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Minireview

Cardiac stem/progenitor cells, secreted proteins, and proteomics

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ABSTRACT

Stem cell-based therapy is emerging as a novel approach for myocardial repair over conventional cardiovascular therapies. In addition to embryonic stem cells and adult stem cells from noncardiac sources, there is a small population of resident stem cells in the heart from which new cardiac cells (myocytes, vascular endothelial cells and smooth muscle cells) can be derived and used for cardiac repair in case of heart injury. It has been proposed that the clinical benefit of stem cells may arise from secreted proteins that mediate regeneration in a paracrine/autocrine manner. To be able to track the regulatory pathway on a molecular basis, utilization of proteomics in stem cell research is essential. Proteomics offers a tool that can address questions regarding stem cell response to disease/injury.

1. Introduction

Embryonic stem cells (ESCs) are defined as unspecialized cells with the capability of dividing and renewing for indefinite periods but under certain physiologic and experimental conditions, they can be induced to become specific cell types performing specialized functions, e.g. beating cells of the heart muscle, oxygen-carrying red blood cells, or electrochemical signaling nerve cells. Although animal and human ESCs have indisputable potential in cell-based therapy after injury and/or in disease treatment (e.g. diabetes, Parkinson's disease, spinal cord injury, heart diseases, etc.) [1–6], there are several limitations in their clinical applications. In addition to ethical limitations involved in ESC research, they have a high proliferation capacity leading to the formation of teratomas and they can be rejected by the immune system. Thus, the identification of adult stem cells (ASCs), also called progenitor cells, in many more tissue types than was originally thought, has led to increased scientific effort and enthusiasm in stem cell and clinical research.

Adult mammalian heart has a special need for regenerative therapy as it has a very limited ability for endogenous renewal, and events such as heart attack or myocardial infarction (MI) result in destruction of the complex cardiac architecture and replacement of myocardium by scar tissue resulting in increased risk of death due to heart failure (HF) or arrhythmia. There is currently no effective therapy to replace dead/damaged myocardium except cardiac transplantation. And thus, stem cell-based therapy with stem/progenitor cells offers the promise of organ repair on demand, and appears to have an immense potential in the treatment of MI and HF. The discovery of cardiac stem/progenitor cells (CSCs), a small fraction of the total cells residing in the heart, opened the possibility for autologous heart repair. And thus, efficient methods for their isolation and expansion to sufficient quantities for therapeutic purposes have been searched and tested, as well as methods for enhancing the activation of endogenous stem cells or converting other cells within the heart into cardiac stem cells. There is growing evidence that proteins secreted from stem/progenitor cells into the vicinity of the injury can modulate the infarct microenvironment and play an important role in functional improvement [7,8]. Proteomics, an approach for the study of proteins and their functions in complex biological systems, is a suitable strategy to obtain new, and otherwise unavailable, information about stem cells. By using proteomics we could better understand the mechanisms driving ASC engraftment and differentiation into cardiac myocytes and vascular cells. This involves understanding the autocrine and paracrine manners of proteins secreted by stem/progenitor cells, and clarifying how the cell-surface receptors and signaling proteins stimulate and influence the differentiation process.

This minireview focuses on ASCs, with a special emphasis on CSCs and their potential therapeutic use in heart disease, and on the role of proteomics in stem cell/cardiac stem cell research.

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2. Adult stem/progenitor cells

Bone marrow ASCs have been used in clinical applications for years, but ASCs were discovered only recently in other tissues including brain, blood vessels, liver, and skin. ASCs are undifferentiated cells that reside, in extremely low numbers, among differentiated cells in a mature organ or tissue [9,10]. Unlike ESCs, which are known to originate in the inner cell mass of blastocyst, the origin of ASCs in mature tissue is highly speculative with many unanswered questions such as: what stopped them from differentiating, why they are still stem cells when most cells have differentiated? A recent hypothesis [11] suggests that ASCs arise from the neural crest, a group of embryonic cells found in the ectoderm during embryo development due to their ability to migrate to numerous locations in the body and differentiate into various cell types. ASCs can regenerate and under proper but yet to be fully defined internal/external stimuli, they can either differentiate into the specific tissue of their origin or transdifferentiate into multiple cell types. Transdifferentiation, or plasticity, was reported for e.g. brain stem cells and bone marrow-derived stem cells [9,12,13]. Bone marrow hematopoietic stem cells have been shown to transdifferentiate into brain cells (neurons, oligodendrocytes and astrocytes), liver cells, and skeletal muscle cells [9,12], while bone marrow stromal cells (mesenchymal stem cells) may transdifferentiate into skeletal muscle cells and cardiac muscle cells [13]. Although the articles claiming little or no evidence for transdifferentiation of adult hematopoietic stem cells have been published [14–16], the first reports of successful transdifferentiation of adult hematopoietic stem cells into cardiac cells appeared in 2001 [10,13,17]. In these reports, adult hematopoietic cells were injected directly into damaged myocardium [13] or their enriched forms were transplanted into bone marrow with subsequent delivery to the heart [10]. When hematopoietic stem cells were injected directly into the damaged wall of the ventricle of infarcted adult mouse myocardium they were able to generate all three major heart cell lineages, cardiomyocytes, smooth muscle cells (SMCs), and vascular endothelial cells [13] based on cell-specific marker expression (cardiac myosin, factor VIII and α-smooth muscle actin). However, the success rate with this invasive approach was only 40% (repair was successful in 12 out of 30 mice). The same research group [17] went on to improve efficiency by administration of cytokines (stem cell factor, SCF, and granulocyte-colony-stimulating factor, G-CSF; injected s.c.), resulting in increased levels and mobilization of adult bone marrow stem cells into circulation with subsequent translocation to the infarcted portion of the myocardium. The volume of tissue repair in regenerating myocardium was measured in three sections and determined as the product of section thickness and the area occupied by restored tissue. Cardiac repair was characterized by a band of newly formed myocardium occupying most of the damaged area. In the absence of cytokines, myocardial replacement was never observed. This noninvasive cytokine-mediated autologous therapeutic strategy resulted in a significant degree of tissue regeneration 27 days later, with 73% of mice surviving. In the latter study [10], highly enriched hematopoietic stem cells (side population cells) from a genetically altered mouse strain were transplanted into the bone marrow of a lethally irradiated mouse subsequently subjected to heart attack. As in [13], hematopoietic stem cells were able to engraft and transdifferentiate into cardiac myocytes and vascular endothelial cells. In a recent study [18], four types of ASCs in a murine model of myocardial ischemia were compared: bone marrow mononuclear cells (MNCs), mesenchymal stem cells (MSCs), skeletal myoblasts (SkMb), and fibroblasts. It was demonstrated that although each cell type has its own advantages and limitations, there is a clear survival and a modest functional benefit of MNCs in the infarcted mouse heart resulting in more robust preservation of cardiac function compared to SkMb and MSCs. There was, however, no evidence of transdifferentiation by donor MNCs into cardiomyocytes.

In addition to murine stem cells, human ASCs from various sources were shown to transdifferentiate into cardiac cells as well, either in vitro or when injected into murine hearts, e.g. human peripheral blood-derived adult stem cells (CD34+, endothelial progenitor cells (EPCs), smooth muscle progenitor cells) [19–21] and human MSCs [22]. And thus, a large number of studies have been performed in small animal models (mice/rats) using both foreign ASCs and pretreated autologous ASCs of various cell types, either injected directly into infarcted area or transplanted/stimulated in other body locations with subsequently induced translocation/mobilization to damaged parts of the myocardium, or injected intracoronarily or intravenously. Despite considerable experimental data with rodents, data from large animal studies have been limited and contradictory. In rabbits, injection of human MSCs into infarcted region has shown their transdifferentiation into cardiomyocytes, regeneration of vascular structures, and functional improvement [23]. Improvement was also observed in a canine model, in which cardiomyogenic growth factor-pretreated bone marrow MSCs had a beneficial effect on functional regeneration of chronically infarcted myocardium [24]. In a porcine model, intravenous delivery of autologous, allogeneic porcine and human MSCs resulted in improved myocardial function for all three types of cell used. However, formation of a non-malignant cardiac tumor containing mesenchymal tissue was observed in one animal treated with human MSCs [25]. Another instance of human MSC transplantation improving cardiac function in swine was reported [26], with markedly greater improvement achieved by cotransplantation of human MSCs and human fetal cardiomyocytes (1:1) [26]. On the other hand, intracoronary delivery of umbilical cord blood ASCs, after induced MI in swine, did not show any improvement in left ventricular function [27], and although intracoronary injection of unselected bone marrow ASCs and MNCs reduced an infarct size, surprisingly, there was little or no functional improvement demonstrated [28]. Initial human clinical results and their outcomes were recently summarized [29]. Most of the transplantations have been carried out using unselected bone marrow and blood-derived progenitor cells in patients with MI or in patients with chronic HF due to previous MI. The individual trials of the patient with MI included groups of 10, 59, 60 and 20 patients at 8, 4.9, 4.8 and 13.5 days after MI, respectively. In chronic HF, groups of 8, 10, and 14 patients were examined, mostly 5–6 years after MI. Collectively, these human studies have shown the relative efficacy of intracoronary progenitor cell transplantation for patient with MI. In chronic ischemic HF treatment, additional questions arose: e.g. whether identifying hibernating myocardium to direct cell therapy is essential to an effective outcome, and whether delivery of skeletal myoblasts to established scar tissue late in the disease improves clinical outcome once patients are protected against potential arrhythmias by an implantable defibrillator [29]. Safety trials in humans [30–33] have confirmed, at least under experimental conditions used, that stem cell-based therapy can be relatively safe and does not at this time appear to be associated with an increase in the number of adverse clinical events [34]. Still, optimization of patient cohort, cell processing methods, cell delivery methods, and cell sources remained to be optimized.

Over time, reviews regarding differentiation of the various ASCs used for cardiac regeneration have been published [35–39], but some questions are still unanswered, e.g. whether cardiomyocytes reported as arising through transdifferentiation could in fact be the result of cell–cell fusion. Thus, some authors have reported

transdifferentiation [13,17,19,20,40] while others have claimed cell fusion, based, e.g. on a hybrid genotype displayed by fused cells [41–43]. According to [44], intrinsic features of stem cells (clonogenicity, self-renewal and multipotentiality) exclude cell fusion as a necessary requirement for growth and differentiation and point to the plasticity of stem cells. Still, the cell fusion theory remained as an alternative hypothesis for the concept of stem cell plasticity [40].

3. Cardiac stem/progenitor cells

The heart was for a long time considered a terminally differentiated organ: it was believed that cardiac myocytes could not self-regenerate after damage and that the only response to injury was hypertrophy of remaining viable cardiomyocytes resulting in an increase in cardiac mass. The first report that ventricular myocytes are not terminally differentiated in adult heart appeared in 1998 [45]. Moreover, the discovery of stem/progenitor cells residing in the adult normal or diseased heart which can undergo mitosis, especially in connection to various types of cardiac damage, and from which new cardiomyocytes can arise, opened the possibility for autologous heart cell repair. Although transplantation of fetal cardiac cells as an optimal cell source into damaged myocardium displayed promising results in functional improvement [46], similarly to ESCs, ethical and immunological issues, and limited quantity and availability, made application in large-scale clinical trials impossible. On the other hand, CSCs could offer many advantages for regenerative therapy, since they are autologous if harvested and grown in vitro prior to transplantation in the patient, and thus, immunological complications and disease transmittal are therefore improbable. CSCs also appear to be more cardiogenic than other ASCs, and trigger robust angiogenic responses after myocardial transplantation [47].

The first reports of CSCs residing in very low numbers in the heart appeared in 2003 [41,48,49]. Although controversial [49], it supported an older observation [50], and showed that increased cardiac mass resulted from a combination of myocyte hypertrophy and hyperplasia in human aortic stenosis and that new myocytes were formed by the differentiation of stem-like cells that expressed stem cell markers (c-kit, stem cell antigen Sca-1, and MDR-1) and telomerase, and that their numbers increased more than 13-fold [49]. The discovery of cell clusters with CSCs making the transition to cardiogenic and myocyte precursors, and very primitive myocytes that turned into terminally differentiated myocytes, provided a link between CSCs and myocyte differentiation, and thus supported the existence of CSCs that amplify and commit to the myocyte lineage in response to increased cardiac workload [49]. CSCs have now been shown to differentiate into all types of cardiac lineages [48,51] and they are self-renewing, clonogenic and multipotent. To date, they have been identified in mouse/rat [48,51], dog [52], pig [53] and human [49,51,53,54] hearts. Recently, several methods for CSC isolation have been developed [41,48,51,53–55]. The first successful isolation of adult CSCs was reported by Messina et al. [51]. In this study, undifferentiated cells, growing either from explants of postnatal atrial/ventricular human biopsy specimens or from murine heart tissues, were able to form multicellular clusters (cardiospheres, CSps) in suspension culture. CSps were created by heterogenous population of cells (cardiac stem cells, differentiating progenitors, spontaneously differentiated cardiomyocytes and vascular cells) and expressed stem cell and EPC antigens/markers. Murine CSps started beating spontaneously after their generation and maintained this function during their lifespan, whereas human CSps did so only when cocultured with rat cardiomyocytes [51]. This was recently overcome by inducing human cardiomyocyte progenitor cells to differentiate into cardiomyocytes and then form spontaneously beating aggregates in vitro by stimulation with 5-azacytidine, without a need for coculture with neonatal myocytes [55]. The original culture method [51] was further modified to improve efficiency, and the addition of a postcardiosphere expansion step allows one to obtain reasonable number of cells for transplantation from the small specimen (biopsy) in a timely manner [53]. It is now possible to routinely replate CSps to yield a monolayer of cardiosphere-derived cells (CDCs). CSps and CDCs, derived from human biopsy specimens and from comparable porcine samples, expressed antigenic characteristics of stem cells at each stage of processing, as well as proteins vital for cardiac contractile and electrical function [53]. Importantly, human CDCs injected into the border zones of myocardial infarcts promoted cardiac regeneration and improved heart function in a mouse infarct model [53]. In another study, the conditions were established for isolation and expansion of c-kit-positive human CSCs from small samples of myocardium [54]. Two methodologies for preparation of c-kit-positive human CSCs were used [54], either an enzymatic dissociation of myocardial samples, from which c-kit-positive cells were sorted with immunobeads and plated at low density to obtain multicellular clones from single founder cells (successful isolation in 8 of 12 cases) or a primary explant technique (successful cell outgrowth in 46 of 70 cases). Human CSCs differentiated predominantly into cardiomyocytes, and to a lesser extent into SMCs and endothelial cells based on specific protein expression. For example, human CSCs generate myocytes positive for cardiac myosin heavy chain, α-sarcomeric actin, and α-cardiac actinin. In addition, when locally injected in the infarcted myocardium of mice/rats, human CSCs created a chimeric heart in which human myocardium was structurally and functionally integrated with the rodent myocardium and contributed to the performance of the infarcted heart. Differentiated human cardiac cells possessed only one set of human sex chromosomes, indicating that human CSCs form human myocardium independently of cell fusion [54]. Taken together, the possibility of human CSCs isolated and expanded in vitro with subsequent autologous application for regeneration of myocardium in patients suffering from HF, appeared to be feasible.

In an interesting paper, hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) were injected intramyocardially after induced MI in a canine model to stimulate resident CSCs. This intervention led to the formation of myocytes and coronary vessels within the infarct, and thus to improvement of cardiac function [52], and further supported the thesis, that secreted proteins are important and may affect the viability of cells. HGF and IGF-1 were also used to stimulate c-kit-positive cardiac progenitor cells in vitro prior to their injection into infarcted myocardium of rats [56]. In this case, cardiac progenitor cells divided and differentiated into endothelial and SMCs and, to a lesser extent, into cardiomyocytes that appeared to be mediated by the up-regulation of hypoxia-inducible factor 1α, which promoted the synthesis and secretion of stromal-derived factor 1 (SDF-1) from hypoxic coronary vessels [56].

Recently, many of the issues involved in cardiac stem cell-based therapy have been addressed: some are mentioned above. They include the establishment of methods for CSC isolation, their pretreatment, culturing, and expansion in vitro, various methods for their delivery to the heart and controlling their growth and maturation afterwards. Even a successful method for stimulation and activation of the CSC pool in vivo, leading to progressive improvement in cardiac function, was also reported [52]. Although all of these advancements undoubtedly indicate a big step forward in cardiovascular disease prevention and treatments, due to their relatively recent discovery, there is an absence of knowledge about long-term outcomes, possible risks involved, and clinical follow-up data before a routine therapeutic CSC use. And so, the

development of suitable monitoring methods is necessary, and the needs to go hand-in-hand with progress in CSC and stem cell-based therapies. Currently, the methods frequently used, e.g. histologic analysis (postmortem) and echocardiography, are limited and insufficient. Direct repetitive visualization of stem cell-based therapy progress in vivo by noninvasive techniques, in terms of identification of therapy cell location, magnitude and length of survival, is still a big challenge. And thus, to address this issue, novel promising methods like magnetic resonance imaging (MRI), nuclear imaging, reporter gene imaging and quantum dot imaging are emerging and further improved for application to stem cells [57] Proteomics, the method for identification and quantification of proteins, can help to clarify the molecular basis of stem cell response to injury: mechanisms leading to stem cell differentiation and maturation and the role of proteins secreted by stem cells in the regenerative process.

4. The role for proteomics in stem cell research

Proteomics is a scientific discipline in which analytical protein biochemistry is applied in a manner that allows simultaneous assessment and characterization of many proteins. This involves quantification of proteins, and the ability to distinguish among the protein isoforms arising from mRNA splice variants or genes. As well, proteomics can characterize the proteins and determine posttranslational modification (PTM) status, including phosphorylation, glycosylation, etc. Proteins are the actual mediators of most cell mechanisms and processes. Proteomics, a method for study of proteins and their functions in complex biological systems, can extend, be complimentary to other scientific approaches and moreover, provide new information about stem cells that would otherwise be unavailable.

The majority of stem cell proteomic studies have relied on two dimensional gel electrophoresis (2-DE), which separates proteins according to pI (charge) and mass (molecular weight), and on mass spectrometry (MS) for protein identifications. 2-DE is particularly well suited for identification of PTMs with pI shifts observed in gels, and for providing the quantitative data. Although widely used, 2-DE does have however several limitations, e.g. solubilization of hydrophobic membrane proteins is problematic, and the identification of low abundant proteins can be difficult. Thus, different strategies have been developed to increase the protein solubility, based mostly on detergents or various combinations of chaotropes. To increase detectability of low abundant proteins, intensive prefractionation of complex sample mixture can be used prior to detection and different methods for enrichment/isolation/affinity purification of subgroups of proteins of interest can be applied. This can be from isolation of a particular organelle or to identify protein chemical property such as pI or PTM. Mass spectrometers are ultimately used to identify proteins, either single MS instruments such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) for peptide mapping (peptide mass fingerprinting) or tandem instruments like MALDI-TOF-TOF or one of many LC–MS/MS instruments which further fragment peptides in order to obtain the amino acid sequences. For quantitative comparison of multiple samples, MS techniques using protein/peptide labeling such as iTRAQ (isobaric tags for relative and absolute quantification) or SILAC (stable isotope labeling by amino acids in cell culture) can be used or even label-free techniques are feasible on high accuracy MS instruments. However, for absolute quantification, spiked in standards must be used, and analysis is often carried out with multiple reaction monitoring (MRM) in a triple quadrupole/linear ion trap MS instrument.

Modern MS instruments and techniques allow identification of proteins in femtomole to picomole range. Other gel-free separation techniques can be used in proteomics, either alone or in various combinations prior to MS detection in multidimensional arrangement (mostly various modes of liquid chromatography). Recently, new techniques have been developed and used for stem cell analysis [58,59]. For example, one approach addressed the problem of identification of proteins occurring in low abundance, in which novel MS-based proteomic method called iterative exclusion (IE-MS) was successfully applied for identification of previously undetectable growth factors in human ESCs present in concentrations of 10^{-9}–10^{-11} g/mL [58]. In this method, the same sample is run many times with excluding of peptides already detected and allowing only new ones to be observed with each subsequent analysis. Another new technique targeted the difficulties associated with analysis of membrane protein as well as their low presence in cells [59]. In this study [59], a strategy for membrane protein profiling by using combination of differential in-gel electrophoresis DIGE technology (CyDye labeling) was described with subsequent membrane protein enrichment by biotin/avidin purification. By this method they were able to discriminate membrane and membrane-associated proteins from intracellular contaminants and identify additional markers during mouse ESC differentiation. In addition to methods aforementioned, numerous proteomic techniques have been developed and improved in recent years to suit specific stem cell research needs [60–63]. Many stem cell proteomic studies have been aimed at human stem cells either ESCs or ASCs [61,62]. They have followed several major directions, as described below, and they are depicted schematically in Fig. 1.

4.1. Global protein screening – what makes a stem cell as stem cell

Global protein profiling has been performed for various cell types with further extension to comparative analyses leading to recognition of cell lineage similarity/diversity either within the same species or for different species compared. For example, a characterization of MSCs [64] or neural stem cells from rat [65] and human [66] was carried out for better understanding of their physiologies. The global proteome identification of murine ESCs was published recently [67,68]. The latter study revealed more than 5000 proteins, thus creating the largest stem cell proteome reported to date [68]. It was shown that ESCs can be SILAC-labeled when grown feeder-free during the last phase of cell culture. Two different preparation approaches were compared: cell fractionation followed by 1-DE separation, and in-solution digestion of total cell lysate combined with isoelectric focusing. Both preparation methods were then followed by LC–MS/MS protein/peptide identification, and resulted in comparable proteome coverage for either approach used [68].

4.2. Differentiation – what are the initiating and control hubs

Another direction included proteomic monitoring of developmental processes during stem cell differentiation. Protein expression changes during stem cell differentiation revealed, for example, a close relationship between mouse ESC-derived cardiomyocytes and neonatal cardiomyocytes and certain similarities with original mouse ESCs [69]. Other studies identified and mapped the protein expression changes during differentiation of mouse ESCs into Sca-1^+ progenitor cells (which are meant to have a potential to serve as precursors of vascular SMCs), and performed further comparisons with the proteome of mouse adult aortic SMCs. It was found that although SMCs derived from ESCs can express smooth muscle markers, the proteome is very different from mature aortic SMCs [70–72]. Heat shock protein Hsp25 was identified as useful marker of early ESC differentiation, with a gradual decrease to levels barely detectable at 4 days following differentiation [73]. Quantitative analysis of the changes in protein expres-

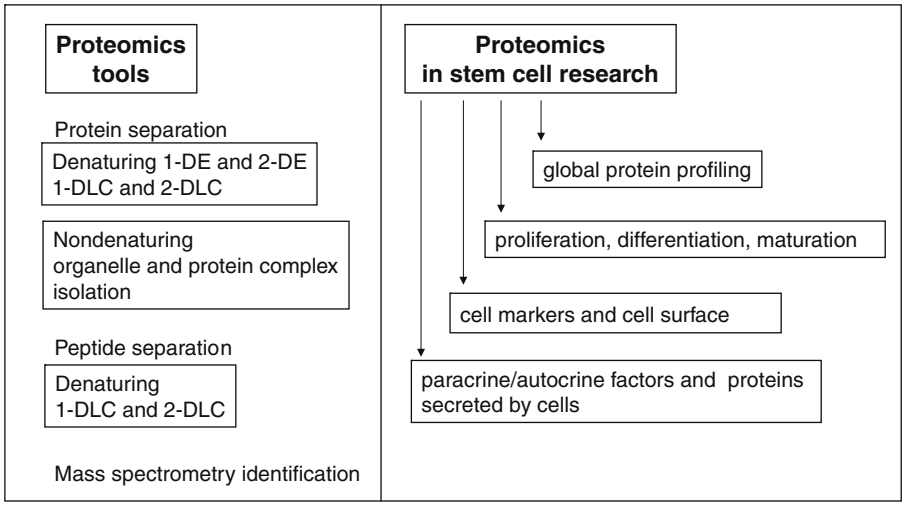

Fig. 1. Schema outlining opportunities for proteomics to address key questions in stem cell research. 1-DE: one dimensional gel electrophoresis; 2-DE: two dimensional gel electrophoresis; 1-DLC: one dimensional liquid chromatography (reversed phase); 2-DLC: two dimensional liquid chromatography.

sion by MS before and after short-term induction of osteoblast differentiation in a cell model of human MSCs, established by overexpression of the human telomerase reverse-transcriptase gene, revealed that expression levels changed by at least 2-fold upon osteoblast differentiation, with an increase for 83 proteins (e.g. alkaline phosphatase, versican core protein and tenascin) and a decrease for 21 proteins (e.g. fatty acid synthase) [74].

4.3. Stem cell markers and the cell surface

Although stem cell-based regenerative and reparative therapies are currently in preclinical and clinical trials, number of specific cell markers is insufficient as well as identification of signaling proteins/cell-surface receptors. The proteins of the plasma membrane are of great importance and interest in stem cell research. Approximately 20–30% of all genes in many organisms encode integral membrane proteins [75]. Cell-surface proteins are involved in basic cellular processes such as signal reception and transduction, internal/external cell communication, interaction, and transportation. They are targets for therapeutic molecules, and thus the discovery of novel proteins (biomarkers) and understanding of their functions, will help in both diagnostics and therapy. Proteomics can provide new valuable information about cell signaling mechanisms [63,76] and add to the number of cell-specific biomarkers already known. Although plasma membrane protein hydrophobicity (less solubility), functional alterations due to PMTs and relatively lower abundance compared to other cellular proteins make proteomic analysis of the plasma membrane challenging, new methods are being developed to overcome these problems. Thus, e.g. subcellular fractionation methods, employing various centrifugation techniques and/or chemical labeling/tagging methods for surface protein enrichment and purification have been implemented. In addition to the example already mentioned [59], in which DIGE technology combined with biotin/avidin enrichment allowed identification and comparison of membrane proteins of mature and ESC-derived SMCs, proteomic analysis of the hydrophobic fraction of human MSCs from umbilical cord blood has yielded 32 protein identifications, of which 2 proteins were shown to be cell-surface proteins, using a combination of centrifugation steps, 2-DE and MALDI-TOF MS analysis [77]. A similar strategy, with centrifugation steps prior to LC–MS/MS, was used for identification of novel membrane protein markers in a human MSC line undergoing osteoblast differentiation [74]. This study identified 148 integral membrane or membrane-anchored proteins, 159 membrane-associated proteins, 29 integrins and cell adhesion proteins, and 20 receptors out of a total of 463 proteins. An enhanced enrichment strategy, using both cell-surface biotin labeling and discontinuous sucrose density gradient followed by MS was used for identification of cell-surface markers and signaling molecules in mouse ESCs [78]. Here, diverse cell-surface proteins were identified with receptors, transporters and cell adhesion molecules as the major identified protein groups. Since many interesting cell-surface proteins can be glycosylated, a cell-surface capturing technology for selective detection of stem cell-surface glycoproteins was recently developed [76], in which less than 15% contamination from intracellular and non-glycosylated peptides/proteins were observed by MS identification. This technique proved to be highly selective for low abundant plasma membrane proteins, compared to chemical tagging glycoprotein and high affinity enrichment techniques.

4.4. Secretomes: paracrine/autocrine factors and other proteins secreted by stem cells

Proteins secreted by cells (secretomes) are important since their secretions are specific for each cell type, and reflect the local environment of the cells (cellular state). It has been documented in studies on stem cell-based therapies that the correlation between the numbers of new cells forming at the site of an injury does not often correspond to the degree of observed functional improvement [53]. And thus, an alternative hypothesis based on paracrine mechanisms was suggested and frequently discussed at present, in which paracrine/autocrine mechanisms mediated by either endogenous or transplanted exogenous stem cells are meant to contribute to regeneration and repair process [7,8]. Paracrine and autocrine factors are defined as proteins synthesized and secreted by a cell, which can diffuse into the near vicinity and induce changes in neighboring cells or in the originating cell itself. In some cases, the autocrine/paracrine protein can affect the originating cell prior to its secretion. Common paracrine factors can be divided into several major families, such as fibroblast growth factors, Hedgehog proteins, Wnt family proteins, and transforming growth factor TGF-ß superfamily. In addition to the families mentioned, there are other paracrine factors, e.g. epidermal and HGFs, neurotrophins, cytokines, interleukins and various factors that play an important role during development.

Table 1
Proteomic application in stem cell-based cardiac research.

Ref.	Cell source	Proteomic method	Finding	Proteins/# proteins identified
[69]	Mouse ESCs,neonatal cardiomyocytes	2-DE	95% of proteins matched between ESC-derived cardiomyocytes and NCs	60
[70]	Mouse ESCs	2-DE, MALDI-TOF MS, MS/MS	Sca-1$^+$ protein database	241
[72]	Mouse ESC-derived SMCs, mouse aortic SMCs	DIGE, MALDI-TOF MS, LC–MS/MS	Comparative analysis; ESC-derived SMCs express smooth muscle markers but are different from aortic SMCs	146 protein differed between ESC-derived SMCs and aortic SMCs
[59]	Mouse ESC-derived SMCs, mouse mature SMCs	DIGE, biotin–avidin labeling	Comparative analysis of plasma membrane proteins; ESC-derived SMCs maintain characteristics of their ESC origin	E-cadherin, integrin alpha6, CD98 (4F2) upregulated in ESC-derived SMCs
[80]	EPCs	MALDI-TOF MS, nanoflow liquid chromatography, DIGE	TP found in conditioned medium from EPCs stimulated endothelial cell migration and promoted angiogenesis	Thymidin phosphorylase (TP)
[81]	Murine ESCs	DIGE, LC–MS/MS, LTQ-FT MS, multidimensional LC	TNFα-primed secretome mediated promotion of ESCs to cardiac commitment	99
[82]	Resident CSCs in infarcted myocardium of mouse	2-DE, MALDI-MS	Differentiation of resident CSCs into diversed cardiac cell types indicated by up-regulation of nestin	26

NCs: neonatal cardiomyocytes; SMCs: smooth muscle cells; EPCs: endothelial progenitor cells; CSCs: cardiac stem cells; TP: thymidine phosphorylase (proangiogenic factor); FT: Fourier transform.

Endogenous cells are usually able to release paracrine factors in response to injury, and it has been suggested that these paracrine factors can signal and activate resident ASCs or induce mobilization and homing of stem cells into damaged tissue from various locations through the circulation. It was observed that progenitor cells can improve cardiac function, under experimental conditions, even with low or absent cardiac differentiation [57]. In cardiovascular research, transplanted exogenous stem cells releasing paracrine factors can influence neovascularization, myocardial protection, cardiac remodeling, and contractility [7]. Nevertheless, if paracrine mechanisms themselves are sufficient or whether their combination with stem cell transplantation or other factors are necessary for satisfactory results, remains an open question. Yet, identification of paracrine factors released by stem cells into surrounding damaged tissue could lead to the possibility to replace stem cell-based therapy by simple protein-based therapy [7] in which a "mixture" of specific proteins, with proper concentrations and timing, could be administered to patients.

In [79], microarray experiments were paralleled with proteomics with significant release of angiogenic factors, vascular endothelial growth factor VEGF, stromal cell-derived factor-1 SDF-1 and IGF-1 proteins into a cell culture supernatant of EPCs. Moreover, conditioned media from EPCs induced a strong migratory response of mature endothelial cells, and significantly stimulated the migration of resident cardiac c-kit$^+$ progenitor cells in vitro [79]. Another proangiogenic factor, thymidine phosphorylase (TP), was identified in proteins secreted by EPCs, and its key role in EPC survival and angiogenesis was confirmed using a combination of RT-PCR, multiplex cytokine assays, and proteomics [80]. In another study, cardiogenic priming of the endoderm with tumor necrosis factor alpha TNFα (a cardiogenic inducer) was investigated by comparative proteomics. It was found, by using DIGE and MS techniques, that 75% of protein species were increased in TNFα-primed compared to unprimed endodermal secretome. Protein–protein interaction analysis revealed a TNFα-centric secretome network with cardiovascular development as the primary developmental function. The function of this cardioinductive network was validated by direct application of the TNFα-primed secretome on ESCs potentiating cardiac commitment and sarcomerogenesis, whereas exclusion of TNFα from the network during its generation demoted the primary ranking of cardiovascular development [81]. Upregulated expression of the intermediate filament protein nestin in the infarcted myocardium, as determined by proteomics, was related to differentiation of resident CSCs into various lineages, including cardiomyocytes [82]. Intracoronary injection of conditioned media derived from porcine autologous EPCs was associated with increased cardiomyocyte size in the infarct territory in conditioned media treated groups as compared to controls. Analysis of conditioned media detected elevated levels of TGFbeta1, a recognized mediator of hypertrophic signaling in the heart. The hypertrophic effect of TGFbeta1 was further confirmed by neutralizing antibodies/recombinant TGFbeta1 added into conditioned media, leading to attenuation/restoration of the hypertrophic effect in vitro [83]. Conditioned media from bone marrow MNCs injected into acutely infarcted hearts resulted in increased capillary density, decreased infarct size, and improved cardiac function compared to controls [84].

The effect of paracrine factors can be increased by genetic modification of stem/progenitor cells. It was documented that genetically modified stem cells may release therapeutic paracrine factors and also show better reparative potential compared to control (untreated) stem cells. For example, MSCs with a hypoxia regulated heme oxygenase-1 vector injected into infarcted hearts led to improved stem cell survival compared to control MSCs [85] and Bcl-2 engineered MSCs inhibited apoptosis, expressed higher amounts of VEGF, and improved heart function in rats [86]. The overexpression of the survival protein Akt in MSCs resulted in secretion of HGF, thymosin-β4, and secreted frizzled-related protein-2, paracrine factors that influence myocardial protection, improve cell survival, and decrease cell death [87]. Paracrine factors secreted from ASCs were extensively listed in [7,8]. Although published applications of proteomics in stem cell-based cardiovascular research are not numerous, some of them are summarized in Table 1.

5. Conclusion

Stem cells hold great promise in cardiac medicine. Moreover, resident CSCs even offer a suitable and autologous source for repair and regeneration. Although methods for their isolation, expansion, and transplantation have been developed, the best options for optimal results remain to be examined and established. From murine to larger animal models to human clinical trials, application of stem cells in cell-based therapy has shown to be relatively safe, and has resulted in higher efficiency results compared to conventional methods used. In addition, based on the paracrine mechanisms of proteins secreted by stem cells, alternative approach of protein-based therapy was discussed and experimental evidence obtained recently. Thus, the possibility of efficient, noninvasive treatments for cardiovascular diseases seems to be even closer. Although proteomics has not been implemented sufficiently in

stem cell studies yet, it has a big potential and can address many issues and obtain information not accessible by other techniques.

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