Stem Cell Therapy

Elena De Falco, Antonella Bordin, and Eleonora Scaccia, Sapienza University of Rome, Latina, Italy

Carmela Rita Balistreri, University of Palermo, Palermo, Italy

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Email addresses: elena.defalco@uniroma1.it (E. De Falco); antonella.bordin@uniroma1.it (A. Bordin); eleonora.scaccia@uniroma1.it (E. Scaccia); carmelarita.balistreri@unipa.it (C.R. Balistreri)

Introduction

By 2030, approximately 20% of the population will be aged 65 or older, and age-related diseases (ARD) will represent a very health problem, with the cardiovascular diseases (CVDs), that will result in 40% of all deaths and rank as the leading cause (**Edwards, 2012**). Consequently, the research of urgent interventions both in preventive measures and biomedicine research is imperative. Some advancements have been achieved in the last years, including primordial prevention based on healthful lifestyle (i.e., Mediterranean diet, lifestyle, and physical activity (**Armanios et al., 2015**). In addition, advanced procedures, such as for examples the percutaneous coronary intervention and coronary artery bypass grafting in management of coronary artery diseases, having higher prevalence and incidence in the world, have obtained a significant success (**Balistreri, 2018**). Despite these efforts, there are no effective solutions now, against the ARDs and their complications. In addition, numerous gaps remain between the knowledge of precise cellular and molecular mechanisms involved in the onset and progression of ARDs, and the identification of disease pathways to apply as appropriate biomarkers and targets for new and more efficient therapeutic treatments, that is, personalized therapies.

Thus, biomedical community is pursuing new ways in trying to face this imposing challenge. Specifically, the latest discoveries and advanced knowledge in the fields of stem cell biology and their ability to provide a cue for counteracting several diseases are leading numerous researchers to focus their attention on the *Reg*enerative *Medi*cine (RegMed), as possible solutions for ARDs (**Balistreri, 2018**). However, a critical analysis of the current status of RegMed field appears unfavorable, by evidencing its unproductive application in clinic therapy. Effectively, RegMed is yet to bring about the therapeutic revolution, that it awaited already before its birth. After about two decades of extremely high expectations and often disappointing returns both in the medical as well as in the financial arena, this scientific field reflects the sense of a new era and suggests the feeling of making a fresh start, although many scientists are probably seeking a reorientation. Much of research was industry driven, so that especially in the aftermath of the recent financial meltdown in the last years it has witnessed a biotech asset yard sale. Despite any monetary shortcomings, from a technological point of view there have been great leaps that are yet to find their way to the patient. RegMed is bound to play a major role in our life, because it embodies one of the primordial dreams of mankind, such as: everlasting youth, flying, remote communication and setting foot on the moon. The scientific journals have been the voice of these developments in RegMed from its beginning, and currently reflect the recent scientific advances in this field. Therefore, the idea of our chapter is in describing the advantages, progresses and limitation in this field, that might just be like looking '*back to the future.'* Thus, the principal message of this chapter stays in suggesting that "we are almost there," being able to produce tissue replacement 'off the shelf,' and soon for everyone in need. However, it currently appears—literally spoken—that we can fabricate constructs that 'look like tissue, smell like tissue and taste like tissue' but not some that also function equally like one. A continuous evolution in the research and application is yet to bring the long-awaited revolution in the health area. More critically spoken, some researchers and physicians have proclaimed that the time must come to '*stop tissue engineering and start engineering tissues.'* In order to achieve this goal, these measures are needed: (1) considering the advances and the limitations of this field; (2) re-examining the data obtained and controversies (3) in order to filter them and (4) to put together only the valid parts (5) for constituting a clear puzzle by using standardized research methods and methodologies; (6) this will be useful for pursuing innovative ways through a strong cooperation among scientists, physicians, administrators, and public officials. Obstacles might be diverse, and the road might be hard and long, but the advances obtained might be numerous and offer us greater and unique opportunities to meet the imposing challenge of ARDs.

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Stem Cell Therapy

Stem cell therapy represents a new class of medicine, and precisely one of the most crucial approaches of RegMed (see **Fig. 1**). Ever since their discovery and initial isolation, the scientific community has embraced stem cells as potential candidates for a therapeutic approach to chronic diseases, ARDs, that moves from disease management toward a regenerative framework. However, this strategy is not new, since it has already been successfully applied in the clinic area in the form of organ transplantation. Organ transplantation provides the possibility to replace malfunctioning organs with unscathed donor organs, that can take over the functions and to improve and extend the life's quality of the patients. However, this approach is severely hindered by the shortage of donor organs, and, therefore, it will never become a viable medical treatment option for most patients. Thus, progression and refinement in terms of both the isolation and culturing procedures of stem cells over recent decades have reopened the door for RegMed approaches, in hope of healing the chronically damaged organs of patients who are on a transplant waiting list (**Heidary Rouchi and Mahdavi-Mazdeh, 2015**). Regarding stem cell therapy, it needs to stress that it has different features from drug therapy, or other types of RegMed therapies. It is based on cells, and they represent the most complex biopharmaceuticals. Protein or gene therapies are based on relatively simple macromolecules, and they are ideally appropriate to target a single defect, rather than eliciting a complex biological regenerative response (for which stem cells seem to be better suited). Cells are more complex, being formed by different and numerous proteins, and by an entire genome. In addition, they are dynamic in their phenotypes and activities, by interacting with their microenvironment and responding to systemic stressors. These features offer limitations in their application. Upon transplantation, the transcriptome, proteome, and even secretome profile of cells can, indeed, change, thereby varying their functional capacities respect with those observed in vitro upon initial culture expansions. All these observations evidence, on one hand, that cells are unique multidimensional therapeutic treatments well-suited for a RegMed approach. On the other hand, it also stresses that cells are a complex and challenging entity that remain to study and apply (**Heidary Rouchi and Mahdavi-Mazdeh, 2015**). Accordingly, stem cell therapy shows unique properties when compared with other RegMed approaches or standardized drug treatments. Firstly, it reduces the capacity to discovery an appropriate pharmacokinetics for each type of cell therapy, that is essential for its outcome. Specifically, the methods of administration vary than those for a drug. The preferred drug way is oral administration. For cell therapies, it is not favorable, because cells do not survive in the acid environment of the stomach, and even less so in the intestine. A more appropriate administration way for cell therapies might be into the circulation, by means of an intravenous injection, such as for the hematopoietic stem cells (HSCs) therapy in cases of leukemia treatment (**Heidary Rouchi and Mahdavi-Mazdeh, 2015**). However, it shows many obstacles prior to the arrival of cells in the specific damaged organ or tissue. Consequently, local strategy might be more advantageous, even if it can be difficult to bring the cells to a preferred location in order to avoid injecting them into a remote region that is too far from the injury or deprived by oxygen and nutrients (**Feyen et al., 2016**). Furthermore, the pharmacokinetics of cell-based therapies show other difficulties, including: (a) the inability to monitor the bio-distribution of stem cells

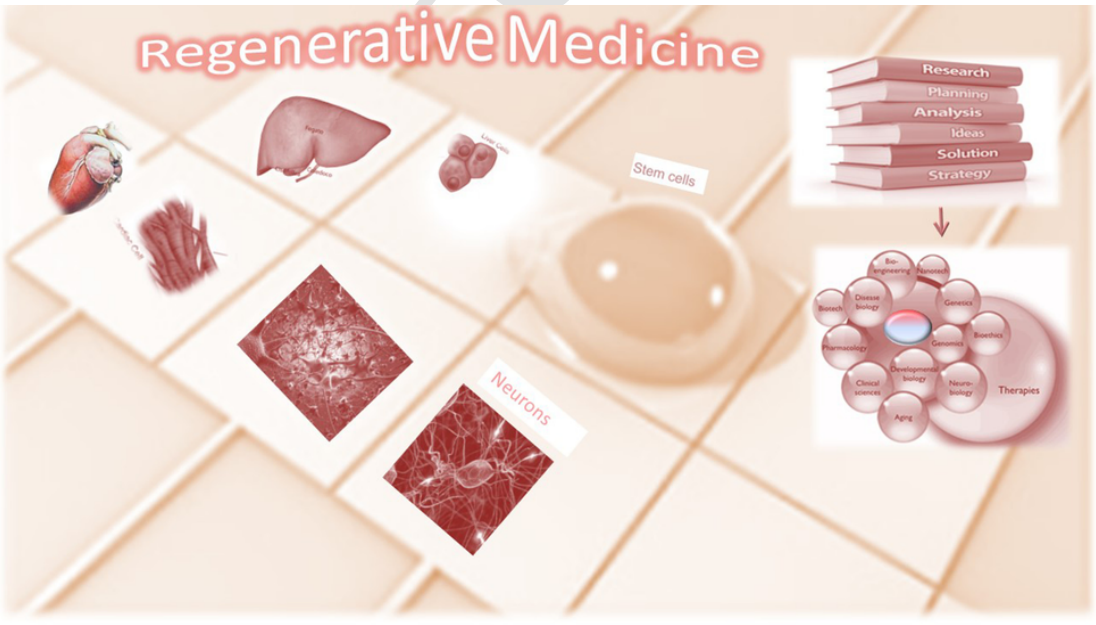

Fig. 1 Regenerative medicine. An emerging branch of translational medicine focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any causes, including congenital defects, diseases, trauma and aging. Stem cells are the cornerstone at the heart of regenerative medicine and might provide the potential solution for human diseases. However, use of stem cells as drugs for diseases, that is *stem cell therapy*, requires several steps as well described in the picture.

after their administration; (b) the survival of stem cells in the damaged tissues, that is very difficult (cellular therapeutics delivered into ischemic myocardial tissue arrive in a hostile inflammatory milieu, and are therefore susceptible to pro-apoptotic signaling); and (c) engraftment and integration (in case for example of cardiac stem therapy, engraftment is only the first step toward remuscularization, since subsequent organization and proper tissue integration are critical for the participation of engrafted cardiomyocytes in heart repair) (**Feyen et al., 2016**). These restrictions have led the researchers to develop delivery strategies to enhance the retention, survival and integration of stem cells. Approaches utilizing pharmacology, genetic manipulation, biological or material incorporation have been implemented to improve these aspects (*see below*).

However, the pharmacokinetic proprieties of cells are not the exclusive aspects to consider for the further improving stem cell therapies, since therapeutic action can also be addressed by modulating the performance of stem cells. All these observations highlight the fact that the early promise of stem cell therapy to repair the damaged organ by injecting different cell types has not yet been satisfied. For improving stem cell therapy, an important feature to consider in the stem cell therapy is the capacity of the injected cells to produce and secrete a pleiotropic repertoire of factors. These factors can influence the cellular micro-environment and thereby help the stressed tissues. As result, stem cell therapy becomes a complex delivery tool for reparative biological drugs. First-generation cells, including bone marrow (BM) stem cells and their secretomes, are mainly aimed at cellular salvage and at stimulating the endogenous repair mechanisms of the damaged organs through pro-angiogenic or pro-survival activities. However, the application of these cells in the clinic area has never achieved the expected level of results. Future studies will need to address the underlying poor pharmacokinetic properties, in order to bolster the effects of these first-generation paracrine therapies. Furthermore, the manipulation of cells to rejuvenate the patients' own cells or to enhance paracrine action are also exploring. This will lead to the application of stem cell therapy as a complex delivery tool in which the slow release of reparative signals is further enhanced. The paracrine effects will boost endogenous repair mechanisms to maintain organ homeostasis. Overall, a shift from the initial pragmatic delivery approaches toward tailored delivery strategies aimed at improving pharmacokinetics by using pharmacodynamics properties, that will consent their clinical application. However, once optimal strategies are developed, they can be coupled with technologies that have been developed over the last decade of stem cell research. Furthermore, recent clinical trials have helped to train clinicians for the delivery of cells into the organs and lay a foundation for cell therapy work in many medical centers around the world. These advances will help expedite the transition of future cell therapies toward patients. Although cellular therapeutics have failed to survive to their initial hype, careful re-evaluation of their mode of action and steps to address the current pitfalls should help to unlock the vast potential of stem cells, and help them to reach the clinic in a timely fashion. Furthermore, the research community is also focusing its attention on progenitor cells as optimal candidates. Among these, bone marrow (BM)-derived endothelial progenitor cells (EPCs) are emerging as candidates for several applications (**Feyen et al., 2016**).

An Overview on Stem Cells

Stem cells and progenitors have been considered as potential candidates for stem cell therapy.

For a better understanding of this topic, it briefly reports what it intends for stem cells. Stem cells are defined as undifferentiated cells with the potential to renew themselves, and to differentiate into any other specialized cell of human body, and, therefore (potentially and theoretically), to create any tissues or organs. Under specific conditions, stem cells can, indeed, differentiate into a diverse population of mature and functionally specialized cellular types. To date, different classes of stem cells are recognized. Among these, in the first class, there are the totipotent cells, which have the capacity to differentiate into embryonic and extra embryonic cell types, thereby generating entire organisms, even if this capacity is limited to cells produced by the first few divisions after fertilization. Pluripotent stem cell types are another class. They can differentiate into all embryonic cell types and form ectoderm, endoderm and mesoderm cell lineages, but not into extra embryonic cell types, and thereby they can form all the cell types of an adult organism. Finally, there is the class of adult multipotent/ unipotent stem cells, often termed progenitor cells, which can only differentiate into several closely related cell types (**Stoltz et al., 2015**).

Several cellular types have been and are currently investigated and applied in RegMed, including BM-derived mononuclear cells (BM-MNCs), peripheral blood mononuclear cells (PBMCs), mesenchymal stromal cells (MSCs), embryonic stem cells (ESC), induced pluripotent cells (iPSCs), and organ-specific stem cells. Among these cells, ESCs and iPSCs exhibit nearly unlimited potential to differentiate in vitro *and* in vivo, but their applications are limited by ethical, legal and political concerns, as well as by scientific and clinical issues of safety and efficacy (**Stoltz et al., 2015**). Therefore, tissue-specific stem cells derived from adults offer alternative approaches that circumvent many of these concerns. However, stem cells for RegMed applications should be consistent with the following criteria (**Stoltz et al., 2015**):

- ✓ Can be found in abundant numbers
- ✓ Can be harvested by a minimally invasive procedure with minimal morbidity
- ✓ Can be differentiated along multiple cell lineage pathways in a controllable and
- ✓ reproducible manner
- ✓ Can be safely and effectively transplanted to either an autologous or allogeneic
- ✓ host
- ✓ Can be produced in accordance with current "Good Manufacturing Practice Guidelines"

Based on these criteria, alternative and more efficient candidates for RegMed applications are represented by cells of human adipose tissue, that can be collected in large quantities (**Lindroos et al., 2011**). Human adipose stem cells (ASCs) are an abundant cell source with therapeutic applicability in preclinical studies in diverse fields, due to their ability to be readily expanded and their large capacity to differentiate in vitro in several cell types, from the adipogenic type to osteogenic, chondrogenic and neurogenic varieties. Furthermore,

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ASCs have been shown to have immune-privilege and to be more genetically stable in long-term culture, when compared with BMSCs. The safety and efficacy of ASCs for tissue regeneration or reconstruction is currently under assessment in clinical trials (**Lindroos et al., 2011**). The number of trials investigating the efficacy of treating conditions such as Type I and II diabetes, liver cirrhosis and regeneration, fistulas, CVDs, limb ischemia, amyotrophic lateral sclerosis and lipo-dystrophy have risen rapidly, even if a very limited number has been completed (http://clinicaltrials.gov). Furthermore, ASCs are also under examination in clinical case studies for graft-versus-host disease, immunosuppression (rheumatoid arthritis, Crohn's disease and ulcerous colitis), multiple sclerosis, soft tissue augmentation and bone tissue repair. Clinical bone tissue reconstruction studies using autologous ASCs are also ongoing (**Lindroos et al., 2011**).

Currently, other potential candidates for RegMed cell therapy are emerging, including progenitor cells from BM or other tissue niches. Among these, EPCs are emerging as new therapeutic agents for several age-related diseases. They also represent the most widely studied adult human progenitor cell subpopulation up to now. The interest of the research community on EPCs arises from advances, over the last decade or so, relating to the discovery of postnatal vasculogenesis (called neoangiogenesis), which is brought about by circulating progenitor cells, capable of differentiating into mature blood vessel endothelial cells. Thus, EPCs and their biology constitute a common point of interest for physicians and basic scientists with the goal of translating their research into clinical application by using the innate reparatory mechanisms of the heart and vascular endothelium, as well as of other organs (**Balistreri et al., 2015**).

Stem Cells as Therapeutic Agents: Limitations and Concerns

The clinical application of stem cells as therapeutic agents has a reduced validity limited by different factors: (a) the small number of patients enrolled in the major number of studies, their randomization not blinded, the involvement of few centers, (b) the exact phenotypic profile of cells used for the treatments which is always not indicated or missing, (c) the different administration ways and methods used, and (d) the safety and feasibility of the treatments not proved by long-term follow-up results. Teratoma formation, immunoreactivity, or other negative effects may represent the adverse effects of these treatments. Accordingly, the genetic and epigenetic instabilities of stem cells present a recurring obstacle to progress in RegMed using this approach. Various studies have stated that these instabilities can transform stem cells when transferred in vivo, developing tumors. Previous research has shown that various extrinsic and intrinsic factors can contribute to the stability of stem cells. The extrinsic factors include growth supplements, growth factors, oxygen tension, passage technique, and cryopreservation. Controlling these factors based on previous reports may assist researchers in developing strategies for the production and clinical application of "safe" stem cells. On the other hand, the intrinsic factors can be unpredictable and uncontrollable; therefore, to ensure the successful use of stem cells in regenerative medicine, it is imperative to develop and implement appropriate strategies and technique for culturing stem cells and to confirm the genetic and epigenetic safety of these stem cells before employing them in clinical trials (**Stoltz et al., 2015**).

In addition, there are other limitations in the large-scale clinical use of stem cells and their progenitors, such as EPCs (**Lindroos et al., 2011**). For example, in the ample number of cases, their progenitors are relatively rare cells, and expansion in enough subpopulations from peripheral blood is hardly possible. Furthermore, in vitro enumeration of progenitor cells for an appropriate quantity for a therapeutic treatment is associated with changes in phenotype and differentiation and risk of cell senescence and it may require artificial cell preactivation or stimulation (**Stoltz et al., 2015**).

Progresses and Novel Applications

To date, stem cells can be considered as a drug and therefore similarly to a pharmacological treatment, as abovementioned. From this point of view, they have been employed for several clinical purposes and mainly in the oncology, cardiovascular and regenerative medicine field (**Siciliano et al., 2015**). Although, several clinical applications have been tested, not all stem cells have provided the benefits hypothesized. This limitation can be likely ascribable to our poor understanding of the biological interactions between microenvironment and stem cells once injected and to a wide range of negative or positive dynamic changes, which often hamper stem cells to properly engraft, differentiate and function within the tissue (**Spaltro et al., 2016**). Besides, immune response can severely influence the exogenous treatment with stem cells, although in some body districts such as retina, ESC-derived retinal neurons have been found integrated within the tissue even after 3 months (**Chao et al., 2017**). The interplay with the recipient's tissue and the alterations of the stemness is more evident for certain stem cell populations as for MSCs reported to both enhance and suppress cancer progression especially by epithelial-mesenchymal transition process (**Gloushankova et al., 2018**). The capacity of stem cells to function as a drug or gene carrier has been largely demonstrated in MSCs and mainly for specific clinical use including wound healing, cerebral cancers, bone reconstruction (**Wu et al., 2019**). Nevertheless, the urgent need to deliver the biological potency of stem cells only where the insult occurs and even if systemically administered, have shifted toward the combination with nanoparticles, synergizing the beneficial effects of both. Organic or synthetic nanoparticles acts as nanocarriers for stem cells or their soluble mediators or protein derived. The small size and the low toxicity of nanoparticles facilitate to decrease the potential immunogenic or tumorigenic reactions of stem cells and to empower their function. In this novel technological and always more sophisticated scenario, some stem cell populations are more investigated than others, based on the natural ability to act as carrier such as blood stem cells (**Kato et al., 2016**). Mesenchymal stem cells are equally and largely employed, due to their phenotypic plasticity, versatility and no induction of teratoma compared to ESCs or iPSCs, as above stressed and evidenced at least so far (**Cooper, 2013**; **Suryaprakash et al., 2019**). Although several clinical trials employing MSCs alone, in combination with growth factors, seeded on scaffolds or gene modified, have been tested, the new frontier of the nanotechnologies attempts to address stem cells or their products directly to the site of injury in a more accurate fashion. To boost the tumor-tropic ability of MSCs, engineered MSCs and nanocomposites have been integrated and tested in a mouse model of glioblastoma showing high retention ability in the tissue and improved chemotherapeutic drug delivery (**Suryaprakash et al., 2019**; **Wang et al., 2019**). The MSC-based

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cancer tropism has been exploited in other tumors, such as lung cancer, where a similar approach based on a combination of MSCs and loaded nanoparticles with specific cancer drugs avoids the sole entrapment of stem cells within the lung and it increases the suppressive effect on cancer compared to the sole injection of nanoparticles (**Wang et al., 2019**). In different lung pathologies, the effect of the all trans retinoic acids in the form of solid lipid nanoparticles has been evaluated over the time, demonstrating a comparable efficacy to MSCs (**Payne et al., 2019**). Metastatic progression has been reduced by gene transfection of MSCs in a model of DNA nanoparticle and spermine pullulan (**Payne et al., 2019**). The versatility and the chemical composition of nanoparticles have become extremely important to drive stem cell behavior. In fact, if nanoparticles are loaded with drugs or specific molecules, they can be incorporated by stem cells trough endocytosis and once in the tissue, cells can release them over the time in a more physiological fashion. This property has been mainly exploited for MSCs and several antitumoral drugs, demonstrating a better efficacy compared to a mere combination of MSCs and drug in situ (**Payne et al., 2019**; **Levy et al., 2016**). The same studies have also highlighted that this approach would not alter MSC stemness. For instance, mesoporous silica nanoparticles (organotic compound) influence the stemness of human melanoma cell line, activating caspase-dependent pathways and modulating stem cell gene expression (**Maksimović-Ivanić et al., 2019**). More importantly, stem cell-derived molecules can be also intrinsically considered as drug delivery methods. The combination of gold nanoparticles and X-ray computed tomography has been applied to track the in vivo path of MSC-derived exosomes in the brain area of murine models with cerebral disorders or stroke, highlighting the relevance of proinflammatory signaling to address homing, tissue regeneration and drug delivery in organs more difficult to treat such as the brain (**Perets et al., 2019**). An even more advanced approach is represented by the stem cell-derived membrane coated nanoparticles, which would allow maximizing the paracrine ability of progenitor cells without their direct injection. This strategy based on the stem cell secretome encapsulated in microparticles made of golden, nanogel or different polymers and proteins but with a biological stem cell membrane obtained, would act as a quantum release of soluble mediators in situ. Injection of stem cell-derived membrane coated nanoparticles (for instance endogenous cardiac progenitor cells) in murine model of myocardial infarction, have successfully demonstrated the recovery of the fibrotic scar, a decrease apoptosis and the enhancement of the angiogenic process (**Avolio et al., 2014**; **Li et al., 2012**; **Luo et al., 2017**).

Bio-Nanotechnologies as Support of Stem Cell Therapies: Stem Cell Organoid Engineering

Bioprinting and Differentiation of Stem Cells

Current strategies employing stem cells in three-dimensional (3D) systems are revealing extremely promising, not only as study models, but mainly as significant tools to better mimic physiological micro-niches as well as pathological scenarios (specifically, oncology and regenerative medicine), often difficult to accurately reproduce in vitro. The need to switch from two (2D) to 3D systems origins from the phenotypic and functional decline observed in stem cell cultures over the time, also hampering the prospect to evaluate the effects due to long-term stimuli. Three dimensional-based approaches seem also to improve metabolism and to enhance gene expression and cell fate, angiogenic secretome and stem cell engraftment in the recipient tissue when transplanted (**Kim et al., 2018**; **Cesarz and Tamama, 2016**), strengthening the importance of geometry and spatial arrangement of stem cells and critical to determine cell–cell signaling and interactions. From a biological standpoint, stem cell organoids are currently studied for two main reasons: drug testing and differentiation studies. 3D models employing stem cells can be useful to predict the pharmacological response before a stem cell-based therapy or simply to design the best drug regimen for patients (**Mawad et al., 2017**; **Perkhofer et al., 2018**). Stem cell differentiation represents a main gold standard of any clinical treatment. Hence, stem cell-based therapies provide tissue regeneration, only when a proper differentiation program is enough enhanced, and engraftment of progenitor cells in the tissue is achieved in order to restore organ function. The microarchitecture within the 3D system parallel to mechanical stimulation has been demonstrated to improve stem cell fate as reported for human MSCs (**Poudineh et al., 2018**). Notably, there are still opposite reports on the need to predifferentiate stem cells before assembling into 3D constructs. No difference has been found between in vitro pre-differentiated and undifferentiated stem cells (**Gruene et al., 2011**). Nevertheless, many reports (on MSCs) have demonstrated that the predifferentiation enhances the efficiency of 3D constructs (**Binder et al., 2014**), suggesting that the type of commitment required, and the nature of the stem cell population might represent a key issue to consider. A mandatory step of stem cell differentiation, often hampered because of the paucity of the starting material, also requires expansion of progenitors. The 3D systems increase either cell proliferation by decreasing senescence and the chance to expand only selected progenitor fractions which are difficult to isolate and to culture by conventional 2D methods (**Underhill and Khetani, 2017**). Accordingly, nano-scaffolds of synthetic materials well support stem cell self-renewal, growth and higher purity compared to 2D models especially for HSCs which normally require a considerable number of cells for clinical applications (**Mehrasa et al., 2014**). Additional genetic manipulations of stem cells in the form of organoids are also feasible. Three-D liver cell cultures have been transduced with adenoviruses on microchip platforms aiming to control gene expression of drug metabolism enzymes (**Kwon et al., 2014**). Genetic modifications are also allowed when stem cells are assembled in 3D organoids. The combination of 3D systems based on ESC spheroids and CRISP/Cas9 technology to knockdown Wtn4, has been recently employed to recapitulate the kidney development in vitro (**Tan et al., 2018**). Other studies have immobilized hepatocytes-based spheroids on specific microarrays for the evaluation of cytochrome P450 (**Fukuda and Nakazawa, 2011**). Interestingly, 3D-based organoids have been also employed to increase the differentiation of a progenitor population from a second source of stem cells, as demonstrated for human ESC spheroids directly differentiated into MSCs (**Yan et al., 2018**). The type of stem cell can also influence the 3D technology. For instance, although iPSCs show an intrinsic ability to generate 3D structures on scaffolds (**Hattori, 2014**; **Lei and Schaffer, 2013**), however they are difficult to culture due to their complex cytoarchitecture (**Konagaya et al., 2015**). Recently, 3D bioprinting-based approaches (which allow to directly mix iPSCs and bioink and fostering a more physiological and enhanced self-assembling) have been developed to facilitate such process, confirming their differentiation toward several phenotypes including the neural, dermal lineage (**Gu et al., 2017**; **Michael et al., 2013**). Bioinks vary from natural ECM components (hydrogels, hyaluronic acid) to synthetic fibers (**Eswaramoorthy et al., 2019**). The bioprinting strategy

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represents an advancement of the conventional scaffold-based methodology, as it can foster multiple or parallel layer-by-layer stratification of ink and stem cells, therefore better reflecting the different cell layers normally present within a tissue also ameliorating the control of stem cell distribution and in situ differentiation in the scaffold (**Gu et al., 2017**; **Michael et al., 2013**; **Eswaramoorthy et al., 2019**) as demonstrated for ESCs, where even the colony size can be modulated (**Dias et al., 2014**). More importantly, the 3D bioprinting would also recreate a more physiological differentiation-prone microenvironment in presence of stem cells. Accordingly, bioinks can be preloaded with soluble molecules or alternatively, growth factors and cytokines can be added to the 3D construct-derived media, therefore challenging the whole system before or after its transplantation. Mesenchymal stem cells (precursors of bone and cartilage formation (**Siciliano et al., 2015**; **Spaltro et al., 2016**), can be cultured in 3D constructs and differentiated toward the osteogenic and cartilaginous phenotype by adding hydroxyapatite, TGF-β3 or BMP-6 (**Siciliano et al., 2015**; **Spaltro et al., 2016**). A further advancement of the 3D bioprinting is based on a laser direct-write, allowing a more controlled design of the size and shape of stem cells during assembling on the constructs and demonstrating that geometry can even influence drug delivery (**Michael et al., 2013**). Interestingly, the 3D systems allow to combine different biological scenarios: for instance, the investigation and the physical synergy between microenvironments of different origin or stem cells with other non-stem cell adult populations or the testing of a wide range of stimuli including mechanical stretch, extracellular matrix components or metabolic inputs (**Gu et al., 2017**). The microenvironment generated in 3D systems represents a main determinant of stem cell differentiation. By improving the raw material composition of matrix where stem cells are seeded in, it is possible to split up several signals generated into the microenvironment and to assess them singularly such as reaction of stem cells to stiffness or coculture and soluble mediators (**Lei and Schaffer, 2013**). Microwell devices made of hydrogels or hard materials have been designed for this purpose and to evaluate the clonal composition of single stem cells in the cultures (**Lei and Schaffer, 2013**). Besides, organoids represent an interesting method to test the functionality of stem cells, as recently demonstrated by transplanting a new engineered magnetic bioprinting seeded with neural crest-derived MSCs able to differentiate in the salivary gland-like phenotype and to suitable engraft in the tissue without inflammatory or adverse reactions in the recipient (**Lei and Schaffer, 2013**). Stem cell function can be also verified by coculture 3D systems. The combination of MSCs and endothelial cells have revealed the ability of the stromal population to switch from non-stem cell function with supporting features on angiogenesis to mesodermal differentiation around vessels of the organoid (**Lei and Schaffer, 2013**).

A New Vision in the Application of Stem Cells: Stem Cell Derived Exosomes

Exosomes can be defined as nanosized membrane-bound extracellular vesicles representing a possible carrier of several biological information (**Zhang et al., 2019**). Exosomes can exert our activity locally where they are produced, playing a key role in cell–cell communication, or even at a distance, reaching faraway areas where they are able to activate a wide range of cells signaling and to influence cellular behavior and to alter tissue metabolism (**Phinney and Pittenger, 2017**). Both mechanisms implement and represent the foundation of their well-known paracrine effect. For these reasons, exosomes have been considered a great tool to empower regenerative therapy approaches, by including stem cell-derived vesicles, which are currently considered as bioactive component of the stem cells themselves (**Khan and Kishore, 2017**). Accordingly, exosomes have been isolated from multiple stem cell sources. Specifically, many studies focus on exosomes secreted by bone marrow, umbilical cord, urine, oral mucosa and adipose tissue-derived MSCs and pluripotent stem cells such as ESCs and iPSCs (**Xiao et al., 2019**). All reports agree that the regenerative potential ascribable to stem cells is mediated by a complex secretome including growth factors, lipids, cytokines, mRNA and microRNAs, where exosomes are recognized as main transporters (**Roşca et al., 2018**). This heterogenous profile of soluble mediators expressed in the exosomes seems to represent a fundamental feature concerning the regenerative effects for their use in regenerative medicine (**Roşca et al., 2018**). Accordingly, a more suitable microenvironment would be reproduced, therefore empowering the efficacy of transplanted stem cells, which would condition the recipient's tissue, as demonstrated in cardiovascular applications where angiogenesis is restored by the indirect support of exosomes (**Xiao et al., 2019**; **Roşca et al., 2018**). Besides, the microenvironment conditioned by stem cell-derived micro-vesicles are more appropriate. However, among different type of stem cells the biogenesis of exosomes, which determines their own function and characteristics, cannot be assumed as homogenous, although this requires to be fully explored. Membrane protein and types of nucleic acids have been reported to be specific to stem cell source. For instance, exosomes isolated from bone marrow express Angiotensin-1 and miR21a, whereas those secreted by hematopoietic stem cells (HSCs) express tissue factor (CD142) and a different range of mRNAs (**Riazifar et al., 2017**). Oppositely, both MSC and ESC-derived exosomes equally contain Pax-6, exert regenerative properties and cell plasticity (**Roşca et al., 2018**; **Riazifar et al., 2017**). Other studies have highlighted that although ESCs and iPSCs exhibit a starting different genetic profile, however they share a similar array of long non coding-RNAs and miRNAs once differentiated into cardiomyocytes, strengthening the relevance of the degree of the differentiation achieved in culture by pluripotent stem cells rather than their intrinsic genetic profile (**Roşca et al., 2018**; **Riazifar et al., 2017**). Although we could question whether a disease-specific exosome profile might exist, many stem cell-derived exosomes are efficient to restore a wide range of functions. Microvesicles obtained from ESCs can endure osteochondral regeneration in a rat model of osteogenic disorder (**Roşca et al., 2018**; **Riazifar et al., 2017**), but also to attenuate doxorubicin-induced pyroptosis in muscle cells (**Roşca et al., 2018**; **Riazifar et al., 2017**). It has been shown that the intraperitoneal injection in murine ischemic heart of MSC-derived exosomes reduces the infarcted zone, by increasing ATP levels, the activation of the PI3K/Akt pathway and decreasing oxidative stress (**Riazifar et al., 2017**). Additional and relevant effects of stem cell-derived exosomes have been associated to stem cell differentiation and reprogramming. Embryonic stem cells-derived exosomes can induce both de-differentiation and pluripotency in retinal glial and Müller cells, therefore implementing the retinogenic differentiation in retinal disorders (**Roşca et al., 2018**; **Riazifar et al., 2017**). In kidney organogenesis, ESC-derived microenvironment has been reported to act as main regulator of tissue architecture (**Roşca et al., 2018**) and to switch from malignant to benign cancer phenotype by reprogramming via proteins and RNA (**Riazifar et al., 2017**).

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Notably, the efficacy of the stem cell-derived exosomal cargo can be often ascribable to specific mediators. It has been reported that overexpression of exosomal 126-miRNA and miR-181-5p-modified promotes functional recovery after stroke in rats by increased neurogenesis and inhibition of neuroinflammation (**Roşca et al., 2018**; **Riazifar et al., 2017**) and prevention of fibrosis via autophagy activation in liver, respectively (**Bae et al., 2018**). Different and selected ESC-derived miRNAs such as miR-291a-3p exert anti-senescent effects in human dermal fibroblasts trough TGF-β receptor 2 pathway and enhance wound healing (**Bae et al., 2018**), suggesting a protective role. Similarly, HSC-derived miR126 can boost the ESC differentiation in the hematopoietic lineage trough the inhibition of Notch1 pathway (**Bae et al., 2018**). To date, the employment of exosomes in regenerative medicine can be conceivable as a cell-free therapy, whose foundation is based on two main strategies: (1) exosomes as vehicles of substances; (2) the cargo or the cell membrane of exosomes is modified in order to target specific functions and/or tissues. The abovementioned approaches have been designed because stem cell-derived exosomes act as excellent carriers and therefore they represent a more suitable biocompatible alternative than synthetic vesicles. More importantly, exosomes seem to be well tolerated and nontoxic to the organism when administered, with long half-life properties in systemic circulation and in absence of potential adverse immune effects (**Bae et al., 2018**). However, different studies have shown that certain types of exosomes express the major histocompatibility complex (MHC), questioning the absence of potential immune reactions (**Bae et al., 2018**).

Intriguingly, hybrid exosomes have been recently engineered to further improve their bio-functionality. Accordingly, studies regarding cellular uptake have shown that the fusion of exosomal membrane with liposomes can modify exosome-cell interaction (**Bae et al., 2018**). These new approaches represent an alternative modality by which several limitations and risks associated to stem cells might be overcome. Despite this, a deeper understanding of the potency of stem cell-derived exosomes should be implemented, in order to fully exploit stem cell-derived soluble mediators.

Conclusions

Although many efforts have been made to clarify the biology of stem cells, additional issues are needed to be solved. Stem cell populations share similar features, such as the ability to proliferate, to differentiate and the paracrine action, therefore altering the microenvironment. Thus, despite the apparent uniformity among stem cell populations, the genetic and protein profile is often dissimilar, as a comparison has proven for iPSCs and ESCs (**Balistreri, 2018**), therefore suggesting that stem cells are not equal and questioning whether in the next future we might not seek for the unique and best stem cell source for all clinical purposes. For instance, pluripotent stem cells (ESCs and iPSCs) easily but differently reprogram compared to adult stem cells (MSCs, HSCs), therefore this should be considered when used. It is also conceivable that the choice of the best stem cell source depends on the pathological scenario. For instance, bone regeneration can be achieved by employing MSCs, however it might not represent the eligible choice if a bone tumor occurs. Thus, the disease, the tissue involved, the physiology and the intrinsic capacity of the organ to regenerate could eventually dictate the choice of the most suitable stem cell source to employ. Accordingly, tissue rejection still represents a critical issue of ESCs employment, therefore limiting their applicability, but also strengthening the relevance of the microenvironment as a main modulator to address stem cell fate and behavior. To date, we cannot rule out that a multi-combined approach (drug, scaffolds, proteins, genetic modifications) aiming to integrate the regenerative potential of stem cells might provide similar beneficial effects. Both heart and brain are extremely and complex organs with a well acknowledged and restricted regenerative potential and where it is important to restore the function and to instruct stem cells to preserve the tissue in case of future complications or insults. Studies investigating the appropriateness of single or multiple doses of stem cells and whether they are enough to contain the damage within the tissue in the long terms, are urgently needed. Additionally, technical issues as the availability of standardized and optimized protocols for isolation and ex vivo expansion of stem cells must be provided in order to assure reproducibility among clinical applications. The heterogeneity of stem cell populations and the lack of stemness and clonogenic ability thorough in vitro passages is a main technical problem while employing adult progenitors (**Stoltz et al., 2015**). Pure stem cell-based cultures have not been reproduced yet as well as the differentiation rate into several lineages has not been maximized. This aspect reinforces the heterogeneity of the ex vivo cultures, increasing the risk of interference of potential and undesirable populations to prevail after injection. Similarly, ESCs and iPSCs can induce teratomas or malignant transformations due to chromosomal aberrancies (**Stoltz et al., 2015**; **Lindroos et al., 2011**), highlighting our current poor knowledge regarding the biology and molecular mechanisms of these cells. Nonetheless, the use of pluripotent stem cells might be limited only to a specific set of high-risk patients and in targeted tissues with severe damages, as demonstrated in age-related macular degeneration (**Lindroos et al., 2011**). Oncogenic mutation in iPSCs have been described hampering clinical trials (**Balistreri, 2018**; **Stoltz et al., 2015**; **Lindroos et al., 2011**; **Balistreri et al., 2015**). The manipulation of stem cells by gene therapy, employed to boost the regenerative potential of progenitors, necessarily causes chromatin remodeling. Researchers need to better understand the modality by which epigenetic might influence the genomic stability and the biology of stem cells. As the knowledge on genome regulation gradually improves, a more relevant role of major epigenetic modulators of stem cell identity such as miRNAs and non-coding RNAs is given. The pattern profile of miRNAs significantly varies among iPSCs, ESCs, adult stromal cells and alongside tissue development and reprogramming (**Balistreri, 2018**; **Stoltz et al., 2015**; **Lindroos et al., 2011**; **Balistreri et al., 2015**). Micro-RNAs play a key role in determining gene expression profile and differentiation as demonstrated in pluripotent stem cells (**Balistreri et al., 2015**), which remain more unstable and uncontrolled than the adult counterpart. Moreover, in many diseases, non-coding RNAs and epigenetic is already deregulated, therefore potentially conditioning the phenotype, the function and the outcome of the stem cell-based therapy. The genomic instability requires to be deeply investigated, especially in defined type of stem cells like the pluripotent populations, where proto-oncogenes are employed for the reprogramming (**Balistreri et al., 2015**). Finally, the control of the immune response in the recipient's tissue requires to be fully explored, specifically when pluripotent stem cells are employed. Induced pluripotent stem cells can alter immune reactions according to the degree of their differentiation state (**Balistreri et al., 2015**), highlighting the variability of the

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reprogramming effect even within the same stem cell population. Stem cell-based therapies still represent a valid option for clinical use. However, their clinical relevance will be maximized only when the knowledge regarding the interaction between modified stem cells, patient's genomic profile and environment will be improved.

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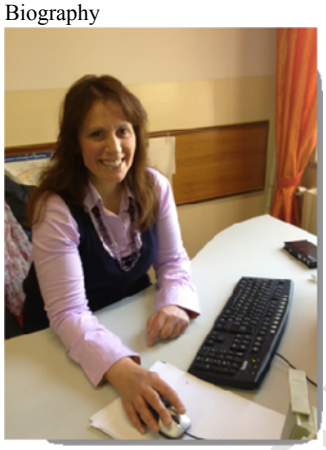
Biography



Carmela Rita Balistreri is an Assistant Professor of Clinical Pathology, and a member of the Bi.N.D Department University of Palermo, Italy. Balistreri received several national and international awards and honors. Her research focuses on immunogenetics, inflammation, aging, longevity, age-related diseases, innate immune pathways, i.e. TLR-4 pathway. She is coauthor of 112 ISI papers. International recognition of her scientific achievements is reflected in frequent citations of her papers (http://www.scopus.com) and the referee activity for the several journals. Her h-index is 27, (http://www.scopus.com; orcid.org/0000-0002-5393-1007). To improve her scientific cultural level and to compare her data with those of other groups, she has attended 80 congresses focused on following topics: Age-related diseases, Immunosenescence, Longevity, Model system, Aging & Wellness and Regenerative Medicine, Cardiovascular diseases. In several congresses she has attended as speaker.

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Elena De Falco is currently focused on the biological properties of human mesenchymal stem cells including the cardiovascular-like commitment, the production of microvescicles and the angiogenic capacity. In particular, the regenerative effects of platelet lysate (an hemoderivate for the ex vivo expansion of human mesenchymal stem cells) as biological tool to restore angiogenesis and decrease cardiac fibrosis during myocardial infarction are also under investigation, as well as the evaluation of a triple regenerative strategy based on the employment of adipose-tissue derived human mesenchymal stem cells, pharmacological regimen and bioscaffolds and/or 3D bioprinting approach. Furthermore, my expertise also includes the role of the circulating endothelial progenitor cell fraction in presence of diabetes and/or cardiovascular diseases.

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