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Doctoral thesis

**“DEVELOPMENT OF MEASUREMENT
METHODOLOGIES IN METROLOGY
FOR CELL BIOLOGY AND REGENERATIVE MEDICINE”**

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**DEVELOPMENT OF MEASUREMENT METHODOLOGIES IN
METROLOGY FOR CELL BIOLOGY AND REGENERATIVE
MEDICINE**

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PhD thesis

Politecnico di Torino and Istituto Nazionale di Ricerca Metrologica

*To my family
and to myself*

Summary

Aim of this thesis is the development of measurement methodologies in metrology for cell biology and regenerative medicine.

Regenerative medicine is a novel branch of medicine based on the use of autologous stem cells and biocompatible medical devices to regenerate and repair damaged tissues of patients, i.e. by using three-dimensional scaffolds¹ to implant stem cells into the tissue to be regenerated.

Stakeholders of metrology for regenerative medicine are: health care providers who require safe, reliable and cost effective treatments, supported by evidence and approved by regulators; regulators who require standard materials and traceable data demonstrating the safety and efficacy of new products and treatments; medical products companies who require advanced and traceable techniques to develop new products and need methods to measure processes, such as cell growth on scaffolds, to ensure quality and efficiency of the medical products implanted into the patients.

Consequently, regenerative medicine has the important requisite of a real time monitoring and not invasiveness neither destructiveness processes to measure the cell-scaffold interactions, in order to preserve the samples from any contamination or modification.

¹ Scaffold: three dimensional structure, natural or synthetic, used to harbour cells *in vitro*. Detailed description in Chapter 1.

Thus non-invasive measurement methodologies need to be developed for analysing the 3D cell culture on scaffolds and, in order to evaluate the uncertainty, highly reproducible measurement procedures are strongly required to minimize the type A uncertainties and to define the type B uncertainties.

The non-invasive and non-destructive measurement of cell-scaffold interactions (i.e. stem cell proliferation and differentiation on scaffolds) is one of the most effective methodology to answer the need of testing the efficacy of the design, production/manufacturing, development and performances of stem cell-scaffold products.

To satisfy the requirements and the needs for metrology in regenerative medicine, for this thesis it has been chosen to develop a measurement methodology for cellular activity (proliferation and differentiation) on 3D Biocoral[®] scaffolds and to conduce a metrological study to evaluate the uncertainty of the methodology.

This thesis has been developed in the Bioscience group of the Italian National Metrological Institute (Istituto Nazionale di Ricerca Metrologica - INRIM).

The main important contributes of this thesis to the metrology in biosciences have been:

- to lay the foundations for a metrological approach to cell biology and particularly to regenerative medicine research and applications;
- to address the filling of the lack of traceability in the metrology for cell biology metabolic methodologies used to evaluate cellular activities in living sample with non-invasive procedures.

The main results and originalities achieved during this PhD work are:

- a metabolic assay, the resazurin/resorufin assay, for the first time, has been metrologically characterized and the uncertainty of the measurement has been evaluated;
- the resazurin/resorufin assay has been for the first time tailored for a 3D cell culture on Biocoral[®] scaffolds and the uncertainty of the measurement has been evaluated;
- it was demonstrated that Biocoral[®] induces osteodifferentiation of stem cells and for the first time it was demonstrated on human mesenchymal stem cells;
- it was demonstrated, for the first time, that the resazurin/resorufin metabolic assay can be a methodology to detect not only the proliferation but also the differentiation of stem cells on Biocoral[®] scaffolds;

A description of the METREGEN regional project, which this thesis is part of, will follow in the **introduction**.

The **chapter 1** will give an overview on regenerative medicine field and its application with scaffolds, particularly referring to the Biocoral[®] scaffold.

The resazurin/resorufin methodology will be deeply described in **chapter 2** with a uncertainty budget evaluation and discussion.

Chapter 3 will present in details a series of experiments made to establish and characterize a hMSCs *in vitro* 2D culture, establish a hMSCs *in vitro* 3D culture on Biocoral, tailor the resazurin/resorufin assay for 3D cell culture on Biocoral and evaluate the hMSCs osteodifferentiation induced by Biocoral scaffolds. All the results have been analysed with a metrological approach to evaluate the uncertainty.

Finally, the **conclusion** will give a recapitulation and some interesting perspective of employment for the resazurin/resorufin methodology to final users, such as the cell factories².

² Cell factories are facilities, working with good manufacturing practice (GMP) procedures, dedicated to the development of cell therapy and cell-based products to be used in experimental clinical protocols for regenerative medicine mainly.

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Introduction

Aim

The thesis has been developed in the Bioscience group of the Italian National Metrological Institute (INRIM) and is part of a wider regional project, “Metrology on a cellular and macromolecular scale for regenerative medicine- METREGEN”. The Bioscience group worked on three PhD theses having the common purpose to develop metrology for biosciences: the main aim is to define measurement methodologies for cellular properties specially studied in regenerative medicine (RM). RM is a branch of medicine that uses stem cells to regenerate and repair damaged tissues and organs. Stem cells are able to repair damaged tissues and organs by reaching the damaged site, by replicating themselves to increase their number and then by differentiating into specialized cells. The main process which RM is based on is here briefly described: cells taken from the patient who needs regeneration are grown *in vitro*³ to increase their number (in order to obtain an appropriate quantity of cells) and then injected into the patient tissue or organ or implanted on a scaffold⁴ for therapeutic purposes and the cells-scaffold complex is then introduced into the patient tissue or organ. Here the cells or the complex cells-scaffold will form new tissue or organ.

Hence, the three thesis works, in a multidisciplinary manner, cooperate for the evaluation of cellular behaviours and functions during the cell-scaffolds

³ *In vitro*: this indicates an artificial environment outside the living organism.

⁴ Scaffolds are three dimensional structures, natural or synthetic, used to harbour cells *in vitro*. Detailed description in the following paragraphs.

interactions in RM applications. In order to test a medical product efficacy before implantation into the patient, operators are used to repeat the measurement, for evaluating the efficacy, several times in order to improve the measurement accuracy, calculate the mean value and the standard deviation and consider them as the accepted value. This could be feasible for non cell-based medical product but is not always possible for cell-based medical products. In some cases, there is no possibility to repeat several times the measurements or there is no possibility to have several samples availability: this happens, for example, when the applications (or the experiments, if we consider the experimental phase) involve expansive materials or rarely obtaining products or time consuming procedures. For cell-based product efficacy evaluation, i.e. in the case of stem cells implanted in patients following a regenerative medicine surgery procedure, it is impossible to collect several samples to have an adequate number of measurements. Cells taken from patient for regenerative medicine are quite precious and need to be cultured *in vitro* being preserved from contaminations, invasive manipulations or any kind of destructive procedures. This imply that cells cannot be manipulated to be, for example, counted several times to have an accurate value of their number. In this case, a reduction of the number of measurements is highly recommended. Hence, how to be sure the quality of cell-scaffold implanted is adequate to ensure a successful regeneration?

The requirement of metrology in RM is due to the need of reducing the number of measurements and obtaining an adequate uncertainty evaluation to be applied on the only single possible measurement and to the need of traceable methodologies and standard materials proper for RM.

Aim of the present thesis work is the development of measurement methodologies in metrology for cell biology and RM by focussing on the

activities and properties of human mesenchymal stem cells⁵ (hMSCs) during their interactions with scaffolds for RM and on the possibility to measure those activities and properties.

The METREGEN project

The METREGEN project addresses the field of metrology in nanomedicine and specifically in regenerative medicine (RM). This is a novel field of research in which metrology is required to enhance the reliability of diagnostic results and to make more effective and efficient the therapeutic techniques and the development of new technologies. The impact of the project falls on the health care improvement, on the patient protection, on the cost limitation and on the competitiveness promotion between laboratories, industries and services in Europe. This metrological approach is required by legislation to improve the knowledge, the application and the utility of more and more substances, methodologies, protocols and techniques. The stakeholders in RM are: healthcare providers (e.g. hospitals, medical centres and cell factories²), regulators and medical products companies.

Health care providers require safe, reliable and cost effective treatments, supported by evidence and approved by regulators.

Regulators require standard materials and traceable data demonstrating the safety and efficacy of new products and treatments.

Medical product companies require advanced and traceable techniques to develop new products and need methods to monitoring in real time processes such as cell growth on scaffold to ensure quality and efficiency of the medical products.

⁵ hMSCs are human stem cells used to repair tissues and organs in RM applications. They will be described in details in the following paragraphs.

The main aim of METREGEN is, in fact, the development of traceable measurement techniques for characterization of cellular and macromolecular processes involving RM products.

At this purpose INRIM, University of Turin (UNITO), Politecnico of Turin (POLITO) and several industries share their expertise on tissue engineering, stem cells biology and genetics, chemistry and on nanoscale measurement techniques to develop research on measurement methodologies and technologies applied or applicable, in RM

More in details, the METREGEN objectives are:

- to develop new measurement technologies and to improve existing measurement instruments (to this aim partnerships with instrumentation companies have been undertaken);
- to validate the following processes: *in vitro* cultivation⁶ of hMSCs, hMSCs seeding and culturing on scaffolds, hMSCs differentiation⁷ and its evaluation;
- to develop methods to produce reference materials (gold standard) and artefacts for calibrating instruments.

The project has been divided into three major topic of RM in which metrology is hardly recommended or required: 1) scaffold characterization in terms of fluids dynamics, mechanical and structural properties; 2) cells characterizations in terms of viability⁸ and functions; 3) macromolecules characterization.

It has been proposed to investigate on a few parameters in order to privilege the study of the used methodologies to the complexity.

⁶ Cell cultivation is the process to maintain living cells *in vitro*. The cell culture is the name given to the system made by the cells, the growth medium and the vessel containing them.

⁷ Differentiation is the process by which a not specialized cell becomes a specialized cell type.

⁸ The cell viability indicate how many cells are living in a specific moment of the cell culture: it is calculated as the percentage of living cells on the total cell number in the culture.

For example, in the cell characterization issue, the cell proliferation⁹ and differentiation and mostly investigated.

On the state of the art, the metrology in nanomedicine and RM is a very recent branch and its activities have been defined at both European level in the Quality of life group of EURAMET¹⁰, and international level in the Bio-analysis working group (BAWG) of the Consult Committee of Amount of substance (CCQM) of the International Committee for weight and Measurement.

In February 2007 EURAMET published a document, the European Metrology Research Program (EMRP), which is the guide line for National Metrological Institutes (NMIs) for the following 15 years. The EMRP defines the metrology for Health, and in particular for nanomedicine, one of the most challenging for the next years and one of the selected project is dedicated to research in metrology for RM as an important added value to medicine products and diagnosis.

INRIM is one of the participants for the development of new methodologies for cell analysis and for the realization of measurement methodologies for certification of reference materials.

⁹ Cell proliferation is the cell growth process. The cells propagate themselves *in vitro* by dividing themselves many times. Each cell division increases the cells number of a 2ⁿ factor.

¹⁰ EURAMET = European Association of National Metrology Institutes

Chapter 1

1.1. The Regenerative Medicine

Regenerative medicine, as defined by Greenwood et al. in 2006 and again in an editorial in the Journal of Regenerative Medicine in 2008 [1, 2], is an emerging interdisciplinary field based on both research and clinical applications focused on repair, replacement and regeneration of human cells, tissues and organs with the purpose of restoring their normal function damaged for various causes, including congenital defects, diseases, trauma and aging.

Regenerative medicine (RM) uses a combination of technological approaches and goes beyond the traditional therapies of transplantation and replacement.

These approaches include the use of specific molecules, gene therapy, stem cell transplantation, tissue engineering and the reprogramming of cells and tissues. In addition, regenerative medicine is perhaps one of the most interdisciplinary sciences covering a range of scientific fields including cell biology, embryology, physiology, biotechnology, nanotechnology, medicine, immunology, biomaterials, the chemical transport and fluids, biochemistry, mechanics, physics and tissue engineering that incorporates other disciplines.

As result of a damage, the repair involves the activation of endogenous stem cells, resident in the organism, by means of biological or pharmacological treatments, in order to support and further stimulate the natural ability of stem cells to self-renew and then to repair damaged tissues and organs: the stem cells are indeed able to maintain tissue homeostasis¹¹ and promote healing of damaged

¹¹ Homeostasis: physical-chemical-biological equilibrium state

tissues [3]. The replacement involves the transplantation of cell-based products¹² from a donor tissue in order to restore tissue homeostasis through receiving the correct functionality of the cells of the donor tissue. Donor and recipient tissue may belong to the same person or to two different individuals. The regeneration process consists in the transplantation of stem cells, which require a growth and differentiation *in vivo* in order to re-establish homeostasis of the tissue and its regeneration [3].

Regenerative medicine combines tissues-substitute materials, created by selecting nonliving but biocompatible materials, called biomaterials, and stem cells that are placed onto the biomaterial to form a complex “biomaterial-cells”: it is implanted in tissue or organ to be repaired, regenerated or partially (or entirely) replaced. The biomaterial has the ability to stimulate regeneration of tissues since it is constructed with structural, mechanical and chemical characteristics similar to those of the tissue itself and it acts as a structure and vehicle for cells to be implanted in a specific site and to reside for the time required for the induction of regeneration.

Regenerative medicine, although it is a relatively new science, in recent years has seen an increasing use of its clinical applications and the tissues and organs involved in regeneration were: skin, cartilage, bones, blood vessels, cornea, heart, some structures of the urinary tract and respiratory tract.

The increase in the number of applications is due to several reasons, including issues associated with transplantation of organs from donors, the first among all is the organs shortage and the risk of rejection of organs from donor [4].

¹² The "cell-based products" are medical products in which one of the components are the cells used for cell therapy or regenerative medicine purposes.

1.2. Mesenchymal Stem Cells

Stem cells are undifferentiated cells, i.e. not specialized to perform a specific function in the organism. However, they have the potential to become differentiated (or to differentiate) giving rise to diverse cell types performing different functions. A red blood cell, for example, is designed to transport oxygen and a white blood cell is designated to fight diseases. The differentiated, or specialized, cell results from a cell division that originates from a stem cell. A stem cell undergoes a division giving rise to two cells, one of which remains generally stem cell and the other undergoes a process of differentiation in order to be specialized to perform a specific function [5]. In this way they ensure the turnover of differentiated cells that have a limited lifespan and die to leave the place for the new differentiated cells or that must be replaced in case of diseases, trauma or injury.

The mesenchymal stem cells (MSCs) are a particular type of stem cell (different types of stem cells exist) called "mesenchymal" because the cells they give origin to are mesenchymal cells. Mesenchymal cells are cells that form the mesenchyme tissue. The mesenchyme is one of the first 3 embryonic tissues and from which other tissues derive: blood tissue, connective tissue, bone tissue, cartilage tissue, muscle tissue [5].

Identified for the first time by Friedenstein in 1970, the terminology by which the MSCs are indicated has undergone a series of changes. They are also defined "fibroblast colony-forming" (CFU-F), to indicate their fibroblastoid shape¹³ and their ability to form colonies of cells from one or a few cells; "Multipotent Stromal Cells" or "Marrow Stromal Cells (MSC) and "Bone Marrow Stromal

¹³ Fibroblastoid shape: similar to that of fibroblasts, typical and more abundant cells of connective tissues (blood, bone, adipose and the cartilage tissues are examples of connective tissue).

Stem Cells" (BMSSC), indicating their origin from the bone marrow and in particular from bone marrow stroma¹⁴ and their ability to differentiate into several ("multi") types of cells. Also *in vitro* studies demonstrate that MSCs can differentiate into cells of the following tissues: bone, fat, cartilage, skeletal muscle, nervous, endothelial [6]. This ability is called "multipotency".

The MSCs reside, besides in the embryo, also in several adult tissues and organs from which they were isolated: primarily they reside in the bone marrow but MSCs have been isolated also from other sites such as adipose tissue, amniotic fluid, the periosteum¹⁵, fetal tissues [6], skin, umbilical cord blood, peripheral blood, circulating in the body through the blood vessels, the placenta, the synovial fluid¹⁶, the articular cartilage as the liver, spleen, thymus, dental pulp, trabecular bone, skeletal muscle [7].

The debate over the use of embryos in order to harvest their stem cells will not be discussed in this thesis. It should be mentioned, however, the discussion of the risks related to the use of embryonic stem cells and the high carcinogenic potential of these cells [8].

In order to be studied and employed in base and applied research for RM, MSCs are isolated from animal and human tissues and cultured, i.e. kept as living cells in laboratory, in a condition called "*in vitro*". MSCs are typically isolated from bone marrow (BM) where are mainly contained in the red BM. However, as already mentioned, they can be isolated from other tissues and organs.

The isolation of MSCs from BM requires a BM aspirate, which contains mainly: immature erythrocytes (i.e. red blood cells), leukocytes (i.e. white blood

¹⁴ Stroma: part of the bone marrow formed from a blend of fibers and reticular cells, such as fibroblasts, that support the hematopoietic marrow production of new blood cells.

¹⁵ Periosteum: a fibrous membrane that covers the bones.

¹⁶ Synovial fluid: fluid that lubricates joints and nourishes non-vascularized tissues.

cells) and platelets and their precursors, i.e. hematopoietic stem cells (that will originate new red and white blood cells and platelets), endothelial stem cells, which will give rise to endothelial cells and MSCs. The aspiration of BM is usually performed from the iliac crest¹⁷ and from the sternum [9].

In the BM, MSCs are present in only minute quantities: about 1 in 100,000 total BM cells is a MSC. Several methods are used to isolate MSCs from BM. One of the most used is the density gradient separation: the collected BM is centrifuged in a tube containing a density gradient medium (e.g Ficoll and Percoll¹⁸) [10] in order to separate the different blood phases containing the different types of cells with different weight and dimensions (fig. 1.1)

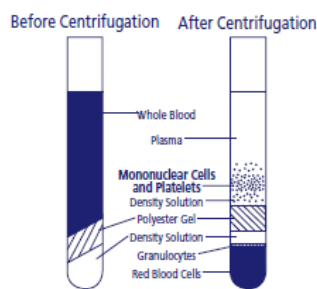


Figure 1.1 : Tube containing a density gradient medium (FICOLL™). The sterile silicone coated glass tube contains: sodium heparin in the top layer, a polyester gen in the middle layer, the FICOLL™ Hypaque™ solution in the bottom fluid layer (made by a polysaccharide sodium diatrizoate solution).

¹⁷ Iliac crest is the top of the ilium, a bone of the pelvis.

¹⁸ Ficoll is a polymer of sucrose with a high synthetic molecular weight, used to separate lymphocytes from other formed elements in blood and Percoll is a suspension of colloidal silica particles to separate cells, organelles, viruses and other subcellular particles. They are commonly used to separate MSCs from the BM other cells.

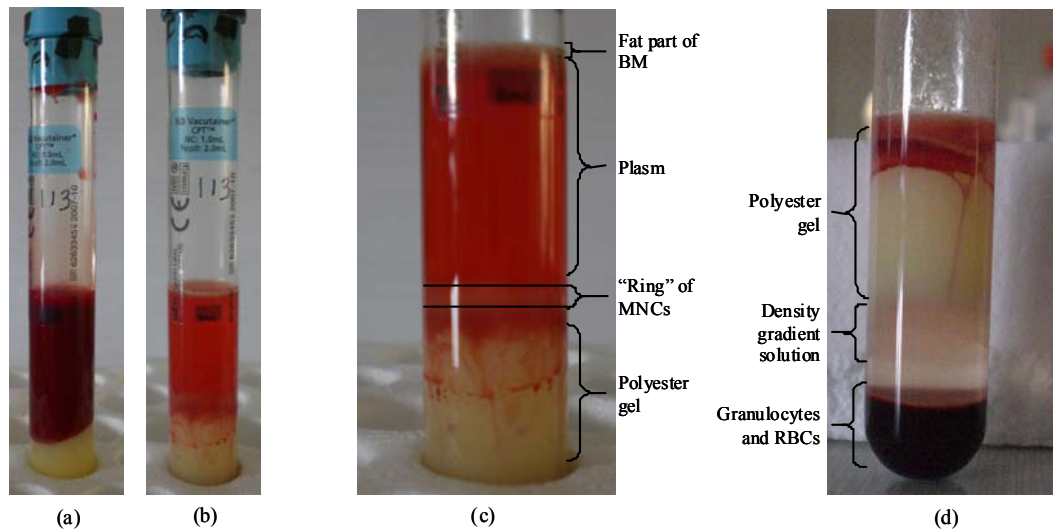


Figure 1.2 : Example of BM aspirate separation on FICOLL™ density gradient. In (a) it is shown the whole BM blood sample before the centrifugation; in (b) it is shown the same BM blood sample after centrifugation and (c) is its enlargement showing the different phases resulting from separation and among them the mononuclear cells (MNCs) ring contains the MSCs; in (d) it is shown the red blood cells confined in the lower part of the testing tube.

After centrifugation in density gradient medium, the phase containing MSCs (the “Ring” of MNCs in fig. 1.2) is deposited on plastic vessels for cell culture (as shown in fig. 1.5).

This will allow the selection of MSCs by their ability to adhere to the plastic. Also other BM cells can adhere to plastic but only MSCs can form colonies and proliferate.

MSCs can be identified *in vitro* by three methodologies based respectively on: the morphological feature, the phenotypic profile and the differentiation potential.

Through a microscopic analysis of cell morphology, once adherent to plastic, MSCs can be recognized because they assume a fibroblastoid morphology, similar to that of fibroblasts: elongated with regular edges, with a wide central body and extensions toward other cells (fig 1.3).

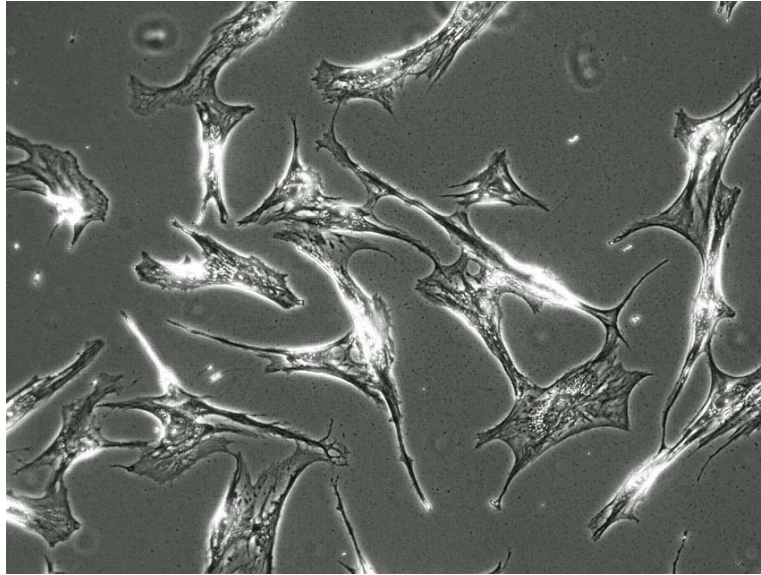


Figure 1.3: Several hMSCs with different shape and dimension but generally with a larger central body and many elongations. Optical microscopy. 20x Magnification.

The identification of MSC by the analysis of their phenotypic expression¹⁹ is commonly used, even if the markers of phenotypic expression are not specific and unique for MSCs and, therefore, a unique phenotype which allows reproducible isolation of a MSCs does not currently exist [9]. However, a combination of positive and negative markers (i.e. present and absent on the surface of MSCs) is today considered commonly acceptable to identify these cells. It is generally accepted that the adult human MSCs do not express the following molecules markers: hematopoietic markers CD45, CD34, CD14 and CD11; signal molecules CD80, CD86, CD40; adhesion molecules CD31, CD18 and CD56; while they express the following surface markers: CD105, CD73, CD44, CD90, CD71, Stro-1, CD106, CD166, CD54, CD29 [6] (Table 1.1).

¹⁹ Phenotypic expression analysis is the analysis of the cell surface antigens determining functions and behaviour of cells within the body.

MARKERS	
Negative	Positive
CD45	CD105
CD14	CD44
CD34	CD29
CD11	CD73
CD80	CD90
CD86	CD71
CD40	Stro-1
CD31	CD166
CD18	CD106
CD56	CD54

Table 1.1 : MSCs phenotype profile

However, it is necessary to point out that for a number of markers listed above, there is a variability of expression due to the tissue of origin, the method of isolation and the *in vitro* culture. Furthermore, there are differences in the expression of some markers influenced by factors secreted by cells during the first steps (in the initial phase) of the *in vitro* cell culture and not always the phenotypic expression of MSCs *in vitro* corresponds with that of these cells *in vivo* [6, 11].

The third method used to identify the MSCs is a functional method based on the differentiation potential of MSCs: these cells are able to differentiate *in vitro* into specialized cells to perform certain functions in different tissues, capacity that

MSCs have also *in vivo* and that is defined "multipotency", as it has been mentioned above. Three MSCs differentiation processes are assayed *in vitro* and are generally accepted to verify the multipotency of MSCs: the three differentiations lead to the chemical induction of bone tissue, adipose tissue and cartilage tissue by treating the MSCs cultures with three different mixtures of chemical compounds. Of recent use is the analysis of the neural, the myogenic and the endothelial potential of MSCs, leading to the formation of nerve, muscle and endothelial cells respectively.

The induction of bone differentiation is obtained by treating the cells with ascorbic acid, β -glycerophosphate and dexamethasone for 2-3 weeks. The cells gradually form nodules of calcium which accumulate over time and increase the levels of gene expression of alkaline phosphatase, collagen type I and other bone-related genes. The nodules are detected by staining methods specific for calcium. The two staining methods most commonly used are the Alizarin red and Von Kossa [12]. See chapter 3 for Alizarin Red staining application.

The adipogenic differentiation is obtained by treating the cells with dexamethasone, insulin, isobutyl-methyl-xanthine and indomethacin. Over time is observed the formation, within the cytoplasm of the cell, of vacuoles (vesicles) containing lipids that are detected by the staining method with Oil Red O. Furthermore, the cells increase the levels of gene expression of proteins typical of adipose tissue including lipoprotein lipase [13].

The cartilage differentiation is induced by treatment with dexamethasone, ascorbic acid, insulin, transferrin, sodium pyruvate, selenious acid and the transforming growth factor-beta (TGF- β). The cells produce an extracellular matrix composed mainly of proteins typical of cartilage tissue, collagen type II and aggrecan. The production of cartilaginous extracellular matrix is evidenced by staining with toluidine blue [13].

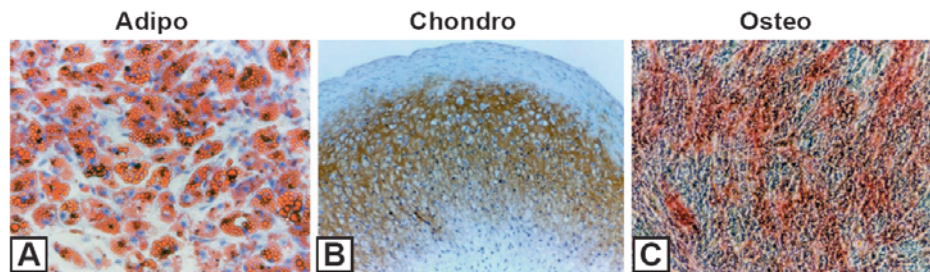


Figure 1.4 : Staining for cells undergone adipogenic (A), chondrogenic (B) and osteogenic (C) *in vitro* differentiation: Oil Red O (A), Toluidine blue (B) and Alizarin Red S (C) stainings.

The three methods used for the identification of MSCs (adhesion/morphological, phenotypic and functional/multipotency) are complementary to each other and are today considered all necessary for the identification of these cells *in vitro*. The first two are affected by variability and are not specific for MSCs. The third one is considered more critical in the MSCs identification.

Summarizing, MSCs are taken by the iliac crest, isolated from the bone marrow by a gradient of density and selected among other bone marrow cells by their ability to adhere to the cell culture plastic vessels. They are cultured *in vitro* in traditional 2D cell culture in flasks or plates and proliferate over time increasing their number by sequential cells divisions and by following a typical cell growth curve up to 40 generations (duplications).

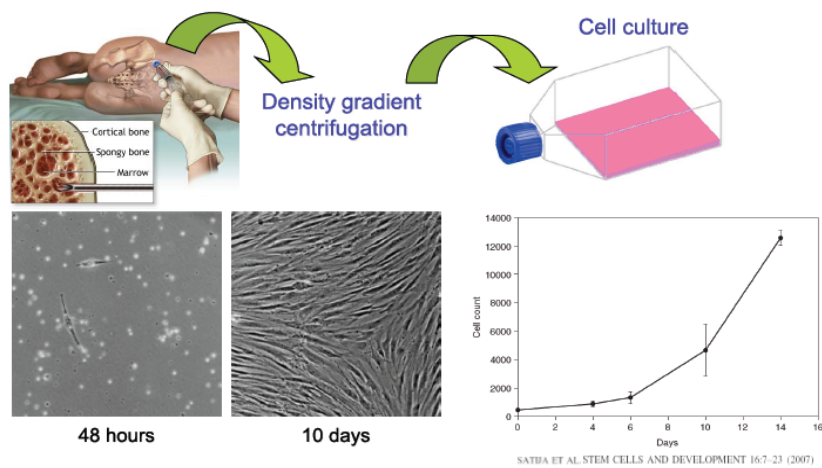


Figure 1.5 : A summary pictures panel shows the steps of MSCs isolation and culture in plastic vessels (a typical flask is shown here).

In *in vitro* culture, cell morphology changes over time in response to the cell state change. In the *high proliferative state*, cells take the fibroblastoid form, described earlier, but after some sequential duplications (process called "passaging"), cells slow the duplication rate and change the morphology becoming flattened and with a larger diameter and enter a *low proliferative state* until reaching the *senescence state*. In the latter state cell replication is almost absent or very slow and the cells assume a polygonal shape, wide and no more elongated. See fig. 1.6

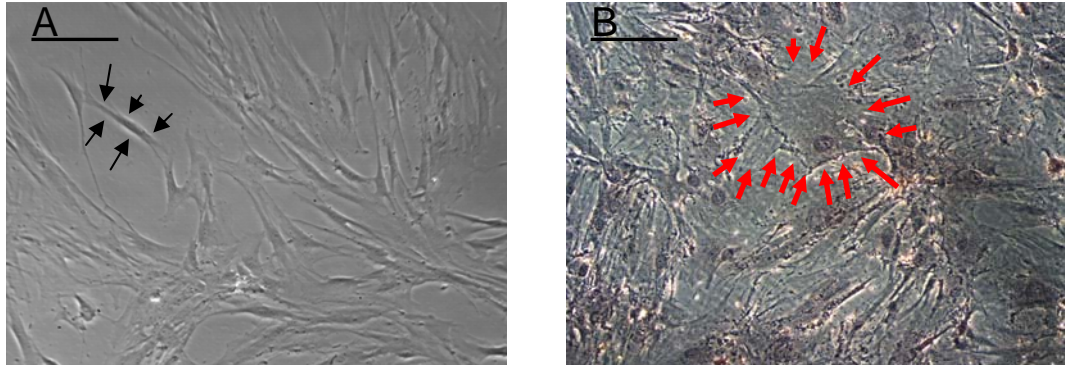


Figure 1.6 : In A the cell culture is at a very early state (Passage 3) and cells are elongated with spindle shape (black arrows). In B cells are in a very late culture state (Passage 17) and their shape is flattened (red arrows). Both cell cultures are hMSCs from INRIM Bioscience group cell laboratory. Optical microscopy. 10x magnification. Bars = 100 µm.

Even MSCs multipotency is maintained over time but is gradually lost with the the passaging. “Passage” is the term used to indicate each step of propagation in a new plate, occurring when cells have no more surface where grow (usually when they occupied the 80% of total growing surface). They are enzymatically detached and seeded in a 1:3 ratio in a new plate or flask: 1/3 of the total amount of detached cells are re-seeded, or all the cells are seeded in a three time more extended surface [11].

1.3. The Bone Tissue

The bone tissue is a specialized form of connective tissue, consisting of different types of cells and an extracellular matrix having the function to protect and support, to provide attachment sites of tendons and muscles, essential for locomotion. Furthermore, the bone tissue forms the main reservoir of many ions such as calcium, phosphate, magnesium and potassium. The mature bone is

composed of two different types of tissues: the cortical bone (external) and the cancellous bone (a trabecular network inside the bones) (fig. 1.7 and fig. 1.8). The cortical bone is formed by cylindrical elements, the “osteons” (forming the so called Haversian system), compounds in turn by concentric lamellae consisting of a central channel (Havers channel) surrounded by lamellae of bone matrix. Within the lamellae reside the osteocytes (mature bone cells) embedded in spaces called lacunae of bone. The Haversian canals contains blood vessels and nerve cells which communicate with osteocytes through canaliculi (fig. 1.9). The cortical bone is covered by an outer membrane, the periosteum, consisting of an outer layer and an inner fibrous having an osteogenic potential and allows bone to grow. The interior of the bone is composed of a trabecular meshwork (cancellous bone) and bone marrow. The trabecular meshwork ensures flexibility and stability to the skeleton. The different structures, cortical and trabecular bone, have different mechanical properties [9].

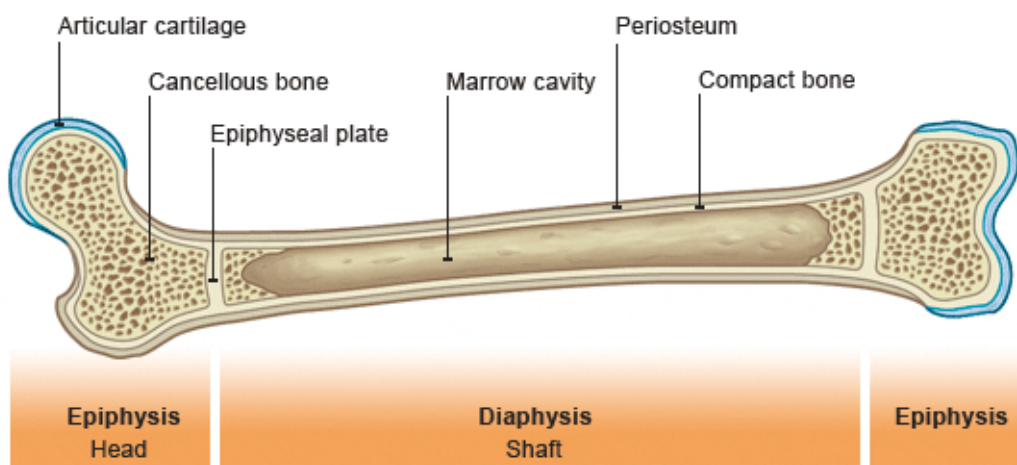


Figure 1.7 : Schematic representation of a long bone.

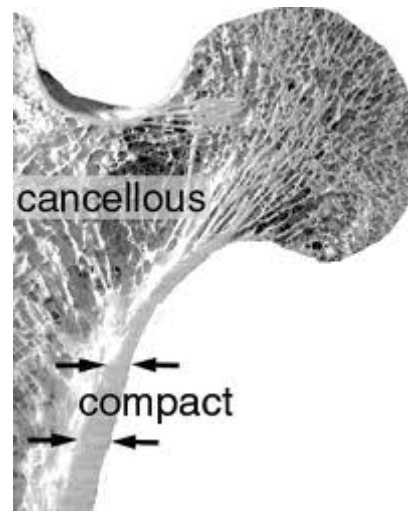


Figure 1.8 : Image of epiphysis of a long bone

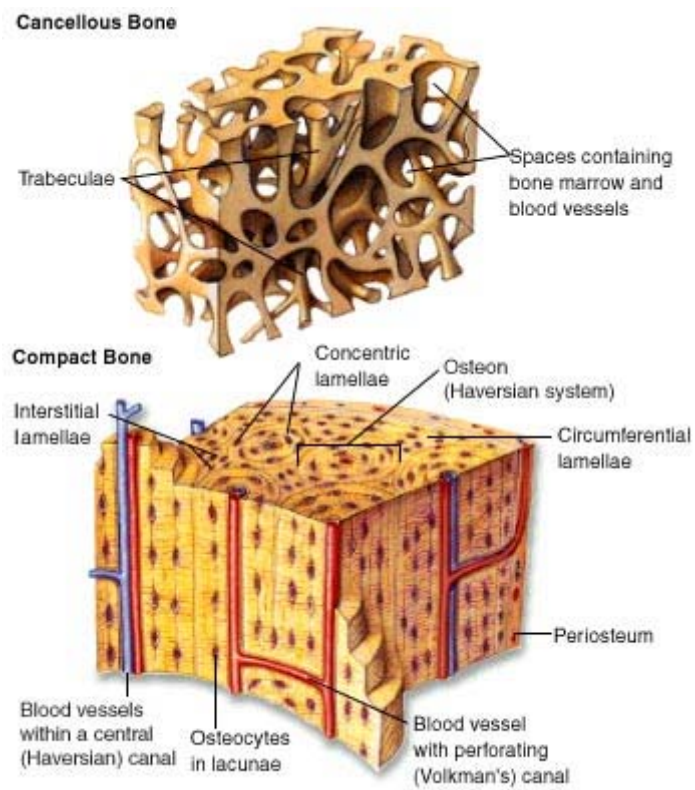


Figure 1.9: Schematic representation of cancellous and compact bone structure.

1.3.1. Bone tissue cells

Bone is composed of different types specialized cells embedded in an extracellular matrix: osteoprogenitor cells, osteoblasts, lining cells, osteoclasts, and osteocytes.

The osteoprogenitors originate from mesenchymal stem cells (MSCs): from MSCs descend the preosteoblasts, from which derive the mature osteoblasts [14].

Osteoprogenitor cells have proliferative capacity especially during the body growth and in the adult if fractures and other bone injuries need to be repaired.

Osteoblasts originate from osteoprogenitor cells when the differentiation process begins. These cells are mainly responsible for the synthesis of bone extracellular matrix and for its mineralization. Osteoblasts have globular or polyhedral morphology and form epithelioid sheets by juxtapositioning one over the other in proximity of the bone surfaces during the bone formation process. Within the osteoblasts the synthesis of organic molecules of the bone extracellular matrix occurs and these molecules are then exocytosed and assembled outside the cell.

Osteocytes descend by osteoblasts that, after the deposition of extracellular matrix, remain trapped within gaps.

The bone lining cells coat the bone forming an encasement. They derived by those osteoblasts that have exhausted the activity of bone formation and remain close to the bone surface. These cells have an important role in mediating the exchanges between the blood vessels and osteocytes.

Osteoclasts are the only cell type non-native of the bone tissue, they in fact originate from preosteoclasts of the bone marrow: they degrade the bone matrix and are responsible for bone resorption [15].

1.3.2. The extracellular matrix (ECM)

The ECM is composed of an organic phase and a mineral phase. The inorganic phase of bone ECM is mainly made of hydroxyapatite: calcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$, is combined with calcium hydroxide, $\text{Ca}(\text{OH})_2$, forming crystals of calcium hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Small amount of calcium carbonate and other minerals are also present in the bone ECM.

The organic component is largely composed of fibers of type I collagen (COL1A1), the most abundant structural protein of bone, forming 90% of the bone matrix. The remaining part consists of non-collagenous proteins such as osteocalcin (OC), osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP), alkaline phosphatase enzyme (ALP), Runt-related transcription factor 2 (Runx2), bone morphogenic proteins (BMPs) and growth factors [16].

Early osteoblastic markers are Runx2, ALP and COL1A1, while during the later stages of differentiation are expressed BSP, OC, OP, and ON.

OC is the most abundant of the non-collagenous proteins and has the function of modulating the morphology of the crystals of hydroxyapatite [17].

This protein is synthesized by osteoblasts during osteogenesis. The levels of serum OC (or GLA protein) are considered to be marker of osteoblastic and bone functionality [18]; these levels are particularly high in patients with increased bone metabolism [19].

ON is a glycoprotein linker between the collagen and the mineral part of bone matrix. It is thought to have a key role in determining the onset of mineralization of bone matrix [20]. It is considered a valid osteoblastic marker.

OP is a sialoprotein that is assumed to have the biological function of allowing the adhesion of cells to the bone matrix and in controlling bone resorption [21]; reduced levels of mRNA of OP in stem cells derived from bone marrow are related to the underproduction of bone, such as in osteoporosis [22].

BSP is a protein probably involved in the formation of hydroxyapatite crystals of the matrix [23].

ALP, the glycosylated protein most abundant in the bone matrix, also participates to the mineralization of the matrix, even though its precise role has not yet been clarified [24].

The BMPs are a group of about 30 multifunctional cytokine and are fundamental in processes of osteogenesis and bone remodeling [25]. Among these, one of the most studied is BMP-2.

Runx2 is one of the earliest genes of osteogenic differentiation [26] and has a key role in the regulation of osteoblast differentiation and bone formation [27].

1.3.3. Skeletal Physiology

The bone is a dynamic tissue due to the continuous removal of portions of matrix followed by newly synthesized matrix apposition. This process is due to the catabolic action of the osteoclasts that break down the existing matrix and anabolic action of osteoblasts that synthesize the new matrix.

During the growth, the activity of osteoclasts and osteoblasts are not tightly coupled: the new bone is deposited in different locations with respect to the bone degraded. This mechanism, called modelling, allows the bone to grow and change shape in response to changes in physiological and mechanical loads to which they are subjected [28]. In the adult, on the contrary, takes place the remodelling, a process in which the activity of osteoblasts and osteoclasts is coupled in space and in time, therefore the newly synthesized bone, produced by osteoblasts, is deposited at the site where the bone has been degraded by osteoclasts. These anabolic and catabolic activities occur within discrete units of bone tissue calls basic multicellular units [29].

The production of bone matrix and its mineralization take place according to a precise orientation: initially the osteoblast lays bone on the side facing the pre-

existing bone surface; subsequently it lays bone on each side around itself, so that each cell progressively move away from surrounding cells due to the interposition of the extracellular matrix. At this point the osteoblast slows substantially its metabolic activity and transforms itself into osteocyte, imprisoned in the gap of the matrix of newly formed bone, while new osteoblasts differentiate gradually from osteoprogenitor cells. When the process of new bone tissue formation is depleted, the osteoblast cells that remain close to the bone surfaces and cease their activities, reduce their organelles and become a membrane of flattened cells, the so-called bone lining cells.

The remodelling allows the bone renewing preventing the accumulation of micro damages.

1.4. Biomaterials and scaffolds

Biomaterials are nonliving materials used in regenerative medicine field in order to replace lost structures, to support existing structures, to promote the formation of new tissues in a receiving body [9]. They are natural, synthetic or semi-synthetic, degradable or non-degradable, but all of them need to be biocompatible to be accepted and not rejected by the receiving body [30].

Natural biomaterials are: collagen, proteoglycans and glycosaminoglycans, fibrin, hyaluronic acid, cellulose, alginates, chitosan, hydroxyapatite of natural origin (e.g. coralline hydroxyapatite), calcium phosphate of natural origin (from bovine, coral and human bones), and others [30].

They are components of the extracellular matrix (ECM) or have macromolecular properties similar to the ECM properties. Among the advantages offered by natural biomaterials there are: biocompatibility, biodegradability, mechanical properties similar to those of human tissues, ability to interact with human tissues in a manner favourable to regeneration (e.g. by supporting the

growth and motility of cells toward sites affected by injury). Disadvantages are: the risk of viral or bacterial infections and in some cases the instability and premature deterioration over time which does not match the timing of natural regeneration of the native tissue.

The synthetic biomaterials are polyesters, polyethylene-glycol, polyurethanes, polyglycolic acid (PGA), polylactic acid (PLA), ceramics, alloys, carbonates and others. They consist of a matrix of synthetic polymers with three-dimensional architecture and generally show high transport properties but exhibit considerable criticality of biocompatibility, especially due to the low content of information and signals to the cells.

The semisynthetic biomaterials are, for example, modified hyaluronic acid, derivatives of hydroxyapatite, chitosan.

A biomaterial is therefore a substance or combination of substances used for treating, improving or replacing tissues or organs. It has to ensure all the requirements the cell needs to produce the tissue, from cell proliferation to tissue production and to transplantation.

The biomaterial is therefore characterized by the following features:

1) Tolerance: it needs to be immunologically inert. In general, any extraneous material which comes in contact with tissues or fluids, generate a specific reaction.

2) Biodegradability: it is the essential requirement for the use of a material and is closely related with the application and location of the biomedical device that is provisional or permanent, as needed.

3) Cell-cell interaction: the biomaterial has to communicate and exchange signals with host cells [31, 32, 33].

Biomaterials are used for the manufacture of scaffolds for regenerative medicine.

The scaffolds are three-dimensional structures, with variable solidity and stiffness, which make possible the implantation of cells on the site of interest in the receiving body: the cells anchored to the scaffold can be introduced into the patient and regenerate damaged tissue by acting directly on the damage site with no dispersion in surrounding areas. It should be noted that the regeneration of new tissue is stimulated through an implant of cells alone. However, it is difficult to cells survive without a surface of adhesion or anchor and the control of the cell adhesion after implantation, in order to maintain their distribution in the site of interest, is quite complex [34, 35, 36, 37]. Therefore, the scaffold primary function is to allow the attachment of cells. The scaffold morphology and geometry must be designed taking into account the space and the external shape of the defect, lacuna or gap to be corrected.

For all types of biomaterials, studied and used in pre-clinical (in animals) and clinical (human) applications, it has been experimentally observed that the characteristics and the surface properties of biomaterials have the ability to affect the initiation phase of cellular events at the interface between cells and the material. Cellular events include, for example, the cellular proliferation and differentiation, stimulated in both the implanted cells, anchored to the scaffold, and in the cells belonging to the tissue in which implantation occurs. The three-dimensional structures of the scaffolds, made of the most disparate biomaterials, have the ability to orchestrate the complete formation of new tissue both *in vitro* and *in vivo*. The scaffold offer to the cells a base structure where they can be anchored and then it supports the growth, differentiation and the three-dimensional orientation for the formation of a 3D construct with cells *in vitro* and *in vivo*. The scaffold structure allows diffusion and convection transport phenomena to ensuring the supply of oxygen and nutrients for cells and the elimination of waste materials. The scaffold architecture influences both properties of single cells (such as vitality, migration and cell differentiation), and

characteristics of the 3D generated construct, which will replace, temporarily or permanently, the tissue of interest. Since tissues have different chemical composition and different characteristics at different levels (e.g. cellular and tissutal levels), it is understandable how different scaffolds, with specific characteristics, are needed to optimally support the regeneration of different tissues [38]. Consequently, an ideal structure and pre-packaged scaffold does not exist because each tissue requires a specific design. For example, bone tissue and cartilage tissues, the two most studied²⁰, require different scaffolds mimicking their chemical composition and their mechanical properties (rigidity, hardness, etc.) and structural (internal architecture, porosity, etc.). As general characteristics, the scaffold must be reproducible products, controlled and with the possibility to host biological components (cells and/or growth factors). Hence the characteristics of the scaffolds, making them different from each other, are:

- Chemical composition
- Chemical properties
- Macrostructure
- Porosity and interconnection between the pores
- Pore size (inner diameter of the channel)
- Surface/volume ratio
- Mechanical properties
- Degradation to allow the remodelling

They all have a relative importance for a specific issue.

For example, the porosity and the channels are important because they provide access to the migrating cells from the outside towards the inside and within in

²⁰ Bone and cartilage tissue are the most studied in the field of tissue regeneration by using mesenchymal stem cells because the ability of these cells to differentiate into cartilage and bone cells both *in vitro* and *in vivo* and for the frequency of diseases and disorders related to these two tissues.

different areas, to the proliferating cells whose daughter cells require new surface to anchor to; in addition they have to allow the capillary passage of blood *in vivo* and of liquids in *in vitro* cultures, they have to provide a proper area available for single cell and for the many cell-cell interactions. In addition, we distinguish micro and macro-porosity. The micro-porosity is fundamental for the growth and inner capillary for the cellular interactions while the macro-porosity is important for the exchange of nutrients and waste and for the removal of dead cells.

The 3D scaffolds are support structures that induce cells to form functional tissues: they represent physical and mechanical support for the cells and the space available in their internal pores allow the cells to develop the tissue, allowing its vasculature during regeneration.

The mechanical support is very important especially in transplants in which forces of compression or traction are developed; sufficient mechanical strength and stiffness are needed to counter forces of initial contraction and further to ensure a perfect reconstruction of the tissue. The biodegradability or reabsorbability of the scaffold is necessary to allow the gradual replacement by newly formed tissue in the long term.

The high porosity and the wide surface area provide high interconnection, structural strength and a three-dimensional surface.

Several techniques have been developed in order to increase the accuracy and reproducibility in the production of scaffolds, ensuring a considerable flexibility in the design phase, because it allows to vary the physical characteristics of the matrices in order to optimize the biological response of the system [39, 40, 41].

1.5. Scaffolds for regenerative medicine in orthopaedics

The biomaterials used in clinical applications to replace portions of bone tissue have been studied over the last 30 years.

They are made of synthetic or natural materials. In both cases their use is motivated mainly by the limited availability of autologous grafts and the choice of material, be it natural or synthetic, is dictated by the following properties: the ability to stimulate osseointegration, the osteoconduction, osteoinduction, the induction of angiogenesis and vascularization, the resistance to mechanical stress and disruption and the reabsorbability. The key element that determines all these properties is the internal architecture of the biomaterial, which makes it, in a unique word, "bioactive".

Another important aspect is related to the use of autologous human cells in tissue regeneration mediated by biomaterials.

Stem cells from the bone marrow are used in the regeneration of bone tissue in clinical applications because of their ability to pass from an undifferentiated state to a differentiated state as a result of chemical, mechanical and biological stimuli and to regenerate the damaged or missing tissue.

Therefore, biomaterials are also selected on the base of their ability to make the scaffold permissive to: cell adhesion, migration and proliferation, cell-cell interactions, extracellular bone matrix formation which provides structural support to the new bone tissue formation.

In addition, the biomaterial is vehicle of biological molecules through its pores interconnected by channels: molecules such as cytokines, including BMPs, insulin-like growth factor (IGF) and TGF β , stimulate the stem cells precursors of bone cells to differentiate into cells producing the extracellular bone matrix.

Finally, in order to better mimic the physiological conditions of a bone or part of it, the biomaterials used in osteogenesis must reconstruct a three-dimensional microenvironment that allow the cells to grow forming new tissue as similar as possible to the natural bone tissue. The biomaterials three-dimensional structure is now considered a requirement of fundamental importance both in experimental studies *in vitro* and in *in vivo* applications. The research of the last 20 years in

regenerative medicine and tissue engineering has seen a growing interest in the study of the cell-scaffold construct, of the interactions between cells and biomaterial, responsible for the tissue regeneration induction and of the possible clinical applications of these cell-scaffold construct.

Osseointegration, osteoconduction, osteoinduction, induction of angiogenesis and vascularization properties, resistance to mechanical stress and rupture, reabsorbability, stimulation of cell differentiation, high internal porosity and interconnection between pores can be made in biomaterials synthesis, but are often naturally possessed by biomaterials of natural origin. Among the natural biomaterials, the ones made of coral are an important example and have been studied and used in clinical applications in orthopaedics, neurosurgery and dentistry [42].

1.6. The Biocoral[®]

Biocoral[®] (Inoteb, LeGuernol, Saint-Gonnery, France) is a natural coral from exoskeleton of Madreporas (fig 1.10).



Figure 1.10 : A Madreporas.

The exoskeleton is made of calcium carbonate in the form of aragonite and constitutes 67% of weight. Several other trace elements are present and are similar to those of mammalian bone. Among them, Magnesium, Phosphorus, Potassium and Sodium.

The Biocoral obtained from Madreporas (fig 1.11) is highly porous, rigid and inert. It has a chemical composition very similar to that of human bone: it is made up to 98% of calcium carbonate in the form of aragonite crystals and other trace elements (fluorine and strontium to 0.7 to 1%, Magnesium to 0, 05 to 0.2%, 1% Sodium, Potassium 0.03%, Phosphorus 0.05%, water 0.5% and 0.025% amino acids). Strontium is able to promote the process of mineralization.

It has a porosity varying from 20% to 50% of the volume with an internal architecture formed by pores having a diameter from 150 to 500 μm and a high degree of interconnection which invests all the material through a network of interconnecting pores of an average diameter of 260 μm (Figure 1.12 and 1.13).

Among the ceramic materials based on calcium, Biocoral is the biomaterial with the best mechanical properties due to its porous structure that is very similar to that of cancellous bone. This makes the Biocoral a good biomaterial to support the formation of new bone [42].

The coral of Biocoral is not altered by the process of production of the product that provides for manipulations such as to confer architectures, shapes and geometries of the most disparate. The geometry cylindrical or truncated cone is the most widely used and depicted in the images below.

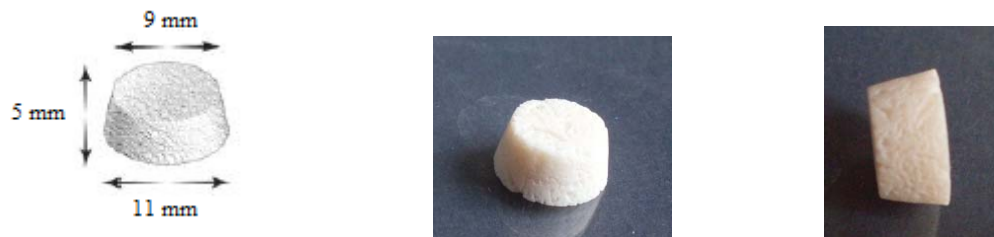


Figure 1.11 : Biocoral (front and side views)

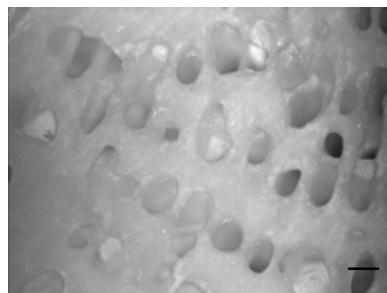


Figure 1.12 : Pores of Biocoral. Images acquired with an atomic force microscope (AFM) at INRIM. It is possible to see the internal connections of the scaffold. Bar: 0.5 mm

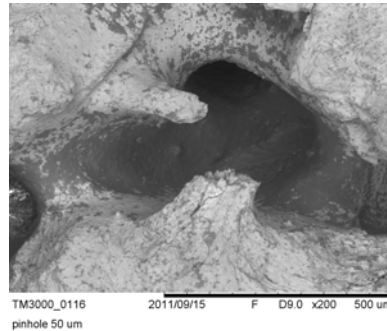


Figure 1.13 : Pore of Biocoral. Image taken with a scanning electron microscope (SEM) at INRIM. Bar: 0.5 mm

Experimental studies and clinical applications in neurosurgery, orthopedic surgery and dentistry for the last 20 years have shown that a material is fully and gradually resorbable, biocompatible and bio-functional: it is well tolerated by both animal and human tissues, its porosity allows the possibility of a good osseointegration, allowing the new bone to grow, followed by gradual resorption which leaves space for the new bone and it has biomechanical properties very similar to those of the bone, also related to the porosity and internal structure. The first experiments and clinical applications date back to the early 90s. The trials have evaluated the biocompatibility of Biocoral with human cells and the ability to support cell growth and an osteogenic differentiation [43], the osteointegration and the resorption of Biocoral in human bone [44] and the ability to convey cytokines [45].

The clinical applications are mainly active in the field of dentistry and periodontal surgery [46].

Chapter 2

This chapter defines the measurand “cell number” and the cell counting procedure, describes the measurement methodology of Resazurin/Resorufin assay and addresses the evaluation of the assay uncertainty. At this scope a series of measurement are described and finally an uncertainty budget is discussed.

2.1 Cell number and cell counting

Cell number is a measurand in many sectors of science: in biomedical and microbiology research for cell-based experiments to determine the cell growth rate and to measure the cell viability, in medical diagnosis to assess the blood cells quantity or to verify the presence of pathogens in samples, in environmental analysis to estimate the microorganisms contaminations [47] and, generally, in all the protocols in which the cell number estimation is required. In biological cell cultures, cell number is one of the parameters necessary to investigate several cell culture features such as cell viability, proliferation, growth, fitness and metabolism which require a monitoring as function of time.

The *cell counting* is a procedure by which it is estimated the number of cells in a given volume in an *in vitro* culture system and is a fundamental procedure in biological research on living cells samples and in all the other fields, named above, in which cell number determination is indicative for understanding a process, estimating a quantity, diagnosing a disease or studying a phenomenon.

This work is focus on the cell number estimation in bi-dimensional cell cultures *in vitro*.

Several methods of cell number quantifying have been developed to follow the life span of an *in vitro* cell culture and different techniques are today in use: from the simplest and cheapest one, based on the use of the hemocytometer (or counting chamber, e.g. Neubauer chamber) or on cell image analysis to more sophisticated ones based on metabolic assays (when the activity is proportional to the number of cells explicating that activity) or spectrophotometry, electrical resistance to the most expansive ones based on flow cytometry.

In the first two cases the object “cell” is really counted by the operator, in all the other cases indirect measurements are performed (e.g. by measuring a metabolic activity of the cells or properties that are only cell-dependent but are not related to cell, such as light and electrical resistance). However, in any case, a low level of accuracy and reproducibility due to several influence parameters of uncertainty, represents a common drawback and obstacle to a reliable consideration of the results.

The lack of traceability needed to compare results from different laboratories or obtained in the same laboratory, but at different times or by different operators, as clearly expressed by EURACHEM/CITAC *Guide, Quantifying Uncertainty in Analytical Measurement* [48], justifies the consideration of quantification of the number of cells as a measurement service required to the National Institutes of Metrology (NMIs). The NMIs raise the question of quantification of cell populations in both traditional two-dimensional (2D) and three-dimensional (3D), mimicking real tissues, culture systems [49].

2.2 Cell counting in 3D cell culture on scaffolds

3D cell cultures on scaffolds *in vitro* mimic real situations occurring in the body, i.e. *in vivo*, because tissues and organs are three-dimensional structures in

which the cells are usually distributed in a three-dimensional matrix (with shapes and characteristics different from tissue to tissue) and are not arranged in a single layer of two-dimensional surfaces as they are, instead, when cultured in traditional *in vitro* 2D cell cultures in flasks, plates or Petri dishes.

As *in vitro* system, the 3D cell culture needs to be checked in terms of biocompatibility. The measurement of *cell viability* and *proliferation* provides important information on the biocompatibility of the culture system, its influence on the state of the cells, on the welfare of cell culture, on cell metabolism and on cell differentiation. In addition, it is very important that by monitoring the *in vitro* proliferation and *differentiation* a great amount of information can be gathered and assumptions or predictions about the behaviour of cells *in vivo* can be made.

Cell viability indicates the amount (usually expressed as a percentage) of live cells on total cells present in the culture system.

Cell proliferation is the process by which cells increase their number by a factor of 2^n through consecutive cell divisions.

In both cases, cell viability and cell proliferation, the *cell number* is the measurand to be quantified.

The possibility to perform sequential measurements on the same cell population is extremely important in the analysis of cell viability and proliferation.

Traditionally, the number of cells is determined by removing the cells from the culture system (2D plates or 3D scaffolds) and counting them by the use of a hemacytometer, which provides for a manual counting, or by the use of an electronic counter. Other methods of counting are based on: imaging, i.e. on the acquisition of images using a microscope coupled with a camera, and on the manual or by software cell count [50]; metabolic tests that assay metabolite activity of cultured cells and that are used when the number of cells is proportional to the number of cells performing that particular metabolic activity.

However, these methods of cell counts are in most cases destructive (manual or electronic counts involve the enzymatic detachment of the cells from the growth system and metabolic methods involve the use of substances toxic for cells or able to alter the cell structure) or invasive (need of making slices or pieces of tissues for imaging), whether they are applied to 2D cultures or 3D scaffolds. As well as a tissue or an organ biopsy, the 3D scaffold needs to be sectioned or sliced, or the cells contained in it must be detached. In any case, this prevents to perform a cell count over time on a single sample. Also, often cells cannot be detached from 3D scaffolds with the classic enzymatic methods.

Therefore, for 3D cell cultures it is necessary to employ non-destructive and non-invasive methods for cell counting.

There are at present some metabolic reaction-based methods in the literature for monitoring animal cell number (giving information on cell proliferation) over time [51, 52, 53]. However, most of them requires the use of substances that are cytotoxic [52], or results in cell lysis to measure the metabolic product [52, 53, 54].

The method chosen for cell number evaluation in this thesis work is the resazurin/resorufin assay based on the reduction of resazurin into the end product resorufin. It is not cytotoxic and does not require any cell damaging steps [55], therefore it satisfies the requirement of non-destructiveness and non-invasiveness.

2.3 Resazurin/Resorufin assay

The resazurin/resorufin (R/R) assay is a fluorimetric and metabolic assay used for determining the number of living cells in a biological *in vitro* system. It is based on a redox reaction: the reduction of resazurin (dark blue in color, redox dye with a slight intrinsic fluorescence) into the end product resorufin (pink, highly fluorescent molecule, excitation wavelength of 579 nm and emission

maximum at the wavelength of 584 nm), made by redox enzymes. Only living cells can perform this reaction because nonviable cells rapidly lose metabolic capacity and do not generate any fluorescent signal [55]. O'Brien et al. (2000) proposed the resazurin dye, the original name of the Alamar Blue dye, to assess the mammalian 2D cell cultures cytotoxicity [56]. Resazurin solution is added to the cell culture medium, containing the cells, as 10% in volume. The resazurin molecule can penetrate cells by passing the cell membrane and into the cytoplasm is reduced by cytosolic, microsomal and mitochondrial redox enzymes producing the fluorescent resorufin (reaction product). Resorufin diffuses out of cells back to the culture medium which alone does not reduce resazurin [57]. The number of cells in the cell culture is considered proportional to the total metabolic activity of cells. The metabolic activity is indicated by the redox reaction (reduction of resazurin) rate and can be calculated by the substrate (resazurin) consumption rate or by the product (resorufin) formation rate. The substrate can have a limiting effect, thus is usually supplied in excess. Thus, the metabolic activity measurement is obtained by measuring the concentrations of the specific metabolic product (resorufin) over time and the reaction rate can be calculated as the variation of product concentration over time.

The product concentration in the culture medium is detected by measuring the fluorescence intensity due to an adequate excitation after a contact time of resazurin solution with cells.

The R/R assay for the first time in this study has been used for 3D cell cultures on Biocoral as non-destructive and non-invasive assay (experimental set up and results have been described in chapter 3). The test measures the metabolic activity carried out by the only viable cells (metabolically active) and quantifies the number of cells under the assumption that all active cells exert this activity and that the average of this metabolic activity is stable over time.

The metabolic activity of a cell varies with the cellular phenotype (for example varies between an undifferentiated or a stem cell and the same cell which is differentiating or is already differentiated) and changes in cell phenotype depend on a series of phenomena that occur in the environment of the *in vitro* cell culture, including the limitation of glucose, changes in pH of the culture medium, the characteristics of the scaffold in the case of 3D cultures such as stiffness, elastic modulus, mechanical properties, etc.

Hence, when the cell number estimation is based on a cell metabolic activity it is extremely important to consider that metabolic assays are affected by various influence parameters and their entire characterization requires a deep and intense analysis of the specific cell phenotype, aiming to evaluate the uncertainty of the methods.

The work described in this chapter is a first approach of uncertainty evaluation of a metabolic method for measuring the cell number. The innovative contribution of this work is because metabolic methods to determine the cell number, although widely used, have never been metrologically characterized, neither in 2D nor in 3D cell cultures.

This study is applied to hMSCs 2D cultures, undifferentiated cells, grown non-differentiating cell culture medium, using the metabolic assay R/R, based on the resazurin conversion to resorufin to evaluate the cell number and estimate the cell proliferation.

2.4 Experimental set up

2.4.1. Cell seeding

The hMSCs were purchased from Lonza Group Ltd (Basel, Switzerland). They are bone marrow derived-hMSCs from donor. hMSCs were expanded and maintained in non-differentiating cell growth aMEM (alpha modified Minimum

Essential Medium - Lonza, Wokingham, UK) with FBS 10% v/v (Fetal Bovine Serum, Lonza, Wokingham, UK), L-glutamine 2mM, penicillin 100 U/ml and streptomycin 100 g/ml (Lonza Wokingham, UK) in Petri dishes. Fresh medium was replaced every 3-4 days until cells reached about the 80% of confluence²¹, then they were washed once with 1X Phosphate Buffer Saline (PBS), detached with 0.25% Trypsin – 0.53mM Ethylenediaminetetraacetic acid (EDTA) solution, counted by means of a hemocytometer and suspended at several concentrations (cells/ml) in 100 µl non-differentiating aMEM growth medium to be seeded and cultured statically in 96 well microplates. Then, cells are posed in the incubator at 37°C with 5% CO₂ overnight to adhere on the surface of the wells. Cells were managed in laminar flow hood (class II) under sterile conditions.

2.4.2. Resazurin/Resorufin (R/R) assay analysis

After cell adhesion, resazurin is added as 10% in volume to the cell culture growth medium in the well containing the cells (adherent on the surface of the well). Fluorescence intensity of the resorufin has been measured after a contact time (0.5 to 6 hours) of resazurin with cells.

Important to note that in the specific case the entire liquid phase (cell culture growth medium containing resazurin) volume has been used to measure fluorescence intensity, i.e. 100 µl. However, cell culture can be established in a different vessel (such as a Petri dish) where the liquid phase volume is much higher. In that case, the volume of resorufin used for fluorescence intensity measurement (typically 100 µl) is taken from the liquid phase (previously mixed to be homogeneous) and placed in a multiplate well (typically in a 96 well plate) for the measurement. Two volumes are therefore distinguished: the total liquid phase volume (V_{LP}) in which the reaction occurs and the volume of aliquot of

²¹ Confluence: the confluence % indicates the % of total growth area in the Petri dish (or other cell culture vessel) occupied by the cells.

liquid phase placed in the well (V_w) in which the measurement is made. In the following measurand definition, both will be taken into account, even if in the specific measurements V_{LP} and V_w are the same entity.

2.4.3. Fluorescence measurement system

The GloMax[®]-Multi Microplate Multimode Reader (Promega) was used to measure fluorescence intensity. Excitation was performed at 525 nm wavelength while emission was measured in the range of 580-640 nm. Microplates with 96 wells were used.

2.5 Measurand definition: *cell number*

The number of cells (N) is measured in a cell culture which is in a certain volume of liquid phase (made by growth medium in which the resazurin is diluted) in cell culture vessel, such as a well of a microplate.

Under the hypothesis that the metabolic activity of the cells is stable, i.e., the measuring time is very lower than cell duplication time, the number of cells N in the cell culture liquid phase volume is proportional to the molar concentration of product in the liquid phase $C_{Prod,t}$ available in the well at contact time τ and to the volume of the liquid phase in the cell culture vessel V_{LF} .

The hypothesis that the metabolic activity of the cells is stable is depending by the cell phenotype that changes, for example, between undifferentiated and differentiated cells. In the following equation, K_1 defines the specific metabolic activity of the cell and depends by the contact time between resazurin and cells (τ)

$$N = K_1(\tau)C_{Prod,t}V_{LF} \quad (1)$$

The product concentration at the contact time τ in the liquid phase is proportional to the fluorescence intensity emitted by the molecules of product $I_{f,Prod,t}$. K_2 is influenced by the liquid phase volume in the measurement well (V_W).

$$C_{Prod,t} = K_2(V_W)I_{f,Prod,t} \quad (2)$$

The cell culture medium and the reaction product resorufin could emit in the same spectrum region and lead to modifications of the fluorescence intensity. It is, hence, necessary to measure the blank (b) in absence of cells but with times and treatments analogous to those of the samples (s) with cells.

$$I_{f,Prod,t} = (I_{f,s,t} - I_{f,b,t}) \quad (3)$$

Hence, the measurand equation can be written as:

$$N = K(I_{f,s,t} - I_{f,b,t})V_{LF} \quad (4)$$

Where $K = f(K_1, K_2)$ and is therefore influenced by τ , V_W and from the metabolic activity of the cells.

2.6 Analysis of quantities influencing the measurement

Each input quantity x_i that appears in equation (4) (V , k , I and the dilution in the measurement procedure) has been considered a quantity influencing the measurement. The estimation of the associated standard uncertainties $u(x_i)$ and of the systematic effects have been given, as shown in table 2.1.

a. Fluorescence

The fluorescence intensity is determined by the instrument used for the measurement, hence the fluorescence measurement needs to be characterized with respect to the adopted measurement system. Several measurement instruments are available for measuring the fluorescence intensity but results coming from different systems are not easily comparable to each other.

In the specific case, adopting the GloMax®-Multi Microplate Multimode Reader, one single measurement system performing up to 96 analysis at the same time and routinely used for diagnostic measures, the fluorescence measurement can be influenced by: the repeatability of the measurement, the position of the well within the plate, the plate re-positioning into the measuring system and the sample volume loaded in the well.

For the sample volume loaded in the well, an analysis of the fluorescence intensity sensitivity needs to be carried out to evaluate the sensitivity coefficients.

The repeatability of the fluorescence measure in a single well has been evaluated lower than 0.5% over the whole fluorescence intensity range of interest. The reproducibility evaluated among the 96 wells on the same plate has been found lower than 1%, even though results from 5-10% of wells were outliers. Thus, it is necessary to work in triplicate in order to eventually identify outliers.

The plate placement into the plate reader could contribute to the reproducibility. Hence, the standard deviation of repeated measurements has been calculated.

The system measures the fluorescence intensity due to excitation produced by an incident ray entering the well. The effective optical path length of the incident beam is influenced by the liquid level in the well, thus the fluorescence intensity has been measured as function of the volume filling the well VW. The fluorescence intensity is highest in the range around 100 μ l. This volume

minimizes the contribution to the uncertainty of the filling-the-well liquid volume fluorescence intensity.

To summarize, the fluorescence intensity uncertainty has a component of repeatability of instrument measure (0.5%) and a component of reproducibility due to the well positions in the plate (1%). A further contribution is due to the volume of liquid filling the well and can be evaluated separately as function of the available sample volume. Sensitivity coefficients of sample and black fluorescence intensity ($I_{f,s,2.5h}$ and $I_{f,b,2.5h}$) are calculated by derivatives of eq. (4) with respect to $I_{f,s,2.5h}$ and $I_{f,b,2.5h}$.

b. Liquid Volumes

The type of liquid phase, namely the cell culture medium, is one of the most influencing factors that can interfere on the reaction rate by increasing or decreasing the cell growth. Hence, two different cell culture media added each one with two different FBS quantities have been analyzed. The difference of fluorescence intensity of the different media as function of the contact time τ_c , i.e., contact between resazurin and cells, for several different cell concentrations (cell number/ml) seeded on wells, has been evaluated. The volume of the liquid phase in the cell culture vessel V_{LF} and the volume filling the measurement well V_w have been measured by a calibrated micropipette. V_{LF} is affected by the residual volumes during liquid replacements, evaporation during the contact time and micropipette uncertainty. In the specific tests the cell culture vessel was the well itself, thus V_w and V_{LF} are the same volume. A 3% total uncertainty has been calculated. The sensitivity coefficient of V_{LF} has been calculated by the derivative of eq (4) with respect to V_{LF} .

c. Slope

The slope of calibration curve (K) can be calculated by regression analysis of experimental data given by cell culture with a nominal value of cells. The cell number can be or estimated from the cell seeding concentration or measured by detaching and counting the cell by means of an hemocytometer, e.g., Neubauer chamber.

The slope of calibration curve K have been calculated by regression analysis of experimental data measured by detaching and counting the cell by means of a Neubauer chamber.

The linear regression minimizes the objective function at the estimated K value [58]:

$$K = \frac{\sum_{i=1,n} \frac{N_i (I_{f,t,i} - I_{f,b,t,i}) V_{LF,i}}{\sigma_{y,i}^2}}{\sum_{i=1,n} \frac{((I_{f,t,i} - I_{f,b,t,i}) V_{LF,i})^2}{\sigma_{y,i}^2}} \quad (5)$$

While the uncertainty of K [58] is:

$$u(K) = \sqrt{\frac{1}{\sum_{i=1,n} \frac{((I_{f,t,i} - I_{f,b,t,i}) V_{LF,i})^2}{\sigma_{y,i}^2}}} \quad (6)$$

where $\sigma_{y,i}$ is the uncertainty of each experimental point and combines the uncertainty of all the measured quantities, i.e., number of cells, volume and fluorescence intensities. Cell counting relative uncertainty, evaluated as the reproducibility of the Neubauer chamber's cell counting, has been calculated to be 5%.

The K uncertainty decreases when the ratio between cell number N and available liquid volume V_{LF} increases and depends on the number of experimental points. The conditions in which the experiments are made to calculate the calibration curve constant should be as similar as possible to the measurements conditions of the cell number. The sensitivity coefficient of K has been calculated by derivative of eq (4) with respect to K . The K uncertainty has been calculated to be 2.2% for time of contact larger than 1 hour and 3% at 0.5 hours of contact.

d. Dilution

The fluorescence intensity measurement instrument has a detection limit of 5×10^5 fluorescence units (or counts). Consequently, the maximum ratio between the number of cells and the volume of available liquid has a limit. The limit value for the different contact times has been analyzed. For values above the detection limit a dilution of the samples is necessary. The optimal dilution is with the blank solution. However, the available volumes of the blank solution are rarely sufficient for each dilution. The water has produced a non linear reduction to the interfering quantities effect. Hence, it has been necessary to dilute also the black solution to obtain a correct linearity of the calibration curve. The dilution of sample and black solutions with a solvent similar to the blank, i.e., fresh medium, has given a good response.

e. Correlation between input quantities

In order to keep the evaluation simple we did not consider correlation between none of the input quantities.

2.7 Uncertainty budget

The uncertainty budget of the cell count by CTB assay is reported in table 2.1. It has been calculated for a contact time of 2.5h and low cell number at the

experimental conditions of the tests. The Significance Index (SI) is the ratio between the contribution of the analysed influence quantity and the maximum contribution to the uncertainty provided by one of the influence quantities. SI lower than 1% indicates that the contribution to the uncertainty is negligible, SI upper than 10% indicates that the contribution is relevant, SI=100% indicates the most relevant contribution.

The most relevant contribution was given by K . The K uncertainty can be reduced by increasing the number of measurement points of calibration curve; in addition, the fluorescence intensity may be enhanced calibrating the system by fluorophores standard solutions.

<i>Component</i>	<i>Unit</i>	<i>Quantity</i>	<i>Source of uncertainty</i>	<i>Standard uncertainty</i>	<i>Combined standard uncertainty</i>	<i>Relative combined standard uncertainty</i>	<i>Sensitivity Coefficient</i>	<i>Uncertainty contribution</i>	<i>SI</i>
X_i	$[X_i]$	x_i		$u(x_i)$	$u(x_i)$	$u(x_i)/x_i$	$ c_i $	$[u(x_i) \cdot c_i]^2$	
$I_{f,s,2.5h}$	counts	$3.8 \cdot 10^5$	reproducibility	$3.8 \cdot 10^3$	$4.2 \cdot 10^3$	1.1%	$2.7 \cdot 10^{-2}$	$1.3 \cdot 10^4$	35%
			repeatability	$1.9 \cdot 10^3$					
$I_{f,b,2.5h}$	counts	$5.0 \cdot 10^4$	reproducibility	$5.0 \cdot 10^3$	$5.6 \cdot 10^3$	11.2%	$2.7 \cdot 10^{-2}$	$2.3 \cdot 10^4$	60%
			repeatability	$2.5 \cdot 10^3$					
V_{LF}	dm^3	$1.0 \cdot 10^{-4}$	pipette calibration	$1.0 \cdot 10^{-6}$	$1.0 \cdot 10^{-6}$	1.0%	$8.9 \cdot 10^7$	$7.9 \cdot 10^3$	21%
K	dm^{-3}	270	regression	5.9	5.9	2.2%	$3.3 \cdot 10^1$	$3.8 \cdot 10^4$	100%
N	-	8910			286	3.2%			

Table 2.1. Uncertainty budget for cell number quantification by R/R assay in 2D cell cultures.

2.8 Discussion

The cell counting methods based on metabolic activities are indirect methods and require a high reproducibility in experiments to calibrate the method and in experiments with samples. The identification of standard measurement units for fluorescence intensities and absorbance are certainly a significant prerogative for methods unification in order to increase their traceability, regardless of the adopted measurement instrument.

2.9 Conclusions

Results, in 2D cell culture, revealed that three are the main components: repeatability and reproducibility of the measurement system and a contribution due to the sample loading step procedure. Uncertainty is around 3,5% in the tested experimental conditions. All the influence quantities give a relevant contribution to the total uncertainty. It means that to reduce uncertainty by one order of magnitude, the uncertainty of all the quantities must be reduced. However the use of appropriate volumes for cell culture vessels and well filling allow to reduce uncertainty. Increasing the number of calibration points on the curve reduce calibration constant uncertainty and gives an opportunity to further reduce uncertainty.

The metabolic methods to the 3D cell cultures requires a deep knowledge of the scaffold-cells and scaffold-metabolites interactions to ensure the independence from transport limitation and to foresee the effective activity of the cells attached to the scaffold. For a 3D system, several other component can be influence parameters.

For example, in the most difficult 3D culture conditions, due to internal porous architecture of the 3D system, usually only the peripheral environment of the 3D

system is available for collecting the culture medium in which the metabolic product is released. Only in that liquid volume the metabolic product concentration can be measured. In addition, in a 3D system, limitation factors of the cell activity are the diffusion of nutrients, such as oxygen and glucose, from the cell culture medium surrounding the scaffold to the cells within the scaffold pores and the diffusion of metabolic products from the inner part of the scaffold to the outer environment. Limitations of the cellular activity influence rate and yield of the cell growth.

This justifies the need to evaluate the uncertainty in a 3D cell culture system. This work is a first approach to the uncertainty evaluation of a metabolic assay in conventional 2D cell cultures and can be the basis for a 3D system in order to increase the traceability of those methods, regardless of the adopted measurement instrument.

Chapter 3

3.1. Summary

This chapter describes the experiments made to tailor the R/R (resazurin/resorufin) cell counting method for 3D cell culture on Biocoral, with metrological approach and with the initial aims to quantify the cell number on scaffolds and to analyze the cell proliferation trend. A series of preliminary operations have been made to perform the experiments, such as the establishment of a hMSCs culture in the biometrology laboratory at the beginning of this PhD thesis work.

Contrary to what was expected, on 42 Biocorals the fluorescence intensity of resorufin, supposed to reflect the cell number trends, did not increase over time but was fluctuating, as detailed explained in the following paragraphs. This evidence and some recent knowledge about the influence of biomaterials on cell behaviour, suggested a different interpretation of the R/R assay results: they do not correlate with the cell number but with the cellular activity (or metabolism) mainly represented by proliferation and differentiation. Thus, new ongoing hypotheses were made. The first one is on Biocoral influence on hMSCs and the second one is on R/R assay: the porous structure of the scaffold induces osteodifferentiation of the cells cultured within it and R/R assay can detect this differentiation. Hence, other experiments were introduced to demonstrate the hypotheses. Finally, it was demonstrated that Biocoral induces osteodifferentiation of hMSCs and that the R/R assay, tailored for the first time in this thesis for a 3D cell culture on Biocoral, can be a methodology to study the

cellular proliferation and differentiation activities on Biocoral because well indicates the dynamic balance between the two cellular activities.

3.2. Experimental set up

The experimental set up consisted in series of preliminary operations to prepare and characterize the samples and subsequent experiments to analyze with a metrological approach the R/R assay results when the method is tailored for 3D Biocoral[®] cell cultures.

Operations and experiments have been performed in order to:

- 3.2.1 establish a hMSCs *in vitro* 2D culture
- 3.2.2 characterize the hMSCs properties and features (proliferation and differentiation)
- 3.2.3 establish a hMSCs *in vitro* 3D culture on Biocoral
- 3.2.4 tailor the R/R assay for 3D cell culture on Biocoral
- 3.2.5 test the R/R on chemically differentiated hMSCs in 2D cultures
- 3.2.6 evaluation of hMSCs osteodifferentiation induced by Biocoral scaffolds

3.2.1 Establishment of hMSCs *in vitro* 2D cell cultures

Materials and Methods

hMSCs (purchased from Lonza Group Ltd) have been cultured in Petri dishes with cell culture medium made by: α MEM (alpha modified Minimum Essential Medium - Lonza, Wokingham, UK) with FBS 10% v/v (Fetal Bovine Serum, Lonza, Wokingham, UK), L-glutamine 2mM, penicillin 100U/mL and streptomycin 100 g/mL (Lonza Wokingham, UK). Petri dishes with a growth area of about 57 cm² have been used. Fresh medium was replaced every 3-4 days until

cells reached 80% of confluence (number of cell/area). Then, cells were further expanded or used for experiments. For expansion, to obtain a suitable total number of cells, hMSCs were detached from the plastic enzymatically by an incubation of 5 min with 0.25% trypsin/EDTA (Invitrogen), counted by using a hemocytometer (or manual cell counter, such as the Neubauer chamber) and then the total cell amount were re-seeded in other 3 Petri dishes. hMSCs are usually expanded with a 1:3 splitting ratio: this ratio is commonly used to support their physiologic (even if *in vitro*) growth rate. A different ratio could inhibit or decrease the normal rate.

Cells were managed in laminar flow hood (class II) under sterile conditions.

Results

hMSCs cell culture were established in the Biosciences cell culture laboratory and a collection of cell samples was made in order to realize a cell bank available for following experiments. hMSCs samples from passage 3 to passage 16 have been made, collected and stored in liquid nitrogen. This operation of cell culture establishment and cell bank production required about 6 months.



Figure 3.1: Petri dishes for cell culture (left), freezing vials containing cells (middle), liquid nitrogen dewar containing vials with cells (right).

3.2.2 hMSCs osteogenic differentiation: induction and evaluation

Osteogenic differentiation induction

Materials and Methods

In order to confirm the stemness and multipotency of hMSCs, cells were treated with chemicals to be induced toward osteogenic phenotype. Cells cultured in Petri dishes were detached from the plastic enzymatically by an incubation of 5 min with 0.25% trypsin/EDTA, counted by using the Neubauer cell counting chamber and 15×10^3 cells/cm² were seeded in 6 well plates having a total growth area of 9.8 cm². Cells were let to adhere over night in growth medium (GM) (α MEM with FBS 2%, L-glutamine 2mM, penicillin 100U/mL and streptomycin 100 g/mL). The day after, cells were treated with osteogenic medium (OM) made by adding the growth medium with the following substances: Dexamethasone 100nM, B-Glycerophosphate 10mM, Ascorbic acid 50ug/ml. The OM was replace freshly every 2-3 days for 21 days.

A parallel control cell culture were treated with GM.

Results

After 21 days treatment, control cells and treated cells have a different morphology, as show in fig. 3.2. Control cells (A) follow a directions in their patter and stratifications are absent.

Treated cells (B) have a disorganized patter, having several overlapping regions in which cells are stratified.

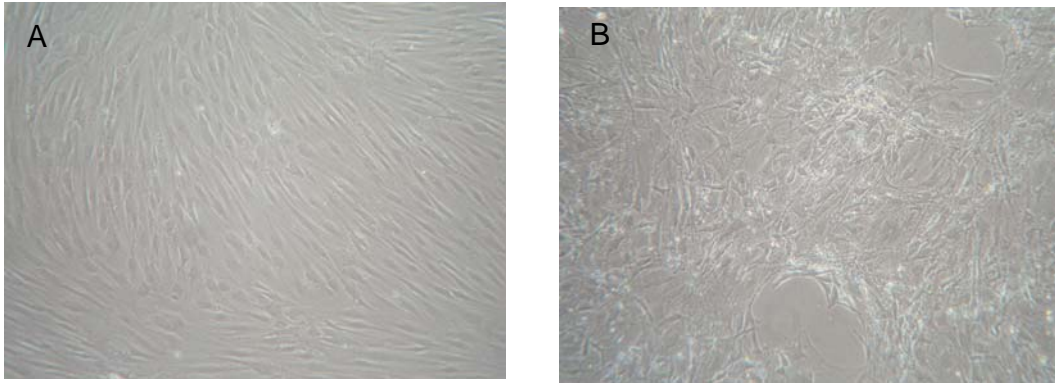


Figure 3.2 : In A control cells and in B treated cells, cultured at INRIM. Optical microscopy with 10x magnification.

Discussion

Control and treated cells have different appearance. However, in order to assess the osteodifferentiation, calcium deposition (marker of osteodifferentiation) needs to be demonstrated and Alizarin Red S Staining (ARS) has been chosen being a well known and used methodology to stain calcium nodules produced by the cells [12].

Osteogenic differentiation evaluation by Alizarin Red S Staining (ARS)

Materials and Methods

ARS stains the calcium deposits produced by differentiated cells. At day 21, control and treated cells were washed twice with PBS and fixed with Ethanol 70% in PBS for 1h. Cells were then stained with ARS 40mM for 15 minutes on a rocker. ARS were eliminated and cells were washed with distilled water 5 times. Finally cells were washed with PBS once.

Results

In treated cell cultures calcium nodules were stained in red as showed in the fig. 3.3. Three upper wells contain control cells which have not being stained with ARS staining and resulted not coloured. Treated cells in the three lower wells reacted to the ARS staining becoming red.

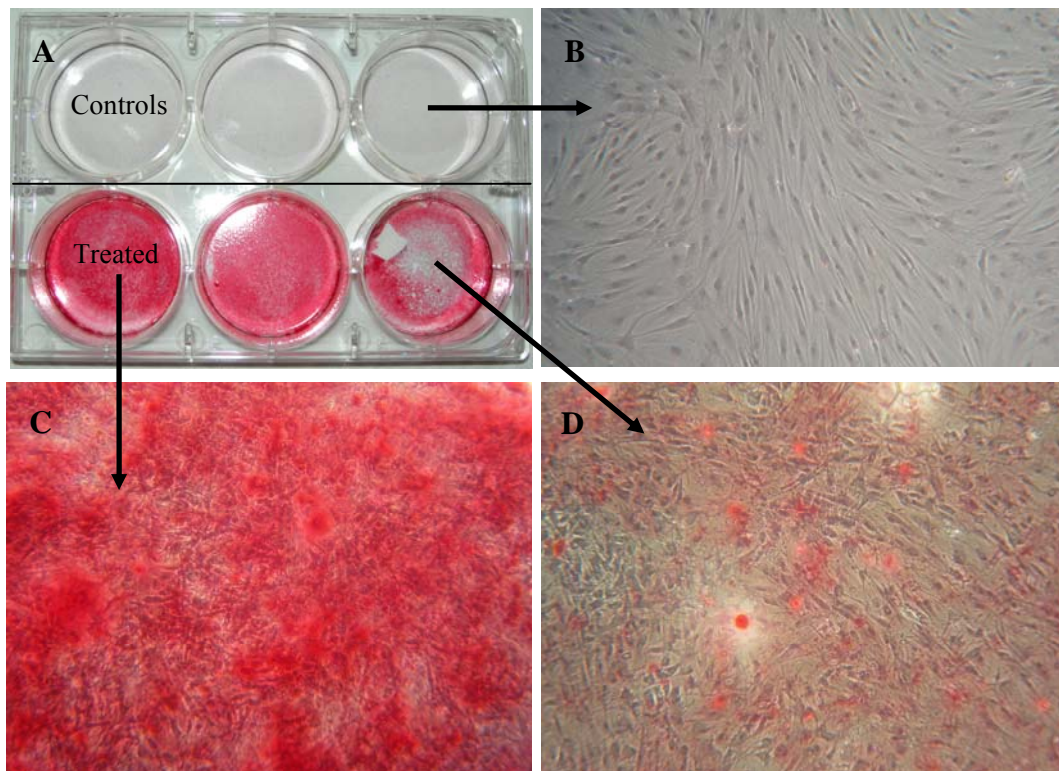


Figure 3.3: Control and treated cells stained with ARS: they appear not coloured and red coloured respectively. Red = calcium deposits (nodules). Images taken at INRIM: a digital camera (A) and an optical microscope with 10x magnification (B, C, D) were used.

Discussions

hMSCs cultures established and grown in the Biosciences cell culture laboratory have been tested for osteodifferentiation potential. They were positive

to the ARS staining confirming the osteodifferentiation. They can therefore be used in osteoinduction evaluation experiments.

3.2.3 Establishment of hMSCs in vitro 3D cell cultures on Biocoral: cell seeding and culture methodologies.

Materials and Methods

hMSCs have been cultured in Petri dishes with cell culture medium (α MEM with FBS, L-Glutamine, penicillin and streptomycin as described above) until they reach the 80% of confluence. Cells have been detached from Petri dishes by using the trypsin/EDTA solution and counted by using the Neubauer chamber. Cells were finally seeded on scaffolds: Biocoral scaffolds were placed into wells of a 24 well-plate (fig. 3.4) and a 100 μ L drop of cell culture medium containing the appropriate number of cells were deposited on the upper base surface of each scaffold. The scaffold with the “cells-drop” were incubated at 37°C and 5% CO₂ for 1 hour to let the drop be absorbed by the scaffold and to allow the cells to adhere on and within the scaffold. After 1 hour, 1 ml of cell culture medium was added in each well (containing one scaffold) to completely cover the scaffold. The 3D cell cultures on Biocoral were maintained for days or for months to conduce several preliminary experiments. Fresh medium was routinely replaced every 3-4 day or every day if necessary for short term measurements. No osteogenic medium or other osteoinductive factors were used.



Figure 3.4 : Biocorals placed in wells of a 24 well-plate. Growth medium will fill the well completely covering the Biocoral.

Results

As described previously, Biocoral has a porous structure, as shown in the following Atomic Force Microscope (AFM) and scanning electron microscope SEM images (figures 3.5, 3.6 and 3.7), which allows cellular colonization. The cells are not visible with an optical microscope because the scaffold is opaque to light.

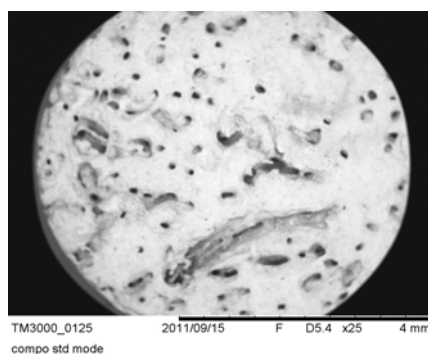


Figure 3.5 : Biocoral upper base surface. Image taken with scanning electron microscope (SEM) at INRIM thanks the kind availability of Dr M. Pisani.

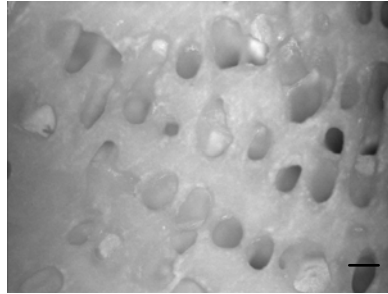


Figure 3.6 : Biocoral porous structure. The scaffold has been fractioned to see the internal pores. Images acquired with Atomic Force Microscope (AFM) at INRIM from Dr E. Bernardi. It is possible to see internal interconnections. Bar: 0.5 mm

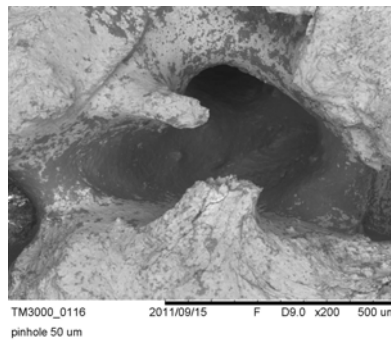


Figure 3.7 : Biocoral pore. Image taken with scanning electron microscope (SEM) at INRIM thanks the kind availability of Dr M. Pisani.

Only cells adherent to the outer surface side are in fact visible in transmitted light with optical microscopy, as shown in fig. 3.8 where cells can be just seen at the edges of Biocoral.

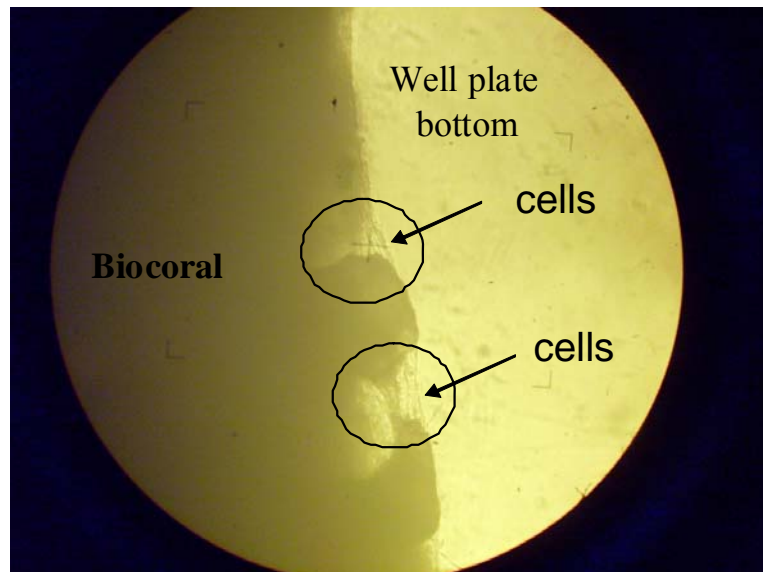


Figure 3.8 : Arrows indicate hMSCs adhered to the external surface of a Biocoral scaffold. They form “bridges” between 2 edges of the scaffolds. Transmitted light optical microscopy.

In order to check the adhesion and the colonization of the scaffolds by cells, a staining of the cells with a fluorescent molecule has been performed. The staining was carried out using the calcein AM (Invitrogen). This molecule is a cell-permeant dye, able to enter the cell and only in live cells is metabolized by cytoplasmic intracellular enzymes: the esterases enzymes, by hydrolysis of the acetoxymethyl ester (AM) group, modify the chemical structure of the molecule that is converted from a nonfluorescent calcein to a green-fluorescent calcein. The green-fluorescent calcein is retained within live cells, producing an intense uniform green fluorescence with excitation maximum wavelength at 495 nm and emission maximum at 515 nm.

As shown in fig. 3.9 the entire cell is evident when illuminated with light at the appropriate wavelength: cells have an elongated shape and seek to create contacts to establish communications between them. However, even by fluorescently colouring the cells, it is not possible to see them inside the scaffold but only on the outer surface or in more external pores (figures 3.9, 3.10, 3.11).

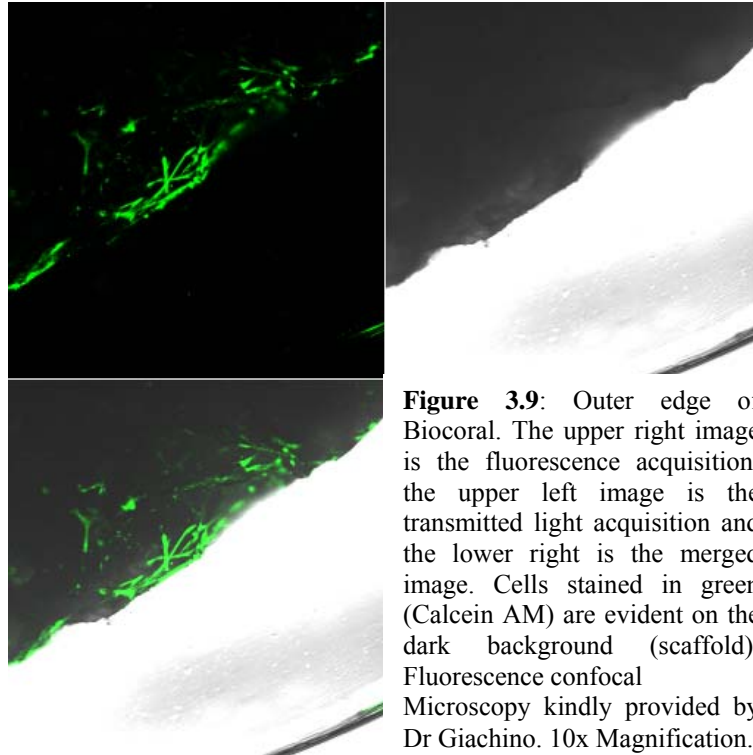


Figure 3.9: Outer edge of Biocoral. The upper right image is the fluorescence acquisition, the upper left image is the transmitted light acquisition and the lower right is the merged image. Cells stained in green (Calcein AM) are evident on the dark background (scaffold). Fluorescence confocal Microscopy kindly provided by Dr Giachino. 10x Magnification.

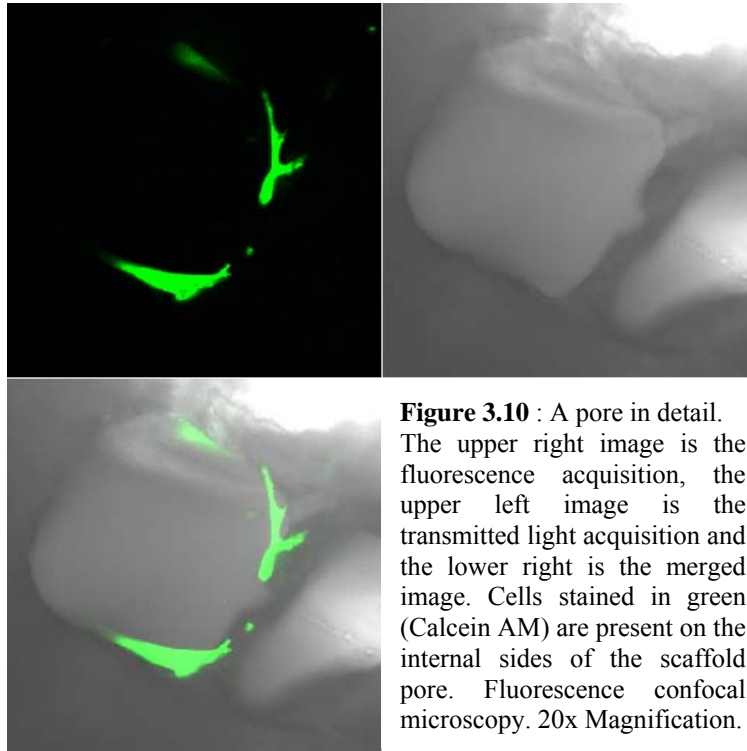


Figure 3.10 : A pore in detail. The upper right image is the fluorescence acquisition, the upper left image is the transmitted light acquisition and the lower right is the merged image. Cells stained in green (Calcein AM) are present on the internal sides of the scaffold pore. Fluorescence confocal microscopy. 20x Magnification.

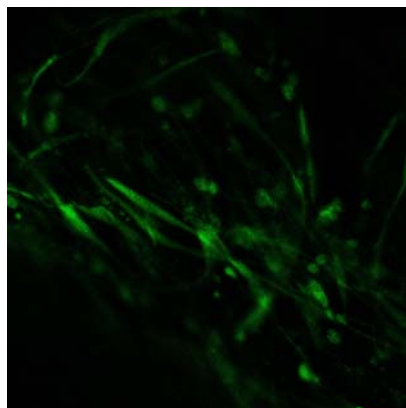


Figure 3.11: Upper face of the Biocoral scaffold. Cells in green (Calcein AM) are visible on the dark background (scaffold) It is the cell seeding site. Fluorescence confocal microscopy. 40x Magnification.

Discussion

Cell seeding methodology on Biocoral resulted in cell attachment to the scaffold pores. Cell colonization by the scaffold, at this stage can only be

hypothesized. However, Mygind et al. in 2007 [59] have made slices of a 3D cell culture on a coralline scaffold and have demonstrated that cells are within the scaffolds, living and colonizing the internal pores. Imagining a situation of cellular distribution within the scaffold, it is important to understand that the diffusion, the transport of nutrients and the elimination of the metabolic products has to be ensured. The structural properties of the scaffolds affect the fluid dynamics properties while the geometry and the chemical characteristics determine the mechanical properties and the substances transport dynamics. Transport through a matrix (scaffold) is linked to highly porous diffusion and convective mechanisms. The biocoral ensures, through its internal architecture, such transport allowing the cells to live inside it for long periods. The images of figures 3.9, 3.10 and 3.11 refer to samples of biocoral scaffolds on which the cells were cultured for 6 months: a sufficiently long time, much longer than necessary, in order to assess the biocompatibility and the ability to allow a cell growth of the scaffold.

The cells, once deposited on the surface of the scaffold, adhere to it, migrate into the more internal pores and colonize the scaffold, proliferating over time.

3.2.4 Cell activity analysis of cell cultures on Biocoral scaffolds: R/R assay for 3D cultures

Materials and Methods

R/R assay, described in Chapter 2, has been used.

Scaffolds were placed in 24 well plates and a known number of cells were seeded on them as described in paragraph 3.2.3. Several cell density (cell number/scaffold) were seeded and the 3D culture were analyzed at several time points (different days of the culture). Cells on Biocorals were cultured for different period of total time (culture lifetime), as summarized in table 3.1.

ID Scaffold	Cells density	n. scaffolds used	Culture lifetime (days)	Time points
A	2.25×10^3	1	10	4
B	4.5×10^3	1	10	4
C	9×10^3	1	10	4
D, E, F	5×10^5	3	21	7
GHI	1×10^5	3	21	7
JKL	3×10^5	3	21	7
M	5×10^4	1	51	8
N	8×10^4	1	51	8
O	9×10^4	1	51	8
P	1×10^5	1	51	8
Q	7×10^3	1	119	12
R	4×10^5	1	119	12
S	1×10^5	1	119	12
T	2×10^5	1	119	12
U	3×10^5	1	119	12

Table 3.1 : Summary of set up for 4 experiments of hMSCs activity on Biocoral with R/R assay.

After 24 hours at 37°C and 5% CO₂ to let the cells to adhere on to the scaffolds surface, scaffolds were incubated for 2 hours with 1 ml of Resazurin 10 mM in growth medium.

After 2 hours, the R/R solution was collected in microcentrifuge tubes.

Scaffolds were washed with PBS and fresh growth medium were added (1 ml/scaffold).

Fluorescence intensity of R/R solution were measured with the GloMax[®]-Multi Microplate Multimode Reader (Promega Corporation, USA). Excitation was performed at 525 nm wavelength while emission was measured in the range 580-640 nm. Microplates with 96 wells were used and a volume of 100 µl for each sample was measured in triplicate.

Results

The following graphs show the trend of fluorescence intensity (I_F), measured in counts, of resorufin after metabolization of resazurin by cells within the scaffold over time (days). I_F is expected to reflect the cell number, hence it should be correlated with the cell number. However the I_F trend allow to hypothesize a different interpretation of R/R results: the I_F detected with the R/R assay does not correlate with the cell number but with the cellular activity (A_C), i.e. the cellular metabolism, as described by the following equation. This new hypothesis will be discussed below.

$$I_F = A_C \cdot k \cdot V_W \text{ [counts]} \quad (3.1)$$

where I_F is the fluorescence intensity measured, A_C is the cellular activity, k is the proportionality constant and V_W is the volume of R/R solution filling the measurement well.

Aim, at this moment, is to evaluate the A_C trend (through I_F values) and not yet to quantify A_C . Thus, k is not quantified.

Error bars on fluorescence intensity values represent the combined standard uncertainty of the measurement, with a coverage factor of $k=1$, equal to the positive square root of the sum of the squared terms repeatability, reproducibility and uncertainty due to V_W , as described by the formula:

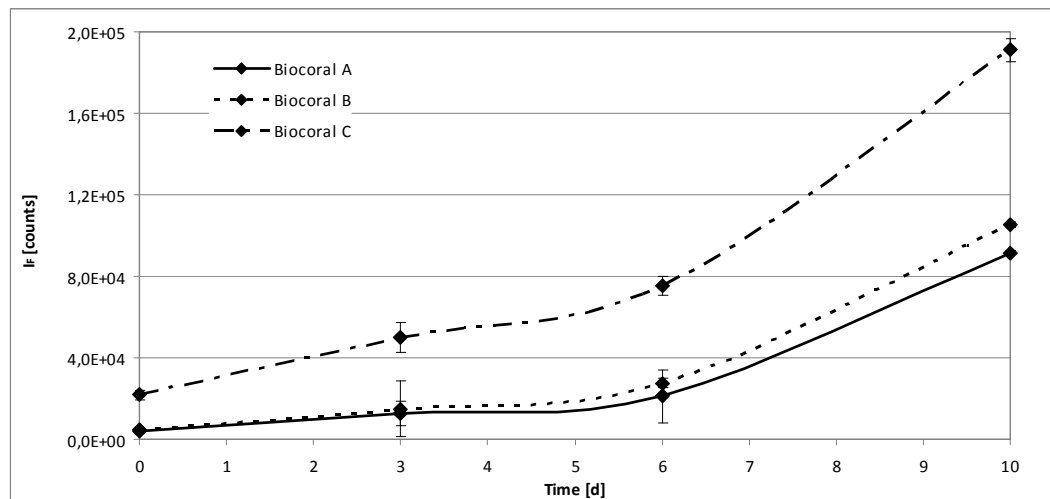
$$u(I_F) = \sqrt{\text{repeatability}^2 + \text{reproducibility}^2 + u(V_W)^2} \text{ [counts]} \quad (3.2)$$

For scaffolds A, B, C, M, N, O, P, Q, R, S, T, U the repeatability is calculated as the standard deviation of the three repeated measurements for each sample and reproducibility has not been calculated because only 1 scaffold was analyzed for each condition.

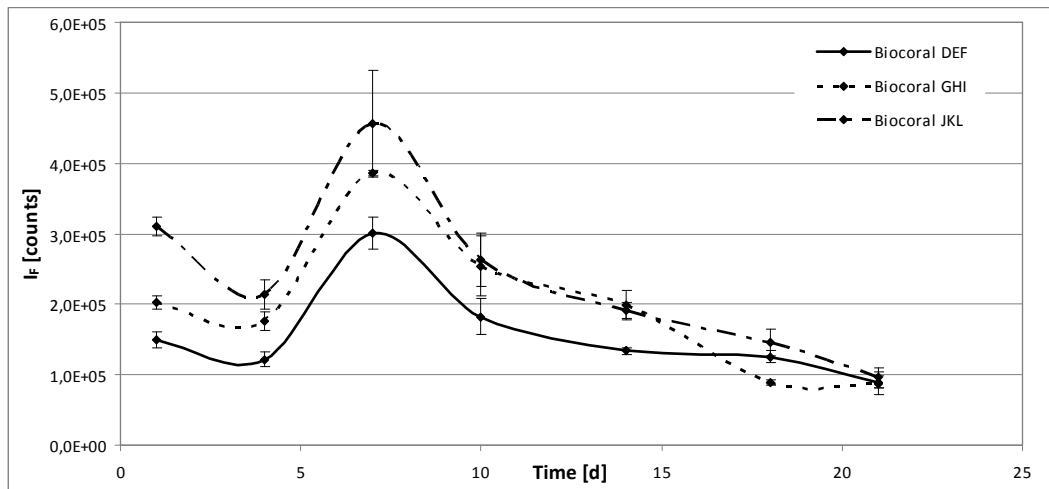
For scaffolds D, E, F, G, H, I, J, K, L the repeatability is the mean of standard deviation of the three repeated measurements and the reproducibility is calculated as the standard deviation of all the 9 measurements.

For each scaffold the V_W is 0.1 ml with an uncertainty due to the pipetting error set as 0.001 ml.

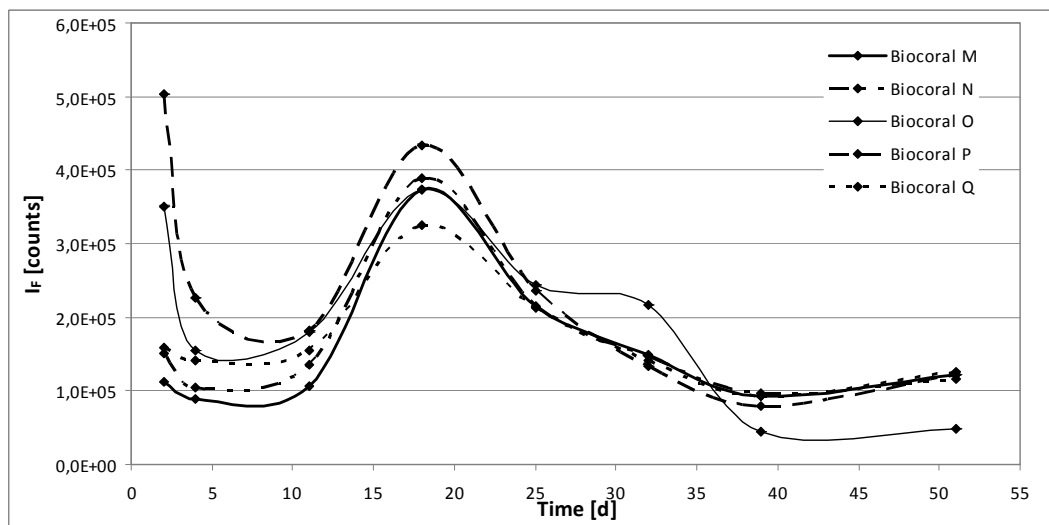
The trend has been analyzed for cells seeded at several density (cells/scaffold) on 21 Biocorals. Cell density and days of culture are reported in table 3.1.



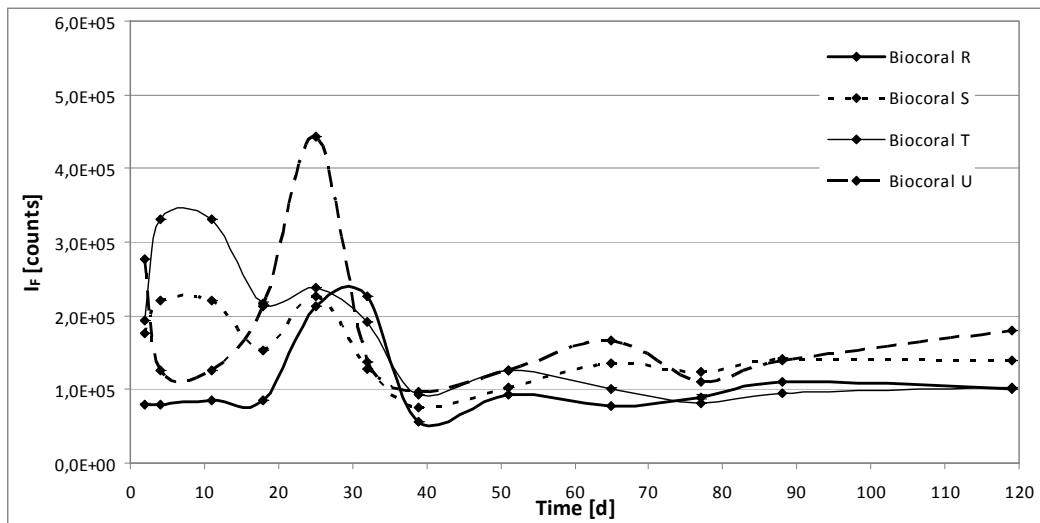
Graph 3.1: I_F increase slowly during the first 6 days and then the increase rate is higher. Cell density at day 0 in A, B and C is very low.



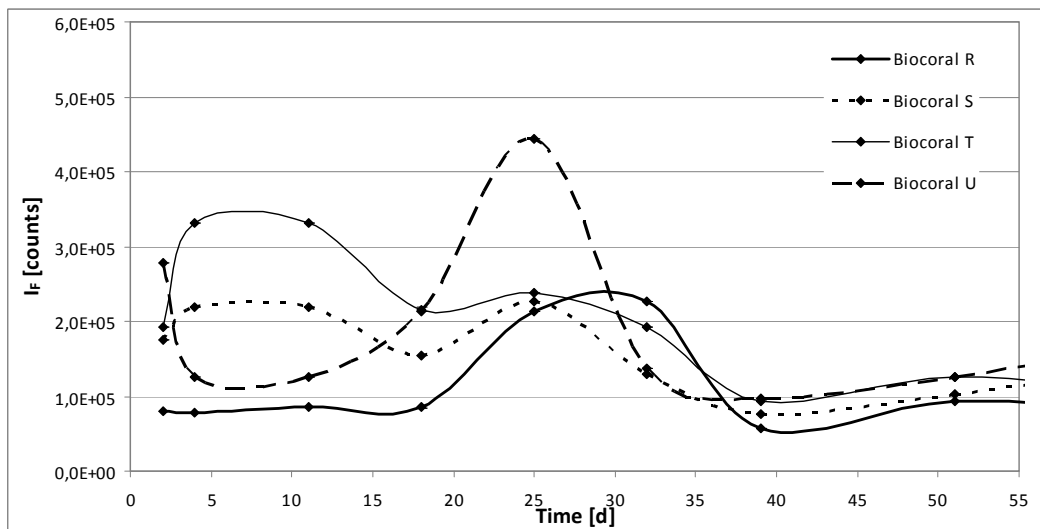
Graph 3.2: I_F values are fluctuating over time. Firstly it decreases, then it increases and again decreases. However, the trend is very similar in all the scaffolds, independently from the cell density at day 0.



Graph 3.3: I_F values are monitored for 50 days. The trend is fluctuating over time reaching the maximum values in all the scaffolds around day 18 of culture.



Graph 3.4: I_F values are monitored for 4 months. The trend is fluctuating over time reaching the maximum values in the first 20 days of culture.



Graph 3.5: By comparing the first 55 days trend of the graph 3.4 with the trend in 50 days shown in graph 3.3, it is evident that Biocoral S very well reflects the trend observed in graph 3.3, even if the maximum value of $I_{F_{IS}}$ is reached later here.

Generally, the R/R fluorescence intensity trend in Biocorals is fluctuating: cells seem to decrease and increase their number, but the following discussion will explain a different interpretation of R/R results.

Discussion

Fluorescence intensity is expected to reflect the cell number. However, the trend observed in all the scaffold, independently from the number of cells seeded on Biocorals at day 0, indicates that I_F , and consequently the cell number, did not increase over time but was fluctuating. This evidence, confirmed in 21 scaffolds, with different cell number seeded at day 0, and some recent works demonstrating that biomaterials induce the cell differentiation, suggested a different interpretation of results of R/R assay and allowed new ongoing hypotheses. The first new hypothesis is that Biocoral influence hMSCs behaviour by inducing an osteodifferentiation. The second is that R/R assay on Biocorals can indicate this differentiation and it does not relate with the cell number. Thus, R/R assay results probably indicate a dynamic process of cellular proliferation/differentiation.

In order to confirm this hypothesis, two experiments have been made: the first is the analysis of resazurin reaction when in contact with cell having two different phenotypes: undifferentiated and differentiated. The second experiment is to demonstrate that Biocoral induce osteodifferentiation of hMSCs.

3.2.5 Testing the R/R on chemically differentiated hMSCs in 2D cultures

Aim of this test is to answer the questions: do hMSCs metabolized differently the resazurin? Hence, does the R/R assay give different responses for differentiated cells with respect to undifferentiated ones?

Materials and Methods

In order to verify the behaviour of R/R metabolic reaction with differentiated hMSCs, 15×10^3 cells/cm² were seeded in 6 well plates. Cells were let to adhere over night in growth medium (GM) (α MEM with FBS 2%, L-glutamine 2 mM, penicillin 100U/mL and streptomycin 100 g/mL). The day after, cells were treated with osteogenic medium (OM) made by adding the growth medium with Dexamethasone 100 nM, B-Glycerophosphate 10 mM, Ascorbic acid 50 μ g/ml. The OM was replace freshly every 2-3 days for 15 days.

A parallel control cell culture were treated with GM.

Control and treated cell were then treated with resazurin to perform the R/R assay.

The incubation time was 24 hours, a time much longer than the standard incubation time (suggested between 0.5 and 4 hours from the manufacturer).

The same samples of control and treated cell were stained with Alizarin Red S staining (as described previously) to mark the calcium nodules. This to directly correlate the R/R assay results and ARS results.

Results

As shown in fig. 3.12, treated cells differentiated forming calcium nodules stained positively with ARS (red), control and treated cells react diversely to the R/R test. Undifferentiated cells (control) reduced the resazurin becoming pink (and fluorescent) while fully differentiated cells (treated), even after 24 hours of incubation with resazurin, did not (or slightly) metabolized the substrate resaruzin and the colour did not change (fluorescence neither).

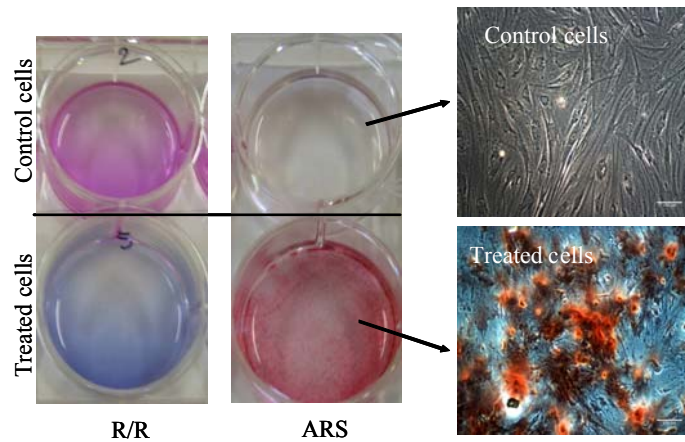


Figure 3.12 : R/R assay results on control and treated cells in the left panel, ARS corresponding results in the central panel and an optical microscopy image of the cells stained with ARS on the right panel, are shown.

Discussion

The results showed that undifferentiated and differentiated hMSCs metabolized differently the resazurin, hence R/R assay give different responses for cells with different phenotype.

The R/R assay on Biocorals does not relate with the cell number. Thus, R/R assay results should be considered as indicating a dynamic process of cellular proliferation/differentiation.

In addition, when cells are chemically induced to differentiate and then undergo to the /R assay, in both samples, control and treated ones, the cells are attached on the well surface but treated cells are embedded in a mineralized matrix that probably prevent the resazurin solution access to the cells. Under this hypothesis, it is plausible that differentiating cells, not completely embedded in the mineralized matrix, can still partially have access to the resazurin. However, experiment does not indicates what is the behaviour of differentiating cells with a mixed phenotype. Hence, further experiments should be done with cells at

different stage of differentiation in order to have a quantitative correlation between R/R assay results and differentiation stages of hMSCs.

3.2.6 Evaluation of hMSCs osteodifferentiation induced by Biocoral scaffold

In order to demonstrate the hypotheses that: 1) Biocoral has an osteogenic potential and induce osteodifferentiation in hMSCs seeded on it and 2) R/R reflect the dynamic process of proliferation/differentiation of hMSCs on Biocoral, an analysis of gene expression was performed.

Materials and Methods

A Real Time Polymerase Chain Reaction (RT-PCR) was performed to study the phenotype expression patten of hMSCs cultured on Biocoral scaffolds and particularly to analyze the cell osteogenic expression patten.

The RT-PCR technique quantifies the expression of specific genes in hMSCs in 2D and 3D cell cultures on Biocoral, contained in the DNA copies. The RT-PCR allows the simultaneous amplification and quantification of DNA by monitoring in real time the intensity of fluorescence released from the amplification product during the chain reaction of DNA polymerase: this is possible by means of the use of fluorescent markers whose accumulation, at the level of the reaction product, follows the same kinetics of PCR.

In order to obtain DNA samples to perform the RT-PCR analysis, the following steps have been carried out:

- *Cells seeding and culture on Biocoral:* 21 Biocoral scaffolds have been seeded with 3×10^5 hMSCs at passage 7 and cultured up to 21 days with normal cell culture medium with no osteogenic or osteoinductive factors.

Three scaffolds for each time point (respectively at day 1, 4, 7, 10, 14, 18, 21) have been used for the gene expression analysis.

- *Cell activity evaluation of hMSCs on Biocoral*: for each scaffold the R/R cell activity assay, non destructive and non dangerous for the RNA, has been performed immediately prior to start the following steps.
- *Biocoral pulverization*: cells, especially the ones embedded in the extracellular mineralized matrix within the Biocoral pores, cannot be detached easily from the scaffold. However, in order to collect the RNA from all the cells within the Biocoral the sample needs to be homogeneous. At this scope, Biocorals are pulverized and homogenized by using liquid nitrogen stainless steel mortars and pestles, designed and made at INRIM, autoclaved to be RNase free (fig. 3.13.) The Biocoral is placed in the compartment of the entire mortar, previously placed in liquid nitrogen. Then the mortar containing the Biocoral is placed in liquid nitrogen. After cooling, the scaffold is pulverized with the pestle. Biocoral powder needs to be as finest as possible.



Figure 3.13 : Mortar and pestles. Mortar is made of stainless steel, autoclavable and liquid nitrogen resistant.

- *RNA extraction*: this step allows the extraction of RNA from cells, both the differentiated ones and the non differentiated (proliferating) ones.

After pulverization, the powder is transferred in falcon tubes of polypropylene, kept in dry ice to ensure the low temperature. The sample was added with 1ml of QIAzol Lysis Reagent (QIAGEN) and vortexed. For phase separation, 0.2 ml of chloroform (for 1 ml of TRIZOL[®] Reagent) were added to the tubes. The samples were shaken vigorously for 15 seconds and taken 3 minutes at room temperature. After centrifugation at 13,000 x g for 15 minutes at 4 °C, the mixture results separated into a phenol-chloroform phase (red), containing proteins and lipids, a middle phase (white) containing DNA and a colorless aqueous phase (transparent). The RNA remains exclusively in the aqueous phase as shown in a demonstrative picture (fig. 3.14).

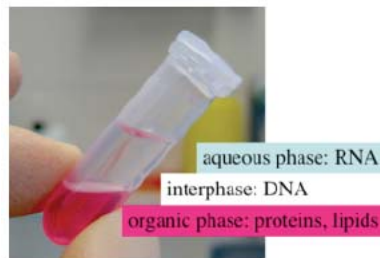


Figure 3.14 : Different stages in the tube after treatment with phenol-chloroform and separation by centrifugation.

The aqueous phase is carefully transferred to a new tube without disturbing the interphase. The RNA obtained in the aqueous phase were added with 0.6 ml of 70% ethanol. The samples were incubated at -4 °C for 10 minutes and then well were re-suspended (avoiding vortexing and centrifugation). RNA is purified using the RNeasy Mini Kit protocol (Qiagen). Briefly, the sample is placed on special columns of purification, centrifuged at 10000 rpm for 15 seconds at room

temperature. The RNA is retained in the column, washed with two buffers provided in the kit and centrifuged at maximum g number of the centrifuge for 1 minute at room temperature to eliminate the buffers. Finally the RNA is eluted from the column with 40 μ l DEPC-treated water in order to remove DNase and RNase.

The RNA samples obtained are quantified using the NanoDrop ND-1000 spectrophotometer.

The RNA purity is secured when the spectrophotometric A260/A280 ratio ≥ 1.8 , where A260 and the absorbance in nanometers relative to nucleic acids, while A280 is the absorbance in the UV and the index of protein contamination.

The RNA integrity is assessed by electrophoresis on agarose gel.

- *Retrotranscription*: the retrotranscription is a reverse transcription reaction producing the molecules of complementary DNA (cDNA) from RNA.

250 ng of total RNA extracted from BIOCORAL and total RNA extracted from hMSCs as control cells are reverse transcribed in a final volume of 20 μ l. A mixture composed of RNA and random primers (1.5 pmol/ μ l) are incubated at 65°C for 10 minutes to facilitate the extension of the primers by reverse transcriptase in the following step. The reverse transcription is performed by using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics): the RNA is incubated in a mixture composed with deoxyribonucleotide triphosphates (dNTPs) 1 mM, RNase inhibitor 20Units and reverse transcriptase 50Units, Buffer 10X and MilliQ water to reach the final volume for 10 minutes at room temperature followed by a at 55°C for 30 minutes and 85°C for 5 minutes. Finally 20 μ l of cDNA are synthesized from RNA.

To check the occurred retrotranscription, 1 μ l of cDNA is used to amplify an endogenous gene control (expressed in all tissues at any time) by Polymerase Chain Reaction (PCR): the endogenous gene amplified for this test is the β -actin

gene. To perform the PCR reaction the following reagents are used: KAPA2G Fast polymerase, 10 μM of forward primers for β -actin (5'-CTAGAAGCATTTGCGGTGGACGATGGAG-3') and 10 μM of reverse primers for β -actin (5'-ATGGATGATGATATCGCCGCG-3'), 5X reaction buffer, 10 μM dNTPs and MilliQ water in a final volume of 25 μl . cDNA is amplified for 30 cycles with annealing temperature of 62°C.

- *RealTime PCR protocol analysis*: The TaqMan Gene Expression Master Mix (Applied Biosystems) was used to perform the amplification. It contains AmpliTaq Gold® DNA polymerase Ultra Pure, uracil-DNA Glycosylase (UDG), deoxyribonucleotide triphosphates (dNTPs) with triphosphate deoxyuridine (dUTP), ROX™ passive reference and buffer components.

To prepare the mixture of the components for the PCR reaction, the following reagents are mixed in tubes RNase-free: 10 μl of TaqMan Gene Expression MasterMix 2X, 0.4 μM final primer, 0.2 l of UPL probe and 50 ng of cDNA, for a final volume of 20 μl per reaction.

The tubes are briefly vortexed to mix the solutions and briefly centrifuged to remove any air bubbles from the solution.

The genes of interest are analyzed on ABI 7500 Real Time PCR platform Fast Real-Time PCR System (Applied Biosystem).

The conditions of thermal cycles are as follows: 2 min at 50°C, 10 minutes at 95°C, necessary to activate the enzyme AmpliTaq Gold, and 40 cycles of PCR consisting of 15 seconds at 95°C for the denaturation process and 1 minute at 60°C for the steps of annealing /extension.

For the analysis of RT-PCR data was used the relative quantification technique that allows to normalize the data with respect to the differences in target gene expression levels compared to a control gene. This gene, called housekeeping

gene, is constitutively expressed, i.e. it is not subject to modulation of the expression due to changes in cell phenotype, and used as an internal control reference. For hMSCs a stable housekeeping gene is RPL13A [60] and for this thesis work it has been used the RPL13A from Roche-Diagnostics (RealTime ready Assay® IDA 102119, No.B Accession NM_012423).

The cDNA quantity is expressed as gene expression (GE) and is calculated as:

$$GE = 2^{-\Delta(\Delta Ct)} \quad (3.2)$$

where $\Delta(\Delta Ct)$ is give by

$$\Delta(\Delta Ct) = (Ct_{RPL13A_{calibrator}} - Ct_{TARGET_{calibrator}}) - (Ct_{RPL13A_{sample}} - Ct_{TARGET_{sample}}) \quad (3.4)$$

Ct defines the amplification cycle at which the fluorescence level crosses a baseline threshold of fluorescence level at which all the samples are in the exponential phase of the amplification reaction; the fig. 3.15 shows the Ct meaning.

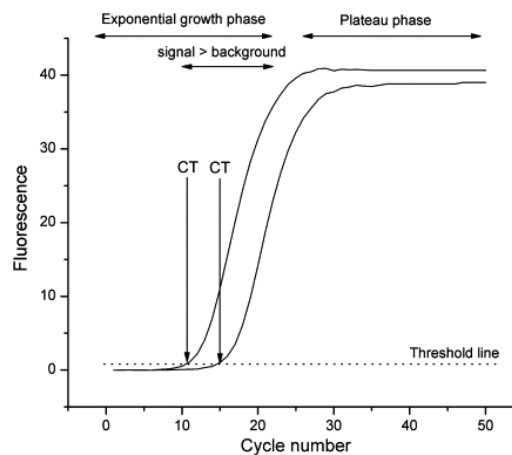


Figure 3.15 : Ct defines the amplification cycle at which the fluorescence level crosses a baseline threshold of fluorescence level at which all the samples are in the exponential phase of the amplification reaction.

Hence, $\Delta(\Delta Ct)$ is the difference between the calibrator ΔCt (CtRPL13A-CtTarget) and the unknown sample ΔCt (CtRPL13A-CtTarget).

The calibrator is a sample of hMSCs cultured in 2D condition prior to be seeded on Biocoral scaffolds. To the GE of the calibrator is arbitrarily assigned the value of 1 unit. The target is the gene of interest which expression has to be checked in the cells.

The relative difference of gene expression (ΔGE) was calculated between hMSCs cultured on 3D Biocoral scaffolds and hMSCs cultured in 2D cultures.

For each gene in both 2D and 3D cell culture sample, ΔGE was calculate using the following formula:

$$\Delta GE = \frac{GE_{t_i} - GE_{t_0}}{GE_{t_0}} \cdot 100 \quad [\%] \quad (3.5)$$

where GE_{t_i} is the GE in the 3D sample at time i and GE_{t_0} is the GE in 2D at time 0 .

Cell activity evaluation of hMSCs on Biocoral

Results

Three series scaffolds (X, Y, Z) have been analyzed with resazurin/resorufin assay in 7 days of analysis by performing the assay on 3 different scaffolds for each day for a total of 21 Biocoral scaffolds in 21 days (7 scaffolds for each series).

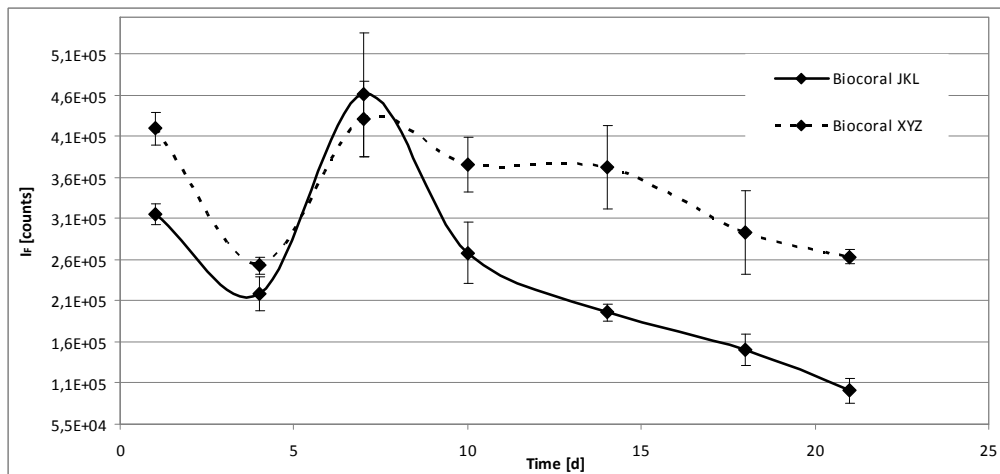
Three scaffolds (J, K, L Biocorals, analyzed also in graph 3.2) have been cultured in parallel as control: on these 3 scaffolds the R/R assay was performed at each time points over the entire period of culture (21 days), therefore the same scaffold was followed over time. The mean values of the 3 scaffolds have been represented in the following graph.

Error bars on fluorescence intensity values represent the combined standard uncertainty of the measurement, with a coverage factor of $k=1$, equal to the positive square root of the sum of the squared terms repeatability, reproducibility and uncertainty due to V_w , as described by the formula:

$$u(I_F) = \sqrt{\text{repeatability}^2 + \text{reproducibility}^2 + u(V_w)^2} \text{ [counts]} \quad (3.6)$$

The repeatability is the mean of standard deviation of the three repeated measurements and the reproducibility is calculated as the standard deviation of all the 9 measurements.

For each scaffold the V_w is 0.1 ml with an uncertainty due to the pipetting error set as 0.001 ml.



Graph 3.6: I_F values fluctuate over time in both set of 3 scaffolds reaching the maximum on the same time. Cellular activity decreases and increases over time on 6 scaffolds where the same cell number was seeded at day 0.

In scaffold set X, Y, Z each point represents the behaviour of cells living within different scaffolds. The behaviour is not exactly of the same cells. However, the same cell population has been divided on the 21 scaffolds and can be considered the same.

In both set of 3 scaffolds, JKL (control) and XYZ, cells have the same fluctuating trend: they decrease and increase the cellular activity until the day 10 and then decrease drastically until the day 21.

Discussion

In this experiment 21 Biocoral scaffolds have been considered as the same scaffold analyzed over time but each scaffold has been destroyed after only one analysis with R/R assay to be used for RNA extraction and gene expression analysis. Fluorescence intensity results of R/R assay, show that cellular activity have a fluctuating behaviour as also occurred in previously experiments on R/R assay tailoring on Biocorals: cells within the Biocoral are a mixed pool of proliferating and differentiating cells and they react differently with the resazurin with a different capacity to metabolize it. Therefore, the fluorescence intensity is fluctuating and indicates a mean cellular activity of the cell population on scaffolds. These results can be correlated with the gene expression results as follows.

RNA extraction

Results

The RNA extracted from Biocorals had a purity in the range of 1.7 - 1.9. RNA integrity has been verified.

Discussion

RNA extraction from cells cultured on Biocoral has not been an easy procedure.

Biocoral has a very stiff and porous structure: two conditions making the accessibility of RNA extraction reagents to the cells within the pores very hard.

Results of RNA quantity was variable from scaffold X, Y and Z at the same time point demonstrating the hardness of the procedure and the difference between different Biocorals in retaining cells within the pores.

To note that the RNA are extracted from hMSCs having three different phenotype: undifferentiated, differentiating and differentiated phenotypes.

Retrotranscription

Discussion

The standard protocol was feasible for the Biocoral RNA extracts and the procedure have not met drawbacks.

RealTime PCR: osteogenic expression patten analysis of cells on Biocoral

Results

The temporal expression of a panel of 5 differentiation genes and of 5 stemness genes were determined by quantitative real time PCR using RNA isolated from 21 scaffolds. The 3 series of scaffolds (X, Y, Z) were analyzed for 21 days at 7 time points. For each point (day of analysis) one scaffold for each series X, Y, Z was used. Results were compared with the housekeeper gene expression RPL13A to normalize the gene expression of different samples and were related to the gene expression in hMSCs in 2D cell cultures placed as 0%. Each sample was run in triplicate.

Results are reported as mean values (mean of X, Y and Z) in one graph for each gene analyzed: each graph for the gene expression is compared with the cellular activity (proliferation/differentiation) coming from R/R assay results on Biocorals (mean of X, Y and Z).

This allows to simultaneously evaluate and correlate the gene expression with the cellular activity of cells on scaffolds.

The following genes have been analyzed:

- for stemness/proliferation: CD29, CD44, CD90, CD105, CD166
- for osteodifferentiation COLIA1, ON, OP, BSP, BMP2

For statistical analysis the raw data of gene expression levels obtained from each experiment of RT PCR (for each sample three replicates) were subjected to the Anova test. The differences between the data obtained were all statistically significant with a p value <0.0001.

The uncertainty of ΔGE has been calculated as:

$$u(\Delta GE) = \sqrt{[c(GE_{ti}) \cdot u(GE_{ti})]^2 + [c(GE_{t0}) \cdot u(GE_{t0})]^2} \quad [\%] \quad (3.7)$$

where $c(GE_{ti})$ and $c(GE_{t0})$ are the sensitivity coefficients of $u(GE_{ti})$ and $u(GE_{t0})$ respectively and are calculated as:

$$c(GE_{ti}) = \frac{\partial GE_{ti}}{\partial \Delta GE} = \frac{1}{GE_{t0}} \quad (3.8)$$

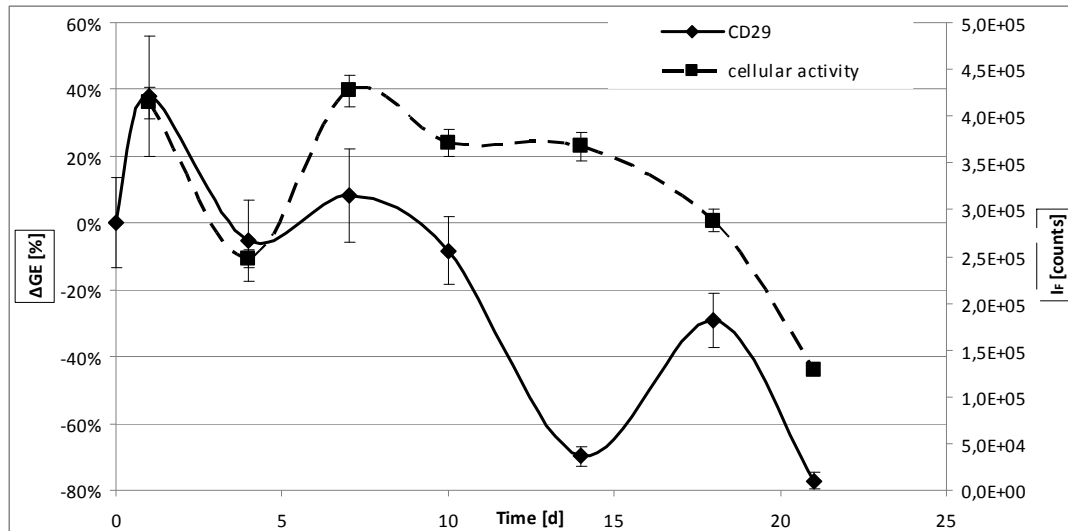
$$c(GE_{t0}) = \frac{\partial GE_{t0}}{\partial \Delta GE} = -\frac{GE_{ti}}{GE_{t0}^2} \quad (3.9)$$

Thus, the eq. 3.7 becomes:

$$u(\Delta GE) = \sqrt{\left[\frac{1}{GE_{t0}} \cdot u(GE_{ti})\right]^2 + \left[-\frac{GE_{ti}}{GE_{t0}^2} \cdot u(GE_{t0})\right]^2} \quad [\%] \quad (3.10)$$

where $u(GE_{ti})$ and $u(GE_{t0})$ are the standard deviations of the GE_{ti} and GE_{t0} triplicate measurements.

CD29



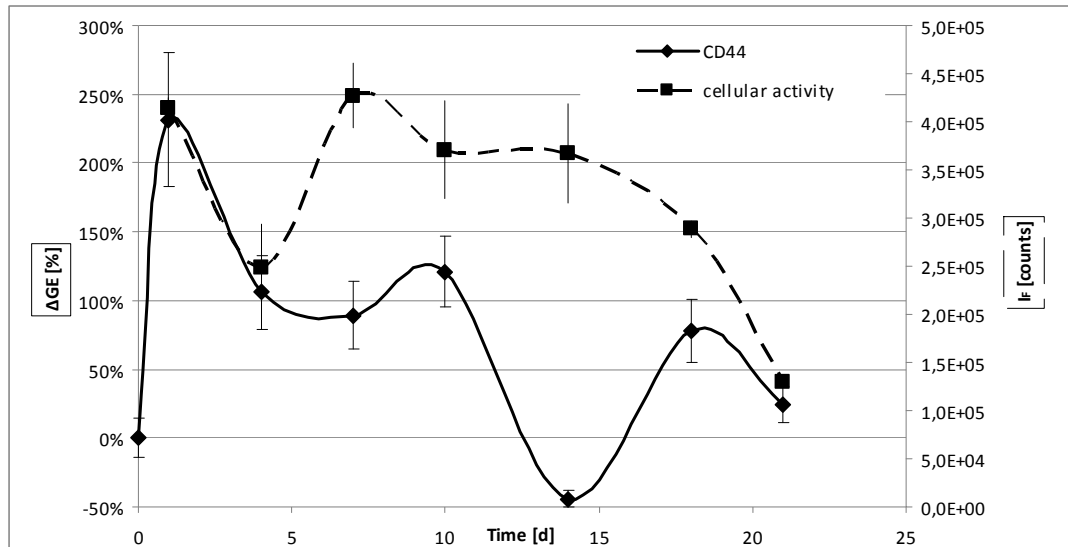
Graph 3.7: CD29 profile of expression and cellular activity on Biocoral.

CD29 expression level in 3D cell cultures is positive between day 0 and day 1 but then decrease during the cell culture on Biocoral. A 20% increment is revealed during the last days in culture. The CD29 expression trend follows the cellular activity trend: when cells are not metabolizing the resazurin and the cellular activity is toward the differentiation, CD29 decrease.

Discussion

The CD29 expression trend after the first days in culture, during which the gene is expressed, decrease with respect to the undifferentiated hMSCs (2D sample at day 0) indicating a change in the cell phenotype: cells are losing their stemness.

CD44



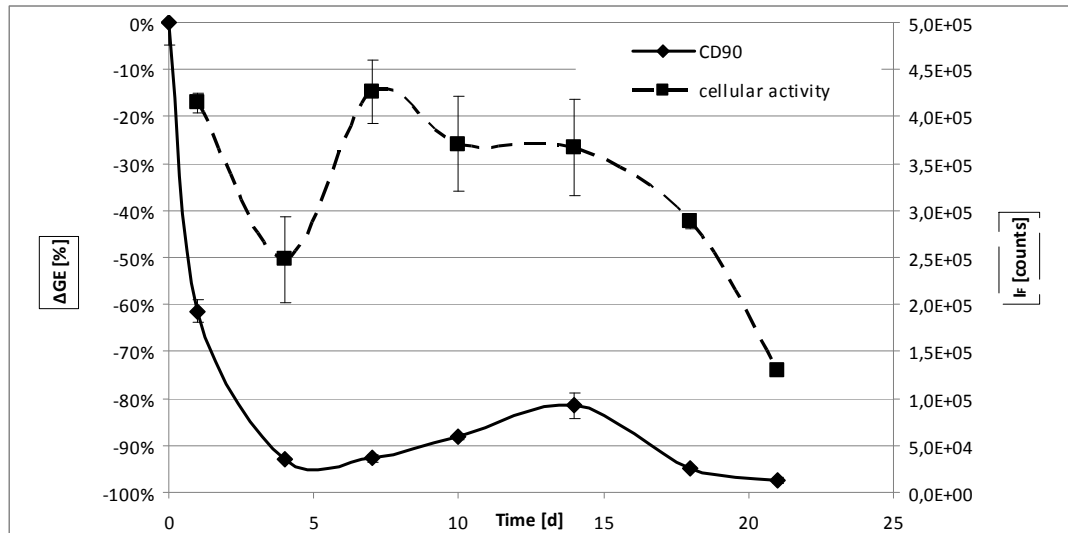
Graph 3.8: CD44 profile of expression and cellular activity on Biocoral.

CD44 expression in 3D cell cultures increases during the first day, then the stemness gene decreases when cell activity goes towards differentiation and increases when cell activity is towards proliferation. Hence, it follows the cellular activity trend.

Discussion

Similarly to the CD29 expression trend, also CD44 expression decrease with respect to the undifferentiated hMSCs (2D sample at day 0) indicating a change in the cell phenotype: this evidence confirm that cells are losing their stemness.

CD90



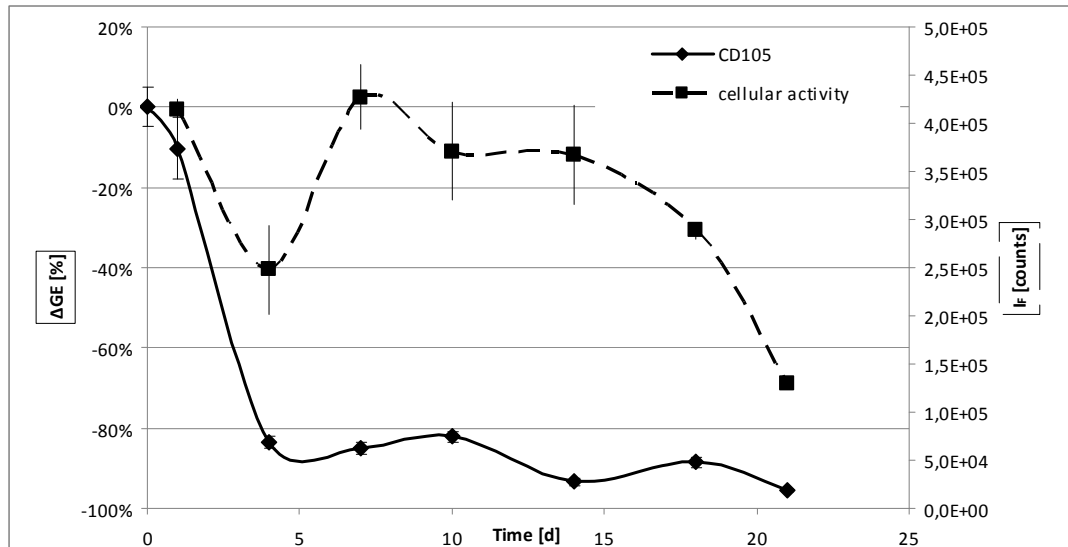
Graph 3.9: CD90 profile of expression and cellular activity on Biocoral.

CD90 expression decreases since from the beginning of the 3D cell culture with a slight increase starting when the cellular activity goes towards proliferation and decreases again when cell activity is substantially towards proliferation after day 14. The expression, therefore, follows the cellular activity trend.

Discussion

Similarly to the CD29 and CD44 expression trend, also CD90 expression decrease with respect to the undifferentiated hMSCs (2D sample at day 0) indicates that cells are losing their stemness when cultured of Biocorals.

CD105



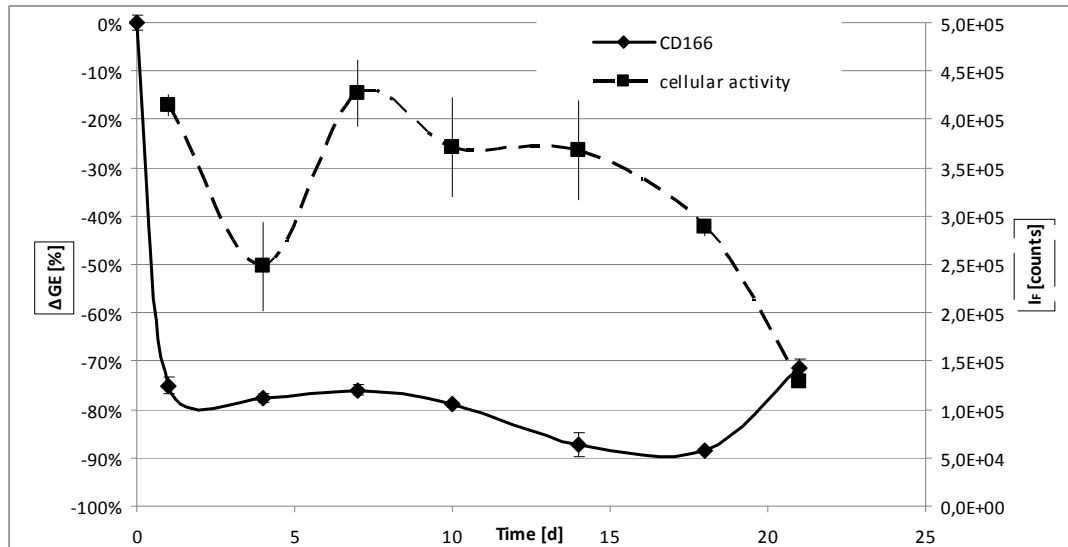
Graph 3.10: CD150 profile of expression and cellular activity on Biocoral.

CD105 expression is very similar to CD90 expression and follows the cellular activity trend: during differentiation, the stemness gene decreases its expression and when cells are mostly during the proliferation phase, the stemness gene expression increases.

Discussion

CD105 expression is a further confirmation of the change in phenotype of the hMSCs seeded and cultured on Biocorals. Cells lose their stemness since the first days of culture on scaffolds.

CD166



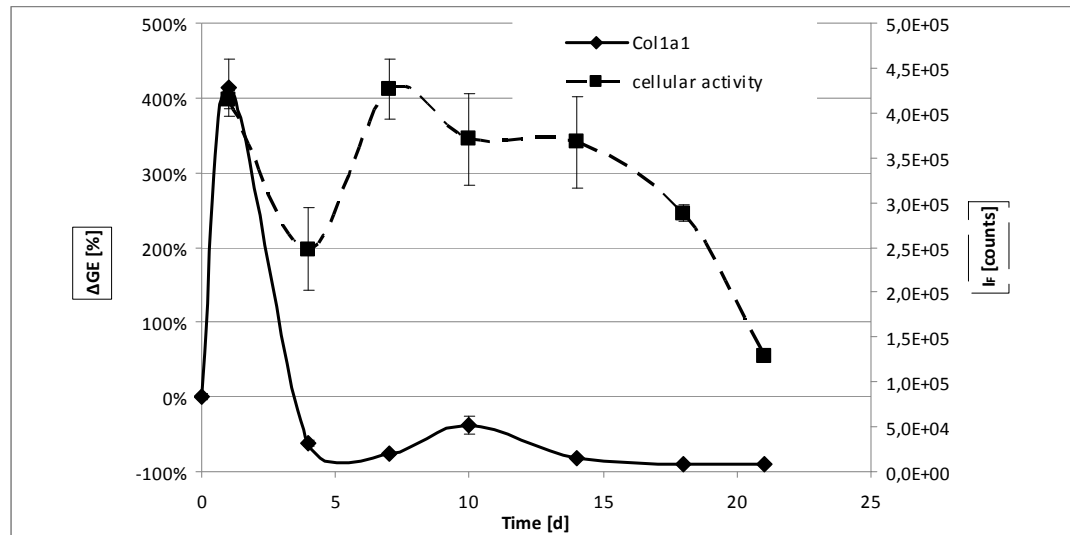
Graph 3.11: CD166 profile of expression and cellular activity on Biocoral.

CD166 expression is very similar to CD105 expression: it decreases since the first days of 3D culture and follows the cellular activity trend: during differentiation, the stemness gene decreases its expression and when cells are mostly during the proliferation phase, the stemness gene expression show a very slight increase. Only during the last days a 20% increment in gene expression level does not follows the cellular activity trend.

Discussion

CD166 expression is the later confirmation of the change in phenotype of the hMSCs when cultured on Biocorals. The slight increase between days 18 and 21 could indicate that a pool of cells are proliferating and their expression of CD166 is revealed while as cellular activity the differentiation is predominant.

Collagene type I, alpha 1 (COLIA1)



Graph 3.12: COLIA1 profile of expression and cellular activity on Biocoral.

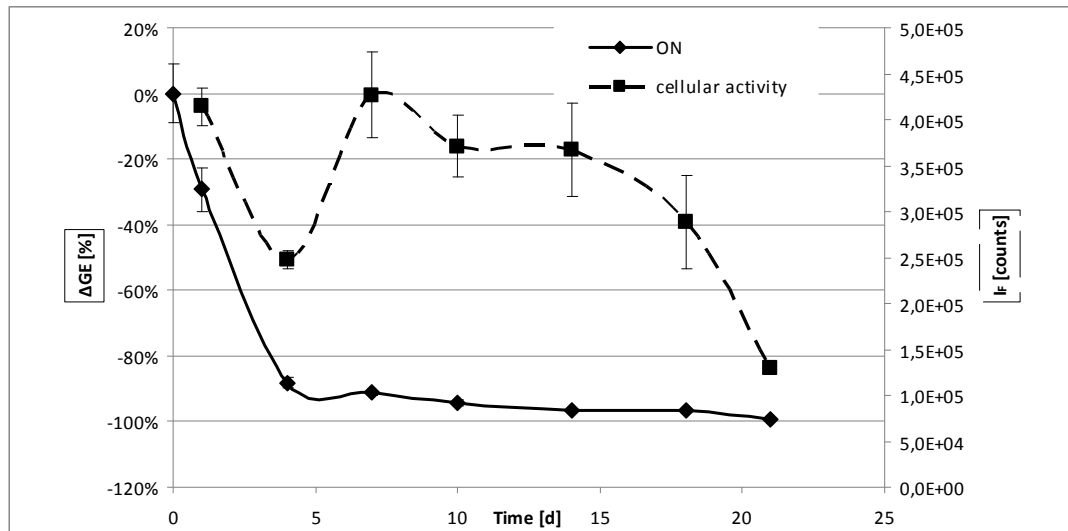
COLIA1 expression increase during the first day of hMSCs in 3D culture on Biocoral and remains at higher level than 2D cell culture during the first three days. Then, its expression decreases during the cell culture remaining at low level of expression. A slight increase is observed when cellular activity trend is towards the proliferation, after day 5.

Discussion

COLIA1 is expressed at very high level during the first establishment of the cell culture within the Biocorals being much higher than in the hMSCs cultured in 2D.

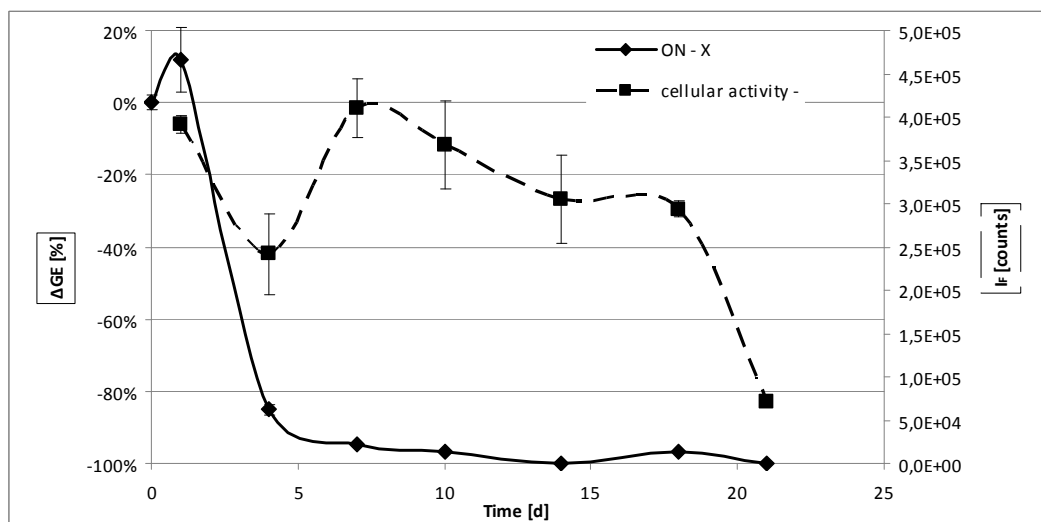
Collagen is the first produced protein in the ECM and the results of this experiment are consistent with this behaviour. It is plausible that Biocoral immediately and strongly induce the collagen production that will be then mineralized by other non-collagenous proteins.

Osteonectine (ON)



Graph 3.13: ON profile of expression and cellular activity on Biocoral.

ON mean expression trend (mean between X, Y and Z series on Biocorals) is always decreasing over time. However, if only scaffold X gene expression trend is considered (graph 3.14) there is a slight increment of expression in the first day of culture.



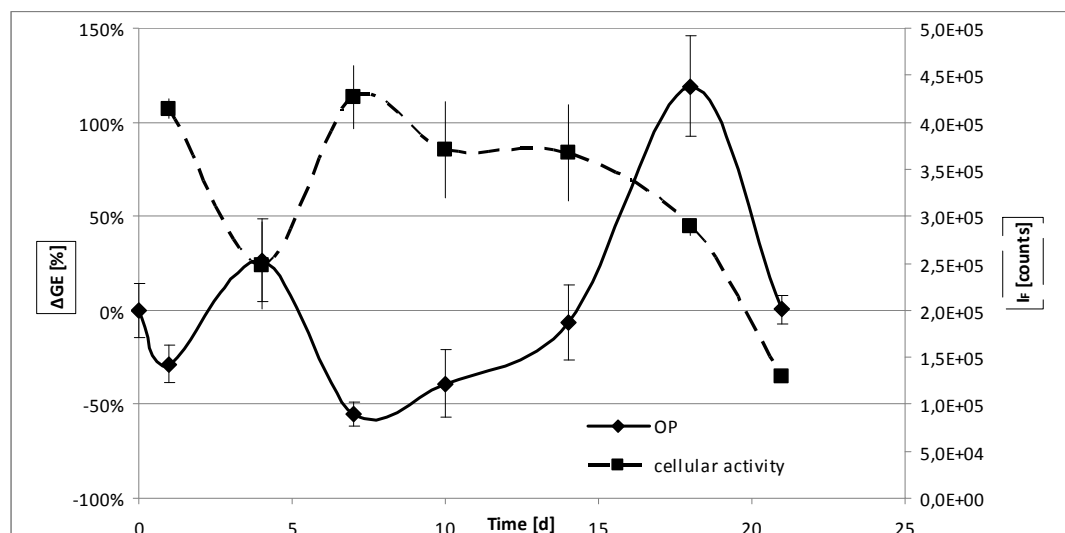
Graph 3.14: ON profile of expression and cellular activity on Biocoral X.

Discussion

The behaviour of ON in cells cultured on scaffold X indicate a rapid induction of ON production simultaneously with collagen, confirming the role of ON in starting the mineralization process and connecting the collagen to the mineral matrix.

However, the mean curve is not showing any increment of ON production indicating a different scaffolds induce different behaviour of cells.

Osteopontine (OP)

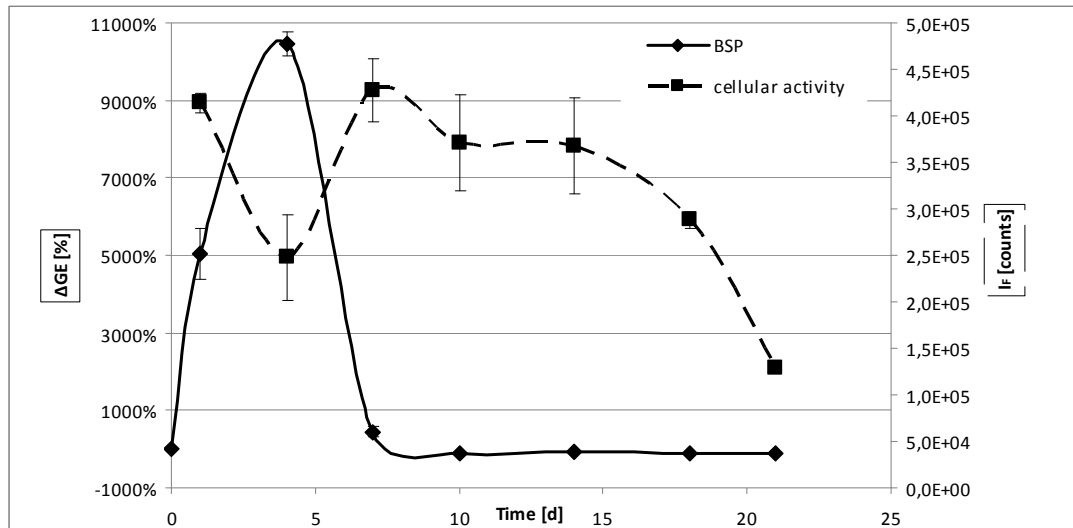


Graph 3.15: OP profile of expression and cellular activity on Biocoral.

The differentiation gene OP shows a fluctuating expression over time: it is lower than 2D cell culture at the beginning of the 3D culture, then it is higher when cellular activity is differentiation and later it decreases from day 4 to day 7 when cells proliferate. Then, again it increases going to day 7 to day 18 while cells differentiate and finally decreases. This last decrease is only visible in

scaffold z. In fact, in X and Y (data not shown) the trend is increasing from day 7 to day 21 while cellular activity is always towards differentiation.

Bone sialoprotein (BSP)



Graph 3.16: BSP profile of expression and cellular activity on Biocoral.

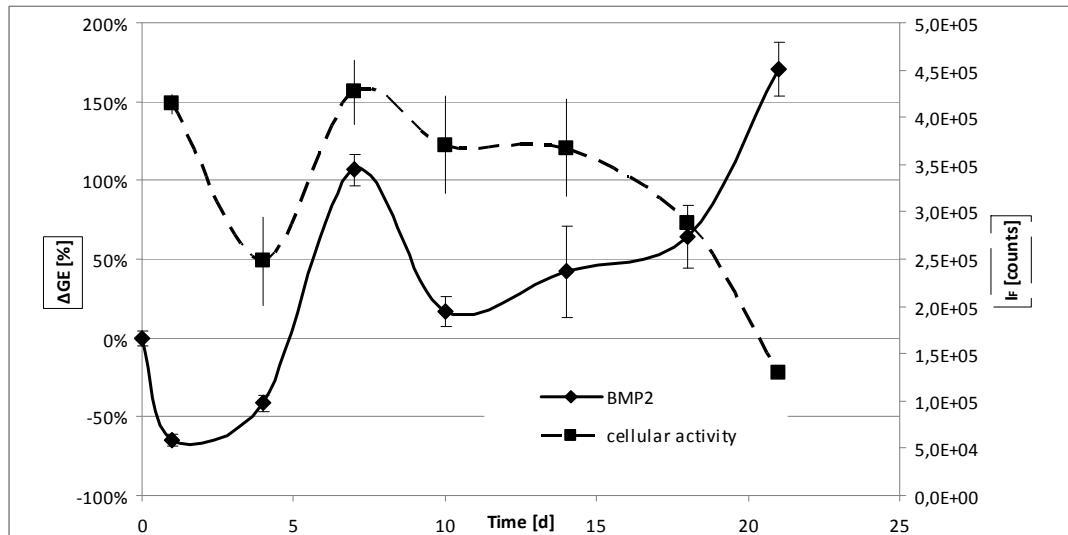
The expression levels of BSP are very high at the beginning of the 3D culture, when cellular activity trend is towards differentiation decreases and became lower when cells proliferation increases. Thus the change in cellular activity is correlated with a decrease of BSP production. After day 7 the production of BSP is reduced to the same 2D cell culture levels. Cells do not produce BSP during the late culture on Biocorals.

Discussion

The correlation between BSP gene expression and cellular activity is very evident in the first period of the culture: when cells differentiate they produce BSP, when cell proliferate the production rapidly decreases. BSP expression starts just shortly after the collagen production has reached the maximum level. This is

consistent with the role of BSP in the formation of mineral crystals of the ECM after collagen deposition.

Bone morphogenic protein 2 (BMP2)



Graph 3.17: BMP2 profile of expression and cellular activity on Biocoral.

BMP2 expression level is higher than in the 2D cell culture only after day 5 when its expression start to follows the cellular activity. It reaches a maximum at day 21, in late stage of differentiation.

Discussion

BMP2 is involved in osteogenesis and bone remodelling. This could be consistent with data of gene expression: the maximum of expression in this experiment is reached at day 21 when cells are in a late differentiation and the scaffold undergo a process of consumption: its edges appear eroded. This behaviour could be an attempt of remodelling by cells differentiating into osteoclasts.

General discussion

To recapitulate, for this study a set of gene expression markers have been chosen: five stemness markers to assess the gene expression of proliferation and stemness-related genes: CD29, CD44, CD90, CD105 and CD166; five osteogenic differentiation markers to evaluate the expression of differentiation-related genes: early differentiation markers Collagen- α type I, Osteonectin and BMP and late differentiation markers Osteopontin and Osteocalcin. The gene expression has been evaluated on 21 scaffolds at 7 time points, on three scaffolds for each time point. Just before each gene expression analysis, a cellular activity assay has been performed on each Biocoral scaffold. Hence, for each scaffold, data on cellular activity and the corresponding data on gene expression have been collected.

It is very well addressed by Lian and Stain in a 1992 review [61], there exists a temporal gene expression during the development of bone cells phenotype *in vivo* and in *in vitro* cultures. Even if the temporal sequence not always coincides between *in vivo* and *in vitro*, because it is influenced by several signals *in vivo* which are not present in *in vitro* conditions, there is a sequence of three principal events always occurring to allow a normal development of cell bone phenotype: 1) proliferation, 2) extracellular matrix maturation and 3) mineralization.

These three periods are characterized by two restriction points to which the cells cannot proceed further without gene regulation and cells signalling pathways: the first is when proliferation is down regulated and this implies that gene expression associated to extracellular matrix maturation is induced and the second is when mineralization occurs. Lian and Stain also claim that a great number of evidences confirm a functional relationship between proliferation and differentiation: in particular, the decrease of proliferation activity involves the subsequent induction of genes associated with extracellular matrix maturation and

mineralization that involves the differentiation induction. When cells decrease their proliferation, they start to produce extracellular matrix and to develop an osteoblast phenotype. This process is normally highly regulated: in transformed osteoblasts and in osteosarcoma cells (tumour cells), the loss of proliferation/differentiation relationship is associated with the loss of cell growth controls and the abnormal development of tissue-specific structure and function.

Summarizing, there is a complex regulatory mechanism associated to a signalling pathway that controls the relationship between osteoblast proliferation and differentiation.

The following two figures represent the temporal gene expression sequence described by Lian et Stain. It very clearly shows the presence of three phases with relative gene expression over a 40 days rat osteoblasts cell culture.

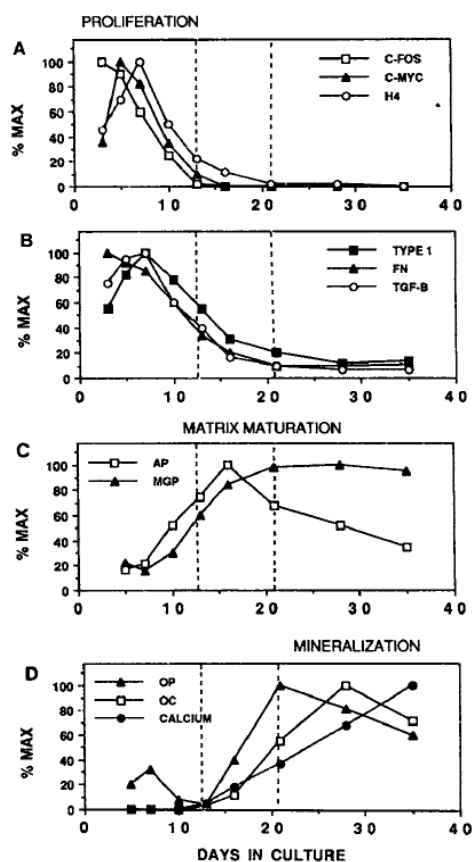


Figure 3.17: Temporal expression of cell growth and osteoblast phenotype related genes during the development of *in vitro* formed bone-like tissue by normal diploid rat osteoblasts. Represented are (A) cell growth-related genes H4 histone (reflects DNA synthesis), *c-myc* and *c-fos*; (B) extracellular matrix-associated genes type I collagen, fibronectin (FN) and TGF- β expressed during the proliferative period; (C) osteoblast phenotype-related genes associated with extracellular matrix maturation are alkaline phosphatase (AP) and MGP; (D) genes induced to high levels with extracellular matrix mineralization represented are osteopontin (OP) and

osteocalcin (OC) along with calcium accumulation. The vertical dotted lines separate periods of maximal expression of cell.

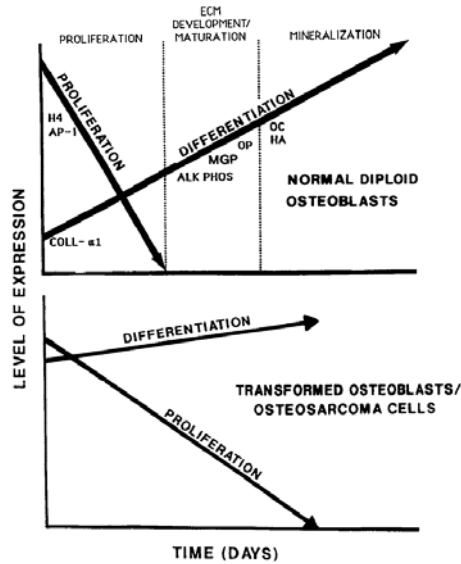


Figure 3.18: Model of the reciprocal relationship between proliferation and differentiation in normal diploid cells during the rat osteoblast developmental sequence and in osteosarcoma (transformed) cells, described by Lian and Stain. These relationships are schematically illustrated as arrows representing changes in expression of cell cycle- and cell growth-regulated genes (proliferation arrow) and genes associated with the maturation (differentiation arrow) of the osteoblast phenotype as the extracellular

matrix develops and mineralizes in normal diploid cell cultures (top panel). Here, the three principal periods of the osteoblast developmental sequence are designated within broken vertical lines (proliferation, matrix development and maturation, and mineralization). These broken lines indicate the two experimentally established principal transition points in the developmental sequence exhibited by normal diploid osteoblast during the progressive acquisition of the bone cell phenotype: the first at the completion of proliferation when genes associated with matrix development and maturation are up-regulated, and the second at the onset of extracellular matrix mineralization. The lower panel schematically illustrates the deregulation of the relationship between growth and differentiation in transformed osteoblasts or osteosarcoma cells. The proliferation vector reflects the continuous expression of the cell growth and expression of cell cycle- and proliferation-related genes. In contrast to normal diploid cells, the constitutive expression of osteoblast differentiation phenotype markers in transformed cells reflects the absence of the two developmentally important transition points observed in normal diploid cells. In osteosarcoma cells, cell growth and tissue-specific gene expression occur concomitantly; thus the relationship between growth and differentiation is deregulated. AP-1 = AP-1 binding activity; H4 = histone; COL-I = type $\alpha 1$ collagen; FN = fibronectin; ALK PHOS = alkaline phosphatase; MGP = matrix Gla protein; OP = osteopontin; OC = osteocalcin; HA = total accumulated

hydroxyapatite. (From G. S. Stein, J. B. Lian, and T. A. Owen : *FASEB J.* 4:311 – 3123 (1990).)

During the proliferation phase stemness gene, such as C-Myc, reach the maximum expression levels; the differentiation begins during final part of proliferation phase when the extracellular matrix production is coming with the expression of extracellular matrix-associated genes such as collagen type I (coll- α 1); finally the differentiation continues during the mineralization phase resulting in the expression of matrix mineralization-related genes such as osteopontin (OP) and osteocalcin (OC) along with calcium accumulation.

In an *in vitro* cell culture of hMSCs, the relationship between cell proliferation and cell differentiation (in general but also in particular into the osteoblast lineage) is influenced by signalling but is also space-dependent. The space limitation in 2D or 3D cell culture system implies a sort of “mechanical” signal (the so called “contact inhibition”) that stimulate the cells to stop proliferation (there is no more space to growth!) and to change their metabolism towards the differentiation.

Taking together the results from cell proliferation and gene expression analysis and the discussion about relationship between proliferation and differentiation, several interesting comment can be made on results obtained from this study.

Under the hypothesis that Biocoral scaffold induce cell osteogenic differentiation and considering that resazurin reacts differently with proliferating cells and differentiating cells, it is possible to formulate a second hypothesis regarding the cell proliferation results.

Results of R/R assay on Biocoral show cells appear to and increase decrease their proliferation rate over time during the 21 days culture within the scaffolds. This fluctuating trend reflects the colonization that cells are performing within the scaffolds. New regions of the scaffold, day by day, are occupied by cells and the cellular activity is heavily influenced by those colonization: when cells reach new

regions with empty pores, they have space to adhere and to proliferate by subsequent cell divisions. When the free space ends, cells stop proliferating and start to differentiate. In each pore of the scaffold, cells create a microenvironment and reach the complete colonization at different times in different pores. Hence, the state of the entire scaffold in terms of proliferation/differentiation continuously evolves over the time until all the pores are colonized by cells and all the cell are differentiating.

Cells that are differentiating, produce an ECM in which step by step they embed themselves and mineralize it to form new bone.

If the measurement is done in a proliferating phase, in which most cells have space to growth, the result is a high fluorescence value. If the measurement is done in a differentiation phase, when only few cells are proliferating because the most part of them stopped to grow and embedded themselves in the matrix, the fluorescence intensity value decrease because cells are not easily accessible to the resazurin solution. They are embedded in a mineralized matrix and are not able to reduce the resazurin as well as they do with free cellular surface not embedded in the matrix.

Collegen type I α 1 (COLIA1)

COLIA1 is the principal structural component of the bone ECM and is very early expressed during bone formation being the base for the following mineralization [9]. In *in vitro* cell culture, it is initially synthesized the proliferation phase and is accumulated during the culture period. The collagen is responsible for collagenous matrix formation and contributes to changes in cell structure, osteoblast differentiation and gene expression reflecting the cellular differentiation. Then, with the increase of collagen production, cells slow till to cease the proliferation at a low cell number [61].

Results of this study on COLIA1 expression reflect the high level of production at the beginning of cell proliferation phase and the continuative production with a slight accumulation during the culture. Cells use the collagen firstly produced to mineralize it and to proceed with the differentiation during the culture.

Osteonectine (ON)

The osteonectin is a glycoprotein linker between the collagen and the mineral part of bone matrix [62]. Is thought to have a key role in determining the onset of mineralization of bone matrix; high concentrations of calcium ion increase its expression as well as that of other markers terminals of osteoblasts, favouring the deposition of matrix mineral. It is considered a valid osteoblastic markers. In addition, it is also involved in cell spreading [61].

Nefussi et al in 1997 [63] have found that ON expression is evident during the first hours of *in vitro* rat osteoblasts cell culture.

Hence, its expression similar in pattern and time concomitance with COLIA1 in all the scaffold series, X, Y and Z is quite interesting and confirm its role in helping the cell spreading and in linking the collagen with the mineral bone matrix. It is possible to hypothesize that within the Biocorals during the proliferation phase, cells produce collagen, start the mineralization process and simultaneously produce ON to connect collagen to the mineral matrix.

Bone sialoprotein (BSP)

The bone sialoprotein is a non-collagenous protein of the ECM involved in the formation of hydroxyapatite crystals of the matrix during mineralization in *in vitro* and *in vivo* [64].

Its pattern of expression in scaffolds confirm its presence during the first two weeks of cell culture having a peak in the middle of first week, just shortly after

the collagen production has reached the maximum level. This is consistent with the role in the formation of mineral crystals of the ECM.

Osteopontin (OP)

The osteopontin (OP) is a sialoprotein that is deemed to have the biological function of allowing the adhesion of cells to the bone matrix and in controlling bone resorption [21]; reduced levels of mRNA in cells of OP stem cells derived from bone marrow (BMSC) are related to the underproduction of bone, such as osteoporosis [22]. High levels in the proliferation phase, low proliferation in the post and then increase again during the mineralization [61].

The reproducibility of these data on scaffolds series X, Y and Z in terms of cellular activity proliferation/differentiation, gene expression levels, temporal sequence of gene expression with a precise correspondence on days is very high and quite impressive. In addition, if considering scaffolds internal structure can influence the cell behaviour and Biocoral are natural scaffolds, hence affected by natural variability, reproducibility of these results is very strong. The uncertainty associated to the cellular activity analysis method (R/R method) allow to consider the trend reliable.

Conclusions

The present thesis is one of the first metrological approaches to cell biology and regenerative medicine aiming to fill the lack of traceability in metrology of biosciences for cellular analysis.

The first issue to face when metrology and uncertainty evaluation need to be applied to living organism, i.e. cells, is the definition of the measurand. This thesis lay the foundations for the definitions of metrological concepts starting from the measurand definition, influence parameters of uncertainty, moving to the selection of one specific methodology, among several with different characteristics, to be analyzed from a metrological point of view, reasoning about the best approach in order to consider living entities that change their nature over time, i.e. differentiating cells, and finally coming to the definition of an uncertainty budget to be applied in different situations to reach standardization among laboratories and in the specific field of the health care.

A method to declare an uncertainty budget for measuring the cellular metabolic activity according to the EURACHEM/CITAC Guide *Quantifying Uncertainty in Analytical Measurement* has been defined for R/R assay methodology. For the first time the methodology was tailored for 3D cell cultures with a metrological approach. 3D cell cultures are fundamental for regenerative medicine research and application on patients and require non invasive methods to be tested in terms of cell proliferation and differentiation over time.

It was demonstrated that Biocoral induces osteodifferentiation of hMSCs and that the R/R assay can be a methodology to study the cellular proliferation and differentiation activities on Biocoral because it well indicates the dynamic balance between the two cellular activities and with the uncertainty budget evaluated in

the thesis, results of the correlation can be stated as accurate and highly reproducible, even if the entire process leading to the results is quite complex and variability is inherent into the sample itself .

Results on 42 different sample of 3D cell culture on Biocoral scaffolds have shown a very impressive level of reproducibility in terms of cellular activity proliferation/differentiation, gene expression levels, temporal sequence of gene expression with a precise correspondence between gene expression and cellular activity. In addition, the uncertainty associated to the cellular activity analysis method (R/R assay) allow to consider the results even more reliable.

Taking together these results, it is highly likely to foresee a broad employment of the metrological approach to other biological assays testing cellular activity or cellular properties not stable over time and difficult to define.

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