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STUDY OF THE MOLECULAR PATTERNING IN STEM CELLS EXPOSED TO BIOPHYSICAL STIMULI: A NEW VISION OF REGENERATIVE MEDICINE

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Έτσι, δεν γνωρίζω

(Ἀπολογία Σωκράτους,VI- Πλάτων 399 BC)

Summary

Abstract

Regenerative medicine is a novel applied field of medical management based on the application of stem cell technology and tissue engineering in order to replace or regenerate human tissues and organs and restore their functions. The regenerative medicine has the prospective to help scientists and clinicians while planning early-intervention treatments for traumatic injury or degenerative diseases, by regrowth or replacement of cells or tissues.

However, the challenges in this field are still countless, as (1) which type of cells are suitable (2) which is the best protocol to commit all the stem cells toward a specific phenotype with the 100% of yield, (3) drop cell manipulation.

At the same time, physical energy were found capable to interact with cell physiology and increase their performance. In the clinical practice physical therapies enhance the quality and time of spontaneous tissues regeneration.

Here, I focused on the effect of physical stimuli by Radio electric asymmetric conveyor (REAC) and Extra corporeal shock wave (SW) and their interaction with cells or stem cell behaviour.

In particular, during the modulation of the main molecular patterning controlling cellular senescence, differentiation toward specific phenotypes and proliferation, in order to figure out the targets of action, to enhance their performance for future therapeutic applications.

Introduction

Regenerative medicine

Historical background

Regenerative medicine is an applied field of medical treatment which is based on the application of stem cell technology and tissue engineering in order to replace or regenerate human tissues and organs and restore their functions [***1***].The term "regenerative medicine" was coined by William Haseltine in 1999 during a conference on Como Lake, describing an emerging field, deriving from different subjects: tissue engineering (TE), cell transplantation, stem cell biology, biomechanics prosthetics, nanotechnology, biochemistry [***2***]. Historically, this term was found for the first time in a 1992 paper by Leland Kaiser, who listed the technologies which would impact the future of hospitals [***3***].

The regenerative medicine has the potential to help scientists and clinicians while planning early-intervention treatments for traumatic injury or degenerative diseases, by regrowth or replacement of cells or tissues. This branch of medicine is highly cross-disciplinary and serves as a bridge between basic science and clinical medicine, however the idea of the production of perfect tissues and organs in a dish is still a big challenge. Several studies and approaches were applied to follow the regeneration of tissues and organs, like growth factors in the damaged site, in order to stimulate the cells to regenerate the tissue, or alternatively the development of biomaterials for tissue engineering, as biomimetic polymers and bioactive three-dimensional scaffolds, capable of inducing specific cellular responses and direct the formation of new tissues to be implanted in vivo.

But, the idea of human regeneration potential was already known in ancient times since the old Greek culture, as demonstrated by the myth of Prometheus, whose liver was eaten by an eagle during the day and it completely regenerated itself overnight.

In Nature, the regeneration of body parts is rather common in reptiles, it is known that the salamander can regenerate an amputated limb in several days, or humans have this "ability" as well to regenerate fingertip but they lose it over the years[1]. Actually the absolute and applicable solution for the regeneration of tissues and organs is really long and it upon small steps that will allow the achievement of amazing discoveries.

In 1954 the transplantation of the first kidney substituted in a human between identical twins, in 1967 it happened the first heart transplant by Christiaan Bernand but without success, the patient pasted away after 18 days. However, a lot of programs directed on organ transplantation started during these years, paving the way to the idea that transplantation of organs and tissues was not absolutely an utopia, but a relevant solution to save patients.

In 1968 the first immune deficient patient with the sibling's bone marrow[2] was successful transplanted. Currently, the organ transplant is widespread and practice which deeply altered medicine, improving the patients' life expectancy.

However, finding compatible donors is not easy and due to the progressively aging population, transplantations will progressively represent a need to replace end-stage diseased organs injured by age-related diseases. That reason encouraged the researchers to direct their force to find a parallel solution besides the organ transplantation and regeneration of damaged tissues.

Relatively at the same time, in 1978 stem cells were isolated for the first time in human cord blood [3], opening a novel chance for patients with leukemia and anemia.

In 1981 for the first time a stem cell line (embryonic) from mice was cultivated in vitro[4]. That pioneering works, paved the way to the world of regenerative medicine and its application with the principal aim to find a solution to organs replacements from donors. The first "product" of application of cells for regenerative medicine was published in 1981 by Burk which using cells seeded on biomaterial composed by collagen, created the first "acceptable bilayer artificial skin" for the treatment of extensive burn injury. Moreover in 1998, Thompson isolated the first human embryonic stem cells from blastocysts showing the ability of these cells to differentiate in the three germs layers[5]. In 1999, in a paper published on the Lancet, the implantation of a laboratory-grown bladder implanted in a patient suffering from myelomengicocele [6] using as scaffolds homologous decellularised bladder submucosa with muscle cells isolated from the same patient, was described.

In 2004, the group directed by Raya-Rivera performed the first study on reconstruction and implantation of urine in five boys, showing that already after 3 months after implantation the engineered grafts developed a normal architecture with normal physiological functions in patients[7], strongly supporting tissue engineering as an effective solution for recovering lost physiological conditions.

In 2006, Takahashi and Yamanaka announced the discovery of the ~~a~~ induction of pluripotency in adult unipotent fribroblast from mouse, recovering an embryonic-like state by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4. The IPS (induced pluripotent stem) exhibit the morphology and growth properties of ES cells and express ES cell marker genes, absolutely

a revolutionary discovery for the applied research in regenerative medicine, introducing the concept of reprogramming, otherwise the possibility to set stem cells back to the embryonic state, by using a few defined factors.

Unlimited studies regard the approaches and methodologies applied to regenerative medicine have been made, like the suitable types of cells to be used, how to isolate and manipulate them, how to obtain the organized tissues, which materials are safe for the patient, which is the best material on for seeding cells, which cells are ethically feasible, a number of questions that still do not have a clear and dogmatic answer. However, in regenerative medicine many questions are still endless.

Strategies used in regenerative medicine

There are different strategies used in regenerative medicine

1. Cell-based therapy

2. Use of materials able to increase repairing processes, cell growth and migration

3. 3D printing cells

4. Use of biophysical stimuli on cells or tissue

1.Cell-based therapy

The multicellular organisms are organized by several types of cells specialized in particular functions, originate from a single cell called "zygote"; during development, all the cells differentiate progressively and acquire specific phenotype, losing their capability to

differentiate into other cells. The ability to differentiate into other cell types is defined as “cell potency or plasticity” and is typical particular cells called “stem cells”.

For this reason, stem cells represent the main tool in regenerative medicine due to their extensive ability to self-renew and to generate differentiated progeny.

Stem cells (SC) can divide and differentiate into different specialized cell types and can self-renew to produce more stem cells. They are capable to develop into organized tissues in the body during early life, and to handle tissue growth and homeostasis during the all life. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of specialized and unipotent cell. In particular, stem cells can be identified by special properties that characterized them, as self-renewal, unspecialized nature and differentiation. They can replicate many times, or proliferate. This particular type of division is called “asymmetric division[8].

Classification of Stem cells

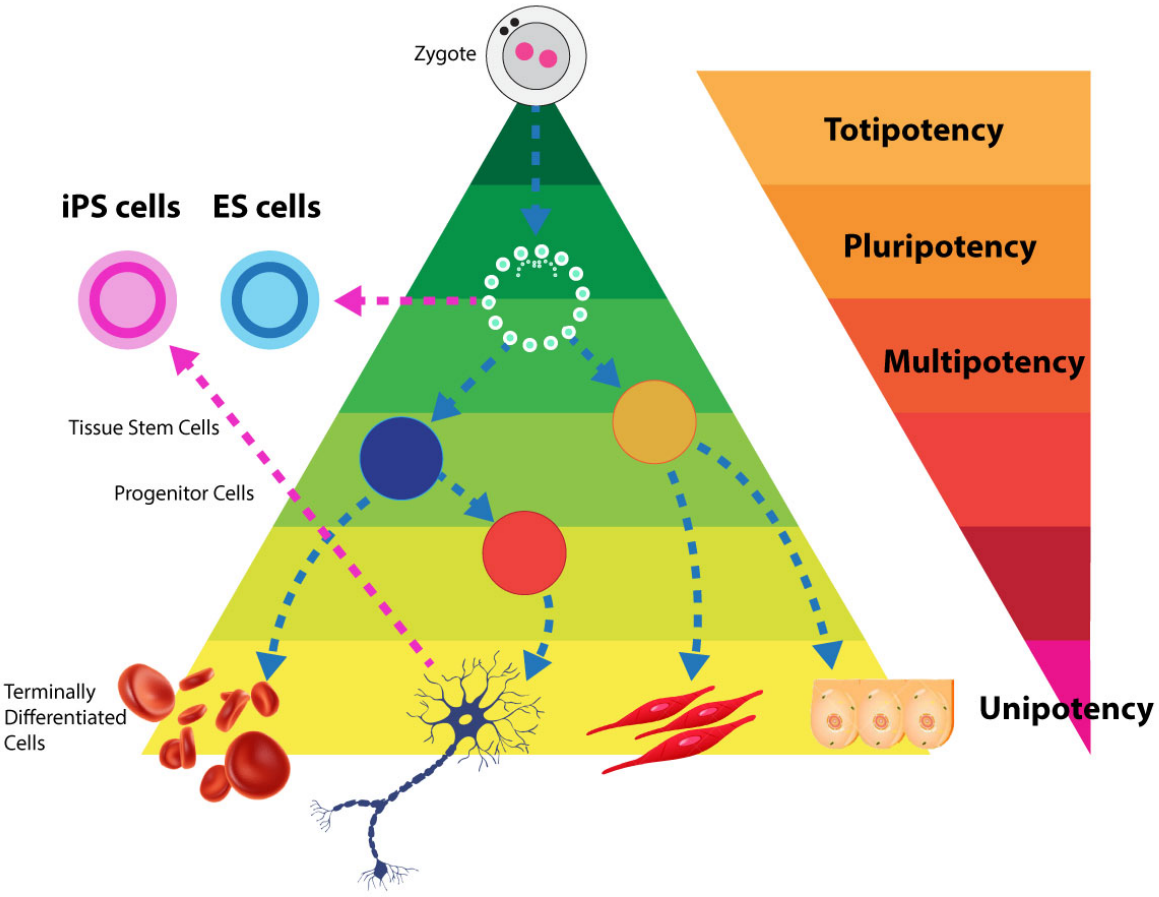

Fig.1 Hierarchic representation of stem cell organization

Classification based on potency

We could classify the different stem cells basically on the ability to differentiate into other cell types. Etymologically, the potency is taken from the Latin term "potens" which means "having power", due to this physiological property, the stem cells are the main actors in the theatre of regenerative medicine.

- **Totipotent stem cells** can differentiate into embryonic and extraembryonic cell types. These cells are produced from the fusion of an egg and sperm cell and develop into a

cell called zygous. The totipotent stem cells give rise to somatic stem/progenitor cells and primitive germ-line stem cells[9]

- **Pluripotent stem cells** are the descendants of totipotent cells and can differentiate into nearly all cells derived from any of the three germ layers, but not in foetal annexes. These pluripotent cells are characterized by self-renewal and a differentiation potential for all the cell types of the adult organism[10].
- **Multipotent stem cells** cells can differentiate into a number of cells, but only those of a closely related family of cells. These are true stem cells but can only differentiate into a limited number of types. For example, the bone marrow contains multipotent stem cells that give rise to all the cells of the blood but not to other types of cells. Adult Haematopoeitic Stem Cells are multipotent as well the Adipose tissue is a source of multipotent stem cells.
- **Oligopotent stem cells** can differentiate into only a few kind of cells, such as lymphoid or myeloid stem cells. For examples the corneal epithelium is a squamous epithelium that is constantly renewing, because is constantly exposed to damages, and is Oligopotent [11].
- **Unipotent cells** can produce only one cell type, their own, but have the property of self-renewal, which distinguishes them from non-stem cells. Most epithelial tissues self-renew throughout adult life due to the presence of unipotent progenitor cells[12].

It is even possible, classify stem cell by their origin:

Types of stem cells

The embryonic stem (ES) derived from embryos at an early developmental stage (day 5–8, called blastocysts) after implantation [5], are considered as totipotent [13] because they have the ability to differentiate into all cell types in the body give rise to cells of all the three embryonic germ layers like ectoderm, mesoderm and endoderm, even after being grown in culture for a long time.

The human ES cell lines (SSEA)–3, SSEA-4, TRA-l-60, TRA-1- 81, and alkaline phosphatase expressed cell surface markers, and are able to produce teratomas after injection [5], this is even an important parameter for testing the potency status. It is known that ESs express high levels of telomerase activity, a ribonucleoprotein involved in maintaining telomere length, which plays an important role in replicative life-span and senescence.

The ES pre-implantation epiblast have an active epigenetic regulation program, which is essential for maintaining pluripotency. They define the cellular epigenetic mechanisms that produce persistent effects in the biological system, without, however, altering the genome sequence. The epigenetic hence enables the modulation of the expression of DNA or structures associated with it by means of special processes, such as DNA methylation and the associated action of Methyl Binding Proteins (Mbds), the modification of histones with proteins belonging the Polycomb Group (PcG) and Trithorax Group (TrxG), the chromatin remodeling [14]. Specifically, the extra- and intra-cellular signals can stimulate the specific signal "propagators"inside the cells, which, can in turn activate defined pathways, producing

long lasting effects, although not altering the genomic sequence. The chromatin structure of mouse ES has been demonstrated to be hyperdynamic, with increased global levels of trimethylated lysine 9 H3 (TriMeK9 H3), a heterochromatic histone modification linked to gene repression, and decreased levels of acetylated histones H3 and H4 (AcH3 and AcH4), modifications linked to euchromatin and the permissively of gene expression. Analyses of global histone modification patterns in ESC has previously suggested that the ESC genome is subject to generalised histone acetylation and lysine 4 H3 methylation[15]. ESC have to act not only to silence such genes in order to maintain totipotency, but also to allow them to remain poised for transcription, so they can be rapidly activated upon the differentiation-induced silencing of NANOG, SOX2 and OCT4. These genes able to interact with epigenetic modifiers, including Polycomb Group (PcG), moreover it was recently discovered that ESCs have a high rate of methylation at CpG site level in Low density CpG promoters (LCPs), usually associated with tissue-specific gene expression. The regulation of these genes occurs upon methylation / demethylation of distal regulatory elements, enhancers or silencers, during the dynamic process of differentiation of ESCs in different phenotypes by the action of active DNA methyltransferases (DNMTs): DNMT1, which is largely responsible for the maintenance of DNA methylation over replication, and DNMT3A and DNMT3B, which generally perform de novo methylation of either unmethylated DNA or hemimethylated DNA to assist in maintenance[16].

However, the overexpression of the pluripotent pathway of Oct-3/4 a master regulator of lineage commitment with SOX2 and Nanog [13], combined with the absence in vitro of LIF (Leukemia Inhibitor Factor)[17] a member of the interleukin-6 family of cytokines that bind

to the gp130 receptor, by activating the Jak/Stat and Ras/MAPK signal transduction pathways of FGF-5 and BMP4 increase the differentiation toward the germinal layers.

Foetal Stem Cells

Foetal stem cells are primitive cell types found in the organs of foetus. The Foetal stem cells can be isolated from foetal blood and bone marrow as well as from other foetal tissues, including liver and kidney[18] The developing baby is referred to as a foetus from approximately 10 weeks of gestation. Most tissues in a foetus contain stem cells that are pluripotent and drive the rapid growth and development of the organs. Foetal blood is a rich source of haemopoietic stem cells, which proliferate more rapidly than those in cord blood or adult bone marrow[19]. Like adult stem cells, fetal stem cells are generally tissue-specific, and generate the mature cell types within the particular tissue or organ in which they are found.

The adult stem cells are Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from several sources such as bone marrow, adipose tissue[20][21], amniotic fluid[22], endometrium, dental pulp and ligament, umbilical cord, Wharton's jelly, epidermis, liver and intestine[23], most of these sources are considered wasting materials after clinical practice but a treasure for regenerative purposes. Adult stem cell are resident in specific zone of the body called "niche", highly dynamic, mainly implicated in the regeneration and homeostasis of the tissues, and have been best characterized in tissues that have a rapid rate of cell turnover.

The easily isolation, selection, characterization, expansion in vitro and the relative easily capability to differentiate into mesodermal lineage such as osteocytes, adipocytes and chondrocytes as well as ectodermal (neurocytes) and endodermal lineages (hepatocytes)[23] endorsed them to become a main candidate for regenerative medicine.

The International Society for Cellular Therapy has proposed minimum criteria to define MSCs, These cells should exhibits plastic adherence, possess specific set of cell surface markers like (CD)73, D90, CD105 and lack expression of CD14, CD34, CD45 characteristic for hematopoietic linages, as human leucocyte antigen-DR (HLA-DR) and must have the ability to differentiate in vitro into adipocyte, chondrocyte and osteoblast. Probably the best efficient population of MSCs has been reported with these features is from bone marrow[24], however the isolation process from the iliac crest is absolutely invasive and comprise several risks for the patient.

The adult stem cell are able to differentiate into adipocytes, osteocytes and chondrocytes, confirmed in vitro, by production of oil droplet, formation of mineralized matrices and expression of type II collagen.

Induced pluripotent Stem cells

The study of adult stem cells and mostly the embryonic stem cell, in term of transcriptomic and gene expression allowed the achievement a revolutionary discovery in the field of regenerative medicine, Takahashi and Yamanaka in 2006 announced the discovery of the combination of a “cocktail of genes” involved in cell reprogramming in adult fibroblast from mice[25].

Induced pluripotent Stem cells (iPSC) are created by inducing the expression of genes that are usually present in embryonic stem cells and that control cell functions. IPS cells are a powerful method for creating patient- and disease-specific cell lines for research and maybe for future application in regenerative medicine. These are not adult stem cells, but rather reprogrammed cells with pluripotent capabilities. Using genetic reprogramming [25][26] cells through the introduction of Oct3/4, Sox2, c-Myc, and Klf4[25], involved in maintaining cell pluripotency at that time by retrovirus[27], actually with episomes[28] or miRNA[29], pluripotent stem cells equivalent to embryonic stem cells have been derived from human adult tissues. A new frontier using this methodology pave the way to the optimization of the use of wasting biological material like urine [30] or from easily isolation material like frozen blood samples[31], opening a new avenue for suitable application. iPSCs are useful tools for drug development and modeling of diseases, and scientists hope to use them in transplantation medicine, however the capability to derive in all cells even to teratomas need to be carful[32]. iPSCs are derived from somatic cells, epigenetically reprogrammed to lose tissue-specific features and gain pluripotency. Similar to hESCs, they can theoretically differentiate into any type of cells[33]. The concept of induced pluripotent stem cells remains an important area of focus for future research and has serious implications for the stem cell cancer theory [32]. The minimal criteria to define a IPs is the expression of pluripotency-related factors, endogenous Oct3/4, Nanog, FoxD3, Rex1, Dnmt3b, and Abcg2[34] as well markers as the protein antigens CD9, Thy1 (CD90), tissue-nonspecific alkalinephosphatase (Tra-2-49 and Tra-2-54), class-1 human leukocyte antigen, and podocalyxin (GCTM2), the globoseries glycosphingolipid antigens stage-specific embryonic antigen (SSEA)-3 and SSEA-4, and the carbohydrate epitopes recognition by the monoclonal antibodies Tra-1-60 and Tra-1-

81[35][26]. IPS must be able to generate teratome in vivo, and develop all cell types. The creation of pluripotent stem cells from adult cells by the introduction of reprogramming transcription factors absolutely raised new hope for future applications like in the production of new disease models and in drug development as well as in transplantation medicine.

Cell in the body or cell in the dish?

The role of cell niche in regenerative medicine

The term 'niche' was first used by Schofield in 1978 to explain the variation in the self-renewal capability of an apparently pure populations of HSCs following transplantation in mice. He hypothesized that the capability of stem cells to self-renew and retain their identity depends on the environment provided by neighbouring, components of the niche, including direct interactions between stem cells and neighbouring cells, secreted factors, inflammation and scarring, extracellular matrix (ECM), physical parameters such as shear stress and tissue stiffness, and environmental signals such as hypoxia and ROS. The niche have been described in a variety of adult tissues, including skin[36], intestine[37] and nervous system[38].

In many adult tissues, the stem cell niche contains a variety of cell types, each with a distinct function. Communication between stem cells and niche cells is either direct, through physical interactions, or indirect, through secreted factors that mediate communication between cells that are not in direct contact by cell-cell adhesion molecules and receptors with membrane-bound ligands [39]. Indirect communication between stem cells and niche cells is mediated by secreted factors. In the clinical practice this phenomenon is routinely exploited, like the

use of granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) to support treatment of haematological malignancy.

Although every stem cell niche is dynamic and exhibits cell turnover, it is useful to distinguish between niche cells that are 'permanent residents' and cells that occupy the niche transiently. Permanent residents include endothelial cells, nerve cells and fibroblasts, the transient ones include immune cells and cells that respond to tissue damage like pathogens or to promote healing.

Another fundamental component in the tissue and in the niche is the extra cellular matrix (ECM) which exhibit an important role, in fact the ECM not only anchors stem cells but also directs their fate[39], the most important ECM receptors are integrins, and their functions can be modulated by biochemical stimuli, such as antibodies, small- molecules as drugs or by biophysical stimuli[40]. The later carry out an important and fundamental role during cell fate and development, as demonstrated there is a systematic relationship between tissue mechanics and differentiation [41] thus modulating the stem cell niche. All these finding in term of regenerative medicine suggest that finding a methods able to interfere with the activation, modulation, fate, differentiation of the cells resident in the niche could open new hopes for the patients.

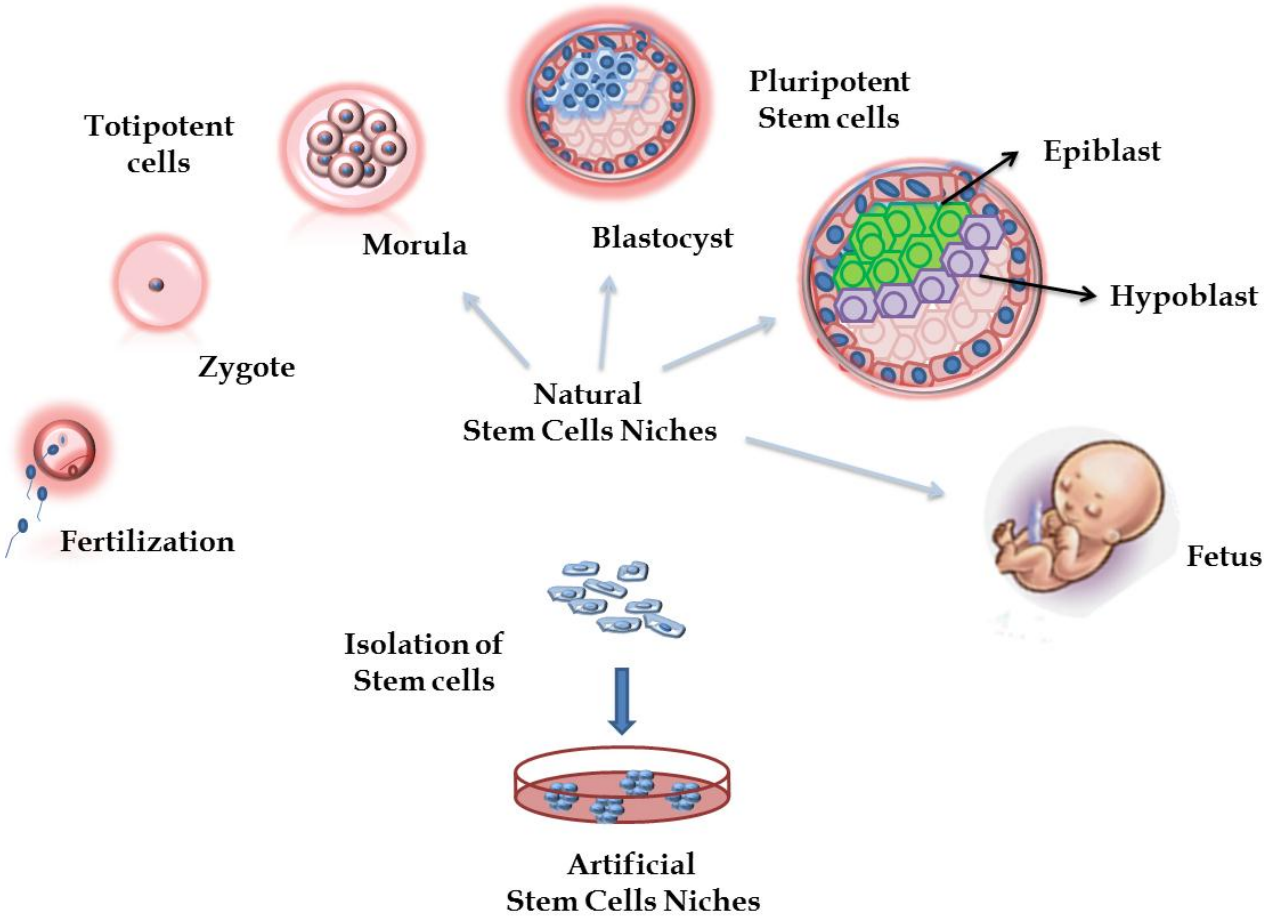

Fig.2 Sources of stem cells

Activation of stem cells and cells

Tissue injury is an inevitable part of life. It is becoming clear that following damage, stem cells switch to producing an excess of proliferating daughters until the wound is sealed, exhibiting high plasticity in their behaviour[42]. There are several studies reporting that stem cells populations respond to injury before turning to the activation of quiescent stem cells in tissues bearing a low turnover of cell proliferation. In fact, in tissues with minimal cell turnover, stem cells are actively maintained in a quiescent state and conditionally activated, , but it is well known and proved that paracrine signalling in the stem cells microenvironment, physical stimuli, hormones are able to switch this behaviour. For example, it was observed

that epidermal stem cells secreted both Wnt ligands and Wnt inhibitory Dkk proteins, resulting in the activation of Wnt target genes in basal cells, and the Wnt/β-catenin signaling is a central regulator of adult stem cells mostly in term proliferation[43]. Autocrine Wnt signalling is thus an essential part of the stem cell niche, allowing permission to cell proliferation [44], while the inhibition of Wnt signals induce cell cycle arrest [43]. Recent studies also demonstrated that stem cells niche is exposed to paracrine signals like the Wnt that mainly regulate the fate of the cells.

Other interesting studies consider the response and activation of the cell is correlated to pro-inflammatory cytokines release as IL-1α, IL-13, TNF-α, and IFN-γ, secreted by T cells in vivo on satellite cells[45]. So far, understanding how cells response to intrinsic factors, hormones, cytokine, cell-intrinsic physical stimuli is not already well understood thus elucidating these mechanism will give new insights in stem cell application in vivo.

In fact the cells, upon an injury or a paracrine signal, or a physical stimuli react by leaving their quiescent status and start to proliferate. The biological mechanism of proliferation is based on cell cycle activation.

Cell cycle

Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Cell cycle is a fine regulated physiological process that can be dived in different phases. Originally, the cell cycle was divides in two stages: mitosis the process of nuclear division and the interphase. But, clear evidence has shown that this process cannot be represented by only two phases. In fact, only the mitosis is composed by different stages that include prophase, metaphase, anaphase

and telophase, as well the interphase (probably the most complicated an delicate phase for the life of the cell) includes G1, S and G2 phases and then S phase, in a perfect and organized cycle [46].

S phase is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. G1, S, G2 and M phases are the traditional units of the standard cell cycle.

However, the cells can be in a not-cycle state state called G0, characterized by not growing, not proliferating cells. The multicellular organisms base their life on the perfect control and regulation of the unit that compose them, the cell. The evolution gives them a sophisticate system in order to continue their development, the organization in tissues, in organs, in the life. Regulation of cell cycle, maybe is one of the most important process for the life, and it cannot be regulated only by activation or deactivation of a gene or by translation in a protein; it must be controlled by a precise mechanism, like a Swiss watch. During cell cycle process, there are many actors, that can be dived in agonist and antagonist. In each phase, defined factors are finely coordinated. These are the cyclin-dependent kinases (CDK), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. Until now, five CDK, acting during the cell cycle are well known, as cCDK4, CDK6 and CDK2 during G1 CDK2 in S phase and CDK1 during the G2 and M.

CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins, whose levels can rise and fall during the cell cycle[47]. The different cyclins are expressed during the cell cycle phases, the three D type cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4 and to CDK6 in order to entry in G1, cyclin E is associates with CDK2

to regulate progression from G1 into S phase[48] and cyclin A bind CDK2 during S phase[49]. In late G2 and early M, cyclin A complexes with CDK1 promote entry into Mitosis is further regulated by cyclin B in complex with CDK1. The activation of the CDKs is based on phosphorylation that induce conformational changes and enhance the binding of cyclins[50].

CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors (CKI) which bind to CDK alone or to the CDK-cyclin complex and regulate CDK activity. Two distinct families of CDK inhibitors have been discovered, the INK4 family and Cip/Kip family[51]. The INK4 family includes p15 (INK4b), p16 (INK4a), p18(INK4c), p19 (INK4d), which specifically inactivate G1 CDK (CDK4 and CDK6). These CKI form stable complexes with the CDK enzyme before cyclin binding, preventing association with cyclin D[52].

The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1, Cip1), p27 (Cip2), p57 (Kip2). These inhibitors inactivate CDK-cyclin. However, p21 is a "famous" protein, that can also inhibits DNA synthesis by binding nuclear complexes. Every time that p21 is expressed, another impotant protein is correlated, p53 the "guardian" of the DNA integrity. In fact, the expression of p21 is under transcriptional control of the p53 tumour suppressor gene. The p21 gene promoter contains a p53-binding site, that allows p53 to transcriptionally activate the p21 gene[53].

During cell cycle, there are restriction point even called ckeck point, that control the commitment of the cell throw the phases. If the DNA for example is demaged, checkpoints arrest the cycle in order to provide time for DNA repair. Usually, DNA damage checkpoints are positioned before the cell enters S phase (G1-S checkpoint) or after DNA replication (G2-

M checkpoint). At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent, Usually, the level of p53 is low but DNA damage can induce upregulation[54].

Moreover, the cell cycle mechanism is finely regulated by an epigenetic control involved in regulation of the genes mentioned upon, by activation of chromatin remodelling complexes SWI/SNF (SWItch/Sucrose Non-Fermentable) [55], histones modification as methylation by methylases(HMTs) and demethylases(HDMTs)[56], acetylation histone acetyltransferases (HATs) and histone deacetylases (HDACs), phosforilation by histone kinases [57][58], control promoter areas (CpG island) by specific DNA metyl transferases (DNMTs)[59]. These last one, the DNMTs play a predominant role during the cell cycle control.

The DNA methyltransferases (DNMTs) are particular proteins that are involved in the establishment and maintainment of methylation in the fifth carbon of cytosine residues in DNA CG dinucleotides (CpG islands) patterns at specific regions in the genome for the regulation (contribute) of gene expression. Easily, we could consider them as "writers of epigenome". DNMT1 is implicated in maintenance of methylated status, while DNMT3a and DNMT3b are recruited for de novo methylation [60]. Recent observations, showed the relevant role of DNMTs during the behaviour of cells. In fact, as observed by Robertson and colleagues, the mRNA levels of all three DNMTs change during the cell cycle, with DNMT1 and DNMT3b decreasing in G1 as well DNMT3a. During G0 phase, conversely the cells expressed high mRNA level of DNMT3a, DNMT3b and DNMT1[61].

The regulation of cell cycle, with its fine and elegant machine represent perhaps, one of the most interesting challenge to understand cell biology and its applications and solutions.

Cell polarity

Adult somatic stem cells have a central role during the homeostasis in the tissues presenting a high cellular turnover like the skin, intestine, and the hematopoietic system. It is thought that polarity is particularly important with respect to fate decisions on stem cell division (symmetric or asymmetric) as well as for the maintenance of stem cell adhesion and quiescence (interaction with the niche). Embryonic and adult stem cells can use their polarity to generate cell diversity by asymmetric cell division, whereas differentiated cells use their polarity to execute specific functions, like epithelial cells that have apical and basolateral cortical domains to guarantee the barrier maintenance, or the fibroblasts that have actin-rich leading edge during cell migration, or the neurons with their axonal and dendritic portion are able to convey the signal[62].

Finding a concise and clear definition about polarity is not easy, however it represent a main characteristic of the behaviour of a cell. In fact, a cell can be defined polarized when the organelles, proteins, RNAs, inside are distributed and maintained in a asymmetrical organization[63]. The polarization can occur in response to extracellular stimuli that induce a redistribution of cellular components to comply a functional need during adhesion, migration, or cell division. During cell migration, the symmetric arrangement is broken, which may be accomplished by (1) repositioning the centrosome, (2) by adding an asymmetric microtubule nucleation site, (3) by reorganizing the microtubules network, or (4) by altering microtubules dynamics [64].The orientation of the polar axis in a cell can be determined by the shape of the cell, the direction of cell protrusions, the orientation of

microtubule and actin networks; for example during migration the protruding front, a retracting rear, and the cell polarity axis is oriented in a define direction[23].

Many recent studies have demonstrated that plasma membrane domains with specialized lipid commonly enriched in cholesterol are distributed asymmetrically in polarized cells. For example, during the migration in lymphocytes it was observed that the membrane-anchored cell surface receptors Inter-cellular adhesion molecule (ICAM) with lipid rafts, together with their respective signal transduction pathway regulate extravasation and crawling.

One obvious hypothesis currently supported by experimental evidence is that polarity establishment during mitosis regulates the mode/outcome (symmetric vs. asymmetric) of stem cell divisions and it is hypothesized that during the mitosis in stem cells, only one daughter cell remains in contacts with the niche, maintaining the ability to self-renew. In a review, Florian explained how Dpp and Hh, the bone-morphogenic protein (BMP)2/4 homo- released from the niche/hub cells are involved in regulating the mode of division and polarity of the cells[63]. It was also recently demonstrated that a planar cell polarity pathway is activated by Wnt receptor Fzd7 and that its candidate ligand Wnt7, controls the homeostatic level of satellite stem cells hence regulating the regenerative potential of muscle [65].

Even the cytokine release is correlated with cell division, by activation of receptors that activate a multitude of intracellular signalling cascades [66].

Recent data indicate a correlation between altered stem cell polarity and stem cell aging. The first pioneering studies were done on animal models, in aged drosophilae, shown a disoriented

centrosomes and thus altered polarity relative to their niche cells. This was correlated with a reduced self-renewal activity caused by aging[67], even in another model.

In yeast the same behaviour was linked to high activity of Cdc42[68]. In the human stem cells, the altered polarity is correlated with aging and with an alteration of cell mobilization, homing, engrafting, and lineage choice[69].

Methodology for stem cell differentiation

Concept of plasticity

Since year, scientists have postulated that some cells in the body appear to participate in the formation of new cells to replace injured, aged, or infected cells in tissues and organs. The cell population which constitutes an organ can be divided into three groups: (static) somatic cells, differentiated, produced during development, which have also lost the minimum proliferative capability, in a slow decay process during adult life; (Transit) cells that produce precursors of differentiated cells, that have a relatively short period of existence in the organ, and their life usually is determined by a suicide at the end of maturation process (apoptosis); (Stem) a type of cells present in a predefined microenvironment, with extensive proliferative capability and plasticity to differentiate.

The current knowledge about the factors that control the biology of a stem cell, or the self-renewal, the maintenance of the undifferentiated state and the ability to take symmetric or asymmetric divisions, are still rather limited. The molecular pathways of stem cells are controlled genetically and epigenetically, so depend on mechanisms able to turn on or off specific genes. The shift in the balance between stem cell and differentiation is influenced by

intrinsic and extrinsic factors that can induce stem cell differentiation or maintenance. Watt proposed that stem cells contribute to the maintenance of homoeostasis, with an average of 50% for the retention of progeny and 50% for the differentiation.

Since years researches in the regenerative field are trying to control in vitro the plasticity and differentiation of stem cells. The numbers of papers produced since the fist discovery of stem cells and their possible management and control in vitro in exponentially growing. It is known, that there are several ways to interact with the commitment and differentiation of the cells throw the different linages (mesoderm, ectoderm, endoderm), below will discuss the principals.

Chemical induction

Mesodermal linages

Differentiation of MSCs into adipocytes is induced by proper media supplementations, which activate transcription factors (genes) responsible for adipogenesis. or adipogenesis, MSCs were cultured in growth medium supplemented with dexamethasone, indomethacine, insulin and isobutyl methyl xanthine for 3 weeks, cells are then analysed by accumulation of lipid droplets and expression of adipocytes-specific genes as peroxisome proliferator- activated receptor γ (PPARγ), adipocyte protein 2(ap2) and lipoprotein lipase (LPL) genes. Adipogenic differentiation is characterized by two phases, during the first one, cells are committed toward pre-adipocytes showing a fibroblast-like morphology thus being difficult to be distinguished from the MSCs, during the second phase pre-adipocyte become mature adipocytes with lipid droplets while changing their morphology.

The most common methodology to differentiate MSCs into osteocytes is based on culturing the cells with ascorbic acid, β-glyceralphosphate and dexamethasone for 3 weeks in growth conditioned media; in a recent work[70] our group described that MSCs isolated from adult dental pulp hDPSCs and exposed to a mixture of hyaluronic, butyric, and retinoic acids (HA + BU + RA) in combination with melatonin exhibited the transcription of genes involved in osteogenesis, as VEGF A, which orchestrate osteogenesis trough by modulating both ZBTB16 and NR4A3 gene expression. The osteogenic differentiation of MSCs in vitro continue with an increase in the expression of osteogenic genes as runt-related transcription factor 2 (Runx2), osteonectin, bone morphogenic protein 2 (BMP2), mineral aggregation, exhibiting an increase in alkaline phosphatase activity at the end of differentiation.

Many investigators have reported that a correlation exists between adipogenesis and osteogenesis [71][72], in fact PPARγ, a key transcription factor implicated in adipogenesis, lipid metabolism, and glucose homeostasis, has shown to promote osteogenesis through enhanced osteoblast formation, by the pathways of Hedgehog, NEL-like protein 1 (NELL-1) [72] and β catenin- dependent Wnt[73][74], the latter being-a Wnt glycolipoproteins directly affecting cell proliferation, cell polarity and cell fate determination during embryonic development, tissue homeostasis, and during differentiation toward the ~~in~~ cardiogenic lineages.

According to the standard protocol for chondrogenesis, it is possible induce using insulin transferrin selenium, linoleic acid, selenious acid, pyruvate, ascorbate 2-phosphate, dexamethasone and transforming growth factor-β III (TGF-βIII) in culture medium. The commitment toward the chondrogenic phenotype resulted in the formation of pre-

chondrocytes which expressed type I and type II collagens, then the pre-chondrocytes differentiate into mature chondrocytes, expressing the typical transcription factors like Sox9, L-Sox5 and Sox6[75]andTGF-β1,the latter interacting with Wnt/β- catenin pathway inhibiting osteoblast differentiation while promoting chondro-differentiation.

MSCs, embryonic and iPS can even differentiate into other mesodermal lineages, the pioneer work by Wakatani on bone marrow derived MSCs from mouse showed the effect of 5-azacytidine cardiogenic commitment [76], moreover recent works show how it is possible to commit stem cells from amniotic fluid (hAFSCs) toward the cardiovascular phenotype in the presence of a mixture of hyaluronic (HA), butyric (BU), and retinoic (RA) acids[77] in human mesenchymal stem from placenta (FMhMSCs) and mouse embryonic stem (ES) cells[78] by an activation on Smad1/4 pathway. However, the most common protocol for cardiac commitment is based on the addition of specific growth factors important for cardiovascular development like fibroblast growth factor 2 (FGF2), transforming growth factor β (TGFβ) Activin A and BMP4, vascular endothelial growth factor (VEGF), and the Wnt inhibitor DKK-1[79].

Ectodermal linages

Despite the mesodermal origin, hMSCs displayed the capability to trans-differentiate into cells from ectodermal lineages. In fact it was demonstrated that trans-differentiation into neuronal cells can occur upon stem cells are exposed to a neural induction media supplemented with cocktails of growth factors, as the nerve growth factor (NGF, able to induce BMSC transdifferentiation into cholinergic phenotype[80], or the sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8 basic fibroblast growth factor (bFGF) , which

can induce neuronal plasticity and dopamine release [81]. Many other studies have shown that factors like insulin, retinoic acid, bFGF, EGF, valproic acid, BME and hydrocortisone support adipose derived stem cells neuronal differentiation [82][83], as well as glial cell line-derived neurotrophic growth factors (GNDF), brain-derived neurotrophic factors (BDNF), retinoic acid, 5-azacytidine, isobutyl- methylxanthine (IBMX) and indomethacin are able to enhance the MSCs differentiation into mature neuronal cells[84]. The dental derived MSCs even called dental pulp stem cells (DPSCs) which originate from neural crest, successfully differentiate into mature neuronal cells [85].

Even the embryonic stem cells as well the IPs are able to commit toward the neuronal linage, because both use the same transcriptional network to generate neuro- epithelia and functionally appropriate neuronal types over the same developmental time course in response to the same set of morphogens treatment as SHH and Wnts[86], or their agonists/antagonists [87].

Endoderm Linages

The differentiation of endodermal linages, is already a challenge for researches, however a lot of protocols and studies comes out during the last years. The discovery that even the MSCs have the capability to trans-differentiate-into hepatocytes and pancreatic cells upon induction with conditioned media supplemented with EGF, bFGF and nicotinamide followed by stimulation with dexamethasone insulin, transferrin, selenium [88]; In other studies, it was shown that valproic acid, which is histone deacetylase inhibitor, up-regulated the expression of hepatic marker through activation of protein kinaseB(AKT) and extracellular signal-regulated kinases (ERK)[89].

Physical energies induction

Recently, alongside the conventional methodologies largely studied by researchers using chemical inductors mentioned upon, new strategies were applied to commit specific cellular phenotypes. Since years, the scientific community, tried some how to reproduce the physiological behaviour of organ, tissue and organs, by physical devices, able to generate specific energies.

In 1974, the group direct by Nucitelli studied the endogenous ionic current and the electric field in multicellular animal tissues, aiming at proving that electric field was able to influence the physiological functions in living organism[90]. Many following studies supported these observations and many protocols were published. Magnetic fields, commonly showed to affect cell proliferation and growth factor expression [91]–[97]. Extremely low frequency (ELF)-PEMF applied to MSCs were able to commit the adipogenic, osteogenic, neural, and glial linage together with conditioned media [98], Moreover pulsed electromagnetic fields (PEMFs) were proved to have a regulatory role in bone marrow derived stem cells (BMSCs) and in adipose derived stem cells (ASCs) differentiation toward the osteogenic phenotype [99]. As well, PEMF of different intensities as 1, 2, and 5mT with a modulation frequency of 750 Hz, a carrier frequency 75Hz and a duty ratio of 0.8, 3 h/day for 4 weeks were able to optimize the process of in vitro endochondral ossification Other study showed how the 50 Hz, 1mT ELF-MFs 5-day exposure implemented NGF-induced PC12 cells neuronal differentiation [97]. [97]. Ventura's group demonstrated that the extremely low frequency (ELF) pulsed magnetic fields (PMF) affected opioid peptide gene expression and opioid dependent signalling pathways in adult ventricular myocytes [100]. Other devices able to

modulate the behaviour of the cells were projected, as the REAC (Radio electric asymmetric conveyor) which produce extremely low-intensity electromagnetic energy circuit, conveyed directely to the tissue or the cell culture, with a frequency of 2.4 GHz and power of only 2mW. It was observed that REAC elicited human embryonic mouse cells differentiation toward , cardiac, neuronal, and skeletal muscle lineages[101]. Even on Lipogems-derived hASCs the radioelectric asymmetric conveyer (REAC) remarkably enhanced the transcription of prodynorphin, GATA-4, Nkx-2.5, VEGF, HGF, vWF, neurogenin-1, and myoD, indicating the commitment toward cardiac, vascular, neuronal, and skeletal muscle lineages respectively[102]. The light therapy, light-emitting diode (LED) at 620 nm and 2 J/cm2 can modulate the fate of human umbilical cord mesenchymal stem cells (hUMSCs) cultured in osteogenic differentiation medium, in term of proliferation and increase of yield of osteogenic differentiation[103], Other studies showed that the modulation of genes involved in osteogengesis in vitro, depended on the LED length frequency used(680-nm, 760-nm and 830-nm)[104]. On human skin-derived fibroblast LED induced an increase in cell behaviour as, proliferation, viability, migration speed and reactive oxygen-species (ROS) generation[105].

The 532 nm green light LLLT performed with KTP laser at 4 J/cm2 influenced the osteoblastic differentiation of mesenchymal stromal cells [106].

However, the basic physics behind al of these instrument is almost the same: a modulation of an electromagnetic signal or a radiofrequency signal that can interact with the biological behaviour-of the cells.

On the other hand, the Extra Corporeal Shock Wave (ECSW or SW) technology, is based on another principle, the ultra sound and cavitation. ECSW have been clinically introduced for lithotripsy since the 1980s. At the beginning, the aim was to brock up the urinary stones by high-energy pulses[107], then several findings demonstrated that changing the modulation of energetic intensity, different effect on tissues can be observed. In particular , SW could affect wound healing with an increase of angiogenesis[108], resolution of tendinitis[109][110] and bone repair increase[111]. SW modulate the differentiation of hASCs toward myofibroblasts[112]

Problems with stem cells

Ethical problems

Scientists plan to differentiate pluripotent cells into specialized cells that could be used for transplantation. However, human stem cell (hSC) research also raises sharp ethical and political controversies because it involves the destruction of human embryos, in fact totipotent stem cell lines can be derived from the inner cell mass of the 5- to 7-d-old blastocyst[113][114]. On the other hand the new discovery by Yamanaka [26] of induced pluripotent stem cells (IPS) has paved the way to new hopes for regenerative medicine and clinical application, because are ethically unproblematic and acceptable to use in humans. Even though IPS cells still exhibit some problems that the researcher are trying to solve, like the possibly tumour formation, immunological reactions, unexpected behaviour of the cells, and unknown long-term health effects[115], based on retroviral integration into the host genome that can create spontaneous and directed differentiations in the expression pattern [116] that cannot really be controlled by researchers.

On the other hand, the MSCs are easier to isolate from many tissues[117], are able to differentiate into many cell types thus representing a starting point of many novel therapies, especially in tissue engineering.

Technical problems

Currently, MSC clinical applications requires the use of high cellular doses (up to several million cells/ patient body weight) together with efficient expansion protocols to generate a large number of cells based on traditional culture techniques[118], meaning culturing MSCs into plastic tissue flasks, a limited process in terms of cell productivity involving at least 2 to 3 cell passages to achieve a clinically relevant cell numbers in an acceptable period of time. On the other hand the effects of extended ex-vivo MSC cells obtained by consecutive cell passaging during long-term cultivation may lead to a senescent state of the cultured cells ultimately compromising clinical safety and efficacy[117]. It is known that after 7 passages BM-MSCs, the proliferative and clonogenic potential is seriously affected, as well as their proteome profile, like cell cycle regulation and apoptosis. [119].

Nevertheless, in practice, cell transplantation does have a number of limitations, adult stem cells or iPSCs, are expensive and need an intensive work, moreover they require specialized facilities for cell collection, expansion, quality control and transplantation as mentioned ATMP standards parameter[120].

Senescence in cells in vivo and in vitro

To maintain tissue homeostasis, stem cells have developed strictly regulatory mechanisms to self-renew, differentiate, and prevent premature senescence and apoptosis. Since years the

researchers observed a limitation of the normal cells unlimitedly proliferation in vitro, showing that human fibroblasts post-isolation stages in culture, at the beginning exhibit a strong proliferation rate, gradually decreasing during passages, and progressively losing the ability to divide[121][122]. That phenomena, at a later time, was called "senescence" by Haflick, who demonstrated that the mechanism was correlated to stressors forces causing the cellular senescence. These stressors factors include dysfunctional telomeres, genotoxic stresses/DNA damage, perturbations to chromatin organization, and strong mitogenic signals. Based on this observation, the scientific community, at that time, proposed two important hypotheses. The first one assumed that the senescence mechanism was a way of the body and the cell to prevent aberrant proliferation like cancers, so was proposed senescence as a beneficial event in order to protect unregulated cell proliferation. The second hypothesis, stated senescence as a phenomenon related to the progressive decline in -tissue regeneration and repair, with age, together with then loss of regenerative capability of cells in vivo. Nevertheless, the understanding of the senescence processes grew and these two hypotheses combined together, brought new insights to the fields of cancer, ageing and regeneration. In the field of regenerative medicine, the senescence is considered a problem, because for stem cell-based therapies a substantial number of cells are needed, requiring extensive ex vivo cell expansion with many related problems.

The primary cells do not grow indefinitely , but only for a limited number of cell division [122]. On the other hand cell therapy protocols usually require hundreds of million cells per treatment, with the need to be expanded in vitro several passages before implantation (http://www.clinicaltrials.gov).

Cell senescence is strictly dependent on another important factor, the patient. It is known, in fact, that the age, together with genetic patterns strongly the quality of the obtained cells, and the of the lifespan in vitro[123][124]. SCs senescence could affect the clinical therapeutic potential, immunomodulatory activity, differentiation potential, and cell migration ability[125]. It was found that senescent cells exhibit striking changes in the expression of genes, as cell cycle activators [126][127] including the cyclin-dependent kinase inhibitors (CDKIs) p21 (also termed CDKN1a, p21Cip1, Waf1 or SDI1) and p16 (also termed CDKN2a or p16INK4a),. These CDKIs are components directly regulated by the p53 and retinoblastoma (pRB) proteins respectively, involved in growth arrest control during senescence. Senescent cells repress genes encoding proteins that stimulate or facilitate cell-cycle progression, as c-FOS, cyclin A, cyclin B and PCNA[128] by modulating, the transcription factor E2F, that is which is in turn inactivated by pRB. In some senescent cells, E2F target genes are silenced by a pRB-dependent reorganization of chromatin.

The senescence can be induced by many stimuli:

- **Telomere-dependent senescence**

  Telomeres are the last part of a linear chromosome and are characterized by the repetition of 5-TTAGGG-3′ (in vertebrates) which are associated proteins that have the main role to protect them from degradation or fusion by DNA-repair processes (during replicative event)[129]. The precise telomeric leght sequence is not known and structurally present in the end a large circular structure like a t-loop[130]. During replicative event in S phase of the cell cycle polymerases loose 50–200 base pairs of telomeric DNA [131] the length of telomere decrease, that is one of the reason why

cells do not proliferate indefinitely. Another event inducing senescence, is the double-strand breaks (DSBs) in telomere; in fact, after a telomere break, the cell can arrest cell-cycle progression and try to repair the damage, otherwise if it is not senescence [132][133].

However, physiologically the end-replication problem can be avoided in cells a particular enzyme, the telomerase. This enzyme contains a catalytic protein component (telomerase reverse transcriptase; TERT) and a template RNA component, that adds telomeric DNA repeats directly to the chromosome ends. The activity of telomerase is strictly correlated with the senescence in cells.

- **DNA-damage induced senescence**

  Severe DNA damage that occurs anywhere in the genome, causes in many type of cells senescence, it was observed that DNA and telomerase damages increase cell senescence through the activation of p53, that in turn up regulate p21 thus causing G1 phase arrest [134][135].

- **Senescence caused by chromatin perturbation**

  The chromatin state determines the extent of genes which are active (chromatin) or silent (heterochromatin), and depends mainly on epigenetic control by histone modifications as acetylation, methylation and remodelling of chromatin associated complexes. Recent observation, demonstrated that chemically-induced inhibition of histone deacetylase (HDAi), which promotes euchromatin formation, induces cell cycle arrest and senescence in fibroblast[136] because HDAi induces p21 and p16 expression and in turn p53 up regulation.

- **Oncogene-induced senescence**

  During cancer transformation, some genes show mutations, thus increasing the seriousness of cellular deregulation. These mutated genes, typical of cancerous cells are known as oncogenes. Usually, cells respond to cancer transformation by activating senescence or apoptosis pathways. For examples oncogenes molecularly activate cell senescence. This phenomenon was first observed when an oncogenic form of RAS, a cytoplasmic transducer of mitogenic signals, was expressed in normal human fibroblasts together with an up regulation of p53 and p16(INK4a)[137]. These finding clearly highlight a defence mechanism established by the cells, in the attempt to counteract abnormal growth ~~e~~ stimulation and cancer formation[138].

- **Stress and other inducers of senescence**

  There are a lot of observations that stressor event as oxidative condition in the cells or chronic inflammatory states induced by cytokines, such as interferon-β may induce a senescent state together with cell growth arrest. Chronic stimulation by transforming growth factor-β in epithelial cell induces senescence by promoting p16–pRB-dependent target genes [139].

In conclusion, cell senescence still represent a problem in the field of regenerative medicine because influences the therapeutic potential of human stem cell that can be used for transplantation by influencing many cellular features as–migratory ability, differentiation, immunomodulation ability, cell expansion and cell quality[140].

Tissue engineering and Market

Most cell-based therapies are currently experimental, with a few exceptions such as haematopoietic stem cell (HSC) transplantation which is already a well-established treatment for blood related disorders[141], or the transplantation of cultured sheets of autologous epidermal or corneal cells to repair burn injuries[142], the used transplantation of ex vivo–expanded autologous chondrocytes to repair cartilage defects[143]in clinical practical, or experimental trials like transplantation of embryonic stem cells for treatment of spinal cord injuries[144] a pilot trial conduct in Australia called Geron Phase 1 or the application of pluripotent stem cells for treatment of blindness on a Japanese woman by Masayo Takahashi group.

Conventionally, cell therapies can be classified by the therapeutic indication and the aim to address (neurological, cardiovascular etc.) or by where are from like the same individual (autologous) or derived from a donor (allogeneic) or most commonly by the cell types, often using the EU regulatory classification, the EU regulatory classification of cell-based therapies discriminates between minimally manipulated cells for homologous use (transplants or transfusions) and those regulated as medicines which are required to demonstrate quality, safety and efficacy standards to obtain a marketing authorization before becoming commercially available (referred to as Advanced Therapy Medicinal Products; ATMPs) which are subdivided into somatic cells, gene therapy and tissue engineered products [145]. However, the majority of cell-based therapies till now are an early stage of development (clinical trial Phases I and II focused on demonstration of safety and early indication of efficacy) with relatively few reaching the later stages of clinical trial and marketing authorization [146].

However, since tissue engineering and regenerative medicine emerged as an industry about two decades ago, a number of therapies have received Food and Drug Administration (FDA) clearance or approval and are commercially available[147]. Carticel, the first FDA-approved biologic product in the orthopaedic field, uses autologous chondrocytes for the treatment of focal articular cartilage defects autologous chondrocytes are harvested from articular cartilage, expanded ex vivo, and implanted at the site of injury, resulting in recovery comparable with that observed using micro fracture and mosaicplasty techniques [148]. Other examples include laViv, which involves the injection of autologous fibroblasts to improve the appearance of nasolabial fold wrinkles, or Epicel that are autologous keratinocytes used for severe burn wounds. Tissue engineering includes materials that are often an important component of current regenerative medicine strategies because the material can mimic the native extra cellular matrix (ECM) of tissues and direct cell behavior, contribute to the structure and function of new tissue, and locally present growth factors[149]. Decellularized donor tissues are also used to promote wound healing (Dermapure, a variety of proprietary bone allografts)[150] or as tissue substitutes the CryoLife and Toronto's heart valve substitutes and cardiac patches[151]. These products provide benefit in terms of healing and regeneration but are unable to fully resolve injuries or diseases[152].

2. Use of materials able to increase the repair processes and cell growth and migration

Tissues generally consist of cells and extracellular matrix (ECM). The main role of biomaterials is mimicking the ECM, giving both structural and functional support. During the last few years, ECM has been shown to play a key role in many different functions, such as gene expression, survival, death, proliferation, migration, differentiation. Therefore, all of

them should be reproduced by biomaterials enriched with bioactive factors, such as growth factors and cytokines.

The biomaterials, called "scaffold" in regenerative medicine, can be either natural or synthetic with different advantages and disadvantages. Synthetic materials can be identically reproduced on a large scale with specific properties of microstructure, and degradation rate. However, they still have some problems of biocompatibility with cells. That problem should be solved using natural biomaterial from living organism, able to integrate themselves with cells, creating decellularized tissue matrices[153], but unfortunately even acellular tissue matrices are not so easy to obtain large quantities according to good manufacturing practice and ATMP standards parameter [120] and in some cases they present cellular components which may induce an immune response [153].

The ideal biomaterial should be biocompatible and biodegradable at the same rate as regeneration well porosity, which allows the exchange of nutrients and wastes, not toxic, not inflammative and may help regeneration in term of time and quality.

3.3D printing cells

The new era of regenerative medicine is trying to solve the problems concerning the use of cells and the biomaterials directly printing all together. The biofabrication technique[154], for example, is based on a photo-induced solidification process, which uses soft biocompatible hydrogels containing living cells and forms one layer of solid structure at a time[154], but in a continuous fashion, by shining light on a selected area of a solution containing photo-sensitive biopolymers and cells Organovo is a medical start-up aiming at

delivering bioprinted organs, like liver, for surgical therapy and transplantation. Cytofuse is a Japanese company that without biopolymer directly prints in 3D articular cartilage and subchondral, starting from 3D adipose tissue-derived mesenchymal stem cells[155].

Principle aim and challenges in regenerative medicine

Stem cells have the capability to differentiate into a wide range of adult cells, the discovery and isolation of them paved the way to new hopes in the regenerative field. But most of the applications of stem cells directly on patient are still under experimental trial phase, except for some procedures actually used in clinical practice (bone marrow transplantation in haematology). Even tissue engineering, one of branch of the regenerative medicine based on the regeneration of novel tissues from cells with the aid of biomaterials and growth factors still have some problems. The regenerated tissues usable by the patients are still very limited, as skin, bone, cartilage, capillary and periodontal tissues.

One of the reason which strongly prevents the use of stem cells to regenerate organs and tissues deal with the need of a large number of stem cells as MSCs for clinical applications.

Most of the time the isolation of tissue could be dangerous and painful for the patient. Then, a proper set up of in vitro MSCs expansion and subsequent cryopreservation and banking are necessary to establish safety and efficacy in transplanted patients. And is well known that expansion of primary cells is strongly influenced by senescence problems, which represent also one of the challenges in the application of tissue engineering. The artificial tissue engineered still have some limitations correlated to the dimensions of the construct, that can not be used for the recovery of serious defects. The other problem is correlated with the

architecture and tridimensional structure, actually the only usable engineered tissues are vases or cave structure like the trachea [156], or tissues not physiologically scattered because the viability of cells seeded on a scaffold gradually decrease with the thickness. On the other hand, the use of growth factors alone or associated with the 3D construct are still not considered completely safe, since it is unknown the the influence that they could have on the environment of the donors. All of these items need to be addressed before cells or engineered constructs can be used routinely in the clinical setting.

The challenges in regenerative medicine are still countless, (1) the safely, for instance the use of stem cell that can differentiate in all types of mature cells include cancer cell, (2) the use of a method that can commit all the stem cell toward a specific phenotype with the 100% yield of final differentiation, (3) the use of a population of cells that can be fast and easily isolated and with high quantity, and safe for the patient. Also for tissue engineering some clues are still open, (4) find the best scaffold, (5) the best bioreactor (6) the best solution for seeding different population in order to have a relevant mature material implantable on patients.

However, since years other technologies were tested on patients in order to modulate some physiological behaviour involved in the homeostasis of tissue and activation of niche.

4.Use of biophysical stimuli on cells or tissue

The modulation of the stem cell niche can influence the function of stem cell population, based on this idea since years the regenerative medical techniques have been improved and focused on the implementation of biophysical stimuli[96].

Physical factors within the local cellular microenvironment, including cell shape and geometry, matrix mechanics, external mechanical forces, and nanotopographical features of the extracellular matrix, can all have strong influences in regulating stem cell fate[157][158]. There are several evidence that regulation of stem cell fate is strongly influenced by the coexisting insoluble adhesive, mechanical, and topological cues contained and dynamically regulated in the stem cell niche[93][159]. Biophysical stimuli can be sensed and transduced into intracellular biochemical and functional responses by stem cells, a process known as mechanotransduction [93]. The sensory machinery of stem cells can sense and integrate multiple signals simultaneously from their niche and convert them into a coherent environmental signal to regulate downstream gene expression and stem cell fate[93][40][160][110].

There are several devices able to interact with cell fate in vivo, directly on patients, on animal models and in vitro on the cells.

Physical devices used in regenerative medicine

For years' scientists tried to drive stem cell fate through the use of chemistry, increase the proliferation using growth factors or fabricate 3D constructs using the combination of stem cell or mature adult cells on natural, artificial polymers.

But only relative recently, some research groups have shown the possibility to use physical stimuli directly on patients, tissue and cells [91].

The use of physical energies for therapeutic purpose is well known, approved by FDA committee and used on patients. Several devices based on different physical mechanisms were studied and observed directly on patients with beneficial effects. The idea behind the use of physical stimuli on tissue and bodies, was proposed already in 1974 by Nucitelli who gained evidence on endogenous ionic current and interaction with electric field in multicellular animal tissues[90]. Nowadays it is possible to explain changes in cellular behaviour, following-electro magnetic stimulation, considering an effect on cell polarity [161] and on stem cell niche in the body [39].

In fact, recent evidence highlighted that the regulation of cell fate by physical stimuli influence the coexisting insoluble adhesive, mechanical, and topological cues inherently contained and dynamically regulated in the stem cell niche. Since then, the use of physical treatment in regenerative medicine progressively raised as an alternative solution.

The **Ultrasound energies** were used for medical purposes since the 1950s for physical therapy in some pathological situations as tendinitis or bursitis[162].

Even the use of **extremely low-frequency electromagnetic fields (ELF-EMFs)** with frequency lower than 100 Hz and magnetic field intensity ranging from 0.1 to 20 mT became a useful therapy for fracture repair, osteoporosis treatment [163] and soft tissue regeneration, like wound healing. The mechanism of action of ELF is not already clear, however several evidence showing that the electric currents can accelerate cell activation [94], and influence

epigenetic remodelling. In particular the use of 50Hz ELF- on GC-2 cell lines decreased genome-wide methylation and the expression of DNMTs [164], in neural stem cells (NSCs) isolated from the hippocampi of newborn mice, moreover the ELF irradiation at 1 mT 50 Hz for 12 days enhances their proliferation and neuronal cell-fate specification by a Cav1-channel-dependent regulation and modification of the histones[165], that result provide a possible correlation with an epigenetic regulation by physical stimuli on fate of cells.

Radio Electric Assimetric Conveyor (REAC)

The REAC is a medical device that generates an emission of microwaves of very weak intensity(2 mW at the emitter and 2.4 or 5.8 GHz frequency), this technology was designed in order to convey asymmetrically radioelectric frequency and microcurents on body. The energy emitted from REAC is resulting from the interaction between the weak electromagnetic field produced by the device and the the cells, like an organized loop auto calibrating (asymmetric). The auto calibration of the signal is permitted by an intern antenna that capture the micro currents produced by cells and re-convey forward them by a probe in a concentrated way. Interestedly, the REAC showed a remarkable biological effects for the improvement of pathological neuroinflammation in mouse model[166], as well in rats model was showed the different neuromodulation in the thalamocortical region after REAC treatment[167][168], in another randomized double-blind study was evaluated on adult patients the effect of 250 milliseconds pulse emitted with radio-electric asymmetric conveyer (REAC) showed cerebellar and ponto-mesencephalic activation in treated patients[169] As well that REAC provided an effect in the resolution of wound healing[170]. In in vitro experiment the application of 2.4 GHz radiofrequency enhanced the yield commitment

towards myocardial, neuronal, and skeletal muscle differentiation in both mouse ES cell[101] and human adipose-derived mesenchymal stem cells (hADSCs)[102], for the first time exposure of hADSCs to REAC-conveyed radioelectric fields was able to counteract the senescence in vitro after several passaging, absolutely a important result for the regenerative applications, but more studies must be perform in order to understand the details of the mechanism of action of REAC.

Extra corporeal shock Waves therapy (ESWT)

Extracorporeal Shock Wave Therapy (ESWT) is another type of biophysical stimuli in the field of regenerative medicine, that could be classify as "mechanotherapy". Initially, in the 80's Shock wave was used in urology (lithotripsy) to disintegrate renal stones[107]. In later times, Shock wave were applied in other fields, showing promising hopes for the treatment of healings and pathological disorders. One of the first applications were in the orthopedic field, but with another purpose. In fact, was not used to disintegrate tissues, rather to induce neovascularization, improve blood supply and tissue regeneration. Gradually, this technology was introduced for the treatment of musculoskeletal disorders[110], tendon pathologies, bone healing disturbances, vascular bone diseases[171], dermatology for the wound healing disturbances, ulcers[172]. However till now the exact biological mechanism of action is unknown. Several studies conducted in vitro, proving the effect of Shock Waves (SW), on the modulation and stimulation of the cells through "mechano-transduction". In a recent study, was provided that SW could activate ADSCs through MAPK, PI-3K/AKT and NF-κB signaling pathways[112], as well in HUVEC cells exposed to SW (1 Hz at an energy level of 0.03 mJ/mm2) was found a upregulation of the expression of angiogenic factor, and caveolin-

1, a constitutive protein of caveolae, has been implicated in the regulation of cell growth, lipid trafficking, endocytosis, and cell migration[108]. The SW treatment act in is dose dependent on the behavior of cells. In a work published by Zhang was found that the cells exposed to low energy (0.04 e 0.13 mJ/mm2) improved the expression of some angiogenic factors, such as eNOS, Ang-1, and Ang-2, on the other hand a high increase of energy reduced the expression of angiogenic factors and increased apoptosis[173]. That findings suggest that the biological effects of shock is strongly correlated with the intensity of energy give on cells, that therefore mechanic-force apply to the cells.

PRINCIPLE OF SHOCK WAVE

Shock waves are generated by an electrohydraulic device that produce underwater high-voltage condenser spark discharge, convoyed by an elliptical reflector on tissue or cells.

There are three main techniques through which shock waves can be generated generated. These are the electrohydraulic, electromagnetic, and piezoelectric. The first one was the first generation devices, the electromagnetic technique is based on electric current passing through a coil to produce a strong magnetic field generating the shock wave, the last one the piezoelectric technique involves a large number (usually > 1000) of piezocrystals mounted in a sphere and receives a rapid electrical discharge that induces a pressure pulse in the surrounding water steepening to a shock wave.

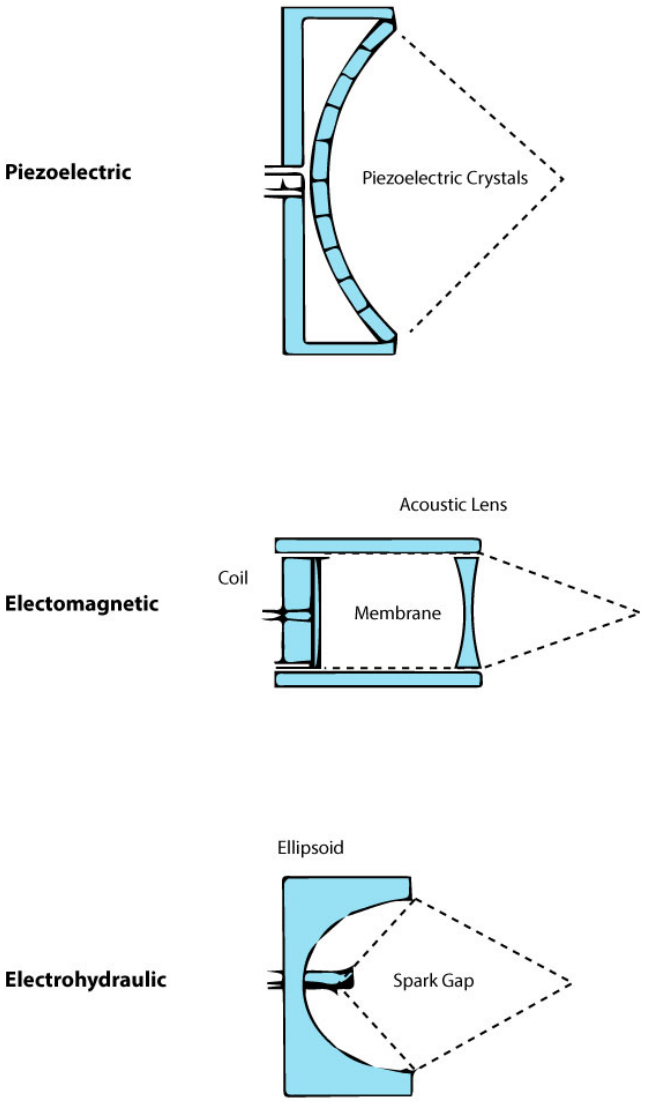

Fig.3 Types of SW generator

However, all these devices maintain the same parameters, that can be modulate or change, base on the needs. That are the pressure distribution, energy density and the total energy at the second focal point. The Shock waves can be convoyed in a specific zone of body, or tissue or cell culture by a applicator.

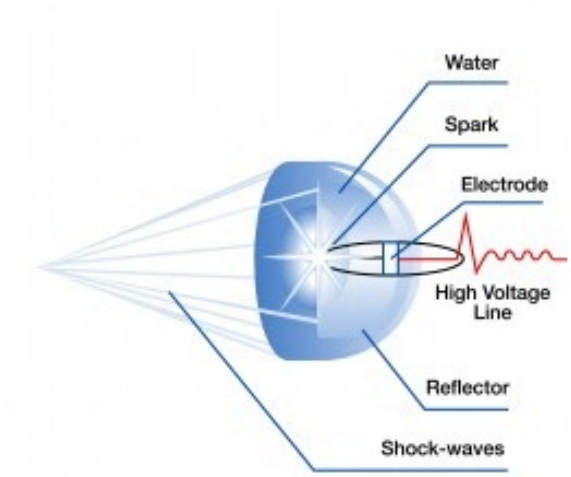

Fig.4 Shock Waves applicator

The "mechanical" waves produced by SW device, are sonic pulsation characterized by biphasic behavior, an initial positive very rapid phase, of high amplitude more than 100 MPa (500 bar), followed after 10 μs and a negative pressure (- 10 MPa), in a frequency spectrum range of 16 to 20 MHz[174].

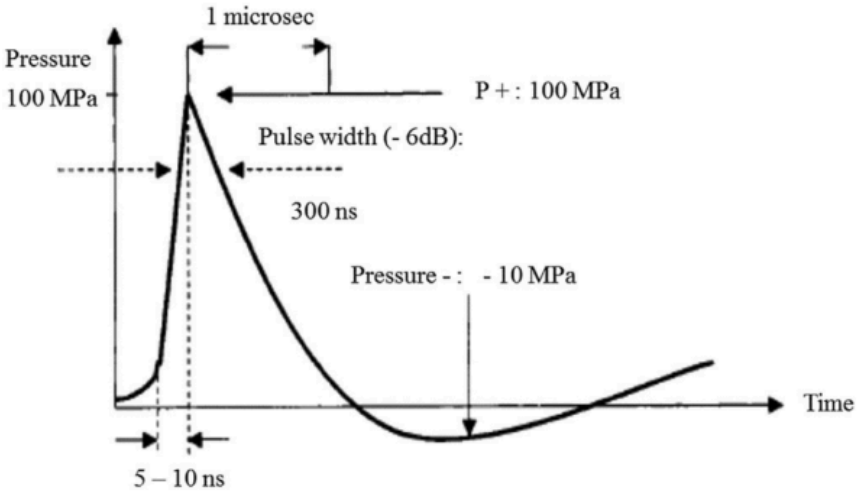

Fig.5 Typical "biphasic" form of the therapeutical extracorporeal shock waves. (from: Odgen JA, Toth-Kischkat A, Schulteiss R. Principles of Shock Wave Therapy. Clinical Orthopedic and Related Research, 2001; 387: 8 e 17)

AIM OF THE PROJECT

The use of direct physical stimuli on tissues, as well on cells, is a novel and suitable method to interact with and control cell fate. Over the years, several studies have demonstrated the ability of Physical stimuli to modulate cells behaviour, including cells that are resident in the niche, activating or changing their physiology.

Physical stimuli, by interacting with cells or stem cell in the niche, modulate their polarity, their thermodynamic status, driving the complex processes of adhesion, proliferation and differentiation toward specific phenotypes promoting tissue regeneration.

However, an understanding of the molecular mechanisms governing such processes is still lacking.

Even if a therapeutic effect is clear to clinicians in applied medicine, the physiological mechanisms behind the effects these stimuli have on tissues and cells remain both unclear and enthralling.

This was the main focus of my PhD project, to try to figure out how biophysical stimuli on cells has an effect on physiological and molecular pathways. I focussed on two devices, the REAC and the extracorporeal shock wave machines.

For years, regenerative medicine has been trying to find a way to obtain the best possible regeneration of tissues or organs that have been severely damaged by chronic inflammatory diseases, tumours or severe trauma. More than three decades have passed from the first suggestion that cells may be grown outside the body, from the moment that, for the first time,

a cell was isolated and grown in an artificial environment, from the moment that researchers tried to create tissues by in vitro seeding on 3D supports called scaffolds, and from the discovery of recombinant proteins and growth factors able to increase cells performance in term of proliferation and differentiation. More than thirty years and researchers still are running for the win. However, the path is still long and the panacea for all ills has not yet been found.

Nevertheless, the idea of taking advantage of the intrinsic properties of cells and tissues in order to control or improve their physiology has always been an objective of researchers. The basic idea was to find a method, as minimally invasive as possible for the patient that could drive tissues towards self-healing. Physical therapies are a well proven reality in routine medicine. The beneficial effect they have on patients has been known for a long time, whether their mechanism is physical, electromagnetic, radio-electric or micromechanical.

At the beginning of my PhD, when shortly after my graduation I “arrogantly” thought that I had learned enough about biology from my university exams, I approached the world of biophysical therapies. Initially I was incredulous; I could not understand how "something that could not be seen and touched" could interact on a finely tuned system that evolution had refined over millions of years.

The aim of this thesis is to demonstrate the effect of physical stimulation on “in vitro” cell systems. Specifically, I wanted to analyse some critical points and mechanisms that still create problems in regenerative medicine, with the hope to clarify them and try to find a solution. I will focus on the senescence pathway and its modulation after physical stimuli with REAC.

As well, I will try to prove the efficacy of REAC during neuronal commitment in PC12, as well the combination of REAC treatment and chemical inductors in urine-derived stem cells toward cardiomyocyte like cells. In the second session, I will try to prove the modulation of cell proliferation and activation after extracorporeal shock wave (ECSW) treatment. The basic hypothesis is that the cells stimulated through a physical stimulus react to it as a differentiation or replication signal, changing the cell polarity. Perhaps acting at the molecular level and on the intrinsic polarity of the cell, this type of allowing cells to be more active and responsive. For convenience I divided this thesis in chapters, based on the different devices applied on the cells.

REAC SECTION

REAC and senescence

One of the most important challenge in regenerative medicine is the use of stem cells for cell therapy or for tissue engineering. It is known that the number of stem cells necessary for bone morrow transplantation is in the order of billions (www.clinicaltrials.gov), moreover if we want to use stem cells from another source like dental pulp, adipose tissue, placenta etc. we will found several troublesome. The quantity of stem cells resident in the tissues or in the niche is limited by many factors. One factor should be correlated to the quantity and the quality of the biopsy that we take from the patient, that could be limited if the starting material is poor. The other could be correlated to the variability between patients in term of gender, physiology, genomic or age. Even the yield of a pure stem cell population isolated from biopsy could be compromised by the presence of other cells. Therefore, prior to be used in translational medicine, even in research the primary cells culture need to be expanded and immune selected. But this actually represent a manipulation and could give rise to secondary issues. The prolonged, expansion of cells in vitro could induce the senescence in them. The senescent cells cannot be used in clinical transplantation because they cannot be proved to be safe, do not reply to self-renewal , stay in a quiescent state and lose the ability to differentiate.

In previous works, my group found that radioelectric irradiation by REAC technology, influenced the gene expression profiles controlling stem cell differentiation in Adipose derived stem cells as well in mouse embryonic stem cells [102][101]. We proved that in

senescent Adipose derived stem cells, aged by multiple passaging in vitro, REAC counteracted the induced senescence phenotype [175], by the modulation of the molecular pattern mediated by p16INK4, ARF, p53, and p21CIP1. The beta-galactosidase staining assays showed a decrease in the number of positively stained cells. Moreover REAC was also able to preserve the pluripotency of Adipose derived stem cells , as assessed by the yield of the adipogenic, osteogenic, and vasculogenic differentiation obtained in REAC-treated cells as compared to control untreated cells belonging to the same culturing passage. We supposed that one of the main mechanism of REAC effect is based on the modulation of cells polarity. Cell polarity is involved in a plethora of physiological cell behaviour, such as potency, plasticity, metabolism, activation of the cells, epigenetic changes and senescence. Based on these issues we tried to figure out some possible REAC target in the stimulated cells. In particular we focused on the expression of BMI, a Polycomb and Trithorax member of the group of repressors, which has been recently shown to be an essential factor for the self-renewal of adult murine hematopoietic stem cells, and neuronal stem cells, acting as a repressor of senescence[176][177].

It is already known, that hyaluronic acid (HA) plays a fundamental role in cell polarity and hydrodynamic processes [178], in this section I will describe the effect of REAC on the modulation of senescence pathways and in term of cell polarity modulation using an inhibitor of HAS2 (hyaluron sintase 2), in order to demonstrate a possible target mechanism of REAC in counteracting cell senescence.

Isolation and culture of hADMSCs

According to the policy approved by the local Ethical Committee from University of Bologna, all tissue samples were obtained after informed consent.

We isolated the cells from human biopsy of subcutaneous adipose tissue obtained from lipoaspiration/liposuction procedures. We choose Adipose derived stem cells (ADhMSC), because actually they represent a promising cellular type in the field of regenerative medicine due to easy procedure of tissue harvesting and their level of multipotency.

After washing, we proceed with enzymatic isolation. The lipoaspirates were digested with 0.2% collagenase A type I solution (Sigma-Aldrich), under gentle agitation for 45 min at 37°C, and centrifuged at 2000 rpm for 10 min to separate the stromal vascular fraction (SVF) from adipocytes. We even treated this fraction with red blood cell lysis buffer for 5 min at 37°C, in order to obtain a pure population without erythrocytes that could be toxic for the cells and interfere with the isolation protocol, then we centrifuged again the solution. The supernatant was discarded, and the cell pellet was re suspended and seeded in culture flasks in DMEM- low glucose (Lonza) supplemented with 20% heat inactivated FBS, 1% penicillin-streptomycin, 2 mML-glutamine, and incubated at 37° C in a humidified atmosphere with 5% CO2.

When the cultures reached 80% of confluence, cells at passage 5,10,15,20 and 30, were detached by treatment with trypsin-EDTA, and seeded in six-well tissue culture plates. The REAC device was placed into incubator and was set at 2.4 GHz. The signal was conveyed by

electrodes for 4,8 or 12 hours to the cells at the different passages. We decided to expose cells for 6, 8 and 12 hours, following previous observation[101] showing that the effect of REAC was time dependent.

Culture of hADMSCs with 4-MU

For the experiment in the presence of the 4-methylumbelliferone (4-MU) , a HAS2 inhibitor cells were seeded in culture flasks in DMEM- low glucose (Lonza) supplemented with 20% heat inactivated FBS, 1% penicillin- streptomycin, 2 mML-glutamine, and incubated at 37° C in a humidified atmosphere with 5% CO2.

As for the previous set of experiments we cultured cells for 5,10,15,20 and 30 passages in order to induce the senescence in vitro. The REAC apparatus was positioned into the CO2 incubator and set at 2.4 GHz, and its probes were immersed for 12 hours into the culture medium at each indicated passage, in the absence or presence of 1 mM 4-MU (Sigma-Aldrich).

REAC SETTING DETAILS

The REAC was set for radio frequency burst of 250 ms with an off interval of 2.5 s. The REAC apparatus, was placed into a CO2 incubator, was used at a frequency of 2.4 GHz, and its conveyer electrodes were immersed into the culture medium of hADSCs. REAC radiated power is about 2 mW, electric field E=0.4 V/m, Magnetic field 1 mA/m, specific absorption rate (SAR) 0.128 μW/g; the density of radio-electric current flowing in the culture medium during the REAC single radio frequency burst is J=30 μA/cm2.

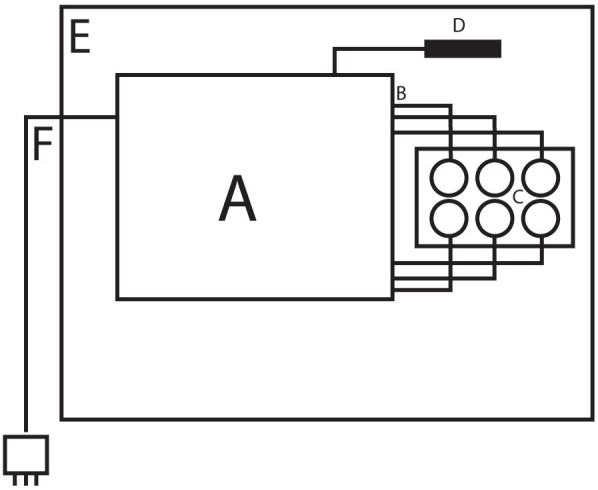

Fig.6 Prospect of REAC and cells inside incubator. *REAC(A) was allocated inside the incubator (E), the cells cultivated inside dedicated tissue culture plate(C) were exposed to REAC by specific probes(B) directly inside the medium culture. A particular antenna (D) detected the frequencies emitted by cells. (F)power supply cable feed of REAC.*

SA-b-Gal staining

SA-b-Gal staining was performed using a ‘‘Senescence-associated b-Galactosidase Staining Kit’’ (Cell Signaling). Briefly, ADhMSCs cultured at passages 5, 10, 15, 20, 25 and 30 were exposed for 12 hours in the absence or presence of REAC in 6-well plates . After treatment with REAC or not, cells were fixed with fixative solution and then processed according to the manufacturer’s instructions. The cells were then photographed under an inverted microscope at 10X magnification for qualitative detection of SA-bGal activity. The number of positive (blue) and negative cells was counted in five random fields under the microscope at 20X magnification and bright field illumination, and the percentage of SA-bGal- positive cells was calculated as the number of positive cells divided by the total number of counted cells.

Analysis of gene expression

Total RNA was isolated using Trizol reagent, according to the manufacturer's instruction (Life Technologies). Total RNA was dissolved in RNAase- free water and. Then quantified using Nanodrop. For reverse Transcription (controlla bene questo termine) PCR, 1 μg of total RNA was used and cDNA was synthesized in a 50-μl reaction volume with MuMLV reverse transcriptase (RT) according to the instruction kit provided (Life Technologies). Quantitative real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad). Two μl of the cDNA solution were amplified in 50-ml reactions using Platinum Supermix UDG (Life Technologies), 200 nM of each primer, 10 nM fluorescein (BioRad), and Syber Green. After an initial denaturation step at 94 C for 10 min, temperature cycling was initiated. Each cycle consisted of 94°C for 15 s, 55–59°C for 30 s and 60°C for 30 s, the fluorescence being read at the end of this step. To evaluate the quality of product of real-time PCR assays, melting curve analysis was performed after each assay. Relative expression was determined using the ''delta-CT method'' with hypoxanthine phosphoribosyltransferase 1 (HPRT1) as housekeeping gene. The mRNA levels of control and REAC-exposed ADhMSCs were expressed as fold of change ($2^{-\Delta\Delta Ct}$), relative to RNA levels of the ADhMSCs at passage 5 before starting the REAC treatment (time 0). We used the primer mentioned in table.1

Immunoblotting analysis

To analyse the protein level and confirm the data observed by gene expression analysis we performed the immunoblot analysis. The cells, were cultured as previously described, at

passages 5, 10, 15, 20, 25 and 30 and then were exposed for 12 hours in the absence or presence of REAC in 6-well plates. From total cell lysates proteins were isolated, then quantified using BCA assay. The SDS-page was performed on 10% Novex Tris-glycine polyacrylamide gels (Invitrogen, CA), in MOPS SDS Running Buffer, using an XCell SureLockTMMini-Cell, according to the instruction provided by the kit. After protein transfer to nitrocellulose membranes (Life Technologies), we saturated the membrane using a solution of PBS-3%BSA, for the washing solution we used PBS-Tween 20 1% , the immunoreaction was carried out for 1 hour at room temperature in the presence of the primary antibody Oct4 (Santa Cruz), Sox2 (Sigma) and GAPDH (Santa Cruz). After additional washing, membranes were incubated with anti-rabbit horseradish peroxidase (HRP) conjugated with secondary antibody (PIERCE). Targeted protein expression was assessed by a chemioluminescence detection system (ECL Western blotting detection reagents were from Amersham Biosciences).

Assessment of telomere length. Genomic DNA was extracted, using Qiamp DNA blood mini kit (Qiagen), from ADhMSCs at passages 5, 15, 20, 25 and 30 cultured in the absence (control) or presence of REAC stimulation for 4, 8 and 12 hours. Telomere length was determined using the Telo TAGGG telomere length assay kit51(Roche), according to the manufacturer's instructions. Briefly, genomic DNA was digested with a mixture of frequently cutting restriction enzymes, digested DNA fragments were then separated by gel electrophoresis and transferred to a nylon membrane by Southern blotting. The blotted DNA fragments were hybridized to a Dygoxigenin (DIG)-labeled probe, specific for telomeric repeats and incubated with a DIG-specific antibody covalently coupled to alkaline

phosphatase. The alkaline phosphatase on the antibody metabolizes CDP-star, a highly sensitive chemioluminescent substrate: this produces a visible signal that indicates the location of immobilized telomere probe (and hence the terminal restriction fragment (TRF) on the blot). The average TRF length has been determined by comparing the position of the TRF on the membrane relative to a molecular weight standard present in the kit. Chemioluminescent signal has been acquired using a BioRad VersaDoc Imaging System. Mean TRF length has been defined according to the following formula:

TRF~S(ODi)positive control (cell at passage1) S(ODi=Li)

Where ODi is the chemioluminescent signal and Li is the length of TRF at position

Assessment of telomerase activity. Telomerase activity was investigated by the aid of TRAPEZE-RT (Millipore, Bedford, MA). This assay quantifies telomerase activity by measuring real-time fluorescence emission with quantitative PCR. Briefly, cells were lysed in 200 µl of CHAPS buffer. Aliquots of cell lysate (1 µ g of protein/well) were assayed in a 96-well quantitative PCR plate. Further wells were set for the generation of the standard curve (TSR8 control template), negative control (no sample), and a PCR amplification efficiency control (TSK, K1). Telomerase activity (total product generated) was calculated by comparing the average Ct values from the sample wells against the standard curve generated by the TSR8 control template 17,52. Assays were carried out with a CFX-96 quantitative PCR apparatus (Bio-Rad).

Data analysis Statistical analysis was performed by using the IBM- SPSS Statistics, version 22. Non-parametric Friedman and Wilcoxon Signed Rank tests were used to investigate,

respectively, differences in treatments across multiple test attempts, and to evaluate, in the same group, the differences (Delta CT) between the data collected over an observational period correlated with treated or non-treated cells. A P value less than 0.05 has been considered as statistically significant.

RESULTS

We cultivated the adipose derived stem cells (hADSCs) for 5, 10, 15, 25, 30 in order to induce the senescence in vitro. Confirming our previous observation [175] REAC remarkably influenced cell the senescence status of the cells during in vitro induced cellular aging. The number of SA-β-Gal- stained cells (blue) was already significantly reduced at passage 15 in REAC treated cells, as compared to control untreated cells at the same passage. Moreover the effect of rEAC on the number of senescent positive cells, was also confirmed in cells cultured for 20, 25 and 30 passages, corresponding to a culturing period of almost 50-days.

BETA GALACTOSIDASE ASSAY

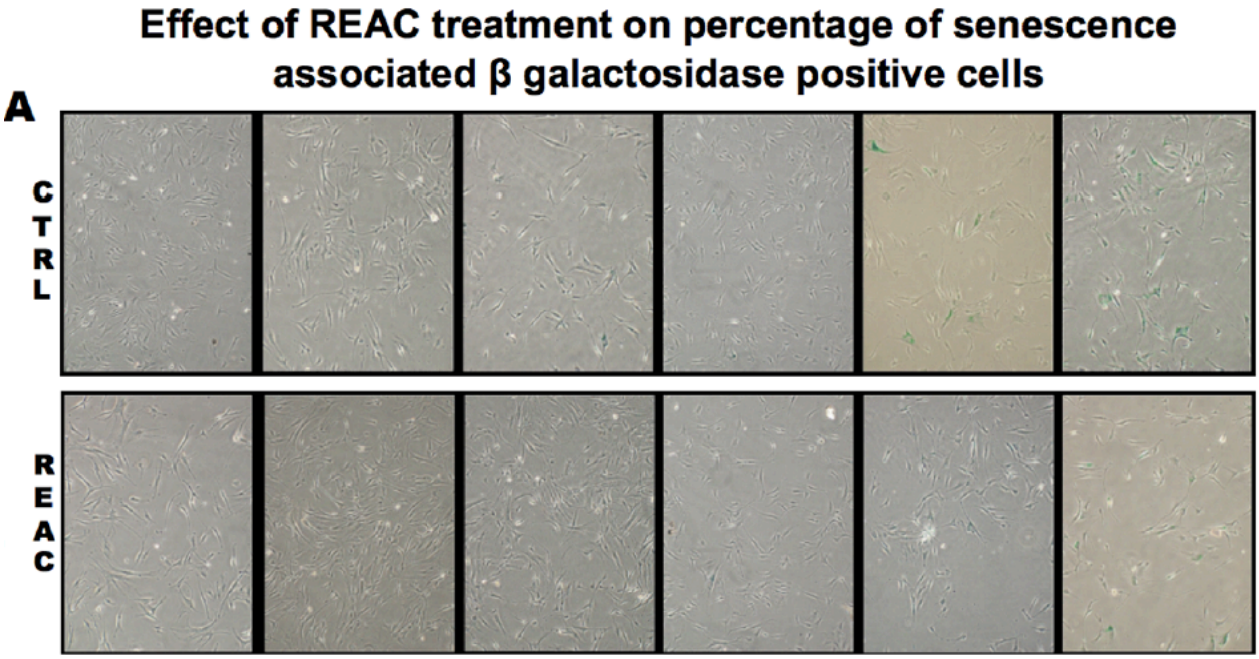

Fig.7 SA-b-Gal staining along multiple culturing passages

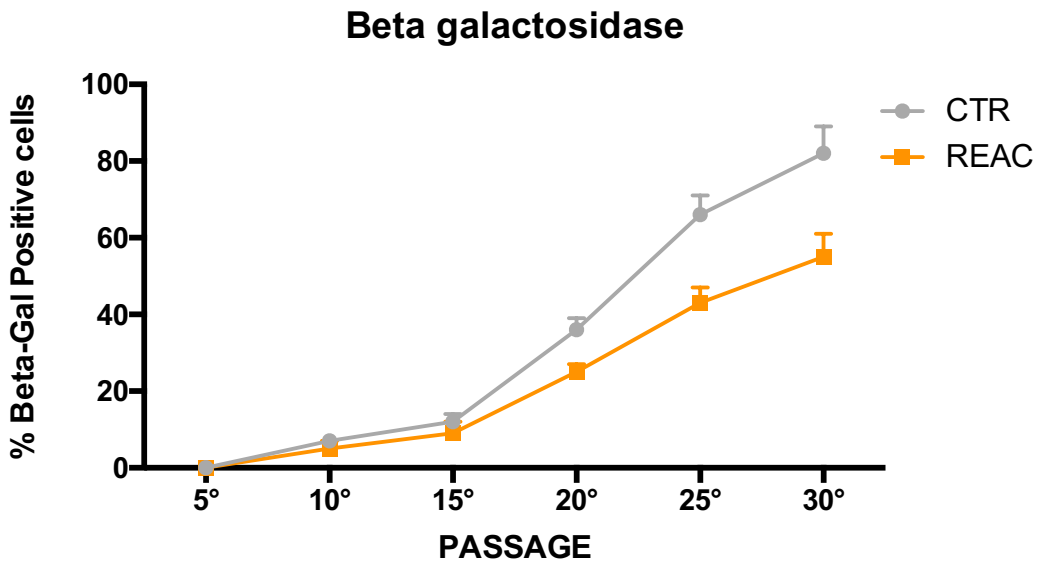

***Fig.8 SA-b-Gal staining along multiple culturing passages**. ADhMSCs at passages 1, 5, 10, 15, 20, 25 and 30, reaching 80% confluence, were exposed for 12 hours in the absence or presence of REAC (panel A, representative of six separate experiments). The percentage of cells positively stained (blue color) for SA-b-Gal was assessed as described in the Methods section below (mean6S.E.; n56; P,0.05).*

the figure 8 shows the number of SA-b-gal stained cells in REAC exposed ADhMSCs.

Already at passage 10° senescent cells were already detectable. The numbers of positive cells was significantly reduced along all the culturing passages in these cells that received a REAC treatment for 12 hours as compared to control untreated cells. In particular this differences between the two cell populations become more evident at late passages (25–30), when the untreated cells showed a 80% of blu positivity for Beta galactosidase, while in REAC treated cells the senescence was reduced at 55%.

GENE EXPRESSION ANALYSIS

We performed the gene expression analysis on Bmi1, genes controlling pluripotency and Tert. In order to have a general vision on the molecular mechanism induced by a 4, 8, or 12h of REAC stimulation.

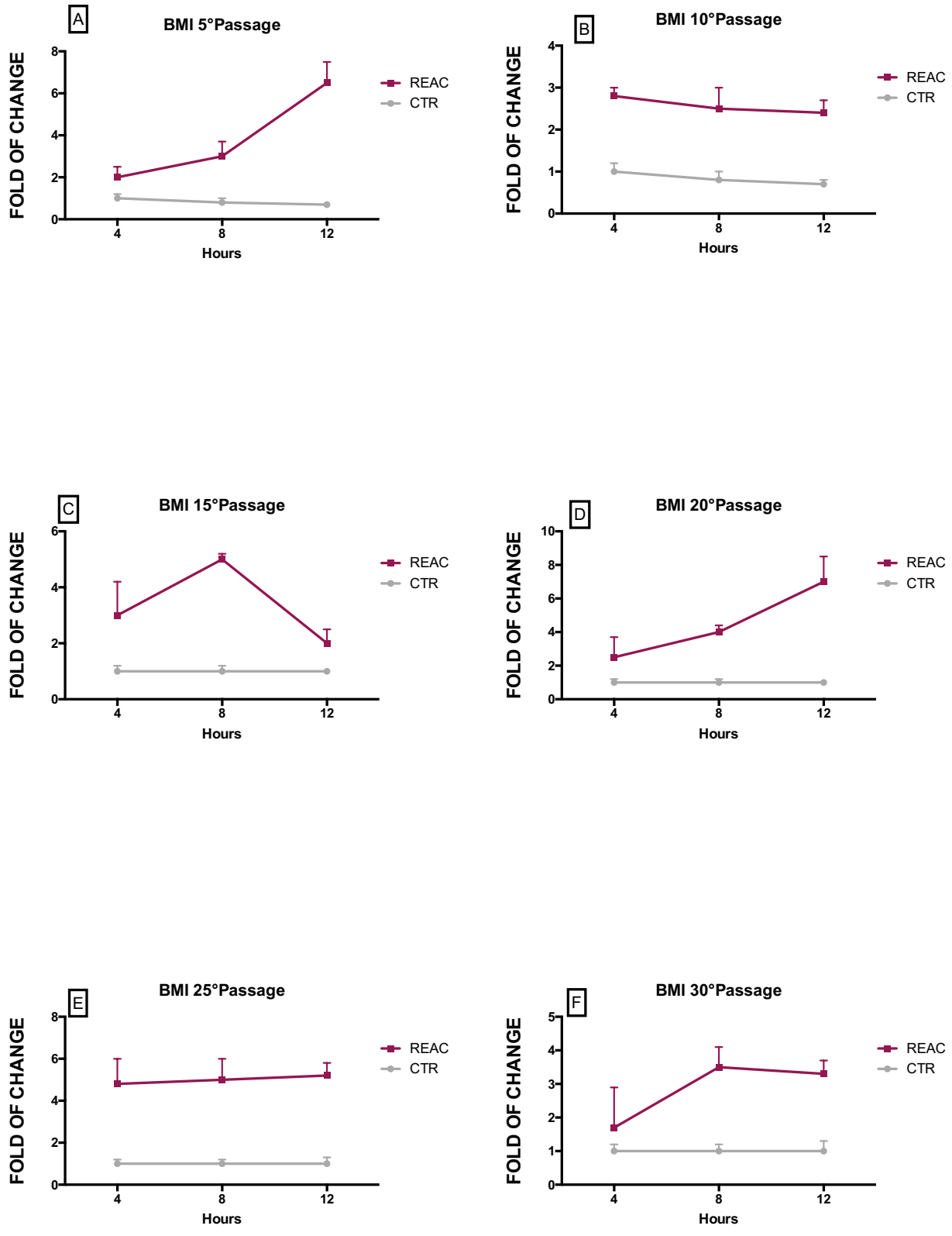

Fig.9 Effect of REAC on the expression Bmi1*. ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D), 25 (panel E) and 30 (panel F), the cells were exposed for 4, 8 or 12 hours in the absence or presence of REAC. The amount of Bmi1 mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells. (mean 6 S.E.; n 5 6) (P , 0.05).*

We studied more in detail, the activation of BMI in ADhMSCs at passages 5, 10, 15, 20, 25 and 30.that had been exposed to REAC for 4, 8 or 12 hours or not (control untreated cells).. The transcript levels of all the investigated genes progressively declined over the culturing time. On the contrary, REAC treatment significantly counteracted the passage-dependent down-regulation of Bmi1 transcription, at all the investigated passages (Figure 9). The use of the different time point exposition differently interact with the expression of BMI during the passages. According to the principle that the senescence status of stem cells decreases their potency, and plasticity, we analysed the gene expression profile of the pluripotency related genes.

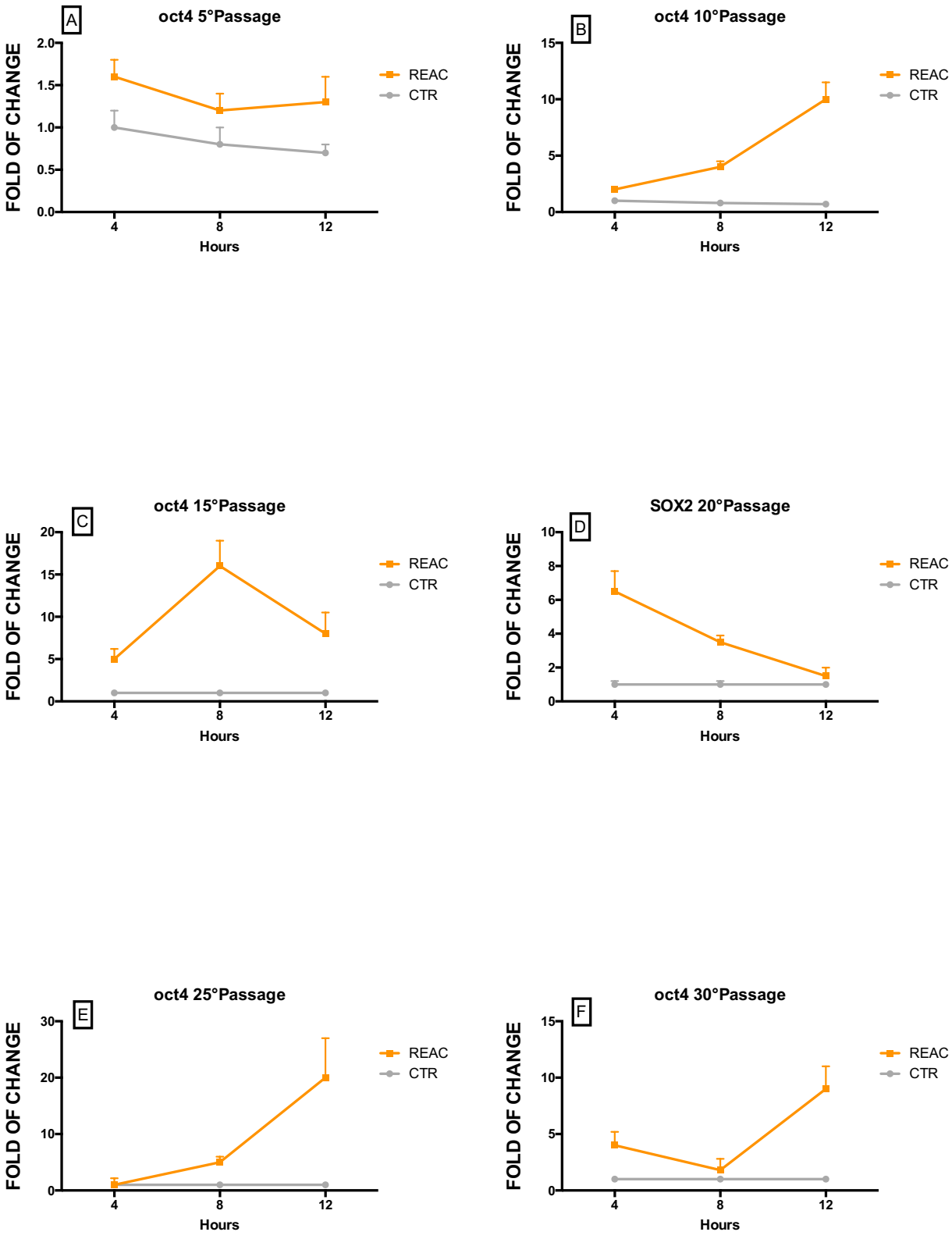

Fig.10 REAC treatment modulates the gene expression of Oct4*. ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D), 25 (panel E) and 30 (panel F) all cells were exposed for 4, 8 or 12 hours in the absence or presence of REAC. The amount of Oct4 mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean 6 S.E.; n 5 6) (P 00.05).*

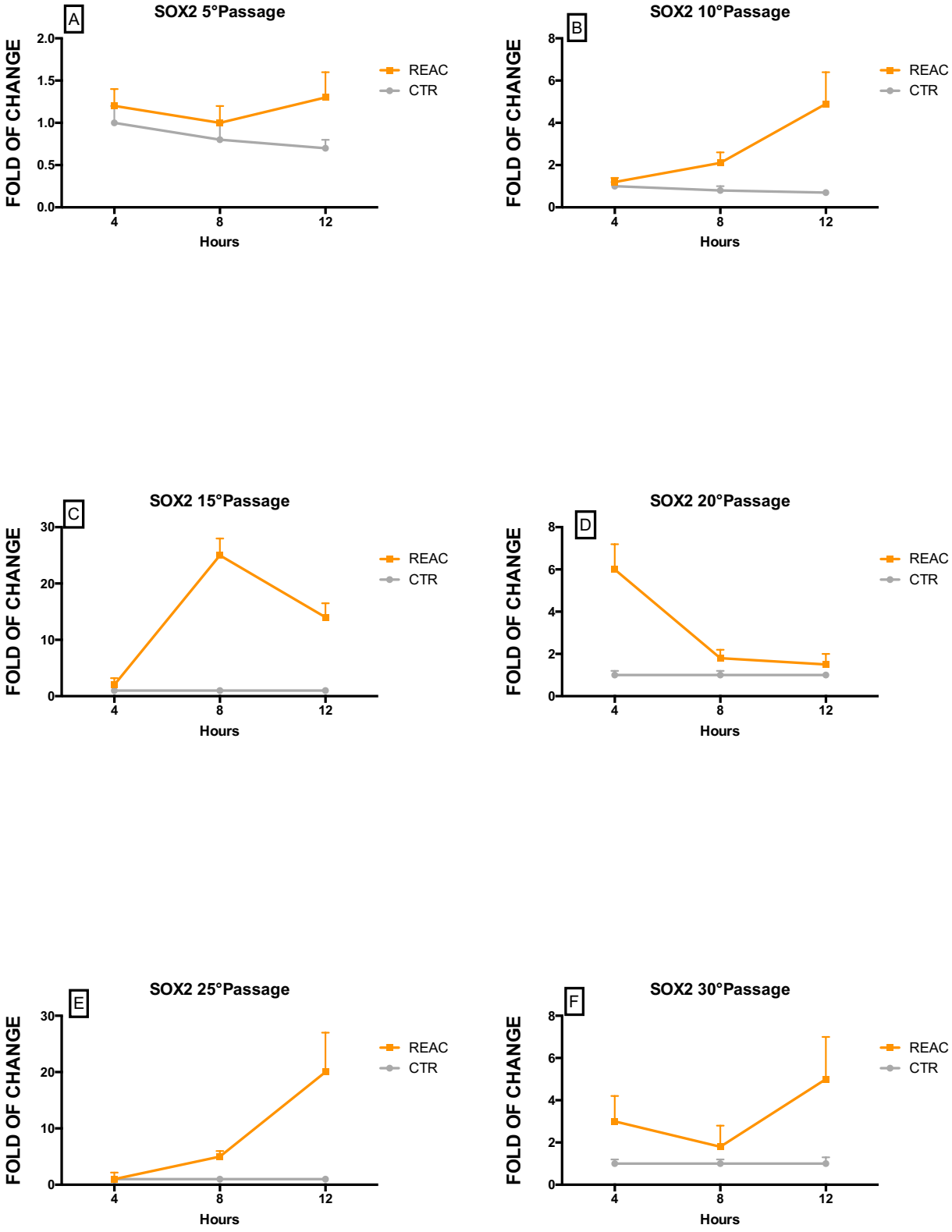

Fig.11 Gene expression of Sox2 with or without REAC treatment. *ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D),*
25 (panel E) and 30 (panel F) for 4, 8 or 12 hours in the absence or presence of REAC. The amount of Sox2 mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean 6 S.E.; n 5 6) (P , 0.05)

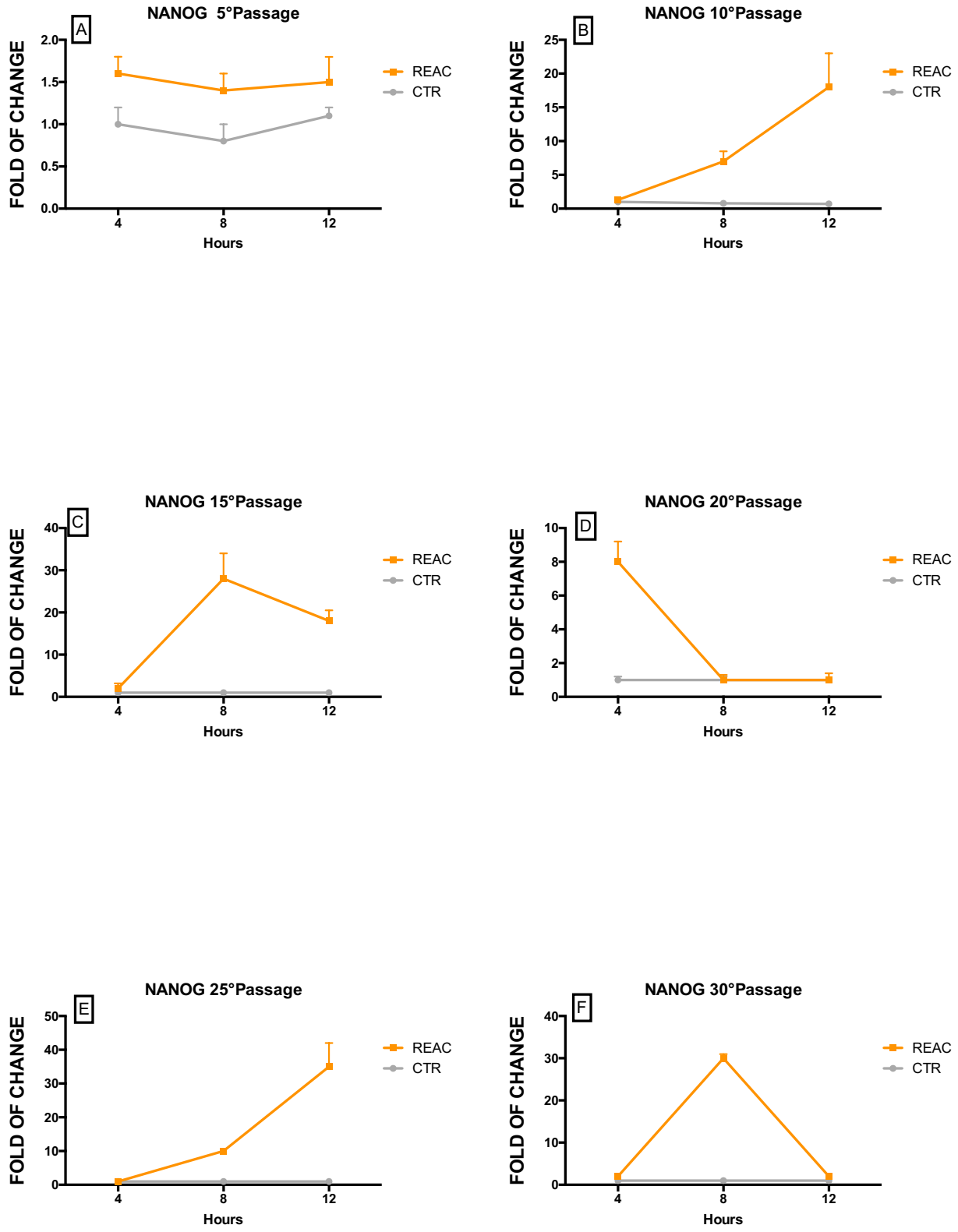

Fig.12 Nanog Expression. *ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D), 25 (panel E) and 30 (panel F), the cells were exposed for 4, 8 or 12 hours in the absence or presence of REAC. The amount of Nanog mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean 6 S.E.; n 5 6) (P , 0.05).*

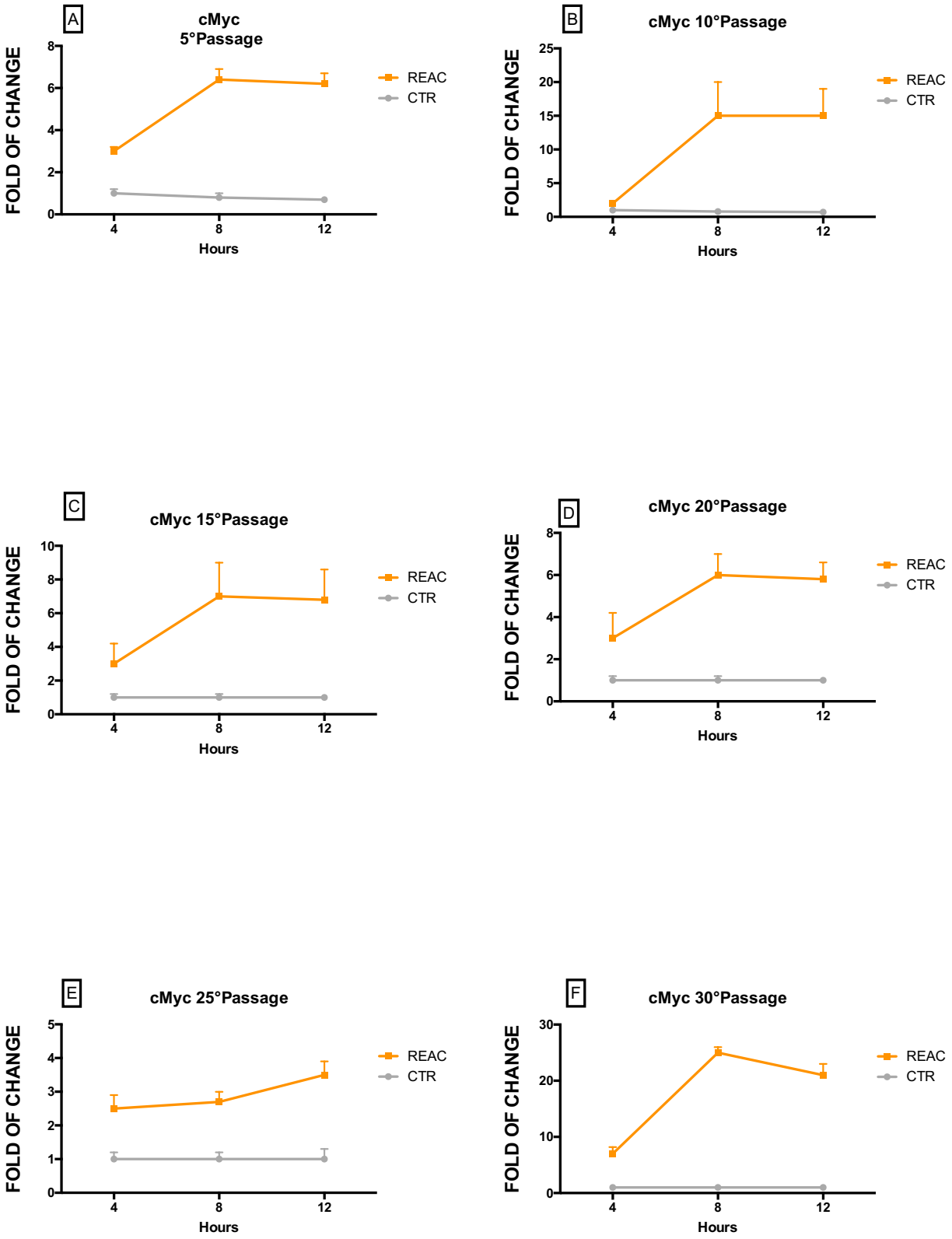

Fig.12 Gene expression of cMyc. *ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D), 25 (panel E) and 30 (panel*

F), after 4, 8 or 12 hours in the absence or presence of REAC. The amount of cMyc mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean 6 S.E.; n 5 6) (P , 0.05).

Transcriptional analysis of OCT4, SOX2, NANOG and cMyc have shown that REAC exposure for 4, 8 and 12 hours was able to finely tune the transcription of master stemness regulators. In particular, during a 4- to 12-hour-treatment, Oct4, Sox2, Nanog, and cMyc gene expression was significantly increased in REAC-exposed ADhMSCs at all passages (10, 15, 20, 25 and 30) as compared to unexposed cells .

This observation was further confirmed by western blot analysis. The modulation of potency in the cells in correlated with the senescence status. In cells cultured for more than 10° passages the level of OCT4 and NANOG proteins progressively declined along the different passages. However cells treated with REAC for 12h hours exhibited higher level of OCT4 and NANOG proteins, as compared to untreated cells at the same culturing passages.

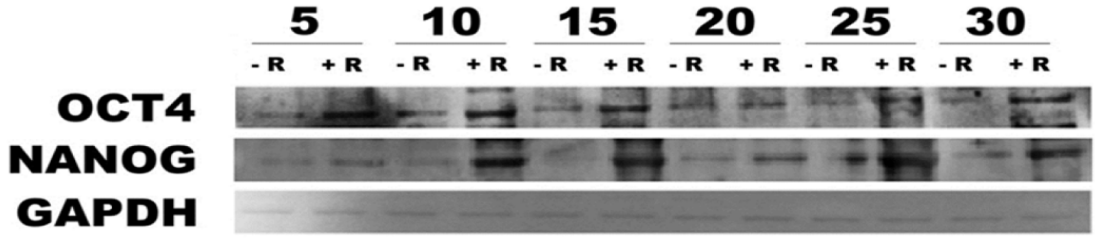

Fig. 13 Effect of REAC on protein expression Oct4 and Sox2*. Total proteins were isolated from ADhMSCs REAC exposed for 12 hours in th (+R) or not exposed (-R). The cells were at passages 5, 10, 15, 20, 25, and 30. The polyclonal antisera against Oct4, Sox2 and GAPDH was used. The sizes of the bands were determined using prestained marker proteins seeblu.. The data presented are representative of five separate experiments.*

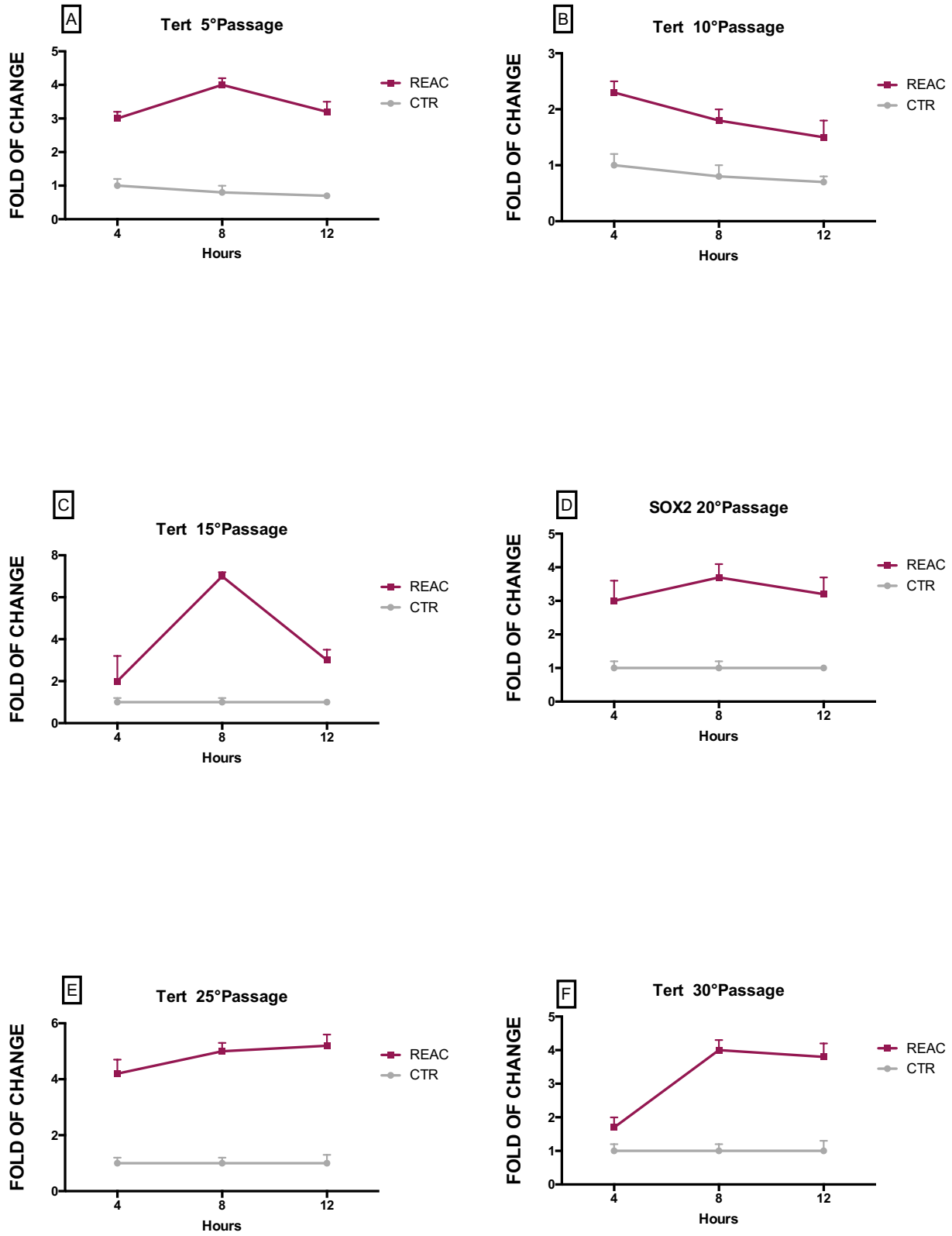

Fig.14 Gene expression of Tert. *ADhMSCs at passages 5 (panel A), 10 (panel B), 15 (panel C), 20 (panel D), 25 (panel E) and 30 (panel F), after 4, 8 or 12 hours in the absence or presence of REAC. The amount of cMyc mRNA from control or REAC-treated cells was normalized to HPRT1, and was plotted as fold change relative to the mRNA expression at time 0, defined as 1. All the REAC-treated cells at each time point were significantly different from each control untreated cells (mean 6 S.E.; n 5 6) (P , 0.05).*

In order to define a possible molecular mechanism through which REAC could exert its effect

on in vitro-induced cell the expression of the Tert was tested. The TERT as well known as telomerase reverse transcriptase is the catalytic subunit of the enzyme telomerase, which, together with the telomerase RNA component (TERC), comprises the most important unit of the telomerase complex.

The gene expression of the catalytic subunit of telomerase (TERT) was assessed by real-time PCR in ADhMSCs cultured at passages 5, 10, 15, 20, 25, and 30 in the absence or presence of REAC energy for 4, 8 or 12 hours respectively. In control cells TERT gene expression progressively decreased throughout the culturing passages. On the other hand, cells exposed to REAC exhibited a different behaviour, with the levels of TERT mRNA being higher as compared to control cells at the same passages. In particular a 4 hour REAC-exposure was not able to induce any effect on TERT gene expression. Nevertheless, at late passages, including passages 25 and 30, a more prolonged, 8–12- hour REAC-exposure was required to achieve the highest enhancement in TERT transcription.

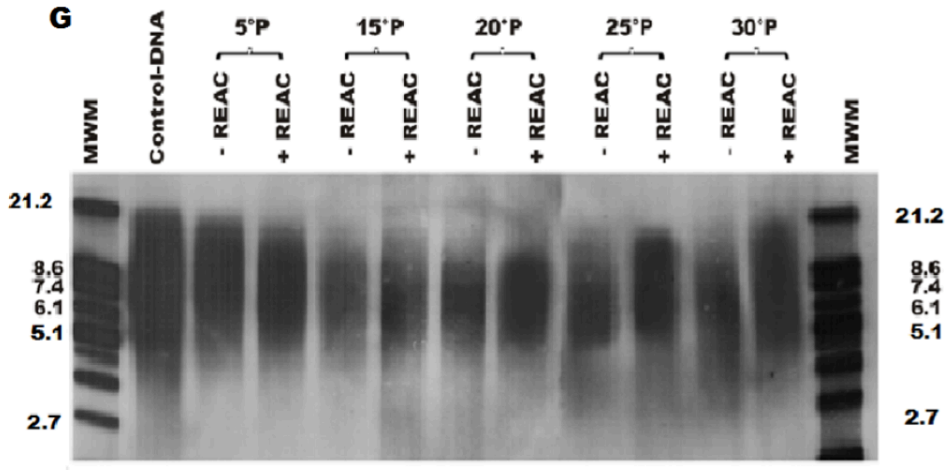

***Fig.15 Telomere length**, the cells at passages 5 (lanes 3, 4), 15 (lanes 5, 6), 20 (lanes 7, 8), 25 (lanes 9, 10), and 30 (lanes 11, 12), were exposed (+REAC) or not (-REAC) for 12 hours with REAC. Lanes 1 and 13 are molecular weight markers (MWM). Lane 2, telomere length assessed at basal conditions (defined as ADhMSCs cultured at passage 1) This figure representative of 6 individual experiments.*

Telomere length was determined using the TeloTAGGG telomere length assay kit, based on

Southern blotting methodology. The membrane with digested telomere DNA fragments shows the difference in length during different passages in culture, it is normal that the telomerase length decrease during cell aging. That it due to the continues DNA replication during cell growing and replication that affect telomere reduction[121]. In this figure 15 control DNA in untreated ADhMSCs (line 3,5,7,9,11) underwent a progressive reduction in telomere length along the different passages, as compared to ADhMSCs cultured at passage 1 (defined as ‘‘basal conditions’’, line 2). Telomere length declined in both untreated and REAC exposed cells between passages 5 and 20 as expected. However, cells at passages 20°, 25° and 30°, exposed to REAC for 12 hours, exhibited higher telomere length as compared to control untreated cells.

REAC SENESCENCE AND HYALURON SINTETASES II

As described above we demonstrated that REAC physical treatment was able to counteract the biochemical and morphological changes occurring in stem cells during aging [175] mainly acting on specific mediator genes, including p16INK4, ARF, p53, and p21CIP1.

Here, in the experiments described below we want to investigate if the effect of REAC on cellular senescence could be exerted by acting on cell polarity. Recent findings demonstrated that in senescent cells there is a down regulation of type 2 hyaluronan synthase (HAS2), that provide a subsequent decrease in Hyaluronic synthesis (HS)[178]. HS plays a fundamental role in cell polarity and hydrodynamic processes that characterized young cells.

In order to demonstrate a possible involvement of REAC on the type 2 hyaluronal synthase (HAS2)-dependent pathway, and thus its related effect on cellular senescence, we stimulated ADSCs at different passages (5, 10, 15, 20, 25, 30), with REAC in the presence of the 4-

methylumbelliferone (4-MU), a potent inhibitor of type 2 HA synthase and endogenous HA synthesis.

RESULTS

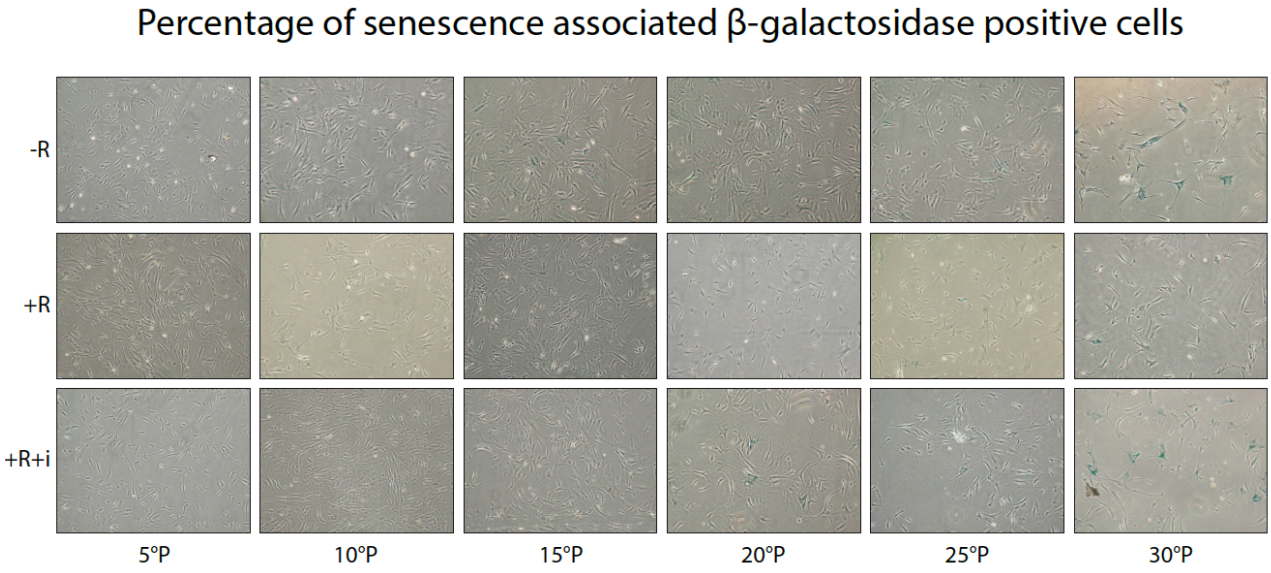

Fig.16 SA-Gal staining in the presence of REAC and 4-MU*. ADhMSCs were exposed for 12 hours in the absence (Control) or presence of REAC (REAC treated), or they were subjected to a 12-hour REAC treatment in the presence of 1 mM 4-MU (HAS2 inhibitor) (REAC treated + 4-MU) during the different passages 5°, 10°, 15°,20°, 25° and 30°. SA-β -Gal staining positive are blue.*

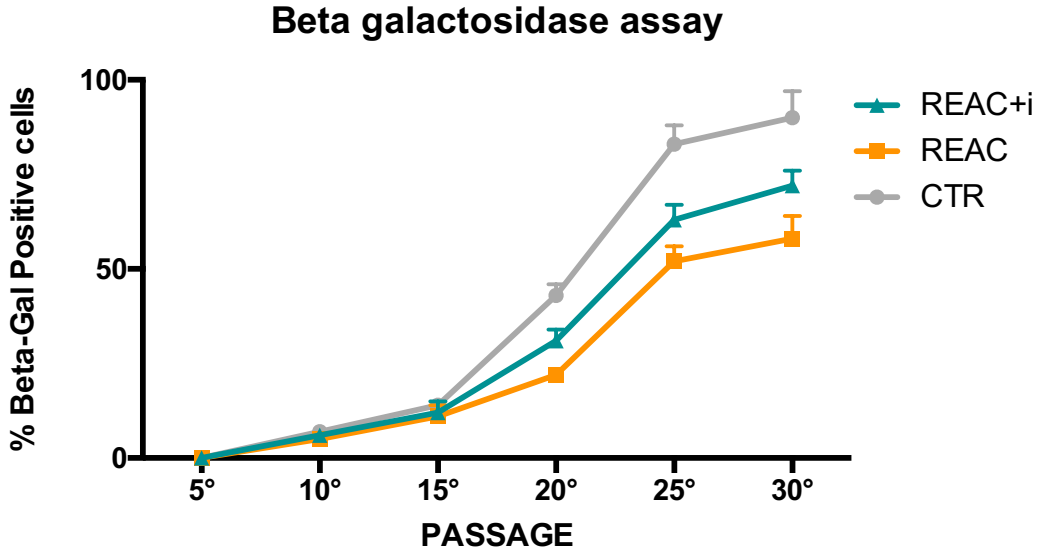

Fig.17 ***Percentage analysis of positively stained cells*** *under each experimental condition, CONTROL gray line, REAC orange line, REAC+I (4-MU) green line. (mean ± S.E.; n = 6; P < 0.05)*

Upon prolonged expansion for 5 to 30 passages, ADhMSCs underwent replicative senescence, as confirmed by the expression of senescence associated β-galactosidase (SA-β -Gal). We confirmed, that the senescence status was detectable already after 15° Passage, and that REAC already at 20°passage provided a beneficial anti-senescence effect, mostly evident during the 30°passage, both in cells cultured in the absence or presence of 4-MU.

GENE EXPRESSION ANALYSIS ON BMI AND PLURIPOTENCY GENES

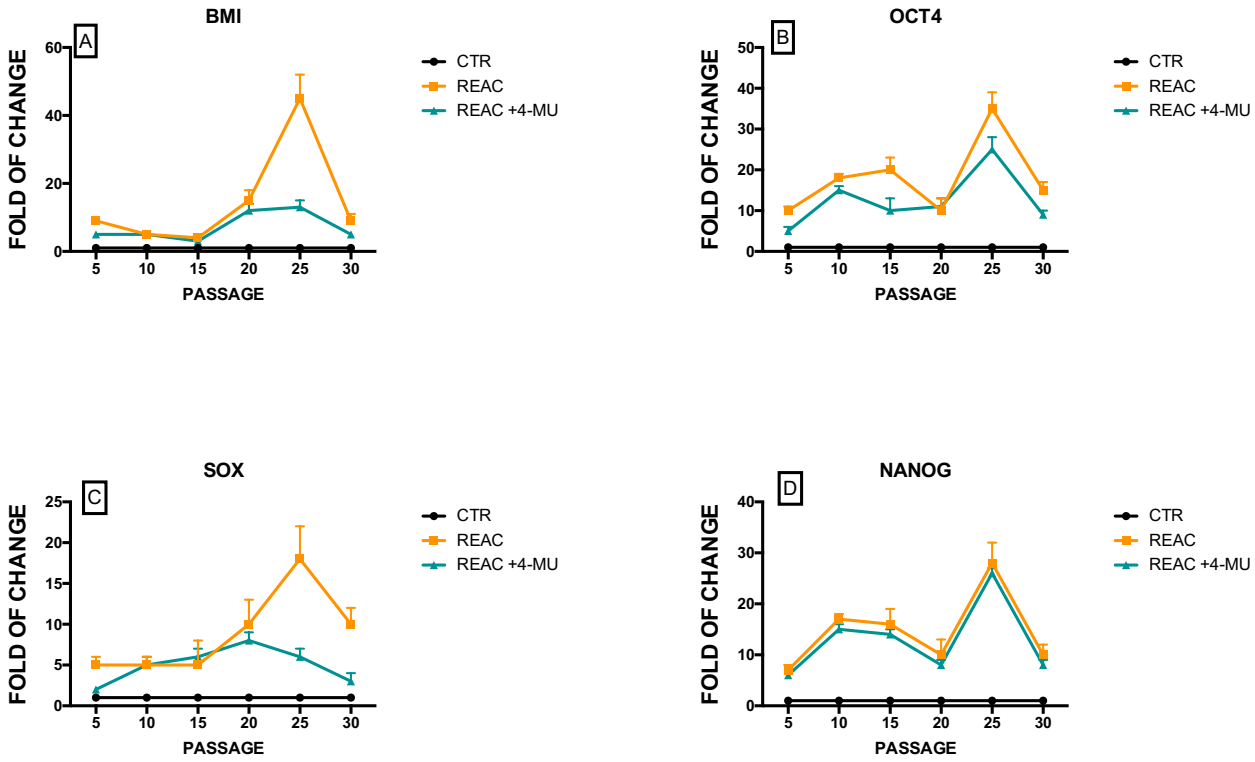

Fig.18 Effect of REAC treatment on the transcription in presence or absence of 4-MU (inhibitor). *At each indicated passage, ADhMSCs were exposed to REAC treatment for 12 hours in the absence (orange line) or presence of 1 mM 4-MU (green line). The mRNA levels of BMI (a), Oct4 (b), Sox2 (B), or Nanog (C) were normalized to HPRT1 and were expressed as fold of change relative to mRNA level at time 0 (unexposed cells at passage 5) black line, defined as 1.. (mean ± S.E.; n = 6; P < 0.05).*

Figure 18 shows that in ADhMSCs cultured up to 30° passage BMI gene expression progressive declined during multiple passages in vitro. BMI is member of a polycomb group (PcG) of repressor, and known as a transcriptional regulator of p16arf and telomere-

independent mechanism involved in senescence,. As previously observed, the REAC treatment, based the on irradiation of radiofrequency at 2.4 GHz, conveyed in an asymmetric loop, was able to interact at the transcriptional level thus interfering with the molecular pathway controlling cellular senescence[175], expression of p16, p53, and p21CIP1. In Physiological conditions, these genes control cell senescence and growth arrest, through their up regulation [179], strictly related to a decrease in BMI gene expression. The REAC treatment, counteracted the BMI-dependent senescence pathway (orange line) , as shown by the upregulation of BMI gene expression both in cells exposed to REAC in the presence or absence of 4-MU BMI (fig.18).

During senescence as well, the pluripotent markers and the potency force turn into drop [121], [138], [179], in previous work we demonstrated that REAC had an effect on OCT3, SOX, NANOG and c-myc [175] gene expression, thus influencing the yield of the adipogenic, condrogenic and osteogenic differentiation processes. In the present work, we attempted at demonstrating the effect of REAC on the preservation of pluripotency markers, in the presence of the HAS2 inhibitor 4-MU. The inhibition of HAS2 is associated with a decrease in HA synthesis in cells, changes in cell polarity, plasticity, potency and associated senescence[178].

As previously described, we observed that the senescence-associated decrease in the pluripotent markers gene expression, was already evident at 10° passage in control adipose derived stem cells, while remaining high in REAC treated cells. Interestingly in cells cultured for multiple passages and exposed to REAC, the presence of 4MU was not able to abolish this effect.

In a second step, we tried to further infer the effect of REAC on the telomerase-associated senescent pathway, by analysing telomerase activity.

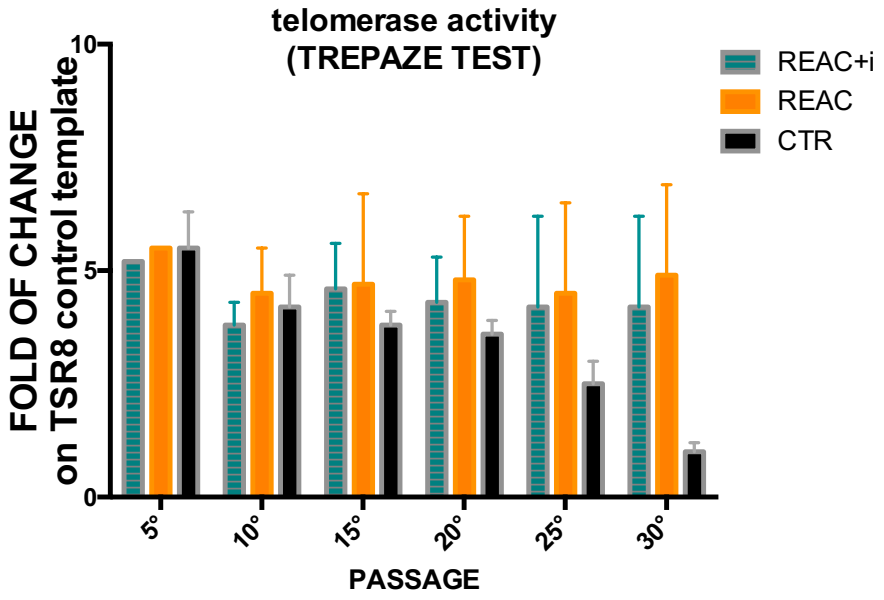

Fig.19 Telomerase activity. *TRAPEZE-RT assay was performed in control untreated cells (black bar) or ADhMSCs or REAC treated for 12 h at the indicated passages in the absence (green bar) or presence of 1 mM 4-MU (orange bar). All data from REAC-treated cells at each time point were significantly different from those in control untreated cells. (mean ± S.E.; n = 6; P < 0.05)*

Telomerase activity from cells at different passages that had been exposed to REAC in the presence of 4-MU was significantly lower than the enzyme activity detected in REAC-treated cells in the absence of the HAS2 inhibitor. As expected, even with this trapeze test based on fluorescence quantification of telomerase enzyme, the activity decreased during the cell passaging and the advance of cellular senescence. These results confirm the strong modulation of cell polarity and of the molecular pathways of senescence, by REAC induced microcurrents and radiofrequency, and highlight a potential role of HAS2 in this anti aging circuit elicited by the machine.

DISCUSSION REAC AND SENESCENCE

One of the most important problem during the regenerative process in vivo and the homeostasis of tissues, in physiological condition is correlated with the wellness of the cells. Several studies demonstrated that senescent cells and stem cells resident in the niche lose their ability to self- renew, to differentiate in specific cell type, and to migrate to the damaged tissues [124], [140], [179]. It is well established that the regenerative capability of stem cells isolated from old patients is lower as compared to cells from younger donors [180]. In the last decades, there has been an increasing effort in exploiting the therapeutic potential of stem cells for the treatment of irreversibly damaged tissues that could not be rescued even by the most advanced pharmacological or surgical treatments. Nevertheless, for the clinical application in regenerative medicine, in cell therapy or tissue engineering, there are still open issues, one of which is the number of cells needed. In fact, in most clinical trials stem cells (mesenchymal stem cells) are exposed to long-term expansion ex vivo, in order to obtain a high yield of viable elements eventually committed toward specific phenotypes with various strategies. However stem cell culturing for multiple passages actually represent a risk because become senescent and lose their peculiar properties. Within this context, the development of innovative approaches capable of counteracting senescence during stem cell expansion in vitro may pave the way to future clinical applications. Identifying a strategy that could revert the molecular cues controlling stem cell senescence in vitro may represent an important progress for future application. In a previous work published by Maioli et al.[175] the effect of REAC technology in modulating stem cell senescence was largely demonstrated. The innovative founding showed that the aging process could be reverted without the aid of viral vectors. The REAC in fact, was able to counteract the biochemical and morphological changes occurring in stem cells during aging[175], as well as to preserve the multilineage

differentiating potential of hADSCs even at late passages. These observation demonstrated that REAC treatment regulated the expression of p16INK4, ARF, p53, and p21CIP1. The inhibition of p16INK4 and ARF is directly correlated with the activity of cyclin-dependent kinase 4 and 6, essential for the G1 progression through the cell cycle, while p53 regulates the expression of p21 CIP1, which inhibits cyclin-dependent kinase2, a regulator of the S phase progression. These evidence, even strongly demonstrated by beta galactosidase assay, opened the path to investigated more in detail the molecular connection and interaction between REAC and cell metabolism during in vitro senescence. The hypothesis behind, is based on a fine epigenetic regulation elicited by REAC on the policomb and tritorax gene expression during the senescence pathway activation [181]. Bmi-1 is a transcriptional repressor belonging to the Polycomb group gene family, the Polycomb group of proteins, and the Trithorax group of proteins, are crucial for maintaining proper gene expression patterns during development and cell growth[182], controlling INK4a locus, which encodes for the p16 and p19ARF (p14ARF in humans)[183].)[182]. In recent findings the expression of BMI-1 resulted essential for the maintenance of plasticity in epithelia cells [184] and in cell fate and polarity[185].

REAC stimulation for 4,8 and 12 hours was able to antagonize the down-regulation of Bmi1 transcription during multiple ADhMSC passages in vitro. That findings suggest the possibility to use a physical milieu to control a part of the molecular patterning that regulates cell senescence throughout the chromatin remodelling processes.

During the senescence process stem cells decrease their ability to differentiate toward the different lineages. It was recently shown that MSCs during senescence, undergo a progressive

decline in the expression of early mesenchymal stem cell markers strongly correlated with BMI, p53, p19ARF and the activity of telomerase[186].

Confirming our previous observations in mouse embryonic stem cells, REAC modulated the expression of Nanog, Sox2, Oct4, and cMyc even in ADSCs cultured for passages 5, 10, 15, 20, 25, 30[101]. In particular even in cells at late passages REAC positively modulated the pluripotency markers. These observations indicate that the REAC technology can actively interfere with a complex molecular circuitry responsible for stem cell self-renewal and cell pluripotency.

Several studies on senescence, control of pluripotency, growth arrest, quiescence in cells demonstrated that a decrease in TERT expression and activity is correlated with a decreased proliferation, and differentiation[187][186], for instance TERT expression and activity is involved in neuronal differentiation during development . Here, we show that REAC exposure during the first passages does not increase dramatically TERT transcription, while a significant increase could be observed at late passages (20–30) as compared to the untreated cells. Interestingly REAC exposure, while not exerting an appreciable effect on the telomere shortening that spontaneously occurs at early passages, was conversely able to counteract the telomere shortening that progresses at late passages, when cell senescence in culture becomes more marked. This behaviour again, could be correlated with the modulation of cell polarity exerted by REAC. Cell polarity could be defined as an asymmetric organization and distribution of biomolecules (proteins, lipids, mRNAs), cellular components (membrane rafts, organelles) and structures (cytoskeletal filaments)and is strongly correlated with differentiation, proliferation, protrusion, motility and cell migration changes[188]. We

believe, that the Radio frequencies emitted from REAC, are able to act on the polarization level of protein, transcription factor, enzymes in the cells. All the molecules, proteins, nucleotides, membranes, organelles in the cells have a chemical charge and electrical properties[189], [188], that could be modulated by energetic forced directly modulated.

For these reason we decided to figure out the possible role of REAC in the modulation of cell polarity during senescence. In particular we dissected a possible involvement of Radio frequency, on the Hyaluronic acid (HA) homing, that plays a fundamental role in cell polarity and hydrodynamic processes, affording significant modulation of proliferation, migration, morphogenesis and senescence[190]. We believe that the modulation of HAS2, the enzyme that produce HA in cells, is correlated with cell polarity and modulation of senescence by REAC.

It is well known, that HAS2 plays a pivotal role in preventing cell senescence, acting as a downstream target at which multiple signaling pathways converge to afford growth factor-mediated maintenance of cell differentiation[191]. For instance, in recent studies it was observed that impairment of differentiation in senescent cells is largely dependent upon the lack of growth factor-mediated induction of type 2 hyaluronan synthase (HAS2), with the subsequent decrease in HA synthesis, and the differentiation potency loss of cells has been shown to be significantly restorable by HAS2 overexpression[192]. HA-mediated signalling is also involved in the regulation of cell polarization which occurs in response to stimuli that promote non-symmetrical subcellular organization to fulfil functional requirements emerging during migration, adhesion, or mitotic spindle orientation[193].

For this purpose, the effect of REAC technology was assessed in ADhMSCs expanded in vitro up to the 30th passage in the absence or presence of 4-methylumbelliferone (4-MU), a powerful inhibitor of HA synthesis acting through HAS2 repression, in order to figure out the implication of REAC in stem cells senescence modulation during HA synthesis control.

According to previous results, REAC was able to modulate the pathway involved in BMI, p53, p16, as well the telomerase activity (TERT) during the aging-induction in Adipose derived stem cells. The strong evidence that REAC can interact with cell fate, plasticity by modulation of cell polarity may disclose unprecedented perspectives to preserve a delicate intracellular nanotopography and revert substantial traits of stem cell aging and disease. This studies shows that REAC technology has an evident force even in the presence of hyaluron synthase 2 inhibitor, and interesting slow down stem cell senescence.

That findings on REAC technology and interaction with the cells in vitro may be useful to counteract the physiological aging in the cells cultured in vitro for clinical application. As well the use of physical stimuli, an absolutely not invasive technology could be directly applied to patient in order to interact on intracellular niche level of stem cell regulation during regenerative processes, even in these old patients which have lost their regenerative capability.

STEM CELL DIFFERENTIATION AND REAC

Previously, we demonstrated the effect of REAC on the Adipose derived stem cells during the senescence pathways. Showing an interesting interaction in the modulation of BMI, TERT activity and preserving the pluripotency expression even in these cells in which senescence was induced with prolonged culturing passages. It was even observed that one of the main modulation acted by REAC was at the cell polarity level.

Cell polarity carry out a fundamental role during cell fate, in terms of proliferation, migration, senescence and differentiation [67], mostly during stem cell differentiation is involved in self-renew, activation and differentiation [68]. One of the most important issue, in regenerative medicine is the proper use of stem cells. For clinical application, there are still countless questions concerning the best type of cells that could be used for clinical purposes. Some studies in animals have shown the usefulness of directly transplanted stem cells, while other studies displayed the possibility to use stem cells previously committed toward a specific cell lineage of interest. Within this context during the years many protocols were developed, in order to obtain a high yield of cell differentiation, with minimal manipulation of the cells, thus minimizing the risks related to prolonged culturing . The way to commit cells to specific pathways are different, and most of the technics are based on the use of chemical inductors or viral vectors. However, other studies demonstrated how the use of physical energies could heavily increase the differentiation of stem cells. For instance, the use of extremely low frequency (ELF)-PEMF was able to commit MSCs toward the adipogenic, osteogenic, neural, or glial linage together with the dedicated media[98], moreover a 50 Hz, 1mT ELF-MFs 5-days treatment induced PC12 cells neuronal differentiation [97]. Ventura's group even demonstrated that the extremely low frequency (ELF) pulsed magnetic fields (PMF) affected

opioid peptide gene expression its related signalling pathways in adult ventricular myocyte[100]. Also the radiofrequency circuit elicited by REAC devices was able to induce the cardiac, neuronal, and skeletal muscle lineages in embryonic mouse cells [101]. Even on Lipogems-derived hASCs the radioelectric asymmetric conveyer (REAC) remarkably enhanced the transcription of prodynorphin, GATA-4, Nkx-2.5, VEGF, HGF, vWF, neurogenin-1, and myoD, indicating the on commitment toward cardiac, vascular, neuronal, and skeletal muscle lineages respectively [102].

To the evidence of these findings, in this part of the thesis I will show how the Radio frequency stimuli can be used to induce specific phenotype, increasing the cell proprieties and the yield of differentiation.

REAC AND PC12 NEURONAL DIFFERENTIATION

To better figure out the REAC effects on stem cells or on cells that have the ability to transdifferentiate toward another phenotype, PC 12 were use as a cellular model. This model was widely used to study neuron functions and to understand the physiology of central dopamine (DA) neurons. PC12 cells are a rat pheochromocytoma tumor cell line, that respond to nerve growth factor (NGF) by differentiating to sympathetic neurons. After differentiation toward neuronal linage, extend neurites, become electrically excitable and express neuronal markers, and cease division[194]. The PC12 are still considered a valuable model through which it is possible to study the mechanism underlining neuronal differentiation. With this claim, radiofrequency produced by REAC were applied, in order to figure out the possible

implication of this device during the commitment toward the neuronal linage, without the interference of other lineages[101], [102].

MATERIALS AND METHODS

PC12 cell culture.

PC12 cells were cultured in tissue plates in Dulbecco's modified Eagle's Medium F12 (DMEM) with 10% HS, 5% FBS and a 1% of Streptomycin/Penicillin, inside the incubator in humidified atmosphere of 5% CO2 at 37 °C. In a separate incubator, with the same characteristics, PC12 were exposed to REAC for 24, 48, 72, 96, 120, 144, 168 or 192 hours, in order to evaluate a possible effect of REAC on the different stages of the differentiation process.

REAC SETTING

The cells were put at a distance of 35 cm from the 2.4 GHz. emitter, we measured a radiated power of approximately 400 μ W/m2. Electric field E = 0.4 V/m, magnetic field = 1 mA/m. Specific absorption rate (SAR) = 0.128 μ W/g. The REAC electromagnetic quantities have been measured with the spectrum analyzer Tektronix model 2754p (TekNet Electronics, Inc., Alpharetta, GA, USA), orienting the receiving antenna for maximum signal.

Cell Viability Assays. PC12 cells were seeded in 24-well plates (60×10^3 cells per well) and then treated or not (control) for 24 h and 8 days (192 h) with REAC. Experiments were done in technical replicates. Cell viability was assessed by the 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5, diphenyltetrazolium bromide (MTT) assay. We are able to evaluate viable cells by using a test based on the conversion of the soluble dye MTT to insoluble (in aqueous media) blue

formazan crystals. Briefly, 1 mg of MTT (200 µ l of a 5 mg/ml stock solution in phosphate buffered saline (PBS)) was added per ml of medium , then incubated at 37 °C for 4 hours. The MTT was removed and the cells were washed with PBS, the supernatant was dissolved in isopropanol an after 5 min the color was read at 600 nm using a Bauty Diagnostic Microplate Reader.

Morphological analysis. Microphotographs of PC12 cells from untreated cultures (control) and from treated ones were taken after 96 hours of REAC TO-RGN treatment to track any morphologic changes. Collected images were loaded into ImageJ (National Institute of Health, USA) and analyzed to measure neurite length.

Gene expression analysis. PC12 cells were cultured in the absence or presence of REAC for 24, 48, 72, 96, 120, 144, 168, 196 hours. Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen). Total RNA was dissolved in RNAase-free water and, for RT- PCR, cDNA was synthesized in a 50-µ l reaction volume with 1 µ g of total RNA using Superscript Vilo cDNA synthesis kit, according to the manufacturer's instruction (Life Technologies). Quantitative real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad). Two µ l of cDNA were ampli- fied in 50-µ l reactions using Platinum Supermix UDG (Invitrogen), 200 nM of each primer, 10 nM fluorescein (BioRad), and SYBR Green. After an initial denaturation step at 94 °C for 10 min, temperature cycling was initiated. Each cycle consisted of 94 °C for 15 s, 55– 59 °C for 30 s, and 60 °C for 30 s, the fluorescence being read at the end of this step. To evaluate the quality of product of real-time PCR assays, melting curve analysis was per-formed after each assay.

Relative expression was determined using the "delta-CT method" with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene.

Primers used for the analysis of neurogenesis (Neurogenin-1 and beta 3 tubulin) and nerve growth factor (NGF) were specific and spanning exons, are reported table 1.

Immunocytochemistry. Cells were cultured for 7 days with or without treatment on glass coverslips (Falcon) coated with 0.2% type III Collagen at low density to allow visualization of individual cells. Aſter 7 days, cells were fixed for 20 min in 4% paraformaldehyde (Life Technologies). The cells were washed 3 times with phosphate-buffered saline (PBS) with 0.1% Triton X-100 in order to permit the permeabilization, and then incubated with the primary antibody overnight at 4 °C. The cells were then incubated with the secondary antibody for 1 hour at room temperature. To verify differentiation toward a dopaminergic neuronal phenotype, the following antibodies were used: mouse monoclonal anti-ß-3 tubulin (Santa Cruz Biotechnology); rabbit polyclonal anti- tyrosine hydroxylase (Santa Cruz Biotechnology). The secondary antibodies used in this study were: Alexa 594-conjugated goat-anti-rabbit IgG (1:400; Life Technologies) and anti-Mouse IgG (whole molecule) − TRITC antibody produced in goat (Sigma-Aldrich). All microscopy was performed with a Leica confocal microscope (LEICA TCSSP5). DNA was visualized with 1µ g/ml 4',6-diamidino-2-phenylindole (DAPI).

Western blotting. PC12 were cultured in the presence or absence of REAC for 24, 48, or 72 hours and for 7 days in 100 mm tissue culture plates. Total proteins isolated with RIPA buffer, were quantified using BCA assay (Roche). 40 ug of proteins were electrophoresed on 10% Novex Tris-glycine polyacrylamide gels (Invitrogen) in MOPS sodium dodecyl sulfate

running buffer using an XCell SureLock™ Mini-Cell (Invitrogen) according to the instructions provided by the manufacturer. After protein transfer to polyvinylidenedifluoride membranes (Invitrogen) by iBlot (invitrogen) the membrane was saturated with 3% BSA-PBS solution and then washed 3 times with PBS-1%tween 20. The incubation of the membrans with specific antibody, antisera against β Tubulin isotype III (Cell Signaling technology), NGF (Santa Cruz Biotechnology, Inc.), TH (Santa Cruz Biotechnology, Inc), Neurogenin (Santa Cruz Biotechnology, Inc), and GAPDH (Santa Cruz Biotechnology, Inc.) diluted to 1:1000 was carried out for one hour at room temperature. After additional washing, membranes were incubated with secondary anti- rabbit (NGF ,GAPDH) or antimouse (β Tubulin isotype III) or antigoat (Neurogenin). Targeted protein expression was assessed using a chemoluminescence detection system (ECL Western blotting detection reagents were from Amersham Biosciences Corporation, Piscataway, NJ, USA).

Statistical analysis. Statistical analysis was performed using the software package SPSS (Statistical Package for Social Science) version 13.0. For this study, were applied to the data collected, the nonpara- metric Kruskal-Wallis test, Jonckheere-Terpstra and Wilcoxon test: the first two to assess the adequacy of treatment, i.e. the differences (DeltaDelta CT) between the data collected in treated and in control. The Wilcoxon test was applied to assess the adequacy of the data of each group in the different observation times. The tests and results with $p < 0.05$ were considered statistically significant. For the adequacy of the data, the results of the Wilcoxon test showed a high statistical significance in all observations (Asymp. Mr. 2-tailed < 0.05). For the congruity of the treatments, the tests were applied to 3 sets, in eight moments of observation, after the treatment and control conditions. The analysis shows a

statistical significance $p < 0.05$ in the treated (Kruskal-Wallis test: Asymp. Sig. 2-tailed < 0.417 and Jonckheere-Terpstra test: Asymp. Sig. 2-tailed < 0.295) and $p > 0.05$ in controls. (Kruskal-Wallis test: Asymp. Sig. 2-tailed = 0.7 and Jonckheere-Terpstra test: Asymp. Sig. 2-tailed > 0.75).

RESULTS

Morphology of PC12

A morphological study of PC12 cells was performed after 96 hours and 192 hours of REAC exposure, unconditioned PC12 cells didn't show any significant change in morphology, during 96 and 192 hours, on the other hand the REAC treatment enhanced neurite-like outgrowth processes. The REAC-exposed group had longer neurite outgrowths and more neurite bearing cells, as compared PC12 control cells, already visible after 4 day under treatment.

The lenght neurites analysis performed with ImageJ showed and increase in neurite length by the fourth day in treated cells compared to untreated cells. Indeed starting from the fourth day of treatment these cells exhibited longer cellular processes (neurites)($P < 0.05$) than those eventually present in the untreated cells, that evidence could be correlated with the different changes in cell polarity elicited by REAC.

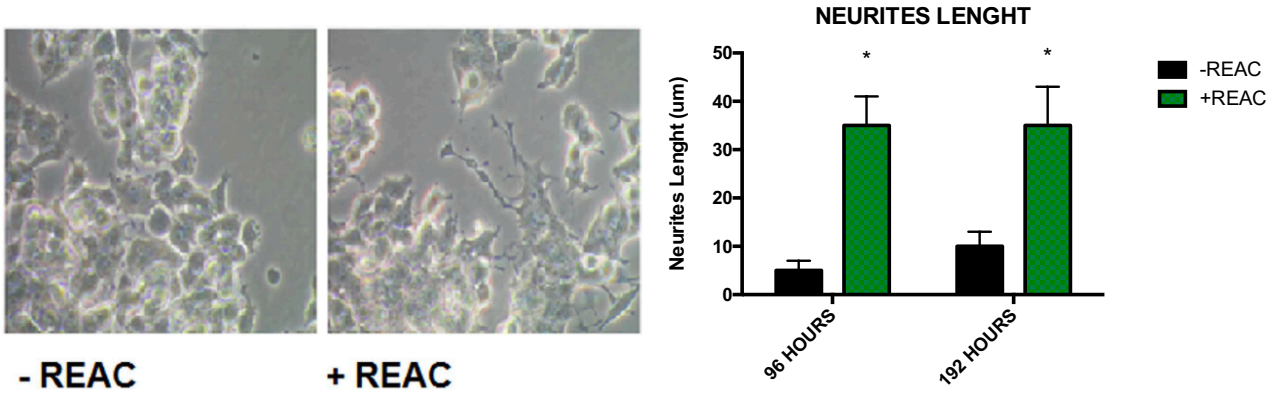

Fig.20 Morphology of PC12 *in phase-contrast microscope picture (already published on SCIREP DOI: 10.1038/srep10439) The picture shows untreated cells (-REAC) and treated cells with neurites at 96 hours(+REAC) . In the right, the Neurite length was measured. The morphological changing as neurite growth is shown in control (black bars) and REAC treated cells (green bars) after 96 and 192 hours.*

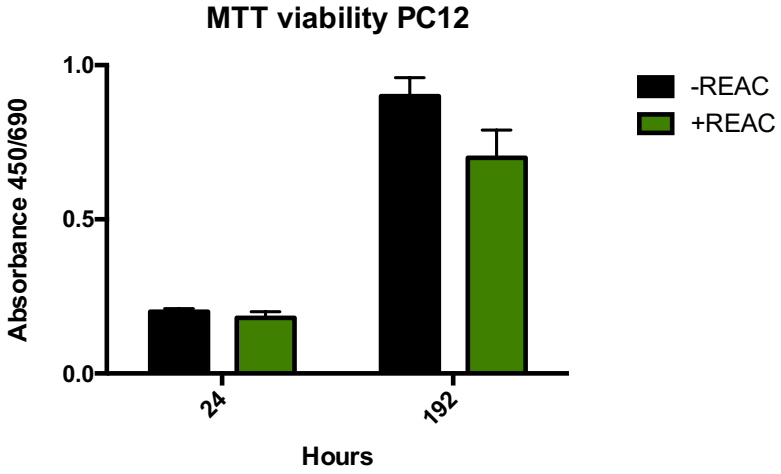

Fig.21 MTT on proliferative cell during differentiation*. Cell proliferation analysis were performed 24 and 192 hours after culturing PC12 cell, control (black bars) and REAC treated cells (green bars).*

The MTT analysis for the 24 hours, didn't show significant difference in the cell viability and growing, however shows that REAC treatment is not toxic for PC12 (fig.21). After 192 hours the number of PC12 treated with REAC, were significantly less compared to controls. That behaviour is strictly connected with the number of cells that are differentiating toward neuronal linage, in fact a decrease in the proliferation activity, is associated with an increase in differentiated cells, as demonstrated in another study, performed on PC12 treated with physical energy force (ELF) with low electromagnetic 11 mT and 50 Hz frequency[97].

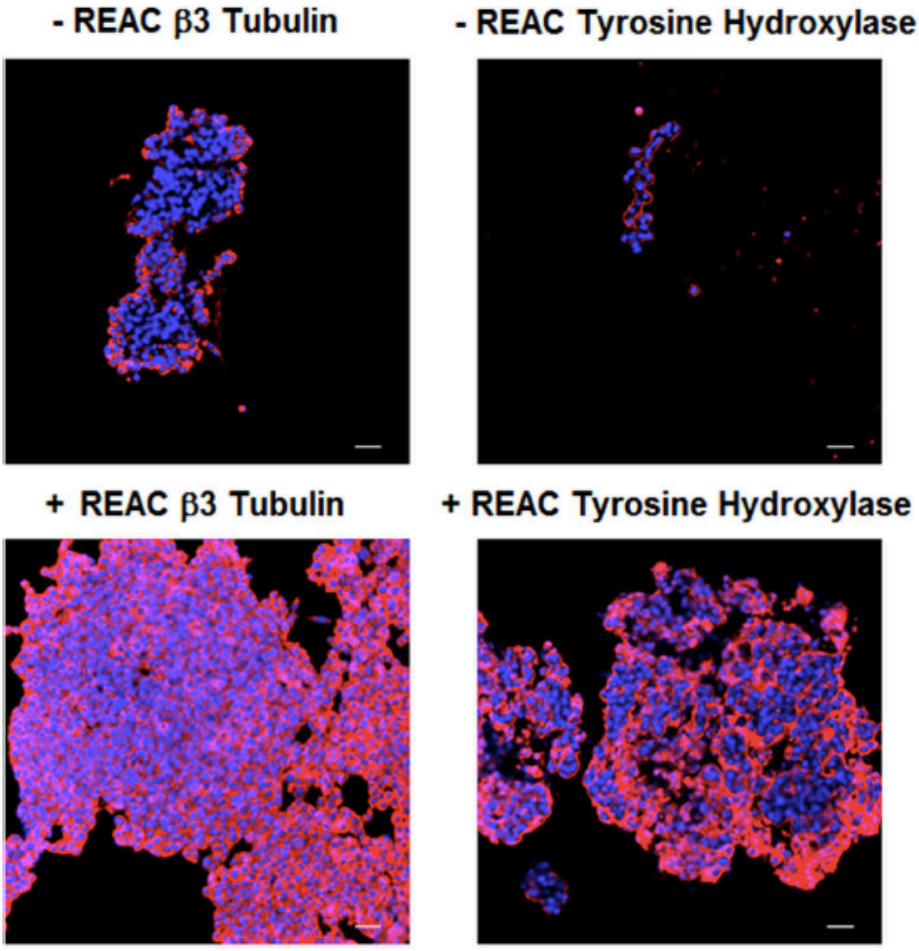

Fig.22 PC12 differentiation toward neural phenotype. Expression of β -3-tubulin and Tyrosine Hydroxylase were assessed in cells cultured in the absence or presence of REAC TO-RGN treatment, for 168 h (7 days). Nuclei are labeled with DAPI (blue). Scale bars are 40 μ m. Representative of five separate experiments. For each differentiation marker, fields with the highest yield of positively stained cells are shown (already published on SCIREP DOI: 10.1038/srep10439)

The immunocytochemistry analysis revealed that PC12 cells treated with REAC, expressed the neuronal marker β3- tubulin and the tyrosine hydroxylase, even when cultured in the absence of NGF, after 7 days. These two enzymes are essential for catecholamine biosynthesis and for the neuronal differentiation, however a weak expression of this markers was revealed even in control untreated cells, that finding indicate that pc12 exhibit an intrinsic trend to commit toward the neuronal phenotype. However, the positively for β3- tubulin and the

tyrosine hydroxylase markers in PC12 treated was dramatically higher as compared to the controls.

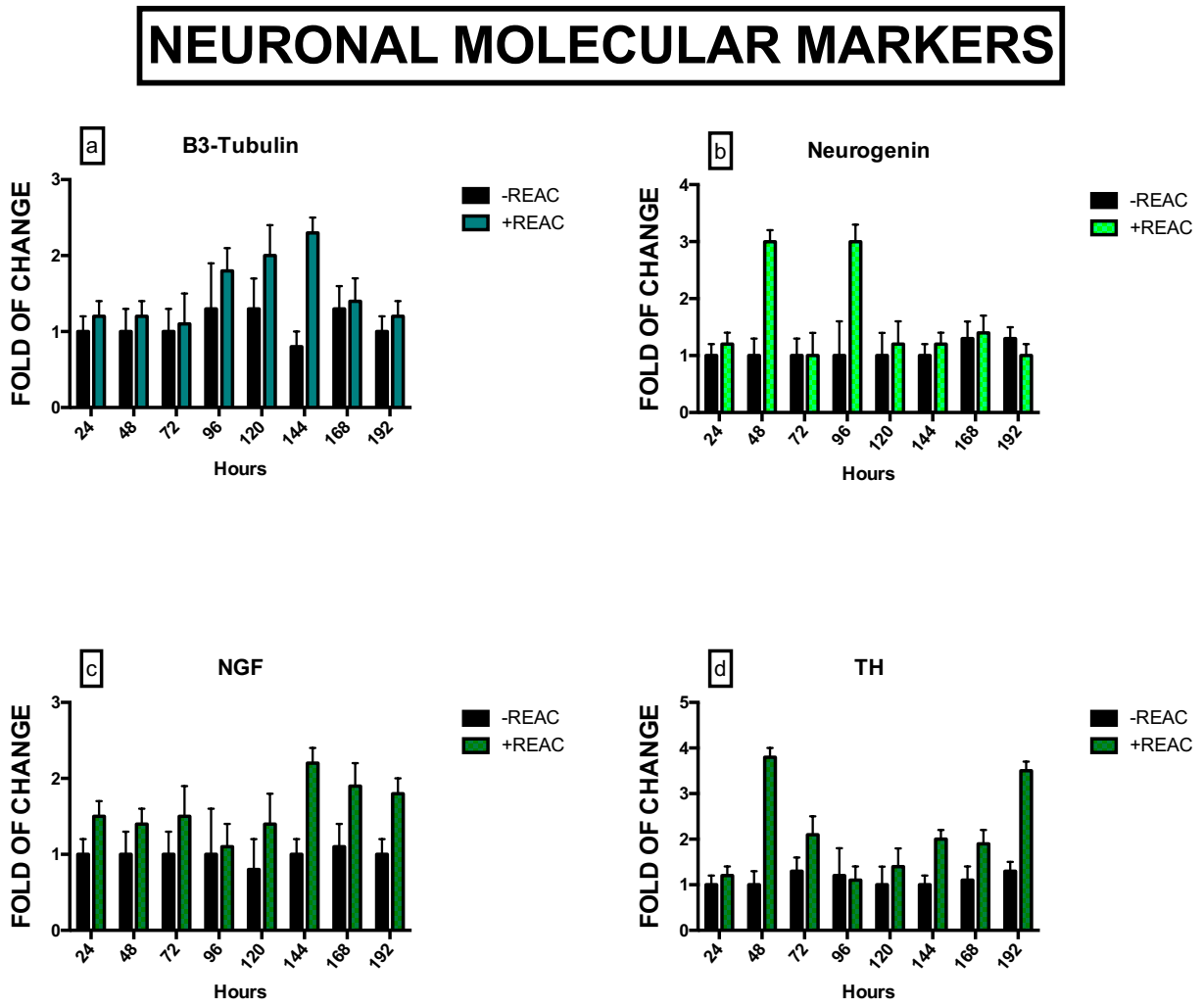

Fig.23 Gene expression during different time point in neuritogenesis regulating genes in PC12 cells. Cells were exposed from 1 (24 h) to 8 days (192 h) in the absence (black bars) or presence (green bars) of REAC. The amounts of β-3 tubulin (a), neurogenin-1(b) NGF(c) and tyrosine hydroxylase (TH) (d) mRNA from REAC or untreated cells were normalized to GAPDH, and the mRNA expression was plotted at each time point as fold of change relative to the expression in PC12 untreated cells cultured for 24 hours defined as 1 (mean ± S.E.; n = 6). All the REAC treated cells at each time point were significantly different from each control untreated cells (mean ± S.E.; n = 6; P < 0.05).

After morphologic analysis, the neuritis formation, typical of the differentiated cells, was observed in cells treated with REAC. In order to confirm the real differentiation of PC12 the expression of beta-tubulin and tyrosine hydroxylase, the most important markers during

neuronal differentiation, was assayed by immunoisthochemistry. Interesting, we found an increase in the expression of these proteins in the cells that received the biophysical stimuli.

However, the gene expression analysis during the different experimental time points, shows and confirm the expression of the neurogenic phenotype associated genes β 3-tubulin and neurogenin-1. The Beta 3-Tubulin expression was higher in cells treated with REAC already after 96 hours, as well the level of TH mRNA was detectable higher 48 hours and 192 hours after REAC radio frequency energy irradiation. The expression of the other most important neuro-markers, NGF and Neurogenin were investigated, in order to strongly corroborate the previous observations.

Nerve growth factor (NGF), is a neurotrophic factor and neuropeptide involved in the regulation of growth, maintenance, proliferation and survival of neurons, first described by Italian nobel prize Rita Levi-Montalcini in 1956[195]. It was found that NGF, was significantly up regulated in PC12 cells exposed to REAC for 24 h (1 day) to 192 h (8 days), as compared to the control. Moreover the neurogenin-1, a transcription factor involved in neurogenesis and neuronal differentiation, was significantly increased in treated as compared to untreated cells, already after 48 hours until 96 hours. That behaviour is typical of the neurogenic commitment, where Neurogenin drives toward neuronal differentiation.

PROTEOMIC VALIDATION

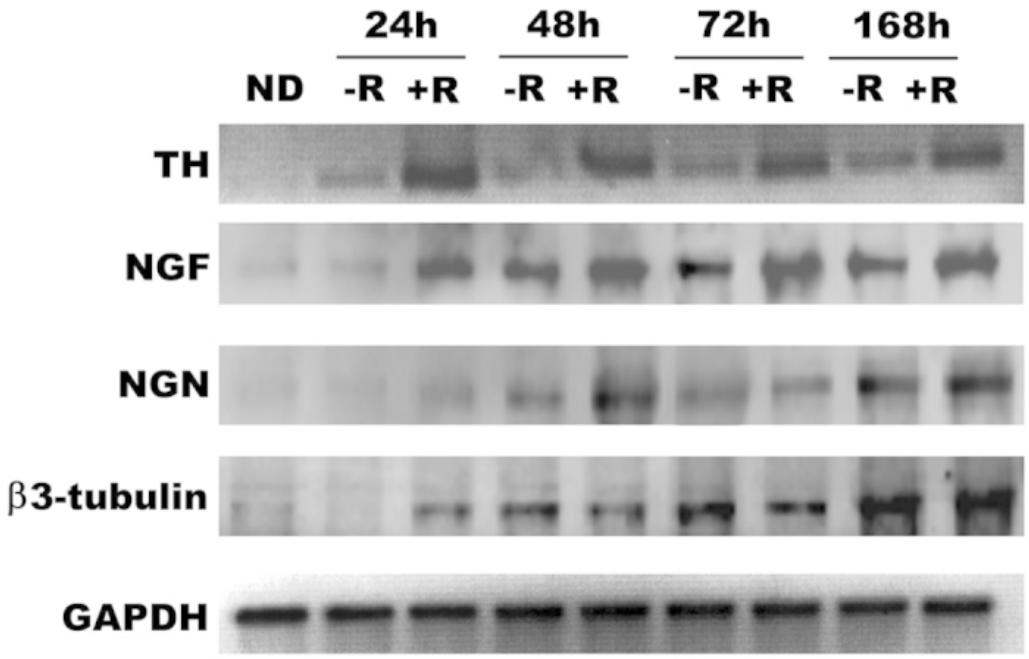

Fig.24 Western blot analysis on total proteins isolated in PC12 cells*. Total lysates were isolated from PC12 cells exposed for 24, 48, or 72 hours and for 7 days in the absence (− R) or presence of REAC (+ R). The antibodies used were tyrosine hydroxylase (TH), NGF, neurogenin 1 (NGN), ß-3 tubulin and GAPDH. The sizes of the bands were determined using prestained marker proteins. The data presented are representative of five separate experiments. Two different membranes were incubated with antirabbit (NGF ,GAPDH) or antimouse (β Tubulin isotype III) or antigoat (Neurogenin). (already published on SCIREP DOI: 10.1038/srep10439).*

In order to verify the protein level translated western blotting analysis was performed on the same genes analysed by Real-time PCR. Tyrosine Hydrosilase and NGF were expressed at 24, 48, 72, 162 hours in both REAC-treated and untreated cells, however in REAC-exposed cells the protein expression was higher. The β 3-tubulin was detected already after 24 hours, strengthening that REAC acted by increasing neuronal differentiation, during the early phases of the neural commitment. Neurogenin (NGN), whose gene expression was higher already

after 48 hours in REAC-treated cells, exhibited even higher protein level at 48 hours of REAC exposure.

DISCUSSION

In several published studies, the use of physical energies and mostly electromagnetic field radiation has been reported to modulate adult neurogenesis and central nervous and neuroendocrine functions [196][197], In addition it was demonstrated the implication of physical stimuli during neuronal differentiation at epigenetic level by the modulation of histones modification and of the transcription factor cAMP response element-binding protein (CREB), NeuroD1 and Neurogenin1[165]. In addition, magnetic fields have been shown to induce neurite outgrowth and neuronal differentiation in PC12[198][199].

Even the radio electric asymmetric conveyor (REAC) technology, was able to induce neurogenic cell differentiation both in cultures of murine embryonic cells [101] and in adipose derived stem cells [102]. In this work we show the effect of REAC effect on PC12, a model cell able to differentiate in neuronal cells, in order to study more in details the neuronal differentiation induced by REAC without other interferences.

Here we demonstrated that PC12 REAC exposure primes cell commitment toward a neurogenic phenotype and induced the appearance of neuron-like cells. The neuritogenesis was more visible in cells treated with REAC, this behaviour could be linked with the microcurrents produced by the device, that in somehow implemented cell polarity organization in cells. The neurogenesis was more relevant in these cells that received the physical stimuli, as demonstrated by gene expression analysis which highlighted higher

mRNA levels neurogenin-1, an important transcription factor regulating neurogenesis[200] , of the specific markers for neuronal phenotype, β 3-tubulin, of TH a specific enzyme involved in the dopaminergic pathway, and of NGF , the latter being the most important neuronal growth factor involved in the viability, maintenance and survival of neuronal cells.

The expression of this neural specific genes, was confirmed by proteomic analysis and assessed by immunofluorescence. Neuronal differentiation, was even associated with another interesting observation in term of cell proliferation.

The number of cell differentiated to neuronal-like cell was related to the increase of NGF, as and inversely related to the number of proliferative cells. In fact, the cells that undergo neural differentiation, decrease their capacity to replicate. This behaviour is strictly connected with neuronal physiology.

The data collected in this study, confirm the effect of the REAC treatments on neuronal differentiation, previously observed in the induction of specific differentiation processes in stem cells[101], [102]. Furthermore, the results obtained in this study, might suggest a possible future application of REAC on the control or increase of the yield of neuronal differentiation in stem cells in vitro, or for therapeutic future application, or to finely tune neuron differentiation used as models to test drug or study the development.

As well this results could open the path for clinical application of REAC as adjuvant for the treatment of neurodegenerative diseases directly on patients.

REAC AND CARDIAC COMMITMENT ON UIPSCs

Radio Electric Asymmetric Conveyer (REAC) has already been shown to enhance the transcription of stemness genes and consistently induce cardiogenesis, neurogenesis and skeletal myogenesis in mouse embryonic stem (ES) cells[101] in human adipose derived mesenchymal stem cells (ADhMSCs)[102], as well the neurogenesis in PC12 cells. This application directly on cells, provide an evident action and modulation of their fate. REAC strongly interact during the differentiation of stem cells, modulating the principal pathways involved during commitment. Based on this evidence, in the present study a possible role of REAC as positive enhancer toward a specific phenotype was investigated. For this reason we used a combination with specific chemical inductors. The iPS cells represented a perfect candidate for this type of experiments. The induced pluripotent stem cells (iPSCs)[25], infact has notably transformed the vision and the future application of stem cell in regenerative medicine. Since this discovery, many investigators have focused their efforts on developing strategies to efficiently and reliably direct stem cell differentiation towards the different linages, toward specific cells of interest, using many strategies based on the use of chemical inductors, as repressor of specific pathways, or agonist of specific proteins, or biological strategies using viruses, retroviruses, plasmids in order to induce the expression of specific protein involved in the commitment. The use of specific miRNA, or episome are strongly demonstrating that can have an effect in the control of stem cells, and IPS differentiation.

However most of the protocols published cannot provide a 100% yield of differentiation, this could rise several issues for cells therapy and regenerative medicine.

In fact, the use of IPs cells, that are not completely differentiated should implicate important risks for patients. The IPs cells, are amazing and promising cells that have the ability to

differentiate into all lineages[25], but can also induce teratome formations [113]. Based on this important point, the control of yield and stability of cells and mostly IPS cells during differentiation is one of the most important issue [146] for future clinical trials and application.

Previous studies, directed on the modulation of physiological behaviour of cells, during senescence and mostly on differentiation, showed the ability of physical devices as ELF and REAC, to commit mouse embryonic stem cells[101] and adipose derived stem cells into heterogenic population composed by cardiac, neuronal and skeletal myogenic lineages. As well, here in this thesis I have show the capability of REAC to increase the differentiation of PC12 cells toward dopaminergic neurons, able to produce typical dopaminergic enzyme as Tyrosine hydrosilase (TH), as well as neurogenin 1 and nerve growth factor.

The present study aims at investigating the effect of REAC technology, in combination with WNT-ihibitor, BMP4, activin A [201] during iPSC commitment into cardiomyocite-like cells, in order to obtain a high-throughput and highly reproducible differentiating outcome, that can be usable in the modulation and control of IPS differentiation.

MATERIAL AND METHODS

hUiPSC culture

The hUiPSC line was developed from urine-derived cells in EverCyte lab according with the published protocol[202]. Cells were routinely grown on Matrigel Matrix (Invitrogen) in mTeSR_1 medium (STEMCELL Technologies) at 37°C degree in an incubator with 5% CO_2

and 3% O_2. Cells were subcultured using STEMPRO ACCUTASE (Life Technologies) and ROCK-Inhibitor Y-27632 (Enzo Life Sciences).

Cardiomyocyte differentiation.

Undifferentiated hUiPSCs were digested into smaller clusters using ACCUTASE and seeded onto Matrigel-coated plates at $3 \cdot 10^5$ cells/10 cm^2 in mTeSR1 medium until they reached 80%–90% confluence. Thereafter, the cells were digested into single-cell suspensions and seeded into ultralow-attachment six-well plates (Corning) and cardiac differentiation was induced as described in Weng protocol[201]. Briefly, cells were kept for 24 hours in mTeSR1 medium with Matrigel (40 mg/mL), BMP4 (1 ng/mL; Invitrogen) and Rho kinase inhibitor (ROCK) (10 mM; R&D) under a hypoxic condition with 5% O2. Then, the first group of cells was washed and replaced in cardiogenic medium (Medium C -REAC), containing: StemPro34 SFM (Invitrogen) with ascorbic acid (AA, 50 mg/mL; Sigma), 2mM Gluta-MAX-1 (Invitrogen), BMP4 (10 ng/mL), and human recombinant activin-A (10 ng/mL; Invitrogen). The second group of cells (Medium C+REAC) was replaced in the presence of the cardiogenic medium and additionally exposed to REAC for 72 hours. On day 4 both groups of cells were incubated with IWR-1, a Wnt inhibitor (5 mM; Enzo Life Sciences). On day 8, all cells were transferred to a normoxic environment and maintained in cardiogenic medium until the end of experiment (day 14). Cardiac mesodermal cells organized in functional contracting clusters were detected as early as day 8.

RNA extraction, cDNA synthesis, and gene expression analysis by real-time PCR.

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Sigma). Total RNA was dissolved in RNAase-free water and quantitated using a Nanodrop.

A twenty-µl reaction volume containing 1 µg total RNA was reverse transcribed using HiCapacity cDNA reverse transcription kit (Applied Biosystem) with oligo (dT) primers. qPCR reaction was performed using Rotorgene machine (Qiagen) with EvaGreen mix (Biolab).

After an initial denaturation step at 95°C for 15 min, temperature cycling was initiated. Each cycle consisted of 95°C for 15 sec, 53-59°C for 30 s, and 72 °C for 15 sec, the fluorescence being read at the end of this step. All primers used in this work were from Invitrogen and are listed in Table 1. PCR products were confirmed by melting curve analysis and electrophoresis. All measurements were done as technical quadruplicate of biological triplicates. Biological replicates were obtained from independent differentiation setup. Relative expression was determined using "2^ΔΔCt method" with glyceraldehyde 3-phospate dehydrogenase (GAPDH) as normalizing expression levels.

Immunostaining.

hUiPSCs (Embryo bodies clusters), cultured in the cardiogenic medium with or without REAC exposure for the first 72 hours, were disaggregated at day 14 by collagenase 1 (Invitrogen) for 1 hour at 37°C, and the resulting suspension was cultured at low density to visualize individual cells. The cultures were fixed with absolute methanol for 30 min and 1 hour at -80°C. Cells were then exposed to antibodies against α-sarcomeric actinin (Mouse, Sigma, Clone EA-53), Cardiac troponin T (Mouse,Thermo scientific, 1F11) GATA4 (Mouse,

Santa Cruz, sc-25310), MEF2C (Rabbit, Thermo scientific, PA5-28247) or to TBX5 (Rabbit, Thermo scientific, PA5-29845) for 1 hour at 37°C cells, followed by staining with the respective secondary antibodies anti mouse Alexa fluor 594 or anti rabbit Alexa fluor 498 at 37°C for 1 h. All microscopy was performed with a Leica confocal microscope (LEICA TCSSP5). DNA was visualized with DAPI (1 μg/ml).

Counting of beating cells

Cells seeded in ultra low-attachment six-well plates (Corning) aggregated in EBs clusters were examined under an inverted live microscope (Leika) at 37°C at different time points. In each well (9.5 cm2) total EBs were counted at a field with an area of 1 mm2. Ten to fifteen fields in each well were randomly chosen and counted. The percentage of contractile EBs was determined as the number of EBs that showed spontaneous contraction divided by the total number of EBs plated.

RESULTS

MORPHOLOGY

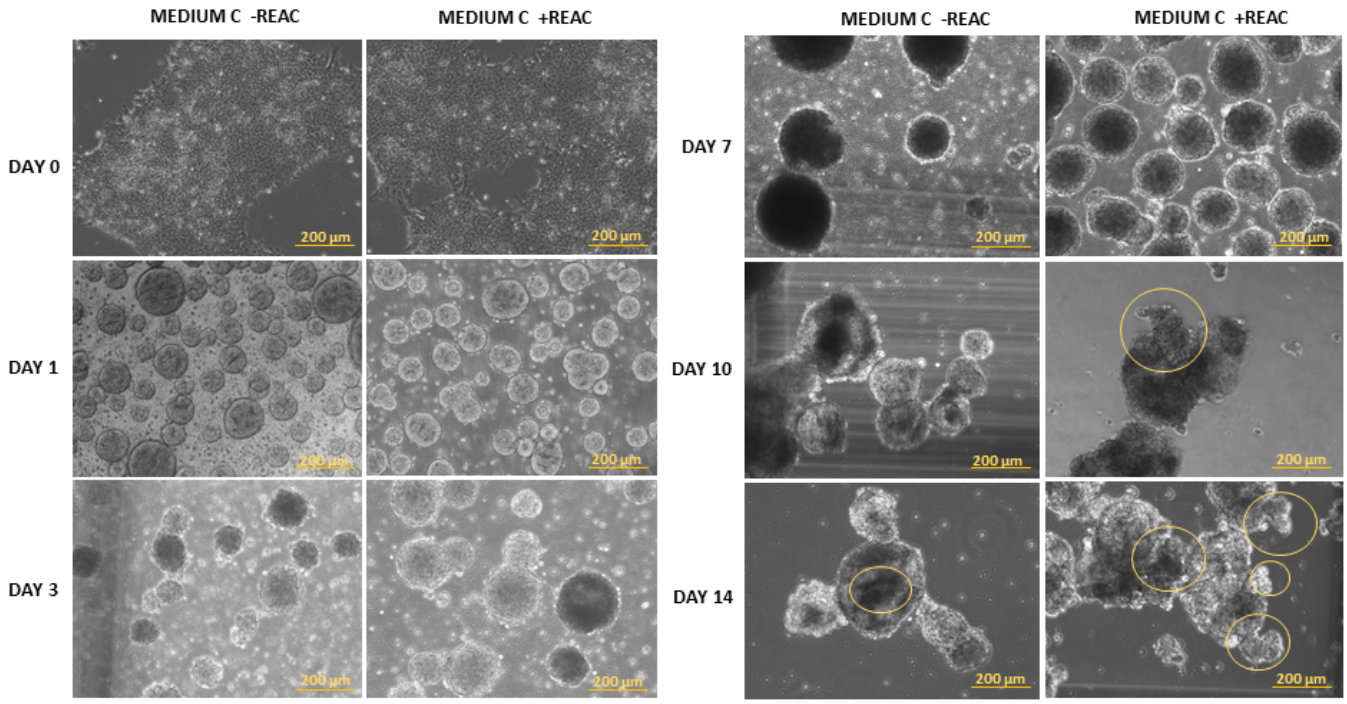

***Fig.25 UiPSCs morphology during different time point observation**, in cells treated with MEDIUM C+ REAC, and without REAC.*

UiPSCs changed their morphology when cultured with differentiation medium with or without REAC exposure, that cells cultured for a period of 14 days progressively changes their morphology, and develop clusters of cardiomyocyte like cells. The morphology of the cells treated with the cardiogenic medium and REAC (Medium C+REAC) appeared brighter and with homogenous size during the first days of embryoid body formation. The beatings clusters were observed at day 8, the cells treated with Medium C+REAC appeared morphologically different in the beating cluster zone as compared to cells treated with Medium C alone, this behaviour could be correlated with microcurrents emitted by REAC that could modulate the polarity inside the cells.

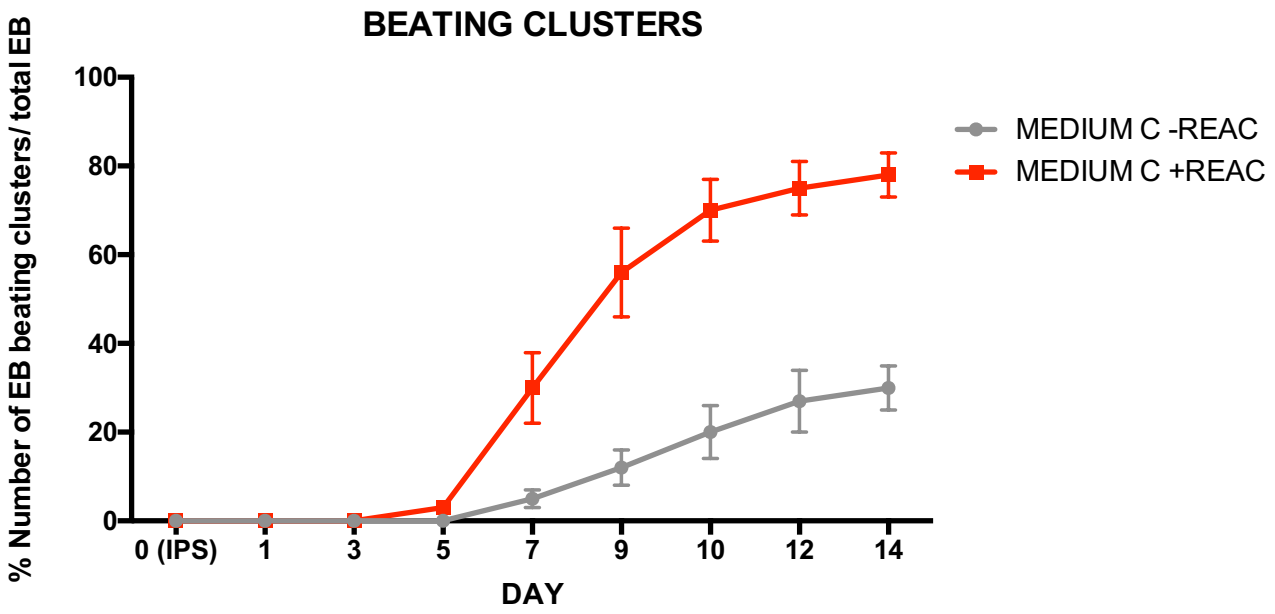

Fig.26 Percent of total beating cluster cells for ultra low 30 mm well. *The percentage of contractile EBs was determined as the number of EBs that showed spontaneous contraction divided by the total number of EBs plated. Medium C+ REAC (Red line) are the UiPSCs exposed to REAC for 72 hours and then observed during the different days, Medium C- REAC are UiPSCs without REAC treatment (grey line).*

REAC treatment maximizes the yield of spontaneously beating cardiomyocytes-like cells.

The cells, were cultured in the same condition and medium in order to drive the cells toward cardiomyocyte differentiation. The chemical inductors, WNT inhibitor, BMP4 synergic committed the UiPSCs to the cardiac phenotype[79], [201], that behavior was detectable in autonomous contraction of Embryoid bodies. However the yield of differentiation in the two population of cells appeared significantly different since the 7th day.

The number of beating clusters were consistent along with the days of culturing under the differentiation stimuli. In particular, at 10th day, more than 65% of beating cluster for well were observed in Medium C plus REAC-stimulated cells (red line). On the contrary, as shown in figure 26 the UiPSCs cultured in Medium C without physical treatment (Grey line) exhibited less beating clusters as compared to cells cultured in medium C together with REAC

. That finding demonstrated that the only use of chemical inductors are able of course to induce the cells to specific phenotype, but the combination with a physical stimulation, that might mimic somehow the physical behavior and physiology of this type of cells, can absolutely enhance the final yield of differentiation Interestingly, we found that the cluster of cells treated with REAC, beated in a organized and synchronic way as compared to the untreated cells. That findings, could suggest that the REAC could be able to drive the UiPSCs to a specific cardiac phenotype, even though further investigation are needed to confirm these results.

GENE EXPRESSION

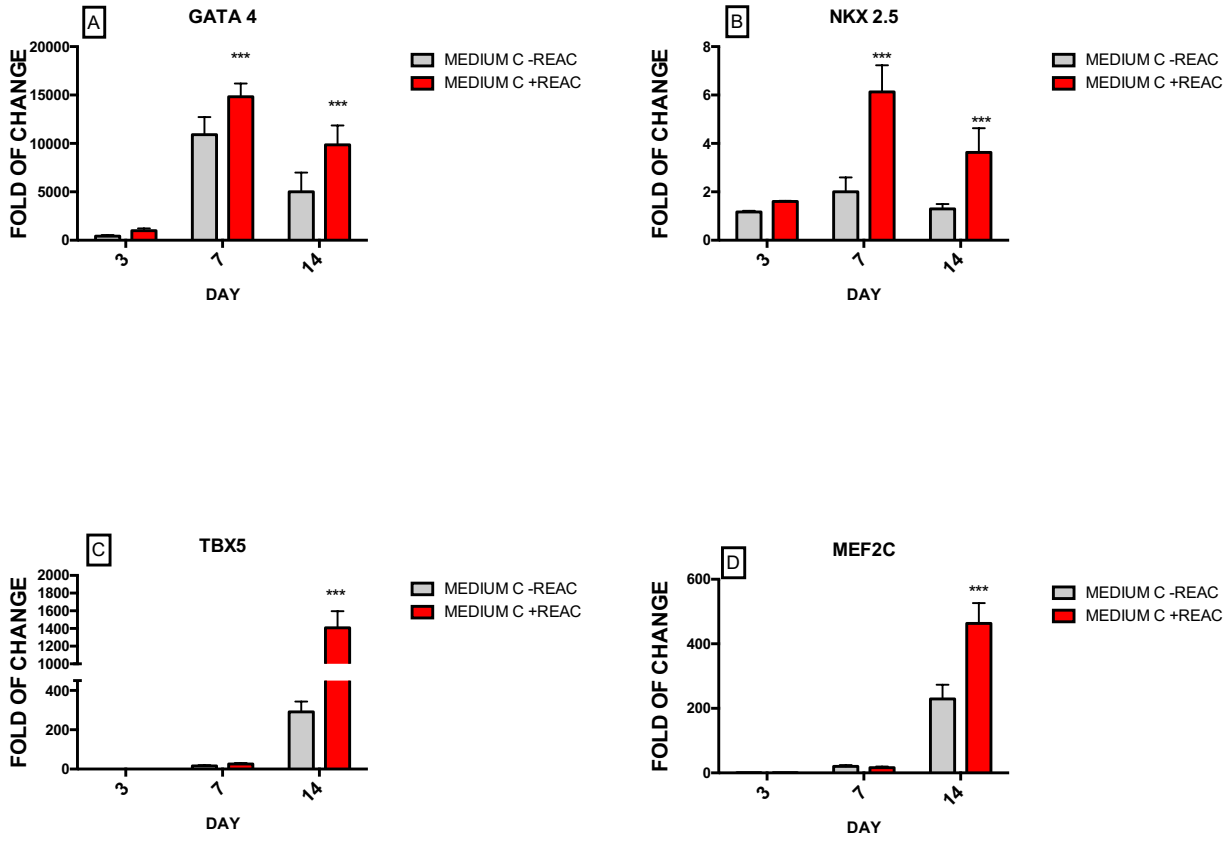

Fig.27 Effect of REAC on early cardiogenic gene markers in hUiPSCs *supplemented with defined medium. Cells cultured in cardiogenic conditioned medium (Medium C) were exposed for 72 h, in the absence (grey bars) or presence (red bars) of REAC. The amount of GATA4 (a), Nkx-2.5(b), Tbx5(c) and Mef2C(d) mRNA was assayed during 3,7 and 14 days and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change*

relative to the mRNA expression in hUiPSCs at day 0. All the REAC treated cells at each time point were significantly different from REAC untreated cells (mean ± S.E; n=4) (P<00.5).

REAC enhances the expression of the cardiogenic and cardiac specific genes.

In hUiPSCs the presence of Medium C enhanced in both cells, +REAC (red bars) and –REAC (grey bars) the transcription of Nkx2.5 and GATA4, encoding a homeodomain and a zinc finger essential in early cardiogenesis, respectively. However the mRNA levels of both of them during cardiogenic commitment was dramatically higher in cells which have been exposed to REAC radiofrequencies for the first 72 hours of cardiac commitment. The same figure shows that the gene expression levels of both T-Box protein 5 (TBX5) and MADS box transcription enhancer factor 2 (MEF2), encoding pivotal proteins during cardiogenesis, were also strongly increased in hUiPSCs that had been cultured in Medium C in the presence of REAC.

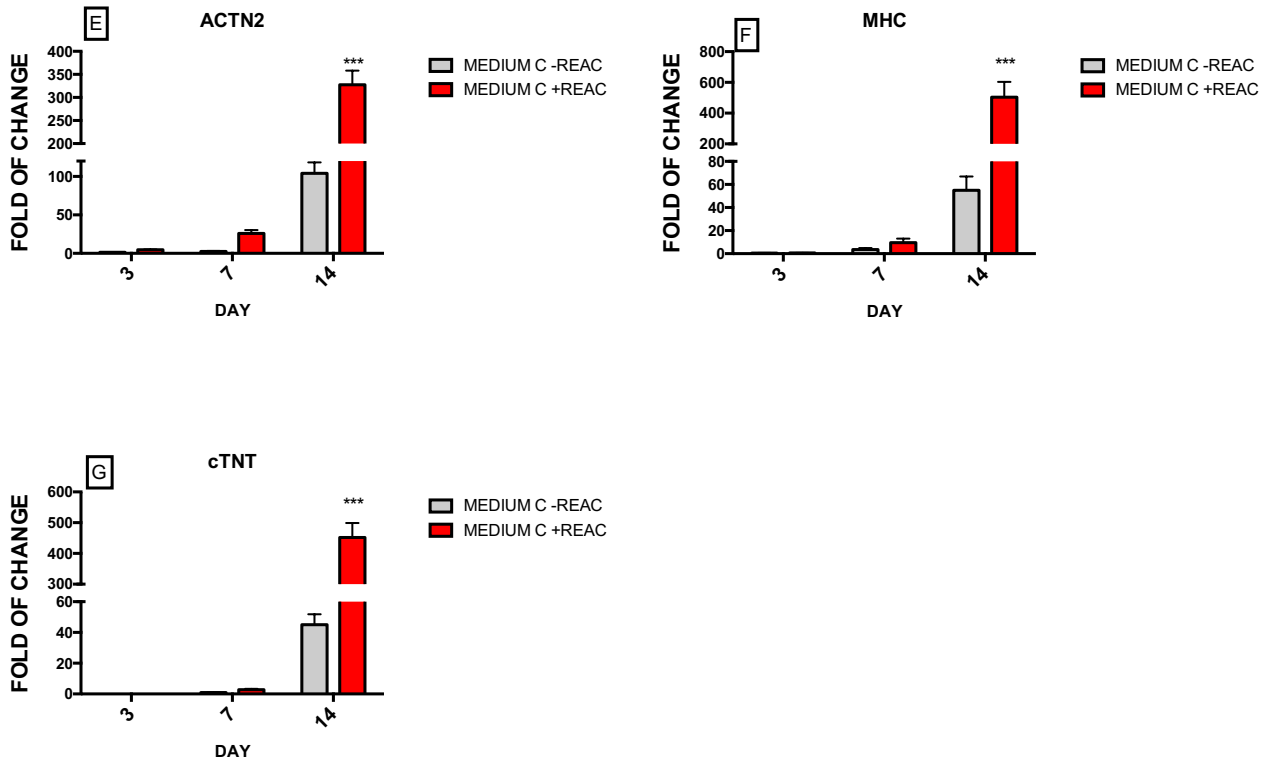

Fig.28 Effect of REAC on later cardiogenic gene markers in hUiPSCs *supplemented with defined medium. Cells cultured in cardiogenic conditioned medium (Medium C) were exposed for 72 h, in the absence (grey bars) or presence (red bars) of REAC. The amount of ACTN2(e),MHC (f) and cTNT(g) mRNA was assayed during 3,7 and 14 days and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in hUiPSCs at day 0. All the REAC treated cells at each time point were significantly different from REAC untreated cells (mean ± S.E; n=4) (P<00.5).*

As well, during the later differentiation phases, the combination of radio frequency and the Medium C described by Weng, supplemented with WNT inhibitor, activin A and BMP4[201] on hUiPSC consistently enhanced the transcription of genes underlying a terminal cardiac differentiation.

ACTN2, encoding an actin-binding protein, was strongly expressed already after 7 days in cells treated with physical stimuli, at 14th day actin-binding protein, in REAC exposed cells was four times higher than control without REAC. As well, the other most important genes, involved in the maturation of cardiomyocites, Alpha-myosin heavy chain (MHC) and the Troponin complex (cTnT), encoding two major cardiac sarcomeric proteins, according with

the other observation exhibited higher expression in cells treated with the combination of physical and chemical stimuli. These effects were detected as early as 72 hand and peaked after 14 days.

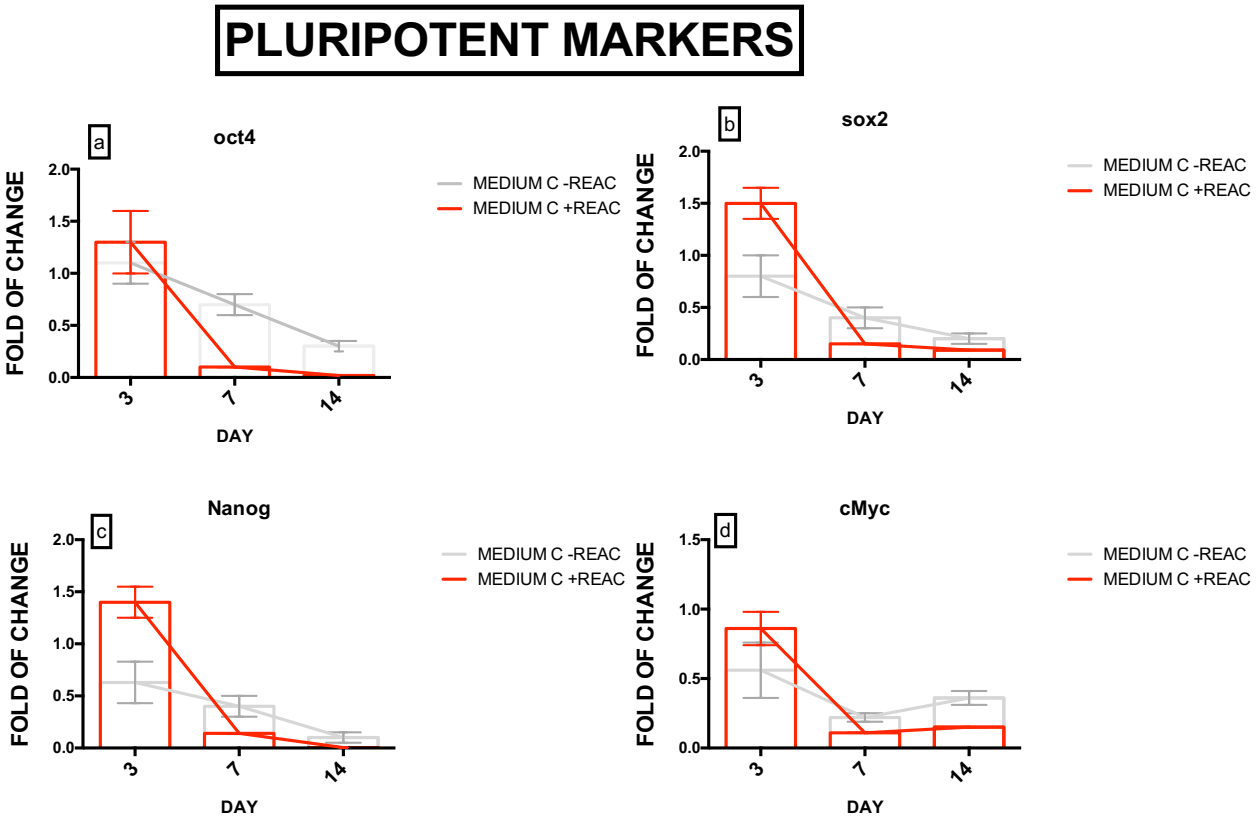

Fig.29 Effect of REAC on pluripotent markers in hUiPSCs *supplemented with defined medium. Cells cultured in cardiogenic conditioned medium (Medium C) were exposed for 72 h, in the absence (grey bars/line) or presence (red bars/lines) of REAC. The amount of OCT4(a), SOX2 (b) and NANOG(c) and cMyc(d) mRNA was assayed during 3,7 and 14 days and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in hUiPSCs at day 0. All the REAC treated cells at each time point were significantly different from REAC untreated cells (mean ± S.E; n=4) (P<00.5).*

As expected during cardiogenic commitment, induced in hUiPSCs using MEDIUM C with or without the combination of radiofrequency energies for 72 hours, pluripotent genes mRNA, gradually decreased in both populations of cells (+REAC and –REAC), being directly

correlated with the up regulation of cardiac specific markers. According to previous observations REAC was able to modulate in a biphasic reversible way the expression of pluripotent genes after 72 hours[105], here we further confirm that REAC treatment can enhance, the expression of the pluripotency genes OCT4, Sox2, Nanog and cMyc during the first 72 hours of treatment,

On the other hand, according to the physiologic behavior of cells during the differentiation, the pluripotency genes were gradually down regulated**.**

IMMUNOFLUORESCENCE ANALYSIS

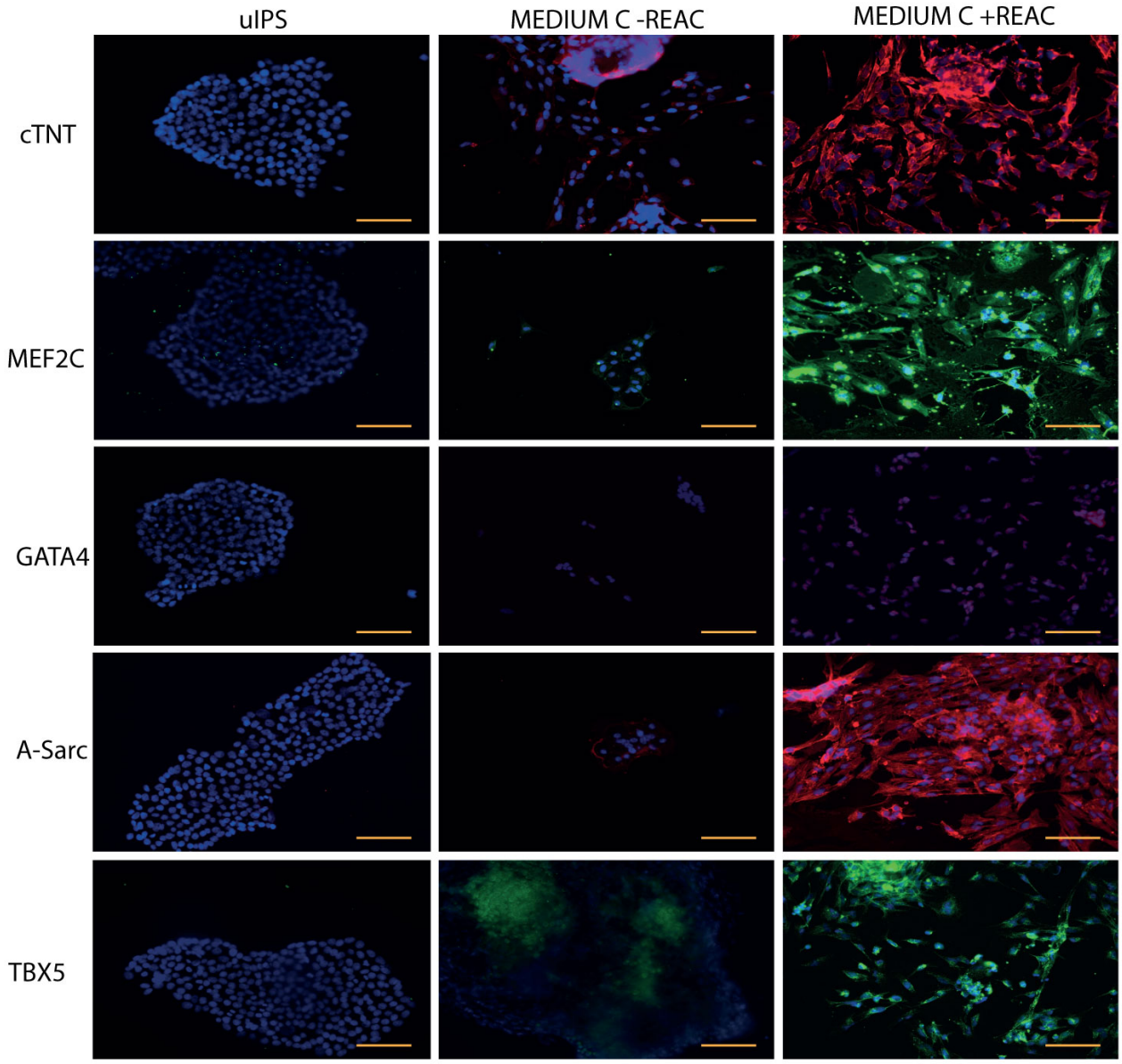

Fig. 30 Immunofluorescence analysis with confocal microscope on cardiac specific proteins at day 14*. hUiPSCs exposed for 72 hours to conditioned medium in the absence (Medium C-REAC) or presence of REAC (Medium C+REAC) were fixed after 14 days from time 0 (undifferentiated hUiPSCs) and processed for immunostaining using specific antibodies directed: Troponin complex (cTnT), MADS box transcription enhancer factor 2 (MEF2C), GATA binding protein 4 (GATA4), alpha sarcomeric actin (A-Sarc) and T-Box protein 5 (TBX5). Confocal microscopy analysis was performed with Leica confocal microscope (LEICA TCSSP5). Nuclei were labeled with DAPI (blue). The yellow scale bars are 100 μm.*

The immunofluoscencence analysis on hUiPSCs was performed at day 0 as negative control (first column) when the cells were in the pluripotent status (UiPS) and after 14 day, in conditioned media (MEDIUM C) specific for cardiac differentiation, exposed (MEDIUM

C+REAC) or not (MEDIUM C-REAC) to radio asymmetric energy. This protein analysis inferred what previously observed in gene expression analysis on the early cardiac markers GATA 4, MEF2C and on the mature cardiac markers cTNT, alfa-sarcomeric and TBX5.

The influence of REAC on cardiogenic commitment on hUiPSCs was strongly relevant. Interestingly, REAC exposure resulted in a consistent increase in the yield of cells positively stained for the cardiac specific marker proteins cTnT, MEF2C, □-sarcomeric and TBX5, as compared to cells that had been cultured with Medium C alone, that showed a weak positively staining. Moreover, the expression of GATA4, an early cardiogenic transcription factor, was only faintly detectable in REAC exposed cells.

DISCUSSION

Self-renewable human induced pluripotent stem cells (hiPSCs) obtained from unipotent cells represent a potential unlimited ex vivo source of human cells for cell-based disease modeling and therapies. The principal characteristic of induced pluripotent stem cells is the ability to propagate indefinitely, as well as give rise to every other cell type in the body (such as neurons, cardiomyocyte, epatocytes ect.)[25], however the published protocols till now, never shown a 100% yield of differentiation.

In fact, the ability of these cells to differentiate toward all phenotypes, rise also considerable problems for the control to a specific cell population. During the years, researchers have published a large number of protocols for differentiation into ectodermal, endodermal and mesodermal line. The principle underlying, is in the use of molecules able to miming or activate specific pathways by the use of inhibitors on common routes, or by the increase only one. For istance, in the protocol proposed by Weng was shown the ability of WNT inhibitor

in combination with BMP4, and activin 4 during the differentiation of IPS towards cardio myocyte[201], all molecules that miming or push a specific pathway. Other protocols use recombinant proteins or induce the expression of specific gene by genetic engineering methods with viruses, plasmids or through specific miRNA.

The main topic of my research group, is the studying how physical energy waves act and interact with cellular systems. It has been shown that the low frequency electromagnetic waves increase cardiac differentiation in murine embryonic cells, as well as the use of radio frequencies to 2.4GHz were able, to induce more phenotypes together in embryonic stem cells of mice and of adipose derived stem cells[101]. The hypothesis is that the application of physical energies, like electromagnetics or radiofrequencies are able to interact directly[90] with the cells that are in "unstable" and “immature” condition, like stem cell, and as much this condition is strong (potency force), is possible use the physical energies to commit the cells to a high yield of differentiation. That behaviour, could be explained if we think about the role that has the polarity[68], [69], [188], [189] during activation and differentiation in the cells.

On this point, this work was carried out to evaluate the efficiency of REAC waves in combination with Wnt1 ihnibitor, activin A and BMP4 during cardiac differentiation on hUIPSc. In order to evaluate how and when the REAC can provide strong advantage during differentiation. The use of physical stimuli on cardiomyocytes was used since long time, it was observed that these ELF stimuli were able to increase the potential performance of this type of cells defined “excitable”, it was clear described how a proper electromagnetic irradiations on mouse embryonic stem cells interacted with β-adrenergic signalling,

indispensable for modulating heart frequency[203], as well was described that electromagnetic fields (EMF) affect intracellular calcium levels in many types of cells[204].In our observation, in this experiment, as well was found that the combinatory use of chemical inductors and physical energies, used during the embryo body formation and for 72 hours constantly, provided significant cardiogenic enhancement. The early cardiac genes GATA4 and nkx 2.5, were already up regulated at 3rd day more in these cells treated with the physical stimulation, that find suggest the importance that the physical stimuli have during the early phases of differentiation as well during the development[205]. The use of physical treatment mostly in IPS strongly commit the unipotent differentiation. As well after the expression of early and inducible cardiac specific genes, the stability of cardiomiocyte derived UiPSCs apperead more strong in these cells treated with REAC, the cTNT that is protein integral to muscle contraction, in skeletal muscle and cardiac muscle was strongly expressed since 7th day and decrease till 14th day observation. MEF2C and Sarcomeric protein alfa, was strongly regulated positively in REAC treated cells. That two proteins, MADS box transcription enhancer factor 2 (MEF2), that play a role in maintaining the differentiated state of muscle cells as well alpha actinin, that is a protein expressed in both skeletal and cardiac muscles differentiated cells, was strongly up regulated after physical treatment. The number of beating clusters, were dramatically more in cells treated with REAC than the other ones, even some changes in morphology, like sharps formation in EBs were detect in these cells treated with REAC. That morphology, could be linked to a polarized disposing of organelles or molecules after REAC treatment, that could be correlate with a change in polarity. All this evidence, regarding the influence that the physical stimuli have on cells, could prompt other researchers to use them in order to influence the cellular behaviour.

EXTRA CORPOREAL SHOCK WAVE THERAPY (ECSWT) SECTION

EXTRA CORPOREAL SHOCK WAVE THERAPY AND CELL PROLIFERATION

Another physical stimuli, that stimulated interest for my project was the Extra Corporeal shock wave therapy. Even trough the physical principle behind the SW is completely different from REAC, many evidence provided common point in the behaviour of cells after both treatments.

The REAC, based its technology on the modulation emitted signal by auto calibration on the frequencies detected from cells, which in turn conveys in asymmetrical manner on them, creating an auto calibrated loop. SW are physical stimuli that act quite differently on cellular systems. In fact, although it is also an electronic device, therefore able to produce electromagnetic energy, SW based its technology on the production of small pressure-acoustic waves that interact directly on cells by mechanotrasduction[174]. Extracorporeal shock wave therapy (ESWT or SW) initially were used to disintegrate renal stones [107], however in later time the use of SW was completely revolutionized into a pro regenerative treatment able to modulate musculoskeletal disorders [206], soft tissue wounds repairment[110], neurological pathologies[207]. It is proposed that shock waves could promote tissue regeneration through mechanotransduction [208]. However the mechanism of action of SW on cells is still unknown and many researchers are working on. One of the most important and common point in the published works about SW, is the pro-activator effect that have on cells. For instance, physical shock wave mediates membrane hyperpolarization and Ras activation for osteogenesis in human bone marrow stromal cells[209], as well provide an activation in macrophages [210], and up-regulation of angiogenesis by the activation of

vascular endothelial growth factor (VEGF), endothelial nitric-oxide synthase, hypoxia-inducible factor 1a, and CD31[211], or as well shown that SW were implicated in the expression of cytokines, chemokines, and matrix metalloproteinases with proangiogenic promoting wound healing [212] and in ligament fibroblast depending on the dose SW changed completely the viability and secretion of of IL-6, IL-8, MCP-1, and TNF-a[213].

In a recent study published by Wheis et al, the SW treatment with different dosage on C3H10T1/2 murine mesenchymal progenitor cells, primary human adipose tissue-derived stem cells and human T cell line was able to increase extracellular ATP released Erk1/2 and p38 MAPK pathways, and enhanced cell proliferation. All evidence that suggest the important implication of physical stimuli on activation and modulation of cellular behaviour[214], for instance in another study it was demonstrated the implication of MAPK activation pathway in mesenchymal stem cells, immune cells, and osteoblasts, resulting in osteogenic differentiation, increase of angiogenesis and T cell proliferation[215].

MATERIAL AND METHODS

Adipose stem cells isolation and cell culture

We received lipospirate biopsy from 2 different consentient donors, from red cross in Linz. After washing, we proceeded with enzymatic isolation. The lipoaspirates were digested with 0.2% collagenase A type I solution (Sigma-Aldrich), under gentle agitation for 45 min at 37°C, and centrifuged at 2000 rpm for 10 min to separate the stromal vascular fraction (SVF) from adipocytes. We even treated this fraction with red blood cell lysis buffer for 5 min at 37°C, in order to obtain a pure population without erythrocytes that could be toxic for the cells and interfere with the isolation protocol, then we centrifuged the solution again. The supernatant was discarded, and the cell pellet was resuspended and seeded in culture flasks in EGM-medium with supplement (Lonza) supplemented with 5% heat inactivated FBS, 1% penicillin- streptomycin, and incubated at 37° C in a humidified atmosphere with 5% CO2. All the cells were used to passages 1-6.

HFF1 cell culture

HFF1 are Foreskin fibroblast cells type 1, purchased from ATCC (Usa). Cells were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 400mM Glutammine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. In the present study cells from passage 2-9 were used. The cell culture medium was refreshed every 3 days. Additional cell group of HFF1 were treated with 1ng/ml LPS (Sigma-Aldrich), as a powerful positive inflammatory inducer representing positive control of an inflammation-induced cell behaviour.

Characterization of Adipose derived stem cells

Flow cytometry analysis was exploited to assess the percentage mesenchymal markers and the purity of population isolated. After a fixation/permeabilization step, cells were incubated with a primary antibody directed against CD73, CD90 (BD Biosciences, San Jose, CA, USA), CD105(Santa Cruz Biotechnology, Heidelberg, Germany), CD45 and CD31 (Sigma- Aldrich) (all at 1 μg/106cells) for 1 h at 4°C and with 1 μg of fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at 4°C in the dark. After washing, cells were analysed on a flow cytometer (CytoFlex, Backman Coulter) by collecting 10,000 events.

Synchronization of cells

In order to test the effect of SW on cell cycle progression, proliferation, ADSC cells were first deprived of serum and all supplements contained in EGM-Medium for 24 h, to induce the synchronization in G0/G1. After that time, normal medium EGM with supplements and FBS was added. Then cells received (0,19 mJ/mm2 100 pulses) or not (CTR) SW treatment.

Shock Wave setting and cell treatment

Shock wave device used was Ortho-Gold100 (Tissue Regeneration Technologies, LLC, manufactured by MTS Europe GmbH). Cells, after synchronization were detached and a number of 1x 10^6 for each population (control/ SW-treated) were placed inside 1 ml medium in a 15-ml poly-propylene tube (falcon 15), shock wave treatment was performed using a water bath setup at 37°C in order to preserve aberrant changes in cellular behaviour. The distance between the shock wave applicator and the 15 ml tube containing the cells were 5 cm. The control group were allocated in a separate water bath without SW applicator, with

the same temperature condition. For SW treatment cells were applied a 100 pulses of shock waves using energy flux densities between 0.03 and 0.19 mJ/mm2 at 3 Hz were applied, according to previous experiments and in vivo studies[214], [215].

***Fig 30 Shock wave device representation**. The cells inside polipropylen tube 15 ml (C), in 1 ml medium were allocated inside the water bath (B) at 37°C. Shock waves device (A) conduct the energy by the applicator connect to water bath, 5 cm far from tube.*

Cell Proliferation with Propidium iodide, flow cytometry analysis

DNA staining was used to specifically determine the amount of cells undergoing S phase, or G1 phase. Cells were detached using trypsin for 5 minutes, then deactivated using medium. The cells were fixed adding ice-cold 85% EtOH during vortexing. Samples were stored at -20 °C for 24 hours. After washing, DNA was stained with 0.25 mg/ml propidium iodide for 30 minutes, cells were washed again and resuspended in 300 ul flowcitrometry load solution.

Viability assay by EZ4U and BRdU assay

EZ4U cell viability assay was performed after 6 and 24 hours of SW treatment. Briefly according to the manufacturing protocol, EZ4U is based on 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5, diphenyltetrazolium bromide (MTT) assay, but could be used and detected directly on the cells. 100 ul of EZ4U solution was added to wells in cells treated or not with SW, after 2 hour of incubation the absorbance at 450 nm was detected.

A BrdU cell proliferation assay (Roche, Germany) was performed 6h and 24h after irradiation. According to the manufacturing protocol, BrdU labelling solution was added to the cell cultures exposed or not to SW at a final concentration of 10μM and incubated for 2 hours. This was followed by 90 minutes incubation in diluted peroxidase-conjugated anti-BrdU antibody. Absorbance was measured with a photometer (Spectra Thermo, TECAN Austria GmbH, Austria) at a wavelength of 450nm.

Reactive Oxygen Species Production – Electron Paramagnetic Resonance on HFF1

HFF1 Cultures were treated with or without 0.19 mJ/mm2 100 pulses Shock Waves, contemporary a positive control group of HFF1 was treated for 24 hours with 1 ng/ml LPS.

After treatment cells were seeded in TC 24 well, with final volume of 250 ul. The cells were observed at 1, 4, 8, 12, 24, 48 hours. 10 minute before every measurement PPH (1-Hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine), a ROS detector, was added at a final concentration of 500μM. 50μl of medium were put into oxygen permeable capillary tubes (Noxygen Science transfer and Diagnostics, Germany) and measured at room temperature with e-scan EPR spectrometer (Bruker, Germany) with following settings: microwave frequency: 9.762741 GHz, modulation frequency: 86.00 GHz, modulation amplitude: 1.4

GHz, centre field: 3487.920 G, sweep width: 200 G, microwave power: 54.10 mW, number of scans: 1, receiver gain: 2.24 x10.

ATP Release in HFF1

Immediately after shock waves pulses or after 15, 30, 60 and 120 minutes' cells were lysed. Lysates were pipetted into a 96 microplate and 100uL ATP Assay reagent (Promega CellTiter-Glo Luminescent Cell Viability Assay, Austria) were added and shaken for 2 minutes. Lysates were left on a stationary surface to incubated for further 10 minutes, after which samples luminescence weremeasured by a photometer (SPECTRAstar Omega, Germany). Sample concentrations were compared to an ATP standard calibration standard.

Enzyme-linked immunosorbent assay (ELISA) on HFF1

HFF1 were cultured for 24h in presence or not of LPS in order to induce inflammation to be used in comparison to SHOCK WAVES cells treated. After SW treatment, cells were seeded in TC24 plate in a final volume of 250 ul. The culture medium was collected at different time points (1, 4, 8, 12, 24, 48 hours) for the ELISA analysis. The levels of IL-6 and IL-8, MCP-1 and TGF-alpha released into the culture supernatant were measured using human Ready-SET-Go, eBioscience. Respectively, and according to the manufacturer's protocol.

RNA extraction, cDNA synthesis, and gene expression analysis by real-time PCR.

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Sigma). Total RNA was dissolved in RNAase-free water and quantified using a Nanodrop.

A twenty-µl reaction volume containing 1 µg total RNA was reverse transcribed using HiCapacity cDNA reverse transcription kit (Applied Biosystem) with oligo (dT) primers. qPCR reaction was performed using i5 Biorad with kapa green (Biolab).

After an initial denaturation step at 95°C for 15 min, temperature cycling was initiated. Each cycle consisted of 95°C for 15 sec, 53-59°C for 30 s, and 72 °C for 15 sec, the fluorescence being read at the end of this step. All primers used in this work were from Invitrogen and are listed in Table 1. PCR products were confirmed by melting curve analysis and electrophoresis. All measurements were done as technical quadruplicate of biological replicates. Biological replicates were obtained from independent cell culture samples at different passages. Relative expression was determined using "2^ΔΔCt method" with glyceraldehyde 3-phospate dehydrogenase (GAPDH) as normalizing gene.

Immunostaining

Adiposed derived stem cells cultured in EGM medium with or without SW 100 pluses 0,019 mJ/mm2 exposure were seeded in chamber slide and after 24 hours were analyzed. The cultures were fixed with absolute methanol for 30 min and 1 hour at -80°C. Cells were then exposed to antibodies against DNMT1(Mouse, Santa Cruz), DNMT2a (Rabbit, Thermo scientific) or DNMT2b (Rabbit, Santa Cruz) for 1 hour at 37°C cells, followed by staining with the appropriate secondary antibodies anti mouse Alexa fluor 594 or anti rabbit Alexa fluor 498 at 37°C for 1 h. All microscopy was performed with a Leica confocal microscope (LEICA TCSSP5). DNA was visualized with DAPI (1 µg/ml).

RESULTS

Immuno characterization of Adipose stem cell isolated

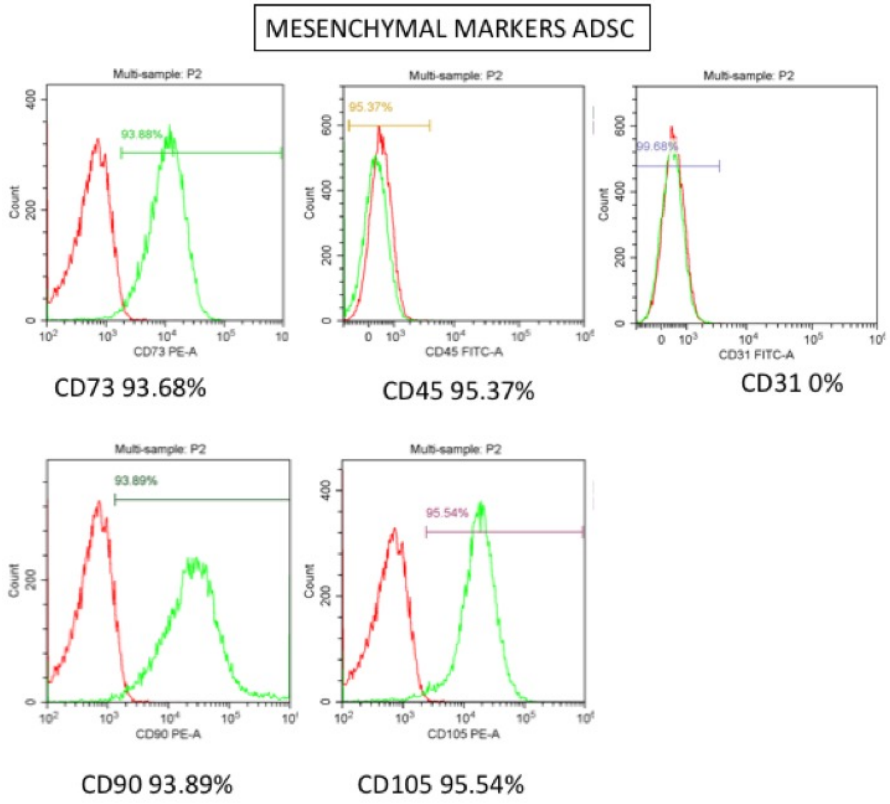

Fig. 31 Mesenchymal surface markers tested in flowcitometry. CD73, CD90 and CD105 were significantly expressed, contrarily CD45 and CD31 typical hematopoietic markers.

According to the International Society for Cellular Therapy that identity MSC markers, here we wanted to be sure that adipose derived stem cells isolated were a pure population. As expected only Adipose derived stem cells were isolated. The CD73, CD90, and CD105, typical mesenchymal markers were positively expressed, while not detected the markers CD31 and CD45 typical for the haematological linage were not detectable.

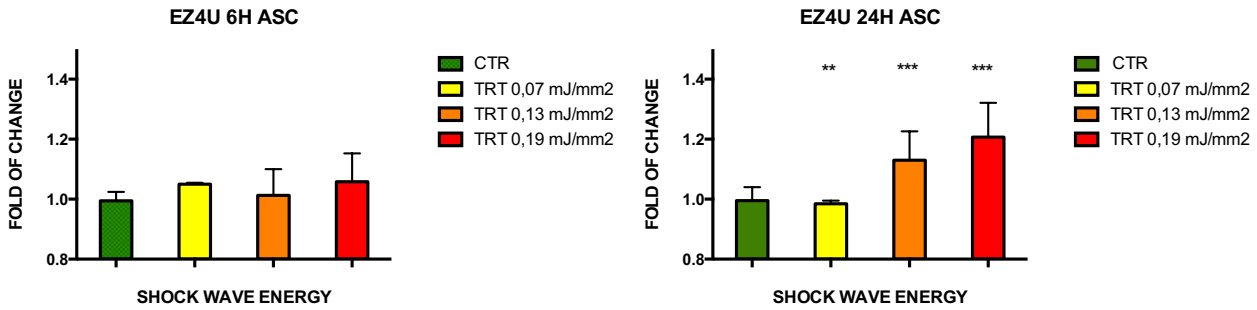

Fig. 32 ADSC Cell proliferation analysis with EZ4U *were performed at 6 and 24 hours after SW treatment 100 pulses with different energy, CTR (green bars), cells treated with 0,07 mJ/mm2 (Yellow bars), 0,13 mJ/mm2 (orange bars), 0,19 mJ/mm2 (Red bars).*

Analysis of viability and proliferation on ADSC.

Adipose derived stem cells, were treated with different doses of SW in order to evaluate how the power influence could the proliferation cell viability. At 6 hours after shock waves treatment we could not see any difference between treated and untreated cells. But this behaviour could be explained with the physiological cell cycle time, the normal cells cannot increase their proliferation in 6 hours. However, this data shows the not toxicity of SW on cells. Interestingly after 24 hours comparing the viability between cells treated with different doses of SW, the 0,13 mJ/mm2 and 0,19 mJ/mm2 dose elicited a better response as compared to control cells or to cells treated with 0,07 mJ/mm2. That finding however confirm previous observation[173].

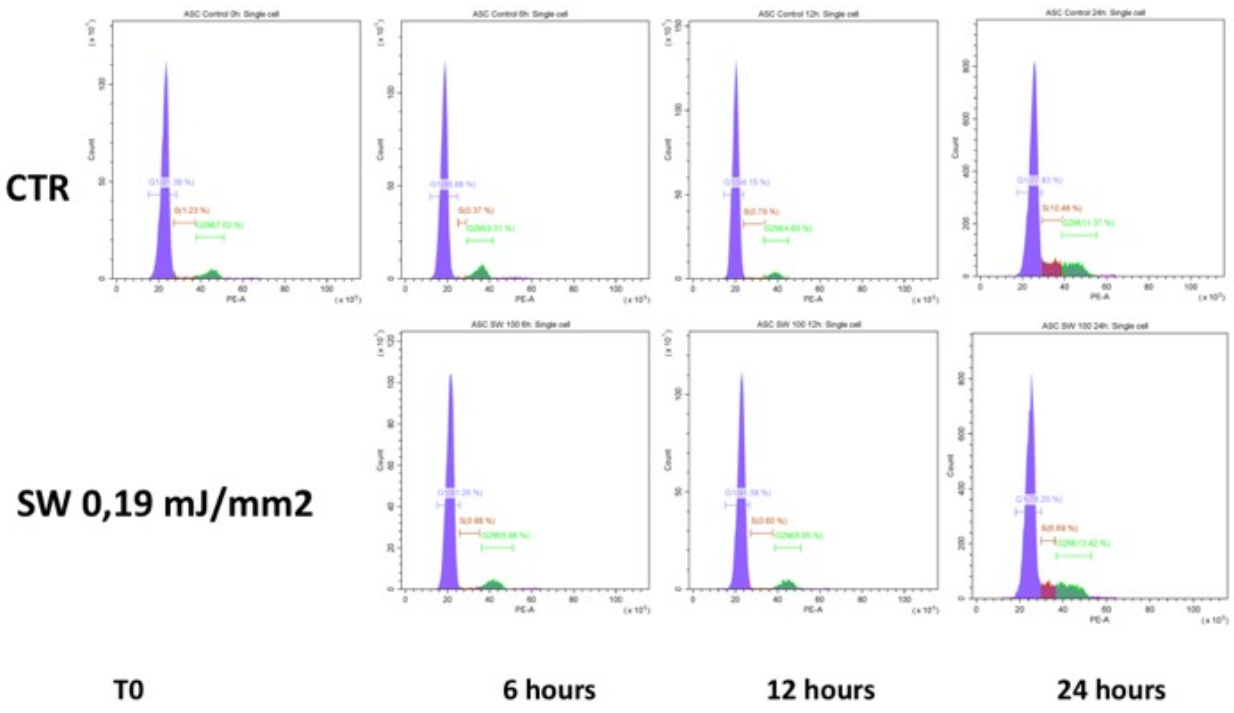

Fig. 33 ADSC cells were synchronized in G0/G1 phase*. Then cells were treated or not with 100 shock wave pulses at 0,19 mJ/mm2 were observed at T0, 6h,12h and 24h.. Untreated cells were treated identically except they did not receive shock wavetreatment.*

Cells were harvested for 24 hours, then treated or not with 100 pulses at 0,19 mJ/mm2 and then seeded in culture flasks and cultured for additional 6h, 12h and 24h. The ADSC were stained with propidium iodide and assessed using flow cytometric analysis (Fig.33). After 12 hours the cell cycle profiles revealed more cells in the S phase in the samples treated with shock waves as compared to control untreated cells.

GENE EXPRESSION ANALYSIS

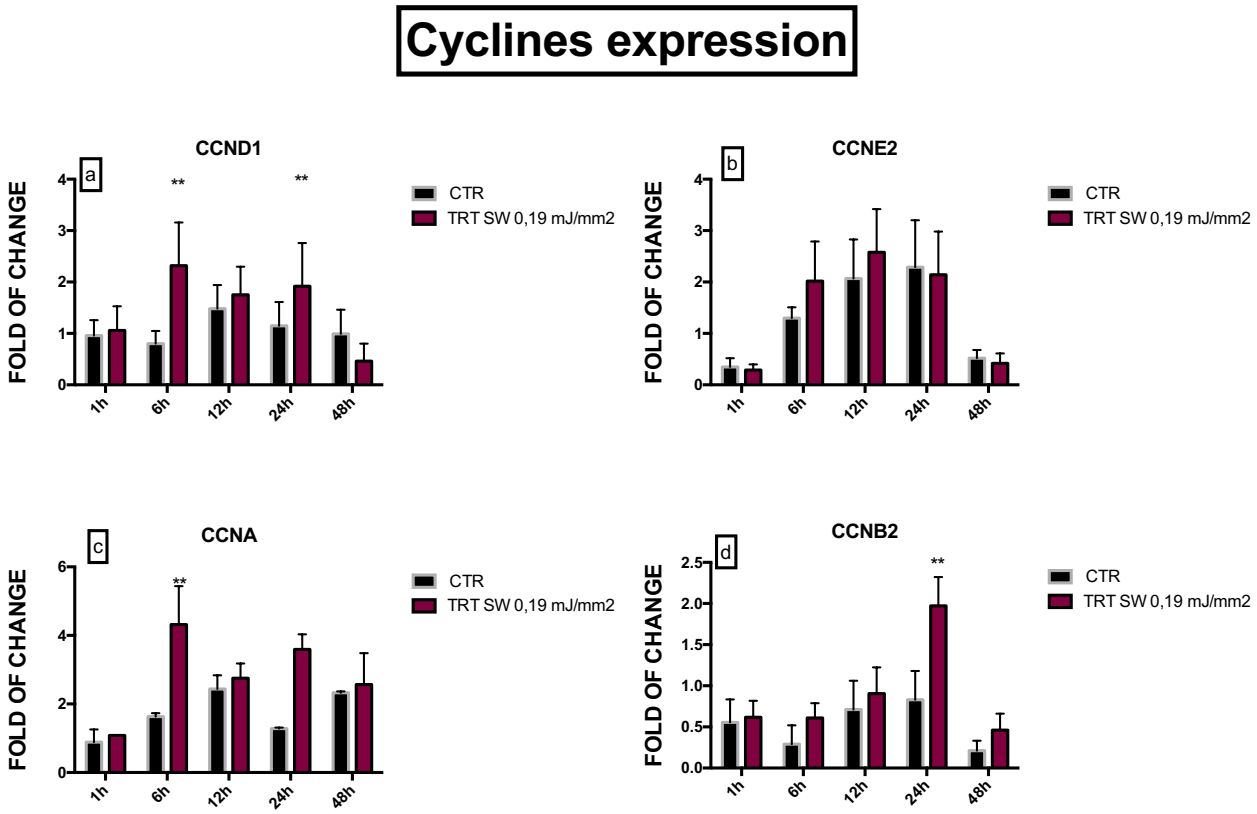

Fig. 34 Effect of Shock wave 100 pulses on cyclin expression in Adipose derived stem cells (ADSC). *Cells were starves for 24 hours without FBS and EGM-supplement, then were exposed (Bordeaux bars) or not (black bars) to 100 pulses 0,19 mJ/mm2 Shock and cultured in normal growth medium .The amount of CCND1(a), CCNE2 (b), CCNA(c) and CCNB2(d) mRNA was assayed during 1,6,12, 24 and 48 hours and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in control cell t0. Statistical analysis was performed with t-test and ANOVA o.w (mean ± S.D; n=6) (P<00.5).*

The gene expression analysis of the main cyclins involved in the cell cycle, confirm previous observations derived from viability test EZ4U as well as from flow citometry analysis. The Adipose derived stem cells were synchronized 24 hour before treatment and then exposed or not to 100 pulses 0,19 mJ/mm2 energy. After 1 hour the mRNA levels of CCD1, CCNE2, CCNA and CCNB2 were detected, and, according to the normal behaviour of synchronized cells, mRNA was extremely low in both cells. After 6 hours the expression of CCND1, CCNE2 and CCNA was increased in both SW treated or untreated cells, a typical behaviour of cells entering in cell cycle[46], interestingly we observed that the SW treatment improved the cell cycle regulation, by acting on cyclin gene expression . Cyclin D1, ususally express in

the cells that are entering in G1 phase, in SW cell treated was more expressed and detectable already after 6 hours culturing following shockwave treatment and then gradually decreases. Cyclin E, also involved in the progression of G1 to S phase in cells was higher expressed in cells treated with SW as compared to control cells, and its expression was retained higher until 24 hours. Cyclin A, as shown cooperate with Cyclin E to the promotion of Synthesis phase in cells, here according to the previous observations, SW-treated ADSC exhibited higher expression levels of this cyclin as compared to control. The same result was also obtained for Cyclin B.

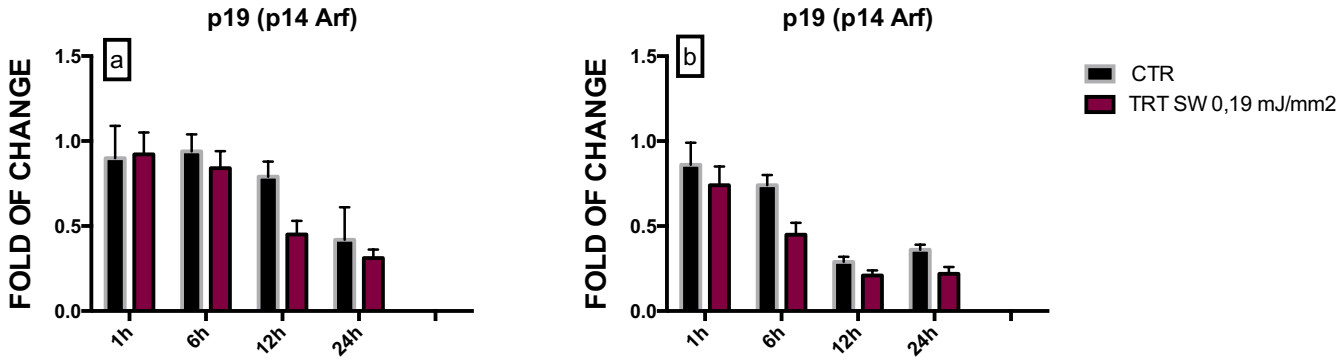

Fig. 35 Effect of Shock wave 100 pulses on DNA Damage markers in Adipose derived stem cells (ADSC)*. Cells were starves for 24 hours without FBS and EGM-supplement, then were exposed (Bordeaux bars) or not (black bars) to 100 pulses 0,19 mJ/mm2 Shock and cultured in normal growth medium .The amount of p53(a), p19(p14 ARF) mRNA was assayed during 1,6,12, 24 hours and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in control cell t0. Statistical analysis was performed with t-test and ANOVA o.w (mean ± S.D; n=6) (P<00.5).*

During cell cycle, there are restriction points even called ckeck points, that have the role to decide the progression of the cells during the different phases. Usually, DNA damage checkpoints are located before the cell enters S phase (G1-S checkpoint) or after DNA replication (G2-M checkpoint). At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent activation pathway[46], [51]. Here we analysed the expression of p53 and p19 in order to confirm the relation between cyclin expression and progression

through cell cycle. Interestingly, in adipose derived stem cells exposed to 0.19 mJ/mm2 100 pulses energy, an increase of this two genes could not be detected, while after 12 hours after treatment the expression of both genes was less as compared to the control. Noteworthy p53 and p19 mRNA levels were not increased after SW treatment, indicating that the treatment doesn't damage the integrity of cells and DNA, even "strong" force is applied.

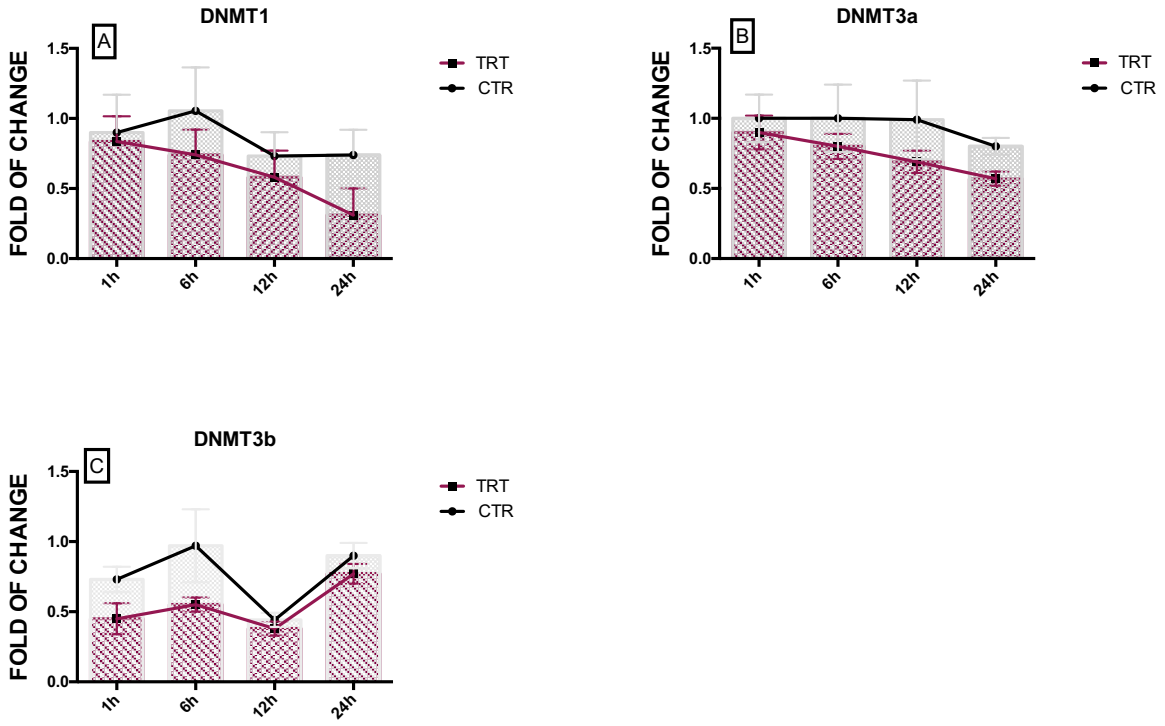

Fig. 36 Effect of Shock wave 100 pulses on DNA methyltransferase in Adipose derived stem cells (ADSC)*. Cells were starves for 24 hours without FBS and EGM-supplement, then were exposed (Bordeaux bars/Bordeaux line) or not (grey bars/black line) to 100 pulses 0,19 mJ/mm2 Shock and cultured in normal growth medium .The amount of DNMT1(a), DNMT3a (b) and DNMT3b (c) mRNA was assayed during 1,6,12, 24 hours and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in control cell t0. Statistical analysis was performed with t-test and ANOVA o.w (mean ± S.D; n=6) (P<00.5).*

Recent observations, showed the relevant role of DNMTs during cellular behaviour. As observed by Robertson and colleagues, the mRNA levels of DNA methyltransferase (DNMTs) change during the cell cycle, with DNMT1 and DNMT3b decreaseing in G1 as well as DNMT3a. During G0 phase, conversely the cells expressed high mRNA level of DNMT3a together with DNMT3a and DNMT1. Here we show how in both cells SW-treated

or not the mRNA levels of DNMTs changed during cell cycle progression, and in these cells treated with SW stimuli, already after 6 hours culturing following shockwave treatment a decrease could be detected as compared to the controls.

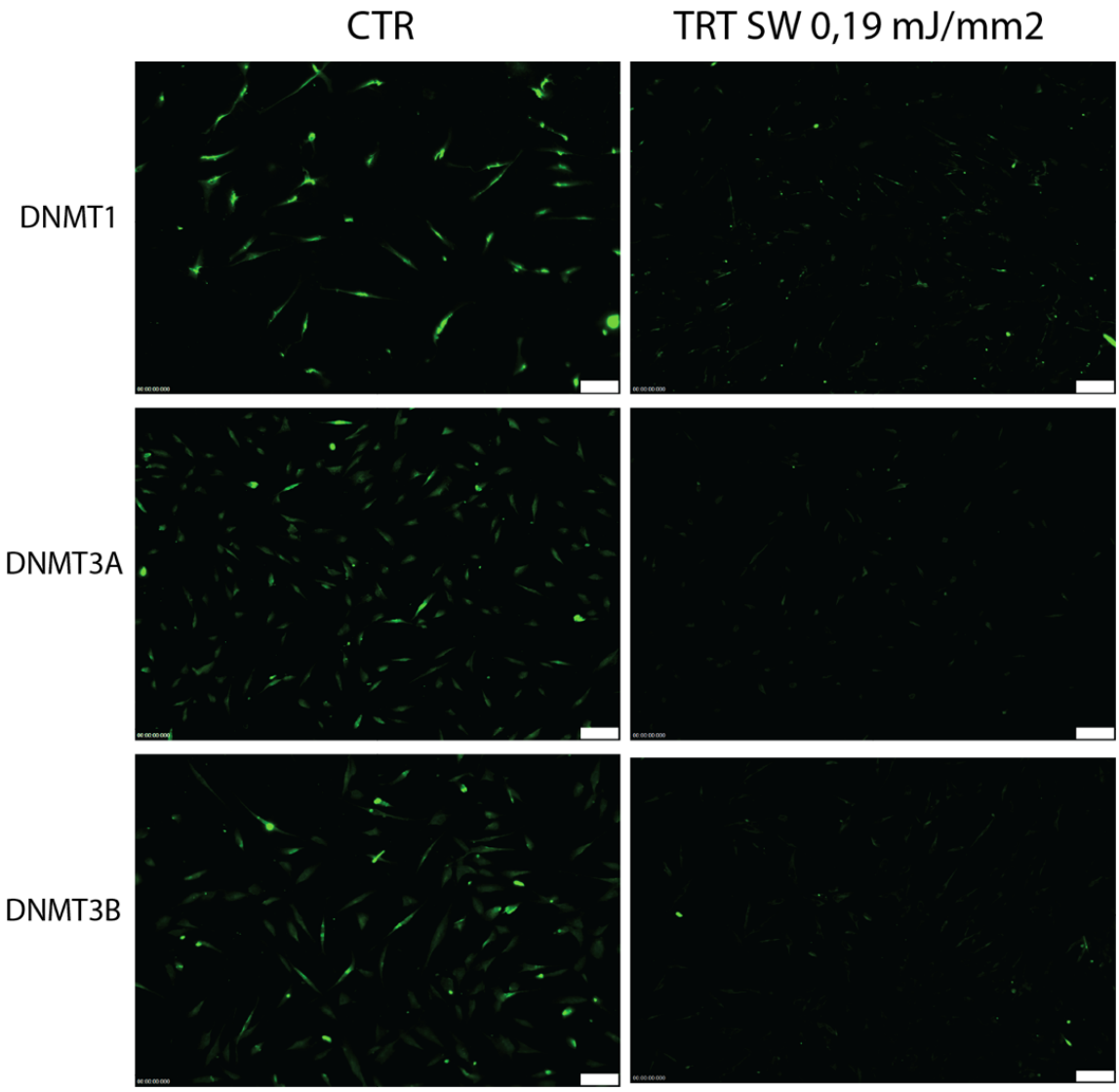

Fig. 37 Immunofluorescence analysis with confocal microscope on DNMTs. *ADSC cells were starves for 24 hours without FBS and EGM-supplement, then were exposed to 100 pulses SW 0,19 mJ/mm2, seeded in chamber slide and after 24h processed for immunostaining using specific antibodies directed:DNMT1, DNMT3a,DNMT3b. Confocal microscopy analysis was performed with Leica confocal microscope (LEICA TCSSP5). Nuclei were labeled with DAPI (blue).*

Immunofluorescence analysis on Adipose derived stem cells after 24 hours of Shock waves treatment showed low positivity for DNMT1, DNMT3a and DNMT3b as compared to the

control cells that did not received Shock wave stimulation. However, in both cells DNMTs was expressed, and as shown in figure 37 SW-treatment could strongly modulate the expression of all DNMTs. That behaviour again, could be connected with the increase in cell cycle progression, induced by Shock wave stimulation.

SHOCK WAVE TREATMENT AND CELL ACTIVATION BY PRO-INFLAMMATORY INDUCTION

SW are "mechanical" waves, characterized by an initial positive very rapid phase, of high amplitude, followed BY negative pressure, producing a "micro-explosion" that can be directed on a target zone (body, tissue or cells) in order to stimulate or modify the cells in their behaviour. Since years is known the effect of this type of physical stimuli on body and cells, moreover it was previously described the effect of shock waves on the cyclins activation and proliferation. Furthermore, the exact mechanism through which cells convert mechanical signals into biochemical responses is not clear yet. Interestingly it was described the mechanism mediated by ATP release and P2 receptor activation that foster cell proliferation and tissue remodelling via Erk1/2 activation[216][108][215], as well PI-3K/AKT and NF-κB signalling pathways[217] and the implication of TLR3 signalling and subsequent TLR4 in SW treatment[207]. Recently the effects of Shock Waves on Expression of IL-6, IL-8, MCP-1, and TNF-a in human periodontal ligament was recently described by Cay and colleagues [213].

Unwittingly, in the same time some experiments concerning cytokine production in human fibroblast exposed to shock waves energies were performed. Even if most of the literature prove the anti-inflammatory effect of SW treatment in Vivo[109], [110], [110], [171], [206],

[218], nobody before have shown the pro-inflammatory effect of SW on cells in vitro, as a pro-activator event mediated by cytokine and chemokine expression. It was supposed that the shock wave impulses on cells were able to create in the cells a pro-inflammatory milieu, mediated by mechanotransduction [216], similar to pro-inflammatory activation mediated by LPS[219]. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, implicated in the induction of a strong immune response in normal animals. LPS exerts its biological effects on the host by binding to Toll-like receptor 4 (TLR4), a pattern recognition receptor (PPR) that is widely distributed among lung parenchyma cells, including macrophages, epithelial cells, and fibroblasts[219], In a recent study it was shown that LPS was able to directly induce secretion of collagen in primary cultured mouse lung fibroblasts via TLR4-mediated activation of the phosphoinositide3-kinase-Akt (PI3K-Akt) pathway thus increasing the proliferation rate[220]. Here we will analyse the effect of Shock wave and LPS in human foreskin fibroblast in terms of proliferation, cytokine production, ATP release and ROS production.

RESULTS

Proliferation assa

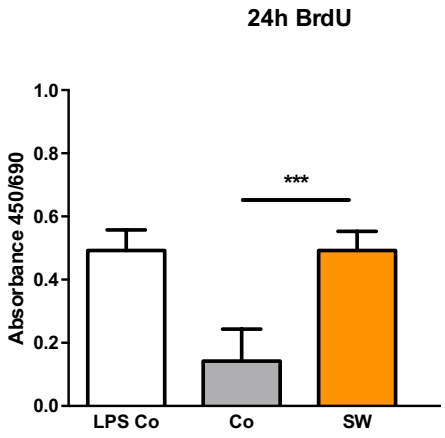

Fig. 38 HFF1 Cell proliferation analysis with BrdU *were performed at 24 hours after SW 0,19 mJ/mm2 treatment 100 pulses (orange bars) or not (grey bars) LPS control (white bars) are positive control. Statistical analysis was performed with GraphPad using ANOVA o.w (mean ± S.D; n=8) (P<00.5)*

To investigate the effect of Shock wave in comparison to LPS on fibroblast proliferation, the BrdU assay was performed to temporally quantify DNA synthesis in HFF1 after to Shock wave 0,19 mJ/mm2 treatment or and LPS exposure. The amount of DNA synthesis in both HFF1 treated with SW or LPS was significantly increased during the following 24 hours as compared to the negative control group. According to previous observations, even SW treated HFF1 exhibited an increase in their proliferation rate as compared to untreated cells. Interestingly, we found that LPS-inflammatory-induced events in HFF1 were able to increase their proliferation. That finding was already showed by He group, who observed the significant proliferation in lung fibroblast exposed to LPS.

ATP RELEASE AFTER SHOCK WAVE TREATMENT

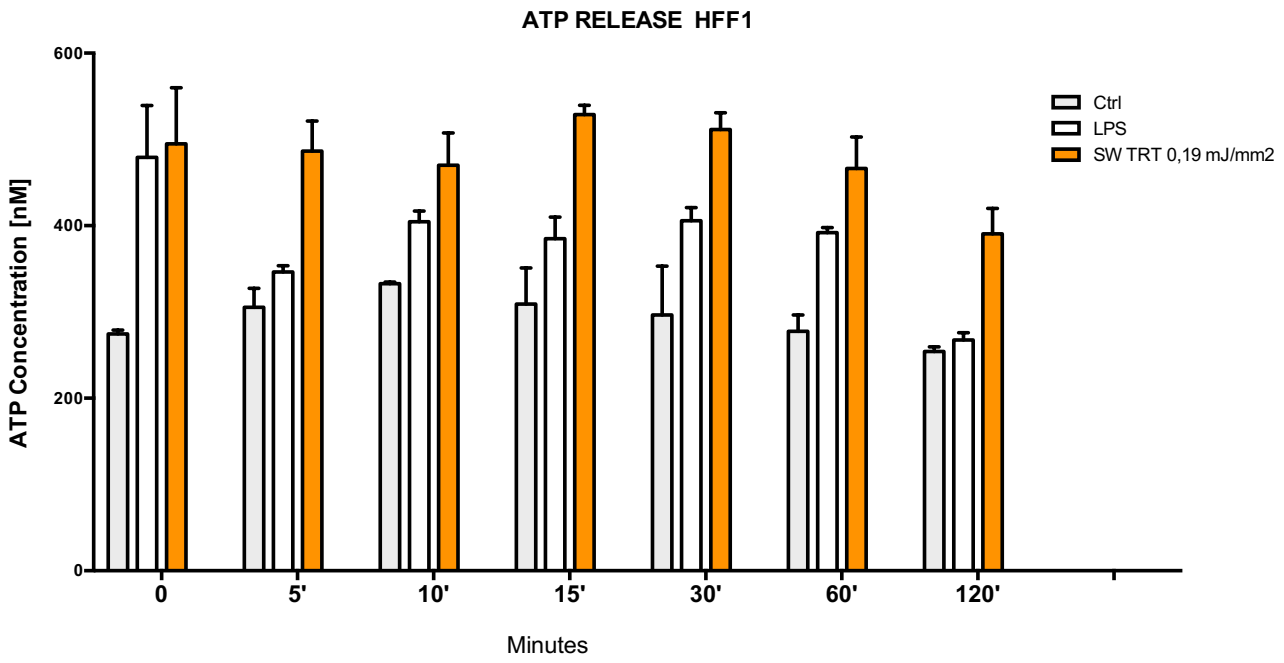

Fig. 39 ATP concentrations *in HFF1 supernatants were determined after 5,10,15,30, 60 120 minutes after SW 0,19 mJ/mm2 treatment 100 pulses (orange bars) or not (grey bars) , LPS control (white bars) are positive control. Statistical analysis was performed with GraphPad using ANOVA o.w (mean ± S.D; n=6 in triplicate) (P<00.5)*

In other studies the ability of SW to induce the ATP release after SW treatment [209], [214], [221], which in turn activates Erk1/2, an upstream effector Mek1/2, and p38 MAPK, was strongly demonstrated. Several other evidence show that lipopolysaccharide promote ATP release as well [222] by the activation of macrophage cells. Here we found that LPS can promote the increase of ATP release in HFF1. Shock waves treatment caused a significant release of ATP during the different experimental time points.

REACTIVE OXYGEN SPECIES (ROS) PRODUCED IN HFF1

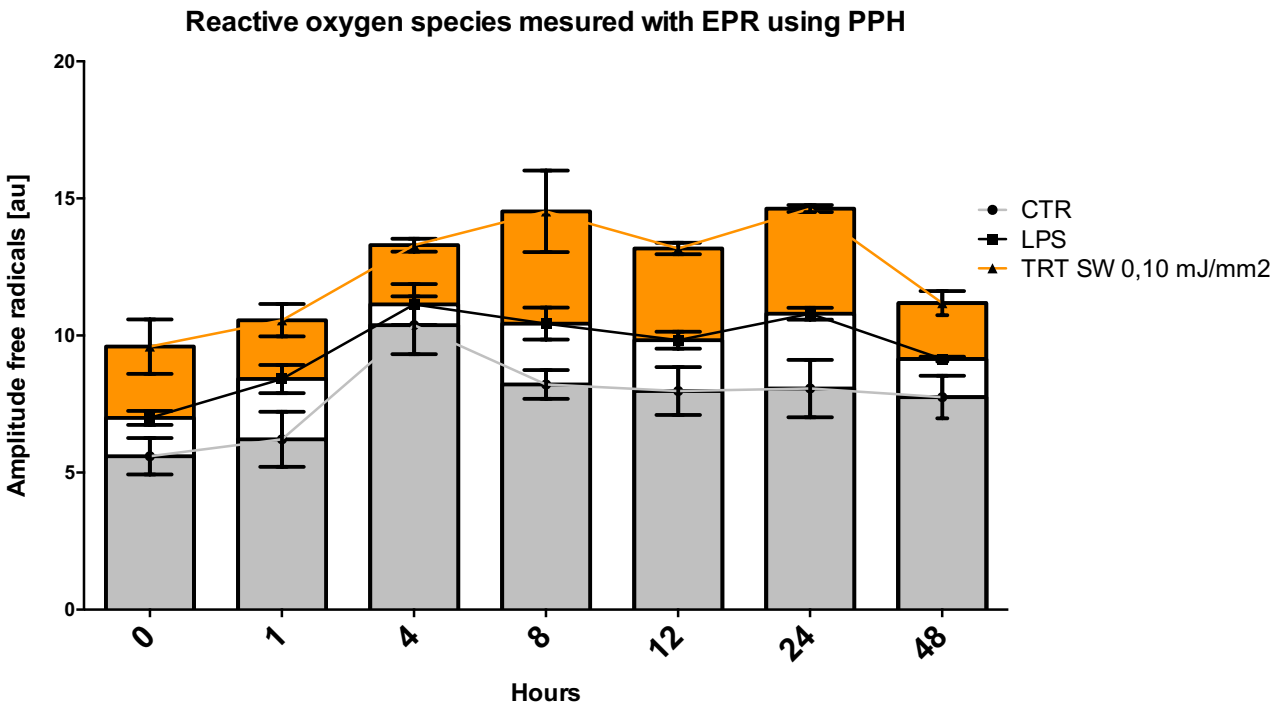

Fig. 40 HFF1 ROS measurement analysis with Electron spin resonance using PPH *was performed at 0,1,4,8,12,24 and 48 hour after SW 0,19 mJ/mm2 treatment 100 pulses (orange bars) or not (grey bars) LPS control (white bars). Statistical analysis was performed with GraphPad using ANOVA o.w (mean ± S.D; n=6) (P<00.5)*

Reactive oxygen species (ROS) have an established role in inflammation, ROS have been implicated in the activation of signalling pathways such as mitogen-activated protein kinases (MAPK), NF-kB, and guanylate cyclase[223]. Recently, a role for ROS arises also in the activation of the NLRP3 inflammasome, one pathway for generation of active caspase-1 and secretion of mature IL-1[224]. Here we analyze the hypothesis that activation of HFF1 depend on the pro-inflammatory reaction modulated by SW. ROS were released soon after 1 hours, increasing at 4 hours till 8 hours, then gradually decreased. LPS-treated cells positively modulated ROS release, SW was able to induce more ROS production as compared to the negative control and than positive control.

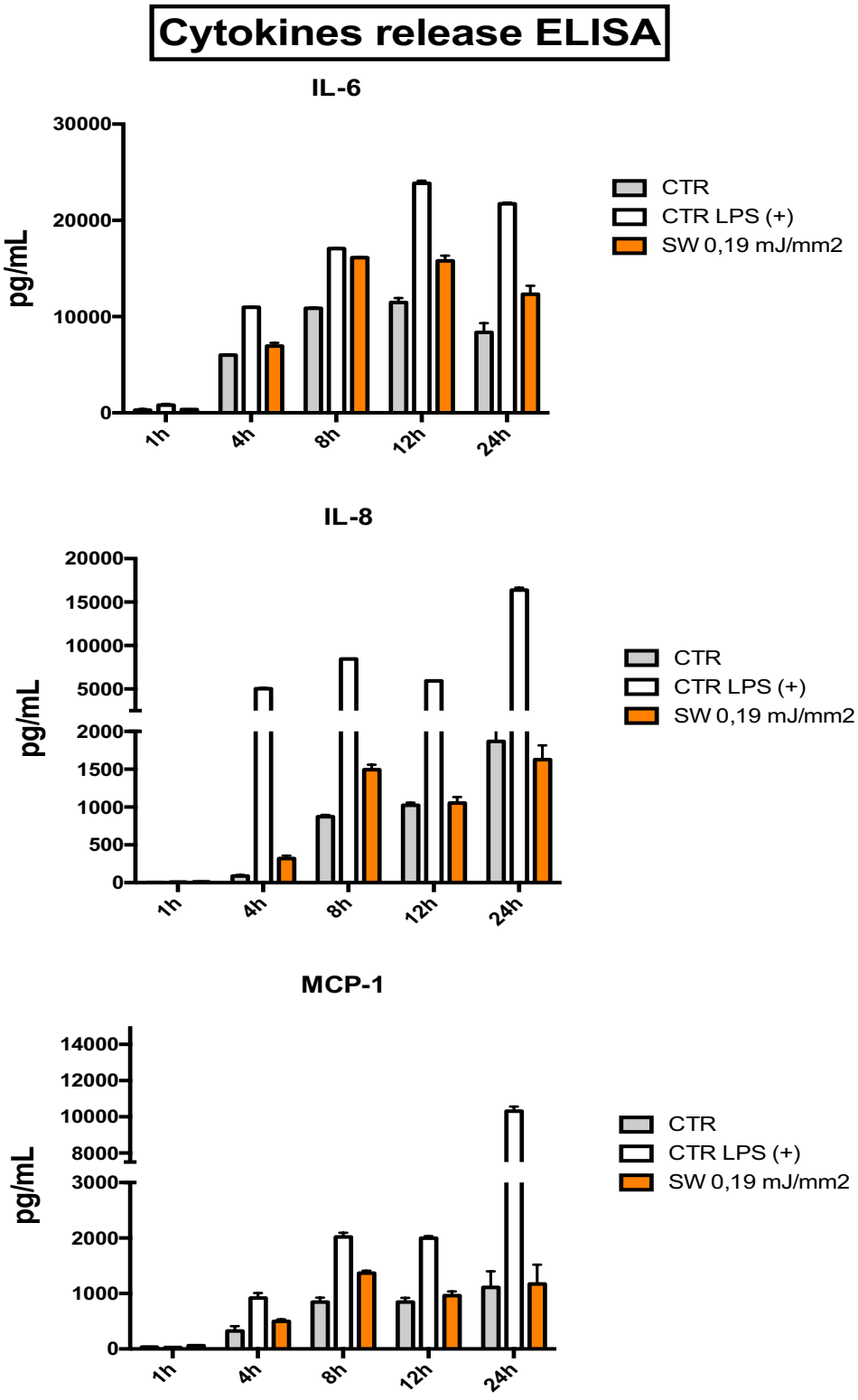

Fig. 41 Effect of shock wave treatment on cytokines production in HFF1 during different time points (1, 4, 8,12 and 24 hours) in response to shock wave treatment. The levels of IL-6 (A) and IL-8 (B), MCP-1 (C) were measured in cell supernatants using ELISA.

Cytokines are hormone-like proteins which mediate and regulate inflammatory and immune responses, ILs are produced by numerous cell types, including macrophages, monocytes, fibroblasts, endothelial cells, and smooth muscle cells. Here we supposed that SW modulated fibroblast proliferation trough a pro-inflammatory induction already after 4hours culturing following shockwave treatment. The mechanic-forces by SW 0,19 mJ/mm2 may induce the HFF1 activation. Here the cytokine production in cells modulated by mechanotransdution was analysed. To be sure about Cytokines production we decided to use LPS at final concentration of 1 ng/ml[225]as positive control.

TGF-alpha was measured on supernatant of confluent HFF, but was not found any differences in cells treated with SW or LPS as compared to controls, (data not shown). Whereas, according to previously published observations[213], [225], [226] IL-6 was detected already after 4 hours in cells treated with LPS, interestingly at that time any significant difference could be detected between SW treated and negative control, nevertheless IL-6 level was up regulated in cells treated with SW after at 8 hours, similarly to what observed in LPS control.

The level of IL-8, was up regulated already after 4 hours of LPS treatment , as well as in cells that received SW treatment, however IL-8 modulation in cells treated with SW was not significant during the other experimental time points as compared to both positive or negative controls.

MCP-1, Monocyte chemoattractant protein-1, similarly to what observed for IL-8 and IL-6 at 4 hours in cells treated with LPS, in SW treated cells a minimal difference was observed with the negative control, but other analysis must be performed

GENE EXPRESSION ANALYSIS

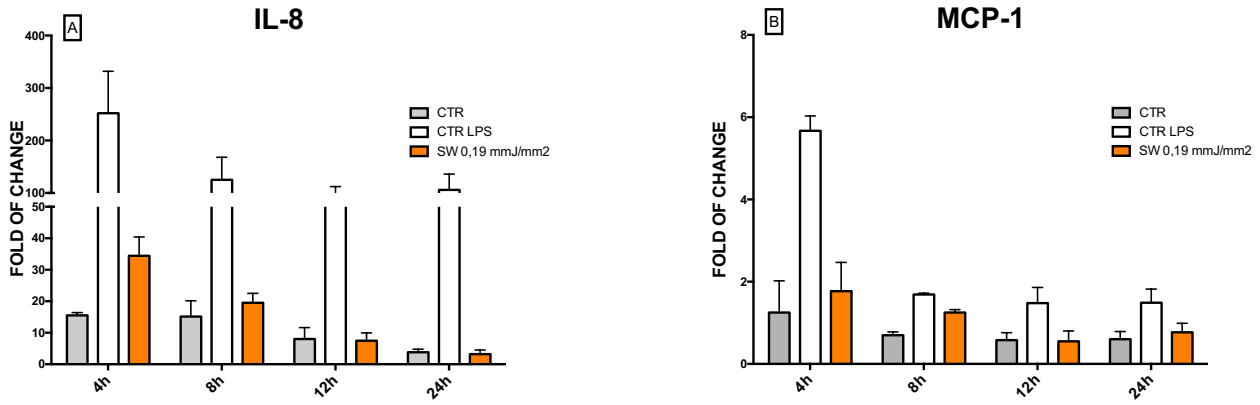

Fig. 42 Effect of Shock wave 100 pulses on IL-8 and MCP-1 gene expression on human foreskin fibroblast (HFF1)*. Cells were exposed (orange bar) or not (grey bars) to 100 pulses 0,19 mJ/mm2, cell treated with 1 ng/ml LPS are positive control (white bar).The amount of IL-8(a), MCP-1 (b) mRNA was assayed during t0,4,8,12 24 hours and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in control cell t0. (mean ± S.D; n=3).*

In order to evaluate the behaviour of HFF1 exposed to 100 pulses 0,19 mJ/mm2 in term of gene expression activation, cytokines mRNA level was quantified on HFF1. IL-8 was strongly expressed in LPS treated cells during 4, 8, 12 and 24 hours, while in SW treated cells IL-8 mRNA levels progressively decline fro 4-24 hours, although being up-regulated, as compared to control untreated cells. MCP-1 gene expression was strongly increased mainly at 4 hours, being upregulated in LPS positive control cells, than SW treated and control.

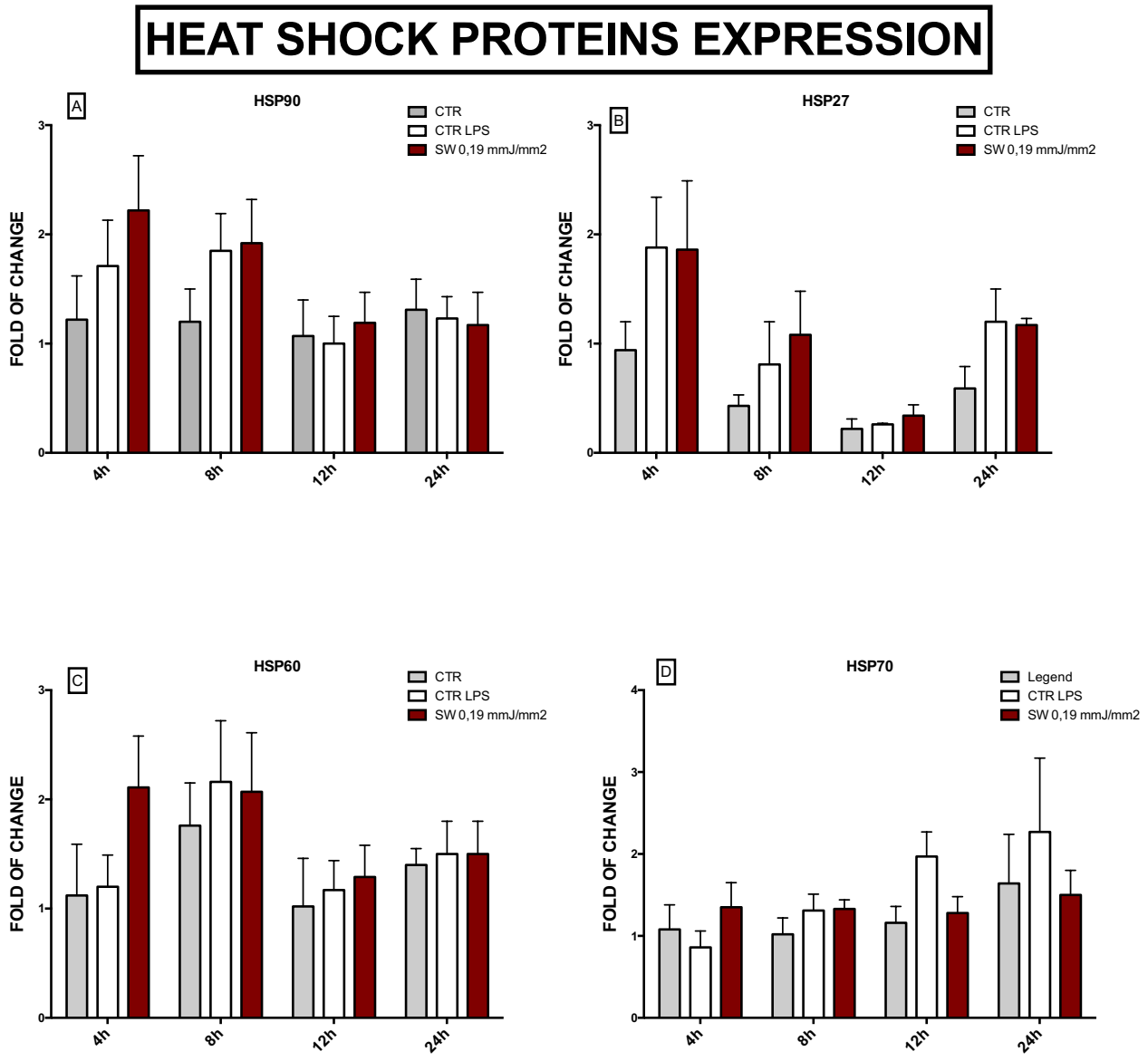

Fig. 43 Effect of Shock wave 100 pulses on HSPs gene expression on human foreskin fibroblast (HFF1). *Cells were exposed (bordeaux bar) or not (grey bars) to 100 pulses 0,19 mJ/mm2, cell treated with 1 ng/ml LPS are positive control (white bar).The amount of HSP90(a), HSP27 (b) HSP60(c) and HSP70 (d)mRNA was assayed during t0,4,8,12 24 hours and was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were plotted as fold change relative to the mRNA expression in control cell t0. (mean ± S.D; n=3).*

Heat-shock proteins (HSPs), also known as chaperones or stress-induced proteins, have a crucial roles during proteins folding/unfolding, assembly multiprotein complexes, transport/sorting of proteins into correct subcellular compartments[227]–[229]. HSPs are involved also in cell-cycle control and signalling, and protection of cells against stress/apoptosis. HSPs are often classified based on their molecular weight: hsp27, hsp60,

hsp70, hsp90 etc. In particular HSP90 is associated with proteins of the mitogen-activated signal cascade, particularly with the Src kinase, with tyrosine receptor kinases, with Raf and the MAP-kinase activating kinase (MEK) (MEK)[229]. Here we found that, the mRNA level of the different HSPs were differently modulated by LPS and by SW. The Hsp90 system is a complicated machinery that is co-activated by Hsp70 and by a large number of cofactors [229], however other studies showed that it can also be modulated alone. Interestingly, HSP90 and HSP60 gene expression exhibited an increase during 4 and 8 hours; HSP90 was increased after 4 hours in HFF1 cells treated with SW as compared to LPS control and tonegative controls, in the other experimental time points HSP90 progressively decreased to a basal expression. Concerning HSP60 expression the SW stimuli provided an up regulation during the first 4 hours, then similarly to what observed for HSP90 expression, progressively decreased. A similar behaviour was detected for HSP27, however the strongly down regulation detected after 12 hours in both LPS and SW treated cell populations need to be investigated more in details. On the other hand, HSP70 did not show any significant change during the experimental time, perhaps this HSPs is not activated by SW during the early times, however in cells treated with LPS, an increase at 12 and 24 hours could be observed. That finding, show how SW are involved in the modulation of HSPs pathways, and how a physical stimuli can interact with cellular behaviour similarly to a biochemical compound like LPS, in inducing cell activation and modification. Obviously the physical energy are acting generally on cell structure, while LPS binding to a specific receptors. The HSPs study is one of the most important pathway regulated by SW, however more evidence and experiments must be performed to further clarify these correlations.

DISCUSSION

If the effectiveness of Shock wave is clear in the clinical application on patients, the mechanism of action of shock wave stimuli on cells is still unknown. However, interesting founding encourage the researcher to continue in the study of physical stimuli on stem cells. Recently it was demonstrated the effect of shock waves on membrane hyperpolarization and Ras activation in human bone marrow stromal cells[209], and in macrophages activation [210], in HUVEC cells promoted the angiogenesis by the activation of vascular endothelial growth factor (VEGF), endothelial nitric-oxide synthase, hypoxia-inducible factor 1[211]. Wheis and colleagues demonstrated that the effect of SW treatment on C3H10T1/2 murine mesenchymal progenitor cells, primary human adipose tissue-derived stem cells and human T cell line, dose-dependently undergo the promotion of cell proliferation and the increase in extracellular Erk1/2 and p38 MAPK-dependent ATP release. Based on these observations, we used the same parameters on Adipose derived stem cells, in order to investigate more in detail the effect of SW during cell cycle activation. The viability analysis by EZ4U as well as the cell cycle detection were confirmed, with the maximum effect at high intensity, 0,19 mJ/mm2 for 100 pulses. The gene expression measurement on cyclins provided more evidence on the effect of SW treatment on adipose derived stem cells compared to control cells that did not receive any physical treatment. The cyclines, were finely regulated during cell cycle progression. The p53 and p19 were inversely expressed, according with cell cycle [52], [54]. Recent observations, showed the relevant role of DNMTs during cellular

behaviour. In fact, as observed by Robertson and colleagues, the mRNA levels of all three DNMTs change during the cell cycle progression, with DNMT1 and DNMT3b decreasing in G1 as well DNMT3a. During G0 phase, conversely cells express high mRNA level of DNMT3a, DNMT3b and DNMT1[61], here was found that physical treatment on Adipose derived stem cells interact with DNMTs protein profile, as well on gene expression. After 24 hours post treatment, SW-treated cells exhibited a significant decrease on DNA methyl transferase activation. That finding could be linked to the promotion of proliferation by SW energy, perhaps correlated with the activation of Erk1/2 and p38 MAPK previously observed in other studies[214], [221].

However, the main hypothesis is that the physical stimuli, shock wave, can activate the cellular metabolism, by a mechanic-transduction mechanism [216] at cellular level. In a second experiment, it was decided to use the fibroblasts cells (HFF1) to demonstrate that one of the basic mechanisms of shock wave, underlining cellular activation is based on the pro inflammatory-like pathway. It is known that during a stress, cells respond with various reactions. These include the inflammation. So we tried to figure out how the shock waves could activate cells through this induction. It was used a positive control with inflamed cells induced by the LPS, as found in the literature[219], [220]. Recently, it was published the effect of Extracorporeal shock wave therapy on the modulation of interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and tumour necrosis factor-alpha (TNF-a) in ligament fibroblast[213]. Moreover TLR3 stimulation and downstream signalling leads to the production of cytokines and chemokines that in turn modulate a macrophage-mediated inflammatory response [230]. In the present in vitro experiments, we

hypothesized that SW 0,19 mJ/mm2 may modulate early pro-inflammatory response (initiation phase). According to previous observation we found that SW mainly enhanced cell proliferation, acting on ATP release[214]. Reactive oxygen species (ROS) that physiologically play a role as secondary messengers during the repairing process, and appear to be important in coordinating the recruitment of white blood cells during healing[231]. The term 'ROS' describes molecules containing O_2 but which have been reduced with added electrons to become highly reactive. Endogenous cellular ROS can arise from mitochondrial oxidative phosphorylation during ATP production from the endoplasmic reticulum or from a class of enzymes known as oxidoreductases[231], here we believe that the increase in reactive oxygen species levels detected in SW treated cells could be correlated with ATP release and cell proliferation, trough the pro-inflammatory activation pathway. That finding were further inferred by the cytokine detected during the different experimental time points, in term of protein as well as gene expression. We believe that the mechanism by which cells convert mechanical signals into biochemical responses, by mechanosensitive molecules recruits the heat shock proteins, that are differently regulated after Shock wave treatment. HSP60 and HSP90, 4 hours after SW treatment were up regulated; it was recently shown that HSP60 plays a regulatory role during pro-inflammatory activation via TLR4-p38 MAPK axis[232], as well HSP90[229]. That findings again, could be strictly correlated with the previous observations rconcerning the involvement of SW in the Erk1/2 and p38 MAPK activation pathways [214], [215], [221] and with the modulatory action of SW on the plasticity of TLRs[230].

However, many other papers describe the main action of SW on patients as an anti-inflammatory treatment [108], [173], [211], [214], [230]. Here we just suggest a hypothesis of SW action on in vitro model during early time after SW treatment. Other studies must be performed in order to prove the anti-inflammatory effect of SW on cells after longer time following SW treatment. No matter how the science must be unique and reproducible, we need model to answer to the infinitive questions. Nevertheless, we have to keep in mind that "in vitro model" is always a model, which cannot be totally superimposable to the complex system that is human body. However, for the basic studies it is necessary to use them in order to figure out in a controlled behaviour what is going on.

CONCLUSION

Regenerative medicine for years aimed at finding new solutions for the regeneration of tissues and organs functioning in clinical practice in these patients with pathological degenerative diseases, with organs defects, tumours or with several traumas. However, till now many concerns are still unresolved. The cell transplantation protocols used are still a consolidated reality only in the haematological practice. The engineering of tissues still, proposed during this years, already suffers from many problems such as the type of cells to be used, and the suitable amount needed. All issues which turn to affects the quality of final product. Nowadays in fact, it is still not possible to produce organized tissues, with suitable dimensions applicable for the regeneration of severe trauma or to replace complete organs. The same use, of cell therapy is limited to haematological practice, the other applications are limited by issues including the amount of cells needed, their manipulations before implanting in patients, ethical concerning and safely guarantee. However, the first trials started a couple of years

ago, as well as the development of new technologies with 3D printing, that directly use cells like polymers to print tissues. These finding and new proposals, are opening up new hopes for the regeneration of tissues and organs, but still, there are many questions and problems to solve.

However, in parallel, in regenerative medicine field are studying other methods based on cellular stimulation with physical energy, which interacts with cellular systems increasing their performance. For a long time, researchers have observed that the cell itself, was perfectly organized in a perfect thermodynamic system, which enthalpy and entropy cooperate to support life. Each molecule, protein, nucleic acid is arranged in a specific order, now definable "polarization". The organization on various levels, from the cell, to the tissue, to the niche, to the body is based polarization. Finding then, a system that can interact with the cells, the tissues, the niches is a goal sought for long time. Over the years have been used and applied several physical stimuli ranging from the use of electromagnetism, ultrasound, photons, shock waves, radio waves. That have shown that after a specific stimulus, the cells, the tissues, healthy or pathological responded. Therefore, the main objective chased was and is the spontaneous induction of regeneration in the body, limiting so the manipulation in vitro of cells and tissues.

The scientific literature shows that physical stimuli make changes in term of physiological cell improvement. The electromagnetic energies, radio frequencies, as well as the shock waves, are able to increase the regeneration of wounds, fractures. The main question that arises is, how is possible interact in a targeted manner with cellular systems? With the tissues? In this thesis was analysed the effect of radiofrequency during modulation of aging in adipose

stem cells, we observed that the physical stimulus was able to interfere with aging at the molecular level by acting on gene expression into specific pathways, as well as on the epigenetic level and in the proteins involved in chromatin remodelling. it was also noted that radio frequencies are able to promote the differentiation of "instable cells" such as PC12, differentiated into dopaminergic neuronal phenotype. IPS along with specific chemical inducers towards cardiomiocyte phenotype, with an high yield of differentiation. Probably the physical waves interacting with the polarity inherent in every cell, promote towards a more stable state.

In the second session was studied another type of physical stimuli, which are based on mechanotransduction. This type of stimulus, shock wave, is used for years in clinical practice with success. Its efficacy was observed during induction of regeneration in various tissues and in several pathologies. However, it was not yet fully demonstrated its mechanism of action. On the “in vivo” cellular level promotes the proliferation, as well in our experimental study was observed that SW was able to increase cell proliferation in dish. The final hypothesis is that these stimuli creating a micro-trauma on cells, induce activation via a pro-inflammatory pathway, at least on the initial phase. Other studies have shown its anti-inflammatory effect during long time. In our studies we focused only on the early post-treatment stages.

On tissue level, we hypothesize, that this type of stimuli probably acts on the cells resident in the niches, activating them. Or probably, inducing the activation of cells that composed the tissue by inducing a number of secondary messengers such as ATP, ROS and cytokines that communicating with other cells promote the activation and initiate the regenerative process.

The study of the mechanism of action of physical stimuli on cellular systems, tissues or body will permit to figure out the targets of action, usable for enhancing performance in a specific way for future applications. Physical stimuli are an excellent solution for regenerative medicine toward spontaneous enhancement of regeneration compare the use of engineered material, or use of chemical or viral compounds on manipulated cells cultivated in vitro. However, the road is still long and the work is still much to do. Evolution has given us almost perfect body and control of physiological processes, the modulation of them is not easy, but it deserves to be studied. Nevertheless, the cooperation of new technologies, tissue engineering, cell transplantation and physical stimuli will pave the way finally at new suitable solution for helping patients.

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1.Table primers used

PRIMER NAME	REVERSE	FORWARD
REAC		
OCT 3	GAGGAGTCCCAGGACATCAA	CATCGGCCTGTGTATATCCC
sox 2	CAACGGCAGCTACAGCATGATGC	CCGTTCATGTAGGTCTGCGAGCTG
Nanog	CATGAGTGTGGATCCAGCT	CCTGAATAAGCAGATCCAT
cMyc	TCTGCCCATTTGGGGACAC	GCACCGAGTCGTAGTCGAG
P16 INK4	CAACGCACCGAATAGTTACGG	AACTTCGTCCTCCAGAGTCGC
P19 ARF 1	GCCTTCGGCTGACTGGCTGG	TCGTCCTCCAGAGTCGCCCG
P53	TGGCCTTGAAACCACCTTTT	AACTACCAACCCACCAGCCAA
Bmi 1	GCCAACAGCCCAGCAGGAGG	TTGGTGGTTACCGCTGGGGC
HTert	GACGTGGAAGATGAGCGTG	GACGACGTACACACTCATC
P21	CAAAGGCCCGCTCTACATCTT	AGGAACCTCTCATTCACCCGA
ACTN2	ACCAGTTTCACCCCTTTGCT	CGCCATGAACCAGATAGAGCC
GATA4	CTCAGATCCTTAGGTGCTAGA	TCCTCTTGCCTGGTAATGACTCC
hMEF2C	AGTGAGCTGACAGGGTTGCT	GCCCTGAGTCTGAGGACAAG
hMHC	TTTGATGCGCCCGAACTCTT	GAGGAAATGAGGGACGAGAGG
nkx2.5	TAATCGCCGCCACAAACTCTCC	TATAACGCCTACCCCCGCCTAT
TBX5	GTGGGGAGCCATGGTTGGCC	CAGAGTCGGCACAGCGGCAA
cTnT	CGTCTCTCGATCCTGTCTTTG	CATGGAGAAGGACCTGAATGA
SHOCK WAVES		
CCNE2 (cyclin E2)	CCG AAG AGC ACT GAA AAA CC	GAA TTG GCT AGG GCA ATC AA
CCNB2 (cyclin B2)	ACT GCT CTG CTC TTG GCT TC	TTT CTC GGA TTT GGG AAC TG
CCND1 (cyclin d1)	GCCGTGGTGTTTCTAGGGAT	GGGGGTTGGGAGTTCGTTAC
CCNA	CTGACTGAGCTGCTGGCTAA	CTCACAGGTCGATATCCCGC
DNMT1	CTCCTTGGGCCGCGCATCAT	GACGTCCGCAGCGTCACACA
DNMT3a	CAGCCGCTCTCTTGTGCGCT	CCAAGGCCGTGGAGGTGCAG
DNMT3b	CGGGCGTAGGGGGTACTGCT	GGCCGCAACCATGTGGACGA
HSP27	AAGCTAGCCACGCAGTCCAA	CGACTCGAAGGTGACTGGGA
Hsp70	ATGTCGGTGGTGGGCATAGA	CACAGCGACGTAGCAGCTCT
HSP60	TAAAAGGAAAAGGTGACAAGG	GGGCATCTGTAACTCTGTCTT
Hsp90-beta	AGTTGGAATTCAGGGCATTG	TTTCTCGGGAGATGTTCAGG
IL-8	GAACTGAGAGTGATTGAGAGT	CTTCTCCACAACCCTCTG
MCP-1	TCTGACTCTAAGTGGCATTC	ATTGTAGCAATGATCTCAACAC
HPRT1	AGCCCTGGCGTCGTGATTA	TGGCCTCCCATCTCCTTCA
GAPDH	GACAAGCTTCCCGTTCTCAG	GAGTCAACGGATTTGGTCGT

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