Proteomics: A Reality-Check for Putative Stem Cells

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Overview: The Maturing of Proteomics in Cardiovascular Research [*Circ Res*. 2011;108:490–498]

Proteomics: A Reality-Check for Putative Stem Cells

Divide and Conquer: The Application of Organelle Proteomics to Heart Failure

New Biomarkers in Cardiovascular Disease: Potential Impact and Strategies for Discovery

The Future of Posttranslational Modifications: From the Simple to the Complex

Jenny Van Eyk, Guest Editor

Proteomics
A Reality-Check for Putative Stem Cells

Marianna Prokopi, Manuel Mayr

Abstract: **The concept of using stem cells for cardiovascular repair holds great potential, but uncertainties in preclinical experiments must be addressed before their therapeutic application. Contemporary proteomic techniques can help to characterize cell preparations more thoroughly and identify some of the potential causes that may lead to a high failure rate in clinical trials. The first part of this review discusses the broader application of proteomics to stem cell research by providing an overview of the main proteomic technologies and how they might help the translation of stem cell therapy. The second part focuses on the controversy about endothelial progenitor cells (EPCs) and raises cautionary flags for marker assignment and assessment of cell purity. A proteomics-led approach in early outgrowth EPCs has already raised the awareness that markers used to define their endothelial potential may arise from an uptake of platelet proteins. A platelet microparticle–related transfer of endothelial characteristics to mononuclear cells can result in a misinterpretation of the assay. The necessity to perform counterstaining for platelet markers in this setting is not fully appreciated. Similarly, the presence of platelets and platelet microparticles is not taken into consideration when functional improvements are directly attributed to EPCs, whereas saline solutions or plain medium serve as controls. Thus, proteomics shed new light on the caveats of a common stem cell assay in cardiovascular research, which might explain some of the inconsistencies in the field. (*Circ Res*. 2011;108:499-511.)**

Key Words: angiogenesis ■ differentiation ■ platelets ■ progenitor cells ■ proteomics ■ stem cells

Driven by the promise of regenerating cardiac muscle and inducing cardiac repair, stem cells have drawn tremendous interest in recent years. Bone marrow mononuclear cells are the most widely studied cell type to date. Although injection of bone marrow mononuclear cells into coronary arteries appears to be safe and may have some moderate benefits particularly in patients with adverse cardiac remodeling, their ability to create or salvage heart muscle was less than expected, and the mixed results reported in clinical trials have not matched initial hopes (as reviewed elsewhere[1]). The therapeutic potential of other readily available and ethically acceptable stem cell sources, including the more recently described resident cardiac progenitors,[2,3] remains to be tested in clinical trials. Despite substantial advances in stem cell research, it is still unclear whether stem cells contribute to regeneration directly or by indirect effects, and there is a

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Non-standard Abbreviations and Acronyms	
2-DE	2-dimensional electrophoresis
CFU	endothelial colony-forming unit
DIGE	difference in-gel electrophoresis
EPC	endothelial progenitor cell
ESC	embryonic stem cell
iTRAQ	isobaric tagging for relative and absolute quantification
LC-MS/MS	liquid chromatography–tandem mass spectrometry
MP	microparticle
PMP	platelet microparticle
SILAC	stable isotope labeling of amino acids in culture
SMC	smooth muscle cell
SPC	smooth muscle progenitor cell
VEGF	vascular endothelial growth factor

serious concern that laboratory research is pushed prematurely into clinical practice.[4] The inconsistent results in clinical trials using unpurified bone marrow mononuclear cells have led to the notion that the assessment of cell number and viability may not entirely reflect the functional capacity of cells in vivo.[5,6] Additional characterization appears to be mandatory before embarking on clinical cell therapy trials, and a proteomics-led approach can help to provide new insights but requires an understanding of the technologies involved to ensure their successful application to stem cells.

Proteomics: General Considerations

In the last decade of the 20th century, genomics and the functional genomic sciences revolutionized medical research. The key discovery was that the genetic specification of a human being, once assumed to be of almost limitless complexity, consisted of just 20 000 protein-coding genes, surprisingly similar to much simpler organisms such as *Caenorhabditis elegans*.[7] Therefore, it is not the number of genes but the processing of the gene products that accounts for the biological complexity. Despite the sequencing of the genome, there remain many unknowns at the protein level. For 35% of the protein encoding genes in the human genome, there is currently no evidence for the existence of these proteins at the protein level. Also, there are ≈75 000 annotated posttranslational modifications, yet only half of them have been experimentally obtained. This shift in perspective led to the advance of "proteomic" sciences, and they are now beginning to influence cardiovascular research.[8,9] With conventional molecular biological approaches, studies on proteins are only conducted on a limited number of proteins. Proteomics aspires to define the totality of protein concentrations. Currently, it is impossible to resolve the entire complexity of the mammalian proteome. Proteomic technologies have advanced rapidly over recent years[10] and enable us to monitor at least a proportion of the thousands of proteins in mammalian cells.[11] For low-abundance proteins, such as plasma membrane proteins, transcription factors, etc, an enrichment step is essential. In this respect, whole-genome arrays constitute a more mature technological platform. Transcript levels, however, are merely an indication of the protein amount. Although protein and mRNA expression is fairly well correlated, there is no correlation between protein and mRNA half-life. Therefore, the transcriptome is not linearly proportional to the proteome and quantifying mRNA levels cannot suffice.[12] Another important consideration is that current research relies heavily on the use of antibodies. Antibody-based techniques only probe for known proteins with the potential caveats of nonspecific binding, epitope masking, and cross-reactivity with proteins from different species. In contrast, mass spectrometry is considered the gold standard for protein identification. It does not rely on a priori assumptions but provides an unbiased overview of protein expression. A mass spectrometry analysis, however, requires sufficient material and as yet cannot be applied to the single-cell level.[13] Neither does mass spectrometry reveal the spatial localization of the identified proteins unless different subproteomes are compared[14–16] or laser microdissection is used for protein collection.[17]

What Techniques for What Output?

In proteomics, the main existing methods are either gel-based (two-dimensional electrophoresis [2-DE], gel–liquid chromatography–tandem mass spectrometry [LC-MS/MS]) or gel-free (shotgun proteomics) (Figure 1). In gel-based proteomics, proteins are first separated by 2-DE or by SDS-PAGE before tryptic digestion and mass spectrometry. In shotgun proteomics, the protein mixture is directly digested with trypsin. Relative quantitation is either performed at the protein (2-DE) or at the peptide level (gel–LC-MS/MS and shotgun proteomics). 2-DE visualizes proteins as discrete gel spots, and spots with differential expression are subject to mass spectrometry analysis for protein identification. For many years, 2-DE has been the workhorse of proteomics.[18] By now, it is gradually being superseded by mass spectrometry–based quantitation techniques, yet it can still deliver important insights and is more readily implemented in most laboratories. In gel–LC-MS/MS, gel bands covering the entire lane are excised and analyzed by mass spectrometry. Without an isotope label, this approach does not allow multiplexing and requires more mass spectrometry time, but the gel–LC-MS/MS approach provides an in-depth characterization of the sample and an estimate of protein abundance when combined with label-free quantitation in simple mixtures. The most popular methods for isotopic labeling in shotgun proteomics are isobaric tagging for relative and absolute quantification (iTRAQ) and stable isotope labeling of amino acids in culture (SILAC).[19] Labeling with iTRAQ is performed at the peptide level. In contrast, SILAC labeling is performed at the protein level. It involves supplementing the culture medium with either the light or the heavy isoform of a particular amino acid. Samples are combined immediately after harvesting thereby minimizing experimental variation. Every method has its advantages and drawbacks. The right choice depends on the complexity of the sample and on the type of proteins to be analyzed.

Sensitivity

2-DE is biased to more abundant, soluble proteins. Very large, very small, and hydrophobic proteins are difficult to

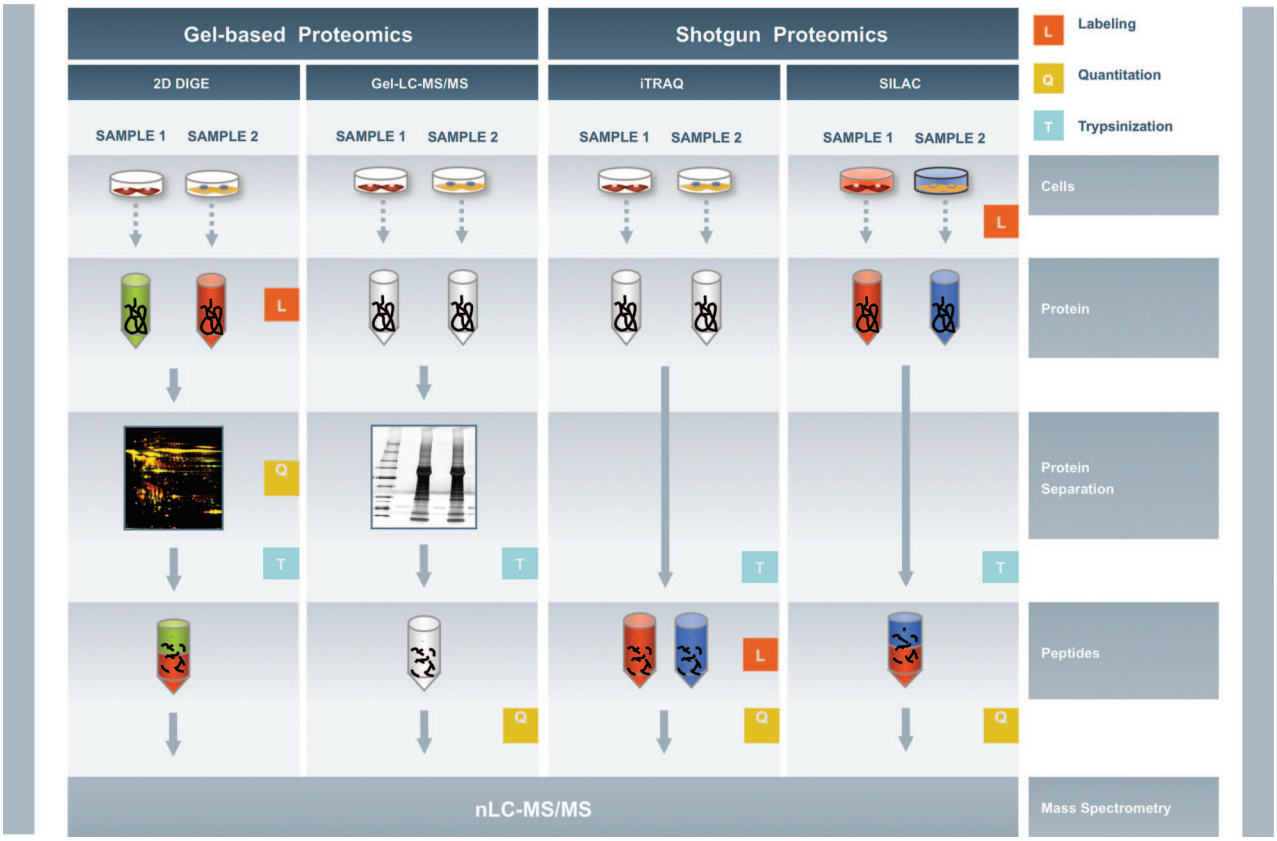

Figure 1. Overview of proteomic methods. The main proteomic methods are gel-based (2-DE, gel–LC-MS/MS) or gel-free (shotgun proteomics). In gel-based proteomics, proteins are first separated by 2-DE or by SDS-PAGE before enzymatic digestion (usually with trypsin) and mass spectrometry analysis. In shotgun proteomics, the protein mixture is directly digested without gel separation. Relative quantitation is either performed at the protein (2-DE) or at the peptide level (gel–LC-MS/MS and shotgun proteomics). At the center of any proteomic approach is the mass spectrometer. The current gold standard for mass spectrometry is nanoflow LC-MS/MS (nLC-MS/MS).

resolve. Therefore certain proteins, including membrane proteins, cytokines, transcription factors etc are underrepresented on 2-DE gels. Shotgun proteomics provides a more comprehensive proteome coverage than 2-DE gel-based approaches but shows its strength at the required sensitivity only in simplified cell extracts. In tissues and whole-cell lysates, shotgun proteomics is confronted with limitations on the dynamic range of detection. Because the peptides are selected for fragmentation based on their ion intensities, the more abundant peptides are more likely to be detected. If the proteome is not subfractionated, the complexity of the resulting peptide mixture can overwhelm the analytic capabilities of the mass spectrometer and interfere with quantitation. Previous quantitative comparisons applying shotgun proteomics to cardiac tissue had to exclude fractions containing myofilament proteins[20] to alleviate the severe dynamic range limitations stemming from the highly abundant contractile components.

Quantitation

The most quantitative 2-DE technique is difference in-gel electrophoresis (DIGE). DIGE involves fluorescent labeling of protein mixtures and reliably quantifies differences as low as 10% in protein expression. Importantly, there is no real limit in the number of replicates that can be compared. Alternative multiplex quantitative mass spectrometry-based approaches enable the simultaneous analysis of up to 8 samples (iTRAQ) or introduce isotope labels by metabolic labeling (SILAC). Although a single peptide can unambiguously identify a protein, multiple peptides of the same protein are required for quantitation. Thus, not all of the identified proteins are reliably quantified in a shotgun experiment. SILAC is considered the best technique to determine relative differences in peptide abundance, but a minimum of 5 population doublings is needed to achieve complete labeling, which limits its use for primary cells; also, metabolic labeling strategies in animals are expensive. iTRAQ can be applied to any specimen, but the labeling step occurs rather late in the proteomics work-flow and may introduce additional experimental variation. In contrast, label-free quantitation is inexpensive and provides an estimate of protein expression based on spectral counts or ion intensities of the identified peptides (Figure 2). With increasing sample complexity, label-free quantitation is subject to quantification errors because of ion suppression (known as matrix suppression), which arises when particular peptides preferentially ionize in a complex mixture. Thus, quantitative changes may be misrepresented as a result of matrix effects, causing either suppression (underestimation) or enhancement (overestimation) of other peptides.

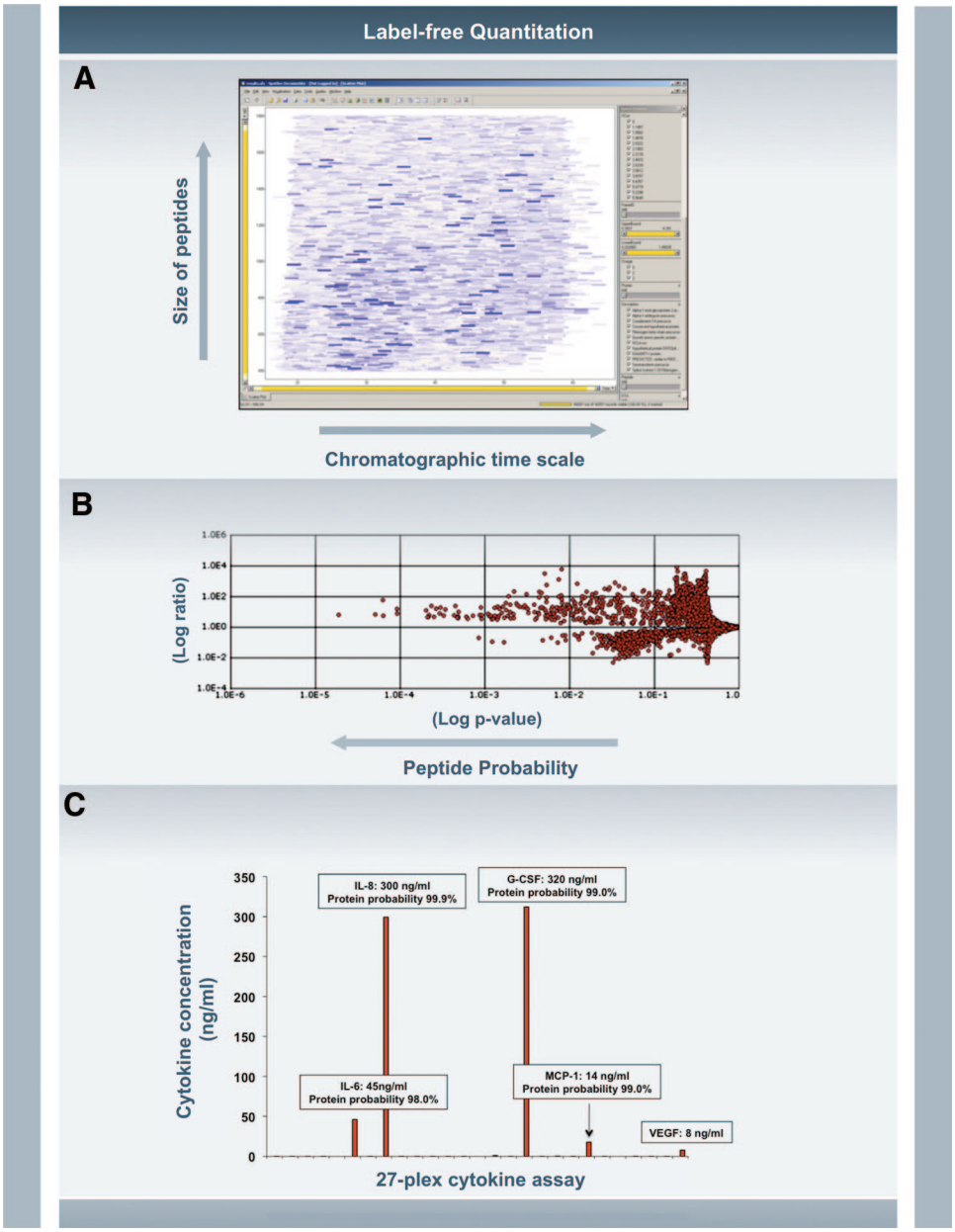

Figure 2. Label-free quantitation. A, By using the chromatographic time scale, size, and intensity of the eluting peptides, commercial software creates a multivariate data space, which consolidates large datasets of complex LC-MS/MS runs in a single view of differential expression. **Blue bars** represent individual peptide frames with different shades of color highlighting different levels of significance. **B,** Volcano plot shows the distribution of peptides according to levels of significance. **C,** The sensitivity of shotgun proteomics. A comparison of the proteomic data on conditioned medium from human aortic SMCs with measurements from a 27-plex cytokine assay revealed that except for VEGF, all cytokines present in the ng/mL range were identified with a protein probability of >95%.

Posttranslational Modifications and Protein Degradation

The gel-based separation depicts posttranslational modifications and protein degradation as a shift in isoelectric point (2-DE) or molecular weight (2-DE and SDS-PAGE). Shotgun proteomics does not provide a map of intact proteins. The available material is usually insufficient to obtain spectra of all modified peptides of a particular protein by mass spectrometry and information on posttranslational modifications can be lost.[10] This problem has been partially overcome by new enrichment methodologies, ie, for phosphopeptides, and technical advancements in mass spectrometry. An alternative peptide dissociation method, electron transfer dissociation, induces a softer peptide fragmentation process and preserves posttranslational modifications[21] that are labile in the customary fragmentation process (collision-induced dissociation). A major concern for the comparison of diseased tissues is protein degradation by enzymes other than trypsin, which will interfere with a quantitation based on tryptic peptides in shotgun proteomics. Hence, there can be a trade-off between sensitivity and quantitative accuracy.

Applications of Proteomics to Stem Cell Research

As described below, one can envision several possibilities how the advent of novel proteomics technology may help the translation of stem cell therapy.[22]

Assessment of Cell Homology

Proteomics can be used to compare cell similarity/diversity leading to the recognition of whether the protein content of stem cell–derived cells recapitulates the protein profiles of their mature counterparts. It is this concern with the whole proteome that distinguishes a proteomics approach from

traditional phenotyping using a selected panel of marker proteins. For example, comparative analysis of early outgrowth endothelial progenitor cell (EPCs), late outgrowth EPCs, monocytes and human umbilical vein endothelial cells by 2-DE, revealed that early EPCs are hematopoietic cells with a protein profile similar to monocytes, whereas the molecular fingerprint of late outgrowth EPCs corresponds to an endothelial phenotype.[23,24] Similarly, a 2-DE comparison showed that after platelet-derived growth factor-BB stimulation, the resident Sca-1 stem cell population from the vasculature shared the proteomic characteristics of a mature aortic smooth muscle cell (SMC) phenotype.[25] In contrast, the proteome of smooth muscle-like cells derived from Sca-1$^+$ progenitors of embryonic stem cells (ESCs) was clearly distinct from aortic SMCs, though the cells expressed a similar panel of smooth muscle markers.[26] Generally, there is a need for new markers that are not only able to assess the stage of the stem cell differentiation process, but to distinguish between mature and stem cell–derived cells. Finally, a DIGE comparison of endothelial colony-forming units (CFUs) and early-outgrowth EPCs identified thymidine phosphorylase to be among the main proangiogenic factors.[27] Thymidine phosphorylase is an intracellular enzyme and highly expressed in certain tumor cells. The proposed mechanism of action is not clear, but it has been suggested that the protective effect of thymidine phosphorylase is mediated by the product of its catalytic reaction (2-deoxy-D-ribose-phosphate). This is in agreement with previous observations that supplementation of this metabolite or thymidine phosphorylase-expressing tumor cells induce endothelial chemotaxis.[28,29]

Identification of Cell Surface Proteins

Many of the accepted markers for the identification and differentiation of stem cells are transcription factors and intracellular proteins. Surface markers are a prerequisite for selecting undifferentiated and differentiated stem cells. By identifying candidate proteins that allow purification of specific cell lineages from live heterogeneous populations of differentiated cells, proteomics would address a clinically relevant need for the translation of stem cells to therapies. Alas, the physiochemical characteristics of membrane proteins render analysis by proteomics challenging and high abundant intracellular proteins hamper the identification and quantification of membrane proteins present in low copy numbers. Additional enrichment steps, ie, by using biotin/avidin labeling of surface proteins[30] or exploiting the fact that a majority of the cell surface proteins are glycosylated,[31] reduce sample complexity and facilitate the identification of membrane proteins by mass spectrometry. Previous proteomic analysis conducted on plasma membrane proteins of mouse embryonic stem cells established a reference catalogue of cell surface proteins expressed on undifferentiated mouse ESCs[32] and differentially expressed during early differentiation.[33] In a recent study, van Hoof et al applied a quantitative SILAC proteomics approach to compare the cell surface proteins of human ESC-derived cardiomyocytes and identified elastin microfibril interfacer 2 as a marker to sort stem cell–derived cardiomyocytes.[34] Similarly, Gundry et al revealed new targets for the characterization of cell intermediates during skeletal myoblast differentiation into myotubes by using a proteomic approach capturing N-linked glycoproteins.[35] In another study by Dormeyer et al,[36] the membrane proteome of human ESCs was analyzed using only 500 000 cells. The method involved an optimized digestion protocol that included a step of carbonate extraction and enzymatic deglycosylation. Although this is not as specific as other membrane purification procedures, 237 plasma membrane proteins could be identified in the human ESC line HUES-7. We have adopted an alternative method and obtained microparticles (MPs) from supernatants of EPC cultures.[37] MPs usually refer to intact vesicles formed from the plasma membrane and can be repeatedly harvested from the same cell culture. This is particularly advantageous for scarce cell populations. MPs have heterogeneous density and size (0.1 to 1.0 μm), are easily separated by differential centrifugation, and originate from many cell types, including endothelial cells, platelets, monocytes, and SMCs.[38,39] The protein composition of MPs has limited complexity and is highly enriched in membrane proteins but can vary dependent on the stimulus. Thus, the membrane protein content in MPs may only be partially representative of the plasma membrane protein profile.

Identification of Paracrine Factors

Free and protein-bound secreted factors are distributed throughout the extracellular and intracellular environments. Modulation of the homeostasis among growth factors, hormones, proteases and extracellular matrix molecules, cell-cell interactions and intracellular compartments is critical in directing the differentiation of stem cells and the formation of tissue-like structures. Little is currently known about the extracellular milieu produced by different stem cell populations. Proteomics is the method of choice for a large-scale analysis of protein secretion.[40] However, many cell types require serum supplements for their survival. Protein concentrations in serum span 9 of magnitude in linear dynamic range.[41] Current proteomic technologies resolve 4 to 5 orders of magnitude. Consequently, they array secreted factors at the required sensitivity only in serum-free medium. Otherwise, classic serum proteins mask the less abundant proteins. To minimize cross-contamination with bovine proteins, the cells have to be washed extensively with plain medium before the secreted factors are sampled. Despite these efforts to ensure that the collected conditioned medium contains no other extraneous proteins, except for the secreted or shed proteins, the cross-contamination with bovine proteins can vary depending on the cell type. Endothelial cells, for example, show a substantial carryover of serum albumin. In a shotgun proteomics analysis of the secretome from human umbilical vein endothelial cells, 12% to 15% of all identified spectra corresponded to albumin peptides.[42] In contrast, SMCs tolerate serum starvation well and can be kept in serum-free medium for longer. In this setting, a shotgun proteomics strategy was able to mine deeper into the secretome and detected all cytokines present at concentrations $>$10 ng/mL (of 27 tested in a multiplex assay). Only vascular endothelial growth factor (VEGF) at 8 ng/mL was not identified (Figure

2C). Similarly, a secretome analysis confirmed high levels of matrix metalloproteinase 9, interleukin-8, and cathepsins in endothelial CFUs,[27,43] previously described as characteristics of early EPCs.[44] To increase the depth of proteomic profiling, Bendall et al designed an approach to strategically identify low-abundance stem cell regulatory proteins of the human ESC secretome. By applying a MS-based proteomic method called iterative exclusion, the group successfully identified previously undetectable growth factors, present at concentrations ranging from 10^{-9} to 10^{-11} g/mL.[11] Another variable is the type of matrix the cells produce. In a recent study,[45] we compared the secretome of smooth muscle progenitors (SPCs) with human aortic SMCs, revealing a substantial overlap among the matrix proteins identified. SPCs, however, selectively retained certain proteins from bovine serum, including pigment epithelium-derived factor, a potent inhibitor of angiogenesis that binds to newly formed collagen and counters the effects of VEGF.[46] Pigment epithelium-derived factor was identified as bovine protein by mass spectrometry in the absence of corresponding mRNA expression in SPCs. Consistent with this finding, SPCs showed reduced invasive capacity and unlike EPCs, their conditioned medium had no angiogenic activity.

Mechanisms of Stem Cell Differentiation

For stem cell–based therapies, it is essential that we gain knowledge on the molecular mechanisms controlling differentiation toward the cardiovascular lineage. The processes of stem cell renewal and differentiation are controlled by intrinsic factors regulated by extrinsic signals, whereby receptors act as transducers of these signals. Proteomics can be used to dissect the mechanisms regulating the proteome of stem cells during self-renewal and commitment to the cardiovascular lineage. Of the different cellular subproteomes, those embedded in the plasma membrane have been of substantial interest as they regulate key biological functions such as cell-to-cell and cell–matrix interactions, transport, and signal reception/transduction. Signaling pathways governing differentiation are controlled by environmental cues, ie, the binding of secreted ligands to membrane receptors. A proteomic approach targeting plasma membrane receptors as well as protein secretion may unravel key mechanisms regulating cardiovascular differentiation. A prerequisite is that stem cells can be expanded to obtain sufficient material for proteomic analysis. Unfortunately, many adult stem cells are scarce. Considering this, our group, like many others,[47,48] opted to work with ESCs to understand molecular mechanisms determining their commitment to the cardiovascular lineage. For instance, Behfar et al used proteomic screens to decipher cardiogenic instructive signals in mouse ESCs that induced the expression and nuclear translocation of cardiac transcription factors.[49] Similarly, Williamson et al revealed the posttranscriptional regulation of mesoderm differentiation to endothelial and hematopoietic precursors, the hemangioblasts, in mouse ESCs.[50] The largest proteome reported in mouse ESC to date was published by Graumann et al.[51] In total, more than 5000 proteins were identified by combining gel-LC-MS/MS and shotgun proteomics with isoelectric focusing of tryptic peptides for prefractionation. The coverage in both methods was comparable and contained key stem cell markers. Importantly, murine ES cells could be fully SILAC-labeled when grown feeder-free during the last phase of cell culture. Of course, large quantities of cells are required to achieve such coverage, ie, up to 10 million. A recently developed proteomics sample processing and analysis platform, termed rare cell proteomic reactor, helped to substantially reduce cell numbers: with this method, as little as 50 000 human ESCs were sufficient to identify more than 2000 unique proteins and quantify significant changes during early mesoderm development.[52] Finally, a phosphoproteomic analysis in human ESCs revealed >10 000 unique phosphorylation sites,[53] among which 5 were localized to Oct4 and Sox2. These 2 transcription factors are known to be important for stem cell pluripotency and to play a critical role in reprogramming adult cell lines to an ESC state (induced pluripotent [iPS] cells).[54]

Controversy of EPCs: Proteomics Provides New Insights

EPCs were first described in 1997 by Asahara and colleagues who showed that purified $CD34^+$/KDR $(VEGFR2)^+$ mononuclear cells from adults can differentiate ex vivo to an endothelial phenotype.[55] This seminal study did not directly test whether these cells also have in vivo vessel forming ability, but subsequent studies showed that EPCs contribute to the recovery of the ischemic cardiac tissue and were proposed as a therapeutic option to rescue tissue after ischemia.[56] By now, a PubMed search on EPCs returns thousands of publications.[57] Despite the immense explosion of interest in this area, there is neither a standard definition nor an accepted methodology for their enumeration.[58] In flow cytometric analysis, CD34, CD133, and KDR are commonly used, but it is unclear whether $CD34^+$, $CD133^+$, and KDR^+ cells represent endothelial precursors or are primitive hematopoietic progenitors.[59,60] Also, circulating EPCs defined by these criteria are extremely rare and difficult to quantify, as highlighted by a methodological comparison of different flow cytometric approaches.[61] In vitro culture methods were introduced as an alternative approach: the endothelial CFU assay was proposed as a surrogate measurement for the number of circulating EPCs in clinical studies,[43] and the culture of early outgrowth EPCs was used as an in vitro expansion method to obtain sufficient cell numbers for mechanistic experiments.[62] In both methods, EPCs were isolated from mononuclear cells by density barrier centrifugation (Lymphoprep, Ficoll, etc) because it is a fast and "stress"-free equilibrium method, while the cell-sorting step for $CD34^+$ KDR^+ cells was eliminated. Therefore, different cell types were assessed in vitro and in vivo[63–65] and at present, there are no specific markers, which unambiguously identify EPCs.[66–68] Subsequent studies challenged the assertion that CFUs and early outgrowth EPCs are bona fide EPCs. Clonal analysis performed by Yoder et al[69] revealed that CFUs are derived from the hematopoietic system, possess myeloid progenitor cell activity, and differentiate into phagocytic macrophages. Rohde et al demonstrated that CFUs formed as a result of a functional cross between T cells and monocytes.[70,71] By now, findings from different groups converged showing that early

outgrowth EPCs fail to incorporate in the vasculature and do not differentiate to endothelial cells (as reviewed elsewhere[72]), but that blood monocytes mimic EPCs and mediate an angiogenic effect in a paracrine manner.[73,74] Yet, it remained unclear how these cells acquire endothelial characteristics and promote angiogenesis.

Proteomics helped to shed new light on the caveats of this common stem cell assay in cardiovascular research: in a proteomics analysis of MPs in the conditioned medium of early outgrowth EPCs, the platelet-specific integrin α-IIb emerged as the most abundant integrin.[37] Conventional methods for isolating mononuclear leukocytes (lymphocytes, monocytes, and natural killer cells) using density barrier centrifugation deplete erythrocytes and granulocytes (mainly neutrophils), but a platelet contamination is commonplace[37,75] and varies depending on the stringency of the washing steps. Most investigators are either unaware of the presence of platelets in EPC cultures or assume that the platelet contamination is of minor importance as the platelets disappear within few days of culture. The platelets, however, just disintegrate into smaller platelet (P)MPs, which are subsequently incorporated by the adherent leukocyte population. Generally, platelets are not considered while performing phenotypic analysis of EPCs. Platelets and PMPs bind *Ulex europaeus* agglutinin (UEA)-1 and the uptake of PMP by the adherent mononuclear cell population can result in a transfer of the "endothelial" markers CD31 and von Willebrand factor. As macrophages also incorporate acetylated low-density lipoprotein,[76] studies evaluating EPCs based on acetylated low-density lipoprotein uptake and UEA-1 binding are not reliable. Similarly, cells staining double positive for hematopoietic and endothelial markers may not be EPCs.[77] Although PMP-induced transfer of marker proteins is unlikely to permanently change a marker expression profile, a PMP uptake is noticeable between day 3 and day 7 of culture (Figure 3A), when most investigators are evaluating the outgrowth of EPCs by immunostaining. Addition of an immunophenotypic marker for platelet proteins would be a prudent measure to avoid misinterpretations of immunolabeling for CD31 and von Willebrand factor. Coincubation of platelets and peripheral blood mononuclear cells dose-dependently increased the number of adherent EPCs.[78] In a large population-based study, platelet and monocyte counts emerged as a positive predictor for the number of CFUs and early outgrowth EPCs.[37] These findings constitute a paradigm shift from the original definition of an EPC phenotype[77] and provide an explanation for the misinterpretation of their cellular progeny.

Furthermore, early outgrowth EPCs and their conditioned media were used for functional experiments, while saline injections or plain medium served as controls. PMPs may, at least partially, be responsible for the observed angiogenic effects. Soluble factors released by EPCs have been previously analyzed using microarrays,[79] but a contamination with platelet proteins would have gone unnoticed, demonstrating the advantages of a proteomics approach. It is well established that platelets and PMPs bind to monocytes/macrophages[80] (Figure 3B) and increase their adhesiveness. In this respect, it is not surprising that platelets and PMPs have a similar effect on EPCs.[81,82] Platelets also contain a range of proangiogenic growth factors, including VEGF, and PMPs are potent inducers of angiogenesis.[83] Meanwhile, it has been shown that MPs contribute to the activation of an angiogenic program in EPCs,[84] that the depletion of MPs reduces the angiogenic activity of their conditioned medium[37] and that PMPs enhance the potential of EPCs to restore endothelial integrity after vascular injury.[82] In the latter study, the effect of PMPs alone was not evaluated in vivo.[82] In a rat model of

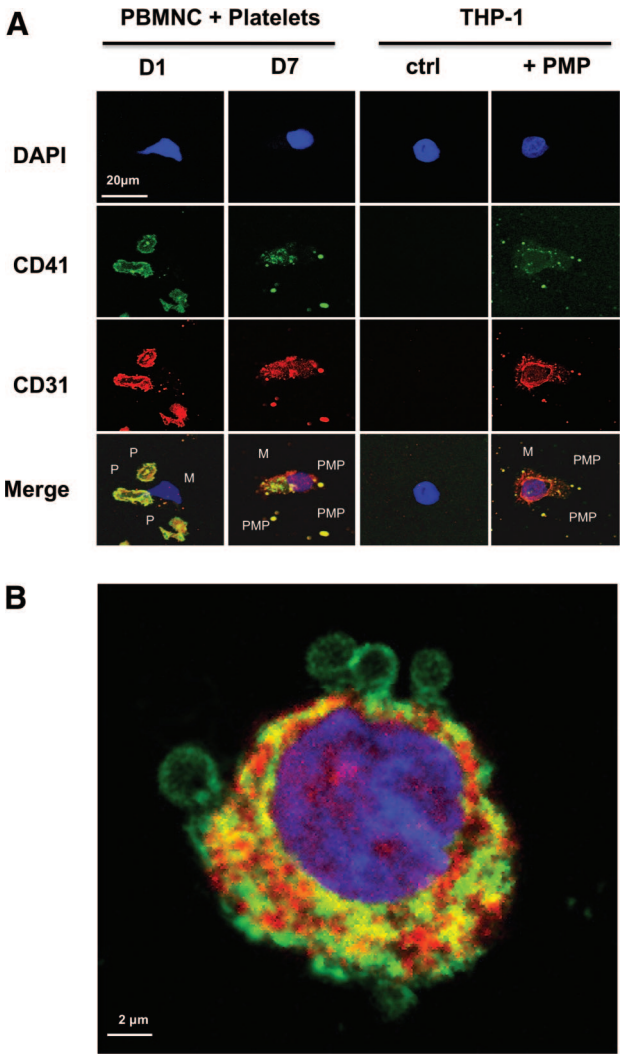

Figure 3. Platelets: a sticky problem for EPCs? A, Intact platelets stain positive for integrin α-IIb (CD41) among peripheral blood mononuclear cells (PBMNCs) counterstained with DAPI on day 1 (D1) of coculture. Platelet proteins are taken up by the adherent cell population and remain detectable in PBMNCs at day 7 (D7) of culture. These findings can be replicated by using PMPs and the monocytic THP-1 cell line. After 2 days, THP-1 cells incorporate PMPs as indicated by CD41/CD31 double-positive staining. Therefore, monocytic cells may have been "masquerading" as EPCs because of contaminating platelets and PMPs. **B,** Platelets and PMPs, known for their role in coagulation, stick to monocytes and exchange membrane components. The image shows this interaction: the platelet surface is stained in **green**, the THP-1 monocyte is stained in **red**, and the **green color** diffuses into the THP-1 monocyte. Some of the membrane markers currently used to identify EPCs, such as CD31 and von Willebrand factor, are not unique to EPCs but also present in platelets.

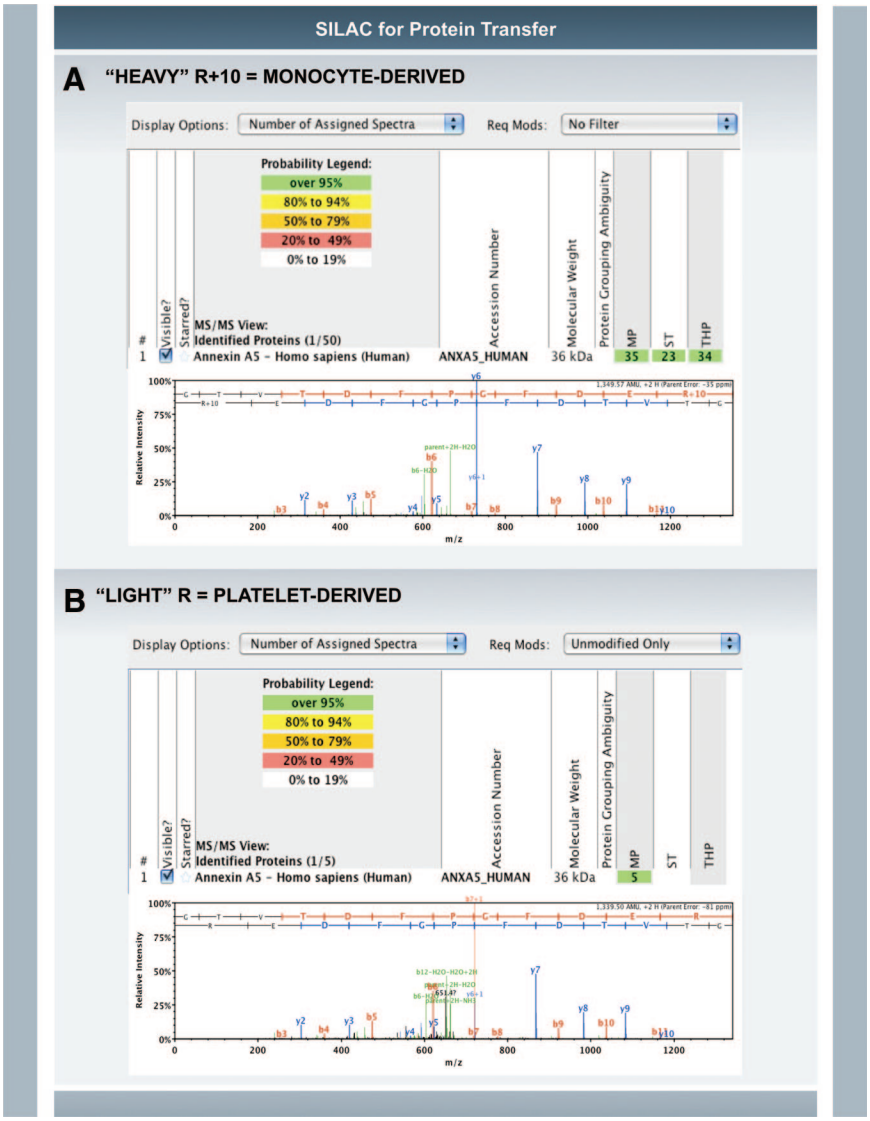

Figure 4. SILAC to study protein transfer. THP-1 monocytes labeled with "heavy" arginine and lysine were incubated with unlabeled "light" PMPs. Untreated THP-1 cells and THP-1 cells treated with PMP-free platelet supernatant served as controls. Despite a similar number of total spectra assigned to annexin A5 **(A)**, unmodified (light) peptides were only identified in THP-1 cells treated with PMPs **(B)**. In the cellular proteome of PMP-treated THP-1 monocytes, 5 of 35 spectra for annexin A5 were platelet-derived, confirming a substantial uptake of PMP proteins. Spectra of a heavy (Arg+10 [R+10]) and a light peptide (Arg [R]) of annexin A5, as identified by LC-MS/MS are shown in the **bottom half of A and B**, respectively. MP indicates THP-1 cells incubated with PMPs; ST, THP-1 cells incubated with PMP-free platelet supernatant; THP, untreated controls.

chronic myocardial ischemia, however, injections of PMPs were sufficient to stimulate postischemic revascularization in the myocardium.[85] In hindsight, functional improvements should not have been attributed to EPCs without an in-depth analysis of the protein content in their conditioned medium. The question whether EPCs are "stem cells of monocytic origin" or "angiogenic macrophages" is not solely semantic and it has been proposed that the term "progenitor" should be retired for early outgrowth EPCs without clonal proliferation and differentiation potential.[72,86] New names, such as circulating angiogenic cells, early angiogenic cells, early outgrowth cells, etc, are currently being introduced but cannot overcome the limitations of this coculture assay. As outlined by Hirschi et al,[86] more stringent criteria are needed for EPC studies and have already been implemented by an American Heart Association journal. There is no general consensus on how to define macrophage phenotypes and the distinction between M1 versus M2 macrophages is an overly simplistic representation of a very complex area of biology. In this respect, the recent findings demonstrating the transfer of mRNA[84] and proteins[82] from PMPs to mononuclear cells, including the chemokine receptor CXCR4, open exciting new possibilities of how platelets may alter macrophage function or influence their angiogenic activity.[67,68,87]

Of course, a protein transfer between cell types also represents an analytic challenge for proteomics. If protein containing-vesicles are taken up by recipient cells, the transferred proteins will not be distinguishable from the endogenous proteins as long as the proteins are expressed by both cells types. This can be addressed by adopting a SILAC approach as illustrated in Figure 4: THP-1 monocytes were SILAC-labeled for 5 population doublings until all endogenous proteins had a "heavy" arginine or lysine. Then, they were incubated with freshly isolated PMPs for 48 hours before their cellular proteome was separated by 2-DE and compared to untreated THP-1 cells. Platelet supernatant depleted of MPs was used as additional negative control to ensure that the observed effects in THP-1 cells are attributable to the MP fraction and not to soluble factors. Differentially expressed proteins were identified by LC-MS/MS. In this case, any nonlabeled/"light" peptides in the cellular proteome of THP-1 monocytes incubated with PMPs should

be platelet-derived. Indeed, the LC-MS/MS analysis of a 2-DE spot containing annexin A5 returned 35 spectra in total (Figure 4A), 5 of which had a "light" peptide confirming a substantial uptake of PMPs (Figure 4B). Thus, it is possible to discern protein exchange from protein expression and determine the cellular origin of proteins in cocultures by using metabolic labeling. Such insights can be obtained by proteomics and not with conventional antibody-based techniques.

Lessons to Be Learned for Stem Cell Research

The bulk of the cardiovascular stem cell literature is based on immunolabeling for marker proteins combined with functional improvements in animal models. Frequently, the detailed mechanisms of these effects remain elusive, and the correct interpretation of the findings relies on the validity of the assumptions described below.

Concept of Marker Proteins: Is Costaining Equivalent to Coexpression?

Stem cells in the cardiovascular system are classically assessed by costaining for a progenitor and a cardiac or vascular differentiation marker. In vitro, marker expression is confirmed at the transcript level, but in vivo studies predominantly rely on immunolabeling. The widely held view is that positive staining for marker proteins is consistent with gene expression, but there might be notable exceptions: in areas of tissue injury where cell death, platelet activation, and inflammatory cell infiltration occur, the possibility of a temporary exchange of antigens between cell types should be taken into consideration. Under these circumstances, the concept of costaining for marker proteins may not be reliable. If we reevaluate the stem cell literature bearing in mind that staining might occur without concurrent gene expression, it is evident that the expression of differentiation markers should be under increased scrutiny. For example, positive staining for CD31 or PECAM-1 (platelet endothelial cell adhesion molecule-1) is widely used to proof a conversion of hematopoietic stem cells into endothelial cells. However, CD31 is not specific for endothelial cells, but also present on platelets and to different degrees on leukocyte subtypes.[67,89] If injected stem cells incorporate platelet material, they could be masquerading as "stem cell-derived" endothelial cells. Similarly, the tie2 promoter has been used extensively to follow the fate of EPCs, but it is also expressed by different non-endothelial cell types, including a monocytic/macrophage cell fraction.[57] With respect to vascular SMCs, staining for smooth muscle actin is insufficient evidence for the differentiation of progenitor cells toward the smooth muscle lineage.[90] The reliance on such nonspecific markers results in an overestimation of bone marrow–derived cells.[91] In fact, a recent time-course analysis in a mouse model of femoral artery injury suggested that the contribution of bone-marrow derived cells to neointima formation is limited to a transient period of the inflammatory response.[92] There was also little evidence for a direct contribution of circulating EPCs to plaque endothelium in apoE-deficient mice.[93] Other studies used cocultures with neonatal rat cardiomyocytes to demonstrate differentiation of bone marrow stromal cells[94] and EPCs into cardiac phenotypes.[95] The conclusion that human EPCs transdifferentiate into functional cardiomyocytes was based on immunostaining for cardiomyocyte markers and the recording of cardiac action potentials.[95] The alternative explanations are that EPCs have incorporated cardiomyocyte markers and the action potentials were inadvertently recorded from neighboring cardiomyocytes or that some cardiomyocytes have incorporated cell material from EPCs. Indeed, several studies failed to detect permanent engraftment and transdifferentiation of transplanted bone marrow–derived hematopoietic stem cells.[96,97] Cell fusion of bone marrow–derived donor cells with recipient cardiomyocytes has been suggested as a potential mechanism,[98,99] but this is contested by others.[100] Notably, membrane vesicles could contribute to an exchange of marker proteins without classic cell fusion events. Therefore, caution should be exercised in the interpretation of immunolabeling, particularly in areas with tissue injury and in coculture systems.

Paracrine Effects: Can the Functional Improvements Be Attributed to Stem Cells?

Progenitor cells have repeatedly been implicated in cardiovascular tissue repair, yet the mechanisms by which they act remain unsettled. In clinical trials, the percentage of retained cells is small and the number of cells that can be delivered via the intracoronary route is limited because of the risk of microinfarction, aggravating rather than repairing the injury. Moreover, some methods of cell labeling for imaging, ie, iron particle-based MRI, have substantial limitations, ie, on death of the delivered cells, the particles can accumulate in macrophages and may not reflect stem cell fate. In view of the poor engraftment and survival rates for injected stem cells, the observed improvements in cardiac function after cell therapy must be explained by mechanisms other than stem cell differentiation. The pendulum was swinging to indirect effects on angiogenesis and functional regeneration of the heart.[101] Paracrine effects are a plausible explanation, but the question arises whether the observed improvements can be attributed to stem cells, if the cell preparation is a heterogeneous population? Arguably, the choice of appropriate controls for stem cell therapies is not trivial. Stem cells may need other cell types and a mixture of cells could be a more potent "biofactory" of paracrine factors than a purified stem cell population. Nonetheless, saline or plain medium are inadequate controls to establish whether the other cells in the mixture actually require the presence of stem cells and whether the paracrine factors are indeed stem cell–derived. By now, positive effects have been reported with many different cell preparations. Clearly, it is the major challenge facing cardiovascular cell therapy to identify the most suitable stem/progenitor cell type for transplantation,[102] but other cell types in unpurified cell preparations must not be ignored.

Concluding Remarks

The fascination with stem cells is derived from their unique capacity for self-renewal and capability of forming at least one, and sometimes many, specific cell types. The fundamental property of stem cells is that they can regenerate the functional capacity of organs by replacing degenerative or

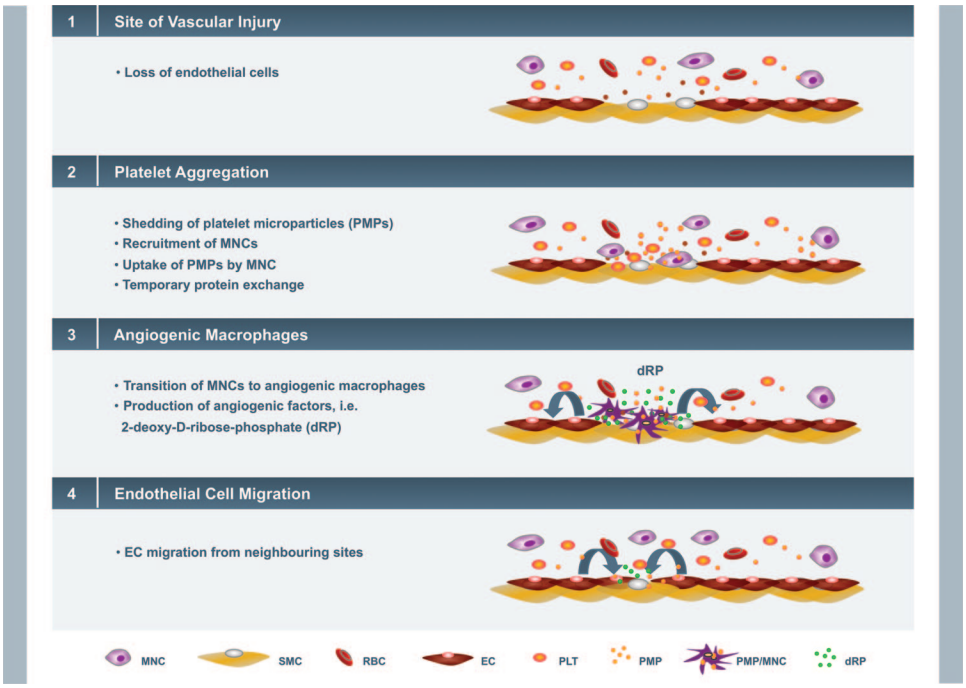

Figure 5. Revised working model for early outgrowth EPCs. Early outgrowth EPCs were supposed to be a type of stem cell that differentiates into endothelial cells and incorporates in the lining of blood vessels. Based on our proteomics analysis of EPC cultures and findings by many other investigators, a tentative model, where vascular repair may be initiated by PMPs that recruit monocytes (MNC) to areas of vascular injury, seems more likely. A PMP-mediated protein and mRNA exchange could alter monocyte function and/or promote the transition to a proangiogenic macrophage phenotype. Together, PMPs and angiogenic macrophages may facilitate the recruitment of neighboring endothelial cells. For example, thymidine phosphorylase (previously referred to as platelet-derived endothelial growth factor) was identified to be among the proangiogenic factors in EPC cultures. Thymidine phosphorylase produces 2-deoxy-D-ribose-phosphate (dRP), an angiogenic metabolite. The expression of thymidine phosphorylase by EPCs may promote endothelial migration to occur along a gradient toward the injury and stimulate wound healing. This alternative concept would help to reconcile the literature documenting beneficial effects of EPCs on cardiovascular function with the recent finding that early outgrowth EPCs are not genuine endothelial precursors and do not incorporate in the vasculature.

dying cells. The key deliverable of cardiac stem cell therapy is the generation of cardiac muscle to repair chronic scars. Long-term success is less likely, if the cells used for therapy have no cardiomyogenic potential but induce angiogenesis while the underlying condition remains a scarred ventricle. The initial concept of delivering bone marrow cells to the injured myocardium was, at least partially, based on the assumption that subpopulations, such as EPCs, can differentiate into cardiomyocytes and functionally regenerate the heart. Meanwhile, the consensus seems to emerge that the functional benefit of bone marrow cell therapy involves stem cell–mediated angiogenesis, not cardiomyogenesis.[81,88] Reports that some previous definitions of EPCs were not reliable and that PMPs contributed to their angiogenic activity, further challenge the concept of using unpurified bone marrow mononuclear cell preparations for therapy. Future studies will need to explore whether variations in platelets and especially in PMPs (which tend to be overlooked because of their small size[103]) can help to explain the inconsistent results in clinical trials.[104,105] After all, progenitor cell–based regenerative therapeutics are now commercially available to treat patients. If PMPs recruit or convert angiogenic monocytes (Figure 5), then the identification and administration of these active components in PMPs may overcome the need for a bone marrow cell-based therapy and ultimately result in novel cell-free therapeutic strategies. In an ironic twist, the very feature of platelet activation, the formation of PMPs that may allow for an enhanced vasoregeneration of EPCs,[82] is inhibited by antiplatelet drugs, and one might consider if a more tailored antiplatelet therapy could preserve some of these beneficial effects of PMPs in promoting tissue repair. Regardless, EPCs and cell therapy are likely to be subject to ongoing controversy in cardiovascular research.[106] Although proteomics cannot be the method of choice for routine quality control, especially given the inherent problems of low stem cell numbers in clinical samples, proteomic technologies are an important research tool that can help to solve some of the fundamental problems that are plaguing the cell therapy field. By identifying surface proteins and defining stem cell–specific markers and secreted factors, proteomics may have a clinical impact for developing new methods for better cell sorting and cell characterization. Ultimately, routine testing of stem cell functionality needs to be done with an easy and inexpensive method that can be performed with very low cell numbers.

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