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The use of physical energy for tissue healing

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Oscillatory patterns permeate the universe and are essential requisite in living cells. Circadian clocks exist at the subcellular and single-cell level. Chromatin is assembled into rhythmic oscillatory domains driving stem cell growth/differentiation up to higher hierarchical embryo development. The cytoskeleton is organized as rhythmically oscillating networks, producing radioelectric fields that may turn local events into non-local, long-ranging paths. For decades, scientists have used chemical tools to affect cell behaviour. However, this view is now deeply challenged. Consonant with a major role of physical forces in living processes is the use of physical energies to modulate cell dynamics. To explore cell biology in the light of physics, we have recently established the Stem Wave Institute for Tissue Healing, within the context of GVM Care & Research—E.S. Health Science Foundation, Lugo, Italy. Here, we will discuss our recent findings that proper delivery of radioelectric fields is able to (i) finely tune stem cell multipotency, (ii) directly reprogramme human skin fibroblasts into cardiac-, neuronal-, and skeletal muscle-like cells, and (iii) revert stem cell senescence. We are dissecting vibrational modes as inherent properties of living cells, entailing nanomechanical signatures that can be used to direct stem cell fate. Mild mechanical forces are deployed to obtain human fluid tissues harbouring stem cells within their stromal–vascular niche. Synthetic molecules are designed to afford stem cell pluripotency. These discoveries prompt a deeper understanding of the interconnections between the physical universe and the living world in the attempt to further approach the information of Life.

Introduction

Chronic degenerative diseases, including cardiovascular diseases, represent a major health problem in modern society, where they occur as widespread diseases within the context of diabetes, obesity, and atherosclerosis.

Although stem cells hold remarkable promise for handling multiple disorders, these cells are currently isolated from the donor tissues by methods that require the use of enzymes and differential centrifugation. Moreover, to obtain sufficient cell yield prior to transplantation, stem cells are usually 'expanded' (cultured) *in vitro* for multiple passages, in an environment that is remarkably different from that experienced in both healthy and diseased tissues. From a regulatory standpoint, these cells are 'subjected to extensive manipulation' and, according to the worldwide established rules by both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA), they are considered as Advanced Therapy Medicinal Products (ATMPs). Accordingly, the chance of translation into clinical settings is remarkably delayed due to the requirement for compliance with cumbersome 'cell manufacturing' in observation of the current Good Manufacturing Practice (cGMP) guidelines.

Nonetheless, it is now increasingly becoming evident that the human body harbours multipotent stem cells within different 'niches', including the bone marrow,

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dental pulp, and adipose tissue. Moreover, resident stem cells with various *grading* of potentiality can be virtually found in any adult organ. These findings raise the larger question as to whether the chance for effective cell therapy should be more closely related to the efforts of promoting the endogenous tissue healing ability by resident stem cells, rather than relying on stem cell transplantation by itself.

For decades, scientists have attempted to drive stem cell fate through the use of chemistry. Challenging this paradigm, we have shown for the first time the possibility to use extremely low frequency magnetic fields to modulate cell growth and gene expression in adult ventricular cardiac myocytes,[1] and afford a high-throughput of cardiogenesis in mouse embryonic stem cells.[2]

More recently, we discovered that extremely low-intensity electromagnetic energy could be delivered to cultured cells with a radioelectric asymmetric conveyer (REAC), an innovative device delivering radioelectric asymmetrically conveyed fields (REACFs) of 2.4 GHz, and a radiated power of only 2 mW, with its conveyer electrodes immersed into the culture medium.[3] The emitted frequency is the most widespread and authorized at international level (i.e. Wi-Fi). At a distance between the emitter and the culture medium of $\sim$35 cm, and with duration of single radiofrequency burst of 250 ms, the specific absorption rate (SAR, the rate at which radiofrequency energy is absorbed by a defined amount of mass of a biological body) was 0.128 μW/g.[3] This value is by far below the SAR limit of 1.6 W/kg, averaged over a volume of 1 g of tissue, for the head, that has been set in the USA by the Federal Communications Commission. In Europe, the limit is 2 W/kg, averaged over a volume of 10 g of tissue.

Exposure of mouse embryonic stem cells to REACF was found to afford expression of pluripotentiality and high-throughput of commitment towards myocardial, neuronal, and skeletal muscle differentiation.[3]

Intriguingly, in our recent studies, we succeeded in the use of REAC–REACF to achieve a direct reprogramming of human dermal skin fibroblasts into cardiac, neuronal, and skeletal muscle lineages.[4] For the first time, human non-stem somatic adult cells were reprogrammed to a pluripotent state without being 'frozen' in such intermediate condition, but rather being rapidly committed to a high yield of fates that have long been pursued as major target lineages in regenerative medicine. These results were achieved without the use of potentially risky viral vector-mediated gene delivery, and without the needs of cumbersome and expensive chemistry.[4] Moreover, this strategy avoided the persistence of stray cells that have not fully differentiated and might have the ability to turn into an unwanted cell type, like a tumour or a cell that just does not fulfil the desired requirement(s) for a targeted tissue repair.

Based on these achievements, we decided to establish a new institute mainly devoted to the study of (stem) cell biology in the light of physics and physical theories, hoping that this approach may pave the way to new frontiers in cell therapy and regenerative medicine.

The Stem Wave Institute for Tissue Healing and its ongoing studies

Harbouring stem cells within autologous/homologous micrografts: a new strategy for tissue healing

Prior to establish the Stem Wave Institute for Tissue Healing (SWITH), we had developed an innovative non-enzymatic physical method to obtain a microfractured human fat tissue product (named Lipogems^{TM}), encompassing an intact stromal–vascular niche with elements of pericyte and mesenchymal stem cell identity.[5] This tissue product was found to be cryopreservable, readily transplantable in humans, and capable of yielding viable human adipose-derived stem cells (hADSCs) even when it was harvested from cadaveric donors within 36 h from death.[5] We have recently shown that the human Lipogems product afforded a significantly higher tissue repair than expanded hADSCs in a rat model of chronic hind limb ischaemia.[6] Worthy to note, although it was delivered as a xenotransplant, the degree of inflammation in the Lipogems-transplanted tissue was considerably lower than in the tissue receiving saline, or expanded hADSCs, indicating remarkable immunomodulatory properties of Lipogems itself.[6]

Consonant with these findings, the activity of SWITH is now involving a continuous effort in developing novel devices and methods that may be able to harvest autologous human stem cell populations within a preserved microenvironment, virtually from any tissue. Our goal is to obtain tissue micrografts prepared according to criteria of tissue homology, starting from the same tissue source that will be subjected to transplantation. The patient will therefore become the donor and the acceptor of a *calibrated micrograft*: bone, cardiac, arterial, venous, skeletal muscle, or endocrine tissues will be processed to provide and receive their own homologous micrograft.

The use of radioelectric fields to modulate stem cell pluripotentiality and differentiation

At SWITH, we are exploiting novel strategies to turn human multipotent adult stem cells into pluripotent elements exhibiting a high-throughput of commitment towards complex cell lineages. Based on our previous experience on the effect of electromagnetic fields on stem cell dynamics,[1–4] we have deployed the REAC technology to expose hADSCs obtained from our Lipogems product to asymmetrically conveyed radioelectric fields of 2.4 GHz. We found that the REAC treatment had no toxic effect on hADSCs, and did not affect significantly the amount of apoptotic or necrotic cells.[7] Interestingly, hADSC exposure to REAC primed a multilineage stem cell commitment. In particular, it induced the expression of the prodynorphin, GATA-4, and Nkx-2.5 genes,[7] which had been previously shown to act as major conductors in cardiogenesis.[8–10] The REAC treatment also triggered a programme of vasculogenic genes, including vascular endothelial growth factor, hepatocyte

growth factor, and von Willebrand Factor (vWF).[7] Radioelectric asymmetric conveyer-exposed hADSCs showed an upregulation of transcripts involved in both neurogenic and skeletal myogenic commitment, including neurogenin-1 and myoD, respectively. These transcriptional responses were associated with a biphasic effect of the radioelectric field on the expression of stemness-related genes, such as Nanog, Sox-2, and Oct-4. After inducing an early overexpression of these pluripotency genes during the first 4–12 h of treatment, the REAC exposure ensued in a significant downregulation of transcript levels below the control value after 24 h.[7] This inhibitory pattern persisted after 72 h of exposure, being still clearly evident even when cells were maintained in culture for additional 7 days in the absence of REAC treatment. This is an important feature of the REAC-mediated response, since it is now evident that after their induction, Sox-2, Nanog, and Oct-4 need to be downregulated to allow cell progression towards a differentiated state.[4,7]

Comparative analysis in REAC-exposed cells revealed that the early overexpression as well as the late inhibition of stemness genes were significantly more pronounced in Lipogems-derived than in enzymatically dissociated hADSCs. Confocal microscopy analysis showed the appearance of tissue-specific markers for cardiogenic (α-sarcomeric actinin and α-myosin heavy chain), neurogenic (β-3-tubulin), skeletal muscle (myoD), and endothelial (vWF) commitment in REAC-exposed, Lipogems-derived hADSCs, indicating that the induction of a tissue-restricted programme of gene and protein expression by radioelectric field delivery converged to the regulation of lineage specification at the intact cell level.[7] On the whole, Lipogems-derived hADSCs were more sensitive to the REAC treatment than enzymatically dissociated cells.

Reversal of stem cell ageing by asymmetrically conveyed radioelectric fields

Stem cells, like any other cell in the body, undergo senescence, which deeply affects their own self-renewal and differentiation potential. Human mesenchymal stem cells (hMSCs) have been isolated from many different tissues, but their number is exiguous in all tissue sources. Meta-analysis of currently available cell therapy protocols shows that hMSCs are transplanted at high doses, between 10 and 400 million hMSCs per treatment (www.clinicaltrials.gov). To fulfil these requirements, hMSCs undergo multiple passages and prolonged time in culture, usually 8–12 weeks. This approach has been shown to be a risk of, and a well-established model for, cell ageing *in vitro*.[11,12] Moreover, prolonged expansion impairs stem cell expression of pluripotency/multipotency, leading to a consistent decline in the multilineage repertoire and in the yield of differentiated cells.

Human adipose-derived stem cells are increasingly used as a source for cell therapy, due to the ease of tissue harvesting and their robust multipotency. Nevertheless, hADSCs also undergo significant senescence after multiple passages in culture.[11,13] This observation suggests that caution should be exercised when using hADSCs after multiple passages and prompts the needs for novel strategies counteracting senescence during the expansion of such a promising source of cell therapy.

A decline in the gene expression of senescence repressor Bmi1, and telomerase, together with telomere shortening, underlay senescence of stem cells cultured for multiple passages. We have recently investigated whether hADSC exposure to radioelectric fields may be capable to counteract the impairment of these senescence-preventing mechanisms. We found that, due to REAC exposure, the number of stem cells positively stained for senescence-associated β-galactosidase was significantly reduced along multiple culturing passages.[14] After 90 days in culture, REAC-treated cells exhibited significantly higher transcription of Bmi1 and enhanced expression of other stem cell pluripotency genes and related proteins, compared with unexposed cells.[14] Transcription of the catalytic telomerase subunit (TERT) was also increased in REAC-treated cells at all passages.[14] Moreover, while telomere shortening occurred at early passages in both REAC-treated and untreated cells, a significant rescue of telomere length could be observed at late passages only in REAC-exposed cells. Thus, REAC asymmetrically conveyed radioelectric fields acted on a gene and protein expression programme of both telomerase-independent and -dependent patterning to optimize stem cell ability to cope with senescence progression.

Directing stem cell fate with sound vibration

On the whole, the above reported findings clearly show the feasibility of using a physical energy to create an ideal 'milieau' capable to resume (stem) cell pluripotentiality, setting a major step forward in the improvement of future cell therapy strategies.

Within this context, we have demonstrated and patented for the first time the ability of cells to express 'vibrational' (nanomechanical) signatures of their health and differentiating potential.[15] Many biological processes taking place inside the living cell rely on the nanomechanical properties of cellular substructures and the cell membrane or wall itself. By the aid of atomic force microscopy (AFM), it is now possible to gain information on the integrity and local nanomechanical properties of mammalian and microbial cellular membranes under normal and stressed metabolic conditions. The AFM is a scanning probe microscope that measures a local property, such as topography, mechanical properties, thermal and electrical properties, optical absorption, or magnetism, with a probe or 'tip' placed very close to the sample. The small probe-sample separation makes it possible to take measurements over a small area. Because the AFM can image biological samples at sub-nanometer resolution in their natural aqueous environment, it has potential for characterization of living cells. Using the AFM, it has been possible to observe living cells under physiological conditions, detecting and applying small forces with high sensitivity.[16] In yeast and bacterial cells, cellular activity, metabolism, growth, and morphogenetic changes were associated with defined nanomechanical activity, merging to the cell surface up to the generation of defined patterns of vibrations.[16] 'Sonocytology' is the term that has been introduced to identify a novel

area of inquiry based on the fact that, in these small cells, after an accurate process of amplification, given the frequency range of nanomechanical motions recorded by AFM, the vibrations could be transformed into audible sounds, providing a thorough assessment of mechanistic cellular dynamics.[16] More complex eukaryotic cells can also be investigated by this approach. For example, stem cells directed to cardiac myocyte differentiation begin to beat at a point in differentiation. This beating motion requires a major reorganization of the cell cytoskeleton and in turn a significant change in cellular nanomechanical properties. Concerning the cytoskeleton, it is now evident that transferring of mechanical vibration to the subcellular environment triggers the mobilization of ionic species and the generation of ionic fluxes and induced microcurrents, ultimately ensuing in the appearance of oscillating electromagnetic fields.[17] Therefore, application of mechanical vibration is expected to generate endogenous electromagnetic fields. Intriguingly, multilevel memory-switching properties have recently been discovered by the aid of AFM and scanning tunnelling microscopy in single brain microtubules.[18]

At SWITH, we plan to investigate cellular mechanical properties as a function of differentiation processes to take a glimpse of measurable characteristics that may allow monitoring progression along specific lineages in individual cells. We are currently working on the hypothesis that application of localized forces, through the use of localized probes, magnetic fields, nanopatterned substrates, or substrates designed to apply localized forces, may inhibit, enhance, or direct cellular differentiation.

Affording epigenetic modulation by novel chemistry

We have previously shown that hyaluronan mixed esters of butyric and retinoic acids act as epigenetic modulators to commit mouse embryonic stem cells and hMSCs towards cardiovascular lineages *in vitro* and enhance the rescuing potential of hMSCs in infarcted hearts *in vivo*.[19,20] We have also provided evidence that small peptides, including endorphin peptides, may act as master inducers of stem cell cardiogenesis.[9,10] In isolated stem cell nuclei, these molecules activated nuclear receptors and signalling recruited in the orchestration of tissue-restricted gene transcription,[10] suggesting that a consistent part of their action on stem cell dynamics may have occurred intracellularly (*intracrine action*).

At SWITH, we are dissecting the epigenetic role of newly developed inhibitors of Class I and II histone deacetylases (HDACs) in vascular homeostasis. In this regard, we have shown that, within Class I HDACs, HDAC1 displays a major role in mediating the proliferation and migration of smooth muscle cells isolated from the pulmonary artery of animal models of pulmonary arterial hypertension.[21] These findings are now promoting the development of novel HDAC1 inhibitors that may act as a potential tool to restore normal vascular dynamics in this devastating disease.

Another area of enquiry is the study of the effects produced by naturally occurring molecules on human stem cell biology. To this end, we have recently shown that developmental stage *zebrafish* extracts are able to finely tune the expression of multipotency and senescence patterning in human Lipogems-derived hADSCs.[22] Studies are in progress to trace a proteomic profiling of early and late developmental stage *zebrafish* extracts and screen factors that may selectively regulate stem cell differentiation, survival, and ageing.

We are also developing novel synthetic chemistry targeted for epigenetic modulation of stem cell commitment and paracrine activity. These studies involve: (i) the synthesis of hyaluronan derivatives bearing small peptides to combine their modulatory action and afford intracellular peptide delivery and intracrine regulation of stem cell fate; (ii) the assessment of the effects elicited *in vitro* by these novel compounds on hMSC differentiation and the secretion of trophic mediators that may promote tissue healing in a paracrine fashion; (iii) the analysis of compound(s) action in animal models of acute myocardial infarction and chronic hind limb ischaemia. Our ongoing activity is also focused on the development of novel synthetic molecules and compounds that may afford substantial reversal of stem cell ageing *in vitro*, acting on both telomerase-dependent and -independent pathways.

Major implications and future directions

A new dynamic vision is emerging in biology, not simply based on the alternation of molecular events, but rather grounded on the essential role of rhythms and oscillatory patterns capable to orchestrate the multifaceted world of gene and protein expression into specific trajectories.

New discoveries in physics, biology, and epigenetics may encourage scientists, who have so far provided a fragmented picture of the living world, to create novel paradigms capable to unify the various disciplines, unravelling all the interconnections between the physical universe and the living world. At the basis of everything in the universe is precise information: it forms the origin that creates the particles and all systems, including the living systems we observe. A new paradigm in Science based on information may prompt a profound change in our perception of man–nature relationship, with an unavoidable shift in medical and therapeutic approaches.

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