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Biological characterization of human MSCs secretome and extracellular vesicles (EVs): from 2D-cultures to robotic systems.

PhD candidate: Maria Elisabetta Federica Palamà
Supervisor: Dr. Chiara Gentili
Co-Supervisor: Prof. Rodolfo Quarto

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Abstract

Human mesenchymal stem cells (hMSCs) are a promising cell therapy candidate for the treatment of several clinical conditions. In recent years, the use of extracellular vesicles emerges for therapeutic purposes. Most of cells indeed release a wide range of extracellular vesicles of different size, content and functions, which interact with target cells by modifying the phenotype or function.

Manufacture of hMSCs requires *in vitro* isolation and expansion to increase the available number of final products to meet clinical demand. Through Autostem (EU-H2020 proposal) European academic and industrial group worked in collaboration to enable large-scale hMSCs production, at clinical-grade quality, by implementing a robotic automated pipeline for cell isolation and culture. The goal is to develop an automated, closed and good manufacturing practice (GMP) MSC cell technology, a "StromalCellFactory" to produce allogenic hBMSCs to use in therapy.

We developed a "donor-to-patient" system for aseptic therapeutic cell manufacturing using a xeno-free medium. We characterized cells, secretome and extracellular vesicles of hBMSCs, isolated in plastic adherence and grown in FBS and in a chemically defined xeno-free medium, compared to hBMSCs isolated in a 3D-bioreactor, demonstrating that our serum-free system allow to select a highly proliferating-hBMSC population with a strong osteogenic potential, both *in vitro* and *in vivo.* These cells have also a secretion pattern characterized by biological factors involved in homeostasis, wound healing and angiogenesis, beside showing a stronger extracellular vesicles secretion.

We validated also the potential therapeutic benefits of secreted factors, conditioned medium and exosomes isolated from hBMSCs cultured in our culture system, for cartilage repair.

In a damaged tissue, the initial inflammatory response plays a key role triggering tissue repair and homeostasis, but can be detrimental in the long term, causing fibrosis. We observed that under inflammatory condition human Articular Chondrocytes (hACs) are able to internalize and recruit more MSC-derived exosomes, compare to the control chondrocytes. We investigated the effects of conditioned medium and extracellular vesicles in the activation of different regeneration pathways (IL6, IL8, COX2 and NF-kB).

The effect of MSCs-derived conditioned media and exosomes revealed to be regenerating and protective for the articular cartilage and they may be a possible therapy for osteoarthritis.

Background

Regenerative medicine has been defined as the process of replacement or regeneration of human cells, tissues or organs to restore or establish a normal function.[1]

The term “regenerative medicine” was introduced by William Haseltine during a 1999 conference on Lake Como, in the attempt to describe an emerging field, which blends knowledge deriving from different subjects: tissue engineering, cell transplantation, stem cell biology, biomechanics prosthetics, nanotechnology, biochemistry. Historically, this term was found for the first time in a 1992 paper by Leland Kaiser, who listed the technologies which would impact the future of hospitals.[2]

The concept of regeneration is commonly observed, but often unappreciated in daily medical practice. The regeneration of body parts is a rather common phenomenon in nature, a salamander can regenerate an amputated limb in several days.[3] Humans have this ability as well, but they lose it over the years: a severed fingertip can regenerate until 11 years of age.[4] Therefore, regenerative medicine uses, when possible, the body's self-regenerative capacities. The normal maintenance of tissues in an adult organism is guaranteed by natural mechanisms of tissue regeneration. In humans, however, the regenerative capacities are often insufficient to face off serious pathological conditions, which lead to a progressive loss of tissue.

The aim of regenerative medicine is to restore normal structure and function following tissue injury and it is defined by the convergent repair triad of replacement, regeneration, and rejuvenation (the R3 paradigm, *Fig.1*). These strategies overlap and conceptualize the scope of regenerative medicine, ranging from transplantation of used parts (“replacement”) to development of new parts (“regeneration”) to induction of self-renewed parts (“rejuvenation”).[5]

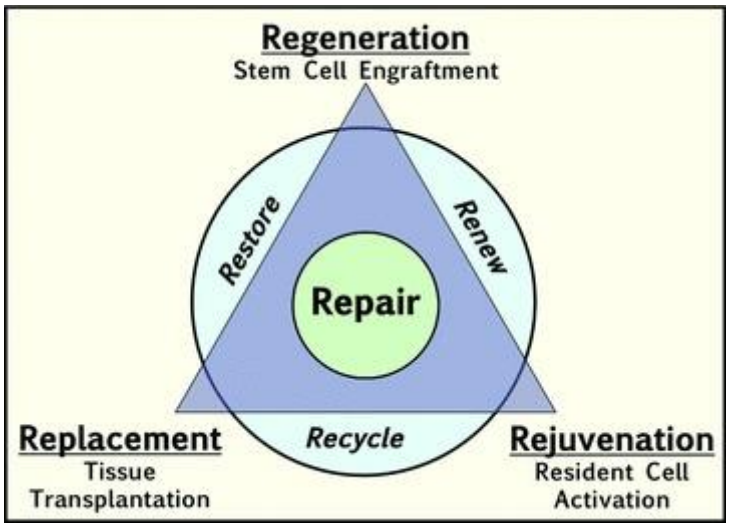

Figure 1 The scope of regenerative medicine. Repair is the central goal of regenerative medicine that encompasses the general strategy triad: replacement, regeneration, and rejuvenation. Replacement is defined as repair of damaged tissue by recycling used parts through tissue/organ transplantation. Regeneration is defined as repair of damaged tissue through differentiation of progenitor cells to replace damaged cells and restore tissue function. Rejuvenation is defined as repair of damaged tissue through activation of endogenous resident stem cells that can stimulate biogenesis and replenish functional tissue. Collectively, therapeutic repair strategies are recognized as the “R3” regenerative medicine paradigm.[5]

Replacement strategy refers to transplantation of a cell-based product that re-establishes homeostasis for the recipient through continuation of the tissue function from the donor.[6] The transplantation is the replacement of an organ or a tissue with another taken from the same organism (homo-transplantation, better referred to as autologous transplantation), from another organism of the same species (allo-transplantation) or from an individual of different species (xeno-transplantation[7,8]). There are different types of transplant, also based on the implant site of the new organ: orthotopic (the original malfunctioning organ is removed, and the organ of the donor is placed in the same anatomical position of the original organ), heterotopic (a new organ is flanked by the old one no longer functioning, but remains in its place as in cardiac surgery where the heart of the donor is sutured on the right side of the receiver's heart that remains in situ)[9–11], or ectopic (a new organ, or more often a tissue, is taken from a certain anatomical area to be implanted in a different one, where it will be able to regenerate the recipient tissue or organ, as in the case of skin transplantation[12–15]).[16–18]

Unfortunately, transplantation brings with it several problems and complications. The issues that arise are related to the disproportion between the demand and the effective availability of organs conforming to the donation, the risks of bacterial or viral infections and the necessary compatibility between the donor and recipient individual. Beyond that, after transplantation it is essential to follow a therapy with immunosuppressive drugs aimed at preventing the phenomenon of rejection. They cause a marked deterioration of the overall patient life condition, causing metabolic imbalances, cardiovascular disorders and greater susceptibility to bacterial and viral pathogens, due to a drastic decline of the body's immune defences.[19–23]

This type of drawbacks could be largely avoided through the new regenerative medicine approaches. Indeed, recent advances in this field have shown that it is possible to stimulate tissue repair mechanisms to improve endogenous tissue regeneration.[24]

Regenerative medicine has the potential to heal or replace tissues and organs damaged by age, disease or trauma.[25] Preclinical and clinical data support the possibility of treating both chronic and acute diseases, including skin wounds, cardiovascular diseases and treatment for some types of cancer, such as leukaemia.[26] Since several decades ago, a wide number of regenerative medicine therapies, including orthopaedics and wound healing applications, have received the Food and Drug Administration (FDA) approval and they are now used in clinical settings.[27]

The field of regenerative medicine includes numerous strategies, including gene therapy, cell therapy and tissue engineering. The innate body repair mechanisms can be exploited to promote endogenous regeneration; indeed, the main objective of regenerative medicine is to refine the methods of using the elements necessary to trigger *in vivo* self-regeneration.[28]

The term "tissue engineering", defined in 1988 by members of the National Science Foundation (NSF), indicates a multidisciplinary field that applies the principles of biology and engineering, for the development of biological substitutes that restore, maintain or improve the tissue functionality.[29]

The similarity between these two fields has allowed their merger into a single field of “tissue engineering and regenerative medicine (TERM)”. TERM makes use of cells (and/or their products), supports and molecular factors to develop bioactive tissue

substitutes. The cells are made to interact with three-dimensional biocompatible matrices, consisting of biomaterials, whose chemical, mechanical and physical characteristics are suitably modulated. The construct thus formed is transplanted into the target organ, where the cells can establish cell-cell and cell-matrix contacts by receiving precise positional information.

The basic elements on which tissue engineering is based are therefore: i) cells (differentiated, progenitor and stem cells), ii) bioactive molecules (responsible for the establishment of the correct tissue microenvironment), iii) Three-dimensional supports (or matrices or scaffolds).[30]

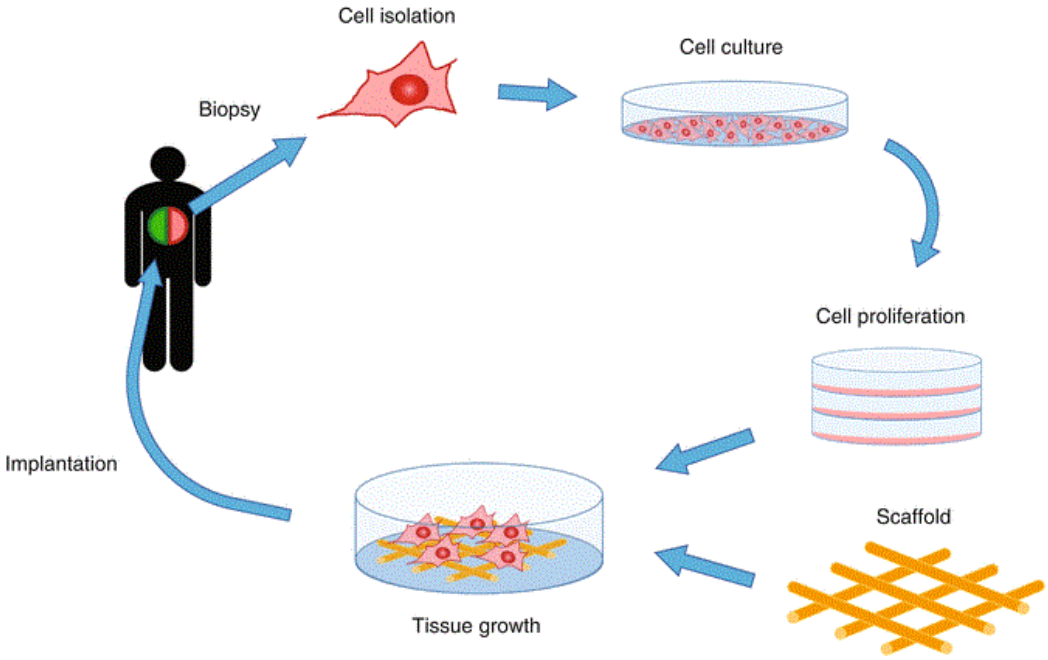

Figure 2 Tissue engineering approaches with a combination of cells, biofactors and scaffolds. Patients derived stem cells, expanded *ex vivo*, are subsequently mixed with morphogens and placed in a 3D scaffold to initiate differentiation. The engineered scaffold will either undergo a period of *ex vivo* pre-implantation differentiation culture or directly implanted *in vivo* for tissue regeneration.[31]

Stem Cell therapy: an overview

Stem-cell therapy is the use of stem cells to treat or prevent a disease or clinical condition. Bone marrow transplant is the most widely used stem-cell therapy, but some therapies derived from umbilical cord blood are also in use. Research is underway to develop various sources for stem cells, as well as to apply stem-cell treatments for neurodegenerative diseases and conditions such as diabetes and heart disease, among others.[32]

The two fundamental historical facts that made cell therapy formulated and feasible were the discovery of pluripotent stem cells of embryonic origin, awarded the Nobel Prize for Medicine in 2007 to Mario R. Capecchi, Martin J. Evans, and Oliver Smithies, and the identification of staminal niches, or tissue regions that host the cell population responsible for repairing the tissue in which they reside.[33]

When we talk about stem cells, it is essential to define several stem criteria: i) *Self-renewal* capacity (the cell must be able to give rise to a more or less defined number of mitotic divisions with the aim of self-maintenance of the healthy and renewed stem population), ii) *plasticity* (the cells derived from the mitotic division must give rise to differentiation in at least one other cell type besides the stem cell) iii) A stem cell population must be able to repopulate the tissue of origin if transplanted to a damaged recipient site, iv) stem cells placed *in vivo* in the absence of tissue damage must be able to contribute to the supply of a differentiating progeny.

The self-renewal is at the basis of the homeostasis of a tissue and it is fundamental for the proper maintenance of the organ over time, as it allows to have a cellular pool capable of providing new cells, which replace the previous ones that have undergone physiological aging. From a mitotic stem cell, it is possible to obtain two identical stem cells (symmetric division), or two identical cells aimed at a differentiation pathway or a stem cell identical to the mother cell and one that will undergo a differentiation process (asymmetric division). The latter system of division therefore seems to be the main mechanism responsible for maintaining a stem cell pool, by generating a poorly proliferating daughter stem cell and a daughter cell in differentiation, able to give new mature terminally differentiated cells, which will compose the tissue.[34]

The second fundamental property of stem cells mentioned above, plasticity, is classically defined as the ability of stem cells to differentiate to cell types both typical of the stem-source district or strangers. In recent years, the possibility that stem cells could generate cell types belonging to tissues different from the one of origin opened new questions and perspectives.[35] The research focused on those cells that have shown a greater susceptibility towards non-classical differentiation and therefore a higher plasticity potential. They are Haematopoietic Stem Cells (HSCs), Neural Stem Cells (NSCs) and Mesenchymal Stem Cells (MSCs). Recently, experiments aimed to obtain alternative cell types are scrupulously evaluated, trying to avoid artefacts, possibly driven by too strong experimental conditions, not *in vivo*-reproducible.

Stem cell is therefore an undifferentiated cell, which can self-renew to replicate itself as well as give rise to the specialized cells under appropriate conditions.[36] Therefore, stem cells have the ability to self-replicate for indefinite periods, perhaps throughout the entire life of the organism. Unlike the large majority of cells of the body that are committed to a specific function, Stem cells are uncommitted and remain as such, until they receive a signal to generate specialized cells.[36]

Stem cells can be classified by the degree of potential differentiation in: i) totipotent (cells that can give rise to an entire organism, including extra-embryonic annexes. A fertilized egg is the only totipotent stem cell), ii) pluripotent (cells with the ability to differentiate into all the three germ layers, endoderm, mesoderm and ectoderm. Examples of pluripotent stem cell are embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst-stage of an embryo, and induced pluripotent stem cells (iPSCs)[37] obtained from differentiated adult cells transfected with at least four key genes, Oct3/4, c-Myc, Sox-2 and Kfl-4, according to a procedure developed by Takahashi and Yamanaka), iii) multipotent (they can differentiate only in some types of cells. Adult stem cells are multipotent stem cells able to differentiate into all the cell types of the tissue), iv) unipotent (they can produce only one cell type).

Adult Stem Cells (ASCs)

Adult stem cells (ASCs) are undifferentiated cells that are found in differentiated adult tissues. During the past decade, ASCs have been found in tissues that were not previously thought to harbour them, such as the central nervous system (CNS). More recently, it has been reported that ASCs from one tissue appear to be capable of developing into cell types that are characteristic of other tissues. Thus, the new concept of ASCs developmental plasticity has emerged.[38] The primary function of stem cells is to maintain homeostasis, and, with limitations, to replace cells dying because of injury or disease. Generally, ASCs are maintained within uninjured tissues in a quiescent and undifferentiated state.[39] Quiescent ASCs are activated upon tissue injury via soluble and mechanical signals emanating from the site of injury, leading to the production of transit amplifying progenitors that in turn differentiate into functional mature cells capable of tissue regeneration.[39,40] A small population of amplifying ASCs exits the cell cycle and returns quiescent to maintain a reserve of ASCs that can respond to future demands.[41] From an evolutionary perspective, quiescence may help to ensure a steady state number of ASCs available for tissue regeneration, and act as a protective mechanism against genotoxic stresses.[42,43]

ASCs have been identified in many animal models and human tissues. The list of adult tissues reported to contain stem cells is growing and includes bone marrow (BM), peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, pancreas, heart, and the CNS. The most abundant information about human ASCs comes from studies on the BM and blood. There are two major types of stem cells found in the BM: hematopoietic stem cells (HSCs) which generate blood cells, and mesenchymal stem cells (MSCs) that support haematopoiesis and can differentiate into multiple tissues of the three germinal layers.[44]

HSCs are the cells that give rise to all the blood cells and derive from the mesoderm. HSC research began in the aftermath of the nuking on Hiroshima and Nagasaki. Investigations of irradiated mice demonstrated that they could be rescued from haematopoietic insufficiency by injection of cell suspensions extracted from haematopoietic organs.[45] In the 1950s, it was demonstrated that the bone marrow cells were able to restore the blood cell system.[46,47] Till and McCulloch in the 1960s quantitatively assessed the sensitivity to radiation of bone marrow cells using a genetic labelling based on pre-irradiation at low dose, inducing non-lethal chromosomal breaks. In this way they observed the presence of multipotent stem cells in the bone marrow, with a clonogenic and self-renewing capacity, and able to generate cellular populations of the blood.[48,49] The applications for this cell type include both autologous and allogeneic transplantation for neoplastic hematological pathologies (such as multiple myeloma,[50] lymphomas and some types of leukemia[51]), solid tumors (such as osteosarcoma[51]), congenital dysmetabolic diseases (such as Crohn's disease[52–54]), immune deficiencies (such as systemic Lupus erythematosus[55,56]) and for the reconstitution of haematopoietic and lymphoid populations after myeloablative treatments.

Mesenchymal stem cells (MSCs) originate from the mesodermal leaflet. With the progress of preclinical studies, MSCs have been shown to be effective in the treatment of many diseases, including immune diseases.[57] These cells, indeed, have shown both the ability to heal lesions and immunomodulatory activity.[58] The first was proved by the good efficacy of application in clinical and preclinical studies for: osteonecrosis,[59] myocardial

infarction,[60–62] liver failure,[63,64] renal failure following ischemic injury,[65,66] wound healing through retrieval of macrophages and endothelial cells,[67] infantile osteogenesis imperfecta,[68] osteoarthritis[69–71] and pulmonary tuberculosis (in cases of severe cross-resistance to drugs).[72]

The importance of immunomodulatory activity, which has emerged in recent years, has led to the formulation of new potential uses. Some therapeutic applications are now found in early phase II trials, including treatments ranging from type-1 diabetes[73] to Crohn's disease[74].

The mechanisms responsible for the properties of these cells are not fully understood but are based on the ability of MSCs to modulate classes of immune cells.[67,75,76] Homing, potential differentiation, production of trophic factors and immunomodulators are the elements at the centre of this research field.

Stem cells in clinical practice: The Good Manufacturing Practice (GMP)

Stem cells are used in regenerative medicine for their self-renewal and plasticity features, i.e. the ability to proliferate without loss of stem potential and to differentiate into specialized cell types. In physiological conditions, stem cells are responsible for normal cell turnover, aimed at the renewal of cell populations residing in their own tissue.

The knowledge of the signals responsible for maintaining the undifferentiated state or, on the contrary, inducing differentiation is fundamental in order to reproduce *in vitro* the essential conditions for the maintenance and expansion of these types of cells. Stem cell behaviour is controlled by the interactions between soluble factors, paracrine signals and molecules of the extracellular matrix that collectively characterize the endogenous niche of the tissue where the stem pool resides.[77,78]

Understanding all these different regulatory systems is therefore the basic condition for expanding and appropriately controlling stem cell differentiation outside the body, both for basic research and for translational studies.

The process for using stem cells in the clinic begins with basic research, to identify, isolate, expand in culture, characterize and differentiate the population of stem cells of interest. Preclinical studies following several phases follow: i) the functional test, which uses animal experimentation to test the regenerative abilities of the cells of interest, ii) the determination of the mechanism of action underlying the treatment and iii) the development of methods for large-scale expansion and cell preservation for clinical trials. After the preclinical tests, the treatment is then submitted to clinical studies evaluating the safety, the appropriate route of administration in the patient population and the effectiveness of the treatment, under the guidelines of Good Manufacturing Practices (GMPs).[79,80] GMPs are a guarantee of quality and sterility of the cellular lot, but also of safety and functionality of cell therapy. GMPs cover all aspects of the manufacturing process: storage, transport, qualified and trained production and quality control personnel, adequate laboratory facilities, approved written procedures and instructions, traceability of the product. The guiding principle of GMPs is that the product has been made every time by the same procedures under the same conditions. Every step is carried out following

approved procedures, written as Standard Operating Procedures (SOPs), describing all the tasks performed in the manufacturing process.[81]

In this context, the problems concerning the use of Foetal Bovine Serum (FBS) as a supplement in culture media have emerged.[79,80] Indeed, although FBS-based culture media are conventionally applied, bovine serum constitutes a source of xenogeneic antigens and a risk of contamination by zoonoses, viruses and prions.[82] In addition, important ethical issues emerged, deriving from the method of collecting whey (because of suffering for the foetus from which it is extracted) and from the world demand constantly increasing (about 700,000 liters per year).[83] For these reasons, the research for an alternative supplement to the FBS is taking great interest in the scientific community. The aim is to find a serum-free *in vitro* culture and expansion system that contains all the components: amino acids, hormones, lipids, vitamins, buffering substances and growth factors.[84] Furthermore, all components used must be animal-free. To replace FBS, numerous studies now refer to “humanized” culture conditions. “Humanized” supplements include human serum (HS) or autologous plasma,[85] or platelet derivatives, such as platelet-rich plasma (PRP) and the platelet lysate (PL).[86]

The first cell therapy products were aimed at the regeneration of portions of coating epithelium irreversibly compromised by serious burns.[87] In particular, the stem cells of the epidermis are taken and are induced in vitro to form flaps of skin that can be subsequently applied on the patient, allowing an almost total restitution to the full functionality of the tissue.[88] A great work was done by the group of De Luca and could constitute a definitive resolution for people affected by epidermolysis bullosa (1,500 in Italy and more than 500,000 in the world). The proposed technique involves the treatment of autologous cells in vitro and manipulation of these with gene therapy aimed at replacing the damaged genes responsible for the pathology phenotype.[89]

Another important application is the use of limbal stem cells, which under normal conditions are responsible for maintaining cellular populations of the cornea. Following burns, radiation or infections it is possible to use this cell type (with autologous modality) to regenerate the corneal epithelium and completely compensate the opacification.[90]

Today the goal of new cell therapies is to formulate targeted treatments that take advantage of the specific characteristics of each cell type. The use of stem cells can play a fundamental role also in the treatment of pathologies currently lacking a definitive and real cure, such as Parkinson's and type-2 diabetes.[91]

A new point of view on stem cells: Extracellular Vesicles

Although it has been demonstrated in recent years that most organ systems of the body have a resident pool of somatic, tissue-specific stem cells, in many cases of traumatic injury or disease, the quantity and potency of endogenous stem cell populations are insufficient to regenerate compromised tissues. As such, research has focused on exogenous or non-tissue-specific stem and progenitor cell sources for tissue repair and regeneration. Initially, efforts to use stem cells for this purpose centered on the directed differentiation of these cells to the intended cell phenotype, and functional improvements in several tissues were noted with cell transplantation and attributed to stem or progenitor

cell differentiation. More recently, however, it has become apparent that many of the functional improvements attributed to stem cells may be due to paracrine actions in the host tissue rather than cell differentiation and repopulation. A resultant shift in research has seen the emergence of studies aiming to elucidate the paracrine mechanisms underlying tissue repair and regeneration with stem or progenitor cell transplantation.[92]

Stem cells can communicate with nearby and distant cells through soluble factors and direct cell-cell contact, as well as via detached extracellular vesicles (EVs). These vesicles possess properties of freely diffusing factors and the extensive membrane and cytoplasmic organization of cells. They range from approximately 30nm to 3,000nm in diameter and have a distinct biomolecular composition and, therefore, functions that depend on EV subtype, cell source, and conditions.[93]

EVs were initially considered cellular debris or a way to excrete unneeded or toxic products from cells, but their ancient evolutionary origins and their conserved mechanisms of generation indicate that EVs perform essential physiological roles in cell-cell communication.[94,95]

EVs are membrane vesicles containing cytosol from the secreting cells enclosed in a lipid bilayer.[96] To date, many subgroups of EVs have been defined. Although specific characteristics have been proposed for each EV subpopulation, there is currently no specific identification of the markers of each subgroup that is widely accepted in order to distinguish these populations. Added to this is the lack of standardized methods of isolation and characterization. Because of the difficulties in the methods of isolation, the multidisciplinary nature of the research field, and the different ways of classification, there is currently no consensus on the nomenclature to be used to identify the various vesicle populations. Currently, three main subpopulations are generally considered: i) exosomes, ii) microvesicles, iii) apoptotic bodies.

The most important criteria for the classification of EV are the dimensions, density, morphology, lipid and protein composition and the subcellular origin.

Microvesicles have a diameter between 100 nm and 1.0 μm[97] and they are formed as a result of the budding of the plasma membrane. Apoptotic bodies are vesicles released by apoptotic cells and they have a diameter between 500 nm and 2000 nm. Exosomes are “cup-shaped” vesicles with a diameter of 30-100 nm, they originate from the fusion of an endosomal compartment, the multivesicular body (MVB), with the plasma membrane, that leads to the release of the vesicles presents in the MVB.

Molecular composition of EVs

As a consequence of their origin, exosomes contain endosome-associated proteins (e.g., Rab GTPase, SNAREs, Annexins, and flotillin), some of which are involved in MVB biogenesis (e.g., Alix and Tsg101).[98]

Membrane proteins often are also enriched on EVs, such as tetraspanins, a family of >30 proteins that are composed of four transmembrane domains.[99] Tetraspanins such as CD63, CD81, CD82, CD53, and CD37 were first identified in B cell exosomes.[100] Other studies confirmed that tetraspanins are abundant in EVs from other sources.[101] EVs are also enriched in proteins that associate with lipid rafts, including glycosylphosphatidylinositol-anchored proteins and flotillin.[102,103] In comparison to the plasma membrane, exosomes from a variety of cells[102,104–106] are highly enriched in cholesterol,

sphingomyelin, and hexosylceramides at the expense of phosphatidylcholine and phosphatidylethanolamine.

A major breakthrough was the demonstration that the cargo of EVs include both mRNA and miRNA and that EV-associated mRNAs could be translated into proteins by target cells.[107,108] Recently, analysis of RNA from EVs by unbiased deep sequencing approaches demonstrated that, in addition to mRNA and miRNA, EVs also contain a large variety of other small noncoding RNA species, including RNA transcripts overlapping with protein coding regions, repeat sequences, structural RNAs, tRNA fragments, vault RNA, Y RNA, and small interfering RNAs.[107,108]

Biogenesis of EVs and cargo selection

Exosomes and microvesicles have different mechanism of biogenesis: exosomes are generated within the endosomal system as IntraLuminal Vesicles (ILVs) and secreted during the fusion of MVBs with the cell surface, whereas microvesicles originate by an outward budding at the plasma membrane.

Exosomes generate in an early endosome: cargo is segregated at the delimiting membrane of the endosome and the endosomal membrane buds inwards forming vesicles, called ILV. The endosomal compartment is now called Multi-Vesicular Body (MVB). The molecular machineries involved in the biogenesis of MVBs consist of conserved proteins assembled into four multiprotein complexes: endosomal sorting complex responsible for transport (ESCRT)-0, -I, -II, and -III, which associate with accessory proteins (e.g., Alix and VPS4). The ESCRT-0, -I, and -II complexes recognize and sequester ubiquitinated membrane proteins at the endosomal delimiting membrane, whereas the ESCRT-III complex is responsible for membrane budding.[107,108]

Exosomes can also be formed in an ESCRT-independent manner, which was revealed by studies showing that MVBs are still formed upon depletion of components of the four ESCRT complexes.[109] Exosome biogenesis and secretion do not require ESCRT function but are dependent on sphingomyelinase, an enzyme that produces ceramide.[110] Ceramide may then allow the generation of membrane subdomains,[111] which impose a spontaneous negative curvature on the membranes. In addition, proteins of the tetraspanin family have been shown to regulate ESCRT-independent endosomal sorting.[112,113] Mechanistically, these proteins form clusters and dynamic membrane platforms with other tetraspanins and with different transmembrane and cytosolic proteins[114] probably acting in the formation of the microdomains that will bud. Tetraspanins also regulate the intracellular routing of cargoes towards MVBs.[115]

Thus, it seems that both ESCRT-dependent and ESCRT-independent mechanisms operate in exosome biogenesis, and their contributions may vary depending on the cargoes, which recruit them, and the cell type.

However, additional mechanisms contribute to the targeting of cargoes to exosomes. For example, the sequestration of cytosolic proteins into ILVs can result from co-sorting with other proteins, such as the chaperones heat shock 70kDa protein (HSP70) and heat shock cognate 71kDa protein (HSC70), which are found in exosomes derived from most cell types.[112,113]

Apart from proteins, extracellular vesicles also carry nucleic acids, including RNAs (mRNAs and non-coding RNAs, including miRNAs) and DNA sequences.[116,117]

Interestingly, miRNAs have been shown to be differentially sorted to exosomes depending on their sequence (presence of specific motifs),[118] which indicates that incorporation of nucleic acids into exosomes is regulated.

The biogenesis of microvesicles, instead, requires several molecular rearrangements within the plasma membrane, including changes in lipid components and protein composition, and in Ca^{2+} levels.[119] Ca^{2+}-dependent enzymatic machineries including aminophospholipid translocases (flippases and floppases), scramblases and rearrangements in the asymmetry of membrane phospholipids (exposition of phosphatidylserine from the inner leaflet to the cell surface), which causes physical bending of the membrane and restructuring of the underlying actin cytoskeleton, which favour membrane budding and formation of microvesicles.[120,121] In addition to lipids, cytoskeletal elements and their regulators are certainly required for microvesicle biogenesis. The activity of the RHO family of small GTPases and of the RHO-associated protein kinase (ROCK), which are important regulators of actin dynamics, induces microvesicle biogenesis in different populations of tumour cells.[122]

It is still unclear how nucleic acids, which are generally found in microvesicles, are targeted to the cell surface. One possible mechanism revealed from studies of cancer cells suggests the involvement of conserved zipcode RNA sequence motifs in the 3'-untranslated regions in mRNA targeting into microvesicles,[123] but the details of this process remain to be discovered.

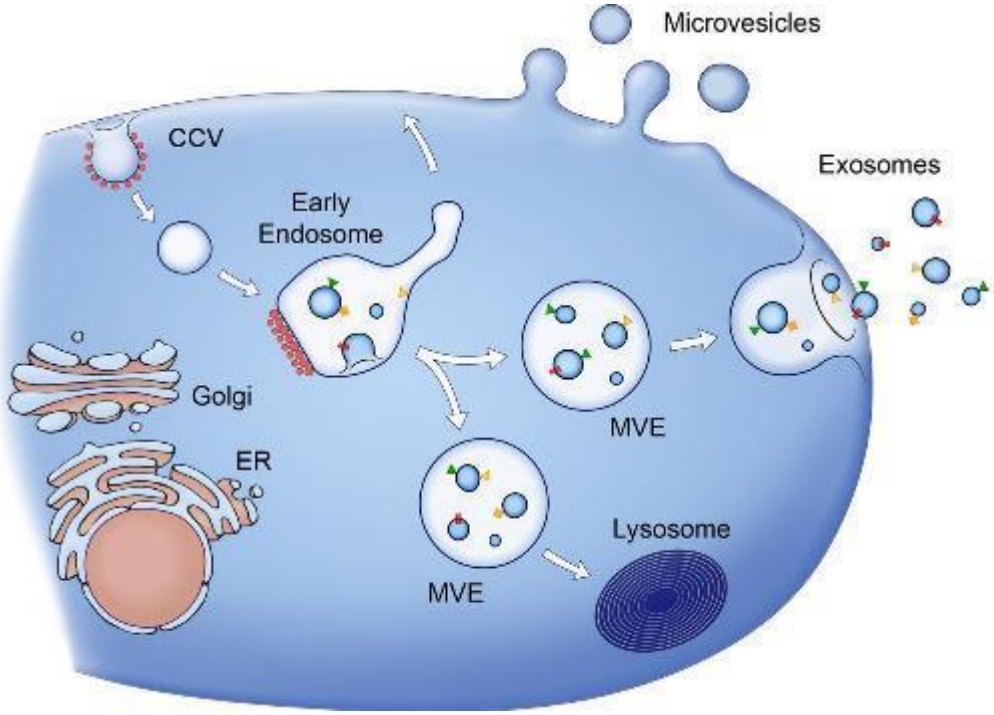

Figure 3 Release of MVs and exosomes. MVs bud directly from the plasma membrane, whereas exosomes are represented by small vesicles of different sizes that are formed as the ILV by budding into early endosomes and MVEs and are released by fusion of MVEs with the plasma membrane. Other MVEs fuse with lysosomes. The point of divergence between these types of MVEs is drawn at early endosomes, but the existence of distinct early endosomes feeding into these two pathways cannot be excluded. Red spots symbolize clathrin associated with vesicles at the plasma membrane (clathrin-coated vesicles [CCV]) or bilayered clathrin coats at endosomes. Membrane-associated and transmembrane proteins on vesicles are represented as triangles and rectangles, respectively. Arrows represent proposed directions of protein and lipid transport between organelles and between MVEs and the plasma membrane for exosome secretion.[96]

Release of EVs

The machineries involved in scission/release of MVs from the plasma membrane and those implicated in the mobilization of secretory MVBs to the cell periphery, their docking, and fusion with the cell surface are still at an early stage of comprehension. These processes require the cytoskeleton (actin and microtubules), associated molecular motors (kinesins and myosins), molecular switches (small GTPases), and the fusion machinery (SNAREs and tethering factors).[124]

The final step of exosome secretion requires, instead, the fusion of MVBs with the plasma membrane to release ILVs as exosomes, a process probably mediated by SNARE proteins and synaptotagmin family members.[125] A SNARE complex known to be involved in the exocytosis of conventional lysosomes consists of VAMP7 on the lysosomes, syntaxin 7 on the plasma membrane and the lysosomal regulatory protein synaptotagmin 7.[126] The process of exosome secretion has been demonstrated in several cell types to be regulated by Ca^{2+}, which may have a role in the activation of the SNARE complexes.

Interactions of EVs with recipient cells

Functions of EVs in physiological and pathological processes depend on the ability of EVs to interact with recipient cells to deliver their contents of proteins, lipids, and RNAs. Specificity of target cell binding is illustrated by the finding that isolated B cell exosomes selectively bind follicular dendritic cells in lymphoid follicles.[127] Similarly, EVs released by a human intestinal epithelial cell line interacted preferentially with dendritic cells rather than with B or T lymphocytes.[128] The cellular and molecular basis for EV targeting is still undetermined, but several target cell–dependent and –conditional aspects are beginning to emerge. Target cell specificity for binding of exosomes (or other EVs) is likely to be determined by adhesion molecules, such as integrins, that are present in EVs. Differences in exosomal tetraspanin complexes also appear to influence target cell selection *in vitro* and *in vivo*,[129] possibly by modulating the functions of associated proteins, including adhesion molecules such as integrins.[99]

After binding to recipient cells, EVs may remain stably associated with the plasma membrane or dissociate, directly fuse with the plasma membrane, or be internalized through distinct endocytic pathways. When endocytosed, EVs may subsequently fuse with the endosomal delimiting membrane or be targeted to lysosomes for degradation.[96]

Exosomes from MSCs

MSC exosomes, like exosomes in general, carry a complex cargo that includes nucleic acids, proteins and lipids. A key requisite of MSC exosomes as a mediator of MSCs' role as stromal support cells would be their capacity to support maintenance of homeostasis within the tissue and yet be responsive to the external environment. This role is particularly important when tissue microenvironment is altered by disease or injury leading to a disruption in tissue homeostasis which in turn compromises normal tissue function.[130]

Tissue homeostasis is influenced by the immune system. While activation of the immune system is critical in the elimination of pathogens and removal of dead or injured cells during tissue injury or disease, its attenuation upon resolution of the injury or disease is equally critical in the well-being of the tissue. Prolonged immune activation beyond the resolution of injury or disease will result in excessive tissue damage or progress to an immune dysfunction disease. Therefore, immune homeostasis is a critical component of tissue homeostasis. MSCs have been widely documented to exert suppressive and regulatory immunomodulatory effects on both adaptive and innate immune cells in an autologous and allogeneic manner.[131]

To rationalize the effects of MSC efficacy against immune diseases, it was postulated that MSCs exert their efficacy through paracrine secretion of trophic mediators.[132,133] Several secreted factors have been proposed, namely interferon-γ (IFN-γ)[132,133] and indoleamine2,3-dioxygenase (IDO),[134] transforming growth factor (TGF)-β1, hepatocyte growth factor (HGF), IL-6, prostaglandin E2 (PGE2).[132,133]

In recent years, exosomes have been increasingly reported as the principal therapeutic agent in MSC secretion that underpins the regenerative and immunomodulatory capabilities of MSCs in tissue repair.[135]

MSC extracellular products: the next generation therapeutics for osteoarthritis

Osteoarthritis (OA) is a type of joint disease that results from breakdown of joint cartilage and underlying bone. The most common symptoms are joint pain and stiffness.[136]

Causes include previous joint injury, abnormal joint or limb development, and inherited factors. Risk is greater in those who are overweight, have one leg of a different length, and have jobs that result in high levels of joint stress. Osteoarthritis is believed to be caused by mechanical stress on the joint and low-grade inflammatory processes.[137]

Treatment includes exercise, efforts to decrease joint stress, support groups, and pain medications.

MSC therapy to treat OA is presently under clinical testing. Based on the large number of clinical trials using MSCs for a wide range of disease indications, it is evident that MSC-based therapies are generally safe. Although MSC has not been approved for OA, the widespread use of MSCs to treat OA in pets and other animals[138,139] provides a compelling rationale to use MSC therapy to treat OA in human.

The discovery that MSC secretome and exosomes mediate MSC therapeutic activity could radically transform MSC therapy by eliminating many difficult issues associated with using living cells as therapeutics. The use of viable cells carries inherent risks such as occlusion in microvasculature leading pulmonary embolism and death,[140] transformation of transplanted cells into inappropriate cell types or cancer, immune rejection, proarrhythmic side effects,[138,139] ossifications and/or calcifications.[141]

A perennial risk of cell-based treatment is that it is considered a permanent treatment as the transplanted cells cannot be removed in event of adverse activity or upon disease resolution. The manufacture of cell-based therapeutics is also challenging as cell viability, potency and transformation have to be monitored and maintained throughout the

manufacturing process, storage, and delivery to patient. Replacing cells with exosomes eliminates many of these challenges such as occlusion of blood vessels and generation of inappropriate cell types as exosomes are not viable and small. Unlike cell therapy, exosome treatment is not a permanent treatment and can be easily suspended when there are adverse effects or when the disease resolves. Exosome production is more amenable to process optimization. For example, the cell source for exosome production could be selected or genetically manipulated to generate a high exosome-yielding clonal cell line with infinite expansion potential to ensure reproducible cost-effective production of exosomes. All things considered, MSC exosome therapy for OA could potentially be a safer, cheaper and a more effective treatment modality than cell-based MSC therapy.

Aim of the thesis

This thesis work is part of a European project that aims to build a robotic and automated system for the isolation, expansion and production of mesenchymal stem cells, according to the rules of GMPs. The bone marrow is taken directly from the patient's pelvis and transferred into a sterile chamber, inside which a robotic arm performs all manipulations, to then provide the cellular product ready for therapy, in a closed system. This, combined with the use of serum-free culture media, could allow to obtain cellular products with reduced risk of contamination by the operator, thus ultimately constituting a material that can be spent in the clinic both for quantities of final product and for techniques of production used.

To date, limitations such as poor cell production, high production costs, incomplete standardization of production processes and the heterogeneity of isolated mesenchymal stem populations are the major obstacle to the clinical translation of cell therapy methods from functional studies.

Cell therapy aims to solve the global problem of chronic diseases, especially those related to advanced age (osteoarthritis, diabetes, cardiovascular disease and osteoporosis), using heterologous or autologous stem cells. Although up to now, their potential has hardly been implemented in the usual clinical application, the MSCs are considered a fundamental resource for cell therapy. These cells are in fact easy to isolate, with high capacity for *ex vivo* expansion and with proven immunomodulatory and differentiating activity.

In recent years, the study of their secretome and the idea that it is mainly responsible for the beneficial effects attributed to these cells *in vivo* has also assumed relevance in clinical perspective.

The aims of this work were:

1. characterize the BMSCs grown in the GMP-facility, in FBS and serum-free medium, compared with BMSCs grown in plastic adherence;
2. characterize the secretome and the extracellular vesicles extracted from the BMSCs cultured in FBS and serum-free conditions;
3. evaluate the immunomodulatory properties of FBS- and SF-hBMSC-CM/EVs on an *in vitro* model of osteoarthritis.

Chapter 1: Biological validation of human mesenchymal stem cells isolated in an automated robotic factory

1.1 Introduction

The presence of non-haematopoietic stromal cells in the bone marrow (BM) able to migrate through the blood steam to injury sites where contribute to tissue regeneration was suggested by the German pathologist Cohnheim in 1867. He proved that these fibroblast-like cells were involved in collagen deposition during the normal processes of wound healing. This role has been widely confirmed many years later.[142–144]

In the 1970s, Friedenstein and colleagues demonstrated the existence of non-hematopoietic stem cells within the bone marrow.[145] After placing whole bone marrow in cell culture dishes, they observed that it was possible to select a population of non-hematopoietic cells thanks to their ability to adhere to the plastic. They also reported that the adherent cells were similar to fibroblasts, spindle-shaped and organized in *foci* of few cells, that began to proliferate rapidly.[146] Since then, these cells were referred to as colony-forming unit fibroblasts (CFU-F). In the following decades, many groups focused their research on this new promising field.[147] The studies unveiled that these cells were multipotent, capable of self-maintenance and able to differentiate in chondrocytes, osteoblasts and adipocytes.[143,148–155] These cells were named by Caplan as mesenchymal stem cells (MSCs) and this term immediately gained wide popularity.[156]

Typically, human MSCs are isolated by plastic adhesion, after plating the whole bone marrow in a cell culture dish. Red blood and other hematopoietic cells are washed away after few days, leaving adherent, fibroblastic-like cells. Although BM is still the main source of MSCs, these cells have also been identified in adipose tissue,[157] synovial membrane,[158] umbilical vein,[159] umbilical cord blood[160] and dental pulp.[161]

Due to the heterogeneity of the isolation methods and the increase in sources of supply, it became difficult to compare studies of different research groups making necessary to establish some guidelines. Thus, in 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed the following minimal criteria to define human MSCs: i) MSCs must be plastic-adherent; ii) phenotypically, MSCs must be positive for the surface markers CD73, CD90 and CD105, and negative for hematopoietic markers like CD45, CD34, CD14, CD19 and HLA-DR surface molecules; iii) they must be able to differentiate in chondrocytes, osteoblasts and adipocytes.[151]

Easy isolation and expansion protocols, good potential of differentiation, low immunogenicity and the fact that they do not require matching in transplantation have placed MSCs as one of the most promising candidates for a wide variety of therapeutic applications.[162–164] Furthermore, MSCs possess immunomodulatory properties, which

confer them the ability to suppress adaptive and innate immune responses through the secretion of anti-inflammatory cytokines such as IFN-γ, IL-1, or TNF-α.[165–167] In addition, they exert trophic effects mediated by numerous growth factors and cytokines.[168] However, the use of stem cells in clinical therapy is braked by several issues arising from the isolation methods and due also to the manipulation of the cells itself . One of the reasons is certainly their low frequency in the tissues of origin that allow to isolate only a very low quantity of cells.[155] Therefore, *in vitro* expansion is required to obtain enough cells to meet the clinical demand, consequently many efforts have been focused on optimizing and improving these *in vitro* methods.[169–171] Moreover, *ex vivo* expansion of MSCs leads to alterations in genome stability,[172,173] epigenetics[174,175] and functional abilities to differentiate into osteogenic cells.[176,177]

The main advantage of MSCs lies in the possibility of using them in both an autologous and an allogenic scenario. For autologous applications, given that less cells are needed, a scale-out approach can be followed. On the contrary, for allogeneic use, a scale-up approach needs to be applied, in which it is possible to produce a large number of cells in bioreactor systems and to create a bank for cell storage.[178] However, many allogeneic processes still require manual intervention that has a significant impact on cost and quality, and the development of reproducible and consistent bioprocesses is still a key challenge. In all cases, the main challenge concerns the manipulation, processing and production in aseptic and closed systems, which should be easy to use and ensure that products are safe and pure, as well as the standardization between different preparations that allows the development of reproducible, robust, and efficient culture processes.[179–181] In this scenario, in 2006 the European Medicines Agency (EMA) wrote the Good Manufacturing Practices (GMPs), a code of standards concerning the manufacture, processing, packing, release and holding of a product for clinical therapy.[81]

In addition to the handling and safety issues, there are other factors which can significantly impact the production of stem cell therapies, as the reagents and the medium employed.[182] The medium is crucial for efficacy and safety of MSC culture and should maintain the phenotype, genotypic stability and functions of MSCs during multiple passages.[181] Fetal bovine serum (FBS) is the most widely used supplement in almost all protocols. The use of animal derivatives for the handling of a clinical-intended product is highly chancy, due to prionic and viral contaminations and immune reactions of the patients.[183] Indeed, the proteins of bovine serum could be internalized by the cells and subsequently recognized as stranger antigens, stimulating immunogenicity in the organism.[184] Unfortunately, the thorough composition of the FBS is not known and there are often significant differences between lots, therefore it is difficult to identify which components are liable of triggering an immune reaction.

Theoretically, using human blood derivatives, the unwanted effects of the FBS constituents should be eliminated or at least reduced. The scientific community has proposed FBS alternatives such as human serum, platelet-rich plasma (PRP) and platelet lysate (hPL) from blood transfusion-secured sources.[185] These products have similar efficacy to bovine serum, as regards the isolation and expansion of human MSCs, and they allow to eliminate all the immunoreactivity issues derived from the bovine derivatives employment. Indeed, the proliferation is even higher than the one achieved by the classical culture conditions and the cell phenotype is almost the same.[186,187] The use of human serum, however, brings back the same problems as bovine serum regarding the

composition variability among the donors, which may negatively affect production efficiency. Furthermore, the functionality of MSCs obtained when changing the medium should be tested, given that the differentiation potential of MSCs could be affected using platelet lysate-supplemented medium. In addition, differences in immunological properties of MSCs produced using FBS or hPL have been reported.[167] In addition, platelet derivatives must also be produced in transfusion centers with GMP approval, with a consequent increase in costs.

For these reasons, it seems mandatory to look for alternative, xeno-free formulations, which allow a culture yield comparable to that one obtained with classical protocols but removing all the problems of contamination and variability, and that can be used in the clinical settings.

Ideally, a suitable substitute for FBS should present a low risk of contamination, low costs, easy availability and long shelf life.[188] Some studies reported the use of a tank bioreactor for the expansion of MSCs in xenogeneic-free conditions with human serum.[189,190] There are already commercial xeno-free media FDA-approved, which guarantee an effective growth of MSCs from adipose tissue and bone marrow grown in plastic microcarrier, as demonstrated in some studies.[191] Unfortunately, the xeno-free media currently used select only a small sub-population of MSCs, which is able to survive under serum deprivation and then began to proliferate in serum-containing medium. This sub-population represent a very early progenitor cells displaying longer telomeres .[192]

In this context, the AutoStem project was proposed. The research is funded by the European Commission Horizon 2020 and it involves ten different European academic and industrial partners. The ambitious goal of this study is to develop a closed and automated robotic pipeline for the scaling-up and GMP-ready production of MSCs, in an aseptic ‘donor-to-patient’ system, practically a ‘StromalCellFactory’.[193]

The study allowed the development of an automated pipeline for allogeneic stem cells: the bone marrow is taken directly from the donor's hip bone and transferred into a sterile chamber, where a robotic arm supports the isolation of the cells, which are kept in culture inside a bioreactor. Although multiple cell production systems are available,[194] two of the major cell culture platforms have been considered, a small stirred vessel bioreactor, with approximately 1 L capacity, and microcarrier systems. Microcarriers provide a significantly larger surface area per unit volume compared to other expansion platforms. The system also includes a steady process of cells amount, microenvironment and other parameters real-time monitoring. Thus, changes in pH, temperature and oxygen are felt by sensors to ensure efficient culture conditions. The development of this platform has appealing advantages, including obtaining substantial quantities of final product, the applicability in clinical therapy, in a short time and reduction of the risk of contaminations due to operator’s manipulation. In addition, this platform can develop bespoke, interlinked modular units which can be reconfigured and adapted to accommodate new processes or take advantage of emerging technologies to facilitate cost- and risk-based continual process improvements.

Another considerable innovation regards the culture medium used, which was a new generation, xeno-free medium, without animal derivatives.[195] This was a chemically defined xeno-free medium, contained known amount of Transforming Grow Factor β (TGF-β1), Platelet Derived Grow Factor (PDGF) and basic Fibroblast Grow Factor (FGF-b).

Bone marrow was obtained from the hip bone of healthy donors with the approval from the University College Hospital Galway Ethics Committee. The samples were transferred to the partners, and the cells were cultured *in vitro* using Standard Operating Procedures (SOPs) developed in a previous FP7 PurStem project.

The goal of our group from University of Genoa, was to compare the traditional plastic adherent isolation and culture of MSCs with the cells isolated and cultured with AutoStem's protocols, using a fully defined growth medium, without FBS. The Plastic Adherent hBMSCs (PA-hBMSCs) were characterized in terms of growth potential, surface phenotype, *in vitro* tri-lineage differentiation potential and *in vivo* osteogenic potential and compared to AutoStem cells. To evaluate the osteogenic potential *in vivo*, we used the MBCP$^{+®}$, a biomaterial already tested in our laboratory in a recent study by R. Pereira. MBCP$^{+®}$ is a scaffold based on calcium phosphate (CaP) with a hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) ratio of 20/80, with a good porosity that allows calcium ion releasing, sustaining the vascularization of the environment and the osteogenic differentiation of the cells.

1.2 Materials and Methods

1.2.1 Human Bone Marrow Stromal Cells (hBMSC) cultures in plastic adherence

Plastic Adherent human Bone Marrow Stromal Cells (PA-hBMSCs) were derived from hip bone marrow aspirates of healthy donors, after informed consent. The human samples were obtained from EU-AutoStem Consortium, after approval by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland and processed at National University of Ireland Galway (NUIG). Briefly, cell nucleated fraction of bone marrow aspirate was suspended in α-MEM-Glutamax medium (Gibco, Waltham, MA, USA), supplemented with: i) 10% Fetal Bovine Serum (FBS, Gibco), 1 ng/ml Fibroblast Growth Factor-2 (FGF-2, Peprotech, London, UK) and 100 U/ml of Penicillin/Streptomycin mixture (Euroclone) for standard culture conditions, ii) or with the AutoStem patented serum-free supplement and 100 U/ml of Penicillin/Streptomycin mixture. The cells were plated at a density of $1x10^6$ cells/cm^2. After at least 5 days, only PA-hBMSCs were attached on the plastic, while all the blood cells and other debris were in suspension. Thus, the medium was discarded, the cells were washed with Phosphate Buffer Saline 1X (PBS) to remove all the refuse and the dishes were refilled with fresh medium. When culture dishes were confluent, PA-hBMSC were detached with trypsin-EDTA (Euroclone) and cryopreserved for the delivery to our laboratory. After arrival, we thawed the cells and 2.5 x 10^5 cells were plated in 100 mm dishes (density of 3000 cells/cm^2) and grown in an incubator at 37°C with 5% CO_2. The medium was changed twice a week.

1.2.2 Analysis of proliferation and Colony Forming Unit-Fybroblasts (CFU-F) assay

To calculate the number of doublings (understood as number of cell replications) performed by cells at each passage in culture, we cultured PA-hBMSCs in α-MEM supplemented with 10% FBS or with serum-free supplement. At the confluence, the cells were detached with trypsin-EDTA, harvested and counted with a Neubauer counting chamber, then they were re-plated, always at the same density of about 3000 cells/cm^2. The base-2 logarithm of the ratio between n° of counted cells and n° of plated cells was the number of doublings performed by the cells in that passage. The cells were maintained in culture for 30 days and, at the end, the data was put on a graph and expressed as n° of cumulative doublings (i.e. every time point was the summation of all previous n° of doublings) vs. days of culture. This experiment was performed for all three primary cultures.

Parallel to normal cell expansion, a CFU-F test was also performed, to test the clonogenic potential. For this purpose, the cells were plated at a very low density (10 cells/cm^2). In this way, they spread on the plate surface as single cells, that form isolated colonies. After 2 weeks, the cells were fixed with 3.7% para-phormaldehide (PFA) for 10 minutes, washed with PBS 1X and stained with 1% methylene blue in borate buffer (pH 8.8) for 45 minutes. The colonies can be counted and observed under a microscope. The images were acquired with a scanner (Epson Scan) and then analyzed for the intensity of pixels and for the colony area with the ColonyCounter plugin of the Software ImageJ.

1.2.3 PA-hBMSC phenotypic characterization by flow cytometry

For phenotypic characterization by flow cytometry, cells cultured in FBS-containing medium and in serum-free medium were trypsinized with Tripsyn-EDTA, counted and divided into several fluorimetric tubes (100,000 cells/tube). Cells were washed with PBS 1X, centrifugated and then incubated with 1µl of specific antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD31-FITC, CD34-FITC, CD45-PE, CD73-FITC, CD105-PE, CD146-FITC, (Biosciences, San Diego, CA, USA), CD90-PE, CD166-PE, CD106-PE, HLA-ABC-PE, HLA-DR-FITC (BD Pharmingen San Diego, CA, USA). The appropriate IgG-isotype controls, conjugated with FITC or PE (Bioscience), were also used to stain the cells. The staining was run for 30 minutes a 4°C in the dark (to preserve the fluorochromes). After this time, the cells were washed with PBS 1X, centrifugated and then resuspended in simple medium or PBS 1X for the analysis. Samples were run on Cyan ADP cytofluorimeter (Beckman-Coulter, Brea, CA, USA). Data were analyzed using FlowJo software and expressed as Log fluorescence intensity versus number of cells. Analysis was performed on all three different primary cultures at early passages (P1).

1.2.4 PA-hBMSCs differentiation assays

PA-hBMSC in vitro adipogenic differentiation and staining protocol

The adipogenic differentiation potential was tested at early stages (P2) by plating the cells at a density of 20,000 cells/cm^2, in growth medium, i.e. α-MEM with 10% FBS or serum-free supplement. Cells were grown in an incubator at 37°C with 5% CO_2, until the confluence was reached. After that, the growth medium was replaced with differentiation medium, containing the specific factors. For adipogenic differentiation, the cells were stimulated for 3 days with an induction medium, represented by DMEM High Glucose with 1µM Dexamethasone, 10µg/ml Insulin, 200µM Indomethacin, 500µM 3-Isobutyl-1-Methyl-Xanthine (Sigma-Aldrich, St. Louis, MO, USA), 1% Penicillin/Streptomycin, 10% FBS, followed by one day of maintenance treatment (DMEM High Glucose, 10µg/ml Insulin, 1% Penicillin/Streptomycin, 10% FBS). Three total cycles of induction and maintenance were repeated, and the cells were monitored over time by microscope observation, to verify the formation of small lipidic drops, looking like dark spot inside the cells.

At the end the cells were fixed with calcium formolo for 20 minutes, washed with 60° isopropyl alcohol for 1 minute, and then stained with 0.5% Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) solution in 60° isopropyl alcohol. After a washing with 60° isopropyl alcohol, followed by a washing with distilled water, it was possible to observe the intracellular lipid drops dyed in red. The images were acquired with an Axiovert 200M microscope (Carl Zeiss) within a few minutes after staining because the dye tends to spread rapidly.

PA-hBMSC in vitro osteogenic differentiation and staining protocol

The osteogenic differentiation was performed at early stages (P2) by plating the cells in a 24 multi-well plate, at a density of 20,000 cells/cm^2, in growth medium. Cells were grown in an incubator at 37°C with 5% CO_2, until the confluence was reached. After that, the growth medium was replaced with differentiation medium, containing the specific factors and the medium was changed twice a week for 7, 14 and 21 days. The differentiation medium consisted of DMEM Low Glucose with the addition of 10% FBS, 100nM Dexamethasone, 10mM β-glycerophosphate, 100µM ascorbic acid-2P (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin. At the end of the treatment, the cells were fixed with 3.7% PFA for 15 minutes and stained with 2% Alizarin S red (Sigma-Aldrich, St. Louis, MO, USA) solution for 10 minutes at the dark (putting the plate in a drawer). Alizarin S is a red dye that binds to calcium deposits. Images were taken with Axiovert 200M.

Also, a quantification of Alizarin was performed by adding 200µl/well of 10% acetic acid and incubating the plate for 30 minutes on a shaker. The cells were subsequently scraped and the whole suspension was transferred in 1.5ml tubes. After vortexing for 30 seconds, the tubes were heated at 85°C for 10 minutes (taking care to seal them with parafilm, to avoid any type of diffusion of acetic acid vapors), incubated on ice for 5 minutes and finally centrifugated at 20,000g for 15 minutes. We transferred 200µl of the supernatants in new tubes and we neutralized the solution by adding about 30µl of 10% ammonium hydroxide (NH_4OH, pH must be between 4.1 and 4.5) (Sigma-Aldrich, St. Louis, MO, USA). We read the Optic Density (OD) of the samples at the spectrophotometer (BioSpectrometer® Eppendorf, Milan, Italy), at 405nm. The data were reported in a graph as OD of the treated (differentiated cells) – OD of the control (undifferentiated cells) vs. time.

PA-hBMSC in vitro chondrogenic differentiation: pellet cultures

For chondrogenic differentiation, cells grown in FBS-containing medium or serum-free medium were trypsinized and 250,000 cells were pelleted in a 15ml tube. Within 24h, cells should form a small pellet capable of differentiating into cartilage, if treated with Chondrogenic Medium (DMEM-HG, Dexamethasone 100nM, Ascorbic Acid-2P 50µg/ml, ITS supplement 1x, 1mM pyruvate sodium, TGF-β 10ng/ml). The medium was changed three times a week for 21 days. After that, the pellets were fixed with 3.7% PFA for 15 minutes, washed in PBS and preserved in 70% ethanol until the histological analysis with paraffin embedding.

1.2.5 Iodide propidium (PI) and a FITC-anti-annexin-V apoptosis detection

PA-hBMSCs cultured in FBS-containing medium or in serum-free medium were analyzed using flow cytometry, after two passages in culture. Double staining for FITC

Annexin-V binding and Propidium Iodide (PI) was performed to determine if cells were viable, early apoptotic or apoptotic using the Annexin-V apoptosis assay kit (BD Pharmingen San Diego, CA, USA), according to the manufacturer instructions.

1.2.6 Karyotype analysis

PA-hBMSCs cultures were tested at 25% confluence. Cells were incubated in Ham's F10 (EuroClone, Milan, Italy) with 0.1mg/mL colcemid (Sigma-Aldrich, St. Louis, MO, USA) for 3 h, which can collapse mitotic spindles and prevent the completion of mitosis, to synchronize the cells and to arrest them at metaphase. The medium was removed and a hypotonic solution (75mM KCl, 17mM Na-citrate) added. Cells were then fixed with a solution of ethanol : methanol : acetic acid (1:2:1) and dried at 25°C and 45% humidity. Metaphase spreads were analyzed after staining with quinacrine (Sigma-Aldrich, St. Louis, MO, USA) for karyotyping using Cytovision 3.93.3 Applied Imaging. Analysis was performed on three different primary cultures counting 20–25 metaphases for each sample.

1.2.7 Assays for neoplastic transformation in vitro and in vivo

To confirm that PA-hBMSCs did not possess malignant properties, *in vitro* colony assay formation and *in vivo* tumorigenesis analyses were performed. For *in vitro* testing, PA-hBMSC cultured in FBS-containing medium and in serum-free medium at P2 were plated at a density of 10,000 cells/35-mm Petri dish in methyl-cellulose media hematopoietic stem cell (HSC)-CFU basic (Miltenyi Biotech, Bergisch Gladbach, Germany). After 14 days of incubation, CFU-cells (CFU-C) were scored using an inverted microscope. The positive control used was the human breast cancer MDA-MB-231 cell line.

In vivo tumorigenesis was assessed with PA-hBMSCs at P2 expanded from two donors in the two different culture conditions. For each PA-hBMSCs preparation, the same dose of cell ($1x10^6$, resuspended in 50µl of physiological solution) was injected subcutaneously (s.c.) into 12 NOD/SCID mice for each condition. The mice were monitored up to 3 months.

1.2.8 In vivo neo-bone formation into ceramic scaffolds with PA-hBMSCs

To assess the *in vivo* osteogenic potential, we associated PA-hBMSCs to a biomaterial for bone formation, the MBCP$^{+®}$. This is a calcium phosphate ceramic scaffolds provided by Biomatlante SA, (Vigneux-de-Bretagne, France). This biomaterial is characterized by granules with a ratio of 20% HA and 80% β-TCP, a total porosity of 70% with a distribution of 25% of micropores (less than 10µm) and 75% of mesopores and macropores (over 10µm, mean size of macropores 300-600µm).

PA-hBMSCs cultured in both serum-free and FBS-containing medium at P2 were trypsinized and 2,000,000 of cells were pelleted in 15 ml tubes. The pellet was resuspended

in 20µl of fibrinogen and loaded onto the biomaterial granules (MBCP$^{+®}$). Then we added 10-15µl of thrombin which polymerizes the scaffold granules together with the cells inside. A construct (about 0.5cm Ø) is obtained that can be implanted subcutaneously in immunodeficient mice.

The ectopic bone formation assay was adopted. CD-1 mice (CD-1 nu/nu; Charles River, Wilmington, Massachusetts, Stati Uniti) were anesthetized with an intraperitoneal injection of ketamine/xylazine mixture, surgical sites were cleaned with Mercurochrome, and subcutaneous pockets for each implant were created in the animal back. Ceramic-hBMSCs constructs were implanted subcutaneously in the pockets and the wound was closed with chirurgical metallic agraphes (7,5 x 1,75mm, Michel). Groups of 6 animals were sacrificed after 2 months by CO_2 saturation and the implants harvested for further analysis. All animals were maintained as required by the Italian Ministry of Health in accordance with the standards of the Federation of European Laboratory Animal Science Associations (FELASA).

1.2.9 Histological techniques

In vivo implants are recovered following the sacrifice of the animals, fixed with PFA 3.7%, decalcified and then included in paraffin.

Decalcification is essential for histologically analyze samples of bone or cartilage, which would otherwise be hard to be included in paraffin. Decalcification were done with a 0.5M EDTA pH 8 solution, which sequester all the calcium, making the sample "softer".

Inclusion in paraffin involved a phase of sample dehydration with ethanol steps at increasing concentration (from 70% up to absolute EtOH), followed by a permeabilization phase in xylene and passages in liquid paraffin to growing temperatures (around 40, 50 and 60°C). At the end of the three phases, the sample was included in paraffin blocks. 4-5 µm serial sections were cut with microtome (Leica RM2165) and sections were stained with specific histological staining.

Hematoxylin & Eosin and Goldner thrichromic stainings

To verify bone formation within the implants, we performed a Hematoxylin & Eosin staining on the sections obtained from our samples. Hematoxylin stains in purple the negatively charged cellular elements such as nucleic acids, membrane proteins, nuclei. Eosin, on the other hand, colors in pink the positively charged components such as cellular proteins, mitochondrial proteins and collagen fibers, thus highlighting the cytoplasm and extracellular matrices.

The sections intended for staining must necessarily be deparaffined and re-hydrated following the descending scale of the alcohols: we started with xylol I, II, III for 7 minutes each one and subsequently we transferred the sections into absolute ethanol I and II, 95% ethanol I and II, ethanol 90% (5 minutes each one) and finally distilled water. After this step of re-hydration, we stained the slides with hematoxylin for 4 minutes (this time is quite variable based on the type of sample to be colored). The sections were washed in running water, then with basic water (distilled water with the addition of some drops of

NH_3), which determined a slight color change of the hematoxylin turning to blue. We stained the slides with eosin for 30 seconds, we washed them in distilled water, and we dehydrated quickly through the ascending scale of the alcohols (ethanol 95%, absolute ethanol, up to the xylols). Lastly, the slides were sealed with cover slides using Eukitt (Bio-Optica, Milan, Italy), an acrylic mounting medium for cytological and histological slides.

We stained the samples also with Goldner thrichromic staining, by using the Goldner thrichromic kit (Bio-Optica, Milan, Italy). We carried out it according to manufacturer's protocol.

Images were obtained with a phase-contrast Axiovert 200M microscope (Zeiss).

Vascular and bone histomorphometric assays

Calcium phosphate ceramics were fixed, decalcified and routinely embedded in paraffin as described above. To visualize and quantify vascular structures in the calcium phosphate ceramics, in the paraffin embedded samples 5µm sections were cut and stained with Mallory's trichromic (Bio-Optica, Milan, Italy) according to manufacturer's instructions. Five nonconsecutive sections per sample were observed in transmitted light. Fiji ImageJ software was used to calculate the number of vessels per ROI through the followed steps: (i) vessel perimeter was manually defined; (ii) vessels were counted; (iii) number of vessels per ROI determined.

To determine bone deposition, 5µm sections were H&E stained. Five nonconsecutive sections per sample were analyzed using Fiji ImageJ software to calculate bone matrix deposition area as follows: bone and scaffold areas were delimitated and the ratio between both areas converted to percentage of bone per Region of Interest (ROI).

Immunohistochemistry

Immunoperoxidase of human osteocalcin was performed using a polyclonal rabbit-anti-human OCN (1:100 in 10% Normal Goat Serum) (Abcam, Cambridge, UK). Immunobinding was detected with biotinylated secondary anti-rabbit (1:300 in 10% NGS) (Dako, Agilent, Santa Clara, USA) and streptavidin-peroxidase (1:500 in 10% NGS) (Jackson ImmunoResearch, Cambridge, UK). Peroxidase activity was visualized by 3-Amino-9-ethylcarbazole (AEC) chromogenic substrate (Sigma-Aldrich, St. Louis, MO, USA).

All images were acquired using a phase-contrast Axiovert 200M microscope (Zeiss).

1.2.10 hBMSCs isolation on carriers in a small stirred vessel bioreactor (i.e. spinner flasks)

Three different bone marrow-derived hBMSC donor lines were isolated from bone marrow aspirates using a bioreactor at University of Aston.

Small stirred vessel bioreactors (i.e. spinner flasks) were used in this study to evaluate the growth of hBMSCs on two different attachment substrates: on Xuri attachment substrates (i.e. waffle macrocarriers; GE Healthcare) and on polystyrene substrates (i.e. SoloHill Plastic microcarriers; Pall Life Sciences).

The spinner flasks were siliconized prior to start the experiments to ensure no cell attachment will occur on the glass vessel and the cells will only attach to the provided attachment substrates (i.e. microcarriers).

Microcarriers were weighed to provide a surface area of 500cm^2 per spinner flask in a total volume of 100ml. The microcarriers were sterilized by autoclaving and prior to cell inoculation, they were initially pre-conditioned in medium for at least 1 hour to allow the protein absorption for facilitating cell attachment. Cells were then inoculated at 6,000 cells/cm^2. The first medium exchange (50%) was performed at day 3 in culture and then every other day. Microcarriers were stirred continuously at the minimum stirring speed necessary to suspend all particles which was assessed to be 30rpm.

The Xuri macrocarriers were acquired from the manufacturer as sterile packs preweighed to provide a 500cm^2 surface area. One pack was used per spinner flask in a total volume of 100ml of medium. Macrocarriers were transferred to the spinner flasks and cells were inoculated at 4,000 cells/cm^2. Medium changes (100%) were performed every other day. A continuous spin/rest cycle of 1minute stirring at 60rpm, followed by a rest of 45 minutes, was performed in order to allow cells to attach to the provided substrates, but also to allow for a homogeneous environment and minimize concentration gradients in the system.

1.2.11 Analytical techniques used for culture monitoring in the spinner flasks

In the macrocarrier culture system, cell attachment and proliferation were monitored by phase contrast microscopy or nuclei staining with DAPI and visualization under a fluorescence microscope. Cell morphology was further assessed by fixing the cells, followed by staining with 1% Crystal Violet solution. Cell growth was assessed by cell counting after enzymatic dissociation. Briefly, waffles with cells could settle, medium was aspirated and replaced with a phosphate saline solution and two consecutive washes were performed. Enzymatic dissociation of the cells from the waffles was done by trypsinization for 10 minutes. Cell dissociation was confirmed by observation under the microscope.

In the microcarrier culture system, culture monitoring was done by taking 0.5ml samples and imaging under either a phase contrast inverted microscope or a fluorescent microscope after staining cells with a Live/Dead viability/cytotoxicity kit (Life Technologies, Thermo Fisher Scientific). For Live/Dead staining, cells-microcarriers samples were first washed once with PBS 1X and then incubated with the Live/Dead staining solution for 40 minutes at 37°C in a humidified incubator. The Live/Dead staining solution was prepared according to the manufacturer's instructions. Post-incubation, the Live/Dead staining solution was removed and the cells-microcarriers samples were resuspended in PBS 1X and visualized under a fluorescence microscope. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely

fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). The other component of the kit, ethidium homodimer (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells.

Additionally, every other day in culture, two independent samples of microcarrier-cell suspension were taken for cell counting on the Nucleocounter cell counter (Chemometec). First, the cells were lysed from the microcarriers and then stabilized and counted on the Nucleocounter following the manufacturer's protocol.

1.2.12 Statistical analysis

In vitro statistical analysis was performed using t-test. Error bars indicate standard deviation (S.D.). *In vivo* statistical evaluation was done by using the data from explants of n=6 animals for each experimental condition. The data were analyzed by t-test. Error bars indicate standard deviation. For all *in vitro* and *in vivo* figures, a p-value 0.05 was considered to be statistically significant. GraphPad software was used as work program.

1.3 Results

1.3.1 PA-hBMSCs grown in vitro in FBS-containing medium or in serum-free medium show different growth kinetics, morphology, and clonogenic potential

The isolation of hBMSCs from bone marrow was performed by our partner at National University of Ireland, Galway (NUIG). The plastic adherent hBMSCs (PA-hBMSCs) obtained from 3 different donors were isolated and cultured in presence of FBS or in serum-free medium. The cell populations obtained after one passage were sent by NUIG to our laboratory. hBMSCs proliferation studies and colony forming unit assay (CFU-F) were performed in both culture conditions.

We observed a slightly higher cell proliferation in serum-free grown populations. The doubling rate in the first 10-15 days of cell expansion was very similar in both conditions (*Fig.4A*), but after 20 days we observed a strong increase of proliferation in PA-hBMSCs grown in serum-free medium (10-12 doublings at 30 days *vs.* 5-7 in FBS group).

Some differences in morphology were found between the two culture conditions (*Fig.4B*). The cells cultured in presence of FBS exhibited a spindle-shaped, fibroblastic-like morphology. In serum-free, already at the first passage, the cells had a less elongated morphology, appeared smaller and less branched.

To test the PA-hBMSCs clonogenic potential, cells were plated at low density (10 cells/cm^2) and cultured for two weeks in both culture conditions. After 14 days, the colonies observed in FBS-containing medium were large, with densely packed cells, while in serum-free medium we observed only a few small colonies (*Fig.4C*).

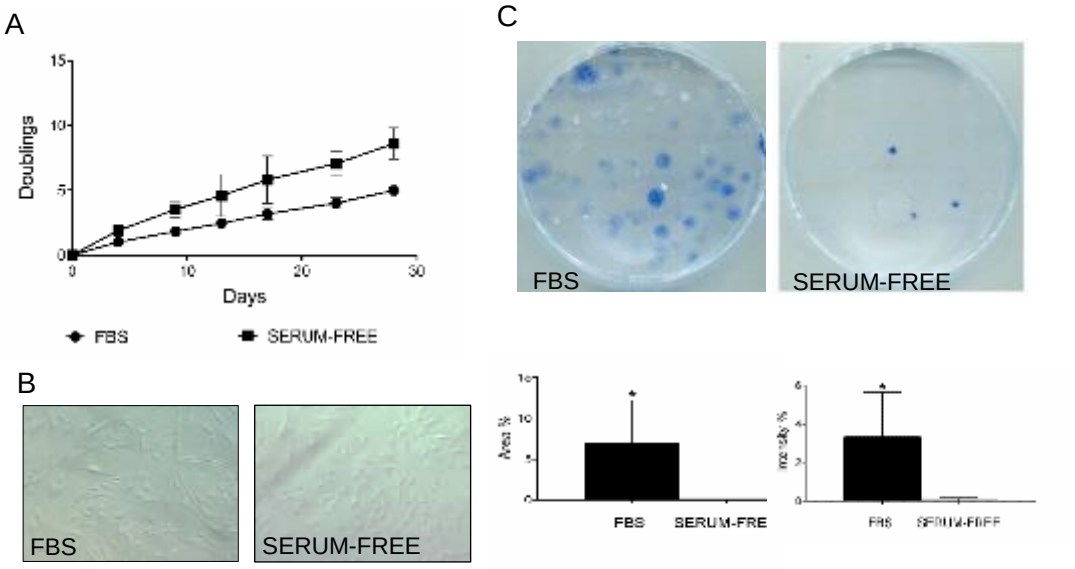

Figure 4 A) Proliferation of PA-hBMSCs growth in presence of FBS or in serum-free medium (3 donors). Growth kinetics is expressed as the number of cell duplications in relation to the culture time.

B) Microscope images showing the morphology of cells. Image acquired with a phase contrast Axiovert 200M microscope.

C) PA-hBMSCs clonogenic potential: CFU-F assay performed in presence of FBS and in serum-free medium. Images have been acquired with scanner Epson 360. The quantification of colony area and intensity was performed with Fiji ImageJ software. The error bars represent standard deviations (S.D). (*) P< 0.05, t-test.

1.3.2 PA-hBMSC cultured in FBS-containing medium or serum-free medium have a similar phenotype with different expression of CD106

A consistent phenotype is also important when isolating and culturing hBMSCs for therapeutic applications. Therefore, we examined the phenotype of PA-hBMSCs cultured in presence of FBS or in serum-free conditions after two passages. This analysis was carried out using flow cytometry to measure markers expressed on the surface of PA-hBMSCs.

PA-hBMSCs cultured in FBS-containing medium and serum-free medium showed little variation in the highly expressed markers such as CD73, CD90, CD105, CD146 and CD166. In agreement with the International Society of Cell Therapy (ISCT) criteria, both cultured MSC preparations were negative for CD45, CD34 and CD31. The markers with the largest differences between different culture conditions were CD106 and HLA-DR, which are completely absent in serum-free cultures (*Fig.5A* and *B*).

CD106 or vascular cell adhesion molecule-1 (VCAM-1) is a member of the immunoglobulin superfamily that interacts with the integrin VLA4. The CD106/VLA4 interaction mediates both adhesion and signal transduction. Although fibronectin facilitates the attachment of the cells to the bottom of the plates, it was previously shown as not necessary for the isolation, attachment and expansion of serum-free hBMSCs and so it was removed from the process. Based on the lower expression of CD106 in PA-hBMSCs culture in serum-free medium reported here, we tried to isolate serum-free hBMSCs in presence of fibronectin to investigate its role in the lack of CD106 expression. hBMSCs were isolated from bone marrow and cultured in absence of fibronectin (W/O), on fibronectin pre-coated plates (FC) or in presence of fibronectin in the medium (FNM). These cells were assessed for their CD106 expression along with the other surface markers mentioned above. Negligible expression of CD106 was observed in these serum-free cell groups and the other markers do not undergo substantial modifications (*Fig.5C*).

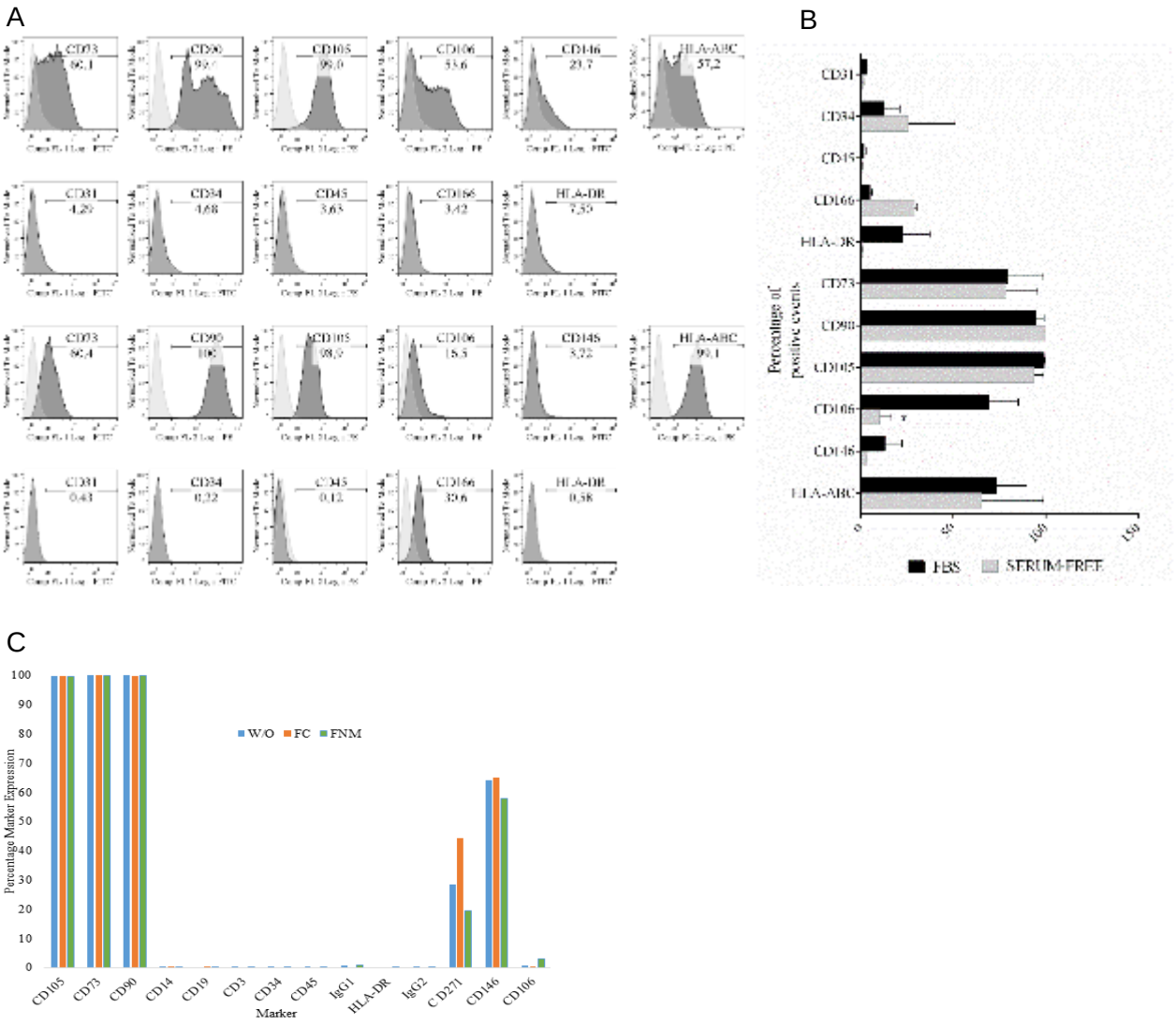

Figure 5 A) Flow cytometry analysis of PA-hBMSCs cultured in the presence of FBS-containing medium (two rows on the top) or in serum-free culture medium (two rows on the bottom). Dark gray-filled histograms indicate antibody staining; light gray histograms showing the isotype control staining are also shown. The percentage of positive events is reported. Results are expressed as log fluorescence intensity a.u. *vs.* number of cells and are representative of three independent experiments.

B) The average percentage of positive events of each marker is reported in a histogram, indicating the mean + SD. (*) P(CD106) < 0.05 t-test Bonferroni.

C) Surface marker expression of serum-free PA-hBMSCs isolated in the presence or absence of fibronectin. W/O: without fibronectin, FNM: fibronectin in the medium, FC: fibronectin pre-coated flasks.

1.3.3 Differentiation potential: PA-hBMSCs cultured in serum-free medium showed a higher osteogenic potential in vitro

To be effective in therapeutic applications, cultured hBMSCs must retain their ability to differentiate into different cell types. Therefore, we assessed the ability of the PA-hBMSCs cultured in presence of FBS or in serum-free conditions to form new bone tissue (osteogenesis), adipose tissue (adipogenesis) and cartilage (chondrogenesis).

To compare the cartilage matrix deposition capacity of PA-hBMSCs cultured in presence of 10% FBS or in serum-free medium, we performed pellet culture with the cells at passage 2. All cell populations formed the typical three-dimensional spherical aggregates. Nonetheless, aggregates formed by the PA-hBMSCs grown in serum-free conditions were larger than those made by cells cultured in FBS. Pellets formed by both hBMSCs cultures presented some metachromatic toluidine blue staining after two passages, suggestive of the presence of cartilage proteoglycans (*Fig.6A*).

In the adipogenic-stimulated cultures, the presence of intracellular lipid granules after 3 weeks of induction was weakly observed in both cell culture conditions. Analysis was performed in triplicate (*Fig.6B)*.

A different scenario is proposed regard to osteogenic potential. Under both FBS or serum-free culture conditions, cells displayed the ability to differentiate toward osteoblasts and exhibited morphological changes. After 21 days of differentiation, calcium mineral deposition was evident, but with some differences between the two culture conditions. The cells grown in serum-free medium deposit bone matrix faster respect to FBS-cultured cells, indeed they start to differentiate already after 1 week of induction, showing a massive mineral deposition after 3 weeks of treatment (*Fig.6C*). These data were confirmed also by the quantification of alizarin by spectrophotometric analysis (405nm, *Fig.6D*).

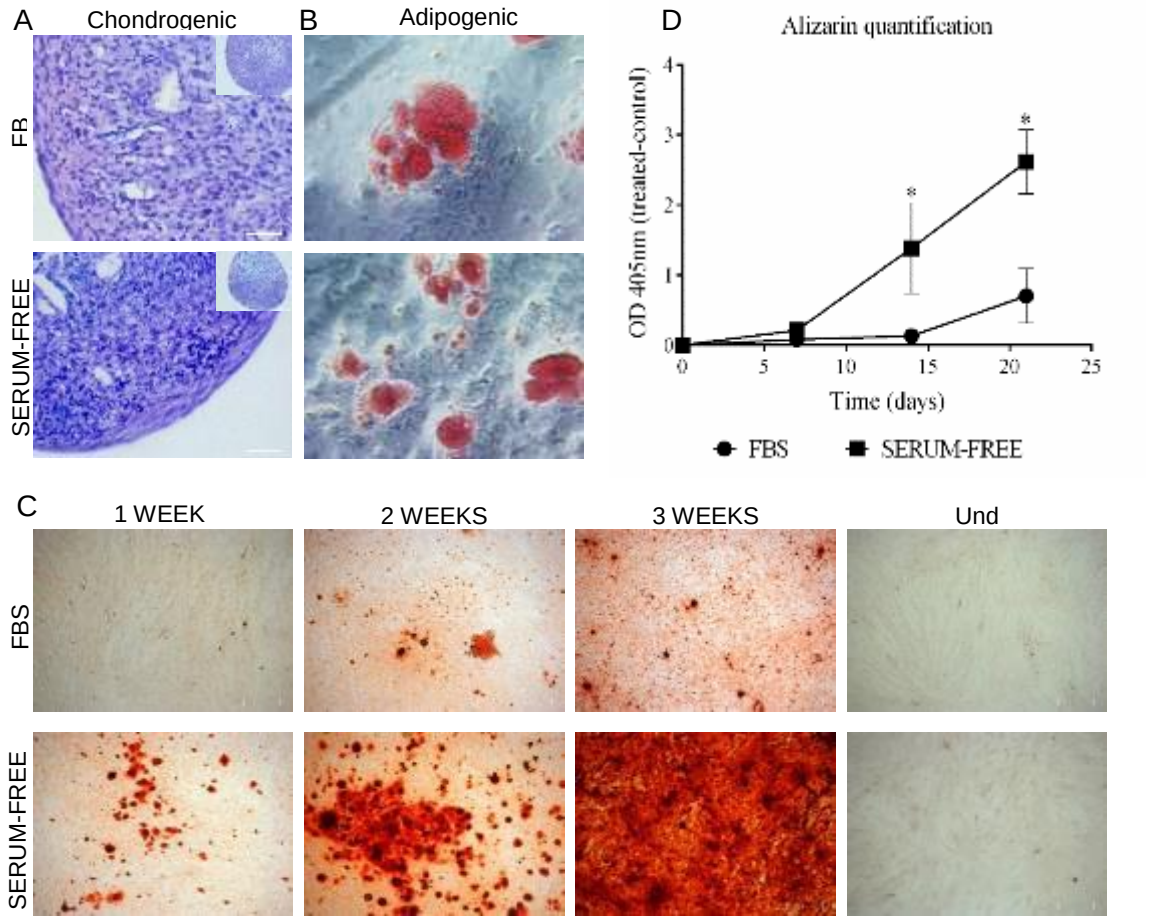

Figure 6 Differentiation assays. A) Chondrogenic differentiation performed by pellet culture, stimulated for 21 days, for the FBS and serum-free cultures. Pellets were embedded in paraffin and stained with toluidine blue.

B) Adipogenic differentiation revealed by formation of lipid droplets stained with Oil Red O in PA-hBMSCs grown in FBS-containing medium or in serum-free medium.

C) Osteogenic differentiation revealed by alizarin staining after 7, 14 and 21 days of induction in PA-hBMSCs cultured FBS-containing medium or serum-free culture medium; negative control (Und), cultured without any differentiation stimulus, is also shown.

D) Alizarin was extracted with 10% acetic acid and the samples were read at 405nm at 7, 14 and 21 days and put on a graph with OD (treated-control) vs. time. The error bars show the SD. (*) P< 0.05 t-test Bonferroni. Scale bar corresponds to 200μm.

1.3.4 Serum-free media culture played a protective role on PA-hBMSCs

To exclude any negative effect on cell viability caused by serum-free medium, we performed a cytofluorimetric assay, using two fluorescent molecules, propidium iodide (PI) and a FITC-anti-annexin-V antibody, so we compared by double staining the percentage of apoptotic cells in both cell culture conditions.

PI is a very voluminous molecule that is unable to cross the plasma membrane. It can enter only dead cells, because following the process of cell death, the plasma membrane is fragmented.[196] Once within the cell, it binds to double stranded DNA by intercalating between base pairs. The annexin is instead a typical marker of apoptosis because it binds phosphatidylserine (PS), molecule which expression in the cell surface is increased during early apoptotic stage. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and PS translocates to the external leaflet. Fluorochrome-labeled Annexin V can then be used to specifically target and identify apoptotic cells.[197,198] Therefore, following the staining, we can have three different conditions: cells positive only to Annexin-V (cells in early apoptosis), double-positive cells (dead cells) or double-negative cells (living cells).

Differences were observed between the cell populations grown in the presence of FBS or in serum-free medium, with a significant increase of alive cells and decrease of apoptotic cells in serum-free samples (*Fig.7A* and *B*).

A cytogenetic analysis was performed on PA-hBMSCs cultured in presence of 10% FBS or in serum-free medium; 20-25 metaphases were analyzed at passage 2. We did not observe any chromosomal abnormality and a normal karyotype was observed in both groups analyzed (*Fig.7C*).

PA-hBMSCs tumorigenic potential was also tested *in vitro* by anchorage-independent growth in methylcellulose media and *in vivo*. PA-hBMSCs grew as adherent cells when plated in FBS-containing medium or in serum-free medium when plated in standard culture dishes, but no CFU-C was detected when both groups of cells were transferred to the methylcellulose semi-solid media (*Fig.7D*). The control tumorigenic human breast cancer cell line MDA-MB-231 formed colonies as expected.

For the *in vivo* tumorigenesis study, $1x10^6$ of PA-hBMSCs for each condition were implanted in 12 NOD/SCID mice, which were monitored for up to 12 weeks. No mouse developed tumors. Mice neither showed any sign of illness, nor macroscopic nodules. Finally, mice were sacrificed, and no anatomical gross abnormalities were observed by autopsy.

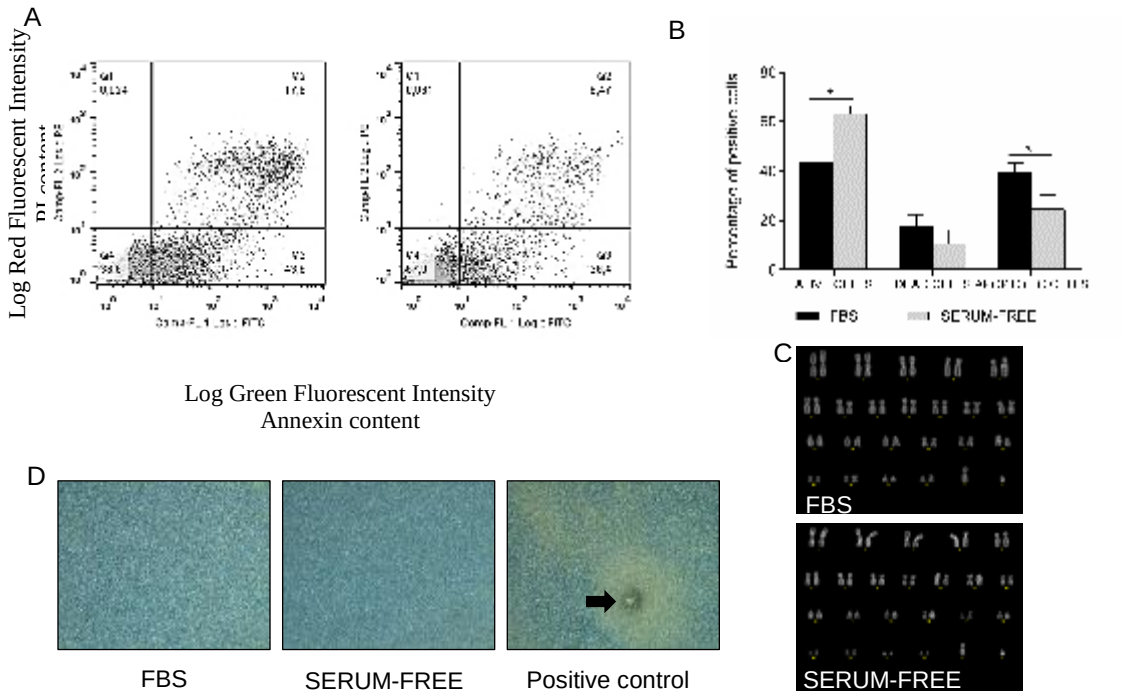

Figure 7 Detection of apoptosis by concurrent staining with FITC-anti-Annexin V and PI. PA-hBMSCs grown in FBS-containing medium or serum-free medium were stained with Annexin V-FITC conjugate and PI and their fluorescence were measured using flow cytometry (A) and quantified (B). The histogram shows the mean + SD, (*) P< 0.05, t-test.

C) Karyotype analysis of PA-hBMSCs cultured in FBS-containing medium or serum-free medium;

D) *In vitro* tumorigenesis test: anchorage independent growth in methylcellulose for PA-hBMSCs cultured in FBS-containing medium or serum-free medium and human breast cancer cells MDA-MB-231 (positive control). Black arrow indicates the colony.

1.3.5 In vivo neo-bone formation into ceramic scaffolds: PA-hBMSCs cultured in serum-free medium showed a higher osteogenic potential in vivo

We tested PA-hBMSC capacity to form bone within engineered constructs based on CaP scaffolds with a specific (HA)/β-tricalcium phosphate (β-TCP) ratio (MBCP+®; 20/80), following the well-established ectopic bone formation model, consisting in the subcutaneous implantation of cell-ceramic constructs in immuno-compromised CD-1 mice.[199] PA-hBMSCs from the two different cultures seeded on CaP ceramic gave rise to tissue-engineered ossicles with mineralized bone detectable after two months. More specifically, the hematoxylin and eosin staining (*Fig. 8B/a,b*) and the Goldner trichromic staining (*Fig.8B/c,d*) of the explants revealed that cells of both culture conditions differentiated in osteoblast (Ob), which organized in an ordered row of cells on the top of the scaffold walls. These cells started to deposit bone matrix to form a dense connective tissue with parallel oriented fibers within which some cells were incorporated to become osteocytes (Oc). However, the explants obtained from serum-free cultured cells (*Fig.8B/d*) presented structures in which the new formed bone is organized around the bone marrow (Bm). These structures were indeed absent in the scaffolds seeded with cells cultured in FBS-containing medium.

Histomorphometric quantification of new-formed bone showed statistically significant differences between the two ceramics, with a higher rate of bone formation in scaffolds seeded with serum-free cells (*Fig.8C*).

To investigate possible mechanisms of the enhanced *in vivo* osteogenesis in CaP scaffolds seeded with PA-hBMSCs, we looked for the presence of osteocalcin taking advantage of antibodies directed against the human protein. Only within the scaffolds seeded with cells grown in serum-free medium there was an upregulation of osteocalcin protein expression, indicating a high process of bone formation and mineralization (*Fig 8B/e,f*).

Detection, visualization and quantification of blood vessels sprouted inside the CaP scaffolds seeded with PA-hBMSCs grown in either FBS-containing medium or serum-free medium were performed using Mallory Trichrome staining (*Fig.85D*). After 2 months, the scaffolds seeded with FBS cells appeared penetrated by a higher number of small sprouted vessels than MBCP$^{+®}$ seeded with serum-free cells (*Fig. 8E)*. However, in the latter the vessels tended to be larger than those found in in the FBS group cells, although the differences did not reach the statistical significance.

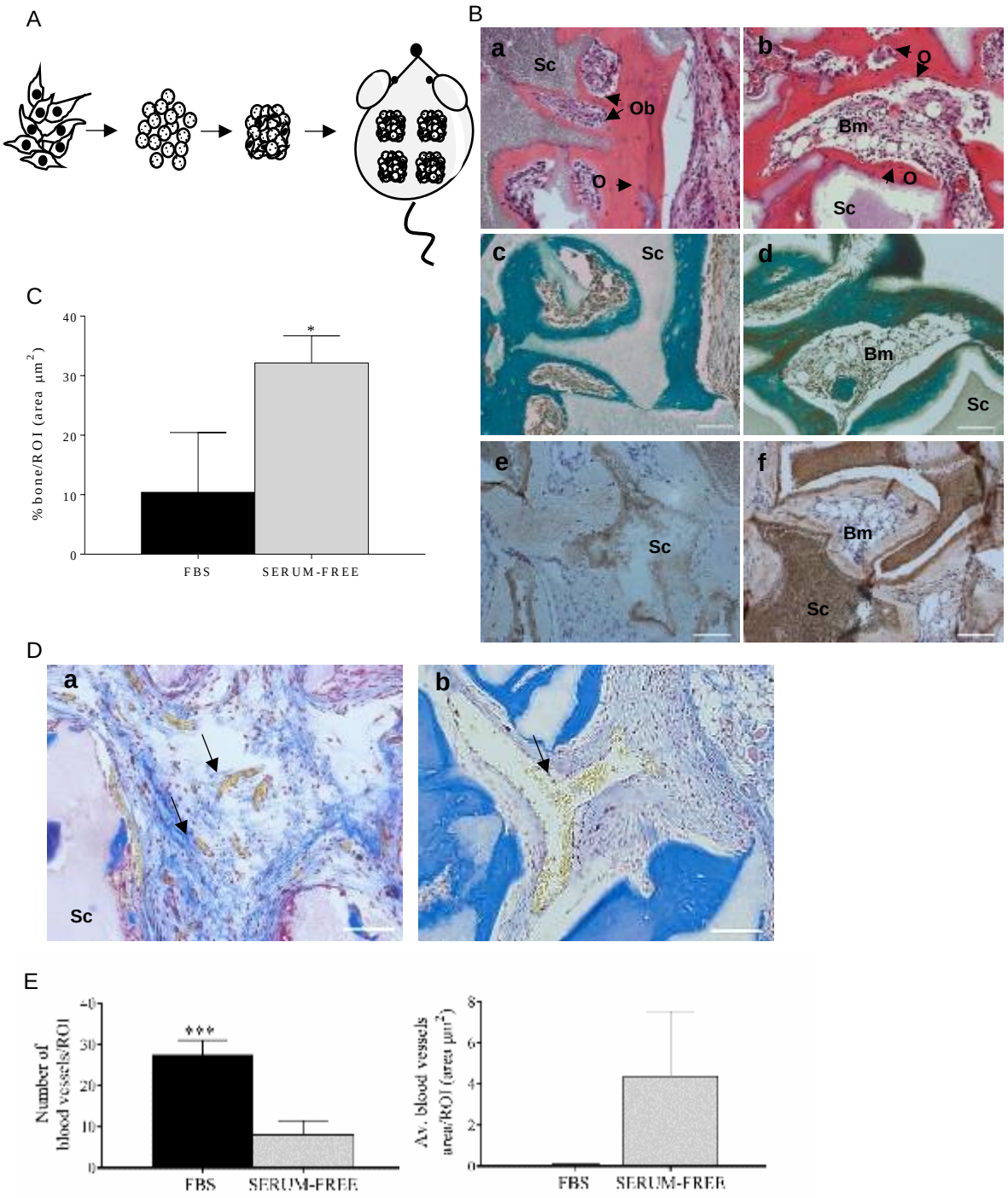

Figure 8 Osteogenic differentiation of PA-hBMSCs on calcium phosphate (CaP) scaffolds.

A) Experimental design: Cells grown in FBS-containing medium or in serum-freemedium were loaded on an MBCP$^{+®}$ ceramic scaffold and implanted subcutaneously in CD-1 nude mice.

B) Histological sections of explants stained with: Hematoxylin and Eosin (H&E), MBCP+® with FBS-PA-hBMSCs (a) and serum-free-PA-hBMSCs (b) after 2 months. Goldner Trychromic staining of MBCP+® with FBS-hBMSCs (c) and serum-free-PA-hBMSCs (d) after 2 months. Osteocalcin immunostaining of MBCP+® with FBS-PA-hBMSCs (e) and serum-free-PA-hBMSCs (f).

C) Quantification of bone area formed by the two different cell populations within the scaffolds. The error bar represents standard deviations. (*) P value < 0.05, t-test.

D) Detection of the presence of vascular structures into scaffolds seeded with the two different populations of PA-hBMSCs. Histological sections of explants were stained with Mallory's trichome: a) and b) are sections of MBCP+® seeded with PA-hBMSCs cultured in FBS-containing medium or in serum-free medium, respectively. Blood vessels are pointed by arrows.

E) On the left, quantification of the number of blood vessels inside both different implants. Error bars represent standard deviations. (***) P value < 0.001, t-test. On the right, quantification of the area of blood vessels inside both different implants.

Scale bar = 100µm. Sc – Scaffold; Oc – osteocytes; Ob – osteoblasts and BM – bone marrow.

1.3.6 PA-hBMSCs growth on waffle macrocarriers in a small stirred vessel bioreactor (spinner flasks)

The type of bioreactor that was chosen to be used in the AutoStem pipeline is a small stirred vessel bioreactor with approximately 1L capacity (*Fig.9A*). Two different types of attachment substrates were tested for cell attachment and growth in agitated conditions in small stirred vessel bioreactors: Xuri attachment substrates (waffle macrocarriers; GE Healthcare) and polystyrene substrates (SoloHill Plastic microcarriers; Pall Life Sciences).

GE waffle macrocarriers are hexagon-shaped, porous structures of approximately 0.5cm in size (*Fig.9B*) made of polystyrene. When inoculated on the macrocarrier waffles, the PA-hBMSCs successfully attached and proliferated when cultured in both FBS-containing and serum-free medium. As the culture progressed, the cell number increased, as shown in *Figure 9C* by the increase in the DAPI-stained nuclei. In addition, cell growth appeared increased when cells were cultured in serum-free medium, compared to those grown in FBS-containing medium. Cell morphology studied after crystal violet staining, when cultured on the waffles appeared to be more rounded and less elongated than the one that is typical for PA-hBMSCs (*Fig.9D*), which is a possible indicator of a stressed phenotype of PA-hBMSCs in the stirred vessel possibly due to agitation.

Overall, cell growth in the bioreactor, shown as cumulative population doublings over 2 passages, was lower than cell growth in monolayer culture, although the difference did not reach the statistical significance. However, this difference in cell growth could become significant with the increase of cell passage. Additionally, an increased cell growth was observed when using a serum-free medium compared to FBS-containing medium (*Fig.9E*).

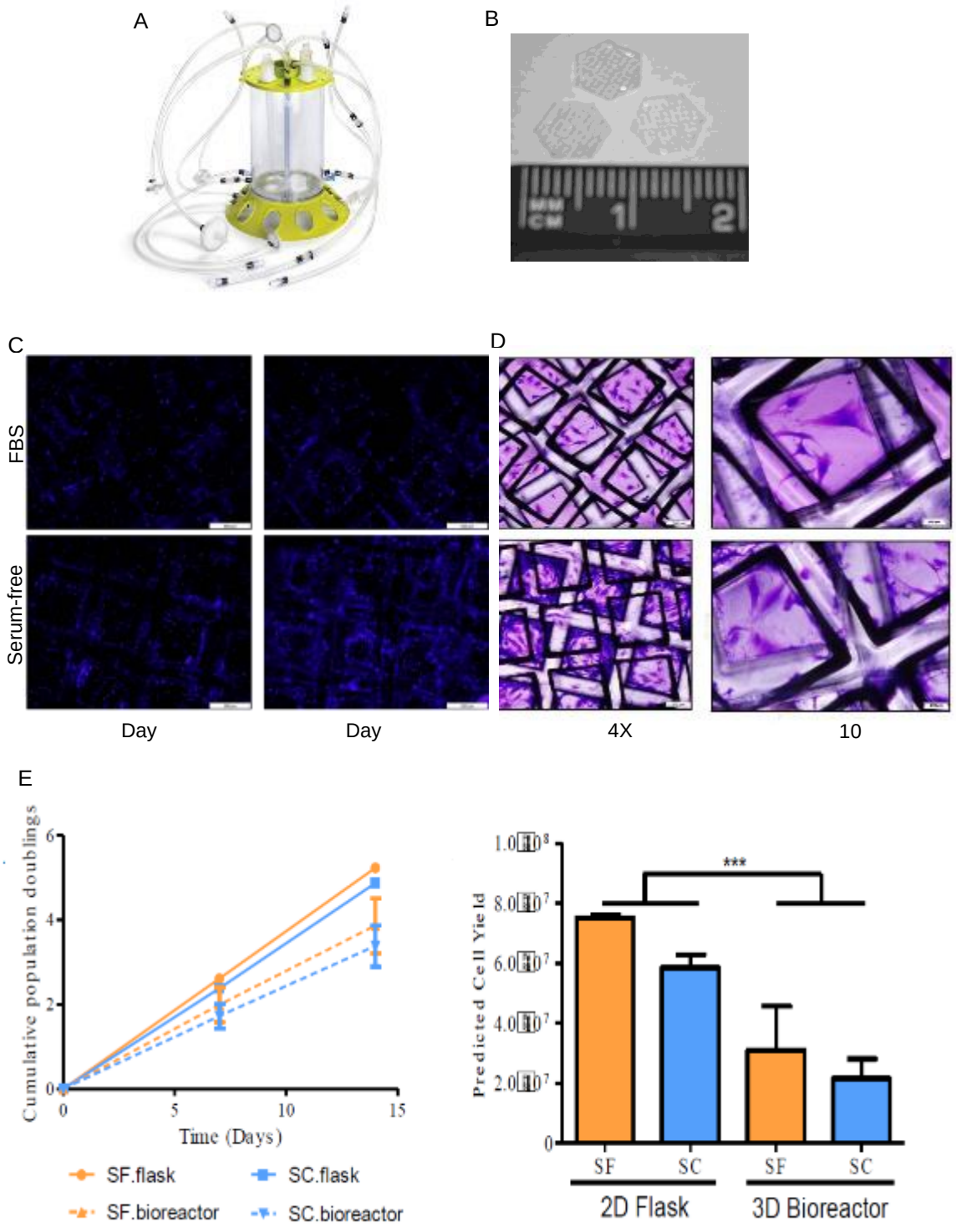

Figure 9 A) Image of the stirrel vessel bioreactor.

B) Representative images of the GE macrocarriers without cells.

C) Representative images of macrocarriers with cells at different time points when cultured in either FBS-containing medium or serum-free medium. Cell nuclei were stained with DAPI and appeared as blue. Scale bars represent 500 µm.

D) PA-hBMSCs morphology when cultured on waffles in FBS-containing medium or in serum-free medium.

E) PA-hBMSCs growth in serum-containing medium (SC) and serum-free medium (SF) over 2 consecutive passages in monolayer culture (i.e. T-flasks) compared to 3D culture in the bioreactor. On the left, cumulative population doublings. on the right, predicted cell yield. ***p≤0.001 as determined using two-way ANOVA and Bonferroni multiple comparisons post-*hoc* test.

1.3.7. PA-hBMSC growth on plastic microcarriers in a small stirred vessel bioreactor (spinner flasks)

Unlike the waffle macrocarriers, the Plastic microcarriers are micrometer sized polystyrene beads of an average size of approximately 180µm and densities slightly higher than water (~1.1 g/cm^3). When inoculated onto the beads, PA-hBMSCs successfully attached and proliferated. Indeed, 4 days post-inoculation the cells attached on the provided microcarriers as demonstrated by the presence of bridges between the cells and the microcarriers (white arrows in *Fig.10A*). Even though not all the microcarriers had visible cells on them or signs of bridging, we cannot state for sure that those microcarriers were not inhabited with cells because the Plastic microcarriers are opaque and don't allow for clear visualization of the cells. However, a better method for cell visualization on these microcarriers, as well as an indicator of cell viability, is a Live/Dead fluorescent staining kit. *Fig. 10B* shows fluorescent microscope images of PA-hMSCs cultured on microcarriers. Live and healthy cells with intact membranes were stained green, while dead or cells with damaged membranes were brightly red.

The cells were kept in culture for 10 days. At day 2 in culture, there was a slight drop in cell number. This decrease in cell density is normal, as during the first 2 days the cells adapt to the new culture environment, which in this case is an agitated environment and the attachment substrates are represented by microcarriers. However, from day 4 onwards, cell growth increased progressively, reaching the peak at day 10 when the cell number was 4.5-fold higher (*Fig.10C*).

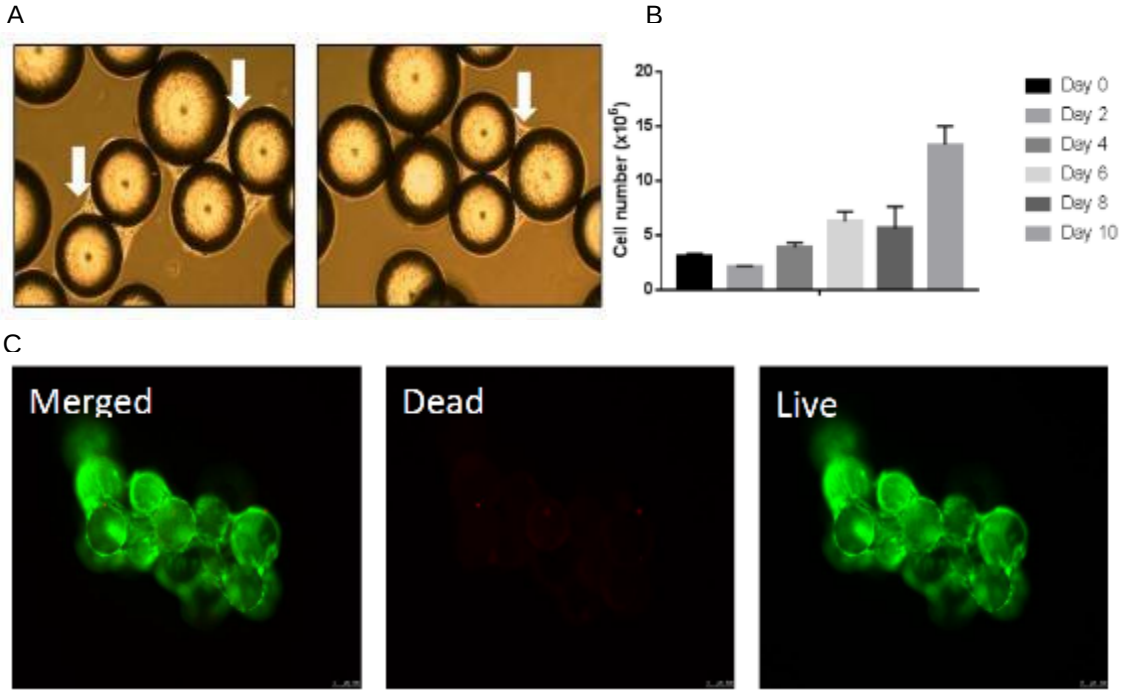

Figure 10 A) PA-hMSCs on Plastic microcarriers at day 4 in culture.
B) PA-hMSCs growth on Plastic microcarriers over 10 days in spinner flasks. Data are expressed as mean + SD.
C) Live/Dead staining of PA-hMSCs cultured on Plastic microcarriers in spinner flasks in FBS-containing medium. Images were acquired at day 5 in culture. Scale bar represents 100 µm.

1.3.8 In vivo neo-bone formation into ceramic scaffolds of 3D-hBMSCs

To validate the AUTOSTEM cell differentiation potency, we received from Aston University hBMSCs cultured in presence of 10% FBS using the Mobius Bioreactor and agreed Autostem protocols. The cells were thawn and cultured for few days (3 days) before their implantation in nude mice.

We tested the capacity of hBMSCs derived from the bioreactor (WP4) to form bone within engineered constructs based on CaP scaffolds with a specific (HA)/β-tricalcium phosphate (β-TCP) ratio (MBCP+®; 20/80), following the well-established ectopic bone formation model, consisting in the subcutaneous implantation of cell-ceramic constructs in immuno-compromised CD-1 mice. We compared the bone formation of these cells with PA-hBMSCs. PA-hBMSCs from the two different cultures (FBS and serum free seeded condition) on CaP ceramic gave rise to tissue-engineered ossicles with mineralized bone detectable after two months. More specifically, the hematoxylin and eosin staining (*Fig.11A*) of the explants revealed that cells of both culture conditions can form bone, which organized in an ordered row of cells on the top of the scaffold walls. These cells started to deposit bone matrix to form a dense connective tissue with parallel oriented fibers within some cells incorporated. However, the explants obtained from serum-free cultured cells presented structures in which the new formed bone is organized around the bone marrow. These structures were indeed absent in the scaffolds seeded with cells cultured in FBS-containing medium. A similar situation is detectable in the explants with 3D-hBMSCs.

Histomorphometric quantification of new-formed bone showed that 3D-hBMSCs had a rate of bone formation similar to FBS cells cultured in plastic adherence (*Fig.11B*)

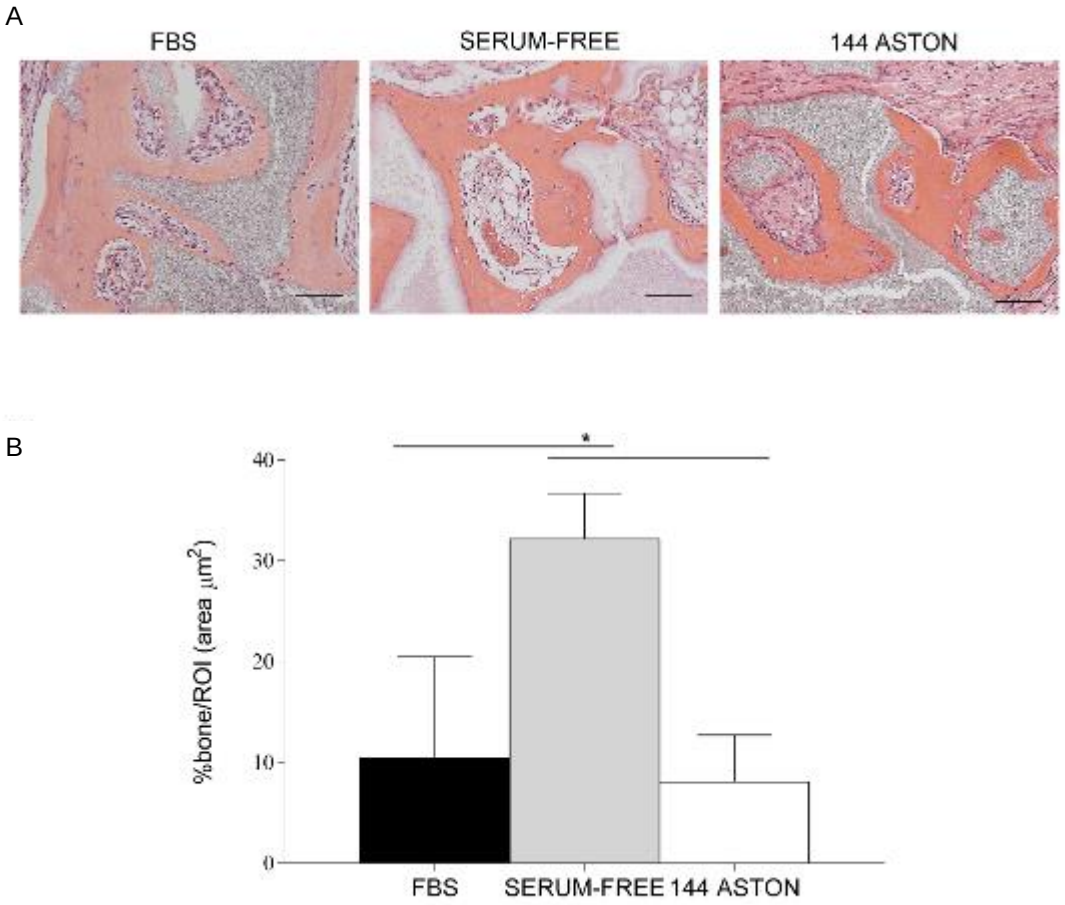

Figure 11 Osteogenic differentiation of 3D-hBMSCs on calcium phosphate (CaP) scaffolds compared to PA-hBMSCs

A) Histological sections of explants stained with Hematoxylin and Eosin.

B) Quantification of bone area formed by the three different cell populations within the scaffolds. The error bar represents standard deviations. (*) P value < 0.05, t-test.

Scale bar = 100µm.

1.3.9 Automated pipeline for PA-hBMSC production in GMP

The basic concept of the AutoStem platform design is shown in *Figure 12*. It basically consists of a sterile chamber, where a robotic arm supports the isolation of the cells, which are kept in culture inside a bioreactor. It is expected two bioreactors, a bioreactor for cell isolation (seeder bioreactor), from which the cells are moved to another bioreactor for expansion (expander bioreactor).

Additionally, there are also area for freezing and storage of cryovials, refrigerators and disposables.

Cell counting will be performed using the NucleoCounter NC-30000 cell counting device. A centrifuge, a pipetting device and a decapper are also present.

The platform and the modules possess functionalities that are analogous to the laboratory equipment used in the manual process. This way the process can be transferred and adapted on the Autostem platform in a straightforward manner.

The automated process can be divided into the distinct steps. Bone marrow that is aspirated from a donor at the clinical site is collected in a 50 mL tube. The bone marrow aspirate is then shipped at room temperature to the production facility, where it is then introduced in the platform (step 1) and seeded into the seeder bioreactor (step 2). After subsequent cultivation in the seeder (step 3) and the expander bioreactor (step 4), cells are harvested and filled into cryo vials (step 5). The product is then stored at – 80°C until pick-up by the user (step 6).

Figure 12 Schematic drawing of the Autostem platform layout. The platform consists of two separate chambers.

1.4 Discussion

The multipotency and anti-inflammatory effects of mesenchymal stem cells (MSCs) make them attractive for cell therapy in regenerative medicine. MSCs are multipotent cells residing in the bone marrow and can, under appropriate conditions, differentiate into mesenchyme-derived cell types (osteoblasts, chondrocytes and adipocytes.[200–203] hBMSCs are harvested and expanded in large quantities without causing donor site morbidity. They represent a rich source of cells to be used in the development and improvement of tissue engineering and regenerative medicine.[204] hBMSCs retain their multi-lineage differentiation capacity until stimulated by specific cytokines and growth factors.[205,206] The use of hBMSCs for the treatment of osteoarthritis and bone defects has been described in large animal models[207–209] and in humans.[204,210–213]

In most cases, hBMSCs expansion must be performed prior to their application for clinical use. However, *in vitro* expansion of hBMSCs could modify cell properties, increase the time to cell implantation and costs, and it is subject to a great deal of regulation.[172,173,175–177] Until now, the field of cell therapy lacks advanced methods for isolation and large-scale expansion of stem cells that are automated, scalable and closed. Choosing the culture system and culture medium used to produce cells are key steps toward a safe, scalable, and cost-effective expansion bioprocess for cell therapy purposes.[189] Present cell culture medium supplements, in most cases based on animal sera, are not fully satisfactory. The adoption of an animal-free culture medium is particularly relevant in establishing culture conditions for isolation and expansion of cells intended for clinical applications.[214]

We proposed here a novel approach to produce MSCs from human bone marrow with xeno-free and aseptic culture system, for therapeutic cell manufacturing and delivery.

It is known that maintenance of a steady population doubling time is critical for amplification of MSCs to treat diseases. Classical isolation by plastic adherence of hBMSCs in our xeno-free medium compared with isolation with conventional FBS-containing medium showed that, although both culture conditions sustain proliferation of PA-hBMSC, the cells grown in serum-free medium presented a higher and significantly stable proliferation rate, suggesting that the culture using the xeno-free medium could harvest more cells in a faster time compared to FBS-containing medium. This data are consistent with that reported for Adipose-Derived MSC (ASCs) and human dental pulp stem cells cultured in chemically defined xeno-free media.[215,216]

Before to move into automated cell production, we needed to set up and validate the culture conditions to be used for AutoStem cell manufacturing. To achieve this goal, we first isolated and expanded PA-hBMSCs in our xeno-free medium and compared these cells with those isolated and expanded using the conventional FBS-containing medium. We found that, although both culture conditions sustain proliferation of PA-hBMSC, the cells grown in serum-free medium seem to have a lower clonogenic potential, as shown in *Fig.1C* in the CFU-F assay. This lower clonogenic capacity of the cells grown in serum-free medium could be related to their decreased expression of the surface markerCD106. The decreased CD106 may lead to a lower adhesion of PA-hBMSC to substrate resulting in a decrease in CFU numbers. It has been reported that the expression of CD106 varies

with the tissue source of MSCs. Thus, while CD106 is expressed by bone marrow-derived MSCs, it is absent on MSCs derived from adipose and other tissues, [217] Therefore, the decrease in CD106 induced by serum-free medium may not affect the efficacy of the PA-hMSCs grown in this conditions on their clinical applications.

Importantly, the absence of expression of HLA-DR observed in cells grown in FBS-containing medium was also observed in those cells grown in serum-free medium. This is a key point given the involvement of this protein in the activation of the immune system and the consequent rejection of allografts.[218] Therefore, these results suggest that the PA-hBMSCs grown in our serum-free medium would not elicit an alloreactive response, making them an excellent candidate for their use in clinical settings, for instance in MSC-based therapy for osteoarthritis. However, it has been shown that MSCs expressing HLA-DR are poorly immunogenic and, in addition, possess immunosuppressive capacity.[167,219] For these reasons, it has been suggested that HLA-DR should not be considered a critical release criterion for MSCs.[220] Therefore, it would be useful to study if cells grown in our serum-free medium retain the capacity of suppressing T-cell proliferation and consequently the immunosuppressive ability.

A second important finding of our research regarding the potential use of our serum-free medium to expand PA-hBMScs intended to be use in clinical settings is that the cells isolated and grown in our medium displayed a strong osteogenic potential. Thus, these cells showed earlier differentiation and faster bone matrix deposition than the cells grown in FBS containing medium, as confirmed by both *in vitro* and *in vivo* differentiation assays. Interestingly, the *in vivo* ectopic bone formation assay suggests that PA-hBMSC cultured in serum-free medium can give rise to an engineered human bone, with wide bone matrix and bone marrow areas (*Fig. 5B*). Also, chondrogenic potential seemed to be higher in serum-free conditions, given that the cells grown in FBS-containing medium formed smaller pellets than those grown in serum-free medium (*Fig. 3A*).
In this scenario, we suggest that serum-free culture conditions allow to expand a PA-hBMSC population that preserve the trilinage potential but with enhanced osteogenic potential respect to its adipogenic and chondrogenic potential.

The safety of the MSCs intended to be used in clinics should be carefully assessed before the administration of the cells to the patients. Karyotyping or other tests evaluating genetic stability are often performed. In our study, the karyotypic profile of the cells cultured in serum-free medium was normal. However, karyotype abnormalities have been reported previously but usually at late passages and the cells enter into senescence without transformation, regardless of chromosomal alterations.[221]

Here we have validated a reproducible clinically-compliant culture method for PA-hBMSCs using a free-serum medium, which allows the production of high quantities of PA-hBMSCs at a low passage (P2). These cells fulfill all MSC criteria, including the ability to differentiate into chondrocytes, osteoblasts and adipocytes and the lack of expression of HLA-DR. In addition, these PA-hBMSCs meet safety criteria given that they retain genetic stability and do not induce tumor formation.The validations described supported the use of the AutoStem platform to isolate hBMSCs in serum-free culture condition.

Chapter 2: Isolation and characterization of secretome and EVs of hBMSCs grown in novel xeno-free system for clinical therapy

2.1 Introduction

Intercellular communication is an essential hallmark of multicellular organisms and can be mediated through direct cell–cell connection or by a paracrine way, with signalling molecules that are released in the extracellular space and caught by other cells to produce a cellular response.[96] In the last two decades, a third mechanism has been brought to light, involving extracellular vesicles (EVs).[96,222–227] EVs are vesicles surrounded by a phospholipidic bilayer containing signaling molecules, including proteins, lipids, DNA, RNA and miRNA. They have reparative and anti-inflammatory properties and they are released from cells into the extracellular space. Although the production of apoptotic bodies during apoptosis has been long known,[228] the fact that also perfectly healthy cells shed vesicles from their plasma membrane has only recently become recognized. Indeed, the release of EVs was initially thought to be only a process to discard nonfunctional cellular components, but increasing evidence suggests that EVs play a critical role in intercellular communication.[229]

The secretion of EVs is a process that appears to be conserved throughout evolution.[96,230] Indeed, cells from different organisms, including all eukaryotes (from amoebae,[231] *Caenorhabditis elegans*,[232,233] and parasites[234–237]) but also prokaryotic cells and virus,[238–240] have been demonstrated to release vesicles into the extracellular environment.

The first observations of the EVs and their relevance occurred simultaneously in various physiological contexts, without the awareness that this form of communication is a universally shared biological structure of the cells. Initially, the EVs were observed in 1946 by Chargaff and West in plasma, as procoagulant particles of platelet derivation[241], and identified as "platelet dust" by Wolf in 1967.[242] First studies also included matrix vesicles identified by Anderson in 1969 in cartilage and correlated with the calcification of epiphysis.[243] Between 1970 and 1980, other studies have identified EVs released in plasma by microvilli cells of rectal adenoma,[244] or virus-like particles identified in bovine serum of cell culture medium[245,246] and vesicles, later called prostasomes, in the seminal fluid.[247,248] Around the same period, first findings were made of membrane fragments acting as pro-coagulants and derived from tumors.[249,250]

A big breakthrough came in 1983 with the work done by Stahl and Johnstone, focusing on reticulocytes maturation in red blood cells.[251,252] These experiments led to the discovery of what is now called transferrin cycle and they allowed to clarify the mechanism of secretion of extracellular vesicles. Reticulocytes begin their journey in the bone marrow as erythroblasts. They discharge their nuclei before taking up residence in the blood where they continue the maturation process for 24 to 36 hours to become bona-fide red

blood cells.[251] As reticulocytes mature into red blood cells, they selectively lose or discard certain membrane proteins, such as transferrin receptors.[253,254] Transferrin (Tf) is a glycoprotein that circulates in the blood of all vertebrates and is responsible for delivering iron to cells. Iron is required by all cells but especially reticulocytes, that contain high concentrations of haemoglobin, an iron binding protein.[255] Each transferrin molecule (apo-transferrin) bonds avidly two atoms of iron (holo-transferrin). Transferrin in turn, binds to transferrin receptor (TfR) with very high affinity.[256–258] Using transferrin bound to gold particles,[252] or anti-TfR antibodies,[259] and using electron microscopy to follow the fate of the endocytosed receptor during its trafficking in the cell and subsequent release, the two groups found that Tf-TfR complex is internalized into a multivesicular endosomal compartment by endocytosis and most of the gold staining corresponding to the receptor was associated with the small internal bodies (approximately 50 nm in diameter). Once in the endosome, iron is reduced, dissociates from its transferrin carrier and is transported by facilitated diffusion into the cytoplasm, where it is incorporated into the heme group of hemoglobin. Tf stays bound to the TfR on the small bodies, which are released upon fusion of the endosomes with the plasma membrane of the cells.[256,257] The net effect of the transferrin cycle is to transport iron into cells, while sparing and recycling the iron carrier protein Tf. These and the following studies suggested that this novel form of secretion was the way in which TfR were discarded from maturing reticulocytes.[260] In 1987, the term exosome was first used to describe small membrane vesicles formed by vesiculation of intracellular endosomes and released by exocytosis.[260]

More than a decade later, Raposo *et al.* showed that these vesicles, isolated from the B lymphocytes transformed by the Epstein-Barr virus, were antigen-presenting particles and they were able to induce a response in T cells.[261]

Following these pioneering studies, EVs were isolated from most types of cells and biological fluids, including blood,[261–264] urine,[265–267] saliva,[268,269] breast milk,[270–273] amniotic fluid,[274–278] ascites,[279–281] cerebrospinal fluid,[282,283] bile,[284,285] and semen.[247,286–289]

A significant forward step in EVs research field was achieved in 2011 by members of the International Society of Extracellular Vesicles (ISEV) aimed at unifying the nomenclature and methodology to isolate the EVs. The data indicated that the content, size and composition of the EVs membrane are very heterogeneous and dynamic and depend on the state of the secreting cell and environmental conditions. To date, many subgroups of EVs have been defined, such as apoptotic bodies, microparticles, microvesicles, shedding vesicles, ectosomes, exosomes and exosome-like vesicles.[95,229]

Although characteristics have been proposed for each EVs subpopulation, there is currently no specific identification of the markers of each subgroup that is widely accepted in order to distinguish these populations. Added to this is the lack of standardized methods of isolation and characterization. Furthermore, isolation procedures generally fail to purify specific types of vesicles but instead produce complex mixtures of various types of EVs. Because of the difficulties in the methods of isolation, the multidisciplinary nature of the research field, and the different ways of classification, there is currently no consensus on the nomenclature to be used to identify the various vesicle populations. For example, vesicles are often called referring to the cell or tissue of origin, for example, dexosomes (exosomes derived from dendritic cells),[290,291] prostasomes (vesicles derived from the prostate),[247,286] vesicles of the matrix (from bone, cartilage and atherosclerotic plaques),[243,263,292–294] or synaptic vesicles (vesicles released by neurons),[295–300] or

referring to their size (microparticles, microvesicles, nanovesicles, nanoparticles),[239,301,302] or to their proposed functions (calcifying matrix vesicles, argosomes, tolerosomes),[303–306] or simply to their presence outside the cells (ectosomes, exosomes, exovesicles, exosome-like vesicles).[307,308] Although the nomenclature is still a matter of debate,[309] the terms ectosome, shedding vesicle, microparticle and microvesicles generally refer to 150-1.000 nm vesicles released by budding from the plasma membrane (PM). The term exosome was initially used to name vesicles ranging from 40 to 1.000 nm released by a variety of cultured cells and carrying 5'-nucleotidase activity.[310] However, this term was adopted after the experiments of the 1980s for small (30-100nm) vesicles of endosomal origin released as consequence of the fusion of multivesicular bodies (MVBs) with the PM.[251,259,260] Since then, the term exosome has undergone a rise in popularity, with increasing numbers of articles choosing this term to designate EVs.

Mesenchymal stems cells (MSCs) and all types of stem cells also secrete extracellular vesicles, which have different therapeutic activities and may represent a breakthrough in clinical stem cell therapy.[305-314]

The paracrine effect of MSCs was first described in 1996 when Haynesworth *et al*. reported that MSCs synthesize and secrete a broad spectrum of growth factors, chemokines and cytokines that could exert significant effects on nearby cells.[324] This funding gave start to numerous studies on the effects of the MSCs secretome which reported that these secreted factors enhance arteriogenesis,[325] protect against ischemic renal and limb tissue injury,[326] promote neovascularization,[327] increase angiogenesis,[328,329] promote human cartilage regeneration *in vitro*,[330] prevent apoptosis induced by perinatal hypoxia-ischemia in neuronal cells,[331] attenuates neuroinflammation and improves functional recovery in a model of acute spinal cord injury (SCI)[332] and protect hippocampal neurons from oxidative stress.[333] These studies have provided the basic foundation of the paracrine hypothesis, according to which MSCs exert their therapeutic function not mainly through their differentiating potential but rather via their ability to secrete certain essential factors.[334–341] Much of the initial efforts to identify the active therapeutic agent in MSC secretion focused therefore on small molecules such as growth factors, chemokines, cytokines and miRNA.[342]

In 2009, Bruno *et al.* demonstrated that MSCs secrete 80nm-1µm microvesicles that protect against acute tubular injury.[343] In 2010, Lai *et al*. subsequently showed that MSCs secrete a specific class of EVs with endosomal origin, a diameter of 40-100nm[344] and expressing exosome-associated markers such as Alix, Tsg101, tetraspanins such as CD9, CD63 or CD81.

MSC exosomes are distinguished from those of other cell derivation for specific proteomic profiles (857 unique gene products) and vesicular RNA (more than 150 identified miRNAs).[135] miRNAs and proteins are involved in many cellular processes, such as communication, inflammation, exosomal biogenesis, metabolism, tissue repair and regeneration.[135,345–353] MSC exosomes have the potential to support the maintenance of the tissue microenvironment. This role is particularly important when the tissue microenvironment is altered by a disease or a lesion leading to failure of tissue homeostasis.[130] On the other hand, microenvironment influences MSCs paracrine signaling (e.g., protein and RNA profile of exosomes). Therefore, controlling MSCs growth conditions by modifying the growth factor composition, oxygen tension and mechanical properties could directly influence the paracrine activity of MSCs[354–356]

Despite the extensive research done in the field of the EVs as therapeutic tools, there are still many issues to be solved. One of the main troubles is related to the isolation and culture method. The vast majority of cell cultures are indeed conducted in media containing foetal bovine serum (FBS), which brings along a large number of extracellular vesicles of bovine derivation, which may represent a contamination in the final sample. The EVs isolation methods currently available do not allow the discrimination between vesicles derived from cells and those arising from the culture medium supplement, precluding the achievement of an accurate and artefacts-free characterization. Moreover, from the point of view of a large-scale production for use in clinical therapy, it is important to have cellular products completely free of animal derivatives. In many studies, FBS was preventively ultracentrifuged to eliminate its vesicular component. However, these methods were not completely efficient, as they do not guarantee the obtainment of a completely exosome-free serum and, in addition, some FBS-deriving extracellular RNAs (exRNAs) can keep in culture and interfere with analysis.[357] In this scenario, it seems important to develop a clean, exosome-free culture system.

In our study we use a novel xeno-free cell culture medium for isolation and expansion of human MSCs in plastic adherence and in bioreactors. As part of an ambitious European project with the ultimate goal of developing one GMP-StromaCellFactory, our goal was to compare the traditional plastic adherent isolation and culture of MSCs with the cells isolated and cultured with AUTOSTEM's protocols, using a fully defined growth medium, without FBS, as previously described. The second important deliverable provided the isolation and characterization of extracellular vesicles in the two different culture systems.

Hypoxia (1% O_2) has been reported to elevate exosome production in a number of cell types in vitro. This hypoxia-induced increase in exosome secretion is controlled by hypoxia-inducible factor-1 alpha (HIF-1α) and is independent of apoptosis.[358] Hypoxia not only affect the quantity of exosomes produced but also the composition of their content and, therefore, their therapeutic potential. Thus, MSCs cultured under hypoxia (< 5% O_2) showed an altered protein expression pattern compared to MSCs cultured in normoxia.[354] Furthermore, in a murine hind limb ischemia model, intra-arterial injection of MSCs cultured under both normoxic or hypoxic conditions promoted revascularization, but the functional recovery of mice that received hypoxia preconditioned MSCs was achieved earlier.[359] These findings indicate that preconditioning MSCs in different oxygen environments may potentiate their tissue regenerative properties.

Plastic-Adherence human MSCs (PA-hMSCs) were cultured under normoxia (20% O_2) or under hypoxia (1% O_2) to enhance EVs production. The hypoxia culture conditions mimic in part the native micro-environment of the cells in case of damage or stress, resulting for example from a stroke: the hypoxic environment that is generated stimulates the stem cells to proliferate and differentiate, as well as to secrete factors that attract other neighboring cells that participate in the repairing of the damage tissue.[360–362] The use of hypoxia therefore allowed us to study the paracrine activity of MSCs, which seems to be more active in the presence of hypoxic stress.

2.2 Materials and Methods

2.2.1 Human Bone Marrow Stromal Cells (hBMSC) cultures in plastic adherence

Plastic Adherent Human Bone Marrow Stromal Cells (PA-hBMSCs) were derived from hip bone marrow aspirates of healthy donors, after informed consent. The human samples were obtained from EU-AUTOSTEM Consortium, after approval by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland and processed at National University of Ireland Galway (NUIG). Briefly, cell nucleated fraction of bone marrow aspirate was suspended in α-MEM-GlutaMax medium (Gibco, Waltham, MA, USA), supplemented with: i) 10% Fetal Bovine Serum (FBS, Gibco), 1ng/ml Fibroblast Growth Factor-2 (FGF-2, Peprotech, London, UK) and 100 U/ml Penicillin/100µg/ml Streptomycin mixture (Euroclone) for standard culture conditions, or ii) with the AUTOSTEM patented serum-free supplement and 100 U/ml Penicillin/100µg/ml Streptomycin mixture. The cells were plated at a density of $1x10^6$ cells/cm^2 in T75cm^2 flasks or 100mm dishes. After at least 5 days, only PA-hBMSCs were attached on the plastic, while all the blood cells and other debris were in suspension. Thus, the medium was discarded, the cells were washed with Phosphate Buffer Saline 1X (PBS) to remove all the refuse and the dishes were refilled with fresh medium. When cells were confluent, PA-hBMSC were detached with trypsin-EDTA (Euroclone) and cryopreserved for the delivery to our laboratory. After arrival, cells were thawed, and 2.5 x 10^5 cells were plated in 100mm dishes (density of 3000 cells/cm^2) and grown in an incubator at 37°C with 5% CO_2. The medium was changed twice a week.

2.2.2 Isolation and culture of human Arthicular Chondrocytes (hACs)

Human Articular Chondrocytes (hACs) were obtained from human femoral head biopsies of healthy patients, after informed consent. First, the sample was processed with the help of a scalpel, in order to withdraw the cartilaginous coating from the joint. The pieces obtained were then cut into smaller pieces of about 3mm x 3mm, then they were transferred in a 50ml tube, washed with PBS 1X and resuspended in 1-2mL of a mix of enzymatic digestion, consisting of 1mg/mL hyaluronidase (Sigma-Aldrich, Saint Louis, Missouri, USA), 400U/mL collagenase I (Wortington Biochemical, Lakewood, NJ), 1000U/mL collagenase II (Wortington Biochemical) and 0.25% trypsin (GiBCo) in Dulbecco's Modified Essential Medium (DMEM) High Glucose (EuroClone) w/o FBS. A first digestion of 30 minutes at 37°C was carried out to relax the matrix and it was therefore discarded. Various serial digestions were then performed, each one for at least 60 minutes at 37°C in agitation, to obtain individual cells. After each digestion, the supernatant was recovered, resuspended in 10mL of DMEM with 10% FBS to block the enzymatic activity and centrifuged for 10 minutes at 1200rpm. The isolated hAC were seeded in 1-2 wells of a 6-well plate, in DMEM High Glucose with 10% FBS 2mM L-Glutamine (EuroClone), 100U/ml Penicillin/100µg/ml Streptomycin, 1mM Na-pyruvate (EuroClone) and 10mM HEPES (EuroClone). In each well a liquid hyaluronidase quota of 8U/mL was also added, in order to break up any aggregates of cells still present in the suspension.

The medium was changed every 2-3 days and the cells were kept in an incubator at 37°C with 5% CO_2. At ~90% of confluence, cells were detached and plated at different densities.

2.2.3 hBMSCs isolation on carriers in a small stirred vessel bioreactor (i.e. spinner flasks)

Three different bone marrow-derived hBMSC donor lines were isolated from bone marrow aspirates using a bioreactor at University of Aston.

Small stirred vessel bioreactors (i.e. spinner flasks) were used in this study to evaluate the growth of hBMSCs on two different attachment substrates: on Xuri attachment substrates (i.e. waffle macrocarriers; GE Healthcare) and on polystyrene substrates (i.e. SoloHill Plastic microcarriers; Pall Life Sciences).

We received the cell from Aston University, we thawed and expanded the cells in dishes for one day. Then we performed the required analysis.

2.2.4 PA-hBMSCs pre-conditioning

PA-hBMSCs and 3D-hBMSCs were cultured until a 70% confluence. Then, the medium was discarder and, after 5-washed with PBS 1X, it was replaced with α-MEM-GlutaMax medium with only 100U/ml of Penicillin/100µg/ml Streptomycin mixture, without any other supplement. Cells were kept in under normoxic (20% O_2 and 5% CO_2 at 37°C) or hypoxic (1% O_2 and 5% CO_2 at 37°C in a hypoxic incubator, Eppendorf, Hamburg, Germany) conditions, for 24 h (for whole secretome analysis) or 72h (for EVs isolation). The PA- and 3D-hBMSCs-conditioned medium (hBMSCs-CM) was collected and processed as described below.

2.2.5 Analysis of conditioned medium content with cytokine array

For analysis of whole conditioned medium, after 24h, hBMSCs-CM medium was recovered and immediately centrifuged at 300g for 10 min, at 4°C to remove any dead cells. Protein content was quantified by Bradford assay and 10µg of proteins were analyzed with a Proteome Profiler Human XL Cytokine Array Kit (R&D, Minneapolis, Toll Free USA, Canada), consist of a nitrocellulose membrane with antibodies spotted in duplicate. The membrane was incubated for 1 hour with a blocking solution and then incubated with the samples overnight at 4°C on a rocking platform shaker. The membrane was washed to remove unbound material followed by incubation with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were then applied, and a signal was produced at each capture spot corresponding to the amount of protein bound. Images were scanned using the Epson perfection 1260 scanner and band densities were quantified using the Fiji-ImageJ software. Gene

Ontology (GO) was also carried out using DAVID tool (Functional Annotation Bioinformatics Microarray Analysis).[363,364]

2.2.6 Isolation of Extracellular Vesicles from PA-hBMSCs (h-BMSC-EVs)

PA-hBMSCs and 3D-hBMSCs were cultured until reaching a confluence of about 60-70%. In order to have a good extraction yield, cells were never allowed to grow until a too high confluence, as this could adversely affect vesicle secretion levels. hBMSCs-EVs were obtained after high-speed differential centrifugation of hBMSCs-CM collected from about 10 x 10^6 PA-hBMSCs cultured in serum-free and FBS-containing medium and starved for 72 hours under normoxic (FBS-hBMSC-EVs_{Normo} and SF-hBMSC-EVs_{Normo}) or hypoxic (FBS-hBMSC-EVs_{Hypo} and SF-hBMSC-EVs_{Hypo}) conditions. hBMSCs-CM were collected in 50ml tubes and cells were trypsinized and counted. The first centrifuge was performed at 300g for 10 minutes at 4°C to eliminate dead cells and debris. Once the supernatant was recovered, a second centrifuge at 2000g for 20 minutes at 4°C allowed to eliminate the apoptotic bodies from the preparation. As these vesicles are very large and their steric encumbrance could trap even the smallest vesicles in the pellet, this was precautionally washed with PBS 1x and re-centrifuged at 2000g for 20 minutes at 4°C. The supernatant was transferred into ultracentrifuge polyallomer tubes (Beckman Coulter, Brea, California, USA), that were placed in appropriate bucket for ultracentrifuge. The supernatant was then ultra-centrifuged at 10,000g for 30 minutes at 4°C, to pellet microvescicles and finally ultra-centrifuged at 10,000g for 90 minutes at 4°C, to pellet the exosomes. The exosome pellet was washed with PBS 1X, previously filtered through 0.22µm filter to remove as much as possible the salts of the solution (because of nanometric size of exosomes). All the exosome pellets were resuspended together into a single clean tube and a last ultracentrifuge was taken at 100,000g for 70 minutes at 4°C, to wash the isolated vesicles. At the end of this ultracentrifugation, the supernatant was discarder and the pellet was resuspended in 100µl of filtered PBS 1X.

A Beckman Coulter ultracentrifuge (Beckman Coulter Optima XPN-100 ultracentrifuge; Beckman Coulter) was used with swinging bucket rotors type SW28 and SW41Ti. A schematic representation of the performed protocol is shown in *Fig.13.*

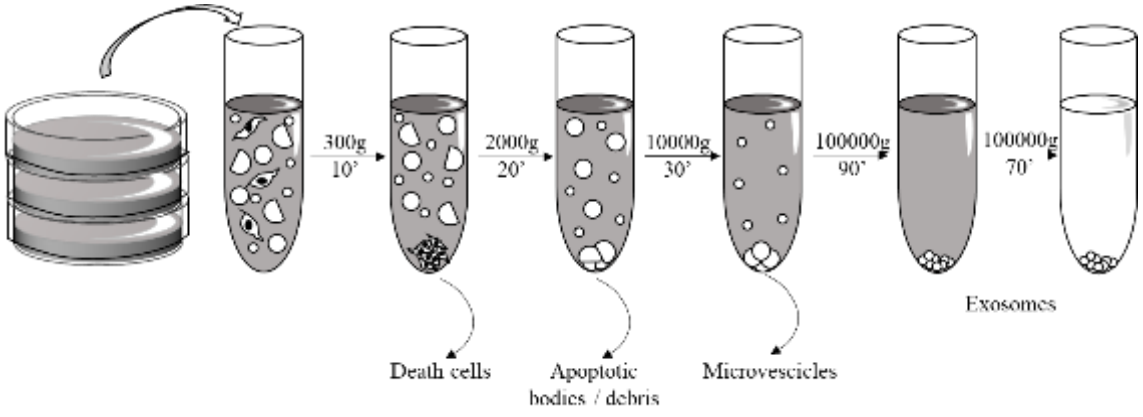

Figure 13 Representative scheme of differential ultra-centrifugation protocol

2.2.7 Characterization of hBMSC-EVs

Protein quantification by BCA assay

The concentration of membrane-bound protein on the surface of freshly isolated, intact hBMSC-EVs was measured using BiCinchoninic Acid (BCA) assay (Thermo Scientific Pierce, Rockford, IL). This method uses a reagent containing bicinconinic acid and is based on the reduction of Cu^{+2} to Cu^{+1} in presence of proteins in an alkaline medium (the biuret reaction) providing colorimetric detection of the cuprous cation (Cu^{+1}). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL). The quantification was performed following manufacturer's instructions. The protein content per microliter was normalized to the number of FBS-hBMSCs$_{Normo/Hypo}$, SF-hBMSCs$_{Normo/Hypo}$ and 3D-hBMSCs ($1x10^6$).

FACS analysis on EVs

At least six independent preparations of both FBS-hBMSC-EVs$_{Normo/Hypo}$ and SF-hBMSC-EVs$_{Normo/Hypo}$ were stained with 1µM PE-Cy5-Cell Trace (ThermoFisher Scientific, Waltham, Massachusetts, USA) in combination with the mouse anti-human monoclonal antibody (mAb) PE-Cy7-CD63 (Clone: H5C6) (BD Pharmingen, San Jose, CA, USA) or the anti-human mAb PE-CD105 (Clone: SN6) (eBioscience, San Diego, CA, USA), both diluted 1: 100 in filtered PBS 1X. Cell Trace is a fluorescent dye analog of the CFSE (Carboxyfluorescein succinimidyl ester), which is cell permeable and covalently couples to intracellular molecules, staining cells, or vescicles, in this case. Thus, we used it to evaluate the vesicular membrane integrity. A set of microsphere suspensions (1.35µm, 0.88µm, 0.45µm and 0.22µm, SpheroTec, Martinsried bei München, Germany) was used as size reference. An unstained sample was acquired to detect the sample autofluorescence and set the photomultiplier for all the three used channels. Forward and side scatter channels (FSC and SSC) were used on a logarithmic scale visualized in bi-exponential mode. The FSC and SSC photomultipliers were set using background noise as the lower optical limit, acquiring a sample of sterile PBS tube. The threshold, set on the FSC channel, was regulated to reduce the noise progressively, allocating dots in low left corner of plot, in order to clearly detect EVs. Samples were run on a FACS Aria II (BD Biosciences, San Jose, CA, USA) cytofluorimeter. At least $5x10^4$ events were analyzed in each instance.

Western Blot analysis

For western blot analysis, the isolated FBS-hBMSC-EVs$_{Normo/Hypo}$ and SF-hBMSC-EVs$_{Normo/Hypo}$ were resuspended in RIPA buffer (1% NONIDET p-40, 0.1% SDS, 0.1% Sodium deoxycholate, protease inhibitor cocktail 1x, in PBS pH 7.5) and protein content was quantified by BCA assay. Afterwards, 2-3µg of proteins for each sample were loaded on 4%–12% NuPAGE Bis-Tris gel (Life Technologies, Carlsbad, California, USA), electrophoresis was performed at 200V and proteins were blotted on a polyvinylidene fluoride

(PVDF) membrane (Millipore, Burlington, Massachusetts, USA) at 350mA (constant A) for 2 h and 10 min. After blocking nonspecific sites with 5% non-fat dry milk (Biorad, Hercules, California, USA) in Tris Buffered Saline with Tween 20 (TTBS, 20mM Tris pH 7.5, 500mM NaCl, 0.05% Tween 20) for 1h at RT, blot membrane was incubated overnight at 4°C with a specific primary antibody (CD9, CD81, or Hsp70, System Biosciences, Palo Alto, CA), prepared in 2.5% non-fat dry milk/TTBS at the dilution of 1:1000. The following day, washes with TTBS were carried out to remove the excess of antibody and the membrane was then incubated with an HRP-anti-rabbit secondary antibody at a dilution of 1:20000, for 1h at RT. The positivity was highlighted by providing the substrates for the chemioluminescence reaction of HRP (Amersham ECL Western Blotting Detection Reagent, GE Healthcare, Chicago, Illinois, USA) and impressing a photographic sheet by autoradiography (GE Healthcare).

Images were scanned using the Epson perfection 1260 scanner and band densities were quantified using Fiji-ImageJ software.

Tunable Resistive Pulse Sensing (TRPS)

Tunable Resistive Pulse Sensing (TRPS) is a technique that allows high-throughput single particle measurements driven through a size-tunable nanopore, one at a time. The technique adapts the principle of resistive pulse-sensing, which monitors current flow through an aperture, combined with the use of tunable nanopore technology, allowing the passage of ionic current and particles to be regulated by adjusting the pore size.[360–362] TRPS provides information about particle concentration, (i.e. number of particles/ml for a specified size range), particle size and accurate number based size distribution, particle charge and number based charge distribution. Analysis was performed on FBS-hBMSC-EVs$_{Normo/Hypo}$, SF-hBMSC-EVs$_{Normo/Hypo}$ and 3D-hBMSC-EVs derived from three different primary cultures using qNano (Izon Science Ltd, Burnside, Christchurch, New Zealand).

Analysis of pre-conditioned cells by Scanned Electron Microscopy (SEM)

PA-hBMSCs were cultured in serum-free and FBS-containing medium on cover glasses and then pre-conditioned for EVs production as previously described. Cells were washed with PBS 1X and fixed with 2.5% glutaraldehyde for 30 min, then rinsed twice in cacodylate buffer solution (Na-cacodylate 1mM). A step of lipid fixation with osmium tetroxide was performed to better discriminate membrane and vesicles. Cells were therefore incubated for 1h with 0.1% OsO_4 (Sigma-Aldrich) and washed at least three times with cacodylate buffer to remove any trace of osmium. The samples were then dehydrated in increasing concentrations of ethanol (50%, 70%, 95%, 100%), transferred to an increasing graded series of hexamethyl–disilazane (HMDS), leaved to dry overnight under a chemical hood and sputtered-coated with a 7nm chromium-film and 20nm aurum-film. The morphology was finally evaluated by SEM (FEI Company, Hillsbora, OR, USA).

2.2.8 Labeling and Internalization of hBMSC-EVs by hACs

EVs uptake can be monitored *in vitro* using a fluorescent dye as PKH67 (Sigma-Aldrich). The PKH67 is a green fluorescent dye with long aliphatic tails, which can be incorporated into lipid regions of the cell membrane. We stained FBS-hBMSC-EVs$_{Normo/Hypo}$ and SF-hBMSC-EVs$_{Normo/Hypo}$ with PKH67, according to the manufacture's protocol. Staining was stopped by adding an equal volume of 1% BSA and the EVs were ultracentrifuged to remove excess of fluorescence. Stained EVs were given at the concentration of 1μg/ml to hAC plated on glasses for cell culture. After 3 hours, hACs were fixed with 4% PFA and marked with Phalloidin to see the cytoskeleton. Nuclei were stained with DAPI and glasses were mounted with an aqueous mounting medium. Slides were observed at different magnifications and images acquired with the Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany).

2.2.9 Confocal Microscopy

The emission signals produced by the fluorochromes, with which the EVs were marked, were observed using an inverted microscope equipped with a confocal system high speed A1 R MP (Nikon, Minato, Tokyo, Japan). With the conventional microscopy, all points of the observed sample are detected simultaneously. In confocal microscopy, the image is obtained by the successive scanning of all the points of the observed field. As a result, a better spatial resolution is obtained, mainly thanks to the delimitation of the focal plane of the image and to a reduced background noise caused by out-of-focus signals, accompanied by a notable increase in contrast. The narrow delimitation of the focal plane of the image and the possibility of varying this plane allow the successive collection of images coming from different planes of the sample, and therefore the observation of thick preparations by means of their optical sectioning. The final three-dimensional image derives from the electronic summation of the single images.

The confocal system used is equipped with different laser sources: Argon laser emitting at 457nm, 476nm, 488nm and 514nm; DPSS laser emitting at 561 nm; two diode lasers emitting at 405 nm and at 640nm.

The sources with λ of 405nm for DAPI, λ of 488nm for FITC and λ of 561nm for TRITC were used for the excitation of the three fluorochromes used in the fluorescence assays described in the previous paragraph.

The emission signal is detected by means of single-channel phototubes with filters that collect the light signal in different ranges of spectral wavelengths (± 25 nm with respect to a central wavelength).

The emission filters used have the following central wavelength λ: 450 nm, 525 nm and 595 nm, respectively for DAPI, FITC and TRITC, respectively.

2.2.10 Statistical analysis

All statistical analysis was performed using one-way ANOVA. Error bars indicate standard deviation (S.D.). A p-value ≤ 0.05 was considered to be statistically significant. GraphPad software was used.

2.3 Results

2.3.1 Secretome profile of PA-hBMSCs: cells grown in serum-free medium showed expression of several cytokines involved in immunomodulation

The cells were cultured in FBS or serum-free medium, until reaching a confluence of about 70%. At this point, the growth medium was replaced with serum-free medium (only α-MEM containing 1% Penicillin/Streptomycin), after five washes with sterile PBS 1X, to remove any serum or supplement residues. The cells were still cultured under these conditions for 24h, parallel in normoxia or hypoxia (1% O2).

The CM obtained were analyzed by Cytokine Array (R&D). The results showed significant differences in the protein content of the conditioned media, with an over-expression of Chitinase-3-like-1 (CHI3L1) in FBS condition (both nìin normoxia and hypoxia) and, on the other side, an over-expression of proteins like angiogenin, pentraxin-3, DKK-1, thrombospondin-1 and VEGF in serum-free condition (*Fig.14*). In almost all cases, hypoxic treatment did not affect in a very significant way the expression of the various cytokines: we noticed that thrombospondin-1, DKK-1 and uPAR decreased in SF-hBMSC-CM_{HYPO} but they remain much higher than in FBS-hBMSC-CM.

CHI3L1, a carbohydrate-binding lectin with a preference for chitin, plays a role in regulation of cell proliferation, adhesion, migration and activation, as well as in tissue remodelling,[365,366] T-helper cell type 2 (Th2) inflammatory response and IL-13-induced inflammation, regulation of allergen sensitization, inflammatory cell apoptosis, dendritic cell accumulation and M2 macrophage differentiation.[367] In addition, CHI3L1 stimulates the production of inflammatory mediators (e.g. CCL2, CXCL2, MMP-9)[368] serving as a pro-inflammatory biomarker.[369] This protein is highly expressed in conditioned media of MSCs cultured in vitro in the presence of FBS.[370]

We observed that in the media of PA-hBMSCs cultured in serum-free condition there are proteins with relevant biological roles which are modulated significantly. This modulation could have important implications from a therapeutic point of view. For instance, pentraxin 3 (PTX3) has a role in inflammation and immune response by interacting with complement components, thus modulating complement activation. PTX3 regulates complement-driven macrophage-mediated tumor progression, acting as an extrinsic oncosuppressor in preclinical models and selected human tumors.[371–373] PTX3 play also an important role in modulating the cardiovascular system[374] and in in bone homeostasis and in proper matrix mineralization during fracture repair supporting the maintenance of the bone mass possibly by inhibiting fibroblast growth factor 2 (FGF2) and its negative impact on bone formation.[375]

Finally, we observed high expression of some angiogenic proteins like angiogenin, a member of the vertebrate-specific, secreted RNASE superfamily which induces vascularization of normal and malignant tissues[376] and VEGF. VEGF is involved in angiogenesis, endothelial cell proliferation, survival and fenestration[377,378] and in the signaling and maintenance of non-endothelial cells, as well as in migration and invasion into the basement membrane, proliferation, survival.[379] VEGF has been involved in many other physiological and pathophysiological process including wound healing and bone repair.[380] VEGF has the capacity to promote both osteogenic and angiogenic differentiation[381] and has been used to enhance bone regeneration due to its promoting effects on angiogenesis, cell migration and mineralization.[382]

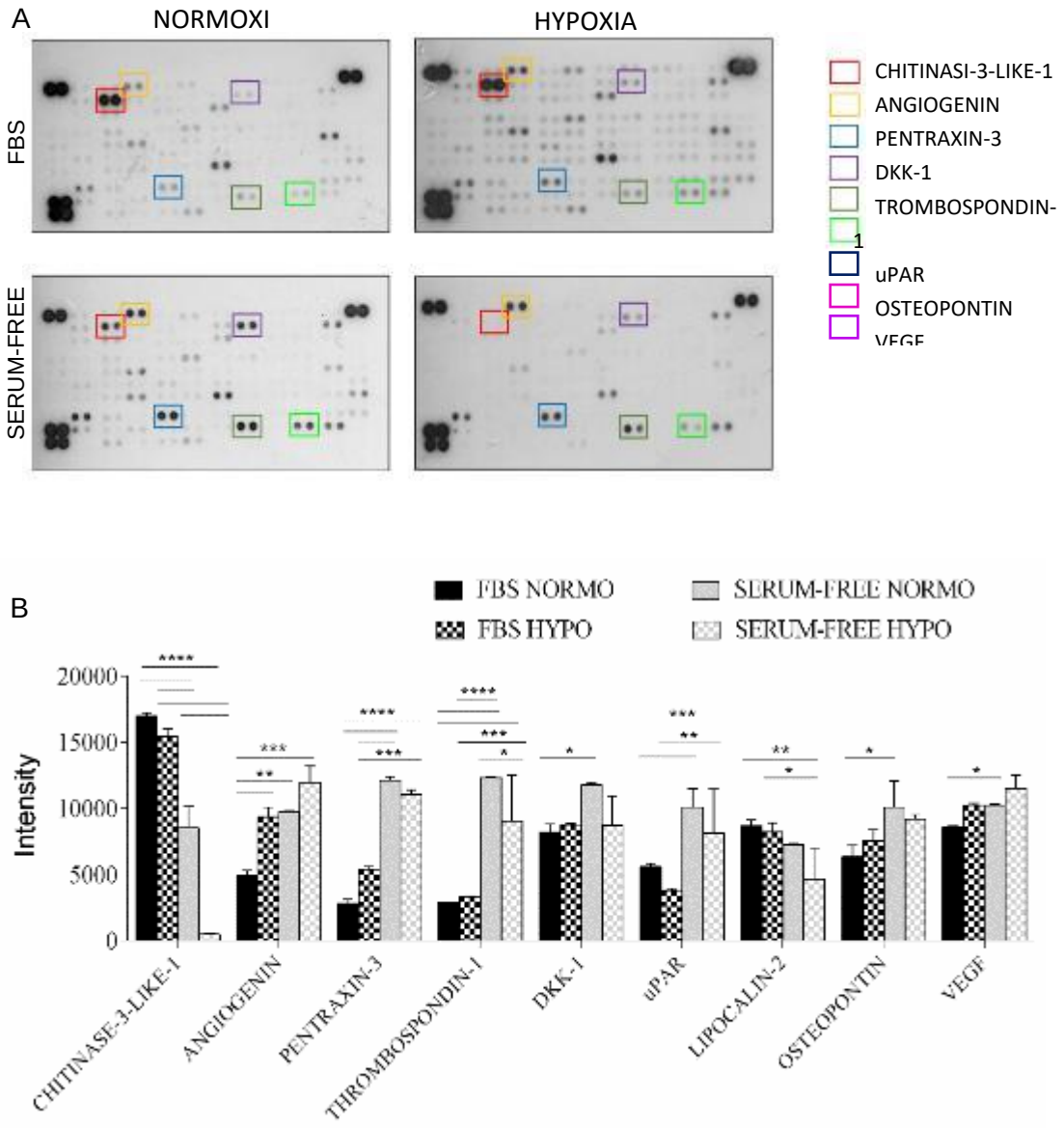

Figure 14 A) Cytokine array assay membrane of PA-hBMSCs-CM (FBS-CM and SF-CM) in normoxic and hypoxic conditions.

B) Quantification of the integrated pixel density for each identified cytokine in the four conditions tested with Fiji ImageJ. Error bars represent S.D. (*) P value < 0.05, (**) P value < 0.01, (***) P value < 0.001, (****) P value < 0.0001, Two-way ANOVA.

2.3.2 Gene Ontology: PA-hBMSCs grown in serum-free medium were more rich in cytokines involved in homeostasis, immune response and wound healing

Gene Ontology (GO) is an enrichment analysis that allows evaluation of the relative frequency of ontology terms (i.e. biological functions) in the proteomic profile of interest. This allows the identification of ontology terms that occur more frequently in each data set when compared with a reference protein dataset. The ontology is divided into three areas: Cellular Component (CC, the parts of a cell or its extracellular environment), Molecular Function (MF, the elementary activities of a gene product at the molecular level, such as binder or catalysis), Biological Processes (BP, operations or complexes of molecular events with a defined beginning and end, relevant to the functioning of integrated living units: cells, tissues, organs and organisms). [383,384]

For our analysis in GO we used DAVID (Functional Annotation Bioinformatics Microarray Analysis), focusing on biological processes. For each of the 4 secretomes we identified several ontologies. *Table 1* shows the ontologies related to the proteins involved, with the relatives p values and the percentage of representation. FBS-hBMSCs-CM exhibited a panel of cytokines involved mainly in defense and immune response, but, although to a lesser extent, also secretion and angiogenesis. SF-hBMSCs-CM showed secretion of proteins influencing immune response (PTX-3, TSP-1), angiogenesis (VEGF, ANG, TSP-1), homeostatic process (VEGF, OPN, ANG) and wound healing (TSP-1, uPAR, OPN).

	CHI3L1	ANG	PTX3	TSP-1	DKK-1	uPAR	NGAL	OPN	VEGF	Count	%	*P*
RESPONSE TO EXTERNAL STIMULUS	*	*	*	*		*	*	*	*	8	88.9	3.3E-6
DEFENSE RESPONSE	*	*	*	*			*	*		6	66.7	2.8 E-4
POSITIVE REGULATION OF METABOLIC PROCESS	*	*		*		*	*		*	6	66.7	5.2 E-3
REGULATION OF PHOSPHORI-LATION	*	*		*	*	*			*	6	66.7	1.8 E-4
REGULATION OF RESPONSE TO STRESS				*		*		*	*	4	44.5	1.9 E-2
ANGIOGENESIS	*	*		*					*	4	44.5	7.7 E-4
ENDOCYTOSIS			*	*	*				*	4	44.5	3.1 E-3
SECRETION	*	*		*					*	4	44.5	1.2 E-2
HOMEOSTATIC PROCESS		*					*	*	*	4	44.5	3.7 E-2
IMMUNE RESPONSE		*	*	*			*			4	44.5	3.2 E-2
RESPONSE TO WOUNDING				*		*		*		3	33.3	2.1 E-3
RESPONSE TO OXYGEN LEV-ELS		*		*					*	3	33.3	9.2 E-3
NEGATIVE REG-ULATION OF CELL DEATH				*		*			*	3	33.3	1.1 E-1
CELL MIGRA-TION		*		*					*	3	33.3	1.1 E-1

Table 1 Gene onthology analysis: Biological processes identified by the cytokine clusterization involved from three to nine of the selected cytokines. CHI3L1: Chitinase-3-like-1; ANG: Angiogenin; PTX-3: Pentraxin-3; TSP-1. Thrombospondin-1; DKK-1: Dickkopf WNT Signaling Pathway Inhibitor 1; uPAR: Plasminogen Activator, Urokinase; NGAL: Lipocalin-2; OPN: Osteopontin; VEGF: Vascular endothelial growth factor.

2.3.3 Hypoxic conditioning of PA-hBMSCs enhances the release of EVs

The cargo and function of EVs depend on their cells of origin, suggesting that intercellular communication through vesicles is a dynamic system, adapting its message depending on the conditions of the producing cells.[354–356,359,385] Changes in oxygen concentrations affect many of the distinctive characteristics of stem and progenitor cells.[359,386] On this basis, we evaluated whether hypoxic conditioning of human bone marrow stromal cells could influence their EVs secretion. Sub-confluent primary PA-hBMSCs cultured in serum-free or FBS-containing medium were maintained for 72 hours in starvation in a normoxic or hypoxic environment. After the starvation period, to exclude any negative effect on cell viability, we performed a cytofluorimetric assay, using propidium iodide (PI) and a FITC-anti-annexin-V antibody, as described in chapter 1 (*Par.2.5*), so we compared by double staining the percentage of apoptotic cells in the four cell culture conditions, each one in normoxia or hypoxia. We observed that about 60% of FBS-hBMSCs resulted viable in both culture conditions (normoxia and hypoxia), but interestingly SF-hBMSCs showed a stronger resistance to starvation and to hypoxia, exhibiting a share of living cells greater than 90% (*Fig.15A*)

The CM collected were subjected to differential centrifugations and ultra-centrifugations, to obtain a pellet containing exosomes, which was then resuspended in filtered PBS 1X. BCA assay was performed to evaluate the protein content on the EVs surface. Hypoxic preconditioning led to a significant enrichment of proteins on the PA-hBMSC-EVs surface in both FBS and SF groups ($1.11 \pm 0.24/10^6 cells_{FBS}$ and $1.63 \pm 0.08/10^6 cells_{SF}$) with almost two-fold increase compared to normoxic cells ($0.54 \pm 0.12/10^6 cells_{FBS}$ and $1.05 \pm 0.22/10^6 cells_{SF}$ *Fig.15B*).

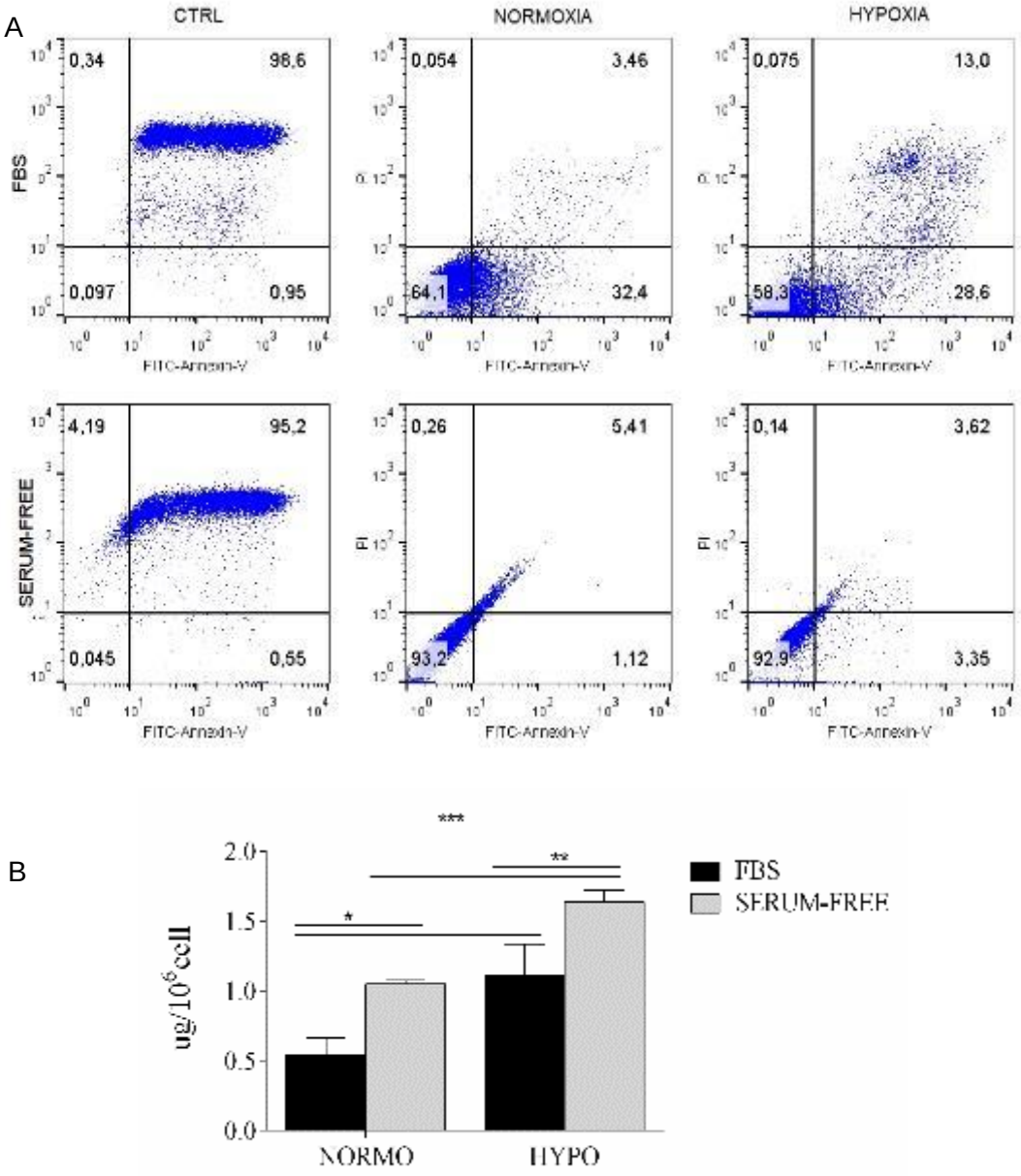

Figure 15 A) Representative flow cytometry analysis of control PA-hBMSCs (dead cells) and PA-hBMSCs after 72 hours starvation in both normoxic (20%) and hypoxic (1%) culture conditions after staining with Annexin V and PI.

B) BCA assay of the protein concentration of PA-hBMSC-EVs$_{HYPO}$ and hBMSC-EVs$_{NORMO}$ released by 10^6 cells. Error bars represent S.D. (*) P value < 0.05, (**) P value < 0.01, (***) P value < 0.001, Two-way ANOVA.

2.3.4 Hypoxic conditioning of PA-hBMSCs promotes the secretion of smaller vesicles

To detect the right particle concentration and the size distribution, FBS-hBMSC-EVs$_{NORMO/HYPO}$ and SF-hBMSC-EVs$_{NORMO/HYPO}$ were further analyzed via TRPS using the qNano (Izon Science, Oxford, UK). Three measurements were run for each sample, in triplicate. We observed a shift in EV size distribution after hypoxic pre-conditioning, in both serum-free and FBS cells.

In normoxic conditions, the ultracentrifuged pellet contained hBMSC-EVs between 100 and 280nm (SF-hBMSC-EVs$_{NORMO}$) or 120 and 360nm (FBS-hBMSC-EVs$_{NORMO}$) (*Fig.16A*), thus including both exosomes and microvesicles. Both FBS-hBMSC-EVs$_{NORMO}$ and SF-hBMSC-EVs$_{NORMO}$ particle dimension showed average size of 160-180nm, likely due to clustering effects previously described for other progenitor-derived EVs.[387]

In hypoxic conditions, instead, the size range of hBMSC-EV was lower, between 80 and 240nm (*Fig.16A*), in both FBS and SF cells including also in this case both exosomes and microvesicles. Both FBS-hBMSC-EVs$_{HYPO}$ and SF-hBMSC-EVs$_{HYPO}$ particle dimension showed average size of 120nm.

The analysis showed a reasonably high number of particles/cell in all the four conditions with almost two-fold increase in SF-derived EVs 303 ± 57.94 particle/cell$_{SF\text{-}NORMO}$ and 328 ± 23.64 particle/cell$_{SF\text{-}HYPO}$) compared to FBS cultured cells (150 ± 3.77 particle/cell$_{FBS\text{-}NORMO}$ and 195 ± 19.03 particle/cell$_{FBS\text{-}HYPO}$, *Fig.16B*). Instead, contrary to what we have seen with BCA quantification (*Fig.9C*), we did not observe any significant differences in particle number between normoxic and hypoxic vesicles in both culture conditions (FBS and SF). Ratio between the number of particles for cell and protein content underlined that in both FBS- and SF-hBMSC-EVs, the normoxic pre-conditioning did not induce a greater secretion of vesicles, but rather a protein enrichment (*Fig.16C*).

SEM analysis also remarked the evidence according to which hypoxic pre-conditioned PA-hBMSCs secreted smaller vesicles in both culture conditions (FBS and SF), although SEM did not allow us to rightly discriminate between microvesicles and exosomes (*Fig.17*).

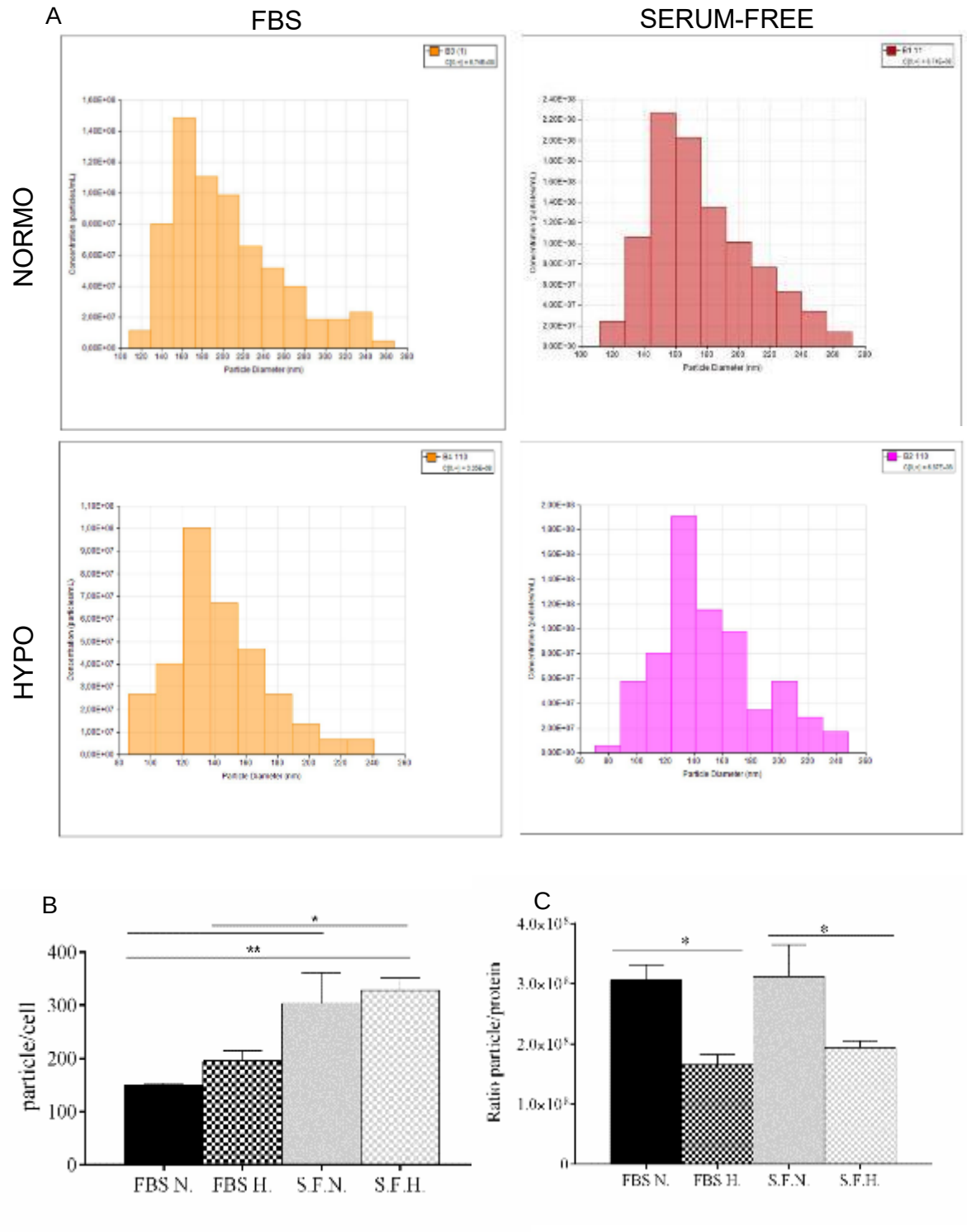

Figure 16 A) Tunable Resistive Pulse Sensing (TRPS) analysis measuring size distribution of FBS-hBMSC-EVs and SF-hBMSC-EVs under normoxia ad hypoxia.

B) Tunable Resistive Pulse Sensing (TRPS) analysis measuring number of particles within FBS-hBMSC-EVs and SF-hBMSC-EVs expressed as particles/cell. Error bars represent S.D. (*) P value < 0.05, (**) P value < 0.011, Two-way ANOVA.

C) Ratio particle/protein for single cell. Error bars represent S.D. (*) P value < 0.05, Two-way ANOVA.

FBS N.: FBS-Normoxia; FBS H.: FBS-Hypoxia; S.F. N.: Serum-free Normoxia; S.F. H.: Serum-free hypoxia

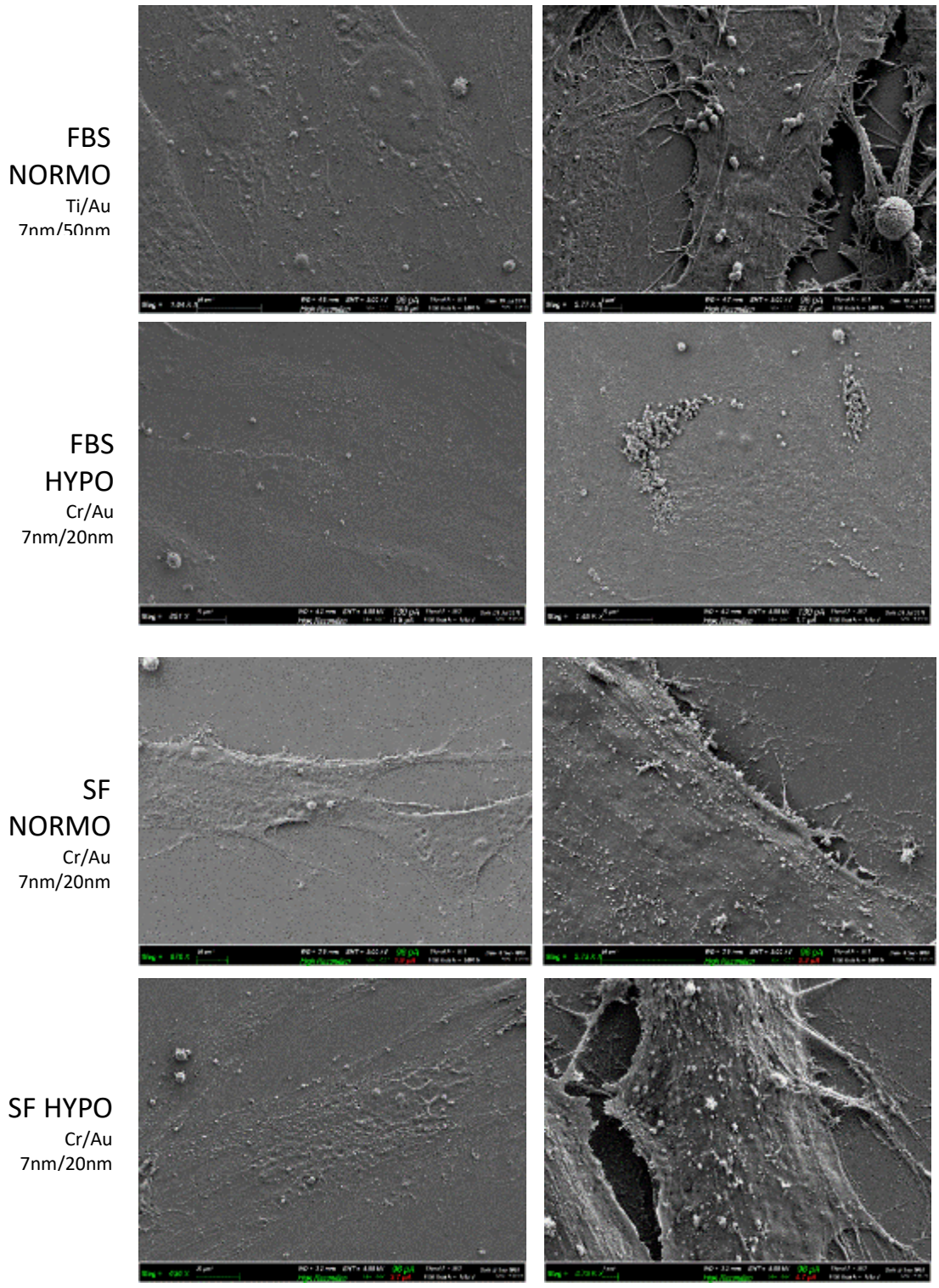

Figure 17 Scanning electron microscopy (SEM) analysis of FBS- and SF-hBMSCs after 72 hours of starvation in normoxia (20% O_2) and hypoxia (1% O_2).

2.3.5 PA-hBMSCs secrete CD105+/CD63+ EVs

In order to characterize isolated EVs, immunoblot and flow cytometry analysis were performed. Western blot analysis revealed that both FBS- and SF-hBMSC-EVs express the specific vesicular proteins CD81 and CD9, members of the tetraspanin family, principally in hypoxia, while the heat shock protein Hsp70 appeared to be present only in FBS-hBMSC-EVs under both normoxic and hypoxic conditions, although it is more expressed under hypoxia (Fig.18C).

FBS- and SF-hBMSC-EVs were further characterized taking advantage of a multiparametric flow cytometry approach. To separate true events from background noise, EVs were defined as events that were included in a dimensional gate which encloses events until 0.88µm, which was established according to a well-defined light scatter profile of beads with absolute size (*Fig.18A*). EVs were tagged with the Cell Trace labeling, an analogue of CFSE: it is a cytoplasmic fluorescent dye, which acts as marker of vesicular integrity. This allowed us to consider only intact membrane structures. We stained Evs with either the mesenchymal marker CD105 or the vesicular marker CD63. All types of Cell Trace labeled-EVs expressed the CD105 and CD63 antigens, without significant differences (*Fig.18B*). However, the preparation derived from cells grown in serum-free was cleaner from debris and larger bodies (grey dots in the picture).

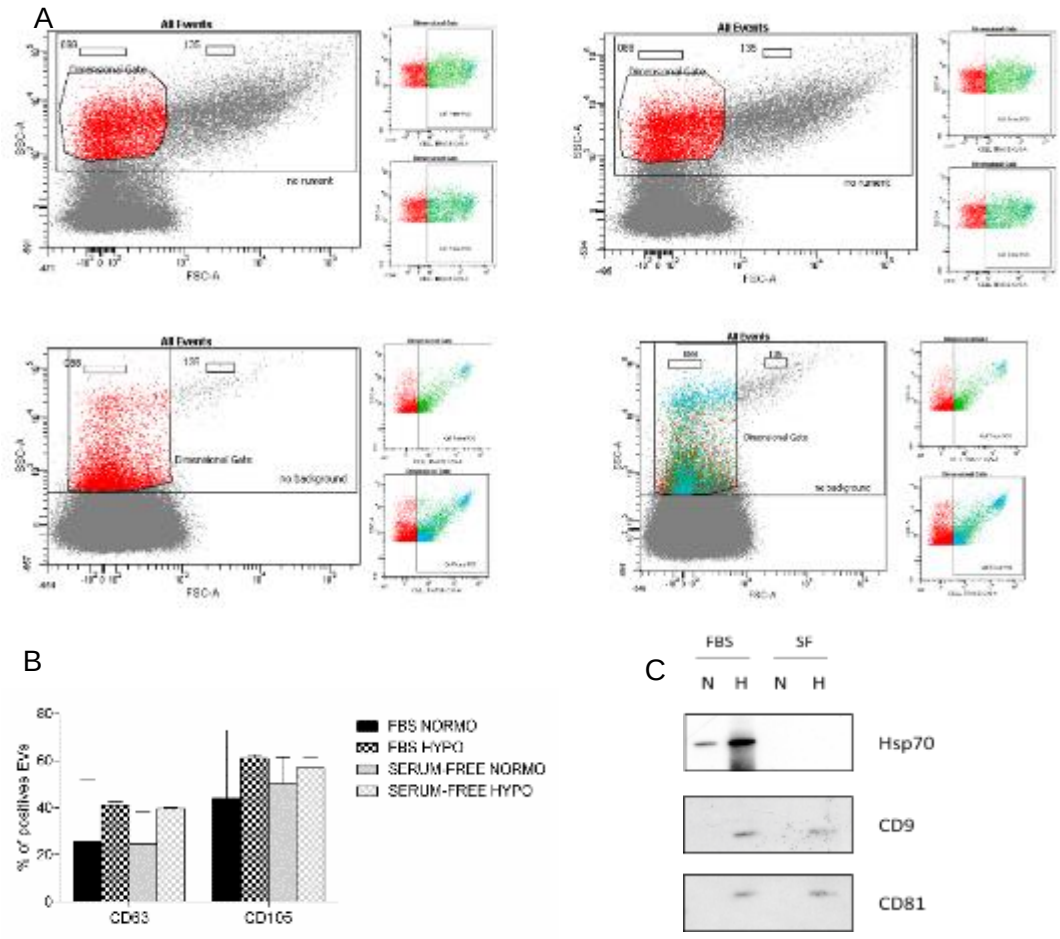

Figure 18 A) Flow cytometry strategy adopted to characterize FBS- and SF-hBMSC-EVs. Vesicles were stained by the lipophilic dye Cell Trace, the vesicular marker CD63 and the MSC marker CD105.

B) Histogram showing the percentage of Cell Trace-pos EVs co-expressing CD63 or CD105. Data are presented as mean ± SD.

C) Representative Western blot analysis for EV specific markers (CD81, CD9 and Hsp70) in the four experimental groups.

2.3.6 Biological effect of hBMSC-EVs on cartilage

Tissue homeostasis is largely influenced by the immune system. Following tissue damage, it is crucial the immune system activation to eliminate dead or damaged cells. However, it is equally important that its activation be attenuated after the resolution in order to have a right damage recovery. Prolonged immune activation beyond the resolution of injury or disease will result in excessive tissue damage or progress to an immune dysfunction disease.[135] Therefore, immune homeostasis is a critical component of tissue homeostasis.

In the case of cartilage injury, the rapid upregulation of pro-inflammatory cytokines including IL-1, IL-6 and IL-8, and MMP-3 contributes to subsequent matrix degradation and joint damage. These pro-inflammatory cytokines and MMPs contribute to the onset and development of Osteoarthritis (OA). MSCs have been widely documented to exert suppressive and regulatory immunomodulatory effects on both adaptive and innate immune cells in an autologous and allogeneic manner.[388–392] MSCs have been intensively investigated as a cell-based therapy or in combination with scaffold in a tissue engineering approach to treat cartilage lesions and OA in both animal and human studies. MSC exosomes was recently reported to mediate cartilage repair and regeneration.

To evaluate the role exerted by EVs in cartilage repair, we began characterizing the interactions of EVs with recipient cells. We tested whether human articular chondrocytes (hACs) were able to internalize both FBS-hBMSC-EVs and SF-hBMSC-EVs incubating them for 3 hours in presence of EVs previously stained with the fluorescent lipophilic membrane-diffuse dye PKH67. hACs efficiently internalized EVs within their cytoplasm (*Fig.19*).

To be sure that EVs were actually internalized and not just stuck on the cell surface, we performed a further analysis by confocal microscopy (*Fig.20A*). The three-dimensional reconstruction demonstrated that hACs really uptook EVs, which were localized around the nucleus, at a similar distance in all the conditions (*Fig.20B/C*). However, calculating the volumetric density (EVs/mm^3), it emerged that SF-hBMSC-EVs were mostly internalized by hACs respect to FBS-hBMSC-EVs, especially those derived from hypoxic cells (*Tab.2*).

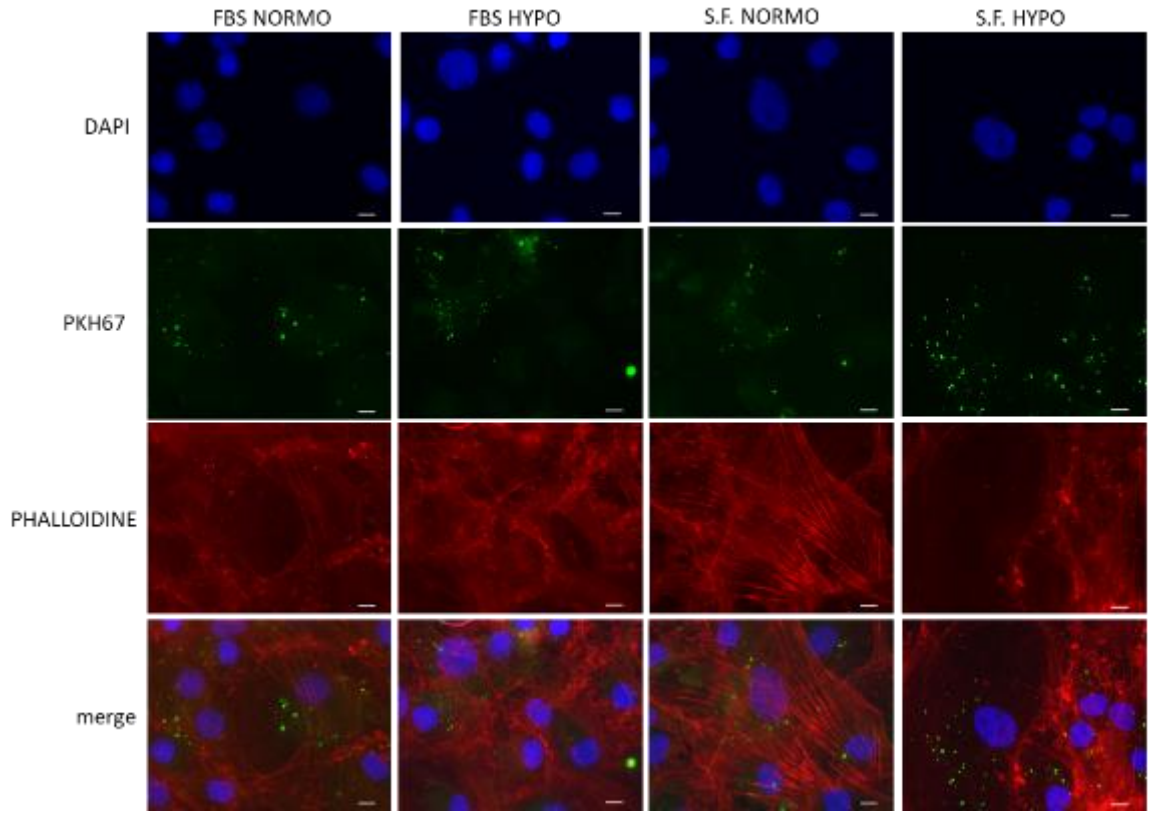

Figure 19 Immunofluorescence analysis of hACs cocultured for 3 hours with normoxic and hypoxic FBS- and SF-derived EVs labelled with PKH67 (EVNormo and EVHypo). DAPI (blue), phalloidin (red), PKH67 (green). Magnification 63X. Scale bar 20μm.

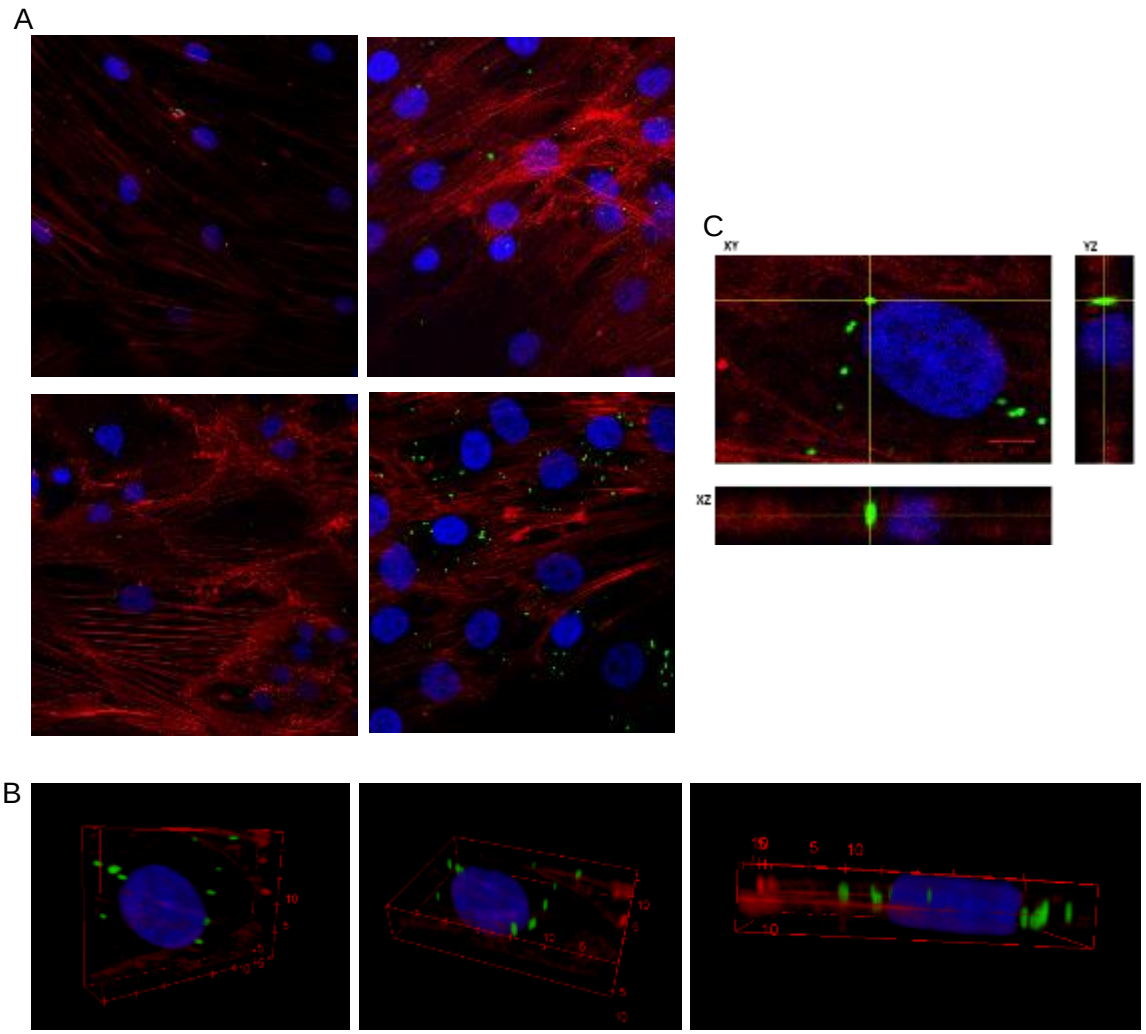

Figure 20 A) Confocal microscopy of of hACs cocultured for 3 hours with normoxic and hypoxic FBS- and SF-derived EVs labelled with PKH67 (EVNormo and EVHypo). DAPI (blue), phalloidin (red), PKH67 (green).
B) Three-dimensional reconstruction.
C) Localization of EVs around the nucleus of recipient cells.

	Average distance (μm)	Volumetric density (n°EVs/mm$^{3)}$
FBS NORMO EVs	12.7	$7*10^5$
FBS HYPO EVs	13.1	$7.5*10^5$
SF NORMO EVs	12.5	$8*10^5$
SF HYPO EVs	12.7	$13.5*10^5$

Table 2 Quantitative analysis based on confocal microscopy.

2.3.7 Characterization of 3D-FBS-MSCs-derived EVs

We received hBMSCs cultured in presence of FBS (3D-FBS) using the Mobius Bioreactor and agreed Autostem protocols (module 1) from Aston University. The cells were thawed and cultured until a confluence of about 70% was reached. At this point, the growth medium was replaced with basal medium (α-MEM containing 1% Penicillin / Streptomycin) after washing with sterile PBS 1X five times to remove any serum or supplement residues (serum may contain vesicles and it could be a potential source of contamination). To mimic the typical environment established during tissue injury, EVs were isolated from the conditioned medium of MSCs harvested under both normoxic and hypoxic conditions. The cells were maintained for 72h in starvation. The conditioned media obtained were subjected to differential centrifugation and ultra-centrifugation, to obtain a pellet containing extracellular vesicles, which was then suspended in filtered PBS (as previously described). The preparation of vesicles was first quantified by BCA assay (*Fig.21A*). Protein quantification has allowed us to note bioreactor culture condition caused a slight not-significant increase in protein content compared to plastic adherent culture.

To detect the right particle concentration and the size distribution, PA-FBS-EVS and 3D-FBS-EVs were further analysed via Tunable Resistive Pulse Sensing (TRPS) using the qNano (Izon Science, Oxford, UK). Three measurements were run for each sample.

We observed a difference in EV size distribution, since PA-FBS-EVs contained vesicles between 120 and 360 nm (*Fig.21B*), thus including both exosomes and microvesicles, while 3D-FBS-EVs contained also higher vesicles, up to 1 μm, likely due to clustering effects previously described for other progenitor-derived EVs.

The analysis showed a reasonably high number of particles/cell in both conditions with almost ten-fold increase in 3DhBMSC-EVs (244.5 ± 7.77 particle/cell) compared to PA-FBS cultured cells (14.95 ± 0.35 particle/cell, *Fig.21C*).

Ratio between the number of particles for cell and protein content underlined that in both plastic adherent and 3D FBS cultured cells the protein content is the same (*Fig.21D*).

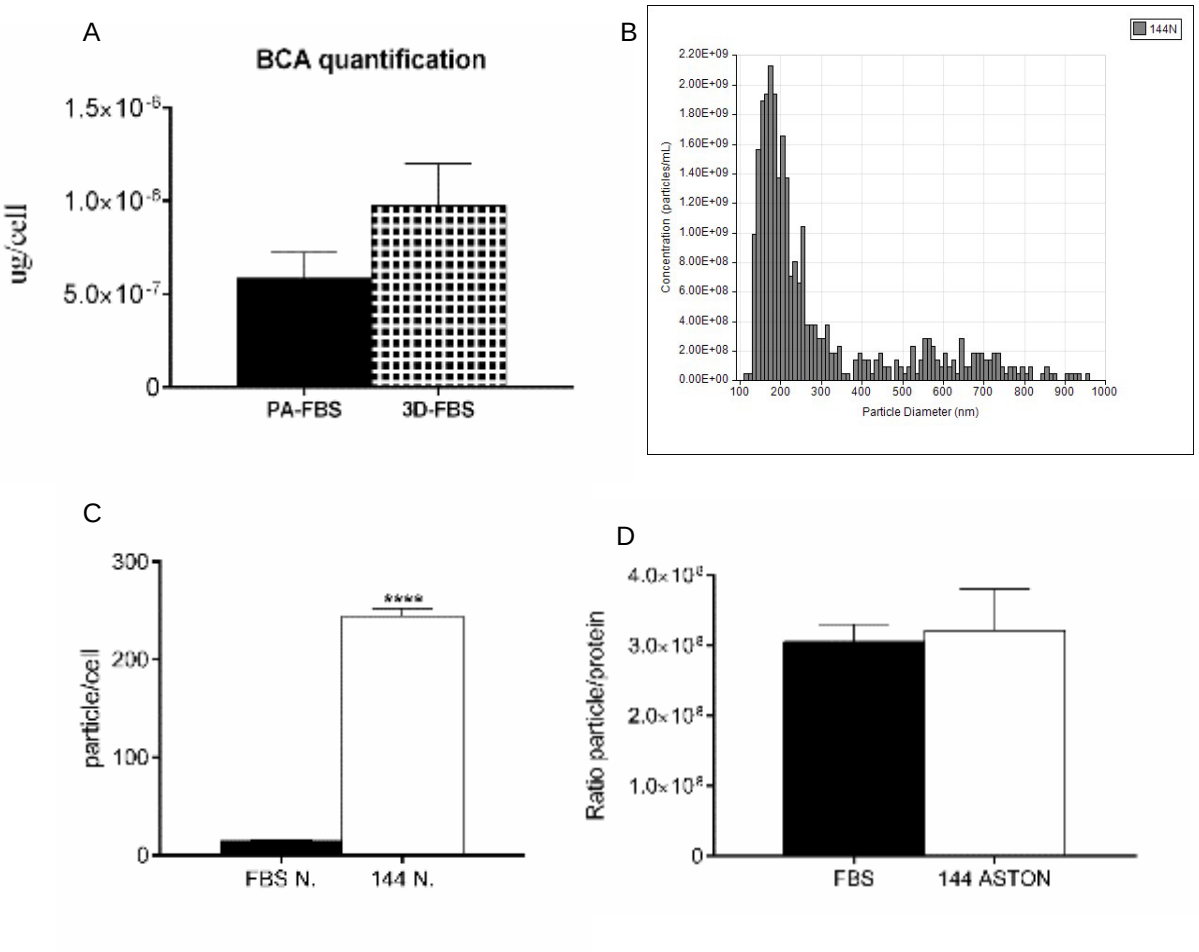

Figure 21 Characterization of 3D-hBMSC-EVs
A) Protein quantification by BCA assay.
B) Tunable Resistive Pulse Sensing (TRPS) analysis measuring size distribution of PA-FBS-EVs and 3D-FBS-EVs. Error bars represent S.D. (****) P value < 0.0001, t-test.
C) Ratio particle/protein for single cell. Error bars represent S.D. t-test.

2.4 Discussion

Regenerative medicine therapies, fueled by advances in stem cell biology and technologies, seek to direct non-healing injuries towards full restoration of tissue structure and subsequent function. Numerous studies have demonstrated that mobilization of endogenous stem cells or exogenous administration of a number of stem cell populations to injured tissues has resulted in structural regeneration of tissue as well as functional improvement.[92]

The use of stem cells and particularly Mesenchymal Stem Cells (MSCs) in clinical practice has increased considerably in the last decade. During this time, the scientific community has tried to understand their biological mechanisms of action in tissue repair and regeneration and unveil their potential in cell therapy and regenerative medicine.[393,394] While the original hypothesis underlying stem cell regenerative therapies was based on functional recovery as a consequence of stem cell differentiation,[393,395,396] it is now clear that other mechanisms of action are at play. A recent paradigm shift has emerged suggesting that the biomolecules synthesized by stem cells may be as important, if not more so, than differentiation of the cells in eliciting functional tissue repair. In essence, it has been proposed an alternative mechanism called paracrine effect, where MSC secrete biologically active molecules that exert beneficial effects on injured tissues[164] by promoting angiogenesis and tissue regeneration and inhibiting fibrosis, apoptosis and inflammation.[397,398] It has also been shown that they have neurogenic, neuroprotective and synaptogenic effects.[399,400] Since the survival and differentiation of MSCs at the site of the lesion is limited, it is proposed that paracrine signaling is the primary mechanism of their therapeutic effects.[401] This hypothesis is supported by *in vitro* and *in vivo* studies showing that many cell types respond to paracrine signaling from MSCs, causing the modulation of a large number of cellular responses, such as survival, proliferation, migration and gene expression.[397]

The secretion of bioactive factors is thought to play a critical role in MSC mediated paracrine activity. These factors and cytokines may be collected in what has been called the conditioned medium (CM), which when transplanted into animal models of different diseases have similar effects to those exerted by the cells, increasing the tissue repair process in acute myocardial infarction,[402,403] wound healing[402,403] and as a neuroprotective agent.[404]

In this study, we characterized CM of hBMSCs cultured in a novel serum-free medium, to produce cells and cellular products ready for the clinical usage, without animal contaminations. We observed that PA-hBMSCs grown in FBS-containing medium appeared richer of proteins like Chitinase-3-like-1 (CHI3L1). The glycoprotein CHI3L1, also known as YKL-40, is a secreted chitinase family protein that has been implicated in tissue remodeling, angiogenesis, and cell survival in both normal and pathological conditions.[405–408] YKL-40 protein is synthesized primarily by classically activated macrophages,[409] neutrophils,[365] *ex vivo* cultured chondrocytes,[410] end stage osteoblasts and primary osteocytes,[411] osteoblasts treated with TNF-α or IL-1,[412] and in lower amounts by other tissues of mesenchymal origin. MSCs differentiate into a number of somatic tissues that express YKL-40, and changes in YKL-40 expression have been noted in differentiation accompanying fetal development in osteogenic and chondrogenic cell lineages.[413] It has

been demonstrated also that undifferentiated MSCs contain significant levels of YKL-40 mRNA but do not synthesize detectable levels of YKL-40 protein. MSCs induced to differentiate into chondrocytes and osteocytes soon began to express and secrete YKL-40 protein, as do *ex vivo* cultured chondrocytes and primary osteocytes.[414] YKL-40 has been proposed also as a new marker during adipogenic differentiation of MSCs.[415] All these findings together led us to suppose that FBS grown PA-hBMSCs could be more committed than cells cultured in serum-free medium, which appeared indeed more undifferentiated.

On the other hand, PA-hBMSCs grown in serum-free medium exhibited over-expression of several bioactive molecules, involved in angiogenesis, tissue homeostasis and remodelling, immunomodulation and wound healing. Interestingly, we observed a two-fold increase of proteins such Pentraxin 3 (PTX3), an essential component of the humoral innate immunity,[416] involved in the resistance against microorganisms[417] and inflammation.[418] It is well established that key activators of the inflammatory and reparative response after tissue injury, such as proinflammatory cytokines and Toll-like receptors, induce PTX3 production in different cell types, including stromal cells.[416,419,420] Moreover, the secretome of adipose tissue derived MSCs has been showed to contain high levels of PTX3 during the early stages of mesengenic differentiation.[421] Another study has shown that PTX3 plays a protective role in the regulation of tissue repair and remodelling.[422] After this finding, using a murine model of skin repair, Capuzzello et al. found that Ptx3-deficient (Ptx3-/-) MSCs delayed wound closure and reduced granulation tissue formation compared with wt-MSCs, demonstrating an essential role of MSC-derived PTX3 in wound repair.

In our study, also a three-fold increase of Thrombospondin-1 (THBS1) emerged in serum-free cultured cells respect to those grown in FBS-containing medium. The main known function of the matricellular protein THBS1 is its anti-angiogenic and anti-inflammatory effect in various models, mainly in cancers and cardiac diseases.[423] In experimental models of infarction, THBS1 was highly upregulated and THBS1−/− mice exhibited inflammatory infiltrates into the infarcted heart.[424] In recent studies it was demonstrated another crucial role of THBS1, its chondroprotective role. Indeed, using two rat models of osteoarthritis, THBS1 was shown to induce regeneration of damaged cartilage.[425] Similar results were described in other inflammatory models of arthritis: in an experimental model of rheumatoid arthritis injection of a peptide-derived from THBS1 prevented joint infiltration and inflammation and was associated with increased levels of connective tissue growth factor (CTGF).[426,427] In rheumatoid arthritis patients, altered expression levels and tissue distribution of THBS1 was observed.[428] In a recent study, it was shown that coculture of adipose tissue MSCs and chondrocytes had a significant induction of THBS1 at mRNA and protein levels. They showed that THBS1 was upregulated at late stages of MSC differentiation toward chondrocytes and that recombinant THBS1 (rTHBS1) exerted a pro-chondrogenic effect on MSCs indicating a role of THBS1 during chondrogenesis. THBS1 exercised also an anti-inflammatory and chondroprotective function in OA animal model.[429]

Our data showed also a slight but anyhow significant increase of factors like Dickkopf-related protein 1, urokinase-type plasminogen activator receptor and osteopontin.

Dickkopf-related protein 1 (DKK-1) is a member of the DKK family and it is widely expressed in many areas including osteoblasts and osteocytes as well as the skin, prostate,

placenta and endothelium.[430] This protein is seen as a well-characterized antagonist of the canonical Wnt signaling. Some studies have shown that activation of the Wnt pathway is associated with cell proliferation and apoptosis inhibition in tumor cells, and suppression of the pathway is associated with apoptosis and growth inhibition.[431] Moreover, dysregulation of Wnt signaling has been involved in the pathogenesis of many diseases such as cancers, bone disorders, autoimmune diseases, diabetes and Alzheimer disease.[431–436] Therefore, considering DKK-1 as a treatment target for these related diseases would be a beneficial strategy. It has been reported that loss-of-function mutations in the Wnt co-receptor, LRP5, can lead to osteoporosis, this report further indicated that there was a correlation between the Wnt signaling pathway and bone biology.[437,438] The Wnt signaling has been shown to participate in bone morphogenesis and homeostasis by regulating the process of mesenchymal cell differentiation into mature osteoblasts; thus, it might play a crucial role in the anabolic component of joint remodelling observed in osteoarthritis.[434,439,440]

The multifunctional urokinase receptor (uPAR) is an important mediator of the anaphylatoxin C5a/C5a receptor (C5aR) complement cascade controlling C5aR expression, C5aR-directed signaling, and related functional effects in kidney mesangial cells and in alveolar macrophages *in vitro* and *in vivo*.[441,442] Our recent findings revealed one further novel function of uPAR as a regulator of differentiation and mobilization of bone marrow-derived MSCs and of their engraftment at the place of injury.[443] MSC, which are adult stem cells retaining self-renewal capability and unique multilineage potential,[444] have emerged as the most promising candidate for bone repair.

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), is a soluble protein that is present in most body fluids. Although OPN may exist intracellularly as a regulator of cytoskeleton dynamics and gene expression, most studies have focused on the secreted form. Extracellular OPN functions through its interactions with multiple ubiquitously-expressed cell surface receptors, including various integrins (αvβ1, αvβ3, αvβ5, α4β1, α8β1, and α9β1) and CD44.[445] Binding of OPN to these receptors can elicit a broad range of functions, such as cell adhesion, survival, migration and immune regulation.[445] Aberrant expression of OPN has been implicated in numerous physiological and pathophysiological processes, including bone remodeling,[446] responses to stress,[447] wound repair,[448] tumor metastasis[449] and autoimmune disorders.[450] OPN is abundantly secreted by MSCs and can be further up-regulated during the osteogenic differentiation of these cells.[451] However, little is known about its role in regulating MSC differentiation.

Our data have therefore shown that PA-hBMSCs grown in serum-free medium could have a chondroprotective, anti-inflammatory effect and they could represent a potential therapy strategy for diseases such as osteoarthritis and for bone repair, since they secreted bioactive molecules involved in these processes, as it also emerged from the gene onthology analysis. Over-expressed angiogenic factors were also founded in conditioned medium of these cells and this could explain their higher osteogenic potential both *in vitro* and *in vivo*.

This study focused on paracrine functionalities of PA-hBMSCs cultured in novel xeno-free culture system for clinical therapy. Thereafter, we characterized also the extracellular vesicles (EVs) secreted by these cells in the two different culture conditions.

EVs represent novel players in various cell communication systems, being involved in the regulation of many routes of signaling pathways and intercellular information

transfer.[95] It is thanks to their vast amount of properties that EVs have been successfully applied in different fields, such as tumor biology, immunology and regenerative medicine.[452] Stem/progenitor cells and in particular MSCs are active biological components of many regenerative medicine therapies.[164]

The cargo and the function of the EVs depend on the cell of origin and are the mirror of its state of health. The cellular state and the microenvironment influence the secreting cell to release vesicles with different contents that can perform different functions and determine a response of various types in the recipient cell.[385] Hypoxic conditioning can change some characteristics of stem cells: growing hMSCs in hypoxia resulted in approximately 30-fold higher hMSC expansion over 6 weeks without loss of multi-lineage differentiation capabilities.[386] It has been already demonstrated that hypoxia could influence also the secretion of EVs in adipose tissue-derived MSCs[453] but also in breast cancer cells like MCF-7[454] and in endothelial cells.[455] We proved here that hypoxic pre-conditioning does not affect the number of EVs secreted, but rather the protein content or at least the amount of protein associated at the EV-surface. We saw indeed that after hypoxia, both FBS- and SF-hBMSC-EVs showed an increase of vesicular markers such as CD81 and CD9, while the EVs number remained unchanged. Interestingly, FBS-hBMSCs-EVs appeared richer in heat shock protein 70 (HSP70). Exosomal HSP70 has been recently shown to have immunomodulatory[456,457] and stress moduling activities.[458,459] In general, these molecular chaperones are usually expressed intracellularly where they support the folding and the transport of a great variety of proteins. In contrast, membrane-bound and extracellular-located HSPs act as potent danger signals.[460] Several pieces of evidence demonstrate that extracellular-located HSPs can be associated to extracellular vesicles[456,461–463] and this molecule contributed to exosome-induced IFNγ production by NK cells.[457] In tumoral cells, exosomes overexpressing membrane-bound Hsp70 have the capability to induce dendritic cells maturation and stimulate $CD4^+$T and $CD8^+$T-cell responses along with the induction of NK cell-mediated immunity in mice[464] and thus they could affect tumor survival and progression.[457]

According with some recent studies,[465] our data demostrated also that the hypoxic preconditioning increase the percentage of smaller vesicles respect to the normoxic treatment, in both cell culture conditions. This could be also due to a different clusterization of the vesicles during the analysis, probably owing to the higher protein concentration: exosomes appeared to be very sticky for their hydrophobic features, our hypothesis is that the higher protein density of EVs_{HYPO} could decrease this characteristic.

In line with the chondroprotective biomolecules founded in SF-hBMSCs-CM we observed that EVs derived from serum-free cultured cells were mostly internalized by human articular chondrocytes, in particular those derived from hypoxic cells. The EV uptake mechanisms involve protein interactions that facilitate subsequent endocytosis.[261,466–469] Numerous molecules having this function have been identified, as proteoglycans, lectins, tetraspanins. Differences in the surface protein kit could explain the different tendency in the uptake of these EVs. Further analysis must be carried out.

Taken all together, our data suggested that cellular products intended as both conditioned medium derived from PA-hBMSCs cultured in serum-free medium, could show a more pronounced osteo-chondro-protective role and they could be therefore employed for clinical therapy in cartilage repair. The new culture systems, without animal derivatives, also make the products obtained safe and ready for use in the clinic.

Ultimately, we demonstrated also that a culture in three-dimensional condition can strongly increase the number of vesicles produced by the cells, according to the literature.[470]

Considering this, it is essential to understand how 3D culture affects the quality of the EVs and it is mandatory to re-create *in vitro* the correct microenvironment that normally surrounds the cells *in vivo*, to be sure that what we are isolating is what is closest to the physiological condition.

Chapter 3: EVs derived from mesenchymal stem cells as a possible therapy for osteoarthritis

3.1 Introduction

In connective tissues, interactions between cells and the extracellular matrix (ECM) govern many aspects of tissue development, homeostasis and cell behaviour, and cartilage is an excellent representative of this paradigm.[87,89] The cartilaginous tissue is a specialized connective tissue, consisting of chondrocytes and a plentiful matrix,[471] consisting in turn of a fibrillar component (mainly collagen), and an amorphous component (proteoglycans, containing hyaluronic acid), which is responsible for mechanical properties of this tissue.[472,473]

This unique structure, including cells and a vast network of cell regulators, gives cartilage a sort of compression rigidity and facilitates the entry of water. Thus, the tissue develops a peculiar power to absorb and dissipate tension forces constantly acting on the system bone. The high degree of hydration and the movement of water in the matrix allow the cartilage matrix to respond to varying pressure loads and contribute to cartilage's weight-bearing capacity.

Throughout life, cartilage undergoes continuous internal remodelling as the cells replace matrix molecules lost through degradation. The structure of the matrix is significant, since it acts as a signal transducer for the embedded chondrocytes. Thus, pressure loads applied to the cartilage as synovial joints create mechanical, electrical, and chemical signals that help to direct the synthetic activity of the chondrocytes.[472,474,475] However, as the body ages the composition of the matrix changes, and chondrocytes lose their ability to respond to these stimuli. In addition to ageing, traumas and diseases of the bones and joints, such as osteoarthritis or rheumatoid arthritis, cause disability in millions of people worldwide,[476] as articular cartilage has limited self-regeneration capacity.[472,474,475] The lesions on the cartilage can be classified into micro-lesions (lesions that do not reach the sub-chondral bone and only superficially affect the tissue) and wounds that reach the bone.[477–479] The first type of injury makes the area of the damage inaccessible to the blood and progenitor cells present in the bone and for this reason there is no self-repair mechanisms.[477] In the case of wounds that reach the bone, the damage is repaired by the mesenchyme stem cells originating from the bone marrow that invade the damaged site and replace it with fibrocartilaginous tissue.[479]

In addition to these methods that occur naturally in the body, a large number of methods have been thought to treat articular cartilage.[480] We can distinguish surgical approaches such as bone marrow stimulation techniques (a subchondral bone perforation is performed to recall the mesenchymal stem cells from the bone marrow to the damaged site),[481,482] osteochondral allograft (a cartilage graft transplantation) joint and its underlying sub-chondral bone from a corpse in the patient's damaged site)[481], autologous chondrocyte transplantation (ACI, the patient undergoes a biopsy and the chondrocytes obtained are implanted in the patient).[483–485] No less important is cell therapy based on

mesenchymal stem cell transplantation. These cells have a good ability to self-renew and a fair chondrogenic potential.[486]

Conventionally, the clinical management of an osteochondral injury involves therefore the use of mechanical symptomatic measures, in most cases associated to the use of analgesics, nonsteroidal anti-inflammatory drugs and/or chondro-protective agents (chondroitin sulfate, sulfate glucosamine, hyaluronic acid). Other protective options involve the use of corticoids, HA, PRP, abrasion, micro fracture, radiofrequency and/or osteochondral grafts.[475,487,488]

It is without doubt that the development of cell-based therapies aimed to repair a damaged cartilage, using either isolated chondrocytes or MSCs as such or in conjunction with biological scaffolds has been an area of intensive clinical research in the last years. The new approaches provide the use of Tissue Engineering techniques, in which autologous chondrocytes are seeded on biocompatible scaffolds to produce neo-cartilages.[476]

Simultaneously, several attempts have been initiated to further improve chondrogenic recovery, exploiting the immunomodulatory properties of mesenchymal stem cells for osteoarthritic therapies.

Osteoarthritis (OA) is the most common form of chronic joint disease affecting 250 million people worldwide. Over 12% of the aging Western population has been reported to suffer from OA, particularly those who are ≥45 years.[489,490] It is presently the fastest growing major health condition. With an aging population and expanding obesity epidemic, OA incidence is anticipated to be the fourth leading cause of disability by the year 2020.[487,488] The pathology of OA is complex involving multiple tissues and processes. It is characterized by the degradation of the articular cartilage, degeneration of menisci and ligaments, thickening of the subchondral bone, and formation of osteophytes.[491] However, it is often a sequela initiated by articular cartilage injuries caused primarily by sports and recreational activities,[492] and aging.[489,490]

OA is triggered by a local inflammatory response caused by joint instability and accompanied by the progressive degeneration of articular cartilage,[493] particularly in sites where stress exceeds the value that can be sustained by the joint. In a damaged tissue, the initial inflammatory response plays a key role triggering tissue repair and homeostasis, but can be detrimental in the long term, causing fibrosis.[494] However, the exact OA pathogenesis remains a subject of debate and research.

During the OA process, a number of inflammatory mediators have been reported.[495] Interleukin (IL) 1β is considered a main inflammatory mediator resulting in the occurrence of OA by damaging articular cartilage via NF-κB pathway activation.[495–497] The destructive effect is also contributed by cyclooxigenase-2 (COX-2), which produces prostaglandin E2 (PGE2), resulting in inflammation and pain in OA.[498,499] COX-2 expression was recently described also in the differentiating growth plate and during chondrocyte differentiation and inflammation.[500–504] COX-2 harms the superficial layers of articular cartilage, and the NF-κB pathway also plays an important role during the process.

Common treatments for OA include nonsteroidal anti-inflammatory drugs, analgesics, locally administered corticosteroids. These medications only provide symptomatic relief to patients, who will require surgical intervention in the end. Nowadays, efforts have been made to reduce the degeneration of articular cartilage, with minimal success.[505]

Recently, human multipotent mesenchymal stromal (stem) cells (MSCs) have entered clinical trials as a novel, less invasive therapy for cartilage defects and OA.[506,507] MSCs

are a promising alternative for current therapies as they are more likely to control the imbalance between anabolic and catabolic processes in an OA joint, thanks to their immunomodulatory and regenerative capacities.[508–510] Although the results of this novel treatment are promising, the fate of MSCs *in vivo* and the molecular mechanism underlying their beneficial effects in cartilage repair remain unclear. Increasing evidence suggests that the therapeutic efficacy of MSCs depends on paracrine signalling[511,512] and more recently their therapeutic potential has been attributed to the secretion of bioactive factors and extracellular vesicles (EVs).[511,512] EVs are secreted by all cell types and range in size from 40-100 nm (exosomes) and from 100-1000 nm (microvesicles). Exosomes are formed by the invagination of the limiting membrane of multivesicular bodies (MVBs), which are part of the cellular endo-lysosomal system. Upon fusion of MVBs with the plasma membrane, exosomes are released into the extracellular environment. Microvesicles bud directly off the plasma membrane.

EVs released by MSC increasingly appear to play an important role in intercellular communication and tissue repair. MSC-EVs have been shown to exert immunomodulatory properties in vitro, and to some extent also possess regenerative properties in a mouse model of myocardial ischemia/reperfusion injury, a rat skin wound model and a rat osteochondral defect model.[511,512]

In this study we investigated the regenerative and immunomodulatory potential of conditioned medium and EVs secreted by human bone marrow derived MSC (hBMSCs) in chondrocytes from OA patients using an in vitro human cartilage repair model.[513] We also investigated the activation of the NF-kB pathways and COX-2 expression and their downstream products interleukin-6 (IL-6) and IL-8.

3.2 Material and Methods

3.2.1 Human Bone Marrow Stromal Cells (hBMSC) cultures in plastic adherence

Plastic Adherent Human Bone Marrow Stromal Cells (PA-hBMSCs) were derived from hip bone marrow aspirates of healthy donors, after informed consent. The human samples were obtained from EU-AutoStem Consortium, after approval by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland and processed at National University of Ireland Galway (NUIG). Briefly, cell nucleated fraction of bone marrow aspirate was suspended in α-MEM-Glutamax medium (Gibco, Waltham, MA, USA), supplemented with: i) 10% Fetal Bovine Serum (FBS, Gibco), 1ng/ml Fibroblast Growth Factor-2 (FGF-2, Peprotech, London, UK) and 100 U/ml of Penicillin/Streptomycin mixture (Euroclone) for standard culture conditions, ii) or with the AutoStem patented serum-free supplement and 100 U/ml of Penicillin/Streptomycin mixture. The cells were plated at a density of $1x10^6$ cells/cm^2. After at least 5 days, only PA-hBMSCs were attached on the plastic, while all the blood cells and other debris were in suspension. Thus, the medium was discarded, the cells were washed with Phosphate Buffer Saline 1X (PBS) to remove all the refuse and the dishes were refilled with fresh medium. When culture dishes were confluent, PA-hBMSC were detached with trypsin-EDTA (Euroclone) and cryopreserved for the delivery to our laboratory. After arrival, we thawed the cells and 2.5 x 10^5 cells were plated in 100mm dishes (density of 3000 cells/cm^2) and grown in an incubator at 37°C with 5% CO_2. The medium was changed twice a week.

3.2.2 Isolation and culture of human Arthicular Chondrocytes (hACs)

Human Articular Chondrocytes (hACs) were obtained from human femoral head biopsies of healthy patients, after informed consent. First, the sample was processed with the help of a scalpel, in order to withdraw the cartilaginous coating from the joint. The pieces obtained were then cut into smaller pieces of about 3mm x 3mm, then they were transferred in a 50ml tube, washed with PBS 1x and resuspended in 1-2mL of a mix of enzymatic digestion, consisting of 1mg/mL hyaluronidase (Sigma-Aldrich, Saint Louis, Missouri, USA), 400U/mL collagenase I (Wortington Biochemical, Lakewood, NJ), 1000U/mL collagenase II (Wortington Biochemical) and 0,25% trypsin (GiBCo) in DMEM (Dulbecco's Modified Essential Medium) High Glucose (EuroClone) w/o FBS. A first digestion of 30 minutes at 37°C was carried out to relax the matrix and it was therefore discarded. Various serial digestions were then performed, each one for at least 60 minutes at 37°C in agitation, to obtain the individual cells. After each digestion, the supernatant was recovered, resuspended in 10mL of DMEM with 10% FBS to block the enzymatic activity and centrifuged for 10 minutes at 1200rpm. hAC thus isolated were seeded in 1-2 wells of a 6-well plate, in DMEM High Glucose with 10% FBS 2mM L-Glutamine (EuroClone), 100U/ml Penicillin/Streptomycin, 1mM Na-pyruvate (EuroClone) and 10mM HEPES (EuroClone). In each well a liquid hyaluronidase quota of 8U/mL was also added, in order to break up any aggregates of cells still present in the suspension. The medium was changed every 2-3 days and the cells were kept in an incubator at

37°C with 5% CO_2. At ~90% of confluence, cells were detached and plated at different densities.

3.2.3 PA-hBMSCs pre-conditioning

PA-hBMSCs were cultured until a 70% confluence. Then, the medium was discarder and, after 5-time washing with PBS 1x, it was replaced with α-MEM-Glutamax medium with only 100 U/ml of Penicillin/Streptomycin mixture, without any other supplement. Cells were keeped in culture for 24 h (for whole secretome production) or 72h (for EVs isolation). The PA-hBMSCs-conditioned medium (hBMSCs-CM) was collected and processed.

3.2.4 Isolation of Extracellular Vesicles from PA-hBMSCs (h-BMSC-EVs)

PA-hBMSCs were cultured until reaching a confluence of about 60-70%. In order to have a good extraction yield, cells were never grown until a too high confluence, as this could adversely affect vesicle secretion levels. hBMSCs-EVs were obtained after high-speed differential centrifugation of hBMSCs-CM collected from ca. 10 x 10^6 PA-hBMSCs cultured in serum-free and FBS-containing medium and starved for 72 hours. hBMSCs-CM were collected in 50ml tubes and cells were trypsinized and counted. The first centrifuge was performed at 300rcf for 10 minutes at 4°C to eliminate dead cells and debris. Once the supernatant was recovered, a second centrifuge at 2000rcf for 20 minutes at 4°C allowed to eliminate the apoptotic bodies from the preparation. As these vesicles are very large and their steric encumbrance could trap even the smallest vesicles in the pellet, this was precautionally washed with PBS 1x and re-centrifuged at 2000rcf for 20 minutes. The supernatant was transferred into ultracentrifuge polyallomer tubes, in turn placed in appropriate bucket for ultracentrifuge. The supernatant was then ultra-centrifuged at 10.000rcf for 30 minutes at 4°C, to pellet microvescicles and finally ultra-centrifuged at 100.000rcf for 90 minutes at 4°C, to pellet the exosomes. The exosome pellet was washed with PBS 1x, previously filtered with 0.22µm filter to remove as much as possible the salts of solution (because of nanometric size of exosomes). The resuspensions were combined into a single clean tube and a last ultracentrifuge was taken at 100,000rcf for 70 minutes, to wash the isolated vesicles. At the end of this ultracentrifuge, the tube was emptied of the supernatant and drained. The pellet was resuspended in 100µl of filtered PBS 1x.

A Beckman Coulter ultracentrifuge (Beckman Coulter Optima XPN-100 ultracentrifuge; Beckman Coulter, Fullerton, CA) was used with swinging bucket rotors type SW28 and SW41Ti.

3.2.5 Labeling and Internalization of hBMSC-EVs by OA hACs

EVs uptake was monitored *in vitro* using a fluorescent dye as PKH67 (Sigma-Aldrich). The PKH67 is a green fluorescent dye with long aliphatic tails, which can be incorporated into lipid regions of the cell membrane. We stained FBS-hBMSC-EVs and SF-hBMSC-EVs with PKH67, according to the manufacture's protocol. Staining was stopped by adding an equal volume of 1% BSA and the EVs were ultra-centrifuged to remove excess of fluorescence. Chondrocytes were previously treated with 200U/ml IL-1α (Peprotech, London,UK) for 16 hours to mimic an inflammatory condition *in vitro* and thus obtain OA-hACs. After this pre-treatment stained EVs were given to OA-hACs plated on glasses for cell culture. After 3 hours, hACs were fixed with 4% PFA and marked with Phalloidin to see the cytoskeleton. Nuclei were stained with DAPI and glasses were mounted with an aqueous mounting. Slides were observed at different magnifications and images acquired with the Axiovert 200M microscope (Carl Zeiss).

3.2.6 Western blot

Confluent HACs (at passage 1) were treated for 16 or 48 hours with a medium supplemented with 200U/ml IL-1a, 200U/ml IL-1a FBS-hBMSC-CM at three different concentrations 1, 10,100µg/ml, 200U/ml IL-1a SF-hBMSC-CM at three different concentrations 1, 10,100µg/ml or without any supplement. Media were then removed, and cells extensively washed with PBS before being transferred to a serum-free medium (no FBS) for 24 h. HAC-Conditioned serum-free culture media were collected and quantified by Bradford assay. 200µl of each sample were precipitated adding 10%TCA (Trichloroacetic acid) and centrifuging samples for 20 minutes at 14,000rpm. The pellet of proteins was resuspended in Sample Buffer and loaded on 4%–12% NuPAGE Bis-Tris gel (Life-Tech, Carlsbad, California, USA), electrophoresis and blot were performed according to a protocol previously optimized in our laboratory. Blot membranes were incubated with specific primary antibodies against IL-6 and IL-8 (1:200, Santa Cruz Biotechnology) (n = 3).

Western blot analysis for COX-2 on cell lysates was also performed. To this purpose, cells were scraped in 30µl of RIPA buffer and quantified by Bradford assay. 10µg of proteins for each sample were loaded on 4%–12% NuPAGE Bis-Tris gel and electrophoresis and blot were performed. Blot membranes were incubated with specific primary antibodies against COX-2 (1:500, Abcam, Cambridge, UK) (n = 3).

To investigate the effect of hBMSC-EVs, confluent HACs (at passage 1) were pretreated for 3 hours with with 1µg/ml FBS-hBMSC-EVs or 1µg/ml SF-hBMSC-EVs in 6-well plates. After 3 hours, 200U/ml IL-1a was added to each well. A negative control without any supplement and a positive control treated only with 200U/ml IL-1a were also carried out. Cells were lived in culture for other 24 hours. Media were then removed, and cells extensively washed with PBS before being transferred to a serum-free medium (no FBS) for 24 h. cells were scraped in 30µl of RIPA buffer and quantified by Bradford assay. 10µg of proteins for each sample were loaded on 4%–12% NuPAGE Bis-Tris gel and electrophoresis and blot were performed. Blot membranes were incubated with specific primary antibodies against COX-2 (1:500, Abcam, Cambridge, UK) and LECT-1 (1:1000, LS Bio, Seattle, Washington, Stati Uniti) (n = 3).

Images were scanned using the Epson perfection 1260 scanner and band densities were quantified using the Fiji-ImageJ software. Different pixel mean densities were analyzed. Means and standard deviations were obtained for statistical analysis (n = 3).

3.2.7 NF-kB nuclear translocation

Chondrocytes were cultured in 24-well plates on glass coverslips. They were treated for 4 or 40 hours with a medium supplemented with 200U/ml IL-1a, 200U/ml IL-1a FBS-hBMSC-CM at three different concentrations 1, 10,100µg/ml, 200U/ml IL-1a SF-hBMSC-CM at three different concentrations 1, 10,100µg/ml or without any supplement. Cells were fixed in 3,7% paraformaldehyde in PBS (pH 7.6) containing 2% sucrose for 10 minutes. After washing in PBS, cells were permeabilized with a solution (20mM HEPES, 300mM sucrose, 50mM sodium chloride, 3mM magnesium chloride, and 0.5% Triton X-100) for 10 minutes at 4°C. Nonspecific binding was prevented by incubation with pure goat serum for 30min at 4°C. Slides were incubated with a rabbit anti-NFκB p65 antibody (1:100; Cell Signaling) overnight at 4°C. After rinsing in PBS, an anti-rabbit fluorescence-labeled antibody Alexafluor-488 (Life Tech) was added for 30min. Cells were marked with Phalloidin to see the cytoskeleton. Nuclei were stained with DAPI and glasses were mounted with an aqueous mounting. Slides were observed at different magnifications and images acquired with the Axiovert 200M microscope (Carl Zeiss).

3.2.8 Statistical analysis

All statistical analysis was performed using one-way ANOVA. Error bars indicate standard deviation (S.D.). A p-value 0.05 was considered to be statistically significant. GraphPad software was used as work program.

3.3 Results

3.3.1 Osteoarthritic chondrocytes are more prone to internalize hBMSC-derived EVs

It has been shown that mesenchymal stem cell exosomes often have an immunosuppressive and anti-inflammatory role. We therefore wondered if our exosomes could have effects in counteracting osteoarthritic processes (characterized by inflammatory processes) in the field of cartilaginous and bone lesions. We hypothesized that BMMSC-EVs interact with chondrocytes from osteoarthritic patients to modulate cartilage regeneration.

Therefore, we started from primary cultures of human articular chondrocytes (HACs), we induced an inflammatory process, using a dose of 200U / ml of IL-1α and we gave exosomes, previously marked with a fluorescent dye, to the cells, at a concentration of 1µg / ml. After three hours, the cells were fixed with 3.7% PFA, treated with DAPI and mounted on a slide for fluorescence microscopy observation.

hBMSC-EVs not only interacted but were taken up by OA chondrocytes after as short as 3 hours of incubation (*Fig.22A*)

A quantitative analysis was also performed counting the percentage of positive cells per ROI (Region of Interest, *Fig.22B*). FBS- and SF-hBMSC-EVs showed a similar internalization efficiency both in normal (30.44 ± 0.56% and 39.95 ± 2.12% respectively) and inflammatory (53.06 ± 6.46% and 54.87 ± 7.1%). Both type of EVs are internalized with a higher efficiency following treatment with interleukin. Although the counts did not find significant differences, the share of SF-hBMSC-EVs for each cell in OA-hAC appeared much higher and the signal is located close to the nucleus, suggesting a localization in the endoplasmic reticulum. Further experiments will be conducted on the subject for clarification regarding the fate of the EVs after the endocytosis.

These data indicate that bone marrow MSC secrete EVs that interact and are rapidly taken up by chondrocytes that come from an osteoarthritic joint more greedy than normal conditions.

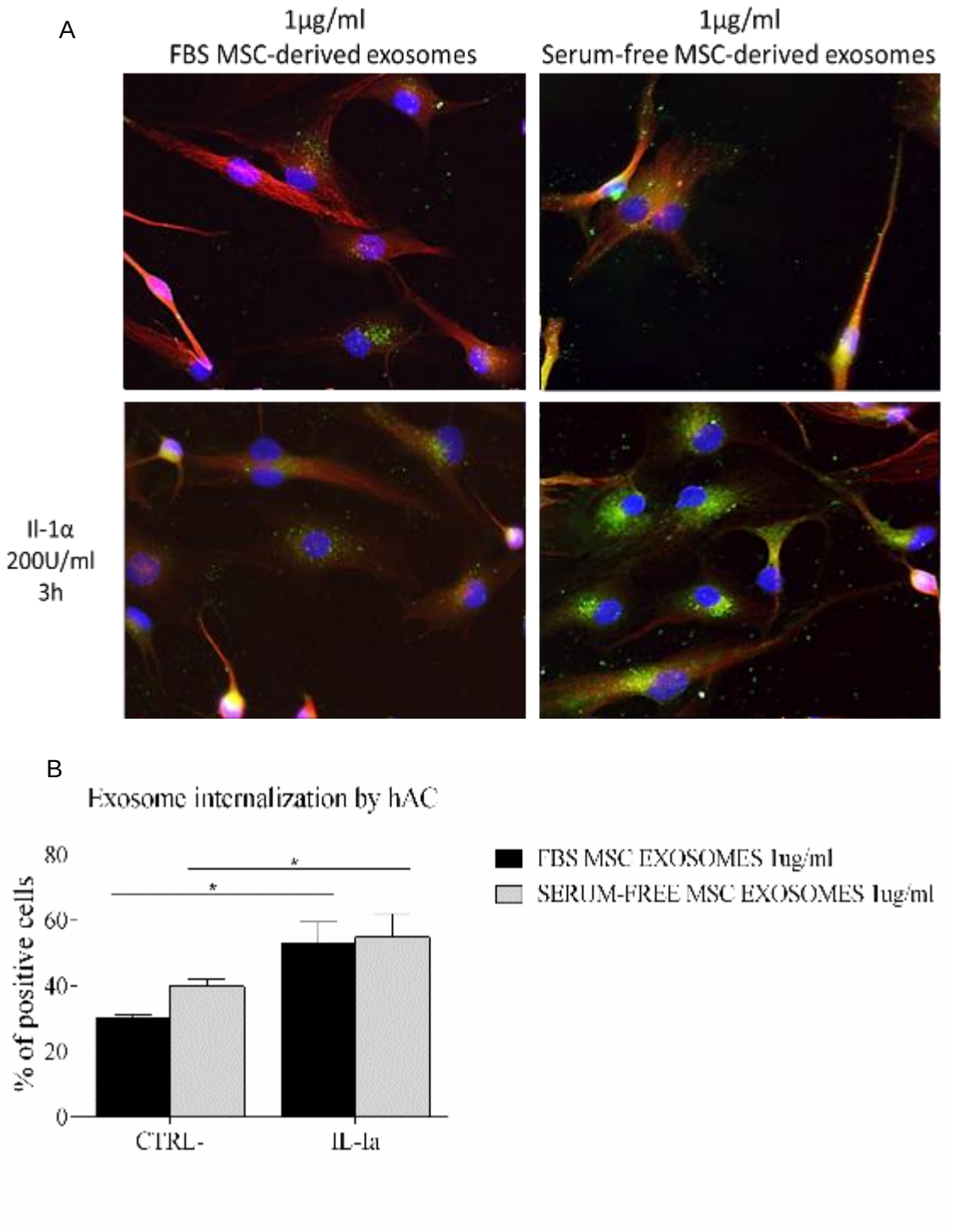

Figure 22 A) PKH67⁺ hBMSC-EVs uptake by hACs and OA-hACs.
B) Quantification of PKH67 positive cells Error bars represent mean ± S.D. (*) $P < 0.05$, two-way ANOVA.

3.3.2 hBMSC-CM inhibited IL-1α-induced pro-inflammatory cytokine secretion and COX-2 expression in osteoarthritic chondrocytes

In a damaged tissue, the initial inflammatory response plays a key role triggering tissue repair and homeostasis, because it serves to recall progenitor cells to start the regeneration. If this inflammatory response retains for a long time, it can be detrimental, causing chronic inflammation and fibrosis.

We evaluated the effect of hBMSC-CM on protein secretion of the proinflammatory cytokines IL-6 and IL-8 in hACs cultured under normal and inflammatory conditions (*Fig.23A/B*). Confluent chondrocytes (at P1) were treated for 16 and 48 hours with the serum-free medium supplemented with 200U/ml IL-1a, 200U/ml IL-1a FBS-hBMSC-CM at three different concentrations 1, 10,100µg/ml, 200U/ml IL-1a SF-hBMSC-CM at three different concentrations 1, 10,100µg/ml or without any supplement. At the end of the incubation time, culture media were removed and replaced with serum-free media (no supplements). After additional 24 h of incubation, media were collected, and secreted proteins were detected by Western blot and quantified.

In a parallel experiment, gene expression levels were measured and quantified, in lysates of the cells after 16 and 48 hours of the different treatments (*Fig.23C*). The secretion of both IL-6 and IL-8 increased after 16 hours when the chondrocytes culture medium was supplemented with IL-1α, as compared to controls (*Fig.23A/B*). Interestingly, when the medium was supplemented with FBS-hBMSC-CM plus IL-1α, the IL-6 and IL-8 levels were significantly higher than with IL-1α alone, in a concentration-dependent manner. An increase of IL-6 and IL-8 was observed also after the treatment with 1µg/ml SF-hBMSC-CM plus IL-1α, while with the treatment at 10 and 100µg/ml the cytokines secretion decreased considerably.

After 48 hours of treatment, while with FBS-hBMSC-CM plus IL-1α and lL-1α the IL-6 and IL-8 remained elevated, indicating the persistence of an inflammatory state, with SF-hBMSC-CM these levels were drastically reduced. In particular, we saw a concentration-dependent decrease of IL-8 levels.

The effect of conditioned media on COX-2 expression was also examined by western blot. The levels of COX-2 were increased in cells treated with IL-1α, as compared to controls (*Fig.23C*), both after 16 and 48 hours. The simultaneous treatment with SF-hBMSC-CM induced an increase of COX-2 in the first hours, but it was then inhibited after 48 hours, indicating the establishment of an acute pro-resolving inflammation and suggesting a role of these CM in inflammation resolution. The same result was not found with FBS-hBMSC-CM treatment.

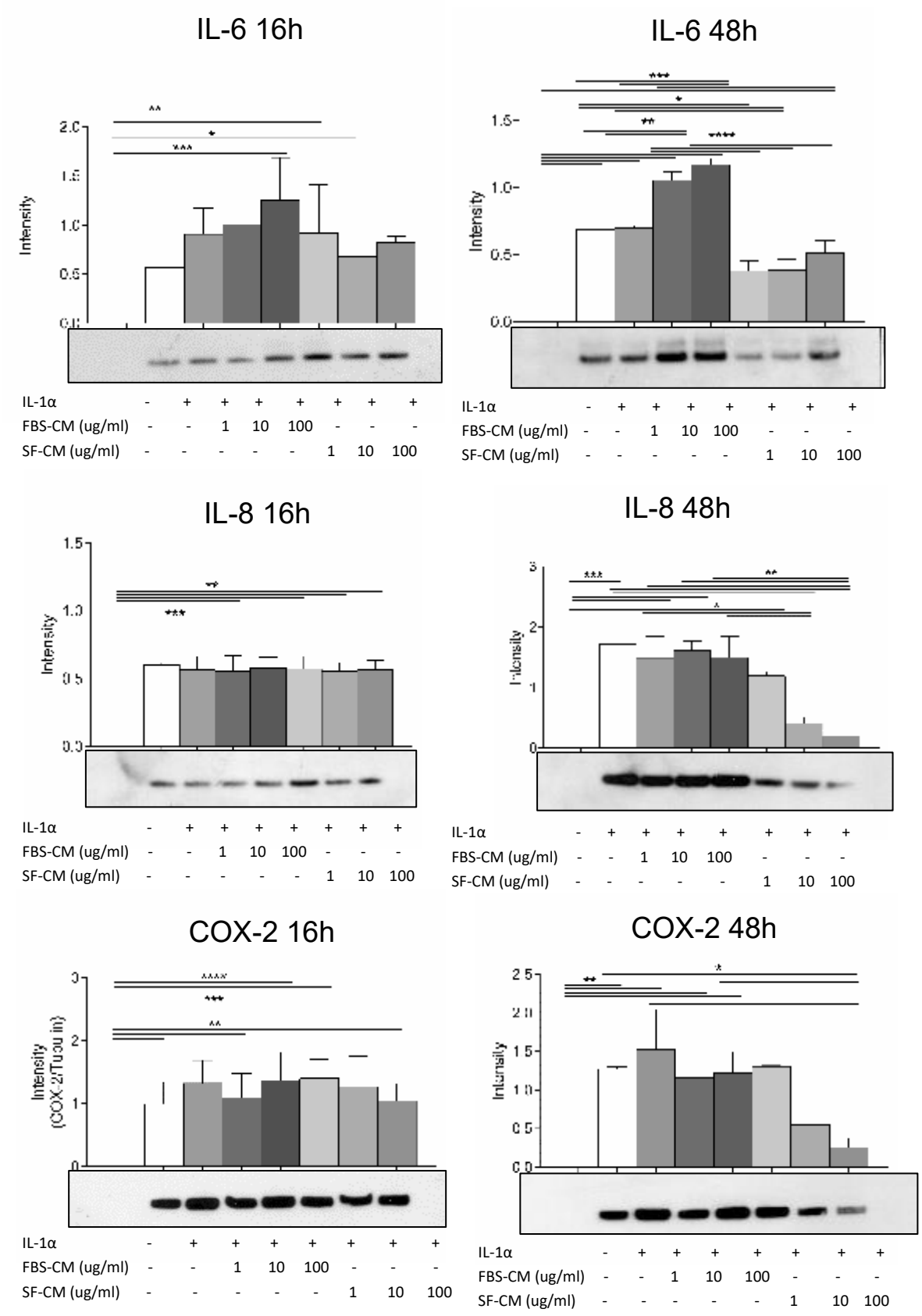

Figure 23 Western blot on conditioned medium derived by hACs treated with IL-1α + FBS- or SF.derived CM, for protreins IL6 (A), IL-8 (B), COX2 (C). Error bars represent S.D. (*) p value < 0.05, (**) p value < 0.01, (***) p value< 0.001, (****) p value < 0.0001, ANOVA.

3.3.3 hBMSC-CM inhibited IL-1α-induced pro-inflammatory NFκB signaling in osteoarthritic chondrocytes

NF-κB signaling is the major signaling pathway activated by TNF-alpha in OA chondrocytes.[514,515]

To understand whether the anti-inflammatory effect of hBMSC-CM could be mediated through the regulation of NFκB activity, we treated hACs for 4 and 40 hours with the serum-free medium supplemented with 200U/ml IL-1a, 200U/ml IL-1a FBS-hBMSC-CM at three different concentrations 1, 10,100µg/ml, 200U/ml IL-1a SF-hBMSC-CM at three different concentrations 1, 10,100µg/ml or without any supplement. We analyzed the subcellular localization of the p65 subunit of NF-κB. As expected, treatment of chondrocytes with IL-1α induced translocation of p65 from cytoplasm to nucleus suggesting NF-κB activation, which could be abrogated by hBMSC-CM from both FBS and serum-free cultured hBMSCs.

Interestingly, after a 4-h stimulation with IL-1α and IL-1α + hBMSC-CM, we detected the upregulation of NF-kB as compared to untreated. After 40 h, the activity of NF-kB in the presence of hBMSC-CM + IL-1α was subverted with a significant reduction as compared to IL-1α alone (*Fig.24*)

These data suggested that both FBS- and SF-hBMSC-CM could potentially affect the inflammatory status of osteoarthritic condition, but SF-hBMSC-CM appeared to be much more effective.

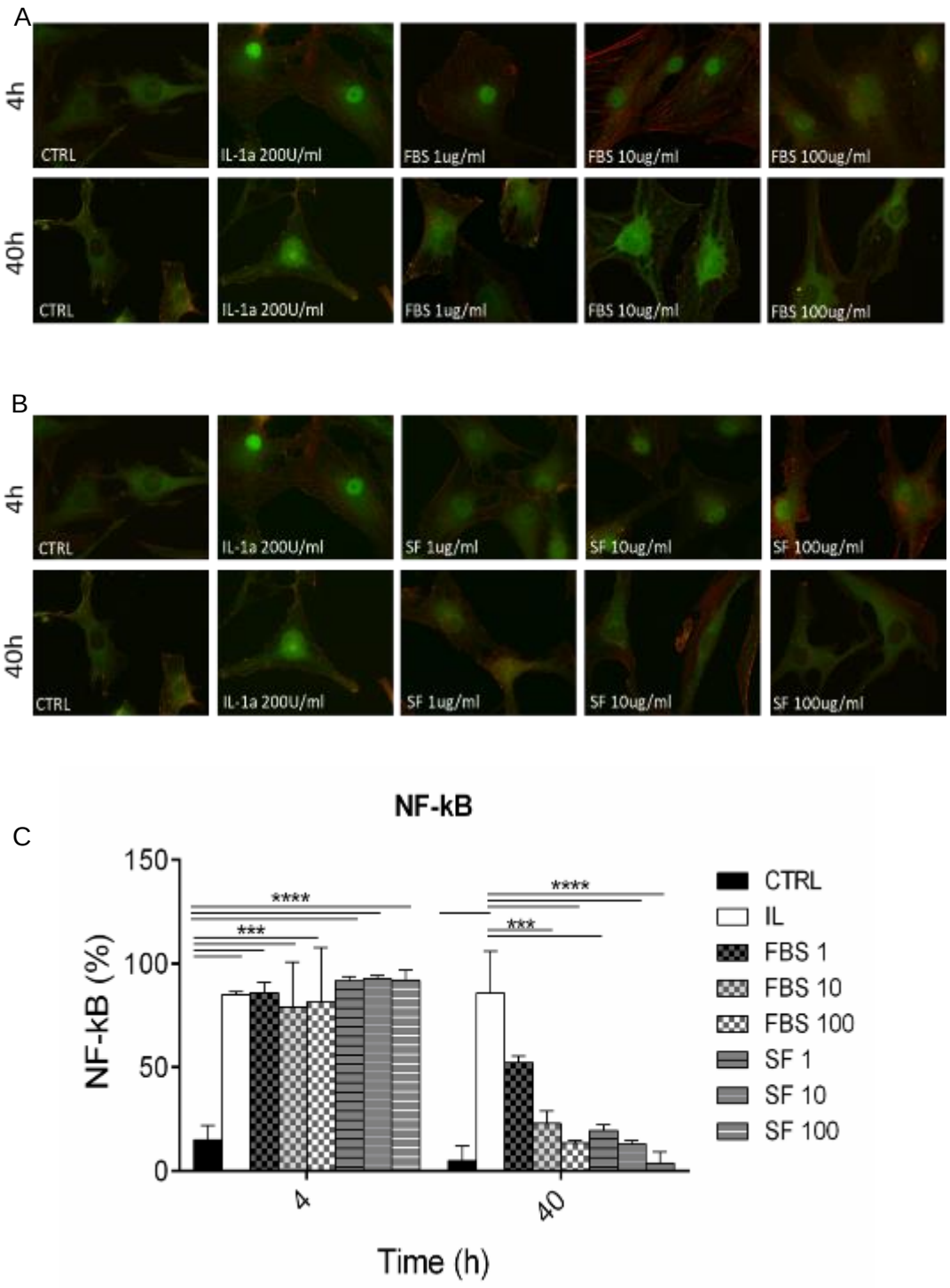

Figure 24 Chondrocytes from three patients were treated with hBMSC-CM from one allogeneic hBMSC donor and with 200U/ml IL-1α for 4 and 40 hours.

(A) hBMSC-CM inhibited IL-1α-induced nuclear translocation of p65 subunit of NF-κB. Nuclear translocation of p65 subunit of NFκB was assessed by immunofluorescence analysis. Representative images of three independent experiments are shown.

(B) Quantification of data presented in A. Data from three independent experiments are shown as mean ± SD. (***) P value < 0.001, (****) P value < 0.0001, two-way ANOVA.

3.3.4 hBMSC-EVs exhibited a chondro-protective role

To investigate the effect of hBMSC-EVs, confluent HACs were pre-treated for 3 hours with 1µg/ml FBS-hBMSC-EVs or 1µg/ml SF-hBMSC-EVs in 6-well plates. After 3 hours, 200U/ml IL-1a was added to each well. A negative control without any supplement and a positive control treated only with 200U/ml IL-1a were also carried out. Cells were lived in culture for other 24 hours. Media were then removed, and cells extensively washed with PBS before being transferred to a serum-free medium (no FBS) for 24 hours.

We analyzed the levels of COX-2: the simultaneous treatment with FBS-hBMSC-EVs induced a decrease of COX-2 and this effect was stronger after the treatment with SF-hBMSC-EVs (*Fig.25*)

We investigated also the effect of LECT-1 expression. Chondromodulin-1 (ChM-1, or LECT-1) is a marker of hyaline cartilage and it has the following biological functions: i) stimulation of the synthesis of DNA and extracellular matrix and promotion of the rapid growth of chondrocytes and cartilage.[516,517] ii) Inhibition of angiogenesis, which can inhibit vascular invasion into cartilage and thus inhibit endochondral bone development and bone ossification.[517,518]

We saw here that the pre- treatment with both FBS- and SF-hBMSC-EVs allowed a recovery of LECT-1 levels, which was inhibited with the IL-1α inflammation.

Taken all together, these data suggested that hBMSC-EVs could have a chondro-protective role and the serum-free culture enhances this peculiarity.

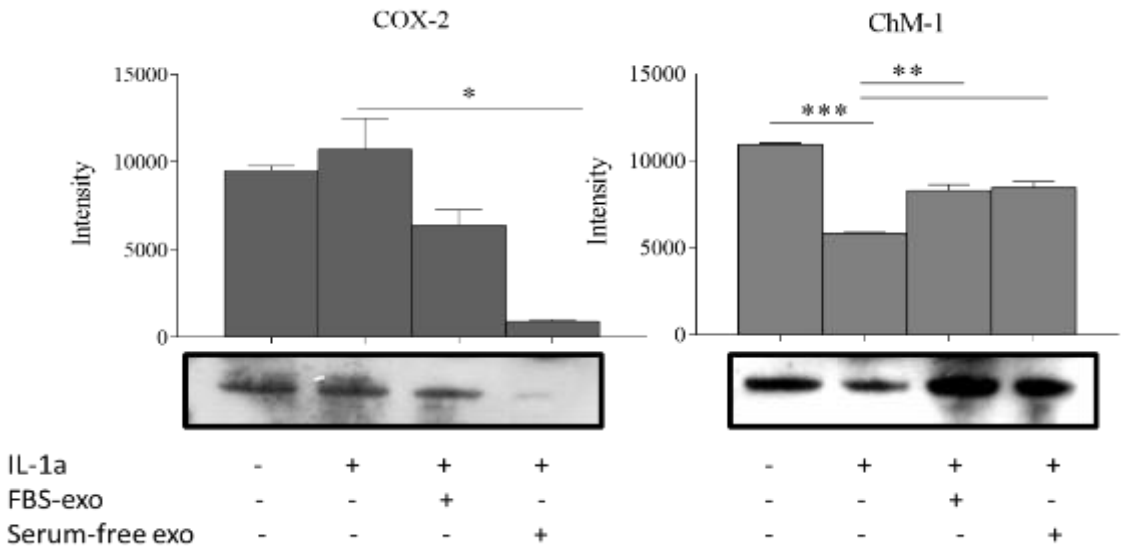

Figure 25 Response of human articular chondrocytes to the hBMSC-EVs treatment in an inflammatory microenvironment. Identification of proteins in chondrocyte was performed after the cell different treatments. Western blot analysis of COX-2 and LECT-1 was performed and the corresponding band intensity was determined by densitometric analysis (n = 3).

3.4 Discussion

In joint, under physiological conditions, chondrocytes, the extracellular matrix, and cell secretome (i.e., the whole joint microenvironment) are responsible for the maintenance of tissue homeostasis. However, when a lesion occurs, profound changes perturb the microenvironment. The proinflammatory cytokine IL-1α produced in the inflamed joints by activated synovial cells and infiltrating macrophages is one of the most potent catabolic factors in knee diseases.[511,512]

This molecule is responsible for the induction of several mediators of cartilage degeneration such as IL-6 and IL-8. In this condition, the cells near to the site of the injury are not able to organize an acceptable regenerative response and, as a consequence, the cartilage normal structure and composition are not restored.

Osteoarthritis is one of the most prevalent joint diseases and a major public health problem. It is characterized by progressive articular cartilage destruction and synovitis.[511,512] Current therapies attempt to relieve the symptoms, but they cannot stop or reverse the ongoing cartilage degeneration.[511,512] The ideal treatment aiming for an optimal OA joint repair should promote regenerative properties of chondrocytes and fight destructive effects of inflammation.

A wide range of evidence indicates that paracrine effects of MSC are a central mechanism of cell therapy promoting tissue regeneration.[511,512]

We compared the effects of hBMSC-CM and EVs on hACs maintained under physiological and inflammatory conditions (i.e., in the presence of IL-1α). The addition of CM derived from hBMSCs cultured in FBS-containing medium itself did not modify the secretion profile of the proinflammatory cytokines IL-6 and IL-8 but caused an increased secretion of these factors enforcing the IL-1α activity. On the other hand, the treatment with CM derived from hBMSCs cultured in our novel well-defined xeno-free culture medium downregulated the pro-inflammatory cytokines IL-6 and IL-8, after an initial increase.

This may have implications for the control of altered chondrocyte metabolism. Interestingly, IL-6 has been involved in OA pathophysiology[519] and increased circulating levels of IL-6 have been associated to radiographic knee OA.[520]

Elevated levels of TNF-α, IL-1 and IL-6 have been found in the synovial fluid, synovial membrane, subchondral bone and cartilage of OA patients, confirming their important roles in OA pathogenesis. Moreover, TNF-α, IL-1 and IL-6 can induce the production of other cytokines, matrix metalloproteinases (MMPs) and prostaglandins and inhibit the synthesis of proteoglycans and type II collagen; thus, they play a pivotal role in cartilage matrix degradation and bone resorption in OA. Activated chondrocytes also produce MMP-1, MMP-3, MMP-13, and aggrecanase 1 and 2 (ADAMTS-4, ADAMTS-5). In addition, IL-1, TNF-α and IL-6 may cause OA indirectly by regulating release of adiponectin and leptin from adipocytes.[521]

Osteoblasts stimulated by IL-1β, TNF-α, and IL-6 become a source of IL-6 and secrete MMPs, thus adversely affecting the cartilage located nearby.[522] IL-6 stimulates normal cartilage and synovial cells to produce large amounts of prostaglandin E and increases collagenase levels. Collagenase degrades collagen, promotes cartilage absorption, and activates MMPs to digest matrix proteins, resulting in loss of matrix water. This abnormal phenomenon causes deterioration of the microenvironment for chondrocyte survival,

making it impossible for the cells to obtain sufficient nutrients, thereby causing self-impairment of function; this process can form a vicious circle, ultimately causing articular cartilage degradation.[523,524]

IL-6 stimulates osteoclast differentiation and growth, promotes osteoclast formation, and increases osteoclast activity, thereby acting in synergy with IL-1β and TNF-α to damage articular cartilage.[522,525]

In OA chondrocytes, canonical NF-κB signaling mediates the induction of inflammatory mediators and catabolic mechanisms as well as cellular differentiation changes which favour the onset and perpetuation of disease.[523,524] A reduction in the activation of this transcription factor by CM and EV could contribute to the downregulation of IL-6, IL-8 COX-2, and MMPs.[526]

The inhibition of NF-kB has been recently reported as actuated by the hBMSC-EVs.[330] Moreover, in primary human osteoarthritic chondrocytes, it was observed that EVs inhibited the translocation of NF-kB to the nucleus. Here, we extend these observations and report that our novel culture system provides cells able to secrete bioactive factors in conditioned medium with the capability to restore the homeostatic status of osteoarthritic cartilage. In primary hACs, hBMSC-CM, in combination with IL-1α, drives the initial transient activation of NF-kB (4-h treatment) with consequent upregulation of IL-6 and IL-8, whereas a longer time of treatment (40 h) causes a reduction of the NFkB activity. Based on these data, we suggest that the activation and the subsequent inhibition of NF-kB is involved in the inflammation response and resolution controlled by CM.

We found that also COX-2, an enzyme activated during the acute-phase response, was significantly reduced. Several studies demonstrated that in the skeletal systems, COX-2 is expressed during fracture callus formation, being its function essential for bone healing.[527] In cartilage, the activation of COX-2 was described not only in the differentiated growth plate, but also during inflammation.[528–530] Ulivi et al. showed that in a chondrocyte cell line, COX-2 was expressed via p38/NF-kB activation and nuclear translocation during both differentiation and inflammatory response.[500] In this context, our observation that hBMSC-CM in the presence of IL-1α was able to reduce not only NF-kB, but also COX-2 expression indicates to an early attempt to obtain the resolution of the inflammation process. We therefore propose that in cartilage, the hBMSC conditioned media initially enhance the inflammatory response, thus promoting a transient inflammation of the HACs, but trigger a cascade of events leading to inflammation resolution.

However, our data also demonstrate that both FBS- and SF-BMMSC-EVs from two independent hBMSC-EVs donors inhibited IL-1α-induced expression of COX2, indicating their significant anti-inflammatory potential in cartilage cells.

Although, BMMSC-EVs stimulated an almost 2-fold reduction in COX2 expression, their relative contribution to the effect of the total MSC secretome was smaller compare to their strong regenerative properties. The other secretory factors present in hBMSC-CM seem to play, next to EVs, an important role in hBMSC immunomodulatory effects on OA chondrocytes.

However, analysis of hBMSC-EVs' impact on immune cells present in synovial fluid and immune cells penetrating joint synovial tissue of OA patients, such as macrophages, should give a more complete picture of hBMSC-EVs anti-inflammatory properties.

Last but not least, pre-treatment with both FBS- and SF-hBMSC-EVs allowed a recovery of LECT-1 levels, which was inhibited with the IL-1α inflammation. LECT-1 promotes

chondrocyte growth and inhibits angiogenesis. This gene is expressed in the avascular zone of prehypertrophic cartilage, and its expression decreases during chondrocyte hypertrophy and vascular invasion. The mature protein likely plays a role in endochondral bone development by permitting cartilaginous anlagen to be vascularized and replaced by bone. It may also be involved in the broad control of tissue vascularization during development.

Conclusions

Taken together, the data presented here demonstrate for the first time that hBMSC-CM and EVs have both regenerative and immunoregulatory properties in human OA cartilage. We demonstrated with our work that we developed a novel serum-free culture system which allows to isolate hBMSCs with high chondro-protective role and the cellular product obtained are safe at clinical grade and devoid of animal derivatives.

The dual potential of hBMSC-CM/EVs makes them a promising candidate for an optimal therapy for OA, which should promote cartilage repair and inhibit ongoing cartilage degradation. Additionally, hBMSC-CM/EVs-based therapy seems to carry less safety risk, since EVs, in contrast to cells, cannot replicate or become transformed. Therefore, hBMSC-EVs could be administered at earlier stages of OA to improve joint homeostasis and prevent OA from further development. This would potentially lower or delay the necessity of surgery for these patients. In cases of advanced OA, BMMSC-EVs could be used to improve the joint condition and might need to be supplemented with other treatment approaches such as cell-transplantations or joint distraction, as there is hardly any cartilage left in late-stage OA joints.

However, BMMSC-EV therapy has little precedence, thus its ethical status needs to be clearly defined before it can be introduced to clinics. This may require more in-depth characterization of BMMSC-EVs regarding their RNA and protein content.

In vivo studies are also underway to evaluate the regenerative potential of these cells and their cellular products in a bone damage system in diabetic mice (the diabetic mouse has a lower ability to self-regenerate a critical bone defect).

Our culture system showed to be suitable to isolated hBMSCs and cellular product ready-to-use for clinical therapy, as it allows to isolate and maintain in culture a large quantity of cells, in a short time, in a culture system devoid of animal derivatives and contaminations due to manipulation, thus obtaining a safe high clinical grade product, compatible with GMP rules.

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