing step with HBSS was performed and cell

membranes were permeabilized with 0.2% Triton X-100 in HBSS for 10 minutes at RT. The blocking buffer was prepared with 1% BSA, 0.2% Triton X-100 in HBSS and the blocking took place during 1 hour at RT. The hydrogels were incubated overnight at 4°C with 1:100 solution of anti-NAMPT primary antibody (sc-393444). As a negative control, a sample was incubated overnight at 4°C with blocking buffer only (without the primary antibody). Then, the samples were incubated with the secondary antibody (1:1000 dilution, 1h at RT), the nuclei stained with DAPI (1:1000 dilution, 5 minutes at RT) and a final washing step with HBSS was performed. Intermediate washes were performed with HBSS for 3 minutes with agitation. NAMPT protein staining was assessed with the Leica SP5 Confocal microscope and the staining area quantified with the ImageJ Fiji Software.

11. Identification of novel anti-miR-195 target candidates

To identify other potential anti-miR-195 targets associated with mechanisms beyond the ECM, the bRNA-seq data was revised. For this, the results were filtered and the transcripts with a *p-value*>0.05 were excluded as performed in section 3. Similarly, only the protein-coding transcripts were considered. The transcripts were ranked accordingly to the Fragments Per Kilobase of transcript per Million mapped reads (fpkm). All transcripts with fpkm values lower than 0.01 were excluded from the analysis [111]. The individual fold-change (FC) values of the fpkm reads were calculated for each donor and the transcripts that presented consistently up- or down-regulated fold-changes were selected. Out of these transcripts, the ones with FC-values constantly greater than 2 or smaller than -2 were selected as the most promising candidates. The role of each of these candidates was investigated by literature research.

12. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 9. Distribution normality of the results was evaluated using the Shapiro-Wilk test and the Kolmogorov-Smirnov test. When data followed a normal distribution, to compare two sets of results paired or unpaired Welch's t-tests were used. When data did not follow a normal distribution, the Wilcoxon test (paired) or the Mann-Whitney test (unpaired) was used when comparing two groups. For multiple comparisons, when data followed a normal distribution, one-way ANOVA tests were performed whereas, when data did not follow

a normal distribution, the Kruskal-Wallis test was used. The ROUT method was used with a significance of 5% to remove outliers. Statistical significance was considered for p-value lower than 0.05 (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$).

IV. Results

1. Anti-miR-195 delivery to MSC targets 19 ECM- and proliferation-related transcripts

Human primary MSC were transfected with either anti-miR-195 or scrambled (SCR) control oligonucleotides and the transfection efficiency was tested for 5 independent MSC donors. As expected, results show a significant 90% decrease in miR-195 levels (Figure 10), hence proving the adopted experimental to be highly efficient without the need for further optimization.

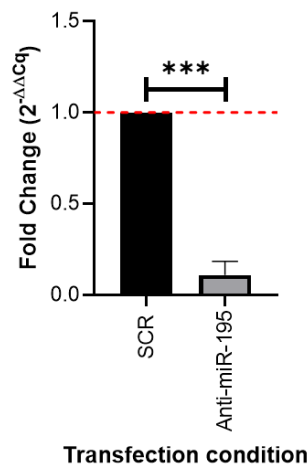


Figure 10. miR-195 expression levels 48h post-transfection of MSCs with anti-miR-195 compared with scrambled control (SCR). The transcript levels were evaluated by qRT-PCR in MSCs isolated from 5 different donors (***) $p < 0.001$).

Next, to identify which transcripts are affected by the decrease of miR-195 levels, bulk RNA sequencing was performed. A data analysis pipeline for the selection of anti-miR-195 target candidates is shown in Figure 11A. Results from the bRNA-seq show that 674 transcripts are differentially expressed when the levels of miR-195 are decreased in MSC from 3 different donors ($p\text{-value}\leq 0.5$) (Figure 11B). Out of those approximately 45.85%, corresponding to 309 transcripts, are protein-coding (Figure 11C), while 54.15% of the differentially expressed genes, corresponding to 365 transcripts, are non-coding transcripts (ncRNAs), despite the use of poly(A) selection method for library preparation. Among those, the long ncRNAs comprise 36.16% of all ncRNAs identified by the bRNA-seq analysis. In the scope of this thesis, only protein coding genes were selected for further analysis. Out of the 309 protein coding transcripts, 200 (64.72%) were up-regulated while 109 (36.33%) were down-regulated in anti-miR-195-transfected MSC compared with SCR-transfected MSC (Figure 11C). Gene ontology and gene enrichment analysis was performed on the protein-coding genes and results show that these transcripts are associated with categories related to extracellular matrix, cell cycle and proliferation, cell survival, angiogenesis and expression in the bone marrow (Figure 11D). In total, 145 transcripts are included in these categories. To narrow down the list of candidate genes, only the ones present in at least 2 categories and/or with a fold change (FC) equal or higher than 2 were considered for further analysis (Figure 10E). This reduced the list of candidate genes from 145 to 113 genes. The next step consisted of individual reviewal of the published literature about each one of those 113 genes (present in 2 or more categories and/or with $FC\geq 2$). This resulted in 19 selected transcripts that had been described to associate with bone and MSC proliferation, survival and osteogenesis. (Figure 11F).

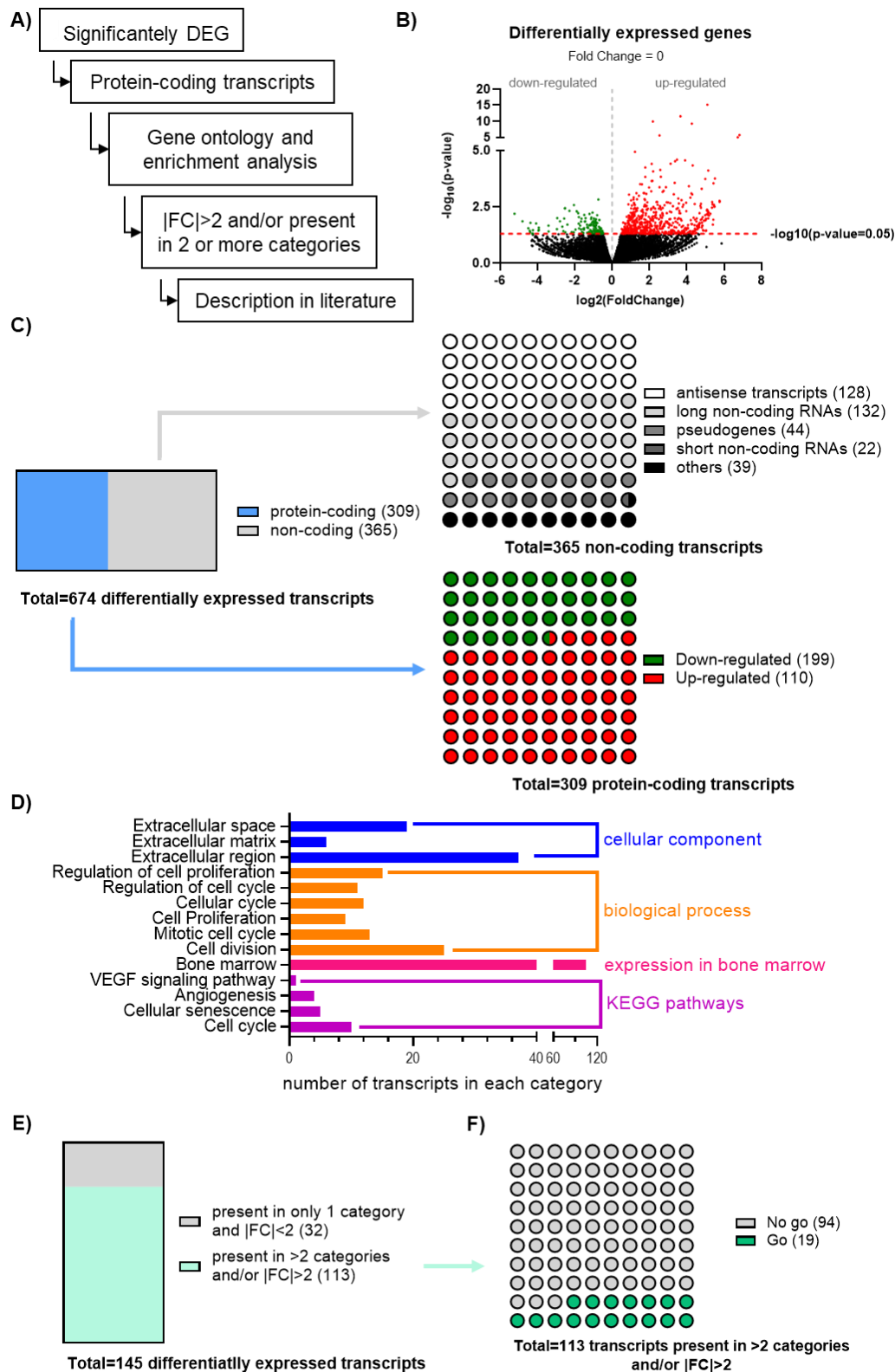


Figure 11. In silico analysis of the RNA-sequencing results. A) Data analysis pipeline; B) Volcano plot of the differentially expressed transcripts between anti-miR-195-MSC compared with SCR-MSC control; C) Number of the differentially expressed coding and non-coding transcripts; D) Gene ontology and gene enrichment analysis; E) Transcripts that are present in 2 or more categories of interest and/or have a $|FC|$ greater than 2; F) Transcripts that received the “go-ahead” due to their impact on MSC proliferation and osteogenesis.

2. The expression of *CCNB1*, *NAMPT* and *IBSP* is regulated by miR-195

To validate the differently expressed genes identified in the previous section, MSC from 7 independent human donors were transfected with anti-miR-195 oligonucleotides or scrambled control. RNA was isolated 48h post-transfection and Table 7 summarizes the concentration and purity ratios of the RNA. The 260/280 ratio indicates the RNA contamination with proteins, whereas the 260/230 ratio measures the contamination with solvents used during the RNA extraction and isolation process. All samples show an 260/280 ratio of approximately 1.90, except for one sample (Donor 1 SCR), indicating low protein contamination. On the other hand, only 4 RNA samples present a 260/230 ratio greater than 1.5, indicating some degree of contamination by the solvents used to extract the RNA.

Table 7. RNA concentration and purity assessment from MSC from 7 different donors.

Sample	RNA (ng/ μ L)	Abs 260/280 nm	Abs 260/230 nm
Donor 1 SCR	579.6	1.78	0.43
Donor 1 Anti-miR-195	375.9	1.89	2.03
Donor 2 SCR	436.4	1.87	1.69
Donor 2 Anti-miR-195	324.6	1.89	1.02
Donor 3 SCR	109.4	1.91	0.78
Donor 3 Anti-miR-195	99.9	1.97	0.92
Donor 4 SCR	69.5	1.90	0.41
Donor 4 Anti-miR-195	144.9	1.90	1.67
Donor 5 SCR	729.9	1.87	0.45
Donor 5 Anti-miR-195	700.6	1.94	0.88
Donor 6 SCR	71.1	1.89	0.23
Donor 6 Anti-miR-195	363.0	1.99	1.70
Donor 7 SCR	431.1	1.86	1.29
Donor 7 Anti-miR-195	421.2	1.97	1.42

Next, RNA integrity was assessed by gel electrophoresis. Non-degraded samples show visible 28S and 18S rRNA. Figure 12A is a representative figure of RNA run in the gel showing visible 28S and 18S bands. Figure 12B shows RNA is not degraded for the first 3 samples (lane 1 to 3) but degraded in sample 4 (lane 4). qRT-PCR was only performed in non-degraded samples.

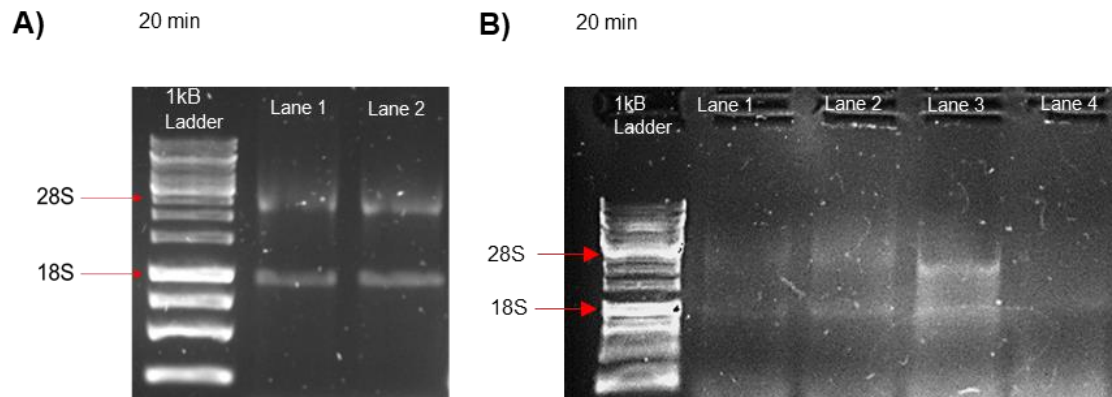


Figure 12. RNA integrity assessment by gel electrophoresis. A) RNA from one representative donor; B) Comparison of gel electrophoresis results from non-degraded (lane 1-3) and degraded RNA (lane 4).

The roles of the 19 genes that passed all the established criteria (Figure 13A) following the bRNA-seq analysis, were reanalysed in the literature for their involvement in proliferation and/or osteogenesis in MSC. Seven with the most promising descriptions were selected for validation by qRT-PCR and divided into 2 categories: 1) proliferation-related genes: *CCNB1*, *NAMPT* and *TGM2*; and 2) osteogenesis-related genes: *BAMBI*, *FND5*, *IBSP* and *CLEC3B*. The average FC-values of these genes are presented in Figure 13A.

Regarding the proliferation-related genes, and in line with the bRNA-seq data, qRT-PCR results show that *CCNB1*, *NAMPT* and *TGM2* are upregulated in anti-miR-195-transfected MSC versus SCR-transfected MSC. However, only *CCNB1* and *NAMPT* show statistical differences (Figure 13B).

Considering osteogenesis-associated genes, results show an upregulation of *FND5* and *IBSP*, but only *IBSP* shows statistical significance, while *BAMBI* shows no differences (Figure 13C). Finally, *CLEC3B* levels were very low or undetectable, with Cq values above 35 cycles (Annex 1, Supplementary Figure 1).

Out of the 7 selected transcripts, *CCNB1*, *NAMPT* and *IBSP* were found to be significantly overexpressed when miR-195 levels are inhibited in human primary MSC.

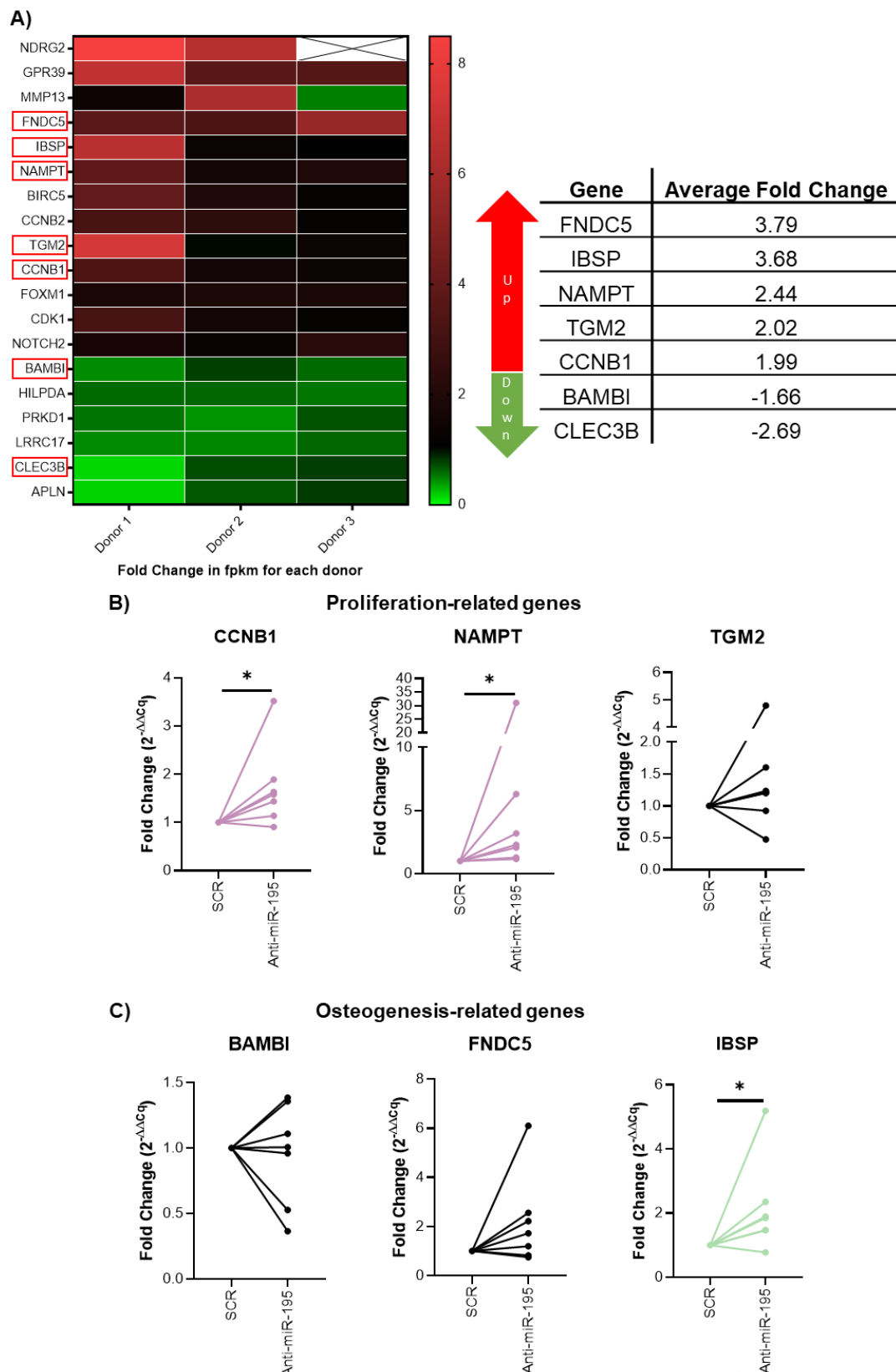


Figure 13. qRT-PCR validation of the 7 selected “go-ahead” transcripts for a first line of screening. A) Heatmap of the 19 “go-ahead” transcripts, where the 7 selected transcripts are identified in red boxes and average fold change values for the selected genes; B) Proliferation-related transcripts qRT-PCR results, and C) osteogenesis-related transcripts qRT-PCR results (n=7) (* $p < 0.05$).

3. Anti-miR-195 increases NAMPT protein levels

3.1. Optimization of CCNB1, NAMPT and IBSP immunofluorescence

The protein levels of CCNB1, NAMPT and IBSP were evaluated by immunofluorescence in 2D MSC monolayers. Firstly, the assay was tested for CCNB1 and NAMPT. The cell line HeLa was used as a positive control, accordingly to the product worksheets supplied by the manufacturer, and non-transfected MSCs were used as a testing condition. Results show a strong positive staining for HeLa cells, both for CCNB1 and NAMPT, whereas no staining was detected in negative control, as expected (Figure 14A). On the other hand, in the MSC, CCNB1 staining was absent, whereas NAMPT stained positive (Figure 14A), suggesting CCNB1 has no or low expression in MSC after 48h in culture. Being a cell cycle protein, this result may be explained by the high proliferative rate of HeLa cells and the low proliferative rate of MSC. For IBSP, a slice of bone tissue, which naturally expresses IBSP, was used as a positive control, since this protein is not found on HeLa cells. Both the bone slice and the MSC showed a positive staining for IBSP, compared with the respective negative controls (Figure 14B).

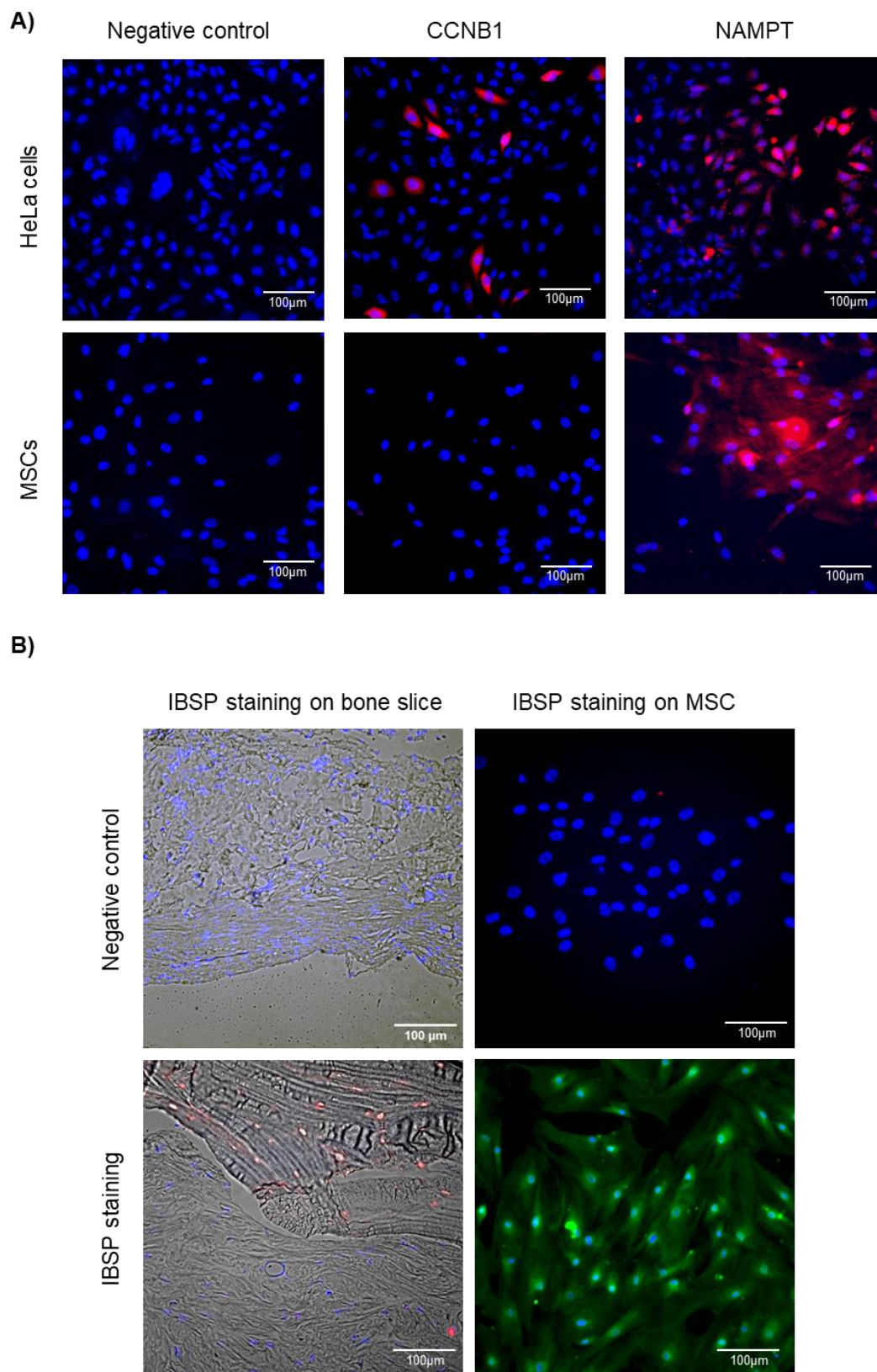


Figure 14. Immunofluorescence assays on non-transfected MSCs grown in basal media and fixed at 48h of culture and respective positive controls to test antibodies. A) CCNB1 (red) and NAMPT (red) on HeLa cells and on MSCs with DAPI nuclei staining (blue); B) IBSP on a bone slice (IBSP: red; nuclei: blue) and MSCs (IBSP: green; nuclei: blue).

3.2. NAMPT and IBSP protein levels are not changed by anti-miR-195 inhibition 48h post-transfection

Next, IBSP and NAMPT protein levels were evaluated in MSC 2 days post-transfection with either anti-miR-195 oligonucleotides or SCR-control, in basal conditions. To allow the evaluation and visualization of multiple fields in a single well in an automated and timely manner, the Operetta CLS High Content Analysis System was used.

Regarding NAMPT, no major differences were observed between anti-miR-195 and SCR conditions in the 3 donors. Figures 15A to C show the images taken for each donor and transfection condition. Considering the absolute staining area of NAMPT (Figure 15D, Table 8), 2 out of the 3 donors, namely Donor 6 and 7, presented no considerable changes in the absolute staining area between transfection conditions. These tendencies were maintained when the staining area was normalized with the number of nuclei (Figure 15E, Table 9). It is worth noticing that there was background staining observed in the negative control (Annex 3, Supplementary Figure 3A-C), which was subsequently subtracted from all quantifications.

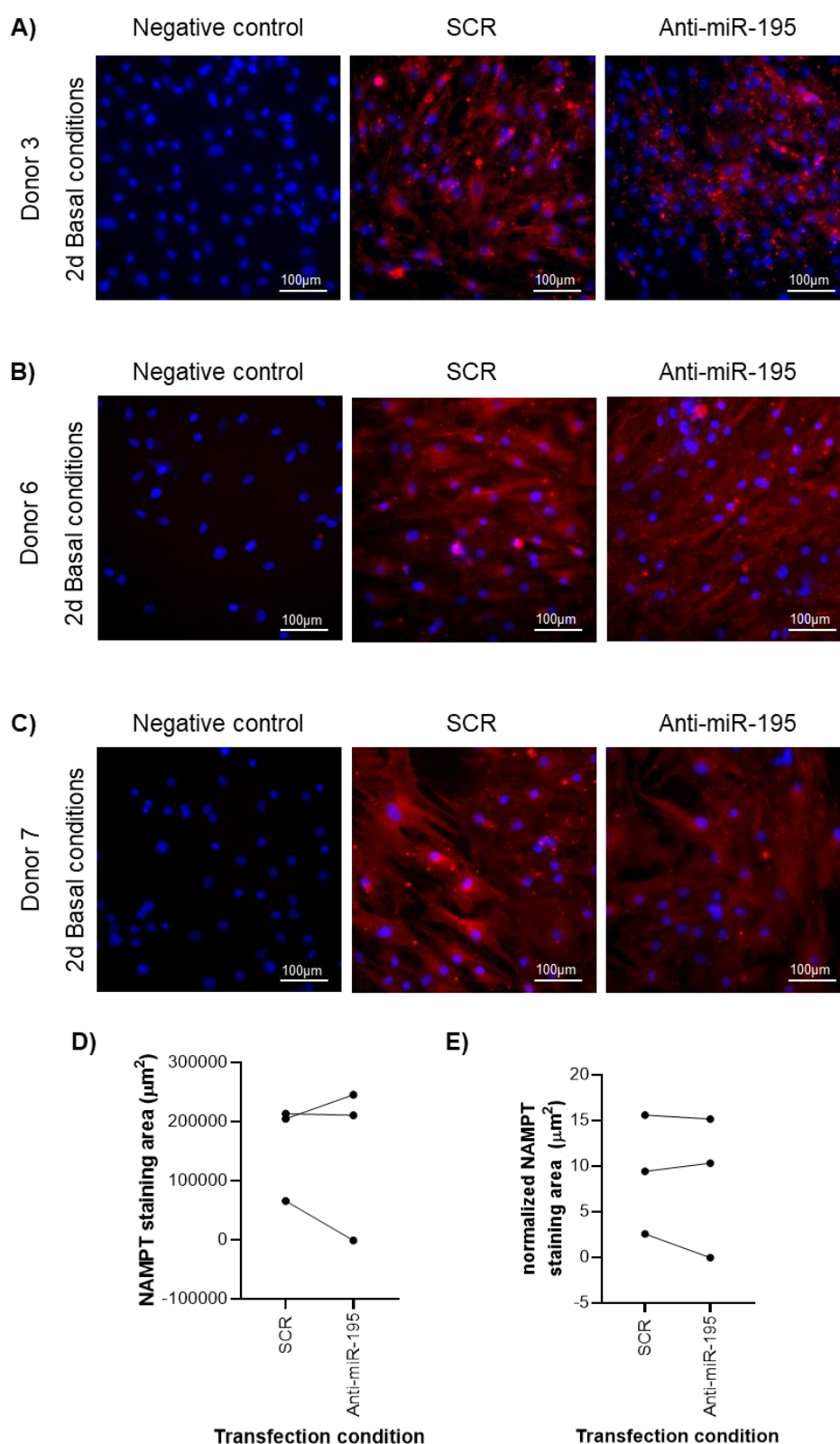


Figure 15. Immunofluorescence assays to validate NAMPT as a miR-195 target in a 2D MSC monolayer, transfected with either SCR or anti-miR-195 and fixed 2 days later. A-C) Images of anti-NAMPT (red) and DAPI nuclear stain (blue) for 3 different donors; negative control: no primary antibody and AF594 anti-mouse secondary antibody (20x amplification); D) Quantification of the staining area (n=3); E) Quantification of the staining area normalized with the number of nuclei (n=3).

Table 8. Quantification of NAMPT staining in 3 different human MSC donors and the variation between anti-miR-195 transfected condition versus SCR control.

Donor	Staining area SCR	Staining area anti-miR-195	Staining area ratio Anti-miR-195/SCR	Variation
3	65375	-1043	-0.016	-102%
6	215548	253731	1.177	17.7%
7	213220	210486	0.987	-1.3%

Table 9. Quantification of NAMPT stained area in 3 different human MSC donors normalized by the number of nuclei and the variation between anti-miR-195 transfected condition versus SCR control.

Donor	Staining area SCR normalized /nr of nuclei	Staining area anti-miR-195 normalized /nr of nuclei	Staining area ratio Anti-miR-195/SCR	Variation
3	2.567	-0.040	-0.015	-102%
6	9.926	10.686	1.077	7.7%
7	15.591	15.150	0.972	-2.8%

Figures 16A to C show the staining performed for IBSP on 3 different MSC donors. Regarding IBSP protein levels, no changes in IBSP staining area between anti-miR-195 and SCR transfected cells were measured in 2 out of 3 donors (Figure 16D and E, Table 10 and 11). In one donor, the IBSP staining area is decreased in the anti-miR-195 versus SCR control (Figure 16D, Table 10). However, in this donor there is a higher cell density in SCR condition than in the anti-miR-195 condition. Therefore, when normalizing with the number of nuclei, the reduction is attenuated (Figure 16E, Table 11).

Taken together, results show no alterations in NAMPT and IBSP protein, 2 days post-transfection with anti-miR-195, in basal conditions.

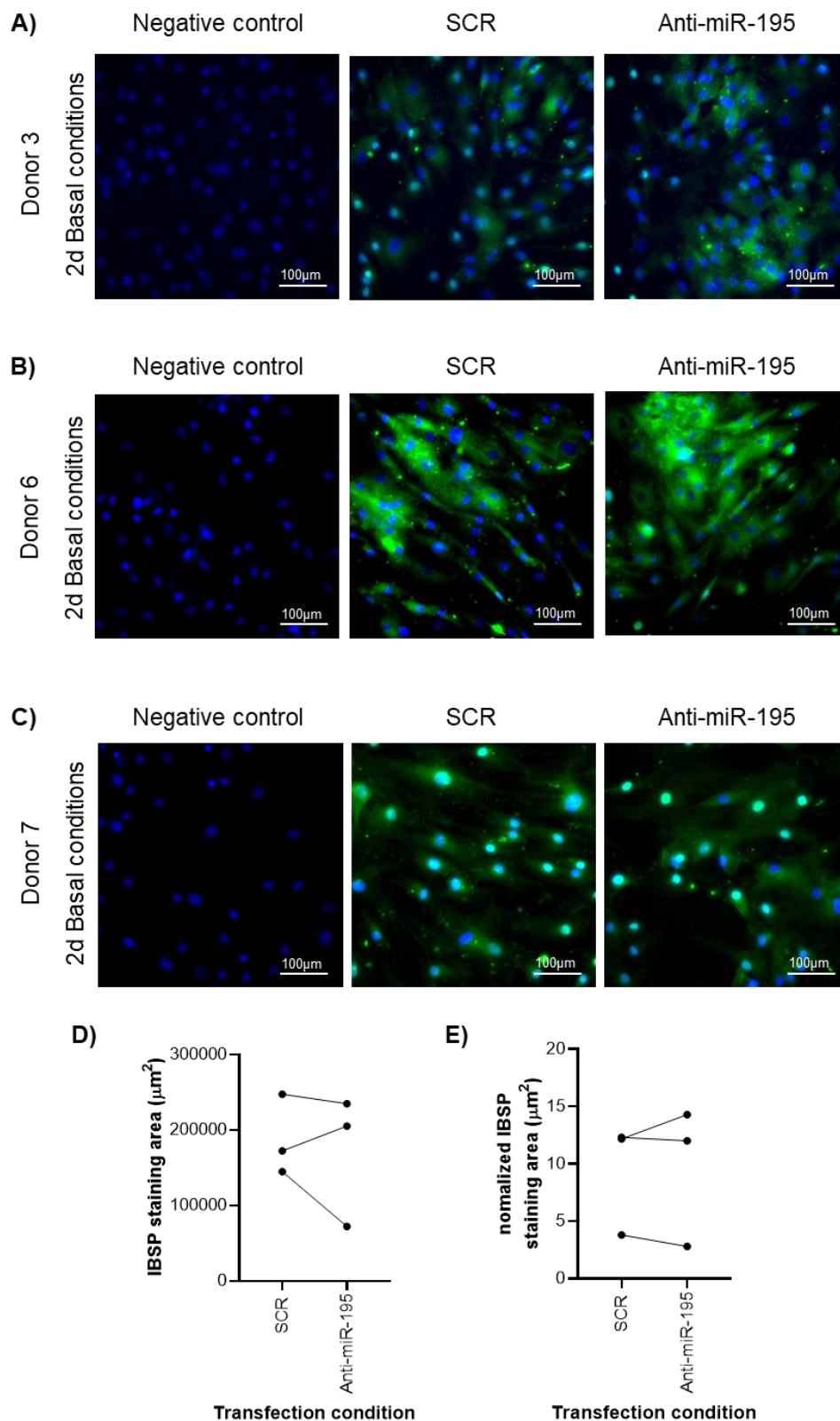


Figure 16. Immunofluorescence assays to validate IBSP as a miR-195 target in a 2D MSC monolayer, transfected with either SCR or anti-miR-195 and fixed 2 days later. A-C) Images of anti-IBSP (green) and DAPI (blue) for 3 different donors; negative control: no primary antibody and AF488 anti-mouse secondary antibody (20x amplification); D) Quantification of the staining area ($n=3$); E) Quantification of the normalized staining area ($n=3$).

Table 10. Quantification of IBSP staining in 3 different human MSC donors and the variation between anti-miR-195 transfected condition versus SCR control.

Donor	Staining area SCR	Staining area anti-miR-195	Staining area ratio Anti-miR-195/SCR	Variation
3	247450	234914	0.949	-5.01%
6	172368	205222	1.191	19.1
7	144876	72172	0.498	-50.2%

Table 11. Quantification of IBSP stained area in 3 different human MSC donors normalized by the number of nuclei and the variation between anti-miR-195 transfected condition versus SCR control.

Donor	Staining area SCR normalized /nr of nuclei	Staining area anti-miR-195 normalized /nr of nuclei	Staining area ratio Anti-miR-195/SCR	Variation
3	12.315	12.014	0.975	-2.5%
6	12.179	14.290	1.173	17.3%
7	3.805	2.815	0.740	-26%

The Operetta CLS photographs of the wells for the immunocytochemistry assays at 48h post-transfection are presented in the Supplemental Material (Supplementary Figures 2 and 3, corresponding to annexes 2 and 3, respectively).

3.3. NAMPT protein is upregulated 7 days post-transfection under osteogenic conditions

Since, overall, no changes in NAMPT and IBSP protein levels could be measured between anti-miR-195-MSC or SCR-MSC at 2 days post-transfection, the experimental setup was changed to evaluate the effects of anti-miR-195 at day 7 post-transfection. For this, cells were transfected with either SCR or anti-miR-195 oligonucleotides and incubated with either basal or osteogenic media.

In basal media, 2 out of the 3 donors presented unaltered absolute and normalized NAMPT staining areas, whereas one donor presented an increase in absolute and normalized NAMPT staining areas greater than 20% in the anti-miR-195 transfection condition versus the SCR control condition (Table 12, Figure 17A-D).

Table 12. Quantification of absolute and normalized NAMPT staining area in 3 different human MSC donors in anti-miR-195 transfected condition versus SCR control in basal media 7 days post-transfection.

	Donor	Staining area SCR	Staining area anti- miR-195	Staining area ratio Anti-miR-195/SCR	Variation
Absolute staining	3	201810	253520	1.256	25.6%
	6	271957	257516	0.947	-5.3%
	7	271931	291748	1.073	7.3%
Normalized staining	3	13.000	15.808	1.216	21.6%
	6	16.721	16.687	0.998	-0.2%
	7	15.670	16.132	1.029	2.9%

The increase in both absolute and normalized NAMPT stained area was also observed in 2 out of 3 donors under osteogenic conditions (Table 13, Figure 17A-C and E). Only one donor presented no alterations between transfection conditions in osteogenic conditions (Table 13, Figure 17A-C and E). This increase in NAMPT expression in anti-miR-195 condition compared with SCR control is in accordance with the bRNA-seq and qRT-PCR results.

Table 13. Quantification of absolute and normalized NAMPT staining area in 3 different human MSC donors in anti-miR-195 transfected condition versus SCR control in osteogenic media 7 days post-transfection.

	Donor	Staining area SCR	Staining area anti- miR-195	Staining area ratio Anti-miR-195/SCR	Variation
Absolute staining	3	205675	255307	1.241	24.1%
	6	233420	281884	1.208	20.8%
	7	265400	235518	0.887	-11.3%
Normalized staining	3	9.928	12.321	1.241	24.1%
	6	14.028	17.008	1.212	21.2%
	7	13.169	12.066	0.916	-8.4%

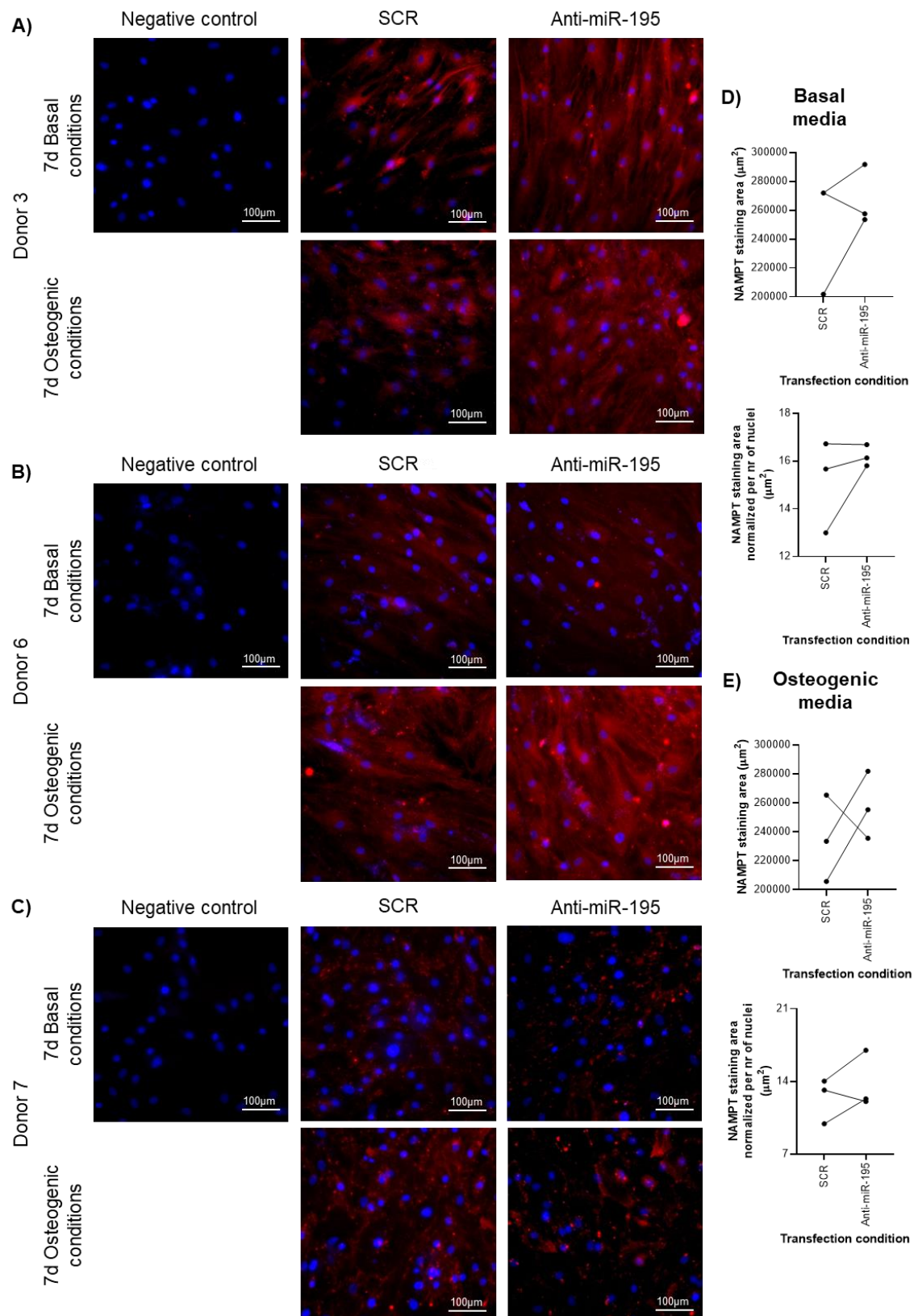


Figure 17. Immunofluorescence assays for NAMPT in a 2D MSC monolayer, transfected with either SCR or anti-miR-195, cultured with basal or osteogenic media for 7 days. A-C) Representative images of anti-NAMPT (red) and DAPI (blue) ($n=3$; 20x amplification); D) Quantification of the absolute (top) and normalized (bottom) NAMPT staining area under basal conditions ($n=3$); E) Quantification of the absolute (top) and normalized (bottom) NAMPT staining area under osteogenic conditions ($n=3$).

Figure 18 summarizes the results obtained for IBSP staining. Considering basal conditions, after 7 days, in all 3 donors, there is no alteration in both absolute and normalized IBSP stained area (Figure 18 A-C and D, Table 14).

Table 14. Quantification of absolute and normalized IBSP staining area in 3 different human MSC donors in anti-miR-195 transfected condition versus SCR control in basal media 7 days post-transfection.

	Donor	Staining area SCR	Staining area anti-miR- 195	Staining area ratio Anti- miR- 195/SCR	Variation
Absolute staining	3	368247	405902	1.102	10.2%
	6	231254	231311	1.000	0.00%
	7	242841	241216	0.993	-0.007%
Normalized staining	3	18.283	18.388	1.006	0.006%
	6	11.135	12.360	1.110	11.0%
	7	11.056	11.062	1.000	0.00%

In osteogenic media, 2 out of 3 donors present no changes between transfection conditions in both absolute and normalized staining area (Figure 18A-C and E, Table 15). However, in the third donor a significant increase greater than 30% in both absolute and normalized IBSP stained area was measured in anti-miR-195 condition versus the SCR control (Table 14). This increase is in agreement with the bRNA-seq and qRT-PCR results.

Table 15. Quantification of absolute and normalized IBSP staining area in 3 different human MSC donors in anti-miR-195 transfected condition versus SCR control in osteogenic media 7 days post-transfection.

	Donor	Staining area SCR	Staining area anti-miR- 195	Staining area ratio Anti- miR- 195/SCR	Variation
Absolute staining	3	332714	341060	1.025	2.5%
	6	269833	360755	1.372	37.2%
	7	254060	263178	1.036	3.6%
Normalized staining	3	20.532	19.766	0.963	-3.7%
	6	15.794	21.676	1.337	33.7%
	7	12.640	12.553	0.993	-0.7%

Overall, there are no differences in the staining area between the different transfection conditions in either basal or osteogenic conditions, 7 days post-transfection (Figure 18A-E, Table 14 and 15).

The Operetta CLS images of the wells for the immunocytochemistry assays at 7 days post-transfection with either anti-miR-195 or SCR oligonucleotides, under basal or osteogenic conditions, for NAMPT and IBSP are presented in the Supplemental Material (Supplementary Figures 4 and 5, corresponding to annexes 4 and 5, respectively).

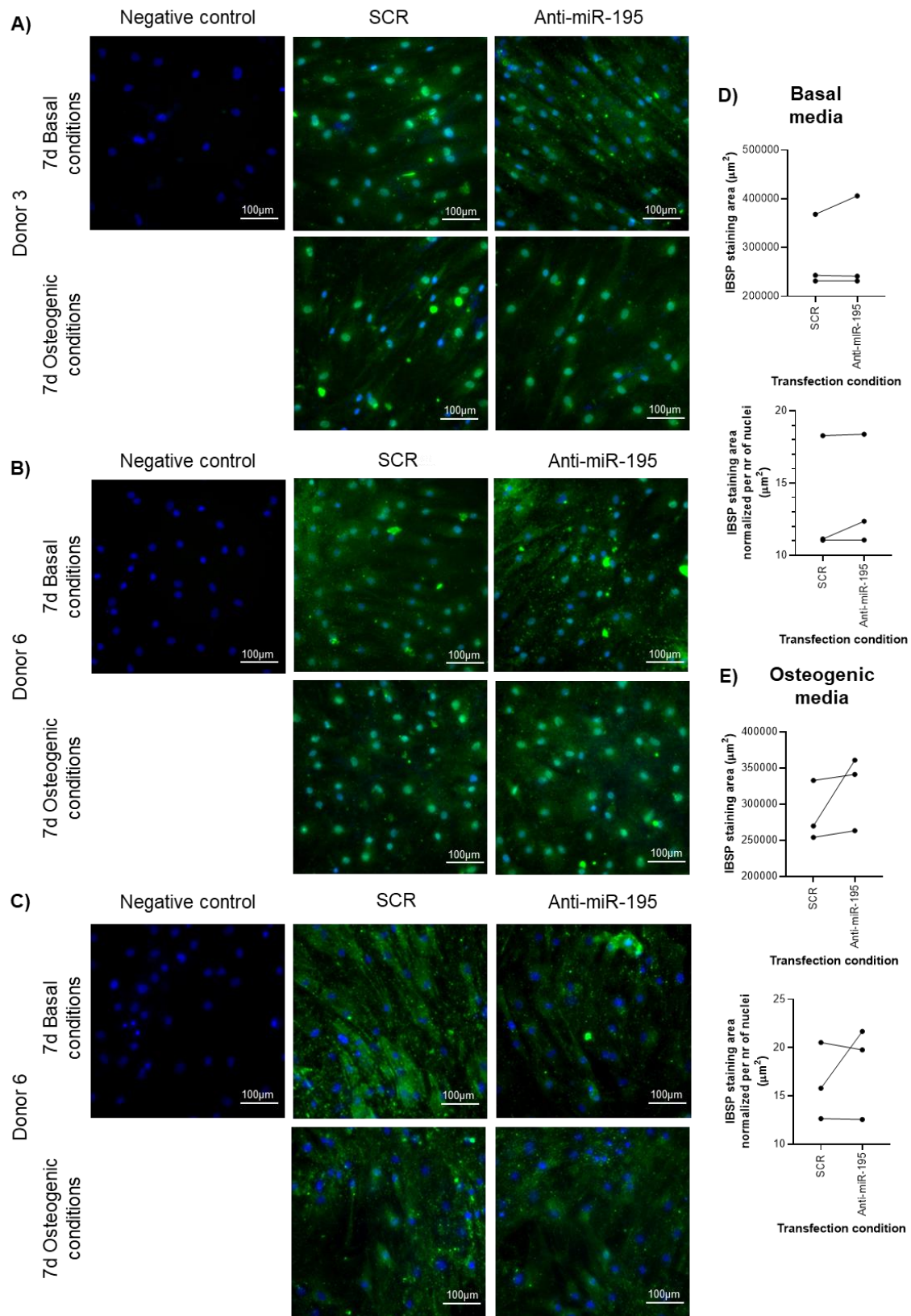


Figure 18. Immunofluorescence assays for IBSP in a 2D MSC monolayer, transfected with either SCR or anti-miR-195, cultured with basal or osteogenic media for 7 days. A-C) Images of anti-IBSP (green) and DAPI (blue) ($n=3$; 20x amplification); D) Quantification of the absolute (top) and normalized (bottom) IBSP staining area under basal conditions ($n=3$); E) Quantification of the absolute (top) and normalized (bottom) IBSP staining area under osteogenic conditions ($n=3$).

4. Optimization of a 3D system for MSC survival and adhesion

To provide a study model that better mimics a 3D microenvironment, an alginate hydrogel system was optimized for *in vitro* MSC culture.

Figure 19A depicts the experimental design used to optimize the cell density and alginate concentration for the construction of MSC-laden hydrogels. For each gel the cell viability, survival and spreading was evaluated. Brightfield photographs taken of the different hydrogels at 48h and 7 days are shown in Figure 19B.

4.1. Increased MSC survival in low density alginate hydrogels

Resazurin assay was used to indirectly evaluate the cell viability of the MSCs seeded within the hydrogels at 24h, 72h and 7 days (Figure 19A). To optimize alginate concentrations, an intermediate concentration of 3.78×10^6 cells/mL MSC was used. Hydrogels containing 2.0% alginate concentration exhibited a significant decrease in cellular viability from 24h to 72h, but showed no significant variation in fluorescence intensity between 72h and 7 days. This indicates that, in the 2% alginate hydrogels, an initial decrease of cell viability occurs in the first 72h, however it stabilizes afterwards. In contrast, hydrogels with a 1.5% alginate concentration, and the same cell density (3.78×10^6 cells/mL), displayed a significant decrease in viability from 24h to 7 days (Figure 19C). In the case of the 1.5% discs, there is no initial steep decrease in cell viability, but rather a continuous and gradual decrease over the course of 7 days. For this reason, the 2% alginate was the selected concentration for testing additional cell densities, specifically 2.5×10^6 cells/mL and 5×10^6 cells/mL.

The lowest cell density hydrogels (2.5×10^6 cells/mL) show no significant alterations in the cell viability during the 7 days, indicating no significant cell death in these hydrogels (Figure 19C).

Considering the hydrogels with highest cell density (5×10^6 cells/mL), they present a significant decrease in cell viability from 24h to 7 days (Figure 19D), exhibiting similar behaviour to the 3.78×10^6 cells/mL, 1.5% alginate hydrogels (Figure 19C).

Live/dead assay results at day 2 and 7 are shown in Figure 19 (Figure 19B: representative images, Figure 19E: quantification). The number of live (green) cells and dead (red) cells was quantified manually in alternating stacks of 3 planes. At day 2 the live/dead assay was not performed for the intermediate cell density hydrogels (3.78×10^6 cells/mL), which is a limitation to this study.

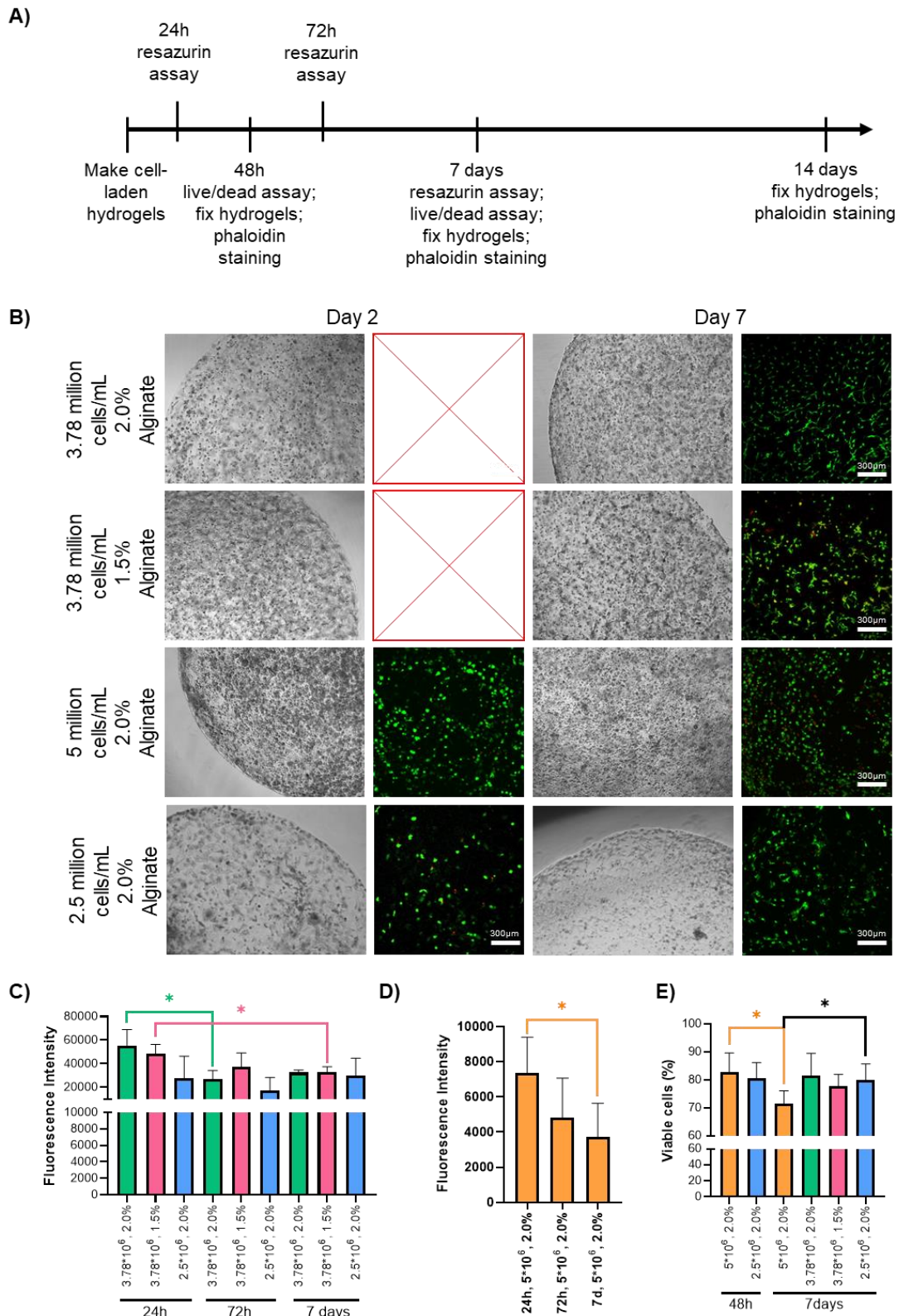


Figure 19. Oxidized alginate hydrogels functionalized with RGD seeded with MSCs. A) Experimental design; B) Brightfield images at day 2 and 7 and representative images of the live(green)/dead(red) cells at day 2 and 7 of the distinct hydrogels; C) Cell survival and viability during 14 days in the intermediate and lowest cell density hydrogels; D) Cell survival and viability during 14 days in the highest cell density hydrogels; E) Percentage of viable cells during 8 days in the various hydrogels (* p -value<0.05).

The highest density (5×10^6 cells/mL) gels present a significant decrease in the percentage of live cells between day 2 and 7 (Figure 19E), indicating that there was significant cell death during the 7 days, which is supported by a significant reduction of the metabolic activity measured in the resazurin assay at day 7 (Figure 19D).

At day 7, for the intermediate cell density hydrogels (3.78×10^6 cells/mL), there is no significant changes in the percentage of viable cells between the two different alginate concentrations, namely 2% and 1.5% (Figure 19E), supporting the resazurin assay results, which showed no alterations in metabolic activity of the MSC (Figure 19C).

Finally, in the lowest cell density hydrogels (2.5×10^6 cells/mL), the percentage of viable cells was similar at both time-points (Figure 19E), supporting the resazurin assay results, that (Figure 19C). Additionally, at day 7, the percentage of viable cells in the 2.5 million cells/mL hydrogels is significantly higher than the percentage of live cells in the 5 million cells/mL gels (Figure 19E).

Thus, at this point, it seems that the MSC prefer the 2% alginate concentration and the lowest cell density (2.5×10^6 cells/mL).

4.2. MSCs spread further during 2 weeks in lower cell density hydrogels

To evaluate cell spreading and the cellular network formed during the 14 days of MSC culture within the distinct hydrogels, the cell morphology was evaluated at day 2, 7 and 14 using phalloidin to stain the cytoskeleton (Figure 19A, Figure 20).

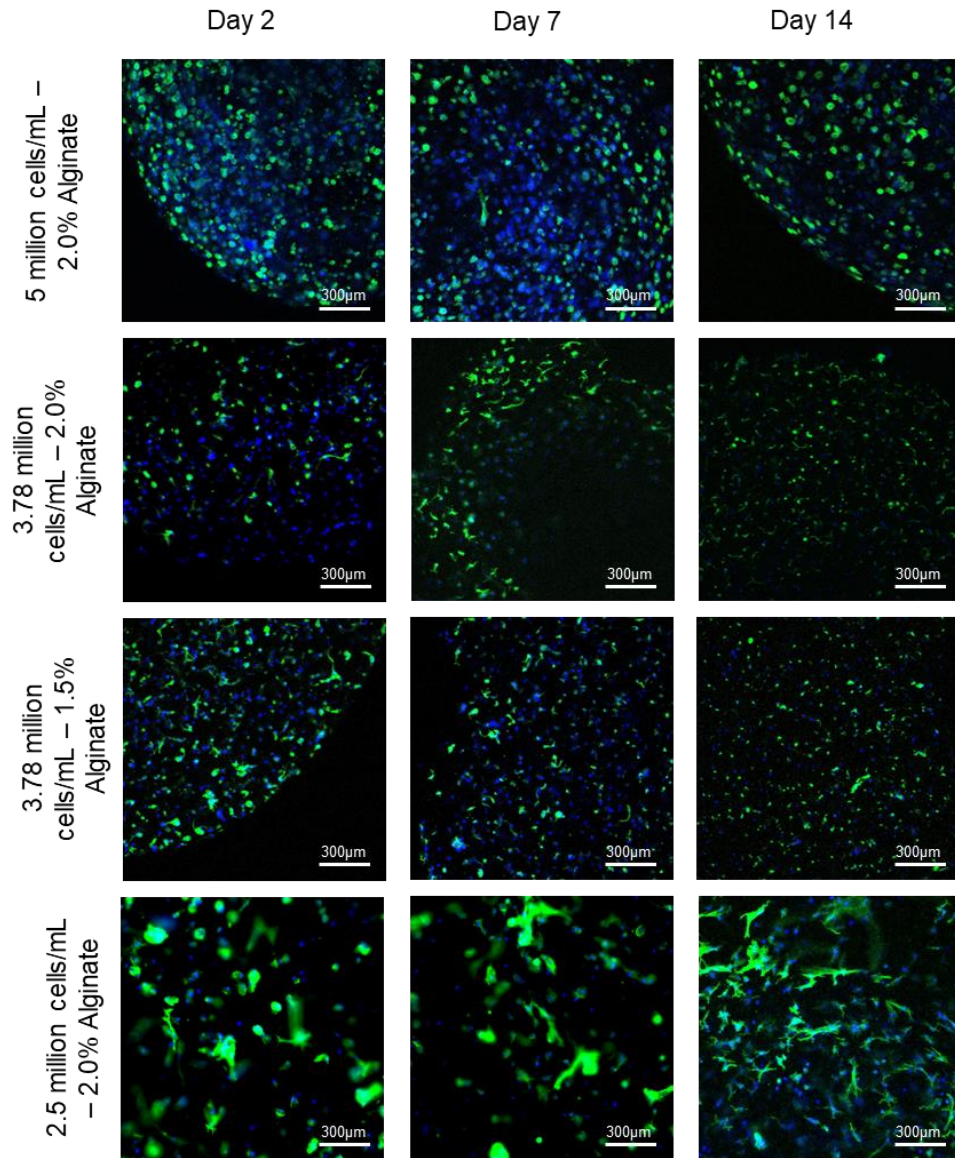


Figure 20. Representative photographs of the phalloidin (green) and DAPI nuclear (blue) staining performed on hydrogels fixed at days 2, 7 and 14.

At day 2, results show no significant differences in the aspect ratio (AR) between the hydrogels with distinct alginate concentrations and distinct cell densities (Figure 21A). However, the roundness of the 2.5 million cells/mL gels is slightly higher than the roundness of the MSC in the 3.78 million cells/mL and 2.0% alginate discs (Annex 6, Supplementary Figure 6A).

At day 7, in the 3.78 million cells/mL gels, there are no differences between the AR of the 2% and 1.5% alginate concentrations. The AR of the highest cell density hydrogels (5×10^6 cells/mL) is significantly lower than the AR of the 3.78 million cells/mL, 2.0% alginate discs, showing that at this time-point the MSC in the intermediate cell density 2.0% alginate gels (3.78×10^6 cells/mL) have spread more than the MSC in

the highest density hydrogels (5×10^6 cells/mL) (Figure 21B). At this time-point, the AR of the 2.5×10^6 cells/mL gels is higher than the AR of the 5×10^6 cells/mL discs (p-value=0.069) (Figure 21B).

At day 14, the 2.5×10^6 cells/mL present a significantly higher AR than all the other gels with the same alginate concentration. Therefore, after 2 weeks, the MSC in the lowest cell density gels have spread the most (Figure 21C). The highest cell density gels present the lowest AR in all time-points (Figure 21 A-C). Curiously, the AR of the 1.5% alginate, intermediate cell density gels (3.78×10^6 cells/mL) is significantly lower than the AR of the 2.0% alginate intermediate cell density gels at 14 days, whereas before, at day 2 and 7, no changes were measured in the AR between the 2% and 1.5% alginate intermediate cell density discs (Figure 21E). This decrease is supported by an increase in the cell roundness (Annex 6, Supplementary Figure 6E).

During the 2-week time period observed, the AR of the 1.5% alginate intermediate cell density gels (3.78×10^6 cells/mL) stays constant in the first week (day 2 and 7) and decreases significantly in the second week (from day 7 to day 14) (Figure 21E). This tendency cannot be verified in the 2% alginate intermediate cell density hydrogels. In fact, in these hydrogels, the AR increases slightly (Figure 21E) and the roundness decreases steadily (Annex 6, Supplementary figure 6E) during the 2 weeks. These measurements indicate that the MSC adhered and spread better in the 2% alginate gels than in the 1.5% alginate discs (Figure 20), suggesting the 2% alginate concentration is better for MSC culture. These results together with the metabolic activity results at day 2 and 7 (section 4.1) emphasize that the MSC prefer the 2% alginate hydrogels instead of the 1.5% alginate hydrogels, further supporting the choice of the 2% alginate concentration for further studies.

Regarding the highest cell density hydrogels (5×10^6 cells/mL), no alterations in cell morphology are visible in Figure 20 during the 14 days. However, a slight statistically significant increase of the AR between days 2 and 7, days 7 and 14, and days 2 and 14 (Figure 21D) was measured, that is supported by a slight decrease in roundness (Annex 6, Supplementary Figure 6D). On the other side, the 2.5×10^6 cells/mL discs present a slight significant increase of the AR between day 2 and 7 and a pronounced significant increase in the AR between days 7 and 14, and days 2 and 14 (Figure 21F), which is supported by the roundness measurements (Annex 6, Supplementary Figure 6F) and clearly visible in the images (Figure 20). Additionally, the MSC in the lowest cell density

gels (2.5×10^6 cells/mL) were the only ones that were able to form a clearly visible cellular network within the hydrogel during the 14 days (Figure 20).

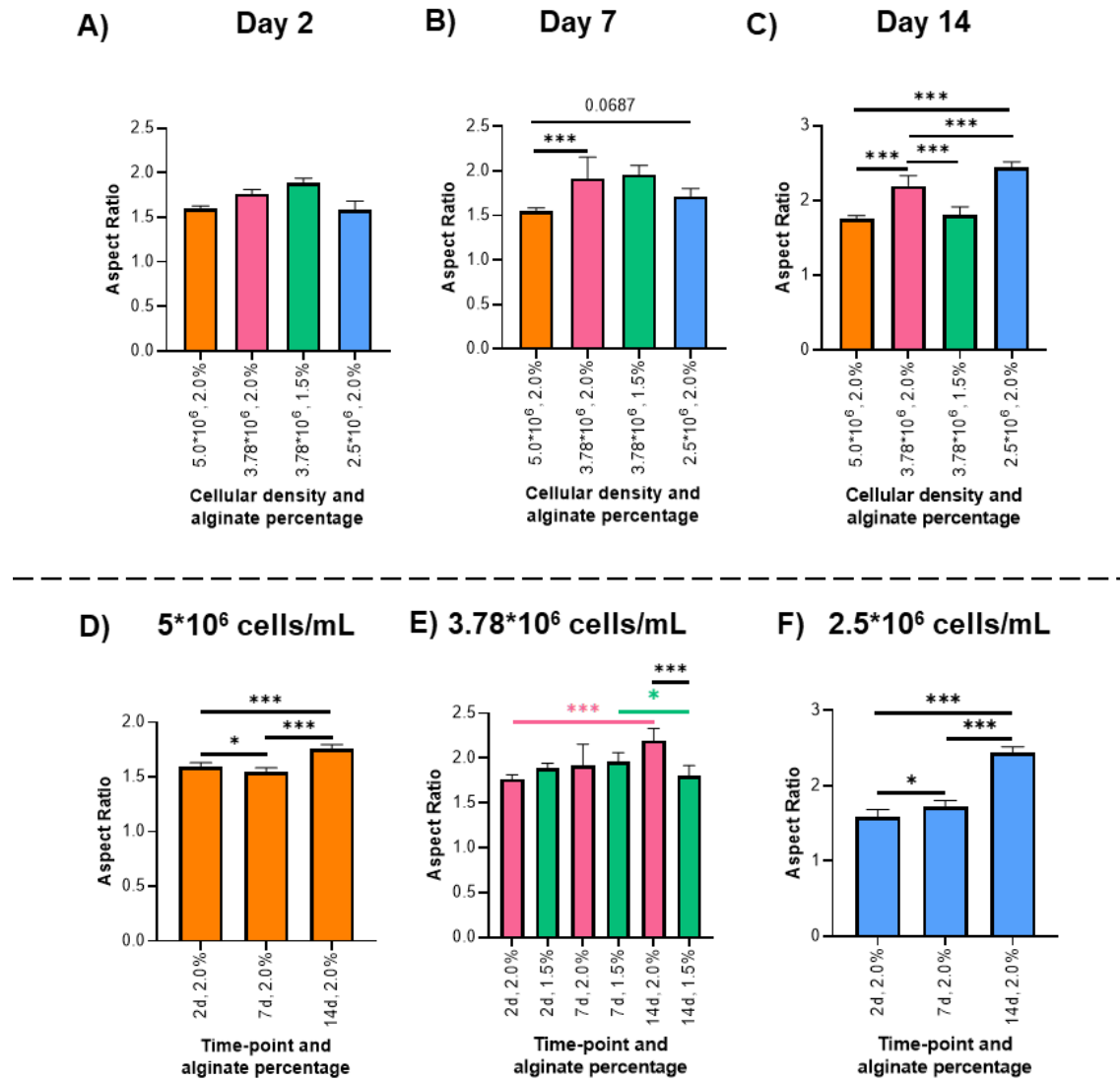


Figure 21. Aspect ratio evaluation at day 2, 7 and 14 performed on the hydrogels with cell densities of 5, 3.78 or 2.5 million cells/mL and with 2.0% or 1.5% alginate concentration. A-C) Aspect ratio evaluation individually for each time-point at day 2, day 7 and at day 14, respectively; D-F) Aspect ratio evaluation during 14 days for each individual cell density, namely 5 million cells/mL, 3.78 million cells/mL and 2.5 million cells/mL, respectively (* p -value<0.05, ** p -value<0.01, *** p -value<0.001).

4.3. NAMPT protein levels in the lowest cell density hydrogels are increased after 7 days in culture

To test the immunofluorescence protocol within the hydrogels, we performed immunostaining of NAMPT on the lowest cell density (2.5×10^6 cells/mL) at day 2 and 7 under basal conditions. The specificity of the staining was confirmed, since no staining could be seen in the negative control, while a specific labelling of NAMPT (red) could be observed at day 2 and 7 of culture (Figure 22A). Additionally, the amount of NAMPT staining was quantified in both time-points and results show that there were increased levels of NAMPT within the hydrogel at day 7 compared with day 2, which was expected (Figure 22 B).

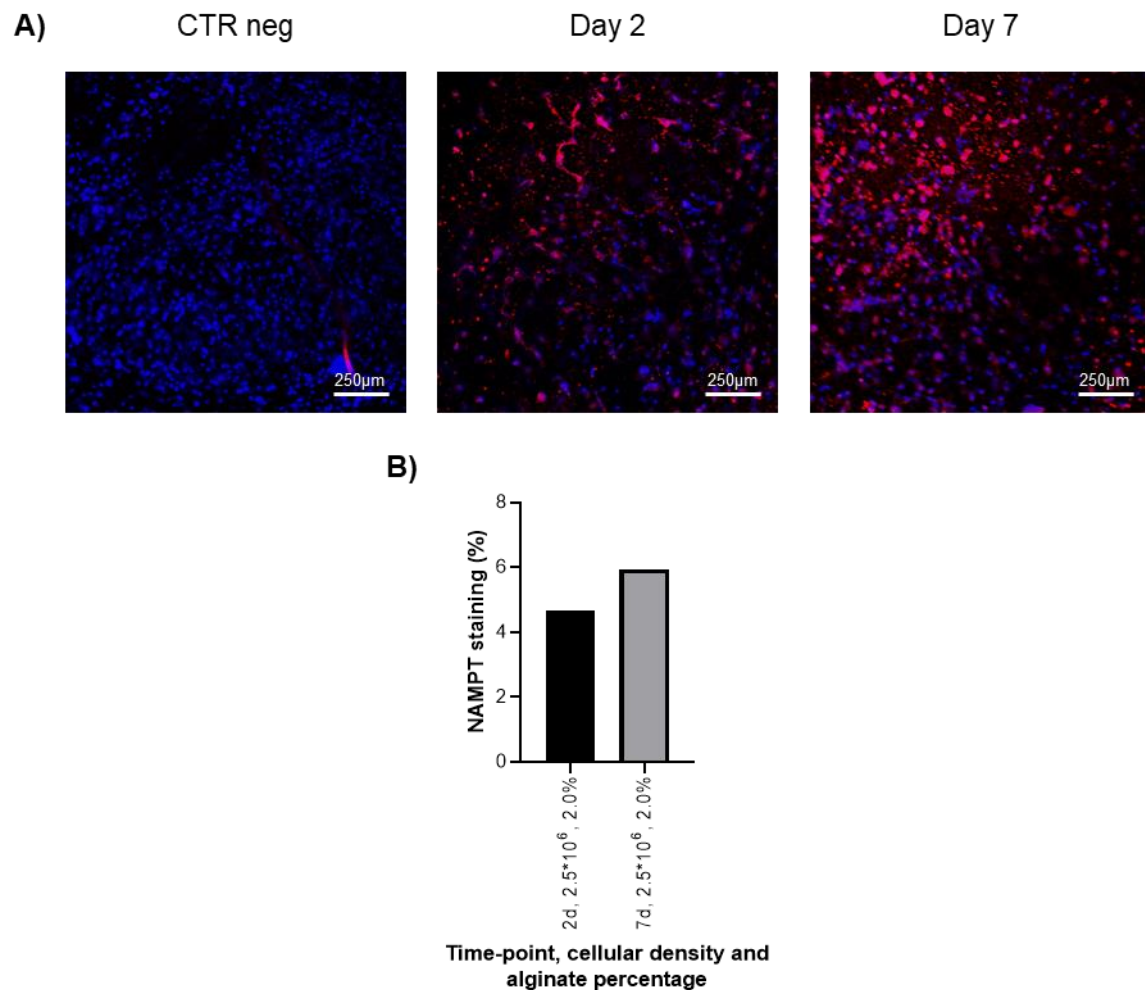


Figure 22. Evaluation of NAMPT expression within the lowest cell density hydrogels (2×10^6 cells/mL) at different timepoints. A) Representative photographs of NAMPT staining; B) Quantification of the percentual NAMPT stained area.

5. Untargeted bRNA-seq results analysis reveals novel transcripts regulated by anti-miR-195

Considering the qRT-PCR assays, results showed that NAMPT and IBSP were differently expressed by anti-miR-195 delivery at the transcript level, but the results could not be validated at the protein level. Therefore, the bRNA-seq data was reanalysed to identify other potentially relevant transcripts. To do so, instead of focussing on particular pathways and gene ontology analysis, the differentially expressed protein-coding genes were reevaluated based on the fpkm values, independently of the pathways in which they were involved. Out of the 309 protein-coding differentially expressed transcripts, 273 transcripts (around 88%) had individual fpkm values greater than 0.01 for each one of the donors and transfection condition (Figure 23A and B). In total 255 genes were found to be either up- or down-regulated in all 3 donors. This gene list consisted of 157 up-regulated transcripts in all 3 donors (around 64%) and 98 down-regulated transcripts (around 36%) in all 3 donors (Figure 23C). Out of the 255 genes, only 18 presented FC-values greater than 2 across all donors and 12 presented FC-values smaller than -2 for all donors (Figure 23D). A final list of 30 genes that are either up- or down-regulated in all donors and have $|FC|$ greater than 2 was identified (Figure 24). Out of these, the first six with the highest FC values, namely *GNRH2*, *KCNG2*, *ZBTB32*, *CCDC168*, *HIST1H1E* and *GPR39*, were selected for future qRT-PCR validation (Figure 24, red box).

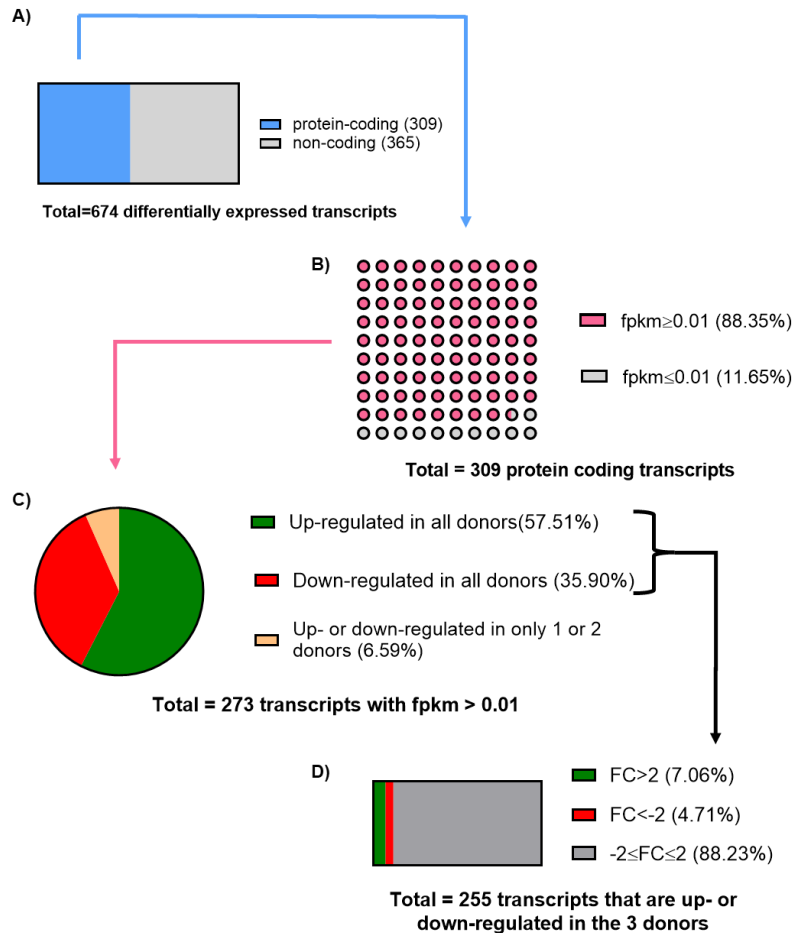


Figure 23. Untargeted reanalysis of bRNA-seq data. A) coding and non-coding transcripts; B) Genes presenting fpkm values greater than 0.01; C) Up- or down-regulated transcripts in the 3 donors D) Transcripts with |FC| greater than 2.

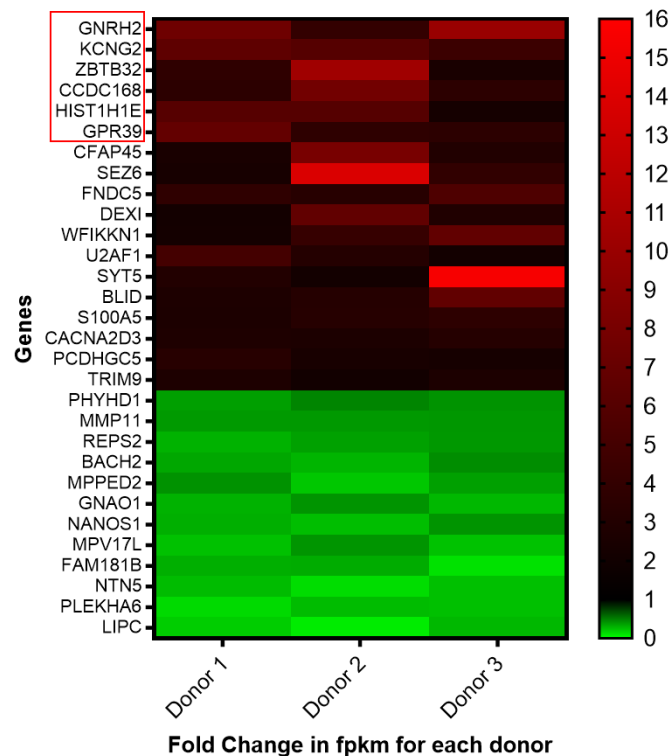


Figure 24. Heatmap of the 30 selected targets for literature review.

V. Discussion

The bRNA-seq is a powerful high-throughput methodology that provides a quantitative and qualitative analysis of the transcriptome with multiple applications, including identification of alternative splicing events, gene isoforms and single nucleotide polymorphisms, and differential gene expression analysis (DGE) [112], [113]. Additional advantages include a lower detection limit than microarrays, high sensitivity, shorter sequencing time and lower price than microarrays [114]–[116]. Specifically in this study, its most relevant advantage for this study is that it allows for a view of the whole transcriptome. On the other side, its disadvantages include the lack of a standardized and optimized protocols for the data analysis and the complexity of the data analysis, which requires extensive computational work [116], [117].

In the RNA-seq library preparation, a poly(A) enrichment method to screen for mRNAs was used. Therefore, it was unexpected to detect 365 ncRNAs in the DGE analysis, including long ncRNAs (lncRNAs). However, this is not uncommon as some ncRNAs can escape elimination, since the poly(A) enrichment method does not specifically eliminate transcripts that do not have a poly(A) tail, instead, it enriches these transcripts, being a limitation of this methodology used. Additionally, some lncRNAs have a poly(A) tail that can be cleaved during their biogenesis, which can partially explain their detection in the bRNA-seq [118]. The detected ncRNAs were not considered for further evaluation, and only the differentially expressed protein-coding transcripts were considered for the gene ontology (GO) analysis.

In the study, 309 protein-coding transcripts were identified as differentially expressed by anti-miR-195 delivery in MSC, thus further reinforcing the impact of miR-195 on the downstream targets. A large number of categories was identified in the GO analysis, the majority of which related to cell cycle, proliferation and mitosis. An interesting line of future work would be to investigate the role of the miR-195 in cell proliferation, not only in MSC but also in bone and bone-marrow cancer cell lines.

In this study, the integrity and purity of the RNA samples was assessed to ensure its quality. Protein contamination was not found in any samples, which indicates that the extraction process successfully avoided RNA contamination with proteins. However, the RNA samples were found to be contaminated with solvents, which could be explained by imprecise supernatant rejection following RNA centrifugation with isopropanol, and/or insufficient washing with ethanol of the formed RNA pellet. So, these steps should be optimized in future experiences. Additionally, RNA degradation could have multiple causes, since RNA is an instable molecule. Possible causes for RNA degradation include leaving the samples at room temperature and/or inadequate manipulation of the RNase- and DNase-free materials and reagents. The low RNA quantities obtained in some donors also enhance the degradation probability and, to avoid this limitation in the future, RNA should be extracted from an increased number of cells. Additionally, the low RNA concentrations for some donors indicates that the number of cells used to obtain the RNA varied for each donor and transfection group (SCR or anti-miR-195), which is not ideal and should be avoided in the future by ensuring that cells have reached confluence before proceeding with transfection, cell lysis and RNA extraction.

From the seven miRNA-195 targets selected for qRT-PCR validation (*FNDC5*, *IBSP*, *NAMPT*, *TGM2*, *CCNB1*, *BAMBI* and *CLEC3B*), *CLEC3B* was found not to be expressed in 2 donors. Out of the remaining six targets, *CCNB1*, *NAMPT* and *IBSP* were successfully identified as upregulated at the transcript level following miR-195 depletion. Thus, this study identified these three transcripts as miRNA-195 targets, but it does not clarify whether they are direct or indirect targets. To address this question, a luciferase reporter assay should be performed.

All the of the 7 selected genes are involved in the bone ECM and/or MSC-osteogenesis and proliferation. Specifically, Fibronectin Type III Domain Containing 5 (*FNDC5* or Irisin) has been found to promote osteogenesis and regulate bone mass in mice [119], [120], and Integrin Binding Sialoprotein (*IBSP*) is involved in bone extracellular matrix (ECM) mineralization, and its expression is induced by Runx2 [121]–

[123]. Additionally, IBSP is involved in the proliferation and differentiation of osteoblasts [49]. Studies found that a reduction in nicotinamide phosphoribosyltransferase (NAMPT) levels in old mice is associated with decreased osteogenesis and increased adipogenesis, whereas NAMPT enhancement has been associated with decreased MSC senescence in rats [124]–[126]. Considering Transglutaminase 2 (TGM2), Fuja et al. found that it promotes stem-like characteristics in osteosarcoma and Kubo et al. described TGM2 as a MSC signature molecule [127], [128]. Additionally, TGM2 has been associated with enhanced MSCs survival and matrix stability in rats [129]. Cyclin B1 (CCNB1) is a specific G2/Mitotic cyclin that has shown to improve MSC proliferative capacity [130], [131]. Finally, BMP And Activin Membrane Bound Inhibitor (BAMBI), has also been described as a osteogenesis promoter, and CLEC3B (C-Type Lectin Domain Family 3 Member B) is involved in mineralization during osteogenesis [132], [133]. Additionally, FNDC5, IBSP, NAMPT and CLEC3B are secreted into the extracellular matrix [134]–[137]. In conclusion, FNDC5, IBSP, BAMBI and CLEC3B are proteins associated with MSC osteogenesis, and, on the other hand, CCNB1, NAMPT and TGM2 are proliferation-related proteins. Therefore, by validating *CCNB1*, *NAMPT* and *IBSP* transcripts by qRT-PCR as miR-195 targets, we further supported the potential of miR-195 as target for osteoregenerative therapies.

To further validate these targets at protein level, immunofluorescence assays were performed at two different time-points. At the later time-point, NAMPT was upregulated upon miR-195 silencing in the majority of the donors in osteogenic conditions, however, IBSP levels remained unaffected by transfection with anti-miR-195 in both basal and osteogenic conditions. At the first time-point (2 days post-transfection), no changes were measured between transfection conditions for both NAMPT and IBSP proteins. Therefore, more donors should be tested to solidify these results regarding NAMPT. Post-transcription modifications and regulatory mechanisms could explain why the changes observed in the transcript levels could not be validated at protein level. Regarding the immunocytochemistry experiments, the background signal in the negative control could be explained by cellular autofluorescence and presence of impurities. In the future experiments, this could be avoided by adding more washing steps into the protocol.

When designing the 3D system, alginate was chosen as a model because of its biocompatibility, low toxicity and favourable gelation properties. It is a natural polymer derived from algae and it consists of copolymers of mannuronic acid and guluronic acid

monomers [138]. Due to their advantageous properties, its applications in biomedical sciences are wide, for example for wound healing, drug delivery and tissue engineering [102]. Particularly in tissue engineering, alginate has been used to provide a 3D structure for cell transplantation and new tissue formation. Native alginate presents low degradation rates *in vivo*. It also degrades in an unpredictable way, originating alginate chains of different lengths and molecular weights. Whereas oxidized alginate has a faster degradation rate, biodegradability and higher ratio of reactive groups when compared with native alginate, allowing for easier functionalization [104]. Therefore, oxidized alginate is indicated for tissue regeneration applications. Additionally, oxidized alginate hydrogels form a mesh with more defects than native alginate hydrogels, facilitating cellular network establishment by MSC. To increase cell adhesion, the oxidized alginate hydrogels were functionalized with RGD, which is an integrin-binding tri-amino acid sequence found commonly in multiple ECM proteins, including bone sialoprotein (BSP or IBSP) [104], [139], [140].

Considering the optimization of an alginate-based hydrogel system to support MSC survival and to assess the expression of target proteins in a complex 3D microenvironment, three different cell densities and alginate concentrations were tested. Results showed that the MSC preferred the 2.0% alginate concentration over the 1.5% concentration and that the 2.5 million cells/mL density was indicated for better MSC survival, spreading and creation of a cellular network. Consequently, the lowest cell density, 2% alginate hydrogels are indicated for future experiments. In the literature, MSC-laden alginate hydrogels have been constructed with various cell densities and alginate concentrations. For example, Panda et al. designed an alginate hydrogel with 2×10^6 cells/mL density and 2% alginate concentration, whereas Reppel et al. chose the cell density of 3.5 million cells/mL and the alginate concentration of 1.5% [141], [142]. Another study reported the use of hydrogels with the cell density of 5 million cells/mL and the alginate concentration of 2% [143]. Therefore, the cell density (2.5×10^6 cells/mL) and alginate percentage (2%) optimized in this work are in line with the published literature.

The protocol for immunostaining of target proteins within hydrogels was also successfully tested and, as expected, NAMPT expression within the 2.5×10^6 cells/mL hydrogels was higher at the later time-point. Future work should aim to create hydrogels with either anti-miR-195-MSC or SCR-MSC to test the expression of target proteins, which were validated in 2D cell monolayers, in the 3D gels.

Multidimensional systems, such as this one, allow for a better representation of the environment that cells are naturally exposed to in the body, which affects their behaviour, survival, differentiation and proliferation. They add new layers of complexity when compared to 2D study models and are closer to the human *in vivo* reality, hence being a very important step in *in vitro* studies. Therefore, the successful optimization of a 3D MSC-laden alginate-based hydrogel was an important goal of this project, which was achieved.

Finally, since it was not possible to validate with certainty any of the selected ECM-, osteogenesis- or proliferation-related miR-195 targets, a new untargeted analysis of the bRNA-seq was performed. This second evaluation was not directed at ECM-related, osteogenesis-related or proliferation-related genes. Instead, it aimed to select targets based on their expression levels and consistency of up- or down-regulation across all donors. After this quantitative screening of the protein-coding differentially expressed genes, the first six with the highest fold change were selected for future validation by qRT-PCR. Their description was investigated in the published literature and, out of the 6, 3 genes had been reported as associated with MSC and osteogenesis (*KCNG2*, *ZBTB32* and *GPR39*), while the other 3 had not yet been described in MSC behaviour (*GNRH2*, *CCDC186*, *HIST1H1E*). *KCNG2* protein is upregulated by BMP-2, which is an important factor during osteogenesis, as previously mentioned [144]. *ZBTB32*, or *FAZF*, expression is also induced by BMP-2 during osteoblast differentiation [145]. Finally, *GPR39* is a zinc receptor required for normal bone matrix production by osteoblasts in mice and a study showed that a *GPR39* agonist promoted osteoblastogenesis and mineralization in MC3T3 cells [146], [147]. So, the untargeted reanalysis of the bRNA-seq data provided 3 potential miR-195 target candidates, that should be validated at transcript level by qRT-PCR and at protein level by immunofluorescence assays in future studies.

Human primary MSCs were used throughout this study, instead of osteoblast cell lines, such as the mouse MC3T3 cell line, which are able to undergo osteoblastogenesis to some degree. This explains the variability between donors observed throughout all experiments. Despite the increased and expected inter-donor variability, the use of human primary MSCs recreates the human clinical setting better than studies with cell lines. Therefore, the results obtained throughout this project are closer to the human *in vivo* setting. To decrease the impact of the inter-donor variability in the future, especially in the immunofluorescence assays, the experiments should be repeated with cells from additional donors.

VI. Conclusions and Future Perspectives

In conclusion, this study shows that modulation of miR-195 levels significantly affects 309 protein-coding transcripts, including genes involved in ECM, proliferation and osteogenesis. Among the tested anti-miR-195 targets, NAMPT, IBSP and CCNB1 expression was validated to be upregulated at the transcript level following miR-195 silencing in MSC. These qRT-PCR results further support and enhance the potential of anti-miR-195 to be included in osteoregenerative therapies and its importance during osteogenesis and bone fracture repair. Out of the selected candidates, NAMPT showed as the most promising as it was increased, at the protein level, following miR-195 depletion, under osteogenic conditions, in 2 out of 3 MSC donors. Additionally, a second analysis of the bRNA-seq results provided 30 consistently up- or down-regulated transcripts with $|\text{FC}|$ greater than 2. Out of those, 3 presented promising descriptions considering their role in MSC and osteogenesis, namely KCNG2, ZBTB32 and GPR39.

A cell-laden alginate-based 3D hydrogel system was successfully optimized and the implantation of the protocol for immunostaining of protein targets was achieved. This system is designed for future target validation in a 3D environment, which is expected to facilitate subsequent research endeavours.

As future perspectives, protein expression validation in the optimized 3D environment through immunofluorescence assays should be performed. Future work

should also determine if NAMPT and other candidate transcripts identified in the bRNA-seq are direct miR-195 targets (by complementary base pairing) or indirect targets. In a later stage, the effect of anti-miR-195 on MSC and bone regeneration should be tested *in vivo*, using the optimized alginate hydrogels as a localized cell delivery and support material.

This study serves as a preliminary exploration of miR-195s potential role in MSC-based therapies for bone repair and regeneration. Our findings have identified potential targets related to the extracellular matrix (ECM) and cell proliferation, although these findings require validation at the protein level. Notably, the validation of NAMPT as a potential candidate shows promise. Additionally, we have uncovered potential targets outside the ECM and proliferation domains.

VII. References

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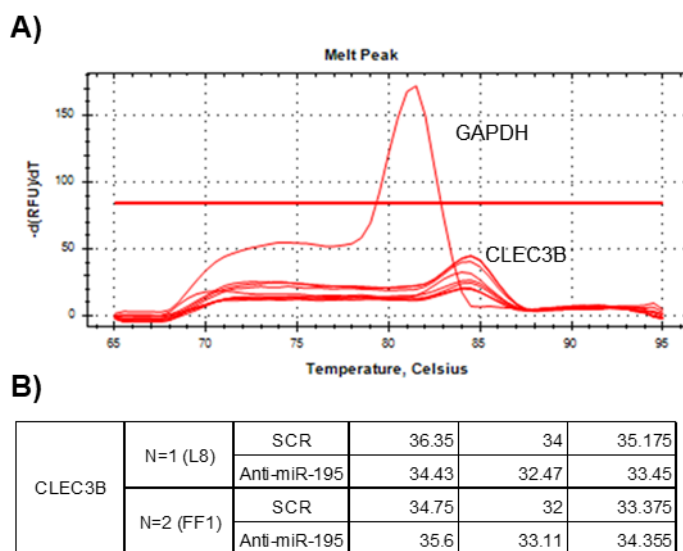
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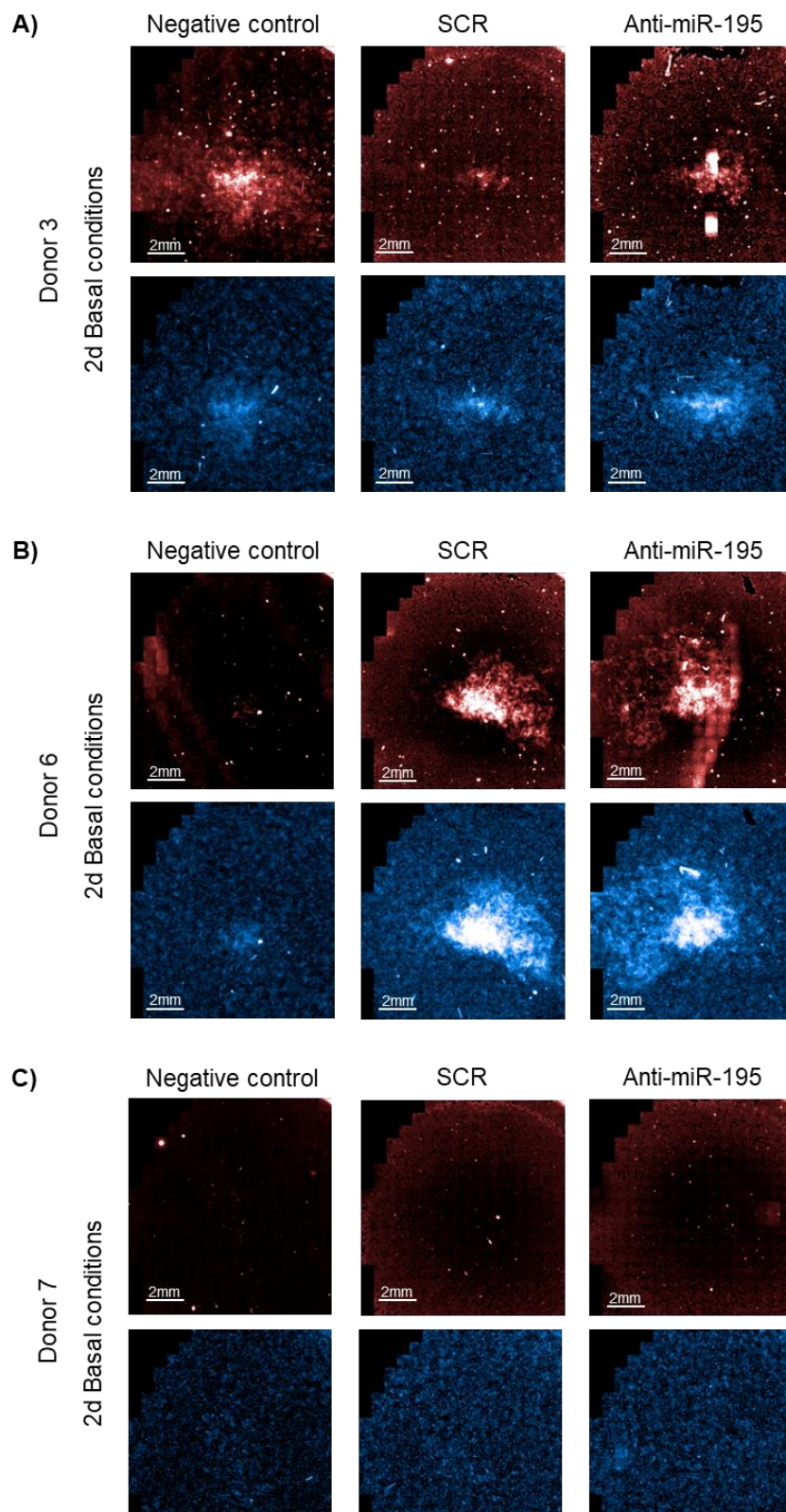
VIII. Annexes

Annex 1: CLEC3B expression



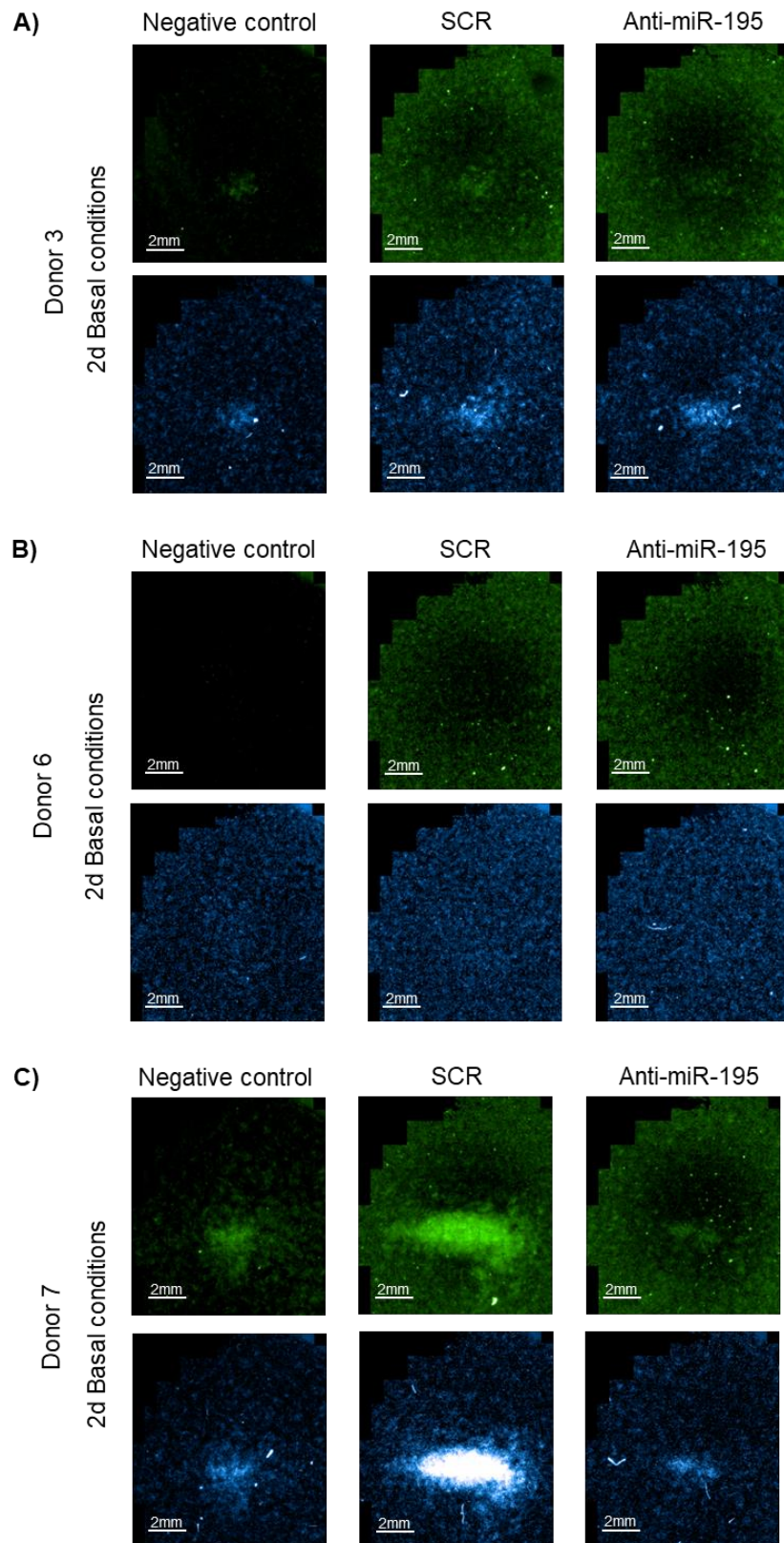
Supplementary Figure 1. A) Melting curve and B) Cq cycles of CLEC3B, which is very low expressed in MSCs from 2 donors (n=2).

Annex 2: NAMPT expression in MSCs 48h post-transfection



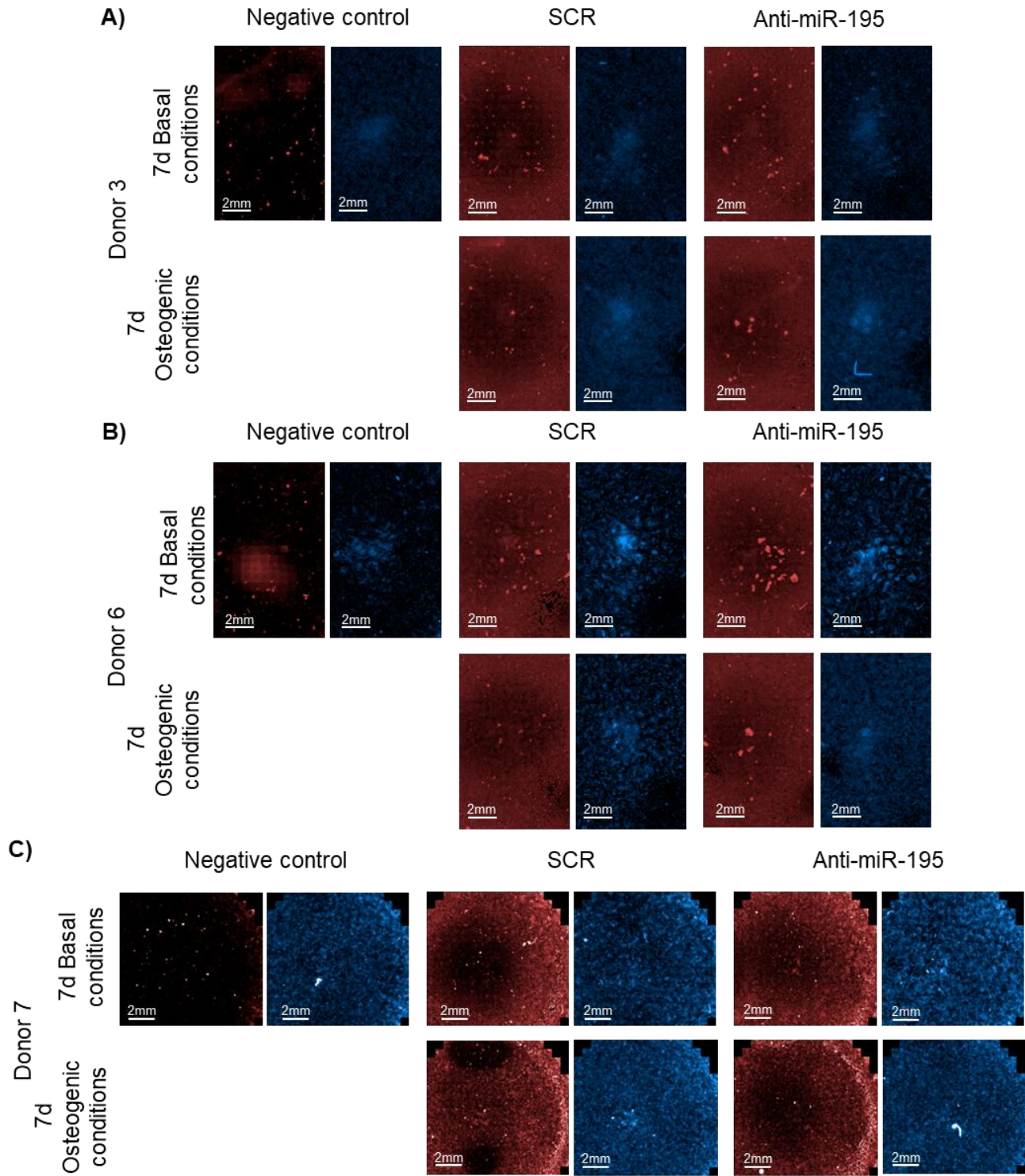
Supplementary Figure 2. Images of anti-NAMPT (red) and DAPI nuclear (blue) staining taken with Operetta CLS High Content Analyzer 48h post-transfection (with SCR control or anti-miR-195 oligonucleotides) for 3 different MSC donors (A to C, respectively) separated by fluorochrome for better visualization (AF-594 on top and DAPI on the bottom).

Annex 3: IBSP expression in MSCs 48h post-transfection



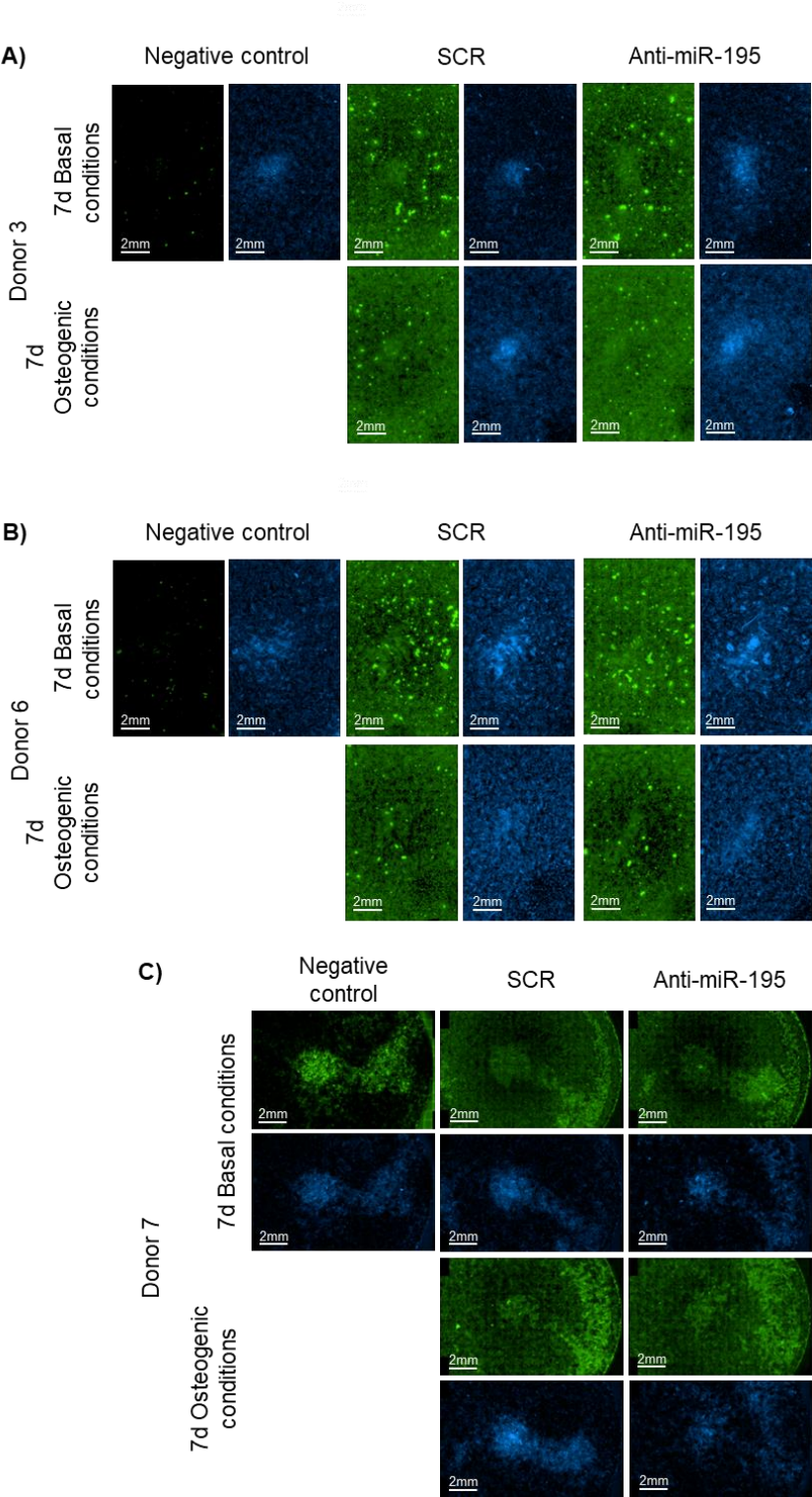
Supplementary Figure 3. Images of anti-IBSP (green) and DAPI nuclear (blue) staining taken with Operetta CLS High Content Analyzer 48h post-transfection (with SCR control or anti-miR-195 oligonucleotides) for 3 different MSC donors (A to C, respectively) separated by fluorochrome for better visualization (AF-488 on top and DAPI on the bottom).

Annex 4: NAMPT expression in MSCs 7d post-transfection with basal and osteogenic media



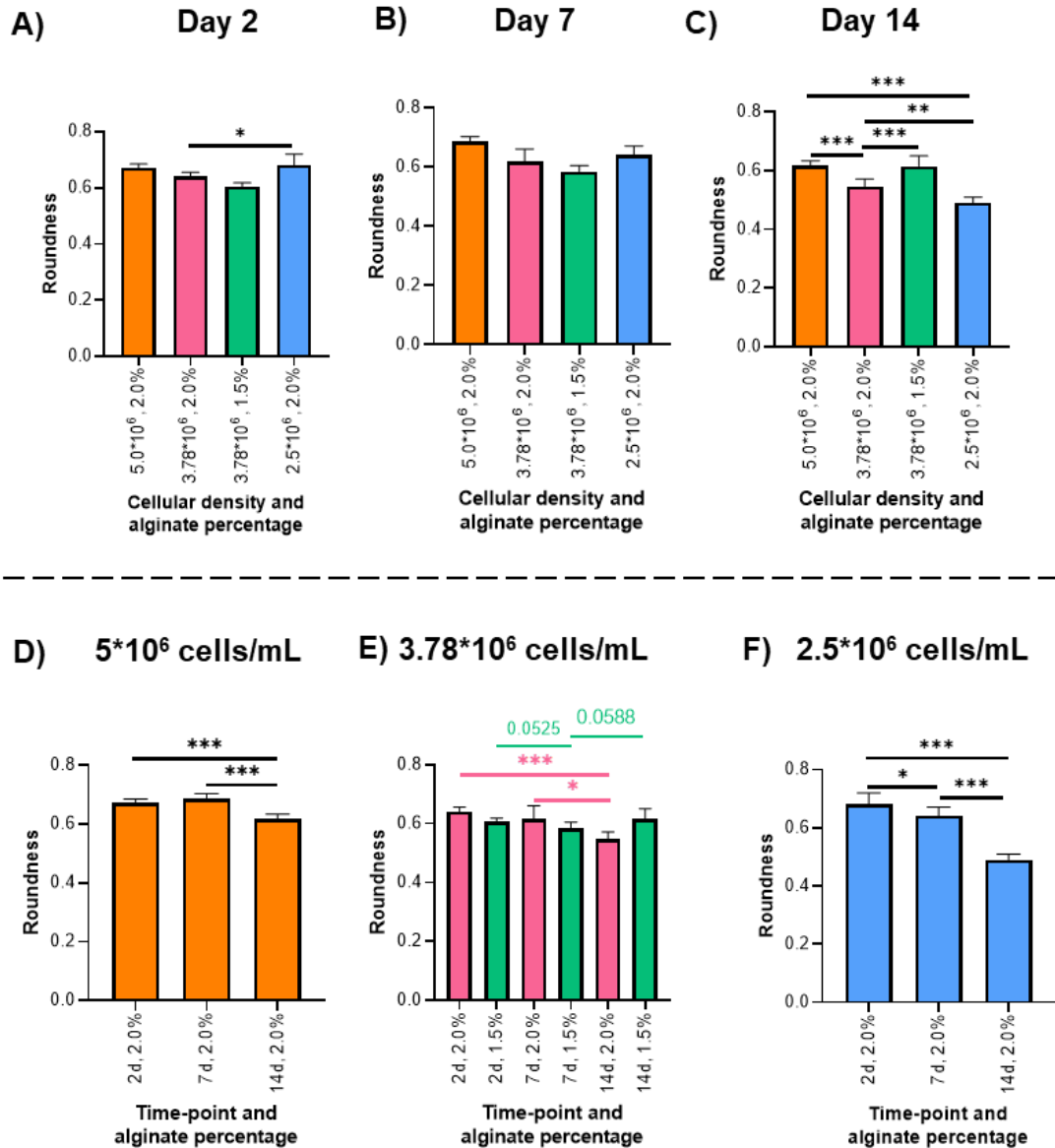
Supplementary Figure 4: Images of anti-NAMPT (red) and DAPI nuclear (blue) staining taken with Operetta CLS High Content Analyzer 7 days post-transfection (with SCR control or anti-miR-195 oligonucleotides) with basal (top) or osteogenic (bottom) conditions for 3 different MSC donors (A to C, respectively) separated by fluorochrome for better visualization.

Annex 5: IBSP expression in MSCs 7d post-transfection with basal and osteogenic media



Supplementary Figure 5: Images of anti-IBSP (green) and DAPI nuclear (blue) staining taken with Operetta CLS High Content Analyzer 7 days post-transfection (with SCR control or anti-miR-195 oligonucleotides) with basal (top) or osteogenic (bottom) conditions for 3 different MSC donors (A to C, respectively) separated by fluorochrome for better visualization.

Annex 6: Roundness evaluation



Supplementary Figure 6. Roundness evaluation at day 2, 7 and 14 performed on the hydrogels with cell densities of 5, 3.78 or 2.5 million cells/mL and with 2.0% or 1.5% alginate concentration; A-C) Roundness evaluation individually for each time-point at day 2, day 7 and at day 14, respectively; D-F) Roundness evaluation during 14 days for each individual cell density, namely 5 million cells/mL, 3.78 million cells/mL and 2.5 million cells/mL, respectively (* p -value<0.05, ** p -value<0.01, *** p -value<0.001).

