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Review

Biochemistry and biology: Heart-to-heart to investigate cardiac progenitor cells ☆

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ABSTRACT

Background: Cardiac regenerative medicine is a rapidly evolving field, with promising future developments for effective personalized treatments. Several stem/progenitor cells are candidates for cardiac cell therapy, and emerging evidence suggests how multiple metabolic and biochemical pathways strictly regulate their fate and renewal.
Scope of review: In this review, we will explore a selection of areas of common interest for biology and biochemistry concerning stem/progenitor cells, and in particular cardiac progenitor cells. Numerous regulatory mechanisms have been identified that link stem cell signaling and functions to the modulation of metabolic pathways, and vice versa. Pharmacological treatments and culture requirements may be exploited to modulate stem cell pluripotency and self-renewal, possibly boosting their regenerative potential for cell therapy.
Major conclusions: Mitochondria and their many related metabolites and messengers, such as oxygen, ROS, calcium and glucose, have a crucial role in regulating stem cell fate and the balance of their functions, together with many metabolic enzymes. Furthermore, protein biochemistry and proteomics can provide precious clues on the definition of different progenitor cell populations, their physiology and their autocrine/paracrine regulatory/signaling networks.
General significance: Interdisciplinary approaches between biology and biochemistry can provide productive insights on stem/progenitor cells, allowing the development of novel strategies and protocols for effective cardiac cell therapy clinical translation. This article is part of a Special Issue entitled Biochemistry of Stem Cells.

1. Introduction

As a leading cause of worldwide morbidity and mortality, heart failure (HF) has pushed basic, pharmacological and clinical research efforts to develop novel effective therapeutic treatments, particularly in the last two/three decades, also for the rising number of affected individuals, parallel to the progressive aging of the global population. The result is that, while seen in the past as an untreatable condition, HF is now considered a chronic disease, nevertheless with a highly demanding human and social cost.

Pharmacological therapies and primary/secondary prevention have traditionally targeted the heart's pump function and the quality of life for end-stage HF patients, without leading to actual replacement of diseased tissue and, thus, without stopping or reversing the progression of adverse left ventricular (LV) remodeling [1]. The use of stem/progenitor cell-based therapy is becoming increasingly important as a powerful strategy to recover damaged myocardium and to promote endogenous repair of cardiac tissue [2,3]. The final aim is obviously the regeneration of functional, vascularized and integrated contractile tissue to compensate for the functional loss of the organ. Although the available data in this area is highly debatable, the potential of cell-based therapy for the treatment of HF remains an alternative option, with the result that widespread laboratory and clinical studies on their use for cardiac repair are ongoing, raising great expectations, as well as controversies. In fact, examining the basic and clinical studies concerning cardiogenic cells used for therapeutic purposes, major efforts have been spent to compare different kinds of possible adult cell sources and candidates (autologous/heterologous, bone marrow/skeletal/cardiac resident biopsy-derived), the delivery methods (intracoronary/direct injection, tissue-engineering combined) and the timing of sampling and intervention. Thus far, cell therapy with ectopic cells has reduced LV end-systolic volume (LVESV), a result that is consistent with a systolic functional benefit, but different from what has been obtained by other therapies. In fact, treatments based on pharmacological intervention or assist devices are able to reverse cardiac remodeling, but still cannot impart long-term benefits for patients.

The mechanism by which cell therapy reduces LVESV is most likely linked to paracrine signaling and the possible cross-talk with the surrounding environment, inducing a more or less prolonged angiovasculogenesis [4].

After the introduction of the first method for the isolation and expansion of cardiac progenitor cells (CPCs) from human heart biopsies

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[5] (Patent number: WO2005012510), multiple basic and preclinical studies have rapidly brought this technology to clinical application, with at least three phase I/II clinical trials almost completed (SCIPIO [6], CADUCEUS [7], ALCADIA [8]; see www.clinicaltrials.gov for details). Better clinical results, in terms of cardiac regeneration, have been reported by Makkar RR et al. [7] (CADUCEUS), who have isolated and expanded CPCs from endomyocardial bioptic samples obtained from the diseased human heart of patients with recent myocardial infarction (MI), followed by autologous retransplantation via intracoronary injection, thus suggesting that this therapeutic approach is feasible and has the potential to provide a treatment strategy for cardiac regeneration after MI. However, improvement in ejection fraction (EF) is not consistent with what was expected from preclinical studies [9,10]. Thus, other factors, such as the beneficial cell paracrine activity, the host tissue or even its cross-talk with the bone marrow, as well as other (still) unpredictable variables, represent open questions. Moreover, methodological bias would be introduced if the individual patient's specific features were not taken into account.

Over the past decade, the shifting of research interest from reversing the remodeling process to cell-based therapies has promoted the rationale of the two approaches being adequately combined together. This conceptual hole needs to be filled by figuring out how patients' specific cardiac remodeling (involving structural changes, such as hypertrophy, fibrosis, and dilation, and multiple abnormalities of cellular and molecular function, as well as inflammatory cytokines and growth factors) should be challenged and integrated with the individual molecular, biochemical and functional modifications occurring to cells during sampling, in vitro growth and in vivo interaction with the evolving microenvironment. These concepts are attracting particular interest from the scientific community, while acquiring potent tools with the integration of "omics" strategies with personalized medicine. The availability of genomic, proteomic, transcriptomic and metabolomic data combined, allows us to revisit the scientific basic and translational potential of these individual tools, which are expected to provide a much extended impact on regenerative medicine, and in other medical and biotechnological fields, as well.

Cardiac regenerative medicine is becoming oriented toward biochemistry, metabolism and related functions, in a more integrated and personalized approach. In order to use stem/progenitor cells for therapeutic purposes, it is important to control their differentiation and regulate their pluripotency and self-renewal. So far most of identified regulators of stem cell fate are growth factors, transcription factors, cell cycle regulators, as well as their associated downstream signaling pathways [11]. Recent evidences suggest that also mitochondria have a crucial role in regulating stem cell fate, both as the center of cellular respiration and as a central platform in the regulation of diverse cellular events [12].

Several regulatory mechanisms are well known that either link cell signaling to the modulation of metabolic pathways or enable cells to sense fuel availability and regulate signaling networks accordingly [13], overall influencing metabolism and gene expression. In the field of regenerative medicine, the importance of these networks is obviously gaining particular interest, to discover and exploit new metabolic key regulators of stem/progenitor cell proliferation/differentiation, in the contest of each individual diseased tissue.

In this regard, taking advantage of the extensive scientific knowledge on embryonic development and cancer biology, as a paradigmatic example of how a shift in metabolic pathways can strongly influence cell biology and functions, a fundamental question can be raised, that is why experts in the field of stem cells biology and biochemists should be mutually interested in their respective expertise, and should share their specialized knowledge. In this review we will attempt to provide some perspectives on this, particularly in the field of cardiac regenerative medicine or under a more general light for topics that have not been thoroughly investigated in cardiovascular biology, by focusing and discussing the following issues:

- stem cells and energy metabolism: external versus internal signaling networks and possible targets for preconditioning pharmacological interventions,
- stem cells and in vitro culture conditions: physical (oxygen), chemical (calcium) and nutrient (glucose) requirements,
- control of regulatory pathways and metabolic changes induced by different environmental conditions, stimulatory factors and small molecules,
- proteomic perspectives to discover novel networks and markers as diagnostic, tracing and therapeutic tools for clinical applications.

2. A brief overview: resident cardiac progenitor cells

The difficulty of regenerating damaged myocardial tissue has led researchers to test different stem/progenitor cell types as possible sources for cell therapy, including embryonic stem cells (ESCs), myoblasts (muscle stem cells), adult bone marrow-derived cells, mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), umbilical cord blood cells and cardiac progenitor cells (CPCs) that naturally reside within the heart. All have been tested in mouse or rat models, while some of them in large animal as well, such as pigs, and in human clinical trials [14–18].

The most appropriate cells for replacing dead cardiomyocytes appear to be cardiomyocytes of fetal or embryonic origin, since they can functionally integrate with the host tissue [19,20]. The ideal cell to be transplanted for cardiac regeneration, though, should probably be in between a highly undifferentiated phenotype (e.g. ESCs) and terminal differentiation (cardiomyocyte). It should be characterized by defined proliferative potential in the host without induction of immune reaction; cardiac commitment and capacity to develop gap-junctions with the host cells, and should preferably be resistant to ischemia, in order to avoid massive cell death and apoptosis, that currently are the biggest hurdles for cell therapy clinical translation.

With these premises, it seems obvious that the best cells to replace lost cardiomyocytes may be cells derived from the heart itself. Emerging evidence suggests that several populations of CPCs are present in the heart, and extensive basic research still needs to be performed to better understand the relationship among these different populations. CPCs are positive for various stem/progenitor cell markers (c-Kit, Sca-1, Isl-1) and have Side Population (SP) properties. In fact, their presence into the heart, the frequent co-expression of early cardiac progenitor transcription factors, and the capability for ex vivo and in vivo differentiation toward cardiac lineages offer the promise of enhanced cardiogenicity compared to other non-cardiac cell sources.

Different methods have been used to isolate CPCs from the heart, based on multiple criteria, and that have been characterized in vitro and tested in vivo in animal models:

- Ability to efflux Hoescht dye (side population, SP).
  SP cells, which have the ability to efflux Hoechst dye (a process dependent on the expression of MDR1, Abcg2 or similar ABC membrane transporters), have been identified not only in the developing, but also in the adult heart of mice [21,22]. These cells are rare and their ability to differentiate into contracting cardiac myocytes or to contribute to functional repair of damaged heart muscle has not been extensively evaluated yet.
- Expression of cell-surface stemness markers (c-kit or Sca-1).
  In the adult heart a distinct population of c-kit+ CPCs has been isolated. These relatively small and primitive cells are negative for blood lineage markers and positive for c-kit, the receptor for the stem cell factor. These cells are self-renewing, clonogenic and multipotent, giving rise to cardiomyocytes, smooth muscle and endothelial cells. When injected into the infarct border zone in adult rats, these CPCs differentiated into newly formed myocardium, including cardiomyocytes, capillaries and arterioles in the infarcted area [23]. C-kit positive cells have been tested in the SCIPIO clinical trial [6].

Moreover, in the non-myocyte fraction of mice hearts, a resident population of CPCs, characterized by the expression of Sca-1, but lacking blood lineage markers or c-kit, has been reported [24,25]. Even though these cells do not spontaneously differentiate in vitro, a small fraction of them demonstrates biochemical evidence of cardiac myocyte differentiation when exposed to 5-azacytidine [24] or oxytocin [25]. After intravenous injection in mice subjected to myocardial ischemia–reperfusion, Sca-1+ cells homed to the heart and differentiated into cardiomyocytes, partially because of fusion with host cells [24].

- Expression of the islet-1 gene (isl1+ cells).
  Another population of CPCs, characterized by the expression of the LIM-homeodomain transcription factor islet-1 (isl1+), has been recently described. These cells reside in the mature heart of newborn mice, rats and humans, and they are negative for c-kit or Sca-1, but express the early cardiac transcription factors Nkx2.5 and GATA4. When co-cultured with cardiomyocytes, isl1+ cells convert very efficiently to mature cardiomyocytes without cell fusion [26]. However, their low abundance and mere presence in very young animals and humans prevent their short-term clinical application.
- Expression of the stage-specific embryonic antigen 1 (SSEA-1).
  Recently, uncommitted cardiac precursor cells (UPCs) have been identified in the heart of adult rats through a typical embryonic antigen, SSEA-1, that is expressed early in heart development [27]. SSEA-1+ cells isolated from adult rats differ from neonatal cells because they do not express cardiac specific transcription factors (Nkx2.5, GATA4). This suggests that only uncommitted stem cells persist in the adult heart. Beating colonies are obtained by culturing UPCs in differentiating media or in co-culture with neonatal cardiomyocytes. UPCs improved ventricular function when injected in infarcted hearts, and SSEA-1+ cells are capable of forming new cardiomyocytes and endothelial cells in the infarct area [27].
- Origin from the epicardium (epicardially derived cells, EPDCs).
  Limana et al. [28] first identified in human and mouse epicardium CPCs outside of the previously described "niche". They can migrate into the sub-epicardium where they generate a population of EPDCs. Authors described two distinct populations of myocardial and vascular precursor cells, expressing c-kit or CD34 respectively [28]. A subset of c-Kit+ and CD34+ cells express cardiac transcription factors (Nkx2.5, GATA4). Their differentiation potential has been demonstrated only for c-kit+ cells when acute MI was induced in the mouse in the presence of an intact pericardial cavity.
- Spontaneous ability of cardiac explant-derived cells to form 3D cardiospheres.
  CPCs can be isolated from explant cultures of adult or pediatric human surgical or endomyocardial biopsies using an intermediate selective cardiosphere (CSp) step [5,29–31]. After few weeks in the primary culture, CSp-forming cells are harvested from the fibroblast-like monolayer of cells that have migrated from the tissue explants. CSps are clonogenic, self-assembling spherical clusters that grow in semi-suspension culture, and that constitute a niche-like microenvironment [32,33]: undifferentiated cells, expressing stemness markers like c-kit, proliferate in the core, while cardiac-committed cells grow on the periphery in a gradient fashion, expressing markers such as CD105, myosin heavy chain (MHC), troponin I (TnI), connexin-43 (Cx43), smooth muscle actin (SMA) and Von Willebrand factor (vWF). The CSp isolation protocol is based on intrinsic and spontaneous functional properties of the cells, which is the ability to migrate from tissue explants and to grow as 3D-structures. CSp-derived cells (CDCs) can be expanded many fold as monolayers on fibronectin (FN), achieving cell numbers suitable for cell therapy. The vast majority of this heterogeneous population is $CD105^+$, and significant subpopulations are $CD90^+$, $cKit^+$, $CD34^+$ and $CD31^+$. CDCs are also $MDR1^-$, $CD133^-$ and $CD45^-$, and negative for a wide cocktail of blood lineage markers, resembling overall a mixture of CPCs and mesenchymal supporting cells. CDCs have been shown [29] to improve left ventricular ejection fraction (LVEF) after 3 weeks in a SCID beige mouse model of acute MI, when compared to mice injected with vehicle or with adult normal human dermal fibroblasts, as control groups. In a porcine pre-clinical model of post-infarct left ventricular dysfunction intracoronary delivery of CDCs has been shown to result in formation of new cardiac tissue, to reduce relative infarct size, to attenuate adverse remodeling, and to improve hemodynamics [9]. The evidence of efficacy of this study offered the fundamental background for human studies in patients after MI and in chronic ischemic cardiomyopathy [7].

3. The role of oxygen in stemness and differentiation

One of the most finely regulated parameters in tissues and cells is O_2 tension [34]. Nevertheless, hypoxic microenvironments normally occur in many developmental and physiological stages in mammals. Various experiments on knock-out mice for molecular regulators of oxygen response, such as members of the HIF family of transcription factors, have shown how disruption of adequate hypoxia signaling networks leads to embryonic lethality or severe post-natal complications [35], and how much "physiological hypoxia" can be important.

For obvious reasons of space impediment and diffusion, tissue O_2 concentrations are much lower than the atmospheric ones. In the human organisms, for example, O_2 concentration varies significantly among tissues, ranging from 14 to 4% from the lung parenchyma and circulation [36–38] to well perfused organs, such as the liver, kidneys and heart [39–41]. In most other tissues which are less irrigated, such as the brain or the bone marrow, O_2 concentration can go as low as 0.5% [42,43], while in the eye (retina, corpus vitreous) it ranges from 1 to 5% [44].

In fact, cellular metabolism of different cell types has adapted to moderate, albeit variable, oxygenation. Therefore, the rationale of in vitro research should be to study cell biology at appropriate O_2 concentrations, approximating the most physiologic in situ normoxia for each cell type. Cell culture, instead, is routinely performed, in most cases, at atmospheric O_2 concentration (20–21%), representing a hyperoxic state for most mammalian cells, excluding mature cells from tissues which are in direct contact with air (e.g. skin surface, mouth and respiratory epithelium).

Together with the need for O_2 as a metabolic substrate, goes the risk of oxidative damage on cellular macromolecules due to ROS generation during oxydative phosphorilation (OXPHOS), and this is why its intracellular concentration is always kept within a narrow range, optimizing the balance between supply and demand. Nevertheless, cells are able to compensate ROS in excess, although such system has limits, beyond which the well-being of cells cannot be guaranteed [45].

The balance between proliferation and differentiation is highly influenced by O_2 concentration, and it has been shown that some stem cells are highly sensitive to O_2 [46] and that there is a strong coupling between intrinsic metabolic parameters and stem cell fate [47].

Thus, it is not surprising that changing O_2 concentration in culture can affect multiple tightly-regulated typical features of stem/progenitor cells [35], such as their self-renewal, quiescence and commitment (Fig. 1), and, other than impairing their therapeutic potency in regenerative medicine [48], genomic alterations of stem cells due to oxidative stress [49] may also increase in vivo carcinogenesis.

As a general rule, hypoxia promotes the undifferentiated state, self-renewal capacity and maintenance of pluripotency in several stem/progenitor cell populations, but the underlying molecular mechanisms remained unknown until recently. Hypoxia not only directly suppresses OXPHOS by reducing the available oxygen, but also activates hypoxia-inducible factors (HIFs), transcription factors which reduce the expression of mitochondrial enzymes and further enhances the shift to glycolysis by upregulating glucose transporters and glycolytic enzymes [35,50,51]. This kind of metabolic shift is strictly involved in the regulation of stemness, as we will discuss in the next sections.

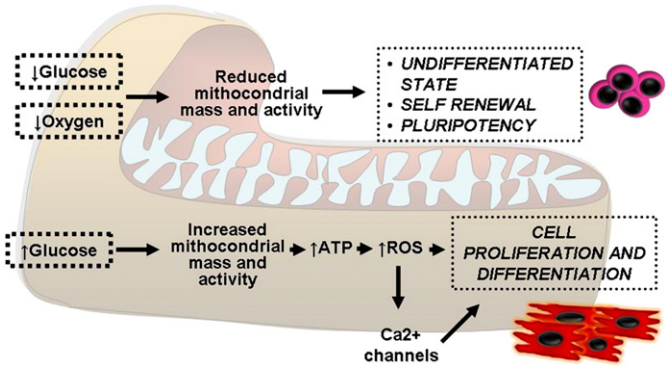

Fig. 1. Glucose and oxygen influence on stem cell functions. Scheme of the key role of glucose and oxygen availability in the metabolic and biological regulations of stem cell fate.

ESCs are able to maintain their pluripotency for longer when cultivated in hypoxic conditions [52], and hypoxia seems to substantially enhance fibroblast reprogramming to induced pluripotent stem cells (iPSs) [53]. In adult stem cells, such as HSCs and MSCs, hypoxia prolongs lifespan, increases proliferation and reduces differentiation in culture [54], probably because the hypoxic culture conditions more closely resemble an in vivo microenvironment. In fact most of the identified adult stem cells occupy hypoxic niches within the tissue, while others (spermatogonial stem cells [55] and brain tumor stem cells [56]) occupy relatively well-oxygenated perivascular microenvironments, thus conflicting results on the metabolic activity of adult stem cells may reflect particular properties of the tissue from which they derive, or may depend on the target lineages into which these cells differentiate.

In addition to inducing a glycolitic shift, hypoxia may also directly act on stem cell fate via other HIF-dependent pathways. A clear link, in fact, has been demonstrated between hypoxia, HIFs and molecules that are crucial for the regulation of the differentiation of stem/progenitor cells, such as Notch, β-catenin, OCT4, and c-MYC [57–64].

In the field of adult resident CPCs, the influence of oxygen tension and ROS on their functions and differentiation has been recently investigated on CPCs isolated through the CSp method [65,66]. The authors tried to reduce the incidence of genomic alterations by culturing CDCs under physiological oxygen (5% O_2) or by culture in a traditional 20% O_2 incubator with the addition of antioxidants to routine culture media. A complex bidirectional effect of intracellular ROS on genomic stability was discovered both in CPCs and ESCs, indicating that optimal physiological levels are required to activate the DNA repair pathway for maintaining genomic stability in stem/progenitor cells. In fact, modest ROS suppression by culture in physiological oxygen (5%) decreased karyotypic abnormalities, but profound ROS suppression by antioxidant supplements paradoxically enhanced genomic alterations.

The authors also tried to improve cell therapeutic quality by expanding human CPCs in physiological low oxygen (5% O_2) conditions. CPCs expanded in 5% O_2 increased cell yield, showed lower senescence and higher resistance to oxidative stress than those grown in 20% O_2. The expression of stem cell markers and CPC phenotype were comparable between the two conditions, as well as the paracrine secretion of selected growth factors into conditioned media. In vivo, the implantation of cells grown in 5% O_2 into mice infarcted hearts resulted in greater cell engraftment and better functional recovery than with conventionally cultured cells [65].

Overall these results suggest that oxygen tension is a key biochemical parameter in both research- and clinical-grade stem cell production, which needs to be carefully considered as potentially affecting cell quality, phenotype and stability.

4. Calcium and cardiac progenitor cells

During the development of the vertebrate embryo, controlled release and/or accumulation of calcium ions is important in a variety of events, affecting cell fate specification and morphogenesis. Wnt, calcium and beta-catenin signaling integrated pathways appear to regulate the formation and organization of polarized cell migratory movements in the early embryo [67].

In the cardiovascular system, the dynamics of gene expression levels of calcium-regulatory proteins reflect functional specification during development [68]. Studies on the pattern of expression of Ca^{2+} handling proteins in mouse embryos from embryonic stage 9.5 to 18.5 and adulthood [69] showed an increased expression of proteins such as the ryanodine receptor 2 (RyR2), the sarcoplasmic reticulum pump (SERCA2), and phospholamban. Consistently, the amplitude of the voltage sensitive Ca^{2+}-current increased with time.

Several studies are beginning to show a role for Ca^{2+} signaling in post-natal stem cell development as well. Human bone marrow-derived MSCs show inositol 1,4,5-triphosphate receptor- (IP3R) and ER-dependent Ca^{2+} oscillations, as well as Ca^{2+} entry and extrusion via plasma membrane pumps and exchangers [70,71]. Further studies found that Ca^{2+} oscillations may depend on an autocrine/paracrine signaling pathway, where secreted ATP stimulates P2Y1 receptors to activate PLC-β to produce IP3 [72]. The trigger of induced Ca^{2+} oscillations promotes the activation of the downstream transcription factor NFAT, whose nuclear translocation instead disappears as MSCs differentiate into adipocytes. Conversely, an increase in intracellular Ca^{2+} results in the inhibition of human adipocyte differentiation [73], suggesting a link between intracellular Ca^{2+} oscillations and the maintenance of undifferentiated human MSCs.

An interesting correlation appears between the above-mentioned calcium-handling regulation of the developing cardiovascular system in the embryo and adult CPCs. In fact, Ca^{2+} oscillations have been identified in c-kit+ CPCs, independently from coupling with cardiomyocytes or extracellular Ca^{2+}. Such oscillations seem to be regulated by release from the endoplasmic reticulum, through activation of IP3Rs, and following Ca^{2+} uptake by SERCA. Importantly, Ca^{2+} oscillations in CPCs are coupled with the entry into the cell cycle and DNA synthesis. Induction of Ca^{2+} oscillations seems also to improve their regenerative effects in animal models of cell therapy [74]. Spontaneous calcium transients occur also in c-kit+ embryonic cardiac stem cells, leading to cell cycle progression, and such Ca^{2+} spikes seem to be associated with the regulation of symmetrical versus asymmetrical division [75], thus possibly being involved in regulating stem cell fate decisions.

Differentiation of a muscle-type Ca^{2+} release mechanism also correlates with cardiac commitment and functional differentiation of CPCs [76]. In particular, the peculiar niche-like 3D-culture stage of the CSp seems to be inductive and/or selective of caffeine responsiveness (indicative of the expression of cardiac-specific Ca^{2+}-handling proteins), leading to muscle-specific functional response and promoting cardiac differentiation of adult resident CPCs. Furthermore, another clue on the role of calcium in cardiac commitment in the post-natal heart comes from experiments on the effects of electromagnetic fields on adult CPCs [77]. Exposure to specific combinations of frequencies, tuned on the calcium ion cyclotron resonance, is able to drive specific cardiac differentiation versus vascular or stem-like phenotypes, and these effects are consistently associated with increased intracellular calcium storage and trafficking among different compartments (Fig. 2).

5. Stemness, reprogramming and glucose metabolism

It has been recently proven that metabolism tightly regulates multiple aspects of stem cell fate. While studies on adult stem cells may give conflicting results [78,79], studies on ESCs and iPSs consistently show that undifferentiated pluripotent stem cells rely mainly on anaerobic glycolysis and are characterized by reduced mitochondrial

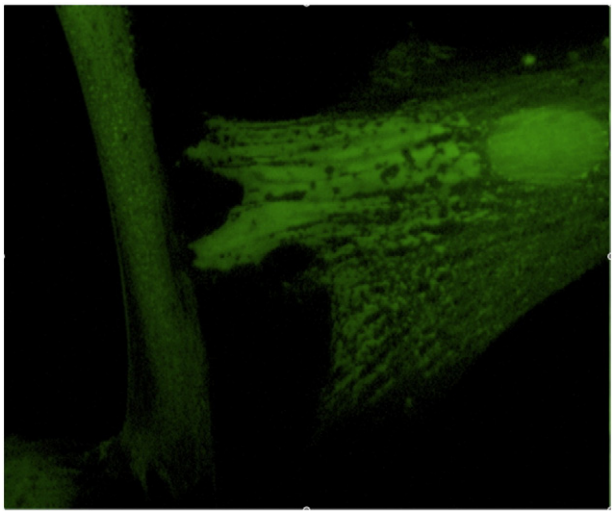

Fig. 2. Oregon Green labeled CPCs after electromagnetic field stimulation. CPCs, cultured as cardiosphere-derived cells, show responsiveness to electromagnetic fields tuned on calcium resonance frequencies. Increased calcium storage, as shown by Oregon Green labeling, specifically correlates to increased cardiac commitment and differentiation.

mass and mitochondrial activity (ATP production, ROS release) compared to differentiated cells [80,81], thus suggesting a metabolic profile similar to cancer stem cells [82]. Instead, cells undergoing differentiation are characterized by an increase in mitochondrial mass, mitochondrial activity and ROS. ROS and other mitochondrial signaling mediators and products, such as ATP and Ca^{2+}, may act as signaling mediators that regulate cell differentiation or self-renewal [12] (Fig. 1).

An example of the basic role of glucose and its metabolism in stem cell biology comes from studies on ESC. ESCs are normally maintained and differentiated in medium containing supraphysiological levels of glucose (25 mM), a condition which is known to result in enhanced cellular ROS formation. When cultured in physiological glucose (5 mM), ESCs maintain their general stemness qualities, but display an altered mitochondrial metabolism, resulting in lower ROS production. Low glucose concentrations also correlated with failure to generate cardiomyocyte structures, and such effect can be mimicked with antioxidant treatments. Therefore, endogenous ROS seem to control cardiomyocyte formation from ESCs, and supraphysiological glucose, by supplying ROS, is absolutely required for efficient cardiac differentiation [83].

A recent striking discovery in the stem cell field has been the possibility of nuclear reprogramming of adult somatic cells into embryonic-like pluripotent cells by exogenous transfer of few genes. The metabolic interplay involved is starting to become clear. It has been previously established that somatic cells primarily utilize mitochondrial OXPHOS for their energy production, whereas pluripotent cells rely on glycolysis [84]. A recent interesting paper [85,86] examined the bioenergetic cellular changes occurring before (mouse fibroblasts) and after (mouse iPSs) reprogramming, and showed how metabolism manipulation can affect reprogramming efficiency. Mitochondria change during reprogramming from a mature cristae-rich morphology in somatic cells to more immature spherical and cristae-poor structures in iPSs. Consistently, glucose utilization and glycolytic enzyme expression are higher in iPSs, compared to their somatic sources of origin, whereas oxygen consumption and levels of electron transport chains are lower. Altering the metabolic balance affects reprogramming: for example, stimulating glycolysis by elevating media glucose increases efficiency, and vice versa. These findings fit the above mentioned model of glycolysis being associated to undifferentiated features and phenotypes, showing how metabolism adaptation occurs in the direction of somatic-to-pluripotency as well, as discussed in reverse for maturation from stem cells to more advanced differentiation stages. This may be of particular interest for CPC cell culture considering that some evidences suggest that prolonged in vitro culture of cardiac cells favors dedifferentiation and redifferentiation [87]. It is still under investigation, though, whether such metabolic changes play a causative role in reprogramming/differentiative processes, or if they are simply a natural consequence of changes in phenotypes and cell functions.

6. Metabolism and signaling intersection: growth factors

Growth factors and extracellular signaling are important regulators of stem cell fate and biology, and their pathways are often integrated as well. The PI3K/Akt/mTOR pathway is a clear example of the integration between signal transduction and metabolic activity. In most mammalian cells, growth is promoted by extracellular ligands which recognize their specific receptor and activate signal transduction pathways, such as the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. Activation of this and other pathways alters the phosphorylation states of numerous targets, which together coordinate the cellular activities that culminate in cell division. A successful transition from a resting state to growth requires a cellular metabolism that is able to sustain the rising demands of proliferation, and this kind of balance is particularly important for a cell type, such as stem cells, in which self-renewal must be under tight control.

Growth factor-induced signaling participates in coordinating these functions, including maintaining a bioenergetic state permissive for growth [88]. In particular, the PI3K/Akt/mTOR pathway stimulates both a rapid increase in essential nutrient uptake and the proper allocation of these nutrients into catabolic and anabolic pathways to produce energy and macromolecules, respectively. Interruption of any of these metabolic effects renders the growth factor ineffective [13].

The knowledge of signals that control stem cell proliferation/differentiation balance can be also used to create specific enriched culture media to promote specific commitment. The manipulation of the main characters of biological signal transduction, like growth factors, chemokines and their receptors, plays an important role in stem cell signaling and functions, such as differentiation, homing and engraftment to target tissue. We will focus our attention mostly on cardiovascular-related issues and perspectives concerning examples of proteins of interest, stem cells and possible approaches to optimize their use in preclinical and clinical protocols.

7. Proteins of interest for stem cells fate modulation and cardiac regeneration

Several studies [89,90] demonstrated that chemokines are upregulated in ischemic cardiac diseases and play a role in post-infarction remodeling, recruiting inflammatory leukocytes and inducing stem cell homing. SDF1 (stromal cells derived factor) is an α-chemokine that binds exclusively its G protein-coupled receptor CXCR4. This interaction leads to several signaling pathways that involve cell motility, chemotactic response, gene transcription and cell adhesion, exerting an important role in cardiogenesis and angiogenesis [91]. Many subsequent studies [92–95] demonstrated that after ischemia SDF1 is upregulated from the damage tissue and in the peri-infarct region. This up-regulation recruits circulating progenitor cells and stimulates stem cell-mediated regenerative response in the injured muscle. Unfortunately this action is very short-lived and tissue recruitment is very low. Novel strategies to prolong and reinforce this effect include the incapsulation of SDF1 into alginate microspheres and incorporation of these within an injectable collagen-based matrix [96]. This way, prolonged in vivo SDF1 release, CXCR4+ angiogenic cell recruitment and increased mobilization of bone marrow-derived progenitor cells were obtained. In these conditions SDF1 is able to increase angiogenic cytokine production and create a more suitable microenvironment for regeneration.

FGF-2, also known as basic FGF (bFGF), is a member of heparin-binding growth factors that bind tyrosine kinase receptors. After receptor binding, autophosphorylation and subsequent activation of the GRB2/

SOS (growth factor receptor-bound protein 2/Son of sevenless) complex occur. SOS, a guanine nucleotide exchange factor, activates RAS, a small G protein, leading to a cascade of phosphorylation inducing the activation of RAF, MEK, and mitogen-activated protein kinase (ERK) kinases. p-ERK phosphorylates target transcription factors to activate gene expression.

The cellular localization of bFGF changes during development, suggesting and reflecting the multiple roles played by this growth factor. FGF-2 participates in the process of tissue repair, in survival, proliferation and differentiation of immature neural cells, shows mitogenic and angiogenic properties [97]. It has been shown that FGF2 also has a fundamental role in the induction of cardiac differentiation on a population of Sca1+ undifferentiated precursors in the non-myocyte population of the mouse neonatal heart [98]. These cells can be expanded in vitro and, after induction, they are able to give rise to functional mature cardiomyocytes. Their differentiation appears to be dependent on the cells' own capacity to produce FGF-2, showing that FGF-2 induces the expression of cardiac transcription factors. This was confirmed by in vivo experiments. Following infusion into immunocompetent recipient mice, these progenitor cells home to the heart, and participate in physiological cardiac remodeling, due to the production of FGF-2 from the surrounding tissues. Nevertheless, differentiation is abolished in the absence of FGF-2 expression in the transferred population or the recipient animals.

A combined strategy for the delivery of autologous CPCs with basic bFGF under controlled release through a gelatin hydrogel is currently under clinical investigation in the ALCADIA trial (see www.clinicaltrials.gov for details). This protocol was validated in a preclinical study, also showing that the therapeutic enhancement to cell therapy efficacy due to FGF was only synergic in combination with CPCs, and not with MSCs [8].

Administration of stem cell factor (SCF) is emerging as a new therapeutic approach for cardiovascular regenerative medicine for MI treatment. SCF binds its receptor c-kit and promotes survival, proliferation, mobilization and adhesion of responsive cells, like hematopoietic and cardiac stem cells [99]. C-kit signaling can promote cardiac repair after MI. The intravenous injection of c-kit+ stem cells into the infarcted myocardium improves cardiac function, enhances angiogenesis and impairs cardiac remodeling. SCF is increased in the bone marrow after myocardial damage leading to EPC mobilization, and improvement in myocardial neovascularization and cardiac function [100].

Unfortunately in the damaged heart SCF expression is decreased [101]. Xiang et al. [102] generated a cardiomyocyte-specific membrane-associated human SCF-overexpressing mouse. They observed increased EPC recruitment to the infarcted myocardium, decreased apoptosis and remodeling, and a subsequent increase in capillary density, cell survivor and myocardial function, due to increased release of soluble factors, such as VEGF-A, FGF and insulin-like growth factor-1 (IGF-1). These results suggest a possible therapeutic potential of SCF in heart failure treatment after MI.

The role of the SCF receptor, c-kit, in CPC biology is debated. Despite being one of the criteria used to identify or isolate putative stem cells, which have been shown to have high regenerative potential [23,103] and have been also tested in the SCIPIO clinical trial [6], several data suggest that it may not be sufficient or comprehensive as a single parameter to univocally define cardiac stem cells, or at least to define the most potent therapeutic cell population [104–106]. Indeed, the notion that a pure homogenous population of cells would not be the best therapeutic tool for cardiac cell therapy is emerging. Future direct comparisons in vivo will provide more and deeper insights.

Vascular endothelial growth factor (VEGF) is predominantly expressed in endothelial cells and acts through its two receptors, FLK-1, a tyrosin kinase receptor, stimulating endothelial cell proliferation, and FLT-1, a FMS-like tyrosine receptor, promoting vascular organization. Many studies demonstrated VEGF influence on angiogenic stimuli, left ventricular remodeling after MI, and its upregulated expression in chronically ischemic myocardium and after myocardial ischemia [107].

A recent work [108] focused the attention on a new method to improve VEGF actions. An MI episode brings to hyperhomocysteinemia that inhibits angiogenesis by the downregulation of 5-methyltetrahydrofolate (5-MTHFR), cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). In normal conditions CSE and CBS catalyze the production of hydrogen sulfide (H_2S) starting from L-cysteine, which promotes smooth muscle relaxation and, consequently, vasodilation. Moreover, during an MI episode the levels of angiostatin, endostatin and parstatin (inhibitors of angiogenesis and endothelial cell proliferation and migration) increase [109,110]. In this study it was demonstrated that, in a MI mouse model, exogenous H_2S administration at the time of MI leads to a cytoprotective and angioprotective action compared to untreated mice. This beneficial action mediated by exogenous H_2S is due, on one side, to a down-regulation in the production of myocardial anti-angiogenic factors, like angiostatin, endostatin and parstatin, and also to CSE stimulation. Opening K^+/ATP channels in cardiomyocytes, stimulates CSE that brings to an upregulation in VEGF, FLK-1 and FLT-1 expressions. This event leads to a down-regulation of inflammatory response and apoptosis, new vessel formation and a considerable limitation of infarct size. These results suggest H_2S therapy like a promising candidate for MI treatment.

VEGF is produced and released by a wide variety of cells, including adult resident CPCs [111]. In this latter model it has been shown to play a direct role in mediating pro-angiogenic and anti-apoptotic effects, and to correlate with tissue viability preservation and capillary density in vivo in a model of cell therapy after acute MI.

Thrombin is a serine protease, obtained from its inactive precursor, pro-thrombin, with multiple actions [112]. It is a fundamental procoagulant factor for its ability to convert fibrinogen in an insoluble fibrin clot. Other important thrombin-mediated cellular actions are due to the interaction with proteinase-activated receptors (PARs). PARs are a G-protein-coupled receptor family, and PAR-1 has the highest affinity for thrombin. Interaction between thrombin and PAR-1 stimulates endothelial cells by expression, release and activation of angiogenesis mediators, in particular VEGF and Ang-2. Thrombin is able to increase mRNA levels of VEGF receptor, VEGFR-2, and consequently enhance its mitogenic activity on endothelial cells [113].

On the other side, thrombin and its receptors are able to yield direct mitogenic effects on endothelial cells by the phosphorylation of extracellular signal-regulated protein kinase 1/2 (Erk1/2-MAPK), mediated by EGF receptor transactivation [114].

Adult CPCs in the form of cardiospheres (CSps) could represent a candidate for cardiac cell therapy. For their formation the composition of the growth medium represents an important step. Thrombin is one of the factors included in CSp medium. The understanding of thrombin's effects on CSps is strategic for the study of the potential of these cells and for GMP translation of the medium formulation. The role of thrombin in different culture conditions of human CPCs cultured as CSps, has been recently investigated, including treatment with PAR-1 agonist TFLLR and antagonist MUMB-2 [115]. In the presence of TFLLR, CSps increase their proliferation activity, associated to a higher phosphorylation level of the cell cycle inhibitor GSK3. Activation of PAR-1-dependent signaling is important to support CSp proliferative potential and does not affect their cardiac and vascular commitment.

Analogous results have been obtained on adipose tissue-derived stem cells (ASCs). The synthetic peptide TP508, representing the receptor-binding domain of human thrombin, known to promote angiogenesis and accelerate wound healing in animal models, is able to stimulate ASC proliferation, by increased Akt phosphorylation, and the increased BrdU incorporation induced by TP508 can be abolished by a PI3 kinase (PI3K) inhibitor [116]. Under these perspectives, the availability of biochemically-defined agonists could represent an improvement for cost-effective, easy-to-handle and translatable synthetic media, avoiding lot variability and contaminants from human and animal additives.

Other growth factors, together with the above mentioned, could be useful to achieve myocardial and vascular regeneration after cardiac damage. For instance, the small secreted peptide thymosin-β4 could be used to inhibit myocardial cell death and stimulate vessel growth, and can activate an endogenous CPCs population [117], perhaps epicardium-derived [118]. EGF seems to be another promising one, and appears to have a great effect on CPC proliferation and migration, even compared to other widely used growth factors, such as VEGF, IGF1, FGF and HGF [119].

Summarizing, the study and selection of specific bioactive proteins, including chemokines, growth factors and enzymes, can help the design of novel experimental strategies to investigate, among other subjects, stem cells and CPC biology, with the final aim of optimizing and controlling their self-renewal and commitment (Fig. 3).

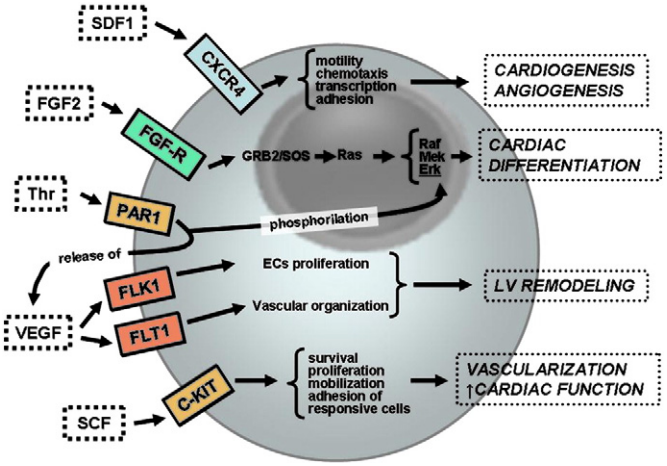

Fig. 3. Overview of selected pathways of interest. Multiple growth factors and proteins play a role in regulating stem/progenitor cell functions and fate. Represented in the figure are examples of proteins (SDF1, FGF2, thrombin, VEGF, SCF) modulating cell behaviors, of particular interest for cardiovascular repair/regeneration.

8. Small molecules for cardiac induction

In the last years, many studies on cardiac regenerative medicine approaches have focused on a new type of biological active molecules, the so called small molecules. A small molecule, in the fields of pharmacology and biochemistry, is a low molecular weight organic compound which is by definition not a polymer, but it is able to bind, with high affinity, a biopolymer such as proteins, nucleic acids or polysaccharides, and to alter their activity or function. Their molecular weight limit is approximately 800 Da, which allows for the possibility of rapidly diffusing across cell membranes, easily reaching intracellular targets and sites of action. Their advantages are manifold: they are easily manufactured, stored and administered, and their effects are mostly specific, dose-dependent, rapid and reversible.

From an economical, productive and versatility point of view, small molecules represent an important tool for research and are a clear example of the need for a very close synergy between biologists and biochemists. Starting from a common central structure, biochemical functional groups can be added based on biological needs, which can act as "switches" for specific molecules or mechanisms. To identify small molecules able to interact with a specific single protein is possible to use screening assays [120]. Normally in these assays the target molecule is in solution, and direct biochemical readouts enable the report of an enzymatic activity or a protein–peptide interaction. In high-throughput screening (HTS) multiple measures can be read, such as the intensity of a fluorescent signal, or, in case of cell-based assays, a reporter fluorescent protein, associated to a biological process within the cell. It is also possible with cell-based assays to exploit image analysis and develop so called high-content screenings (HCS), which offer the possibility to analyze many cellular processes and to discover compounds that cannot be associated to a simple readout.

Concerning stem cell therapy for MI, the high rate of inefficiency and the risk of tumorigenicity following transplantation (especially considering ESCs and iPS) require caution for clinical translation. Therefore, developing strategies to direct the differentiation potential of stem cells to a cardiac phenotype is of great importance. One of the best models to study and test cardiogenic induction protocols is of course ESCs. To generate cardiomyocytes from human ESC, first formation of mesoderm is required, then its subsequent maturation toward a cardiogenic mesoderm, and finally the last step of maturation into early cardiomyocytes.

The Wnt/beta-catenin pathway is a major regulator of heart development and stem cell self-renewal. The Wnt family comprises cysteine-rich glycoproteins, of approximately 350–400 amino acids, that contain an N-terminal signal peptide for secretion. When Wnt binds its seven-pass transmembrane receptor (frizzled or Fzd) and the LDL receptor-related proteins 5 and 6 (LRP5 and LRP6), a signal cascade is activated resulting in displacement of the multifunctional kinase GSK-3β from the APC/Axin/GSK-3β-complex. Normally this complex promotes beta-catenin proteolytic degradation, but Wnt binding prevents this degradation, and beta-catenin is able to reach the nucleus and interact with the TCF/LEF family transcription factors to promote specific gene expression by displacement of Groucho–HDAC co-repressors.

Mercola et al. [121] developed a human ESC-based HCS assay that allows small-molecule screens in serum-free conditions. Screening approximately 550 pathway modulators, they identified small-molecule inhibitors of the Wnt pathway (IWR-1, IWP-3, 53AH, XAV939). They showed that Wnt inhibition, most of all due to IWP-3, was sufficient to drive ESC-derived mesoderm to a cardiac fate in the absence of other signaling modulators, and no other inhibitors had comparable activity, not even the natural Wnt inhibitor DKK1 [122].

Further confirming that inhibition of Wnt/β-catenin signaling appears to be crucial in cardiomyocyte formation across many models, H. Wang et al. [123] showed that XAV939, another synthetic Wnt/β-catenin inhibitor, can induce cardiomyogenesis in mouse ESCs. XAV939 stimulates β-catenin degradation by stabilizing Axin, a component of the β-catenin degradation complex. XAV939 treatment of ESCs from day 3 to 5 of differentiation led to an increase in the expression of several cardiac genes like cardiac myosin heavy chain gene (Myh6) and Nkx2.5.

A similar result, in terms of cardiac commitment, was described by Parson et al. [124] using nicotinamide (NAM), a natural small molecule. Nicotinamide, also known as nicotinic acid amide, is the amide of nicotinic acid (vitamin B3). It is normally used in medicine for its anti-inflammatory actions in patients with inflammatory skin conditions (*acne vulgaris*). In their work Parson et al. found that nicotinamide, under defined culture conditions, is able to induced pluripotent hESCs toward a cardiac-lineage commitment, and up to beating cardiomyocytes, with high efficiency (>95% embryonic cardiac precursors and >50% beating cardiomyocytes). NAM appears to trigger cardiac induction promoting the expression of Nkx2.5, a cardiac-specific transcription factor, in a process that might emulate the specification of cardiac mesoderm from the pluripotent epiblast in human embryonic cardiogenesis.

As previously said, small molecules represent an important tool, showing not only that exogenous factors can be important for efficient differentiation, but also how endogenous cellular signals and pathways can be modulated to control differentiation. For this purpose, it is important to enhance our understanding of cell-fate mechanisms, from both biological and biochemical points of view. In fact, it is very difficult to know how to improve a particular signal to obtain a specific result, without a prior synergic work of these two branches, which could be translated into greater clinical success for cardiovascular cell therapy.

9. Protein biochemistry and stem cells: proteomic perspectives

Human adult CPCs have the ability to differentiate into cardiomyocytes, endothelial and smooth muscle cells. Also, the processes of stemness maintenance and differentiation entail changes in types and amount of proteins expressed by stem cells. Thus, in order to discover and track regulatory pathways and molecules involved in the many aspects of stem cell biology, the utilization of proteomics as a screening strategy seems to be essential [125,126]. Proteomics, in fact, is an approach for the simultaneous study of many proteins and their functions in complex biological systems through analytical protein biochemistry, and is a powerful tool to obtain a huge amount of new, otherwise unavailable, information about stem cell biological regulators. It allows the quantification of proteins and also distinguishing among isoforms arising from mRNA splice variants or genes, as well as characterizing their post-translational modification (PTM) status (phosphorylation, glycosylation, etc.), responsible most of the times for the regulation of their functions. Different strategies have been developed in recent years to optimize this kind of approach, in particular related to increasing protein solubility or detectability (for low abundant proteins), mostly optimizing detergent solutions, or pre-fractionation of complex mixtures, or methods for enrichment/purification of subgroups of proteins of interest. Mass spectrometry (MS) is obviously the ultimate tool used to identify proteins, either single MS or tandem instruments.

Proteomic approaches have been applied from multiple perspectives. First, global profiling aims at characterizing the complete physiology of a specific cell type, as published, for example, concerning MSCs [127], neural stem cells [128,129] or ESCs [130], providing so far a wide library of stemness-related proteins. From this profiling strategy, comparative studies can be performed as well, in order to identify protein modulation related to commitment and differentiation progression. Important clues can be derived on intermediate stages, gene activation and specific regulatory pathways controlling the maturation from stem cells to progenitors, and to functionally differentiated cells [131]. These studies are important for both basic developmental biology and translational regenerative medicine. One example is the study of Baharvand et al. [132], who compared mouse embryonic stem cells with neonatal-derived cardiomyocytes, and identified a large number of proteins involved in protein synthesis, processing, and trafficking. Such finding suggests that human ESCs are able to maintain the undifferentiated state until signals of lineage determination are received, upon which they quickly change phenotype and produce the necessary proteins. Moreover, a large number of proteins, particularly the highly abundant ones, were identified as chaperones, heat shock proteins, ubiquitin/proteasome, and oxidative stress responsive proteins; such high expression levels fit the interesting ability of ESCs to resist oxidative stress, consistently with their required long life-span.

A second proteomic approach is directed toward the discovery of novel markers for the identification and definition of stem cell populations, in particular surface markers. Cell-surface proteins are involved in basic cellular processes such as signal reception and transduction, internal/external cell communication and transportation. They are often targets for therapeutic molecules, and thus their study and understanding might help in both diagnostics and therapy. Proteomics, in fact, can provide valuable information about cell signaling mechanisms [133,134] and add to the number of cell-specific biomarkers already known. Multiple difficulties exist for the study of membrane proteins, such as hydrophobicity and low solubility, alterations due to PTMs and relatively lower abundance compared to other cellular proteins. Therefore new methods are continuously being developed (e.g. subcellular fractionation, chemical labeling/tagging methods for surface protein enrichment). Upon protocol optimization, many information can be obtained from this kind of studies, for example diverse cell-surface proteins have been identified in mouse ESCs, with specific receptors, transporters and adhesion molecules as the major identified protein groups [135].

A third study perspective concerns the analysis of the panel of secreted proteins, the "secretome", of stem/progenitor cells. Humoral signals are important since their secretions are specific for each cell type, and reflect the cellular state. It has been shown in studies on stem cell-based therapies that the number of new cells forming at the injury site does not often correspond to the observed functional improvement [29] and that indirect beneficial mechanisms are also involved in the therapeutic effects of cell therapy, even with resident cardiac committed CPCs [111]. Thus, the so called paracrine hypothesis has been introduced, supporting the notion that paracrine/autocrine mechanisms, mediated by various exogenous stem cells, contribute to tissue preservation and activation of endogenous regeneration [4]. In the case of non-cardiac adult stem cells, paracrine effects are most likely the only responsible for the short-term conflicting results observed, both in pre-clinical and clinical models [2,3], while for resident CPCs both direct and indirect regenerative effects occur [111]. Released paracrine factors can influence neovascularization, myocardial protection, cardiac remodeling, and contractility. Therefore it seems logical as a combined strategy to boost the efficacy of cell therapy, to study and possibly improve the paracrine potency of candidate therapeutic cells. Under this respect, the proteomic study of the panel of secreted proteins released by stem cells can serve the purpose of both discovering novel or distinctive regulators and pathways, and assessing the possible enhancement of paracrine potency.

Several groups have investigated paracrine/autocrine factors secreted by adult stem cells, such as MSCs, and their effects on cardiac functional improvement [136], which can be mediated also by concentrated conditioned media alone [137,138]. Recently, a first secretomic profile of resident CPCs has been obtained by comparative analysis of their conditioned media with that of neonatal rat ventricular myocytes (NRVMs) by RPLC analysis and identification by MS [139]. The specificity and functionality of both secretomes were investigated, considering that proteins secreted from either or both CPCs and NRVMs in vivo can build an interactive network. 83 unique proteins were identified, of which many were NRVM-specific (49%) or CPC-specific (23%), while 63% were integral plasma membrane and/or known secreted proteins, providing many hits for candidate regulatory molecules. From wide database and literature search, multiple potential functions have been identified, such as positive/negative regulation of the cardiac system, and structural/functional regulation of the extracellular matrix, which could further contribute to the understanding of pathologic alterations of heart function. Furthermore, most of the identified proteins that met the criteria for paracrine/autocrine factors had not been previously linked to stem cells, confirming the powerful discovery contribution of this approach to stem cell biology. Among the selected proteins that met the criteria of paracrine factors, some were of particular interest. ST2 is a member of the IL-1-receptor family and it is expressed in CPCs in a membrane-bound or in a soluble decoy isoform. Increased quantities of ST2 are known to occur with neurohormonal and biomechanical stress activation, and increased serum levels of ST2 are predictors of mortality and clinical outcome in patients with MI [140–142]. IL-33 has been identified as a specific ligand for ST2 [143] and IL-33/ST2 signaling has been shown to be a cardioprotective paracrine system between fibroblasts and cardiomyocytes activated by mechanical stimuli [144]. ST2 acts as an autocrine factor, being secreted into conditioned media by CDCs and exerting an anti-proliferative effect on rat CDCs themselves. In this same study two other molecules were identified as potential paracrine factors acting on CPCs, that is connective tissue growth factor (CTCF) and atrial natriuretic peptide (ANP), which had opposite effects on CPC proliferation.

Finally, secretomes are a potential rich source of biomarkers, as they reflect various specific cellular states that may mark disease development, and therefore may be used for diagnosis, prognosis, risk stratification and therapeutic monitoring [145]. They can provide a complementary in vitro approach to direct blood/plasma/fluids analysis of patients, providing identification and pre-selection of candidate

protein biomarkers for subsequent validation in clinical samples. In fact, blood and other body fluids consist of proteins derived from all tissues and organs. Thus, a preliminary screening in vitro can serve the purpose of focusing only on candidate molecule of particular interest for the system under analysis, e.g. the cardiovascular system.

Overall, proteomics may allow the understanding of multiple aspects of stem cells biology, and in particular for adult progenitor cells, providing insights on the definition of different population, their physiology and their autocrine/paracrine regulatory and signaling networks.

10. Conclusions and future perspectives

The integration of different scientific expertise seems to be an increasingly pressing need for science. Interdisciplinary approaches can provide novel and precious insights on rapidly evolving research areas, further pushing forward discovery and optimization. Regenerative medicine is a fast-pace field, encountering great interest and excitement for future effective personalized treatments, where the cooperation and sharing of common interests between biology and biochemistry represent a remarkable example of productive scientific integration. In the field of cardiac regenerative medicine, many hurdles still need to be overcome for cell therapy, mostly related to low delivery, engraftment and differentiation efficiencies of injected therapeutic cells. Moreover, indirect paracrine effects seem to play a significant role on the overall beneficial outcome observed, both in preclinical and clinical settings. Thus, efforts are needed in order to discover and modulate possible regulatory pathways for boosting the regenerative potential of promising stem cells candidates, particularly resident CPCs. Sensible targets may be related to multiple biochemical networks, such as calcium trafficking, glucose metabolism, oxygen tension response and small molecules, affecting different signal transduction pathways and modulating the balance between stemness and differentiation. Novel integrated approaches are needed to discover and optimize protocols for these purposes, with the ultimate goal of finely regulating CPC commitment and potency for exogenous and endogenous tissue repair.

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