Assessment of cell response to microcarriers biofunctionalisation with a bi-functional fusion protein

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To my parents, for the blind faith

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1. Abstract

Achieving suitable yields of cell therapy products without compromising the cell quality remains a great challenge for biochemical engineers. Microcarriers seem like a promising solution for scalable production of adherent cells as they enable cell culture similar to the fermentation process and can provide biochemical and physical cues for the cells. Engineered from implantable biomaterials with a fine-tuning rate of degradation allow expansion and transplantation avoiding the cell harvesting step. They can also be doped with metal oxides such as TiO_2 and CoO_2 which can promote interesting cell responses: osteodifferentiation and angiogenesis, respectively.

To promote cell adhesion calcium phosphate microcarriers doped with 5% TiO_2 were tethered a fusion protein, fibronectin-osteocalcin, FN-OCN, benefitting of osteocalcin's affinity to calcium ions and fibronectin's role in cell adhesion. Also beads with 5% TiO_2 + 2% CoO2 doped, also coated with FN-OCN, and were tested to determine cell response to CoO_2.

The Thesis work involved expressing and purifying the bi-functional fusion protein, fibronectin-osteocalcin (FN-OCN), and then subsequently testing its ability to support responses of human cells (MG63 osteosarcoma) seeded onto microcarriers composed of a proven bioactive and biocompatible material, calcium phosphate (CaP), doped with 5% mol TiO_2 (titanium dioxide) and 2% mol CoO_2 (cobalt dioxide). The results show an increase in cell adhesion in coated 5%TiO2 doped beads and suggest the same for 2%TiO_2 + 2%CoO_2 doped beads however further studies should be performed to confirm such results. There is also evidence that raw 5%TiO_2 +2% CoO_2 beads promote more adhesion that 5% TiO_2 doped beads.

Keywords: Bone regeneration, Microcarriers, bioactive calcium phosphate material, fibronectin-osteocalcin

Resumo

A expansão celular com manutenção da qualidade continua a ser um grande desafio para os engenheiros biológicos. Os *Microcarriers* parecem uma opção promissora para produzir células aderentes pois permitem uma expansão semelhante ao processo de fermentação e podem sinalizar bioquímica ou fisicamente as células. Construídos de biomateriais com degradação controlada (ex: fosfato de cálcio) permitem a expansão celular e a transplantação em conjunto. Adicionando óxidos de metais como TiO_2 e CoO_2 acrescenta-se características de osteodiferenciação e angiogénese, respectivamente.

Para melhorar a adesão celular e*microcarriers* de CaP com 5% de TiO_2 (dióxido de titânio) foi-lhes adicionado uma proteína bi-funcional à superfície, fibronectina-osteocalcina, FN-OCN, beneficiando da afinidade da osteocalcina para iões de cálcio e da função da fibronectina na adesão celular. Outro tipo de *microcarriers* que além de 5% TiO_2 tinham também 2% CoO_2 (dióxido de cobalto), também com FN-OCN à superfície, foi testados e determinado o seu efeito na adesão celular.

Esta tese envolveu a expressão e purificação da proteína FN-OCN e a avaliação do seu efeito na resposta celular de MG63 (células humana de osteossarcoma) em *microcarriers* de CaP, com 5% mol de TiO_2 e 2% mol de CoO_2.

Os resultados mostram mais adesão celular em microcarriers com 5% TiO_2 e sugerem o mesmo para o segundo tipo. No entanto será necessário repetir as experiencias para confirmar esses resultados. Existe também evidência que microcarriers sem proteína à superfície com adição de %5% TiO_2 + 2% CoO_2 promovem mais adesão celular que os mesmos só com dição de 5%TiO_2.

Palavras-chave: Regeneração do osso, Microcarriers, material bioactivo de fosfato de cálcio, fibronectina-osteocalcina

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3.1. List of abbreviations

CaP: calcium phosphate

TiO_2: titanium dioxide

CoO_2: cobalt dioxide

FN-OCN: fibronectin-osteocalcin

FN: fibronectin

OCN: osteocalcin

ASCs: Adult Stem Cells

MSCs: Mesenchymal Stem Cells

SBF: Simulated Body Fluid

ECM: extracellular matrix

VEGF: vascular endothelial growth factor

HIF-1α: hypoxia inducing factor-1a transcription factor

HA: hydroxyapatite

SiO_2 : silicon dioxide

MBG: Mesoporous bioactive glass

GF: growth factors

LB: Lysogeny broth

A_{600}: Absorbance at 600

TAE: Tris-Acetate- Ethylenediamine tetraacetic acid

SOC: Super Optimal broth with Catabolite repression

TRIS: tris(hydroxymethyl)aminomethane

Trp: trypsine

Tyr : tyrosine

Cys: cysteine

PBS: Phosphate Buffered Saline

CCK-8: Cell Counting Kit-8

WST-8:2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt

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Figure 14- a) and b) 2nd experiment with 4 and 24h FN-OCN protein coating, respectively, with 0, 1, 5 and 10 μg/mL of protein concentration

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4. Introduction

At present, current therapies for bone regeneration include the use of autologous bone grafts. However, this procedures often result in treatment failure, long recovery times, long-term complications and major cost to the patients and the health system (Damien & Parsons, 1991; Kon et al., 2012).

In the last decade, the paradigm of tissue engineering has been to also stimulate new functional tissue rather than just the replacement for new tissue or organs (Kon et al., 2012). Stem cells have been suggested as a promising solution to promote such regeneration, namely Adult Stem cells (ASCs), like Mesenchymal (MSCs) (Kon et al., 2012) due to their ability to differentiate into several lineages of connective tissue (bone, cartilage, muscle, tendon, adipose tissue and haematopoiesis supporting stroma), supporting tissue function (Deschaseaux, Pontikoglou, Sensebe, & Sensébé, 2010) and enhancing immunoregulatory and regenerative function at injured sites (Caplan, 2007).

As other target tissues for Cell Therapy, bone tissue engineering also straggles with the need of cells which largely exceeds the available supply. Since MSCs are able to proliferate, differentiate onto osteoblasts and/or modulate bone engraftment given suitable and reproducible protocols, cell culture platforms using this cell source might represent a good solution. Since MSCs are able to proliferate, differentiate onto osteoblasts and/or modulate bone engraftment given suitable and reproducible protocols, cell culture platforms using this cell source might represent a good solution. However, MSCs-based treatments still face several limitations such as donor to donor variability, the loss of multipotency and senescence due to extended culture (Banfi et al., 2002) and bioprocess forces (cycles of trypsinization, centrifugation, pipetting…) which interferes with cell surface (Mallucci & Wells, 1972) and can induce early differentiation by activating intracellular metabolic pathways (Brindley et al., 2011). Therefore, a reproducible and

efficient strategy to expand MSCs ex vivo is needed to meet the high cell numbers required for therapy.

Microcarriers, porous and non-porous small beads used to grow cells, have been shown to successfully expand MSCs (Eibes et al., 2010; Hewitt et al., 2011; Kehoe, Schnitzler, Simler, Dileo, & Ball, 2012; Schop, Borgart, Janssen, & Rozemuller, 2010).

In case of anchorage-depend cells growth cell attachment can be modulated by changing several microcarriers properties such as chemical composition or surface topography (Martin, Eldardiri, Lawrence-Watt, & Sharpe, 2011). By offering a suitable substrate where cells can grow on while presenting the necessary factors, microcarriers can direct cell fate namely proliferation and differentiation (Hollister, 2005). Moreover, microcarriers provide a large surface area for cell growth in bioreactors (Nienow, 2006), can be transplanted with the cells as an injectable product and allow viable cell count (T. A. E. G. Park & Ph, 2008).

It has been proven that direct cell delivery to the injured sites can restore function in several tissues (Bruder & Fox, 1999; Patrick, 2001; Schmidt & Leach, 2003). Stem cells, especially MSCs, can be easily expanded and are able to differentiate into several cell types capacity (Bernardo, Pagliara, & Locatelli, 2012) allowing the possibility to grow the necessary amount of cells and reproducibly differentiate them designing cell therapies to provide cells to the target sites.

The combining knowledge of basic Cell Biology and Material Science employs microcarriers as a suitable substrate where cells can grow on, while providing a direct scaffold for cell transplantation, offering a promising bone regeneration strategy and the therapeutic cells would become a direct injectable product without the need of harvesting.

Although the field of biomaterials exists since the 60's, only recently has emerged a concern regarding bioactivity and resorbability, rather than just biocompatibility of inert biomaterials, in order to improve local cell response. (Perez-Sanchez, Ramirez-Glindon, Lledo-Gil, Calvo-Guirado, & Perez-Sanchez, 2010). Glass ceramics bioactive biomaterials like calcium phosphate that had been shown to form a bone-like apatite layer when in contact with simulated body fluid (SBF), demonstrating its bioactivity. The topography of the apatite layer mimics the endogenous microenvironment and is able to signal cells to mobilize to the target site and start proliferating, differentiating and producing the Extracellular Matrix (ECM) and factors necessary for healing (Kokubo, Kim, Kawashita, & Nakamura, 2004).

It has previously shown that the addition of TiO_2 to calcium phosphate glass ceramics enhances bone-specific gene expression and bone forming capacity in MG63 cells (human sarcoma) by effectively controlling its degradation (Abou Neel, Chrzanowski, & Knowles, 2008). Moreover, the addition of ionic cobalt in low amounts into bioactive glass has been shown to increase VEGF (vascular endothelial growth factor) protein production, HIF-1α (hypoxia inducing factor-1a transcription factor) and bone-related gene expression (Wu et al., 2012).

As mentioned before the new trend of biomaterial science for tissue engineering is to build constructs with the ability to influence cell response by mimicking the target tissue microenvironment: its topography, mechanical load or surrounding ECM molecules. Among the latters, fibronectin, a dimeric glycoprotein essential to vertebrate development, has a central role in bone regeneration as allows cell adhesion and it has been shown to regulate osteodifferentiation (Globus et al., 1998; Moursi et al., 1996).

Another protein relevant to bone regeneration is osteocalcin. Osteocalcin is the most abundant non-collagenous protein in bone tissue and it has a high affinity to calcium ions

present in hydroxyapatite (HA), mineral similar to the one secreted by osteoblasts (Hoang, Sicheri, Howard, & Yang, 2003).

Recently, taking advantage of these interactions, a bifunctional protein has been engineered which tethers the RGD sequence (Arg-Gly-Asp) of fibronectin and the calcium binding site of osteocalcin in order to improve cell adhesion to bone tissue engineering scaffolds (Kang, Kim, Yun, Kim, & Jang, 2011; Lee et al., 2013).

In this project the aim was to enhance mesenchymal stem cell growth and differentiation into osteoblasts by promoting their attachment to CaP beads with 5% mol TiO_2 coated with a fibronectin-osteocalcin (FN-OCN) bifunctional fusion protein with a specific cell binding amino acid sequence, the nineth-to-tenth type III domain of human fibronectin (figure 5) and full length mouse osteocalcin protein as described by Kim, Park, Kim, & Jang, 2007 and Lee et al., 2013 in which fibronectin is expected to promote cell adhesion and osteocalcin to regulate osteogenic differentiation (Lee et al., 2013).

Despite TiO_2 doped calcium phosphate glass bioactivity and biocompatibility, recent studies show that primary mouse fibroblasts growth on microcarriers surface is impaired comparing to tissue culture plastic (Guedes et al., 2013). In order to improve cell adhesion this study was focused on evaluate cell response, namely cell attachment, of MG63 cells, here used as a model for bone precursor cells, on 5% mol TiO2 and 5% mol TiO2 + 2% mol CoO2 calcium phosphate glass beads coated with the bifunctional protein FN-OCN (Lee et al., 2013).

5. Literature Review

5.1. Stem cells

Stem cells are a population of cells that have the ability of self-renewal and differentiation into several cell types. Embryonic cells, pluripotent stem cells, are able to differentiate into all the three germ layers which offers a tremendous potential for therapeutic and clinical use. However, ethical and legal concerns impelled the exploring of progenitors and adult stem cells pools (Lin, 1997).

Adult stem cells can be found in several tissues from bone marrow to hair follicle bulge. Nevertheless, the adult stem cells are rare in the human body and differ highly in concentration between donors and between different aspirates from the same donor especially with age. A popular source of adult stem cells is bone marrow and adipose tissue aspirates and its mesenchymal source allows differentiation into cartilage, bone, adipose tissue and blood vessels. However this is a small population of cells that need expansion to achieve workable cell numbers (Barrilleaux, Phinney, Prockop, & O'Connor, 2006).

5.1.1. Mesenchymal Stem Cells

A specific Adult Stem cell population is called Mesenchymal Stromal cells and they are found in several mesenchymal tissues, like bone marrow, adipose tissue, umbilical cord blood and placenta. This cells are anchorage-depend, meaning they need a surface to grow on and have several features including differentiation in bone, cartilage, muscle and fat (see figures 1 and 2), support of several tissues, production of regenerative and immunoregulatory factors (Caplan, 2007).

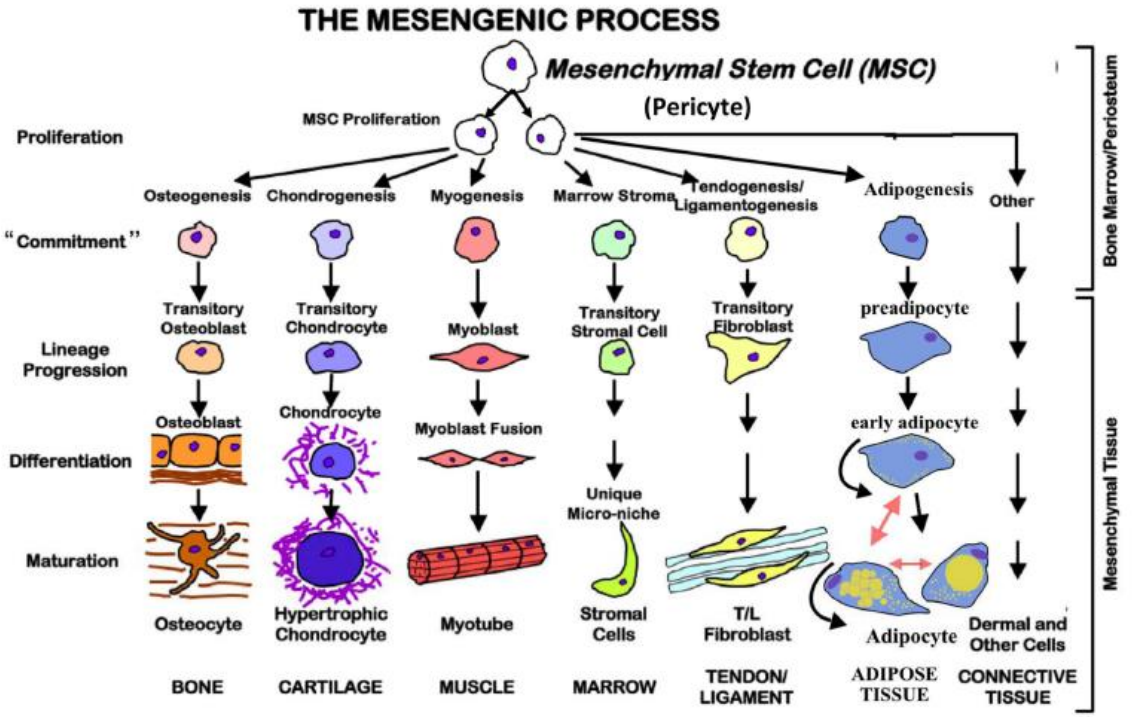

Figure 1- The mesengenic process. Mesenchymal stem cells are multipotent and possess the ability to proliferate and commit to diferente cell types based on the environmental conditions. They also may be redirected from one lineage to another (Dimarino et al., 2013)

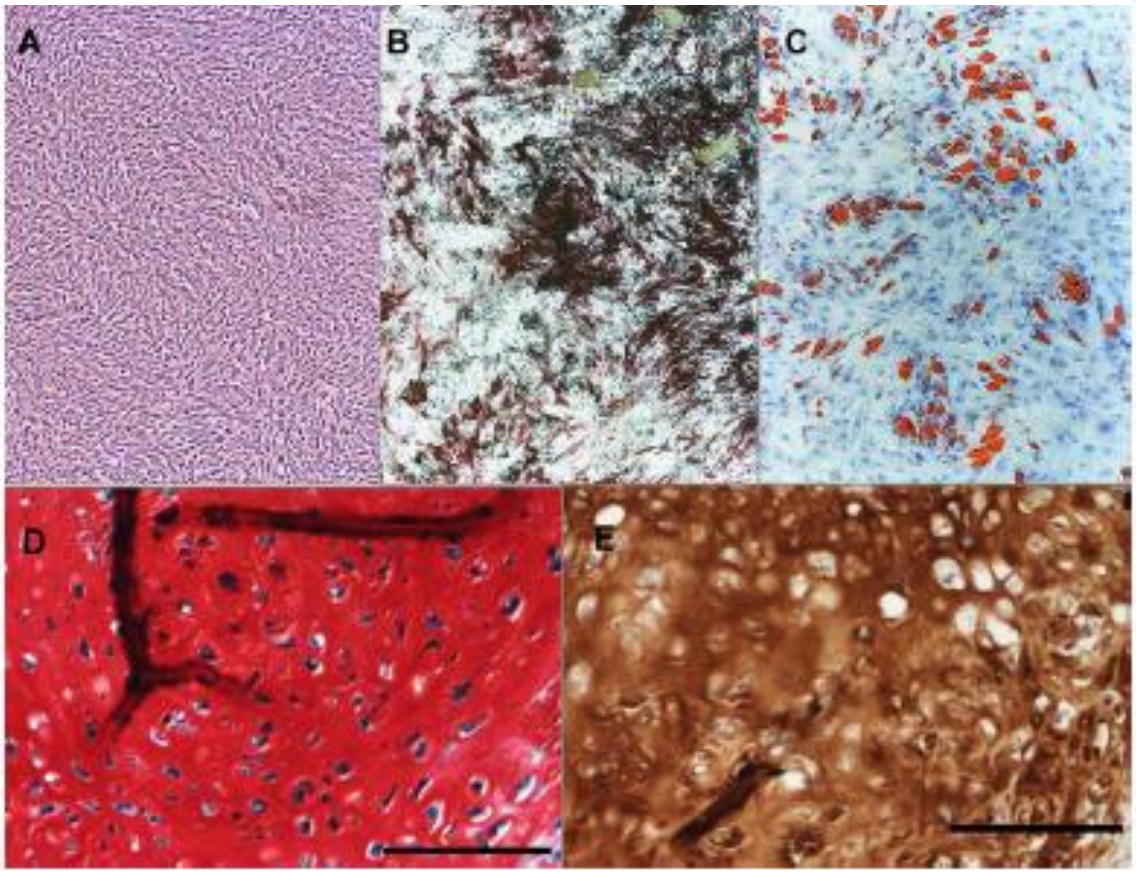

Figure 2-Undifferentiated MSCs grown in monolayer culture (A) and after differentiation along the osteogenic (B), adipogenic (C) and chondrogenic pathways (D) and (E). Cell differentiation is these cultures was observed following staining with von Kossa (B), Nile red O (C), Safranin O (D) and by immunostaining with an antibody specific for type II collagen (E). (Barry & Murphy, 2004)

5.1.1.1. Culture Systems

Animal cells are by nature more sensitive than microbial cells because they lack a rigid cellular wall which makes them more vulnerable to shear stress, osmolarity changes and damage due to bubbles. Moreover, most animal cells require a surface to grow on which makes its expansion much more complex and challenging. (Da Fonseca & Teixeira, 2007)

There is two main kinds of tissue culture systems: 2D culture on plastic and 3D culture on microcarriers. The 2D culture consists on a plastic surface where the cells can grow on a two dimensional environment.

Microcarriers are small beads with diameters ranging from 100 to 400 μm where adherent cells can grow on and are made of several biocompatible materials like cellulose, chitosan, collagen, dextran, gelatin, glass, polyethylene, and polystyrene. They can be porous and non-porous. The use of porous microcarriers offers the possibility of rapidly expand cells on bioreactors in a 3D environment in vitro mimicking more closely their niche in vivo. Moreover, cells grown on microcarriers can be transplanted *in vivo* and its capacity of regenerating tissues evaluated. Cell attachment on microcarriers depends on its chemical composition, surface topography, degree of porosity and charge density. Moreover diameter and resulting surface area of the carrier may also influence the affinity of cells on the microcarrier surface (Martin et al., 2011). Cell attachment is also influenced by cell seeding density, microcarrier concentration, cell to microcarrier ratio, agitation speed and agitation profile (Nam, Ermonval, & Sharfstein, 2009).

One of the main advantages of microcarriers is the surface area available for cell growth per unit of volume. The possibility of culturing high amounts of cells in a reduced space makes this strategy more cost-effective and less time consuming when compared with 2D culturing in tissue cukture plastic. It also allows tight control of all critical culture parameters like shear force and media composition (Martin et al., 2011)

5.2. Tissue Engineering

Tissue injury and organ failure is a major health problem. Despite the existing solutions: transplantation, surgical repair, artificial prosthesis, mechanical devices and occasionally drug therapy, sometimes the extent of the injuries unable the healing and long-term recovery of tissues and organs (Devices, 2000). Tissue engineering, as it was defined, about 20 years ago (Langer & Vacanti, 1993), appeared as a possible solution or complement and soon had several studies with major breakthroughs like the implantation of a human ear in the back of a mouse that open an all new world of possibilities to complete regeneration of tissues. The possibility to assemble, scaffold and induce cells to perform a given function of interest, using principles of Biology and Engineering, allows the design of healing and/or reconstruction therapies to replace injured or diseased tissues and organs (Langer & Vacanti, 1993).

The commercialization of cell-based products to treat organ and tissue failure require large scale production to be able to provide products at reasonable prices to healthcare clients (Davie, Brindley, Culme-seymour, & Mason, 2012). Therefore, a reproducible manufacturing process producing a significant number of high quality cells and suitable protocols to differentiate them into the cell type of interest are needed. Knowledge acquired from Cell Biology, Immunology, molecular biology, materials science, transplantation biology and clinical expertise of the disease being treated can be used to understand cell growth and differentiation and the role of ECM in such processes, to design cells to avoid rejection by the immune system, to produce scaffold constructs mimicking ECM and transplant them into the patient (Gage, 1998; Langer & Vacanti, 1993). Moreover several issues must be considered when producing a cell-based product like cell source, cell line or primary tissue from the patient himself, other human donor, animal source or fetal tissue always taking into account efficacy and ethical and safety issues; cell preservation, cryopreservation, for instance worked well with same cell types, but its procedures must be extended to other tissues (Langer & Vacanti, 1993).

Since in vitro expansion and differentiation of cells means their growth on an artificial environment changes will occur inevitably in the cell micro environment and this results in modification of their morphological and functional characteristics, so it is important for cells to maintain their functionality to the extent that they are need in the cell therapy (Brindley et al., 2011).

One of the main obstacles to cell product manufacturing, specially MSCs-based treatments, besides donor to donor variability, lies in the fact that long term culture of cells leads to loss of multipotency and senescence (Banfi et al., 2002) due to several forces during bioprocessing such as centrifugal, hydrodynamic, capillary flow shear stress. Also intermittent interruption of cell to ECM adhesion through trypsinization in each passage of the cells may affect cell functionality by affecting the cell surface (Mallucci & Wells, 1972).

Since most of bioprocess forces are related to cell harvesting such as shaking of flasks to aid cell detachment after trypsinization, forces produced during centrifugation to obtain a cell pellet or pipetting to resuspend cell pellets (Brindley et al., 2011) the rationale would be to avoid separate the cells from their growing surface. Strategies that allow the transplant of therapeutic cells coupled with a biocompatible scaffold are the ones in which it becomes part of the tissue being reconstruct or after transplanted it degrades in a controlled manner in a way it allows tissue engraftment to occur yet providing support during the process also preventing long term rejection problems (Langer & Vacanti, 1993).

5.2.1. Bone Regeneration

Bone defects due to trauma and pathological and physiological bone resorption are a major health problem. Although bone tissue is able of self-renewal at some extend throughout our life as a response to injury as well as during skeletal development (Kon et al., 2012), the healing process of bone defects due to fracture or disease can impaired due to several factors, namely age, with a decrease in the production of mediators and hormones necessary in healing pathways, smoking and anti-inflammatory drugs which interfere with the inflammation phase of

bone healing (Arvidson et al., 2011). The bone healing process includes the phases of haematoma, inflammation, angiogenesis, chondrogenesis to osteogenesis and finally bone remodelling (Arvidson et al., 2011) being the last two phases similar to normal development of the skeleton during embryogenesis (Deschaseaux et al., 2010).

After bone injuries, several molecular mechanisms are responsible for bone repair from stem/progenitor cells. Inflammation factors attract regenerative cells which expand and differentiate in order to replace the bone (figure 3).

In Bone Regeneration, there is several options such as biomaterial prosthetics to provide support in case of bone loss (Valauri, 1982), autografts, bone graft from the patient himself, have been the primary choice to replace bone defects like no-unions, delayed unions, filling of none cavities, replacement of bone lost due to trauma or tumour removal. However, they have low availability, long and invasive procedures and several possible complications for the patients. Allografts, grafts from a different person, on the other hand, have the disadvantage of risk of rejection and contamination. To prevent contamination some allografts are subjected to extensive procedures that may destroy its integrity and endanger its osteogenic and osteoinductive capability. (Damien & Parsons, 1991)

However in both cases the cell availability is limited and unlikely to fulfil the need for a robust bone regeneration treatment. Strategies for bone regeneration enhancement can have two different approaches: improving cellular capacity with population enrichment, expansion, differentiation or targeting to injured site or improving the extracellular environment for cell-mediating regeneration with matrices/ scaffolds providing the necessary chemical, physical and biological cues.(Dawson, Kanczler, Tare, Kassem, & Oreffo, 2014)

5.2.1.1. Osteoblasts

Osteoblasts originate from mesenchymal precursor cells and are responsible for bone formation and maintenance namely deposition of bone matrix and osteoclast regulation. Along

their differentiation they acquire the ability to secrete bone matrix and eventually some of them became surrounded and differentiate into osteocytes. The formers act as sensors to regulate bone formation and resorbance by osteoblasts and osteoclasts, respectively (Caetano-Lopes, Canhão, & Fonseca, 1998).

In cell culture osteoblasts have a fibroblast-like appearance. In fact, they only differ by only have two specific transcripts: a transcript encoding for Cbfa1 identified as a controller of osteoblastic linage and another encoding for osteocalcin, which is only expressed when osteoblast are fully differentiated (Caetano-Lopes et al., 1998).

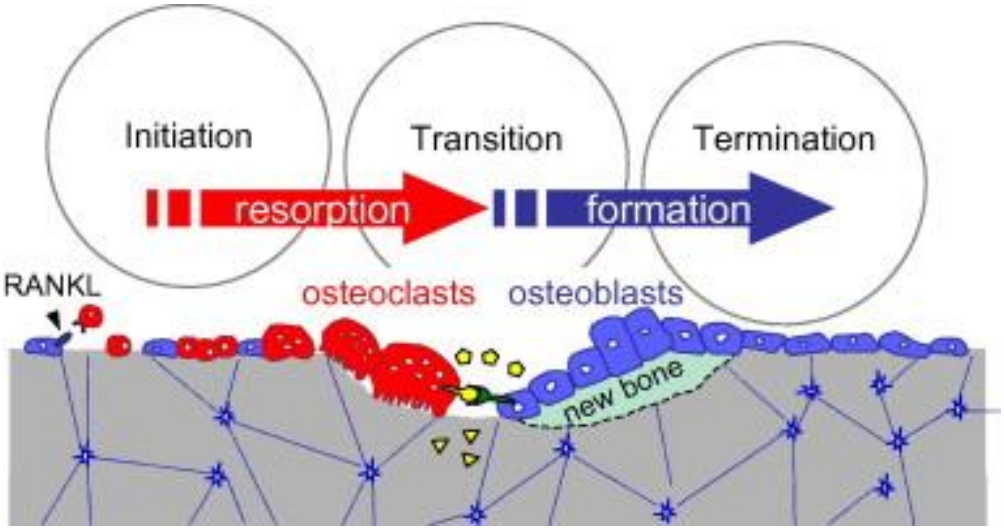

Figure 3 -Three-phase model of bone remodeling. Initiation starts with recruitment of hematopoietic precursors. Osteoclast (red) differentiation is induced by osteoblast (blue) lineage cells expressing osteoclastogenic ligands such as RANKL. Osteoclasts become multinucleated and resorb bone. Transition is marked by switching from bone resorption to formation via coupling factors, possibly including diffusible factors (yellow pentagons), membrane bound molecules (yellow lollipops), and factors embedded in bone matrix (yellow triangles). The termination phase ensures that the resorbed lacuna is refilled due to the bone-forming activity of osteoblasts, and osteoblasts flatten to form a layer of lining cells over new bone (Matsuo & Irie, 2008).

5.2.1.1.1. MG63 cell line

MG63 cell line is a human osteosarcoma cell line that can be used as an osteoblast model to study cell adhesion, integrin subunit expression and osteocalcin production (Clover & Gowen, 1994).

5.2.1.2. Biomaterials

Although tissue engineering field did not progress as fast as expected several clinical successful achievements were made in the last few years like the substitution of skin in burn victims, treatment of diabetic ulcers or implantation of an engineered trachea in a woman with failed airway all using decellurarized matrices. However it is believed that biomaterials can have an important role in Tissue Engineering as well, especially in more complex tissues like bone, myocardium and neural where ECM-derived scaffolds cannot meet its requirements of coordinated presentation of biological cues. (Burdick & Mauck, 2011)

Instead of the traditional design of scaffolds with concern only for degradation times, stability and toxicity new trends tend to enhance scaffolds to guide and direct cells into tissue regeneration and cell therapies. (Burdick & Mauck, 2011)

The strategies envisaged by tissue engineering for bone regeneration are the use of synthetic grafts made from biomaterials since they can be design to have both osteoinductive and osteogenic capacity, are readily available, sterile and have reduced rejection problems. The biomaterials used can be divided into four groups: metallic implants such as titanium and stainless steel implants, ceramics like calcium phosphate, alumina and glass, polymers like polymethylmethacrylate and polyurethane hydrogels or a combination of some of them. (Perez-Sanchez et al., 2010) Although several advances have been made in this field, namely in materials science, no biomaterial has yet full field all requirements necessary for a bone substitute (Arvidson et al., 2011).

5.2.1.2.1. Bioactive glass ceramics

Some of these biomaterials like glass and glass-ceramic compounds form bone-like apatite layers on their surface in contact to human body fluid through which they bond to the a living body demonstrating its bioactivity. The formation of an apatite layer only in bone tissue is due to the high activation energy of homogeneous apatite nucleation in human body fluid. Any biomaterial that has groups with an active site to lower the activation energy will form an apatite nucleus growing spontaneously in contact to the body fluid consuming its calcium and phosphate ions (Kokubo et al., 2004). In order to test its bioactivity, if a bone-like apatite layer is formed, these materials are submerged in a solution called simulated body fluid, SBF.

Some ceramics allow high amounts of material to be produced as small cell-carrying units that can then be transplanted as a whole after expansion (Guedes et al., 2013). Beads of this material can also be coated with metal oxides to add some useful features or improve the existing ones. It has been proved, for instance, that titanium coatings, also proven bioactive materials, like TiO2, titanium dioxide, enhance cell response when compared to CaP, calcium phosphate, component of some phosphate based glass beads, and SiO2, silicon dioxide coating (Mozumder, Zhu, & Perinpanayagam, 2011; Verket et al., 2012). Moreover, it has been shown that calcium phosphate beads doped with 5% mol TiO2 are good candidates for use as support materials in bone tissue engineering applications (Lakhkar et al., 2012).

Hypoxia (low oxygen pressure) is known to enhance angiogenesis. The ability to mimic such environment in a scaffold in of major importance in tissue engineering. Low amounts of ionic cobalt (< 5%) incorporated into mesoporous bioactive glass (MBG) scaffolds was shown to significantly enhanced VEGF protein secretion and HIF-1α factor expression which induces hypoxia-like response. The results also shown that an increase in bone-related gene expression in BMSCs, and also that the Co-MBG scaffolds support BMSC attachment and proliferation. (Wu et al., 2012).

5.2.1.3. Biofunctionalization

Several advances in the area have include finding biocompatible and bioactive materials and tailor them with suitable chemical and biological cues in the form of small molecules to direct cells towards a certain end (Firestone & Chen, 2010; Maia, Bidarra, Granja, & Barrias, 2013).

In osteogenic differentiation from mesenchymal stem cells (MSCs) these molecules include growth factors (GF), ECM proteins, hormones and several chemical compounds. There are several immobilization approaches that can be used to present these molecules to the cells including covalent bonding, physical adsorption and entrapment. From a wide range of chemical groups the more suitable properties for each cell niche can be chosen. In case of bone, negatively charged phosphate groups are present in its mineral part. (Maia et al., 2013)

Several biological and physiological processes are highly dependent of specific amino acids sequences present in peptides and proteins in the ECM which makes the use of artificial equivalents of peptide sequences a clever way of avoiding expensive and complex use of full length proteins, unspecific binding as well as immunogenic and cellular side effects responses. (Maia et al., 2013)

5.2.1.3.1. Adhesion proteins

Fibronectin, a dimeric glycoprotein, is known to be interacting with more than ten different integrin receptors and is essential to vertebrate development. It is produced mainly by stromal cell types and organized into fibrils that can be recognized by the tenth region of the monomers of its dimers possesses an RGD sequence (Arg-Gly-Asp) recognized by cell adhesion molecule integrin (figure 4). Intregrin is an essential specific cell receptor that binds cells to the extracellular matrix (Xu & Mosher, 2011).

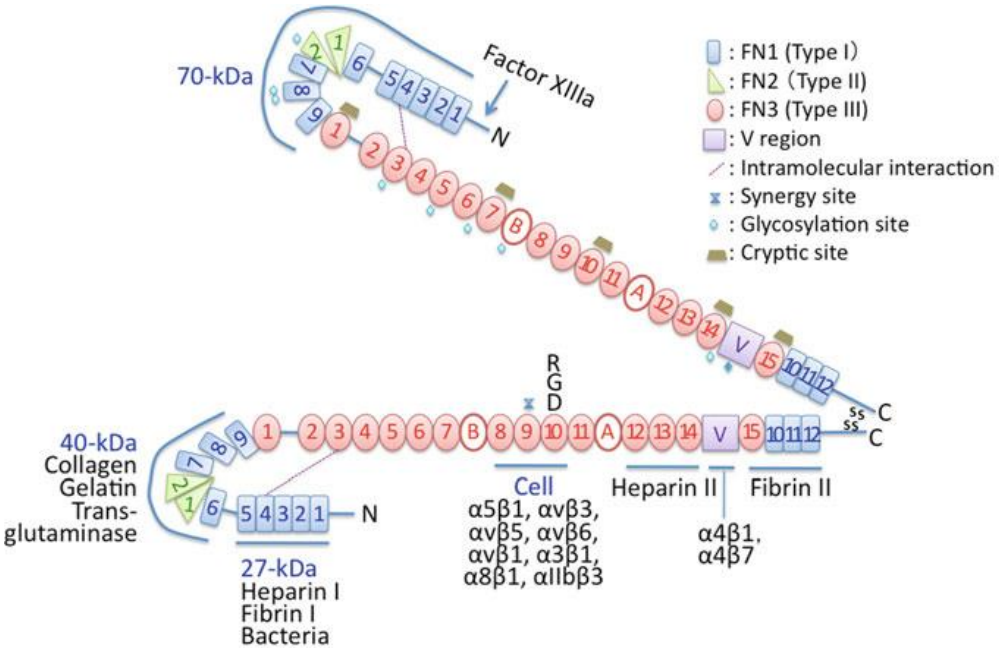

Figure 4 -Diagram of the modular structure of fibronectin. Each fibronectin dimer is composed of two monomers linked at the C-terminus by a pair of disulfide bonds. 12 type I modules (blue rectangles) termed FN1, 2 type II modules (green triangles) termed FN2, and 15–17 type III modules (salmon ovals) termed FN3. The number of FN3 modules varies due to the presence of AFN3 (EDA) and BFN3 (EDB) based on alternative splicing. The alternatively spliced V region is shown as a purple square. Proteolytic 27-kDa, 40-kDa, and 70-kDa N-terminal fragments and the protein-binding sites on fibronectin are underlined with receptors listed.(Xu & Mosher, 2011)

5.3. Aims and objectives

Considering previous results with mouse fibroblast growth onto 5% TiO2 doped calcium phosphate glass microcarriers reduced compared to tissue culture plastic (Guedes et al., 2013), this project major goal was to enhance mesenchymal stem cell growth and differentiation into osteoblasts by promoting their attachment to a functionalized bioactive material, CaP beads with 5% mol TiO2, as this substance was shown to promote bone formation and differentiation (Mozumder et al., 2011; Verket et al., 2012) coated with a fibronectin-osteocalcin (FN-OCN) bifunctional fusion protein with a specific cell binding amino acid sequence, the nineth-to-tenth type III domain of human fibronectin (figure 5) and full length mouse osteocalcin protein as described by Kim, Park, Kim, & Jang, 2007 and Lee *et al.*, 2013 in which fibronectin is expected to promote cell adhesion and osteocalcin to regulate osteogenic differentiation (Lee et al., 2013).

In this project we wanted to assess whether MG63 cell line expansion and osteodifferentiation could be promoted by using ad hoc functionalized microcarriers. In the specific, we wanted to promote MG63 cell attachment on 5% mol TiO_2 and 5% mol TiO_2 + 2% mol CoO_2 CaP beads by using a bi-functional chimeric protein composed by mouse osteocalcin and the nineth to tenth domain of human fibronectin which will connect cells and beads.

The main aims included production of FN-OCN chimeric protein encoded by the plasmid pBAD/His-FNIII9–10/OCN; validation of microcarrier coating with OCN-FN; in vitro MG63/FN-OCN microcarriers adhesion assays.

Figure 5 - Type III tenth module of fibronectin (Xu & Mosher, 2011)

6. Materials and Methods

6.1. FN-OCN production

6.1.1. Transformation of pBAD/His-FNIII9-10/OCN plasmid into electrocompetent *E.coli* TOP10 cells

It was provided the plasmid pBAD/His-FNIII9–10/OCN (map of the plasmid figure 6) which contained the sequence for the protein FN-OCN. It was run an agarose gel to verify the integrity of the plasmid. Transformation into E.coli TOP10 electro competent cells *(E.coli* TOP10, *Invitrogen, Life Technologies*, date of arrival: 13/02/2014) was performed by electroporation. Transformants were selected in an LB-Ampicillin media plates (NaCl, *Fisher Chemical*; Tryptone *Fisher Chemical*; Yeast extract, *Fluka Analytical*, *SIGMA-ALDRICH*; Ampicillin, *SIGMA-ALDRICH*).

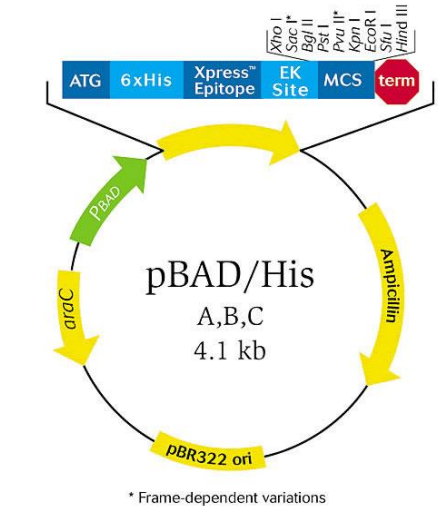

Figure 6 -pBAD/His plasmid map

6.1.1.1. *Agarose Gel*

The Agarose gel separates DNA molecules according to their weight by applying an electric field. A volume of 50 mL of Agarose gel was done at 0.07% (w/m) agarose (*SIGMA Life Science*) dissolved in TAE buffer by heating for 1.5 minutes in the microwave. Then Siber green was added and the mixture was put in the running set up and pBAD/His-FNIII9–10/OCN plasmid was added. The run was performed at 70 volts for 45 minutes.

6.1.1.2. *Transformation by electroporation*

Electroporation is a method for transformation of genetic material into a cell. To perform electroporation transformation on electrocompetent cells 30 µL were added to 1 µL of pBAD/His-FN-OCN plasmid on cuvettes in ice, subjected to electroporation (*BIORAD MicroPulser*), added 1 mL of pre-warmed SOC media (*Invitrogen, Life Technologies*)) and left one hour at 37ºC and 250 rpm to allow the gene expression. Then they were spread at LB-Ampicillin media plates to allow the selection of transformants.

6.1.2. Expression of FN-OCN

Expression of FN-OCN fusion protein was done after growth at 37ºC overnight in LB-Ampicillin media and was induced by 0.02% (w/v) L-arabinose (*Fluka BioChemika, SIGMA ALDRICH*) at A600 around 0.6 as described by Lee *et al.* 2013. The absorbance was measured every hour. After induction samples were taken every hour until 6 hours of fermentation. The harvesting of cells were done by centrifugation (*UNIVERSAL 320R Hettich Zentrifugen*) at 5000 rpm during 10 minutes.

6.1.3. Purification of FN–OCN bifunctional protein

After harvesting the pellets of cells were ressuspended in TRIS buffer 50mM, pH=8 (*CALBIOCHEM*). Then the cells were sonicated in cycles of 10 seconds on and 10 seconds off 10 times (*Soniprep 150 MSE SANYO*), centrifuged (*SORVALL SUPER T21*) for 1 hour at 13 000 rpm and 4ºC and the supernatant recovered.

Following the manual of the manufacturer, 1 mL NTA-Ni column (*QIAGEN, Ni-NTA Superflow Cartridge)* equilibrated with 10 mL of NTI-10 buffer at a flow rate of 10 mL/min, were loaded with the recovered supernatant at the same flow rate. Afterwards the columns were put in the freezer for 10 minutes to improve binding. Then they were washed with 10 mL of NTI-20 buffer and the protein was elute with 10 mL of NTI-250 buffer. The columns were washed with 10 mL (10 volumes of column) of demineralized water and 10 mL of ethanol (PROLABO) at 20% (v/v). Afterwards 2.5 mL elution sample was desalted in a desalting column (GE Healthcare) to remove

the imidazole after equilibration with one column volume of TRIS, 50 mM, pH 8 and before addition of 3.5 mL of the same.

It was run a polyacrylamide gel (gel: novex by Life Technologies; set-up: XCell SureLockTM) to verify the expression of the protein of interest with 125 V for 90 minutes, stained with Coomassie Blue (InstantBlueTM, expedeon) for 30 minutes.

6.1.4. Protein quantification by NanoDrop method

The FN-OCN fusion protein was quantified by the NanoDrop method using *Thermo Scientific NanoDrop ™* 1000 spectrophotometer. This method quantifies purified protein concentration by measuring Trp, Tyr residues or Cys-Cys disulphide bonds absorbance at 280 nm (UV range) making absorbance spectroscopy a fast, convenient method for the quantitation of purified protein preparations.

6.2. Coating of CaP glass beads with FN–OCN protein

5 mg of 5 % mol TiO_2 beads and 5 % mol TiO_2 + 2% mol CoO_2 beads were put into wells of low attachment 96 well plates in order to have a monolayer.

100 µL of of protein solution were added to several wells.

At each coating time point the samples were washed once with PBS and fixed with 4 % (v/v) paraformaldehyde (PFA) for 20 min and then washed once with PBS.

6.2.1. FN-OCN imunostaining

FN-OCN fusion protein was incubated with 70 µL of rabbit anti-osteocalcin (anti-OCN) polyclonal antibody (*MILLIPORE*) 1:500 overnight at 4ºC and a goat anti-rabbit Rhod (abcam) labelled antibody was added afterwards for 45 minutes after washing three times with PBS for development of fluorescence signalling.

6.3. Functional cell adhesion

6.3.1. Culture of MG63 (human osteosarcoma cell line)

A vial with 1 mL and $1x10^6$ MG63 cells with 8 passages (P8) was thaw from -80ºC freezer and split in two 75 cm^2 flasks with 10 mL of media and expanded for two days.

6.3.2. MG63 cell adhesion assays on microcarriers

15000 MG63 cells P9 were added to each well of coated beads for 1h in low-attachment microwell plates.

6.3.3. Cell adhesion assay with CCK8 detection

. Cell Counting Kit-8 (CCK-8) is a sensitive calorimetric method for viable cell counting that uses a highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt] producing a water-soluble formazan dye upon reduction in the presence of an electron carrier, in this case the dehydrogenases in cells. The amount of formazan dye formed is proportional to the number of viable cells.

Cell attachment quantification was done after 1h cell attachment and 1x wash with PBS by adding 10 µL of CCK-8 solution (SIGMA-ALDRICH) to each well with 100 µL of media for 2h. The signal was detected at 490 nm of wavelength.

7. Results

7.1. Production of FN-OCN fusion protein

Integrity of the plasmid was confirmed using gel electrophoresis (figure 7). Plasmids integrity and no degradation was confirmed by two bands on the fifth and seventh rows corresponding to the two forms of the plasmid: supercoiled and unfolded.

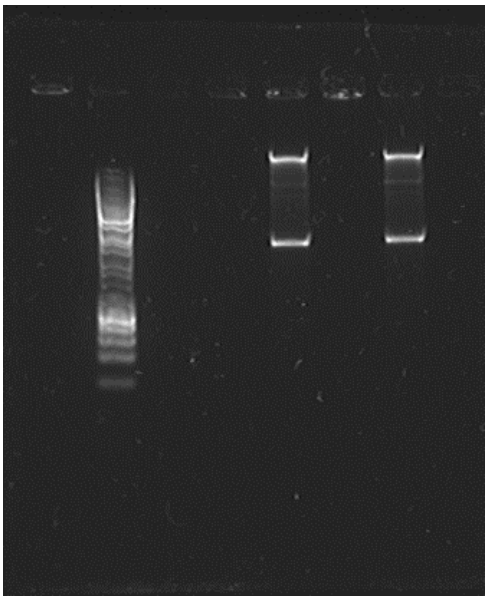

Figure 7- 0.7% Agarose gel in TAE buffer of both plasmids pBAD/His-FN-OCN. The second row is a ladder. The fifth and seventh rows are the two plasmids. The two bands of each row correspond to both forms of the plasmid: supercoiled and unfolded.

7.1.1. Transformation of pBAD/His-FN-OCN plasmid into E.coli TOP10 electrocompetent cells

E.coli TOP10 electrocompetent cells were transformed and selected in a LB media with 50 µg/mL of ampicillin. Control plasmid (pUC19) and the negative control grew and all plates with cells transformed by electroporation had significant growth (figure 8).

Cells transformed by electroporation were inoculated in Falcon tubes with 50 and 100 µg/ mL ampicillin or scratched onto LB-ampicillin media plates containing 50 and 100 µg/ mL ampicillin and left overnight at 37 ºC and 250 rpm (figure 9).

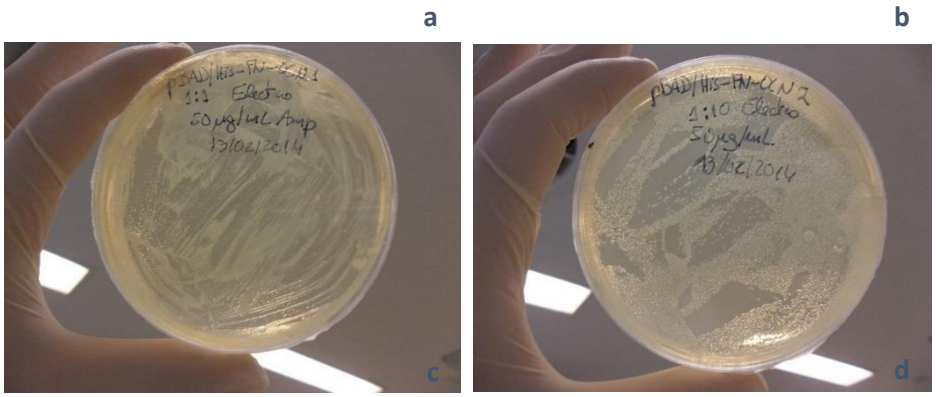

Figure 8-Plates of electrotransformed E. coli TOP10 electrocompetent cells selected in in a LB media with 50 µg/mL of ampicillin: a) no dilution b) 1:10 dilution(17/02/2014)

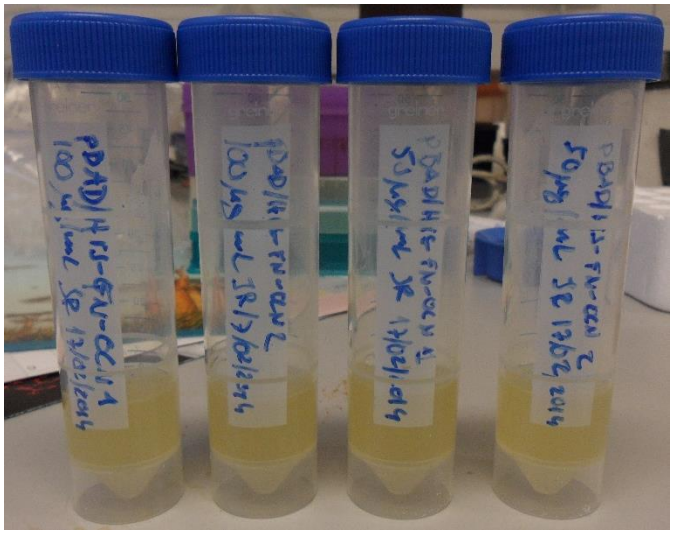

Figure 9 Inoculums grown at 50 and 100 L/mL of ampicillin for both plasmids pBAD/his-FN-OCN (18/02/2014)

7.1.2. Fermentation

For protein production a larger fermentation volume was performed (1.6 L in four 2 L flasks with 400 mL each). Absorbance at 600 nm values for each flask along the fermentation are shown below (table 1).

Table 1- Absorbance at 600 nm values of the fermentation of electrocompetent E.coli TOP 10 pBAD/His-FN-OCN transformed cells. After 0.6 absorbance cells were induced to produce the protein of interest.

Time (h)	1	2	3 (induction)	5 (10x dilution)	6 (10x dilution)
A600	0.126	0.399	0.977	0.188	0.294

7.1.3. Purification of FN–OCN bifunctional protein

The protein was expressed and purified using NTA-Ni column chromatography. The second row in figure 10 corresponds to the 2.5 mL elution volume and it has a band between 43 and 34 kDa ladder bands as expected (the protein has 40 kDa (Lee et al., 2013)).

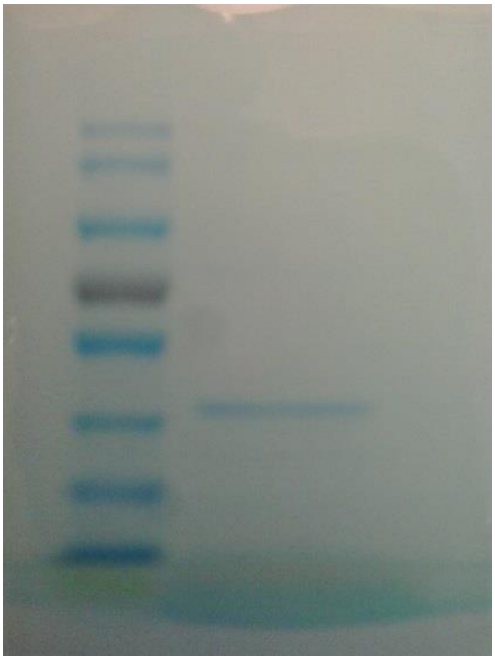

Figure 10-Polyacrylamide gel of the ladder on the first row and FN-OCN fusion protein on the second row. The second row corresponds to the 2.5 mL elution volume of the purification step on the NTA-Ni column.

7.1.4. Protein quantification by NanoDrop method

The protein quantification was done using the NanoDrop method by measuring protein absorbance at 280 nm. The protein concentration was 0.4075 µg/mL.

7.2. Coating Imunostaining of CaP doped with 5% TiO_2 glass beads with FN–OCN protein

Fluorescence intensity of FN-OCN coated onto microcarriers with three different coating times, 0, 4 and 24h (figure 11 a), b) and c) respectively) is shown. The corresponding well with the same coating time but treated with just secondary antibody is also shown (figure 11 a'), b') and c')).

There is an obvious increase in fluorescence intensity with coating time, which cannot be seen in the control well treated with just secondary antibody.

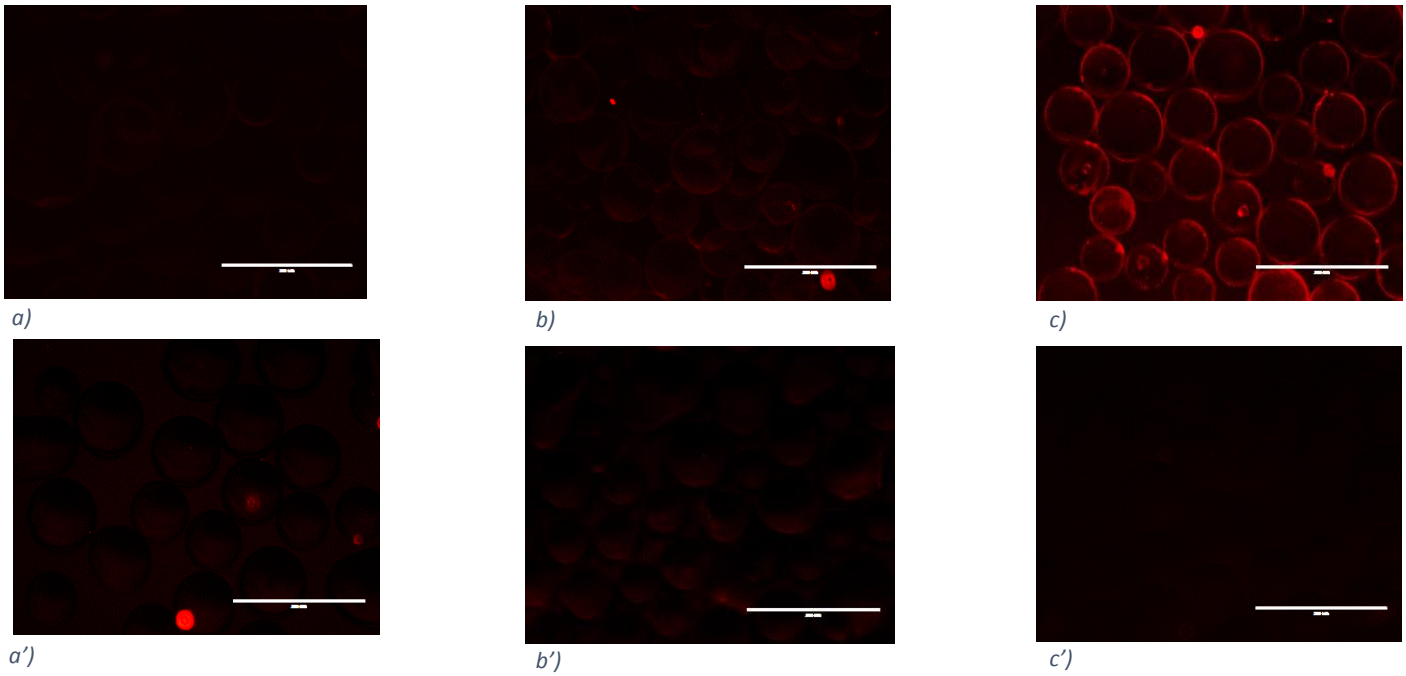

Figure 11- Fluorescence intensity after a) 0h b) 4h c) 24h coating. a'), b') and c') are the correspondent secondary only controls. The staining was done through affinity binding of a rabbit anti-osteocalcin primary antibody and a goat anti-rabbit anti-osteocalcin secondary antibody for fluorescence development.

7.3. MG63 adhesion assays on microcarriers coated with FN-OCN fusion protein

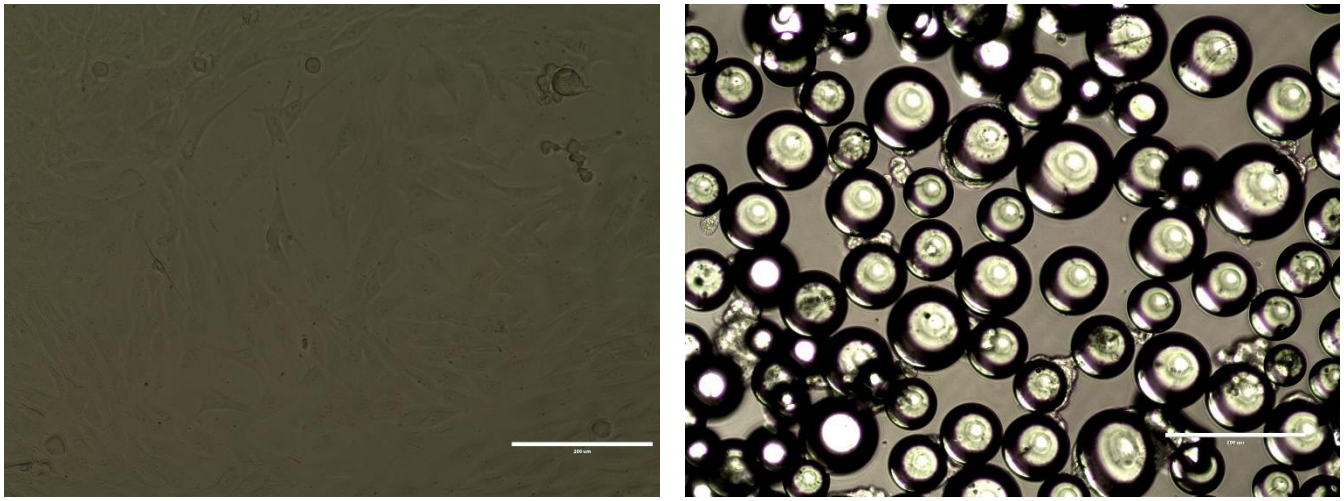

Figure 12- a) MG63 cells expanded on tissue culture plastic b) MG63 cells seeded onto 5% TiO2 CaP microcarriers (pictures are courtesy of PhD student David De Silva-Thompson)

MG63 cells were expanded on tissue culture plastic (figure 12 a)), passaged and seeded onto microcarriers, then left for 1h (figure 12 b)).

Cell attachment after 1hr was measured using the CCK-8 colorimetric assay and data are presented in the following graphs (figures 13 and 14), comparing both kinds of beads: 5% mol TiO_2 and 5% mol TiO_2 + 2% mol CoO_2 CaP; and with different protein concentrations (0, 1, 5 and 10 µg/mL) and coating times (4h figures 13 and 14 a) and 24h figures 13 and 14 b)). Each condition had three replicates and it was measured three times and error bars correspond to the data standard deviation.

The first experiment (figure 13 a) and b)) showed that FN-OCN stimulated a clear increase in attachment to the 5% mol TiO_2 microcarriers but not the 5% mol TiO_2 + 2% mol CoO_2 microcarriers. However, in a second run of the assay (figure 14), this result is not replicated and so further work is needed to confirm these results.

The results indicate an increase in signal intensity with FN-OCN fusion protein concentration during coating for the 4h coating experiments (figure 13 and 14 b)), suggesting there are more cells attached after one hour. The same is observed for the 24h experiment (figure 13 and 14 b))

except for the 10 µg/mL protein concentration, so perhaps a threshold is reached after which the protein would not support cell attachment. Such result can be explained by a saturation time in which there is no more coating no matter how much protein is there in the solution. Also cells consistently attached more on raw 5% mol TiO_2+ 2% mol CoO_2 beads than to raw 2% mol TiO_2 beads.

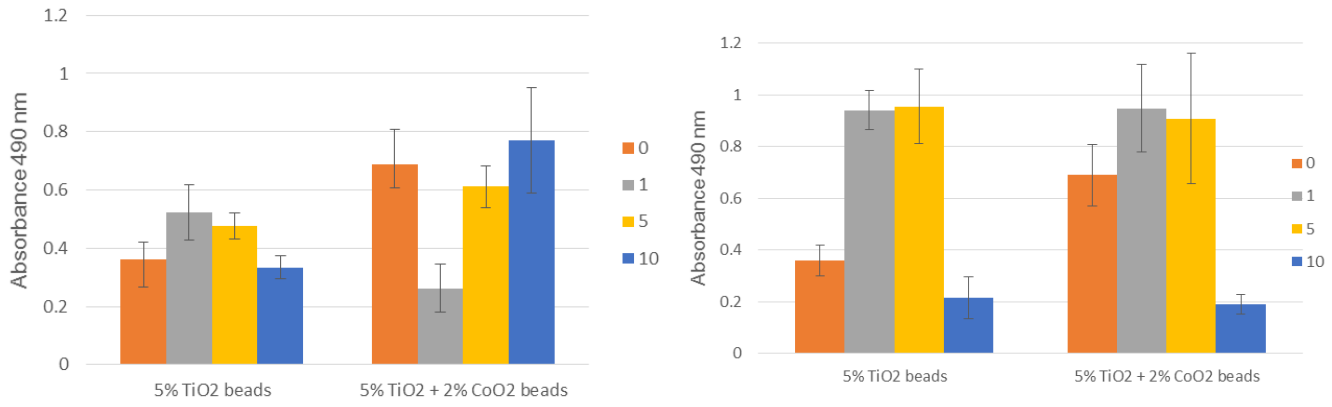

Figure 13- a) and b) 1st experiment with 4 and 24h FN-OCN protein coating respectively, with 0, 1, 5 and 10 µg/mL of protein concentration The cell adhesion assay was performed by adding 15 000 cells to each well, let them adhere for 1 hour, wash 1x with PBS and adding of CCK-8 solution. Each condition had three replicates and error bars correspond to the data standard deviation.

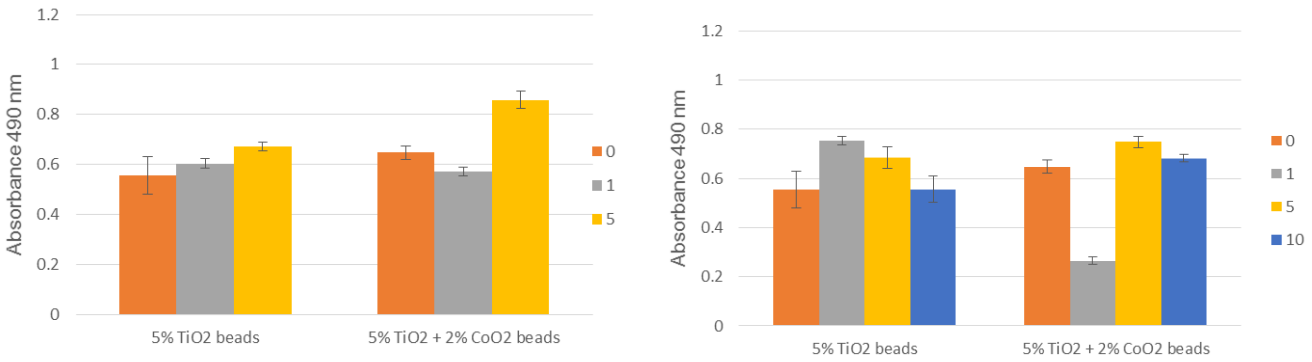

Figure 14- a) and b) 2nd experiment with 4 and 24h FN-OCN protein coating, respectively, with 0, 1, 5 and 10 µg/mL of protein concentration. Each condition had three replicates and error bars correspond to the data standard deviation.

8. Discussion

Due to several limitations in bone grafts used in bone regeneration like treatment failure, rejection, contamination risk, efforts have been made in order to replace by osteoinductive and osteoconductive materials with or instead of bone (Damien & Parsons, 1991)

Although the bone possesses an intrinsic regeneration capacity in face of injury or during development and adulthood, some cases require a great deal of bone regeneration which lead to tissue engineering direct its research towards enhancement of new functional tissue (Kon et al., 2012).

Mesenchymal Stromal Cell have been shown as a promising alternative for cell therapy, namely bone (Kon et al., 2012). However, aspirates from patients provide little number of cells required for cell treatment (Barrilleaux et al., 2006). Therefore it is crucial to find strategies that expand cells to achieve a workable number without compromising cell integrity and function (Brindley et al., 2011). As most animal cells, MSCs are anchorage-depend cells, meaning they need a suitable surface to grow on for efficient proliferation (Caplan, 2007). Microcarriers provide large surface area in a 3D environment for cell attachment and proliferation (Martin et al., 2011).

The aim of this project was to provide a scalable and reproducible strategy for expansion and further use in cell therapy for bone regeneration of mesenchymal stem cells. In this study two kind of CaP beads, doped with 5% mol TiO_2 and doped with 5% mol TiO_2 + 2% mol CoO_2, were coated with a bi-functional protein, FN-OCN, in order to access cell response to the surface of the beads.

Coating studies with imunostaining techniques revealed an increase in coating, meaning more protein on the surface of beads, with coating time. This is consistent with previous work assessing biomaterial scaffolds coated with FN-OCN fusion protein (Lee et al., 2013).

Biocompatibility was accessed by cell adhesion assays using an osteoblast cell model, an osteosarcoma cell line, MG63. Cell adhesion assays revealed a higher cell adhesion to raw CaP beads doped with 5% mol TiO_2+ 2% mol CoO_2 and an increase with FN-OCN concentration in coating for both kinds of beads. Higher protein concentration seems to increase coating for smaller coating times, based on the results from the cell adhesion assay.

Ultimately this research main objective was to create a platform of cell expansion and differentiation on the large surface area of microcarriers microunits that would avoid cell harvest and support cell growth and differentiation into bone tissue throught dopping with TiO2 and CoO2 known to induce osteoblastic differentiation and angiogenesis, respectively, and biofunctionalisation with a bi-functional protein with a portion of an adhesion molecule to increase initial cell adhesion. This results show an affinity of the fusion protein FN-OCN to the CaP beads doped with 5% TiO_2 and suggest biocompatibility and higher initial osteoblast-like cell attachment of the both coated beads doped with TiO_2 and CoO_2. The present study was conducted in static conditions to demonstrate proof of principle that the TiO_2 and CoO_2 doped microcarriers surface coated with FN-OCN protein are adequate for cell adhesion.

However these results were obtained in static conditions, which for producting cell-carrying microunits is not feasible because of the inexistence of a homogeneous culture media. Media changes in static conditions could detach and wash away weakly attached cells. A dynamic culture method must be developed in order to maintain a homogeneous environment. Overall, due to time limitations, it was not possible to optimise the cell attachment assays and fully characterise the effect of FN-OCN on cell attachment and proliferation on the microcarrier surfaces. The data indicates that there may be a positive effect, but much more work needs to be completed, starting with assessment of protein stability on the microcarrier surface and then progressing towards well-defined and reproducible assays to quantify cell attachment and then growth over a period of 14 days.

Also, translating the system to dynamic culture conditions that are more reflective of industry systems will aid development of the tools.

A building block cell therapy in which cell loaded microcarriers would be injected onto the bone injured site would allow a perfect fit scaffold to support bone regeneration while gradually degrading.

9. Conclusions and Future Work

In summary the doping with CoO_2 and biofunctionalisation with FN-OCN bi-functional protein of microcarriers increases cell adhesion reinforcing the idea that microcarrier strategy is a reliable alternative to existing methods in bone regeneration (Lakhkar et al. 2012; Guedes et al. 2013; Park et al. 2013).

Being cell delivery an important strategy in bone regeneration, microcarriers such 5% mol TiO_2 and 5% mol TiO_2 + 2% mol CoO_2 doped CaP glass beads represent a promising alternative to provide temporary support since TiO_2 provides controlled degradation and enhancement of osteogenic differentiation and CoO_2 mimics an hypoxic environment to enhance angiogenesis. Furthermore, biofunctionalisation suggests an increase in initial cell adhesion which is the limiting step in cell expansion. After proliferation and differentiation cells can be transplanted with the scaffold to fulfil bone injuries and restore function avoiding the need of cell detachment as in previous microcarrier strategies.

For further use in cell therapy a complete description of the bi-functional protein must be performed in order to fully characterize it and develop a reproducible protocol for its production. Also for the beads production, maybe the standardization of the beads diameter and shape in could possibly decrease variability in the results. The fact that only a part of the beads is available for coating in a monolayer can influence greatly the results in cell adhesion assays. Maybe a cell adhesion assay in which the cells are left for longer than 1h in contact with coated beads can give us a stronger signal and decrease the noise in the results.

Future work for this project could include long term cell culture in order to evaluate cell behaviour throughout expansion and differentiation, if besides support the studied beads could improve cell expansion and produce more early features of differentiation in comparison with other biomaterials and to monolayer culture. To access this changes cell number and osteodifferentiation features like ALP activity and osteocalcin mRNA expression could be

measured (J.-H. Park et al., 2013). Since the most promising strategy for bone regeneration is expansion and direct differentiation of MSCs in spinner flasks the next thing to do would be characterize the spinner flask culture system to develop a suitable cell/scaffold culture system to mimic *in vivo* environment with the appropriate flask and impeller geometry, size and velocity, medium quantity, microcarrier type, its properties (elasticity and surface roughness for instance) and its size and quantity, cell concentration, flow rate, and culture methods to provide the right mechanical stimulation and shear stress (J.-H. Park et al., 2013)

Lastly *in vivo* biocompatibility and bone formation induction testing must be performed for the doped and coated microcarriers in mouse cranial defect models, for instance. By evaluating the extend (are of formed bone) and location of bone formation (through X-Ray photograph and histological analysis) one can prove the biomaterial stimulation and scaffolding roles (J.-H. Park et al., 2013).

It can be concluded that doped CaP glass beads functionalized with bi-functional protein represent a promising alternative to previous microcarriers-based strategies since it has the potential to overcome several limitations.

An important can be envisaged in the use of this cell loaded microcarriers like the invasive treatment to take the product to the injured site which would have to be injected directly rather than intravenous injection which in that case there should be cues for the cells to move towards the injured site, adding more complexity to the cell product.

Future work in material science and stem cell biology is in order to produce tissue engineering products to be used in cell therapy.

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