MULTICELLULARITY IN THE CARDIOVASCULAR SYSTEM

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MULTICELLULARITY IN THE CARDIOVASCULAR SYSTEM

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EDITORIAL
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Editorial: Multicellularity in the Cardiovascular System

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Editorial on the Research Topic

Multicellularity in the Cardiovascular System

Many cell types exist within the heart and vessels and play key roles in the physiological function of the cardiovascular system. It is unsurprising therefore that successful regeneration of cardiovascular tissues after injury also require the controlled interaction of these cell types. While many functions of non-cardiomyocytes (endothelial cells, smooth muscle cells, pericytes, fibroblasts, and immune cells) in the heart or non-endothelial stromal cells (smooth muscle cells, pericytes, and fibroblasts) in the vessel wall have been clarified *in vivo*, the role of multicellularity in creating physiologically relevant tissues remains mostly unknown. Several factors should be addressed in engineered tissues including humoral signals, cell-to-cell interactions, and matrix materials assessed for their effects on cell survival and enabling cell-to-tissue organization. The purpose of this Research Topic is to provide our readers with the most recent ideas relating to the behavior of these multiple cell types in the cardiovascular system.

There is a growing interest in creating 3-dimensional microphysiological models of the heart and vessels as tool to understand significance of the various cellular and extracellular components. By recapitulating the complex microenvironment that exists in the native tissues, cardiovascular microphysiological systems are proposed as new platforms that could bridge the gap between currently available models and the human body. It is believed that beyond facilitating therapeutic tissue engineering, these systems will enable new insights into tissue morphogenesis, pathogenesis, and drug-induced structural and functional remodeling. Perbellini et al. provide an extensive review of the different components of the heart and their structural, functional, and molecular organization. Zamani et al. report current approaches to create 3D scaffold containing cardiac cells and supporting cells, including bioprinting and organ-on-a-chip echnologies. Tsifaki et al. give a thought-provoking summary on reprogramming approaches toward the cardiovascular regeneration. They also show how to generate a multicellular 3D *ex vivo* model by using these cell products to improve our understanding.

Within this Research Topic, we also collected *in vitro* strategies for physiologically and therapeutically relevant multicellular cardiovascular systems, including stem cell-based approaches. As an example from one of us, Sweeney et al. give an overview how interruption of the interaction between endothelial cells and perivascular cells causes a great variety of genetic and acquired vascular disease. We also gain insight here how novel approaches like derivatives of induced pluripotent stem cells in co-culture can provide potential therapeutic options.

Endothelial cells and cardiomyocytes are the main contributors of the heart multicellularity. The mini-review from Talman and Kivelä describes links between endothelial cells and cardiomyocytes, which uses several means to establish a functional cross-talk instrumental to normal homeostasis and adaptation to stress. Interestingly, the authors report conditions where promotion of angiogenesis are followed by cardiac remodeling. Likewise, cardiomyocyte hypertrophy may result in local ischemia if not accompanied by a parallel increase in surrounding capillaries.

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The list of factors implicated in this cross-talk comprise cytokines, growth factors, and genetic material. With increasing recognition of cell heterogeneity, it would be relevant to determine if different type of endothelial cells are present in the coronary vascular bed, exerting differential influences according to their topographic (endocardial-epicardial), phenotypic (arterial-venular), expressional, and functional characteristics. This followed by an important paper from Gomez et al. who summarizes cross-talk between cardiomyocytes and immune cells, namely resident and circulating macrophages. Importantly, the article provides a deep insight into the heterogeneity and flexibility of macrophages in response to cardiac injury. This reflects a persisting difficulty in establishing therapies able to block the negative actions of macrophages while preserving the positive actions.

The complex mechanisms of cardiovascular cell-to-cell communications is further showcased by Ontoria-Oviedo et al. They present a significant paracrine crosstalk between cardiomyocyte-derived extracellular vesicles, fibroblasts and endothelial cells under physiological as well as in cardiac remodeling after an ischemic insult. Massaia et al. go further down to individual cell level and explore the advantage of single cell gene expression technologies for studies on cardiovascular development and disease models. The approach unravels heterogeneities, identifies new cell subgroups and helps in organizing cellular hierarchies. The image that emerges from this nice review is different from the views that were widely accepted a few years ago. The most noteworthy change has been the recognition the dynamic nature of molecular interrelationships between cell populations within the heart. This can lead to particularly important findings when investigating spatiotemporal lineage diversification during mammalian development. Along these lines, Malandraki-Miller et al. discuss the importance of metabolic adaptation and the role of substrates in medium during cardiac differentiation process of stem cells and during cell transplantation into the heart. Regenerative cardiology is a large and complex field, we hope that readers of this series of papers in the Frontiers of Cardiovascular Medicine will benefit from several sources of information on current advances and future directions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Multicellular Interactions in 3D Engineered Myocardial Tissue

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Cardiovascular disease is a leading cause of death in the US and many countries worldwide. Current cell-based clinical trials to restore cardiomyocyte (CM) health by local delivery of cells have shown only moderate benefit in improving cardiac pumping capacity. CMs have highly organized physiological structure and interact dynamically with non-CM populations, including endothelial cells and fibroblasts. Within engineered myocardial tissue, non-CM populations play an important role in CM survival and function, in part by secreting paracrine factors and cell-cell interactions. In this review, we summarize the progress of engineering myocardial tissue with pre-formed physiological multicellular organization, and present the challenges toward clinical translation.

Keywords: engineered myocardium, cardiovascular tissue engineering, co-culture, cardiomyocyte, endothelial cell, fibroblast, stem cell

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INTRODUCTION

The myocardium is a highly organized complex organ with multicellular structure. Besides the contractile cardiomyocytes (CMs), other important non-myocyte (non-CM) populations include endothelial cells (ECs), fibroblasts (FBs), vascular smooth muscle cells (SMCs), neuronal cells, and immune cells (1). In general, myocardial tissue is composed of 30–40% CMs and 60–70% non-CMs, although the cell population ratio varies regionally in the myocardium and across various species, as well as during cardiac development (2–5). Each cellular component has a distinct role in cardiac function. CMs mainly contribute to the conduction of electrical impulses and contraction, while non-CMs are responsible for vascularization, secretion of extracellular matrix (ECM) components and responding to myocardial injuries caused by oxygen depletion during ischemic heart diseases such as myocardial infarction (2). Apart from their individual functions, CMs and non-CMs interact with one another to regulate cellular organization, differentiation, viability and function. By mediating the production and transmission of the electrical, biochemical and mechanical signals in the cardiac microenvironment, non-CMs can regulate the function of CMs (2). The intercommunication among CMs and non-CMs is mediated through cell-cell interactions, soluble signaling proteins secreted by neighboring cells known as paracrine factors, and ECM-mediated crosstalk (6). Understanding the multicellular interactions in myocardial tissue is critically important, not only to provide insight into healthy and diseased cardiac microenvironments, but also to enable the development of cardio-mimetic tissue culture systems for cardiovascular regeneration applications. This review presents the recent advances in the development of functional cardiac tissue, with emphasis on the heterocellularity of the engineered constructs, toward resembling the multicellular structure of the myocardial environment (**Figure 1**). The importance of the cellular components as well as the physicochemical properties of the ECM in recreating native heart-like tissue are explored.

IN VITRO CO-CULTURE SYSTEMS

Recent *in vitro* studies on generation of implantable cardio-mimetic units have shown great potential for cardiac regeneration, by developing engineered constructs with cellular, extracellular and biomolecular composition resembling native heart tissue. With respect to the cellular composition, various CM and non-CM cell types with different origin, age and activation/differentiation state have been employed for developing cardio-mimetic tissue constructs (7). Due to difficulties associated with long-term maintenance of adult CMs *in vitro*, neonatal CMs, embryonic stem cell-derived CMs (ESC-CMs) and induced pluripotent stem cell-derived CMs (iPSC-CMs) have been extensively explored in cardiac regeneration (8, 9). The use of these less differentiated cell sources signifies the importance of developing a multicellular microenvironment *in vitro*, in which the interactions with non-CM cells can improve the maturity of CMs in the culture system. Here we describe the progress in recreating the cardiac heterocellular microenvironment using CM-EC and CM-FB co-culture systems, and the importance of non-CMs in recapitulating a more biologically relevant microenvironment. We also present an overview of stem cell-based approaches toward developing multicellular tissue constructs, using stem cell-derived CMs/ECs/FBs. Although other cell types like neural cells and immune cells are important in controlling the CM contractility and hypertrophy, these cell types are discussed in greater detail elsewhere (10, 11).

Fibroblasts (FBs)

FBs comprise the major non-CM cellular composition in the adult heart, where each CM is bordered by at least one FB (5, 12, 13). FBs play an essential role in maintaining myocardial structure and mediating CM function by depositing ECM components (6, 14), secreting soluble paracrine factors such as platelet-derived growth factor-β (PDGF-β) and basic fibroblast growth factor (bFGF), and propagating electrical signaling through gap junction proteins (e.g., connexin-43), in cell-cell contacts (15, 16). FBs can transmit electrical impulses between CMs with over 100 μm distance through intercellular gap junctions, contributing in synchronized CM contractions (15, 17, 18). Under the ischemic conditions, the inflammatory mediators such as transforming growth factor-β may induce ECM synthesis by FBs, resulting in cardiac fibrosis (11).

It has been demonstrated that CM-FB co-culture can preserve the polarized morphology of CMs and induce the long-term expression of CM gap junction protein connexin-43 that is responsible for CM-FB heterocellular coupling, resulting in long-term synchronized contraction of engineered cardiac tissue (17). There is evidence that FBs can also modulate the biological properties of CMs through the secretion of various paracrine factors, without direct cell-cell contact. For example, secretion of periostin by FBs has been shown to induce the proliferation of CMs both *in vitro* and *in vivo* (19). Soluble vascular cell adhesion molecule-1 (VCAM-1) secreted by cardiac FBs induced proliferation of mouse ESC-CMs, resulting in higher number of contractile cells and better propagation of extracellular electrical impulses (20). In addition to cell-cell contact signaling and paracrine factoring, the ECM components (e.g., fibronectin, elastin, and glycoproteins) and matrix metalloproteinases (MMPs) produced by FBs also work interdependently to regulate ECM homeostasis *in vivo*, with a determining role in cardiac remodeling and scar tissue formation after myocardial infarction (21, 22). Although CM-FB co-culture can improve CMs contractility and function, the abundance of FBs needs to be optimized, since increasing the FB population may impair CM maturation and reduce cardiac conduction speed, action potential propagation and membrane resting potential by the secretion of paracrine factors (23, 24).

Endothelial Cells (ECs)

As a highly metabolic cell type, CMs reside within 2–3 μm distance from nearest capillary (25, 26). CMs require ECs in the capillary network to provide oxygen and nutrients. ECs have a pivotal role in protecting CMs against ischemic injuries by secreting neuregulin and PDGF-β and reducing fibrotic tissue formation after myocardial infarction (27, 28). An important goal of CM-EC co-culture is to create a vascularized cardio-mimetic tissue. ECs have also shown to be a dynamic mediator of CM spatial organization, contraction, survival and function in a complex heterocellular physiological platform (26, 29–31). ECs in co-culture can promote neovascularization by forming cellular networks within the engineered tissue unit and producing angiogenic cytokines such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF), which can eventually enhance the capillary density of the implanted endothelialized cardiac tissue *in vivo* (27). ECs can also interact with CMs through autocrine and paracrine signaling factors, such as neuregulin and PDGF-β to enhance CM survival (28, 29, 31), and nitric oxide and endothelin-1 to improve CM contractility (26, 29, 32). Besides paracrine signaling, ECs direct spatial organization of the CMs through cell-cell contact. CMs formed interconnected organized structures along capillary-like network in the co-culture system, but this organization was not observed when CMs were cultured with EC-conditioned medium (29).

Pluripotent Stem Cell-Derived CMs/FBs/ECs

Recent advances in efficient differentiation of pluripotent stem cells to cardiac cells represented a new unlimited source of cells for developing cardiac tissues, particularly due to the limited availability of donor primary CMs. However, the survival rate, maturation, and function of these less differentiated cells are highly dependent on the biochemical, mechanical and topographical properties of their microenvironment. The co-culture of both embryonic and induced pluripotent stem cells-derived CMs (ESC-CMs and iPSC-CMs) with non-CMs of different sources demonstrated to preserve the CMs phenotype and function through cell-cell contact and biochemical cues (33–35). The scaffold-free patches created from human ESC-CMs, ESC-ECs and fibroblasts exhibited electrically-paced contraction and more myocardially-relevant passive mechanical properties,

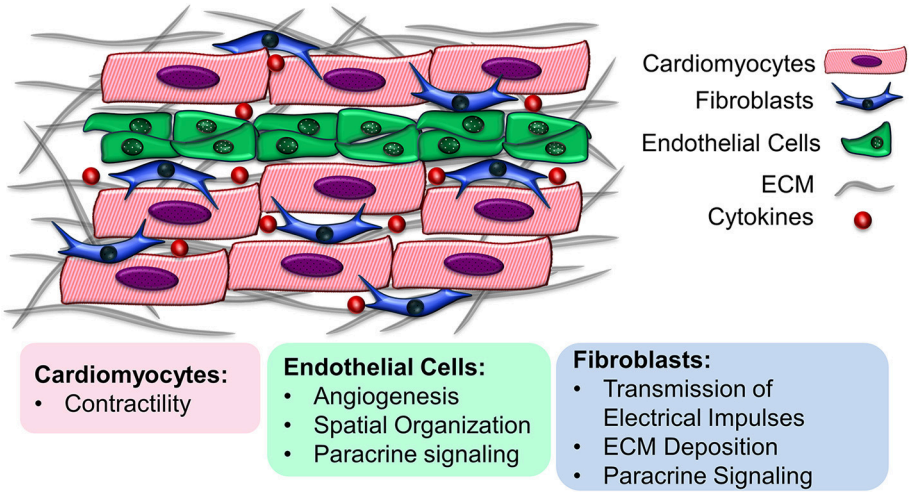

FIGURE 1 | Multicellularity of the myocardial environment**.** Cardiomyocytes interact with support cells such as endothelial cells and fibroblasts to maintain their cell function. The underlying extracellular matrix also provides instructive cues that regulate cell survival and function.

compared to ESC-CMs in monoculture. Transplantation of multi-culture pre-vascularized tissue constructs showed high engraftment and anastomosis to the animal's coronary artery, unlike the patches consisted of pure ESC-CMs that did not survive after implantation (36). Entirely iPSC-derived cardiac patches composed of iPSC-CMs and iPSC-ECs were developed using aligned nanofibrous scaffolds. The multicellular cardiac patches demonstrated improved maturity of iPSC-CMs, as indicated by increased sarcomeric length and myosin heavy chain gene expression that mimicked mature adult CMs (33). Entirely autologous iPSC-derived multicellular cardiac tissues can potentially address the problems associated with allogeneic cardiac tissues for future clinical applications. Although multicellular interactions are critical for promoting pluripotent stem cell survival and function, the ratio of the non-CMs need to be optimized in the tissue construct. A high fraction of non-CMs may adversely affect iPSC-CMs electrical conduction and maturation through the production of various ECM proteins, i.e., collagen, fibronectin, laminin (33, 34).

ENGINEERING FUNCTIONAL CO-CULTURED TISSUES

The formation of a functional cardiac construct comprising multi-cellular components requires the cells to be exposed to an *in vitro* environment that can efficiently support their proliferation, differentiation, organization, and function. Taking advantage of advances in the field of tissue engineering, multi-cellular cardiac constructs have been fabricated over the years using various cell culture techniques and scaffolding materials, to optimize the regenerative ability of the engineered tissue. In general, engineering a biomimetic cardiac tissue *in vitro* aims at generating a highly aligned, contractile, and vascularized cardiac construct in a three-dimensional (3D) microenvironment. In this section, we present some of the main techniques developed for fabrication of 3D co-cultured cardiac constructs to enhance their cellular organization and function.

Cell Sheet Engineering

Cell sheet engineering, the technique for constructing 3D functional tissues by layering two-dimensional (2D) cell sheets harvested from thermo-responsive cell culture surfaces, is an efficient approach for developing layered CM sheets co-cultured with non-CMs. A 3D cardiac tissue made through cell sheet technology may resemble dense cellular population and tight interconnection with gap junctions, which facilitates the exchange of biomolecules and ions resulting in electrically synchronized contraction. Synchronized contractions was observed for the layered CMs, indicating the electrical communication between layers of CMs in 3D construct (37). However, sufficient oxygen and nutrient diffusion into the layered CMs sheets is critically important, particularly due to the dense cell density in 3D constructs (38). Multicellular 3D platforms were also developed either by layering sheets of heterogeneous cells (27) or mono-cultured layers of different cell types (39). When EC-CM heterogeneous sheets were layered to develop pre-vascularized 3D constructs, it significantly enhanced capillary density in the graft, as compared with mono-cultured cell sheets *in vivo* (27). Alternating sheets of neonatal CMs and FBs could also form gap junctions between heterogeneous layers, resulting in synchronized beating of the 3D tissue (39).

Polymer Scaffolds

Polymer porous scaffolds with a wide range of biochemical, mechanical and topographical properties have been developed for creating cardiac tissues, with the aim of providing a temporary ECM-like support to guide the cells growth, organization

and function. The native ECM in the heart is a highly anisotropic (parallel-aligned), elastic and fibrillar network, and significant effort has been made to recapitulate this structure (40, 41). Polymer scaffolds can mediate the cross-talk between the cells in co-culture through its chemical, mechanical and electrical properties, as well as the architectural design of the scaffold which determines the cellular spatial patterning, cell-cell spacing, and the transmission of biomolecular/electrical signals. One advantage of polymer porous scaffolds over cell sheet technology is the ease of engineering 3D constructs. Nanofibrous scaffolds possessing fibrous ECM-like architecture with interconnected pores have been widely explored for generating cardiac tissues (17, 40, 42, 43). The CM-FB and CM-EC co-culture systems on chitosan nanofibers resulted in polarized CMs morphology and maintenance of long-term function (17). Conductive nanofibrous yarns containing carbon nanotubes were incorporated in GelMA hydrogel to produce 3D composite scaffolds mimicking native cardiac ECM. The co-culture of CMs and ECs on the nanofibers yarn and hydrogel construct respectively resulted in aligned CMs morphology in a uniform ECs network, capable of developing pre-vascularized tissue for myocardial regeneration (44). 3D porous scaffolds were also developed through other techniques such as salt leaching (35). Poly(l-lactic acid)/poly(lactide-co-glycolide (PLLA/PLGA) porous sponges seeded with a tri-culture system (i.e., ESC-derived CMs/FBs/ECs) showed higher density of functional vasculature integrated with host myocardial microvasculature, when compared to sponges consisting of mono-cultures of ESC-CMs (35).

Since CMs have highly organized physiological structure for driving efficient electromechanical coupling and contractility, much attention has been placed to recreate endogenous CM spatial patterning using instructive polymeric biomaterials. Spatial patterning of CMs on electrospun fibrous scaffolds (45) with aligned organization led to CM alignment along the direction of the fibers, increased contractile strength, and synchronized beating, compared to cells grown on the randomly oriented scaffolds, owing to improved intercellular crosstalk and exchange of ions via gap junctions (46). CM function was further improved when the aligned scaffolds were co-cultured with FBs or ECs. Similar effects of spatial patterning and improved contractile strength has also been reported using iPSC-CMs (33). It was also demonstrated that the alignment of fibrous scaffolds made of elastomeric polyurethane can improve the parallel organization of ESC-CMs and sarcomeric length in a CM-FB co-culture system (42). The roles of cell-cell contact and paracrine factors were further examined when anisotropic scaffolds seeded with rat CMs were co-cultured with scaffolds seeded with fibroblasts with either direct contact or indirect contact (paracrine interaction only). Interestingly, regardless of direct or indirect contact, the presence of FBs improved CM phenotype and aligned sarcomere organization, suggesting that both spatial patterning and paracrine factors are important for CM function (43). Together, these studies suggest that polymer scaffolds and anisotropic spatial patterning promote heterocellular cultures for improved CM function.

Hydrogels

Hydrogel systems mainly derived from natural sources such as Matrigel (47), fibrin (7), collagen I (48), gelatin methacrylate (GelMA) (49), and peptide hydrogel (29), have been used as substrates to prepare 3D heterocellular units for cardiac regeneration. To develop hydrogel-based 3D constructs, a solution of gelling material mixed with cellular components is usually casted into a mold where the gelation process occurs. Although the mechanical properties of hydrogel systems are lower compared to polymer scaffolds, they are normally more compliant than polymers (23). Cellular spreading, the ability to deform the hydrogel, and nutrient exchange are important factors in obtaining a functional contractile tissue construct (50). Studies show that, unlike ESC-CMs cultured in a fibrin-based hydrogel, ESC-CM/FB co-cultures could successfully compact and remodel the hydrogel substrate to generate macroscopically synchronized action potential propagation (23). It was also demonstrated that geometrical properties of the micropatterned hydrogel platform can influence the synchronized contraction of neonatal CMs-FBs co-culture in a GelMa hydrogel (49, 51). The authors found that the presence of FBs led to elongation of CMs, increased integrin expression levels, and increased connexin43 gap junctions, compared to CM-only cultures.

EMERGING TECHNOLOGIES

In recent years multicellular systems design has been improved with the emerging bioprinting and organ-on-a-chip (OOC) technologies that provide new approaches for basic research and drug discovery. Since the geometry and spatial arrangement of different cell types are important for their function, 3D bioprinting is a useful technology for turning digital designs into precisely organized 3D cultures. Bioprinting uses ECM proteins such as collagen, gelatin, hyaluronic acid, alginate, and de-cellularized ECMs as bioinks (52) to construct digitally designed 3D cultures. To overcome the problem of degradation of scaffolds *in vivo*, bioprinting has also been used to create cardiac tissue consisting of multicellular aggregates and omitting biomaterials altogether (53). Ong et al. created cardiac patches using spheroids of CMs, ECs and FBs that were 3D printed on a needle array and allowed to fuse. They showed that the resulting patch was electrically active and upon implantation *in vivo* resulted in vascularization and engraftment (54).

Recent research on organoid cultures showed that stem cells have the intrinsic capacity to self-assemble and differentiate into 3D tissues that can replicate some of the properties of the native organ, which suggests that it may not be necessary to 3D print the entire tissue. The cardiac organoids developed so far remain in the more immature stages and resemble fetal heart (55). Bioprinting can be employed to guide the cardiac organoid formation by creating patterns and guidance cues in its microenvironment (56).

Some of the limitations of traditional static 3D cultures such as lack of geometric organization, absence of a clear tissue interface, and the accumulation of secreted compounds into spent media can be overcome in a microfluidic platform.

Microfluidic platforms enable tissues of different cell types to be separated from one another by porous membranes to allow for molecular crosstalk. These OOC platforms allow for longer term cultures, owing to the circulating factors, nutrients and oxygen in the microfluidic system. The circulation of media in the capillaries give rise to mechanical factors such as fluid shear stress and allows for the monitoring of secreted factors and metabolites in real-time. The microfluidic chips also make it possible to investigate changes in the morphology and migration of cells (57). This approach has been used to create myocardial OOCs using CMs and ECs derived from human iPSCs to create models for drug screening and disease modeling (58, 59).

LIMITATIONS AND FUTURE DIRECTIONS

In vitro co-culture systems play a significant role in advances in cardiac tissue engineering over the last years. Using heterocellular engineered cardiac tissues, we are closer to developing functional vascularized tissues by taking advantage of complex and dynamic crosstalk between various types of cells and their interactions with ECM. However, extensive studies are yet required to fully understand the distinct roles of each individual cellular component and their interactions, to optimize engineered vascularized cardiac tissue. The *in vitro* co-culture systems are capable of creating physiologically relevant cardiac tissues for regenerative purposes. However, the read-out of multicellular systems is highly complexed and more innovative cell culture systems and characterization techniques are still required to precisely understand the heterocellular communication at a single-cell level to progress in the field. Although 3D scaffolds may better recapitulate the native heart ECM network, the difficulties associated with oxygen and nutrient diffusion, cell infiltration and viability, vascularization, and degree of scaffold remodeling may impair the clinical translation of multicellular constructs made by using 3D scaffolds. The source of the cells as well as the age, differentiation state and cell ratios of the co-culture platforms are important considerations influencing the functions of engineered vascularized cardiac tissues. Despite advances in employing ESC-CMs and iPSC-CMs for cardiac regeneration, it is critical to design cellular microenvironment to maintain characteristics of these differentiated cells by introducing relevant electrical, molecular, mechanical and topographical cues in long-term culture systems. The use of advanced materials such as conductive scaffolds may also facilitate the electrophysiological communication among the cells of multicellular environment. As more advanced techniques emerge in the field of tissue engineering, more biologically relevant native myocardial-like tissues will be developed for regenerative applications.

AUTHOR CONTRIBUTIONS

MZ and NH conceived the content of the manuscript. MZ and EK analyzed the literature and wrote the manuscript. NH critically reviewed and revised the manuscript. All authors read and approved the submitted version.

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REVIEW
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Heterocellularity and Cellular Cross-Talk in the Cardiovascular System

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Cellular specialization and interactions with other cell types are the essence of complex multicellular life. The orchestrated function of different cell populations in the heart, in combination with a complex network of intercellular circuits of communication, is essential to maintain a healthy heart and its disruption gives rise to pathological conditions. Over the past few years, the development of new biological research tools has facilitated more accurate identification of the cardiac cell populations and their specific roles. This review aims to provide an overview on the significance and contributions of the various cellular components: cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells, pericytes, and inflammatory cells. It also aims to describe their role in cardiac development, physiology and pathology with a particular focus on the importance of heterocellularity and cellular interaction between these different cell types.

Keywords: cardiac fi broblast, endothelial cells (ECs), macrophages (M1/M2), multicellularity, myocytes, cardiac tissue, pericytes and vascular smooth muscle cells, inflammatory cell

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INTRODUCTION

The development of multicellular organisms required millions of years of evolution, starting from simple prokaryotic cells, with no intracellular, or rudimentary organization, to eukaryotic cells, with more specialized, sophisticated cellular systems. Species evolved to include multiple specialized cells with distinct roles and functions. Populations of highly specialized cells form a variety of tissues, which allows the formation of organs capable of highly complex functions. Thus, multicellularity and the specialization of cells have driven evolution. The human body is one of the most studied multicellular systems and is comprised of more than 200 different cell types. Among these, the heart has been at the center of investigation not only because of its role in physiology but also because cardiac diseases are the number one cause of death in developed countries. The orchestrated function of different cell populations in the heart, in combination with a complex network of intercellular circuits of communication, is essential to maintain a healthy heart and its disruption gives rise to pathological conditions. Our knowledge of the precise factors involved in the orchestrated function and regulation of the heart is still incomplete. The different cellular components that form the heart, particularly the non-myocyte populations, have only recently been described in detail (1), making cardiac multicellularity a novel/topical target for cardiovascular research. Being at the center of the circulation, the heart is closely regulated by systemic and local signaling of chemical and mechanical nature, and this is also a very important area of investigation. Finally, the relentless electromechanical activity, which is unique to the heart, is also capable of regulating both cardiomyocyte and non-myocyte populations. This adds a crucial element of complexity that has limited our ability to investigate and understand cardiac behavior, particularly from

the multicellular/heterocellular viewpoint. In this review, we will provide an overview of cardiac multicellularity and how both intercellular physical interactions and cell-cell signaling are fundamental in cardiac development and adult cardiac phenotype homeostasis.

Cardiac Multicellularity *in vivo*

The heart is composed of several cell populations, each with specific functions and regulatory roles. Cardiomyocytes being very large cells make up most of cardiac tissue volume (2), but they only account for ≈25–35% of all the cells in the heart (3–5). Using genetic tools and cellular markers, it has recently been shown that endothelial cells make up >60% of the non-myocyte population, making them the most prevalent cell type in the adult heart (1) (**Figure 1**). A consensus is still lacking regarding the remaining stromal cell population composition. Previous studies (4, 6–9) have suggested that fibroblasts constitute the majority of non-myocytes, however, it is now known that they only account for <20% of the non-myocyte population (1, 5) (**Table 1**). Vascular Smooth Muscle Cells, pericytes, and hematopoietic-derived cells make up the rest of the non-myocyte population however a consensus on their respective percentage in cardiac tissue is still debated.

THE ROLE OF CARDIOMYOCYTES

Cardiomyocytes are the muscle cells of cardiac tissue and their synchronous contraction is required to pump blood throughout the body. They are the most physically energetic cells in the body, repeating their relentless contraction cycle over 3 billion times in the average human lifespan (10). They are very large cells, typically 100–150 μm in length and 10–35 μm in width. Their cytoplasm is packed with sarcomeres, the contractile units of muscle cells, and mitochondria, which are needed to satisfy their high energy requirements and account for ∼35% of cardiomyocyte volume (11). Cardiomyocytes are cylindrical in shape with end-to-end connections called intercalated disks. These highly specialized cell-to-cell connections ensure mechanical and electrochemical coupling (11). They help to stabilize the positions of the cells relative to each other and maintain the 3D structural integrity of the tissue (12). The intercalated disks are also the preferential method of cardiomyocyte cross talk. They contain intercellular channels called gap junctions, made of connexins. Ions, small molecules, and small peptides are capable of crossing these junctions. Disorganization of the intercalated discs can make gap junctions more susceptible to improper intercellular transfer of molecules and impulse propagation (12). The expression and distribution of junctional components are often altered in cardiovascular disease. It has been reported that mutations in the gene encoding connexin 40 GJA5 induce altered electric coupling and lead to increased arrhythmogenesis (13). Cardiac-specific loss of murine N-cadherin leads to a modest dilated cardiomyopathy with impaired cardiac function before sudden cardiac death (14). Cardiomyocyte regulation is also controlled by other cell types through paracrine signaling, however cardiomyocytes are also able to secrete soluble factors to interact and communicate

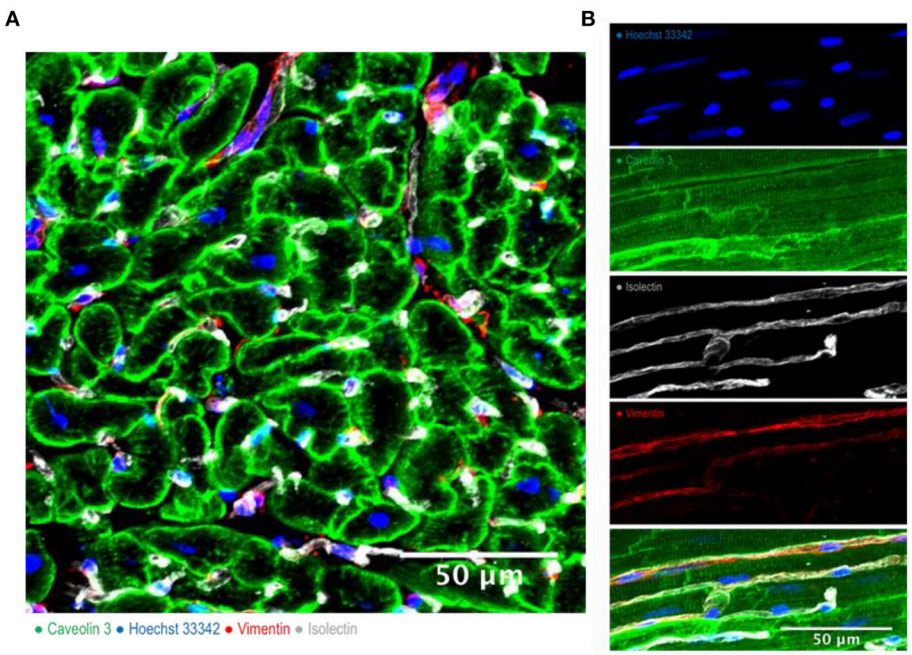

FIGURE 1 | Cardiac multicellularity *in vitro*: Immunohistochemical staining and confocal microscopy were used to identify cardiac cells in a transverse section **(A)** and in a longitudinal section **(B)** of freshly prepared dog myocardial slices. Cardiomyocytes were labeled with caveolin 3, fibroblasts were labeled with vimentin and endothelial cells were labeled with isolectin. Nuclei were labeled with Hoechst 33342. Scale bar = 50 μm.

TABLE 1 | Cellular composition of the myocardium.

Cell types					Species technique		Markers	Date	References
CMs (%)	ECs (%)	FBs (%)	ICs (%)	PCs (%)	Other (%)				
41	33				26	Rat	Histology (Safranin-O)	Stereological analysis based on morphological differences between cells	1980	(6)
30–35					65–70	Rat	Transmission electron microscopy		1980	(7)
27					68	Human	Histology	Heidenhain's Azan staining was used to determine cell boundaries	1991	(8)
52		37			11	Mouse	Immunohistochemistry	α-MHC (CMs) DDR2 (FBs)	2007	(9)
18	24				58	Human	Immunohistochemistry	Connexin-43, Dystrophin, PCM-1 (CMs) UAE I (ECs)	2015	(4)
30	52	10	6	3		Mouse	Immunohistochemistry	α-Actinin (CMs) Isolectin B4 (ECs) GFB targeted (FBs) Lin2 (ICs - Leukocytes) PDGFRβ (VSMC/Pericytes)	*2016*	(1)
38	48				14	Dog	Immunohistochemistry	Caveolin 3 (CMs) Isolectin B4 (ECs) Vimentin (stromal cells)	2017	(5)

CM, cardiomyocytes; EC, endothelial cells; VSMC/PC, vascular smooth muscle cells/pericytes; FB, fibroblasts; IC, inflammatory cells.

PCM-1:pericentriolar material 1
DDR2:discoidin domain receptor 2
UEA:Ulex Europaeus lectin 1
Lin2: hematopoietic lineage cocktail
PDGFR6: platelet-derived growth factor receptor beta

with other cell types, particularly during inflammation or cardiac injury. A recent study by Roy et al. has shown that cardiomyocytes are also able to produce and secrete acetylcholine (ACh), a parasympathetic nervous system neurotransmitter. This non-neuronal source of ACh increases parasympathetic cholinergic signaling to counterbalance neural sympathetic activity regulating cardiac homeostasis and therefore plays a fundamental role in healthy heart activity (15). Inflammatory cytokines such as IL-6 are released by cardiomyocytes during hypoxic stress, suggesting an important role in the progression of myocardial dysfunction observed in cardiac ischemia-reperfusion injury (16). Although IL-6 has been reported to have cardioprotective effects (17), clinical studies suggest that prolonged and/or excessive synthesis of IL-6 is detrimental to the heart (18, 19). Cardiomyocytes have also been shown to produce and secrete TNF-α under certain conditions such as treatment with lipopolysaccharide (LPS). The presence of LPS contributes to the cardiovascular collapse and death observed in patients with sepsis. TNF-α stimulation on cardiomyocytes results in inotropic and pro-apoptotic effect which results in defective contractility and relaxation of the myocardium (20, 21). TNFα is another example of signaling molecule released by cardiomyocytes during myocardial infarction. TNFα release has been shown to be controlled by the hypoxia-inducible factor1α pathway and to be mediated by exosomes release by cardiomyocytes (22, 23).

THE ROLE OF ENDOTHELIAL CELLS

In the healthy myocardium, a dense network of capillaries facilitates the distribution of oxygen and metabolic substrates to cardiomyocytes. Each cardiomyocyte is in contact with at least one capillary and endothelial cells outnumber cardiomyocytes by ≈3:1 (1, 24). This architectural arrangement also allows a mechanical and paracrine cross-talk between cardiomyocytes and endothelial cells to exist, which plays pivotal roles in cardiac development and the regulation of cardiomyocyte function (**Figure 2**). Several factors released by endothelial cells, including neuregulin, neurofibromatosis type 1 (NF1) and platelet-derived growth factor-B (PDGF-B), and by cardiomyocytes, including vascular endothelial growth factor-A (VEGF-A) and angiopoietin 1, have been implicated in these processes (25–27). Endothelial-cardiomyocyte interactions play fundamental roles in the regulation of cardiac function by both autocrine and paracrine mechanisms. Both endothelial cells and cardiomyocytes are able to synthesize Nitric Oxide (NO) with three different nitric oxide synthase isoenzymes (eNOS, iNOS, and NOS). eNOS expression in endothelial cells is four times greater than in cardiomyocytes (28). Nitric oxide affects blood vessels, its complex effect results in vasodilation due to relaxation of vascular smooth muscle, and reduced contractility in cardiomyocytes, leading to an attenuation of contraction (29). ET-1 is also released by endothelial cells, in addition to release by cardiac fibroblasts, and acts in both an autocrine and paracrine manner binding to cardiomyocytes via ET_A receptors and endothelial cells via ET_B receptors (24). ET-1 effects on cardiomyocytes include the induction of hypertrophy and remodeling. There is also

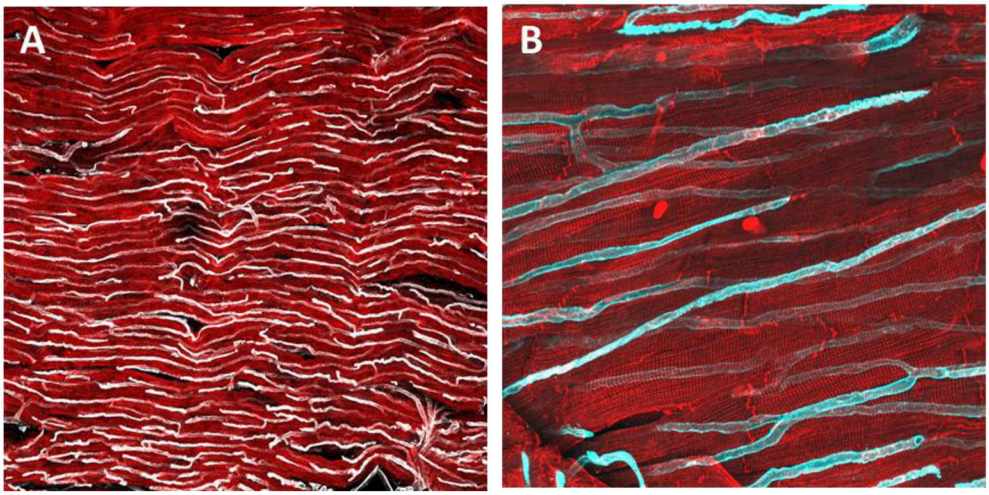

FIGURE 2 | Immunohistochemical staining and confocal microscopy were used to identify endothelial cells distribution in a freshly prepared dog myocardial slice **(A)**. Higher magnification of capillaries and their location in proximity to cardiomyocytes **(B)**. Cardiomyocytes were labeled with caveolin 3 (red) and endothelial cells were labeled with isolectin (White and cyan).

evidence that endothelial cells promote cardiomyocyte survival, with neuregulin found to be a pro-survival factor (30) and less cardiomyocyte apoptosis observed when cardiomyocytes are cultured with endothelial cells *in vitro* (31).

The importance of endothelial factors during cardiac development has also been demonstrated by a number of cell-specific gene knockdown (KO) experiments. Mice lacking either neuregulin or its receptors, erbB2/4, die during mid-embryogenesis due to lack of cardiac trabeculae and cardiac cushion development (32). NF1 KO results in developmental defects in both the myocardial and endocardial cushions, resulting in myocardial thinning and ventricular septal defects. Defects do not occur in cardiomyocyte-specific KO models, indicating that signaling from endothelial cells is crucial for development (33). Endothelial-specific KO of PDGF-B results in cardiac abnormalities, including myocardial thinning, chamber dilation, hypertrabeculation, and septal defects, alongside vascular and glomerular abnormalities (34). Thus, molecular signals from endothelial cells are crucial for development but reciprocal cross-talk between cardiomyocytes and endothelial cells is also required. Mutations in both VEGF-A and its receptor, VEGF receptor-2, result in failure of both the endocardium and myocardium to develop. Cardiomyocyte-specific KO of VEGF-A results in defective angiogenesis and ventricular wall thinning (35). The angiopoietin-Tie-2 system is also fundamental to cardiac development and is primarily responsible for maturation and stabilization of the neovasculature (35). Mice with mutations in this pathway have an underdeveloped endocardium and myocardium, while cardiomyocyte-specific overexpression of angiopoietin-1 results in embryonic death (35). These findings demonstrate that sensitively controlled bilateral paracrine communication between endothelial cells and cardiomyocytes is fundamental to normal cardiac development.

THE ROLE OF CARDIAC FIBROBLASTS

Cardiac fibroblasts are often considered the most abundant stromal cell type and they play a crucial role in extracellular matrix deposition, maintenance and remodeling. They are characterized by a secretory phenotype with an elongated, spindle-like morphology, a granular cytoplasm, and an extensive rough endoplasmic reticulum (36). In the heart they are diffusely distributed throughout the myocardium, localized in the interstitial space that separate cardiomyocytes and in close proximity to capillaries and larger vessels (**Figure 3**). To date, there is no agreement on appropriate markers to identify resident fibroblasts within the heart. The markers available (Vimentin, CD90, DDR2, FSP1, Sca1, Periostin, etc.) target different fibroblast-like cells suggesting that resting fibroblasts are a mixture of cell populations (5, 36). This hypothesis is further reinforced by the notion that cardiac fibroblasts come from two separate developmental origins. Two independent groups showed that fibroblasts residing in the interventricular septum and right ventricle do not form from the epicardium, but instead have an endothelial origin, constituting roughly 20% of the myocardial resident fibroblast (37, 38). Taken this into consideration, modern techniques of single cell analysis and genetic lineage tracing seem to show comparable gene expression profile of different cardiac fibroblast populations suggesting that they may not be as diverse as previously thought (37, 39).

The study of cardiac fibroblasts and their interactions with beating cardiomyocytes *in vivo* is problematic; fibroblast function is complex and multifaceted and there may be several direct and indirect mechanisms of cellular interactions. These include interaction via alteration of extracellular matrix (ECM) quantity and composition, vascular maintenance, paracrine signaling, conduction system insulation, and electrotonic coupling (36). The ECM is a complex, dynamic scaffold, composed of collagens,

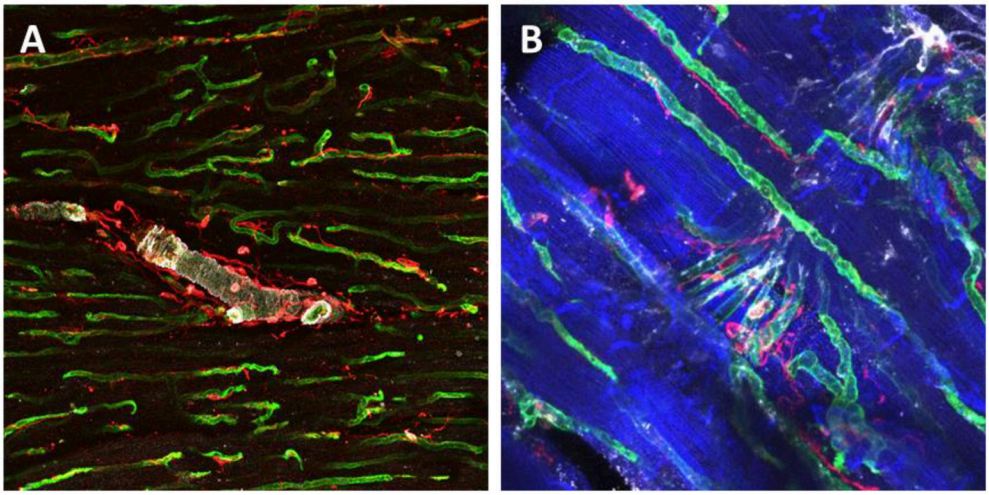

FIGURE 3 | Immunohistochemical staining and confocal microscopy were used to identify different cardiac populations in a dog myocardial slice. Large vessels were identified for α-Smooth Muscle Actin expression (white), endothelial cells were labeled with isolectin (green) and fibroblasts were labeled with vimentin (red) **(A)**. Higher magnification of capillaries and their location in proximity to cardiomyocytes and fibroblasts **(B)**. Cardiomyocytes were labeled with caveolin 3 (blue), endothelial cells were double labeled with isolectin (green) and Von Willerbrand factor (white) and fibroblasts for Vimentin (red).

proteoglycans, and glycoproteins (10). Cardiac fibroblasts are involved in the synthesis and maintenance of the ECM and are responsible for the 5% turnover of the ECM each day (11). Cardiomyocytes are physically linked to the ECM via integrin molecules (12), allowing it to influence cardiomyocyte function through kinase signaling cascades and direct mechanical interaction with intracellular structures (13). As such, regulation of the ECM by fibroblasts indirectly influences cardiomyocytes. In addition to maintenance of the ECM, cardiac fibroblasts secrete a vast array of bioactive substances. These molecules are secreted into the interstitium, where they act in both an autocrine and paracrine fashion (40). The extensive array of soluble mediators released results in functional cross-talk between several cardiac cell populations, including cardiomyocytes. Of the numerous factors released, transforming growth factor beta (TGF-β), interleukin 6 (IL-6), and endothelin 1 (ET-1) have significant effects on cardiomyocytes. Secretion of TGF-β, typically induced by changes in mechanical loading (41), results in cardiomyocyte hypertrophy (42) and profound electrophysiological changes (43). At the whole heart level, these changes are initially protective, but ultimately result in maladaptive remodeling (44). IL-6 is also associated with cardiomyocyte hypertrophy, alongside diastolic dysfunction and reduced expression of SERCA2a (45). ET-1 induces a potent hypertrophy in cardiomyocytes and its expression directly correlates with ventricular remodeling (46, 47). Paracrine interactions can also be achieved via non-soluble mediators, such as extracellular vesicles and microRNAs (miRs). Fibroblast secretion of miR-21in exosomes has been shown to induce cardiomyocyte hypertrophy (48). The presence of all 3 isoforms of connexin (Cx40, Cx43, and Cx45) (49) and electronic coupling between cardiomyocytes and fibroblasts *in vivo* has been demonstrated using optogenetic techniques and tunneling nanotubes between the two cell types have also been observed (50). These findings have implications for cardiomyocyte electrophysiology and cardiac conductivity but the physiological importance of these interactions and their role in cardiac disease remains to be established.

A large variety of stimuli such as cytokines, cardiomyocyte death, or changes in mechanical load can activate cardiac fibroblasts into their pathological phenotype, known as myofibroblasts (40). Activated fibroblasts have a different morphology with increased cytoplasm, well-defined endoplasmic reticulum and Golgi complex and microfilament bundles, which are often identified with αSMA antibody. They also have altered functions which include decreased ECM degradation and excessive secretion of matrix proteins, including collagen Type 1, and pro-inflammatory cytokines which play a crucial role in scar formation and fibrosis (51). Previous studies have indicated that myofibroblasts can derive from pericytes, bone marrow progenitor cells, monocytes, and though endothelial to mesenchymal transition (51). The contribution of these various cell types is still debated, however, a recent and comprehensive study by Kanisicak et al. (39) using genetic lineage tracing, identified resident cardiac fibroblasts as the main source for activated myofibroblasts in the injured heart (39). Several studies have reported different roles of fibroblasts and myofibroblasts during physiology and disease in regulating myocardial function via soluble mediators (43). A study by Cartledge et al. have shown a smaller cardiomyocyte Ca^{2+} transient amplitude when cultured with myofibroblasts compared to fibroblasts suggesting an important bi-directional regulatory role of TGF- β (43). Similarly to fibroblasts, myofibroblasts are also capable of forming functional gap junctions in the diseased myocardium suggesting that myofibroblasts might contribute to arrhythmogenesis by direct electrotonic modulation of impulse

propagation and increased mechanosensitive channel activation (41, 51–53).

THE ROLE OF VASCULAR SMOOTH MUSCLE CELLS AND PERICYTES

Vascular smooth muscle cells (VSMCs) are stromal cells and constitute the vascular wall of large and small vessels. By contraction and relaxation, these cells can alter the vessel luminal diameter and, as a consequence, they are responsible for the regulation of blood pressure and blood flow. VSMCs not only regulate vessel diameter for short periods but they can also be subjected to long-term stimulation which results in physiological (such as pregnancy or exercise) or pathological vascular remodeling (54). VSMCs are normally classified as contractile or synthetic. This simplification only represents the two ends of a spectrum which includes several intermediate phenotypes. These two cell types are different in terms of morphology, function, gene expression, marker profile, and gap junctional/adhesion molecules. Contractile VSMCs are elongated, spindle-shaped cells with contractile filaments and with a low proliferative and migratory capacity. Synthetic VSMCs on the other hand have a rhomboid or cobblestone morphology, high number of organelles and a high proliferative and migratory capacity (55, 56). Smooth-muscle myosin heavy chain (SM-MHC) and smoothelin are the two most common marker proteins used to identify contractile VSMCs, whereas syntethic VSMCs express Smooth-muscle-emb/non-muscle MHC and cellular retinol binding protein (CRBP-1) (54). Although VSMCs phenotype seems to be genetically programmed in relation to their developmental origin (57), as shown in different species such as rat (54), pig (58), and humans (59), local environmental stimuli can significantly modulate VSMCs characteristics and function. These includes physical as well as biochemical factors which act in combination with the extracellular matrix composition. Tensile stretch and shear stress, induced respectively by the blood pressure and blood flow, affecting VSMCs contractile state, can induce vessel wall remodeling (54). The cells of the endothelium are able to sense the shear stress and respond with nitric oxide release and with direct cell-cell interaction with the VSMCs (58, 60). Endothelial cell proliferation and dysfunction, associated with altered production of vasoactive mediators, such as nitric oxide, entothelin-1, serotonin and prostacyclin, are reported to alter VSMCs behavior and contribute to pulmonary arterial hypertension (61). PDGF molecules play a crucial role in cellular cross-talk, they are produced by endothelial cells, perivascular inflammatory cells and smooth muscle cells. During pulmonary arterial hypertension PDGF-A and PDGF-B are overexpressed, they induce fibroblasts activation and a synthetic phenotype in VSMCs with increased cellular proliferation and migration promoting pulmonary arterial remodeling (61, 62). TGF-β signaling is also involved in VSMCs regulation promoting a contractile phenotype on cultured adult smooth muscle cells (63). Recent studies revealed an important role of cellular cross-talk between macrophages and VSMCs and this phenomenon seems to plays an important role during atherosclerotic plaque formation. This communication, principally mediated by Toll-Like receptor pathways, can alter the ECM synthesis and deposition, increase the production of metalloproteinases and increase the production of angiogenic chemokines such as VEGF and IL-1 (57, 64, 65).

Pericytes are also an important contractile cell of the body. They are closely associated with the microvasculature, particularly with pre-capillary arterioles, capillaries, and post-capillary venules (65). Pericytes are normally embedded in the basal membrane in close contact with endothelial cells. In larger vessels of the myocardium, a sparse layer of pericytes separates the endothelium from the VSMCs and the elastic structures of the vessel (66). Morphologically they can be distinguished for their thin and elongated cytoplasm, numerous finger-like projections and the rounded nucleus (67, 68). A range of surface (PDGFRβ, CD146, CD13 and NG2) or cytoplasmic markers (αSMA, desmin, vimentin, and nestin) are commonly used to identify this specific cell population (68–70). The number of pericytes seems to be organ dependent and their number in cardiac tissue is still debated, with groups reporting pericytes to be the second most frequent myocardial cell with a ratio with endothelial cells of 2:1 or 3:1 (71). However, in light of more recent studies on cardiac cellular composition this numbers might be an overestimation (1, 5). If pericytes number is still uncertain, much more is known about their function. The cytoplasmic expression of contractile proteins, such as αSMA or vimentin, is a clear indication of their vasomotion regulatory role. Their main function is to regulate the homeostasis and permeability of the vasculature and to control the blood flow in the micro-circulation. They also play a role in the removal of cell debris and to monitor the maturation of endothelial cells (72, 73). Pericyte's cytoplasmic protrusions connect to cell membrane invaginations of endothelial cell though connexin43 mediated and N-Cadherin adherence junctions. These connections are used to sense mechanical forces, such as stretch and shear stress, and to exchange electrical (66) and biochemical signals (both ions and small molecules) (74, 75). The active cross-talk between pericytes and endothelial cells has been shown to be fundamental for the maintenance of the endothelial barrier, principally mediated by TGFβ and angiopoietin1 (76, 77) and the formation and deposition of collagen I, IV and fibronectin in the basal membrane (78). They also play an active role in the process of new vessel formation. They can induce quiescence and maturation in activated endothelial cells though Angiotensin I secretion (66) or bridge the temporary gaps formed between sprouting endothelial cells (66). In pathological conditions, particularly following ischemic damage, pericytes receive signals from resident cells and infiltrating inflammatory cells and play an active role in angiogenesis and collateralization, reparative fibrosis, tissue remodeling, and regeneration (66). It has been reported that macrophages secrete galectin-3 which stimulates pericyte's proliferation and secretion of protocollagen1 which eventually lead to collagen accumulation and cardiac fibrosis (66). Pericytes can also be activated followed injury via PDGF stimulation, which results in their migration to the interstitium, change into a myofibroblasts phenotype and increased release of

ECM (66). During ischemia, cardiomyocytes release pro-Nerve Growth Factor which binds to the $P75^{NTR}$ on pericytes inducing cytoskeletal changes, disrupting their interaction with endothelial cells and provoking vascular permability (66).

THE ROLE OF OTHER CARDIAC CELL POPULATIONS

The immune cells form another important cardiac cell population. Of these, the role of macrophages has been most extensively explored over the last few years. Macrophages are an important component of the innate immune system and constitute a first line of defense against invading pathogens. They are large, round or spindle-like cells that contain a central round nucleus, have abundant clear, often vacuolated, cytoplasm with far-reaching protrusions, they are found in the interstitial space interspersed between cardiomyocytes, fibroblasts and endothelial cells (79). In the mouse heart it has been estimated that they can be up to 10% of non-cardiomyocytes cells and humans may have similar numbers (80). Following cardiac injury, an expansion of their population occurs through both local proliferation and monocyte recruitment, and is essential for myocardial repair (81). For several years macrophage heterogeneity was oversimplified into two main groups: M1 and M2. Macrophages that encourage inflammation are called M1, whereas those that decrease inflammation and encourage tissue repair are called M2 macrophages (35). Beyond having different functions, they also have distinct gene expression and surface markers profile (35). In the past few years it been shown that this classification does not adequately describe the spectrum of macrophage populations and several studies are now further investigating these differences (80, 82). The role of macrophages in the regulation of cardiomyocytes has been less well explored. A recent study by Hulsmans et al. has demonstrated that macrophages can form gap-junction with cardiomyocytes, via Cx43 expression, thus modulating their electrical activity (83). Furthermore, photo-stimulation of channelrhodopsin-2-expressing macrophages was able to improve atrioventricular conduction (83). Liu et al. (84) using a hypoxic mouse model and acyanotic vs. cyanotic patients, showed that postnatal hypoxia promoted cardiomyocyte proliferation and that cardiac resident macrophages may be involved in this process (35). Macrophages also communicate with other cell types in the myocardium, particularly with cardiac fibroblasts via the leucocyte surface antigen CD40 or the up-regulation of ICAM-1 and VCAM-1 (85, 86). Fibroblasts have the ability to participate in the maintenance of an inflammatory response via the expression of chemokines; on the other hand macrophages are the leading producers of TGF-β which is considered the most significant pro-fibrotic agent involved in the progression of chronic fibrotic diseases (87). It has long been recognized that macrophages can support angiogenesis, through both cell-to-cell contact with endothelial cells and the secretion of proangiogenic factors (88). Activated macrophages secrete a large variety of growth factors and inflammatory cytokines such as VEGF-A, VEGF-C, IL-1β, FGF2 etc., which induce endothelial activation, proliferation, spouting and survival (89, 90). Soluble proteases and matrix remodeling activity induced by macrophages also play a role in vessel sprouting and vascular growth (91, 92). Recent studies have shown that macrophages physically interact with sprouting endothelial cells to support and promote new vascular intersections in a process mediated by angiopoietin receptor, TIE2 and neuropilin-1 (88, 93). The interaction of macrophages and endothelial cells is bidirectional as endothelial cells can also promote the expansion and differentiation of proangiogenic macrophages. He et al. showed that endothelial cells can induce expansion and differentiation of hematopoietic progenitor cells toward an M2-macrophage phenotype (94). Gene marking studies, using Tie2-GFP reporter lentiviral vectors, frequently show clusters of immature Tie2-GFP+ cells monocytes in association with blood vessel sprouting (95, 96).

Although the interaction of macrophages with the other cardiac cell types has been quite extensively investigated, much less is known about the role of the other inflammatory cells (97, 98). Recent evidence suggests that T cells are also involved in the regulation of cardiac remodeling, particularly in the attenuation of hypertrophic response and cardiac dysfunction following myocardial infarction (99). TNFα overexpressing mice develop cardiomyopathy overtime, however the administration of anti-CD3 antibody to neutralize T cells reduced inflammatory cell recruitment and stopped hypertrophy (100). The depletion of T cells in Rag2 deficient mice, which develop pressure-overload induced hypertrophy, has also been shown to reduce macrophage infiltration and fibrosis together with attenuated cardiac dysfunction (101). Neutrophils are the most abundant leukocytes in humans and they also migrate to damaged areas following acute injury, such as myocardial infarction or ischemia. In literature very few studies can be found where their role has been investigated, particularly in hypertrophy and cardiac remodeling. A recent study from Wu et al. showed that the neutralization of S100A9, a molecule secreted by neutrophils, decreased angiotensin-II induced cardiac hypertrophy (102) suggesting a role of this cell type in cardiac remodeling. Several studies have also indicated an important role of T cells in the development of heart-specific autoimmune myocarditis (103–105). α-Myosin Heavy Chain specific (α-MyHC) CD4+ T cells have been found in mouse models of myocarditis as well as in human patients with inflammatory cardiomyopathy. Various CD4+ T cell subsets, particularly Th1 and Th17, have been shown to play an important role in the maintenance of tissue immune homeostasis and in modulating disease phenotypes (103–105). A better understanding of their role might therefore provide new approaches for the development of new therapeutic strategies. The number of mast cells in the heart also increases following cardiac injury. Mast cells have been implicated in maladaptive cardiac remodeling, but the mechanisms by which they contribute to this are yet to be fully elucidated (97). It has been shown that mast cells can release several bioactive molecules including growth factors, cytokines, histamine which affect other cell types (106–108). Changes in the concentrations of these factors can induce cardiomyocytes apoptosis as well as fibroblast proliferation and ECM deposition. Mast cell paracrine secretion of IL-4 has pro-fibrotic and immunomodulatory effects (106).

CONCLUSIONS

Cellular specialization and interactions with other cell types are the essence of complex multicellular life. In recent years, the development of new research tools has enabled the identification of various cell populations within the myocardium. Interactions between different cell types in the heart have been identified as playing major roles in cardiac development and the maintenance of adult phenotype in both healthy physiology and pathological conditions. It is difficult to study cellular interaction *in vivo* and the data collected using current *in vitro* approaches are often oversimplified and do not recapitulate *in vivo* heterocellular complexity. Although progress is evident in the study of multicellularity and cellular interactions, key questions regarding multicellular interactions in an electromechanical stimulated environment remain to be answered. This review has summarized how chemical cross-talk can change cardiac cellular function. As the heart is subjected to electromechanical stimuli which affect cellular function, comprehensive studies and new models that incorporate mechanical, electrical as well as chemical signals need to be developed. New *in vitro* 3-dimensional heterocellular models that can recapitulate adult cardiac physiology are necessary in order to bridge the gap between existing *in vitro* and *in vivo* models. A more in-depth understanding of the role of cardiac microenvironment and heterocellular cross-talk is fundamental in the advancement of other research areas in cardiac biology, such as regenerative medicine and cardiac tissue engineering. The knowledge acquired will be fundamental to develop novel therapeutics with specific biological targets for treatments of patients with heart disease.

AUTHOR CONTRIBUTIONS

FP wrote the first draft of the manuscript; SW and CT wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Single Cell Gene Expression to Understand the Dynamic Architecture of the Heart

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The recent development of single cell gene expression technologies, and especially single cell transcriptomics, have revolutionized the way biologists and clinicians investigate organs and organisms, allowing an unprecedented level of resolution to the description of cell demographics in both healthy and diseased states. Single cell transcriptomics provide information on prevalence, heterogeneity, and gene co-expression at the individual cell level. This enables a cell-centric outlook to define intracellular gene regulatory networks and to bridge toward the definition of intercellular pathways otherwise masked in bulk analysis. The technologies have developed at a fast pace producing a multitude of different approaches, with several alternatives to choose from at any step, including single cell isolation and capturing, lysis, RNA reverse transcription and cDNA amplification, library preparation, sequencing, and computational analyses. Here, we provide guidelines for the experimental design of single cell RNA sequencing experiments, exploring the current options for the crucial steps. Furthermore, we provide a complete overview of the typical data analysis workflow, from handling the raw sequencing data to making biological inferences. Significantly, advancements in single cell transcriptomics have already contributed to outstanding exploratory and functional studies of cardiac development and disease models, as summarized in this review. In conclusion, we discuss achievable outcomes of single cell transcriptomics' applications in addressing unanswered questions and influencing future cardiac clinical applications.

Keywords: heart, gene expression, single cell, cellular landscape, transcriptomics, qRT-PCR, RNA-seq

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INTRODUCTION

Each single cell of our body has a unique position in space and time and is, therefore, exposed to a unique set of specific signals and stimuli. However, until recently, our capacity to study single cells was limited to handfuls of genes or proteins and the vast majority of gene expression studies were done in bulk. Single-cell gene expression analysis started with the development of methods to study targeted transcripts including quantitative RT-PCR (1–3) but also single-molecule FISH that allows to maintain the spatial information (4–7). Approximately a decade later, the first paper proving the feasibility of single cell RNA-sequencing (scRNA-seq) was published, evidencing its superiority to single cell microarrays in the study of single mouse blastomeres, with 75% more genes detected and a level of resolution permitting the identification of more than 1,700 previously unknown splice

junctions (8). Thus, a new era started and a complete change of perspective, permitting the analysis of gene expression and, consequently, cell function and intercellular networks from a cell-centric viewpoint.

There has been rapid development of technologies to reduce noise, improve sensitivity and, notably the throughput for single cell transcriptomics (9–17). Indeed, the number of high-impact studies using scRNA-seq is rapidly growing in many fields. Pioneering studies were conducted on developing embryos, even at the pre-implantation stage, circulating cells and also solid tissue including human disease samples such as cancer (8, 9, 14, 18–23). The first single cell gene expression studies on the heart relied on single cell qRT-PCR and scRNA-seq studies lagged for a few years (24, 25). The initial work focusing on cardiac development as well as adult heart demonstrated feasibility with some useful explorative studies and more recent ones proving the unique value of single cell studies in identifying pathways' dysregulation in disease models (26–29).

The advantages of single cell gene expression analysis offsets any associated technical difficulty. Three fundamental features can be defined at the single cell level for the expression of each gene and, more relevantly for patterns of gene expression predicting a specific cell type: prevalence, heterogeneity, and co-expression. In particular, for single cell transcriptomics based on each cell's own global gene expression, the classification of cell types is uniquely quantitative and data-driven, allowing the discovery of markers defining a tissue or cell type without prior knowledge (17, 19, 30–38). In fact, the sensitivity and specificity of whole-transcriptome signatures is above technical noise and biological variability, and therefore is sufficient to discriminate novel and rare cell types and previously unappreciated markers during development, and disease progression (14, 17, 19, 22, 36–42).

The use of scRNA-seq to unravel heterogeneities, identify novel cell populations and organize cellular hierarchies, represents the first explorative phase enabling a dynamic vision with functional implications that would otherwise be masked in bulk analysis (17, 21, 36–39, 42–46). From a computational perspective, the elaboration of this rich information using unsupervised clustering reliably allows to group cells according to their subtype or state, as shown in experiments deconvoluting the cellular composition of complex tissues (brain, spleen, intestine, ear, retina, and tumors) but, also, to characterize transitions along pseudotemporal trajectories and reconstruct lineage decisions (37, 41, 47–51). Indeed, integrated data analysis entails subsequent deconstruction/reconstruction iterations to define the regulatory and signaling mechanism governing cellular decision that result in the definition of specific cell types and/or functional states. Remarkably, single cell transcriptomics allows the investigation of gene regulatory networks, which can be seen as the ensemble of active transcription factors and the genes they target (52), aiming to predict the effect of disruptions and manipulations to such network. In addition, previously masked by whole-tissue or pooled cell analysis, scRNA-seq also demonstrated the bimodal (on/off) distribution of gene expression in individual cells (22, 53). Ultimately, as described in more detail below, inferences go beyond the boundaries of single cells permitting the extrapolation of intercellular regulatory gene networks (38, 54–56).

In summary, single cell transcriptomics provides a global and unbiased view of tissues' cellular demographics from bottom up, both at steady-state and in dynamic processes like development, differentiation, and progression of disease. In the first section, we provide a detailed overview of the practicalities to design single cell RNA-seq or targeted gene expression experiments, and discuss methods including computational analysis; in the second part we cover the advancements brought by these new technologies in the cardiac field, the foreseeable successes in resolving ambiguities of heart biology and the potential applications in clinical settings.

Abbreviations: CM, CardioMyocyte; DE, Differentially Expressed; ERCC, External RNA Controls Consortium; FACS, Fluorescence-Activated Cell Sorting; FISH, Fluorescent *In Situ* Hybridization; GRN, Gene Regulatory Network; HCA, Human Cell Atlas; iCM, Induced Cardiomyocytes; iPSC, Induced Pluripotent Stem Cell; LCM, Laser Capture Microdissection; MARS-Seq, MAssively parallel RNA Single cell sequencing; mESC, Mouse Embryonic Stem Cell; MSC, Mesenchymal Stem Cell; PCA, Principal Component Analysis; qRT-PCR, Quantitative Real Time PCR; RPKM, Reads Per Kilobase per Million; RPM, Reads Per Million; SCRB-seq, Single-Cell RNA Barcoding and sequencing; scRNA-seq, Single Cell RNA-sequencing; Smart-Seq, Switch Mechanism At the 5' end of RNA Templates sequencing; snRNA-seq, Single Nuclei RNA-sequencing; tSNE, T-distributed Stochastic Neighbor Embedding; UMI, Unique Molecular Identifie; ZIFA, Zero-Inflated Factor Analysis.

EXPERIMENTAL AND COMPUTATIONAL APPROACHES FOR SINGLE CELL GENE EXPRESSION ANALYSIS

Design of Single Cell Transcriptomics Experiments

Single cell RNA-seq technologies involve a number of steps (**Figure 1**) employing different strategies for cell capture, reverse transcription, cDNA amplification, and the increasing number of options available raises practical questions for first-time users.

The first decision concerns the number of cells to be captured and the transcriptome coverage needed, i.e., the number of genes detected per cell. The numbers required for an accurate representation of the reality will vary according to the specific biological context and the heterogeneity expected within the cell population under study. For example, if the objective is to capture all the cell types present in a tissue, it is advisable to consider methods that yield thousands of single cells, even with lower coverage per cell, because differences between cell-types involve so many genes that even a shallow characterization will be enough to distinguish them (57). On the other hand, if the focus of the study is the in-depth characterization of a specific cell-type that shows subtle differences in "state," capturing more genes per cell can be more informative and accurate (57), even if only hundreds of cells are analyzed.

The number of cells that can be captured might also be conditioned by the available tissue size and/or the abundance of the cell types of interest. Approximate estimation of the number of cells required for a given experiment can be based on prior knowledge about the biological setting; alternatively, a more

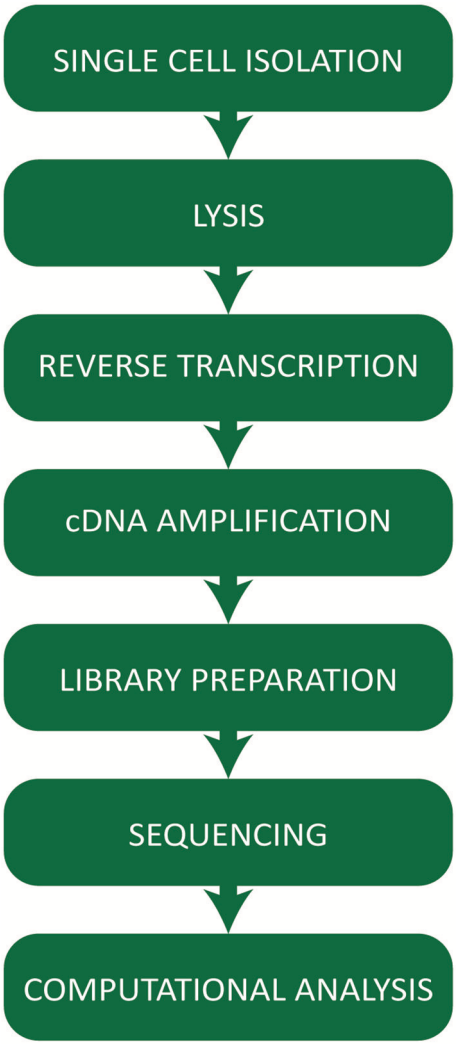

FIGURE 1 | Workflow summarizing the critical steps of a typical single cell RNA-sequencing experiment.

precise estimate of expression mean levels and dispersion as well as dropout rate can be obtained by specialized tools like powsimR (58), that predicts power and sample size requirements to detect differential expression by fitting a polynomial regression to data provided by the investigator, or applying user-generated parameters.

To choose the method for single cell isolation and capturing, the desired experimental outcome needs to be weighed against the availability and source of tissue and/or cells. Indeed, the source of cells, ranging from 2D cell cultures, to small cell aggregates, tissue sections, small fresh or frozen biopsies and large tissue samples, crucially determines the process to follow (**Table 1**). The availability of tissue sections and the need to preserve tissue structure and single cell localization, restricts the choice to laser capture microdissection (LCM) (59–61). This is a time consuming and very low throughput method, requiring staining to define cell boundaries and/or types which is useful to capture single cells in a specific micro-niche (62). Direct single cell trapping from the tissue was used in a variety of settings, including to study the unique identity of mouse and human motor neuronal populations based on their position along the spinal cord and most importantly to uncover the paracrine effects of mesenchymal stem cells (MSCs) on the myocardium of infarcted mouse hearts (63, 64).

All other techniques require a single cell suspension to be used as input. In this regard, it is important to consider that mechanical and/or enzymatic cell isolation can affect cell viability and potentially gene expression profiles, thus, exposure to enzymes (e.g., trypsin or collagenase) and higher temperatures should be minimized. A number of different methods are available to capture single cells (**Table 1** and **Figure 2**).

Micromanipulation allows to manually pick single cells in suspension derived from culture or tissue using an inverted microscope and glass micro-pipettes (67, 69). Even if this method is time consuming, it can be useful to isolate single cells from samples with very few cells, such as early embryos or for large cells like CMs that cannot be unbiasedly selected by current flow sorters or most microfluidic apparatuses, and finally can be also used to select single nuclei (69). The purity of cells obtained will depend greatly on the operator.

The use of flow cytometry for cell capturing has the advantage of selecting and sorting single cells based on their expression of surface markers, fluorescent reporter proteins and/or fluorescent dyes defining their functional status (e.g., viability markers, cell cycle staining), allowing single cell multi-parametric, high throughput sorting into plates (e.g., followed by Smart-seq2) or in a tube for droplet-based methods, Massively parallel RNA single cell sequencing (MARS-Seq) (21), or virtually any other scRNA-seq application. Additionally, unique advantage of FACS is to perform index sorting, allowing the record of the fluorescence information of each parameter analyzed for each single sorted cell and to index it with the position of the sorted event. This enables the retrospective interrogation of flow cytometric parameters of unbiasedly sorted cells for which gene expression profiles has been acquired, providing a deeper understanding of the mechanisms involved in the function of that given cell, and potentially leading to the identification of new markers for populations of interest (70, 71). Importantly, FACS efficacy, accuracy and purity of >95% has been widely demonstrated (72, 73). The major limitation appears to be the relatively large amount of starting cells required (more than 10,000) and the size of sortable cells (19). Indeed, larger cells cannot be accurately and unbiasedly selected by FACS nor by most droplet-based methods. This is an important limitation for the study of single CMs, which reach a length of 150 μm in healthy hearts and even longer in certain disease states. A relatively new instrument, ICELL8, has the capacity to process cells of any size, although with medium throughput [up to 1,800 cells (68)]. The system is based on the use of a nano-dispenser that delivers cells to a chip containing 5,184 nanowells, each one preloaded with oligos which contain oligo-dT, barcodes and unique molecular identifiers (UMIs; as described in the next section); it integrates imaging to discriminate wells containing a single cell vs. multiplets and

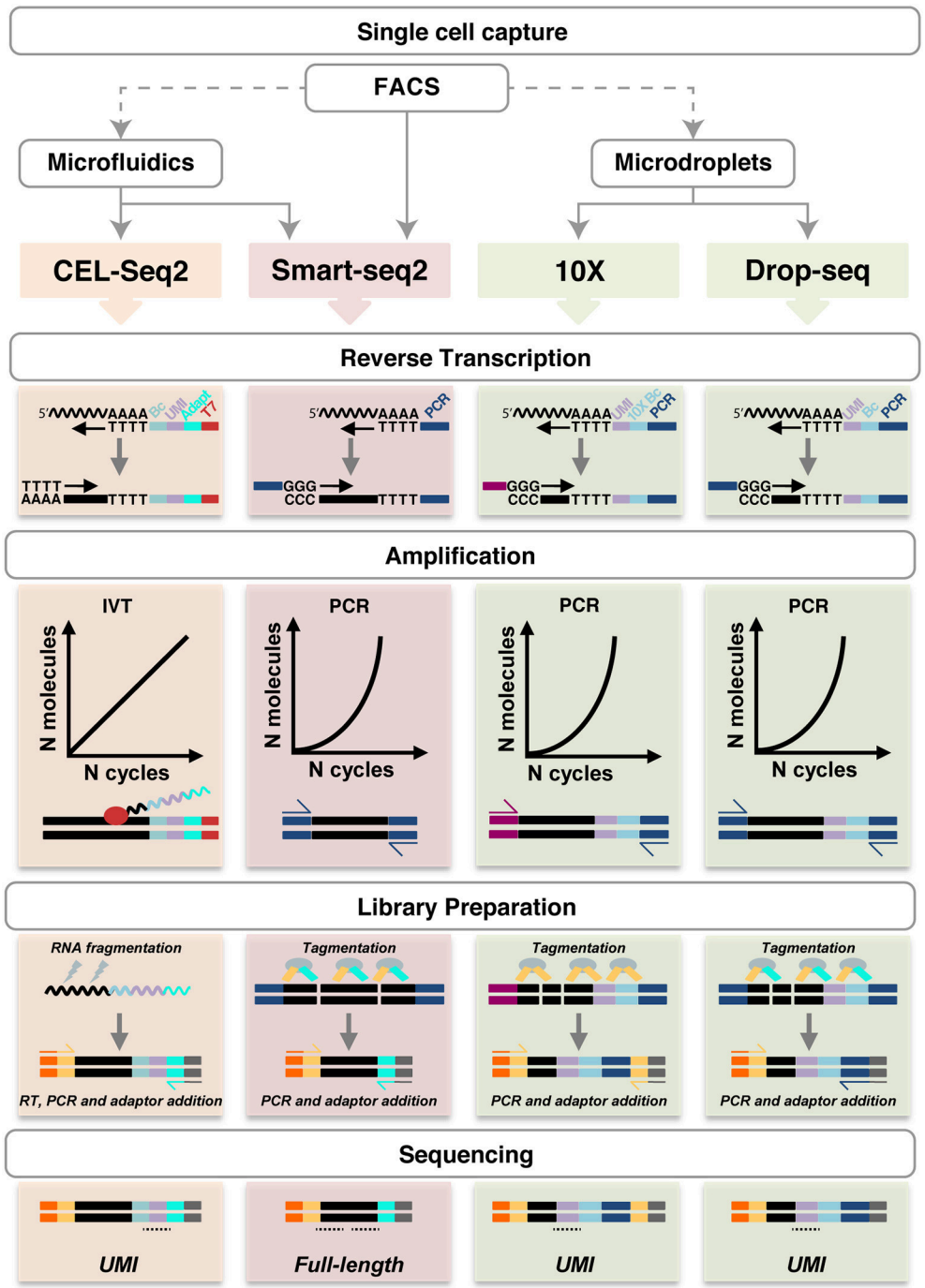

FIGURE 2 | Schematic representation of single cell RNA sequencing experimental pipelines. FACS (with or without Index Sorting), microfluidics and microdroplets are the main methods used for single cell capture. Notably, FACS can be performed as a preparatory step before the other capture techniques. CEL-Seq2, Smart-seq2, 10X Genomics and Drop-seq are shown. Single cell capture is followed by lysis and reverse transcription using oligo-dT primers, which also introduce UMIs (lilac), barcodes (light blue), adapters (cyan), the T7 promoter (red) or PCR primers (dark blue and magenta), as shown in each specific method. Smart-seq does not include early barcoding. Second-strand synthesis in performed by poly(A) tailing (CEL-seq2) or by template-switching (in the other methods). The cDNA is then amplified linearly by IVT using the T7 promoter, or exponentially by PCR. During library preparation, the amplified molecules are fragmented by physical (CEL-Seq2) or enzymatic means (Smart-seq2, 10X and Drop-seq). Fragments are ligated with adaptor sequences required for cDNA amplification (yellow and cyan) and sequencing (orange and gray); in CEL-Seq2, this requires an initial RT step. The resulting sequencing libraries allow UMIs counting or full-length coverage. UMI, unique molecular identifier; Bc, barcode; IVT, *in vitro* transcription. Undulating lines represent RNA, solid blocks DNA, ovals enzymes, dotted lines sequencing reads. For more details, see https://teichlab.github.io/scg_lib_structs/.

TABLE 1 | Comparison of the main features of commonly used single cell capturing techniques.

	Plate-based (13, 16)	**Valve-based microfluidics (65)**	**Droplet-based microfluidics (66)**	**LCM (63)**	**Micromanipulation (67)**	**ICELL8 (68)**
Need for dedicated equipment	No	Yes	Yes	Yes	No	Yes
Samples	Cells in suspension or dissociated	Cells in suspension or dissociated	Cells in suspension or dissociated	Tissue sections	Cells in suspension or dissociated	Cells in suspension or dissociated
Volume	Microliter	Nanoliter	Nanoliter	Nanoliter	Microliter	Nanoliter
Starting cells (minimum)	>10,000	Thousands	2,000-10,000	NA	Any	Hundreds
Number of cells captured	Hundreds	Hundreds	Thousands	Tens	Tens	Hundreds

Need for dedicated equipment refers to the necessity for equipment exclusively designed for single cell RNA-seq capturing; samples refers to the source of single cells; volume indicates reaction size; starting cells reports the typical minimum number of cells required (NA, non applicable); number of cells captured refers to the typical number of events selected by the indicated technique.

live/dead cells based on labeling with fluorescent dyes (68). Alternatively, large cells can be investigated by single nuclei RNA-seq (snRNA-seq), which was reported as sensitive and specific for the identification of CMs subtypes and an effective mean to profile expression dynamics in previously inaccessible frozen tissue (69, 74).

Additional approaches for capturing single cells are microfluidic-based devices and their combination with micro-droplets methods. Microfluidic systems enable sorting into individual compartments, and in the case of the valve-based Fluidigm C1, visual inspection is possible before further processing of the cells (12, 65). The device requires an input of minimum 1,000 cells with a throughput of 96 cells per chip and cell recovery can be low (67, 75).

The combination of microfluidics with micro-droplet methods (droplet-based microfluidics) offer even more advantages, such as lower sample consumption and contamination risks, ultimately, reducing volumes of reagents used and therefore costs (11, 76). Drop-seq was one of the first methods developed that enabled highly parallel analysis of individual cells by RNA-seq via encapsulation of cells in nanoliter droplets with DNA-barcoded beads allowing to analyse 44,808 cells from the retina and identify 39 transcriptionally unique cell types (77). Similarly, the indexing droplets (inDrop) method is based on capturing cells in barcoded nanoliter droplets which, in this case, contain a hydrogel carrying photocleavable combinatorially barcoded primers. The system was initially used to demonstrate a heterogeneous differentiation potential after leukemia inhibitory factor withdrawal in thousands of single mouse embryonic stem cells (78). Within this space, the 10X Genomics system is of recent development and is becoming a first choice for many researchers because of its flexible workflow and high-throughput (66). This micro-droplets system is based on the encapsulation of 500 to 20,000 single cells (or nuclei) thanks to their solution to generate nano-droplets. The process has a capturing efficiency of more than 50% thanks to single Poisson distribution loading and is significantly faster compared to inDrop or Drop-Seq allowing to capture eight samples within minutes with massive throughput results (66). One potential disadvantage is the risk of capturing cells' doublets (or multiplets), nevertheless, contamination's rates of the preparations can be empirically predicted mixing cells derived from different species or, in the absence of internal controls, a more challenging approach is to use computational methods (21, 22, 66, 77, 79). This system has been used in many areas of research demonstrating the power of single cell transcriptomic analysis, which goes well-beyond the mere cataloging of novel cell types. For instance, in a recent study of nearly 100,000 single cells from human lung cancer, 52 stromal cell subtypes were defined, including novel subpopulations hitherto considered to be homogeneous and for which novel functional roles were identified (30). These included fibroblasts expressing different collagen sets, immunomodulating endothelial cells, and disease associated changes in T-cell subtypes pointing to novel immunotherapy targets as well as new biomarkers (30).

In summary, decision-making for the best suited cell capturing platform requires the evaluation of four parameters: number of cells needed to answer a given biological question, transcriptome coverage needed, input of cells vs. cells' availability, cell size (67, 79).

Reverse Transcription and cDNA Amplification: Available Options

After the isolation and lysis of single cells, scRNA-seq requires the conversion of their RNA into cDNA and its amplification to generate libraries with a signal above sequencing sensitivity threshold. Following single cell capturing and consequent cell lysis, virtually all protocols select poly-adenylated RNA and generate cDNA by using poly-(dT) primers (**Figure 2**). Smart-seq (switching mechanism at 5′-end of RNA template) does not include early barcoding. Thus, it requires each sample to be processed individually, but has the advantage of enabling full transcript sequencing obviating to coverage biases caused by incomplete reverse transcription that occurs using poly(A) tailings (8, 14, 31, 80) (**Figure 2**). In 10X Genomics, long fragments of DNA (>50 kb) are encapsulated in a droplet, where they are labeled with semi unique barcode for sequencing by

Illumina technology (66). The barcode presence determines the relative spatial orientation of the tags, creating a map with linked reads, in order to combine information from several tags. Sequencing of this small material requires amplification that can be performed by either polymerase chain reaction (PCR) or *in vitro* transcription (IVT). PCR allows for improved sensitivity, but does not retain strand information (13, 14). The optimisation of Smart-seq2 from Smart-seq increased the sensitivity and library yield as well as reduced the costs and the duration of the process down to 2 days (13, 16). Drop-seq was shown to allow the analysis of 10,000 single cells within 12 h, being among the fastest and more cost-efficient, especially to analyse large number of cells (77, 79). The CEL-Seq method was the first to use IVT, obviating to the requirement for a template-switch step and related reduced efficiency (9). Second generation CEL-Seq2, with optimized primers, reagents, clean-up, and library preparation steps has higher sensitivity, lower hands-on time and costs as well as being adaptable to various platforms, including Fluidigm C1, Drop-seq and inDrop (20). The major limitation remains the lack of information on most instances of splicing because of the 3′-bias (20), which can be overcome barcoding individual cells and pooling them for a linear amplification using only one RT round (9, 20). Ensuring full-length coverage across mRNA is essential to analyse alternative splicing forms as well as identification of single-nucleotide polymorphisms, regardless of the orientation of amplification (at the 3′- or at the 5′-end) (14, 81). Ideally, the samples can be barcoded during RT reaction, such as in CEL-Seq, or during the following step of sequencing and library preparation (Smart-seq/Smart-seq2). In order to reduce noise created by duplets and improve efficiency in gene expression analysis, UMIs have been introduced as internal validation controls; thus, randomly barcoding each individual mRNA molecule during the reverse transcription reaction can be used to determine the absolute amount of mRNAs in a targeted single cell (12, 20, 22, 42, 77, 78).

The choice of the ideal method needs to take in consideration sensitivity, accuracy, precision, power, and efficiency of the cDNA conversion and its amplification plus the throughput of the libraries generated. Work was done to compare methods in parallel experimentally and computationally (79, 82). An integrated framework to analyse scRNA-seq methods performances was developed by comparing 15 protocols computationally from 28 published single cell studies and 4 protocols experimentally (82). Sensitivity in detection of gene expression, tested using spike-ins, was generally high and largely dependent on sequencing depth. Nevertheless, accuracy did not depend as much on sequencing depth and was lower in CEL-seq, and MARS-Seq, possibly because of higher performance variability of these methods (82). Importantly, endogenous RNA was shown to be much more efficiently captured and amplified than ERCC spike-in molecules with consequent underestimated measures of sensitivity (82). Obviating to the limitations of a comparison based on exogenous spike-ins, captured at lower efficiency than endogenous transcripts, a comparison based on endogenous mRNAs found Smart-seq2 to be the most sensitive and accurate method. Indeed, Smart-seq2 has a lower drop-out rate and detects a common set of over 10,000 genes in more cells compared to UMI-based methods. However, CEL-Seq2, Drop-seq, MARS-Seq, and SCRB-seq (single-cell RNA barcoding and sequencing) have lower amplification noise (79). Finally, as cost can be a factor in deciding which approach to choose, Drop-seq is more cost-efficient for transcriptome quantification of large cell numbers, while MARS-Seq, SCRB-seq, and Smart-seq2 have higher efficiency when analyzing fewer cells as shown by power simulations (79).

A Few Considerations About Sequencing Depth

As mentioned above, sequencing depth is an equally important aspect of the design. Higher depth clearly allows a higher resolution in describing the cellular landscape under study based on single cell gene expression with an upper limit determined by the technology in use. Ultra-low coverage sequencing (<10,000 reads/cell) have been used to identify cell types in heterogeneous tissues, however, this number heavily relies on the diversity of the cellular landscape under investigation and finer distinctions and resolution of the genes set explaining variation require moderately shallow sequencing depth (50,000 reads/cell) (65). Recently, a comparison of different single cell RNA-seq technologies based on the detection of exogenous spike-ins, showed that sensitivity (the minimum number of detectable RNA molecules) is strongly affected by sequencing depth, with only a slight improvement after 10^6 reads/cell and saturation at $\sim 4.5 \times 10^6$ reads/cell (82). These data suggest that one million reads per cell is a good target. On the other hand, accuracy (the correlation between known input molecules and quantified expression values) showed saturation at 250,000 reads/cell.

Computational Methods for Single Cell Transcriptomic Analysis

The increase in protocols and applications for single cell RNA-sequencing has been paralleled by the development of a multitude of computational tools and methods for single cell data. Some methods initially designed for bulk analyses can be applied to single cell experiments. However, single cell data tend to be noisier given the small amount of starting material, and several specific tools have been developed [reviewed in Stegle et al. (83)]. Here, we give an overview of the most common tools and methods employed in single cell transcriptomics' workflow analysis (**Figure 3**). We will not cover, however, tools and pipelines developed to be used in conjunction with proprietary technologies and systems (e.g., Cell Ranger by 10X Genomics or Singular by Fluidigm). Finally, we point the reader to the exhaustive archive at https://github.com/seandavi/awesome-single cell, which contains an exhaustive list of tools for single cells analysis, plus links to several in-depth tutorials, journal collections, and various resources.

Raw Sequencing Data Processing

Following demultiplexing, the earliest steps of the analysis pipeline (**Figure 3**) include assessing the quality of the data and preparing them for downstream analyses by filtering out low

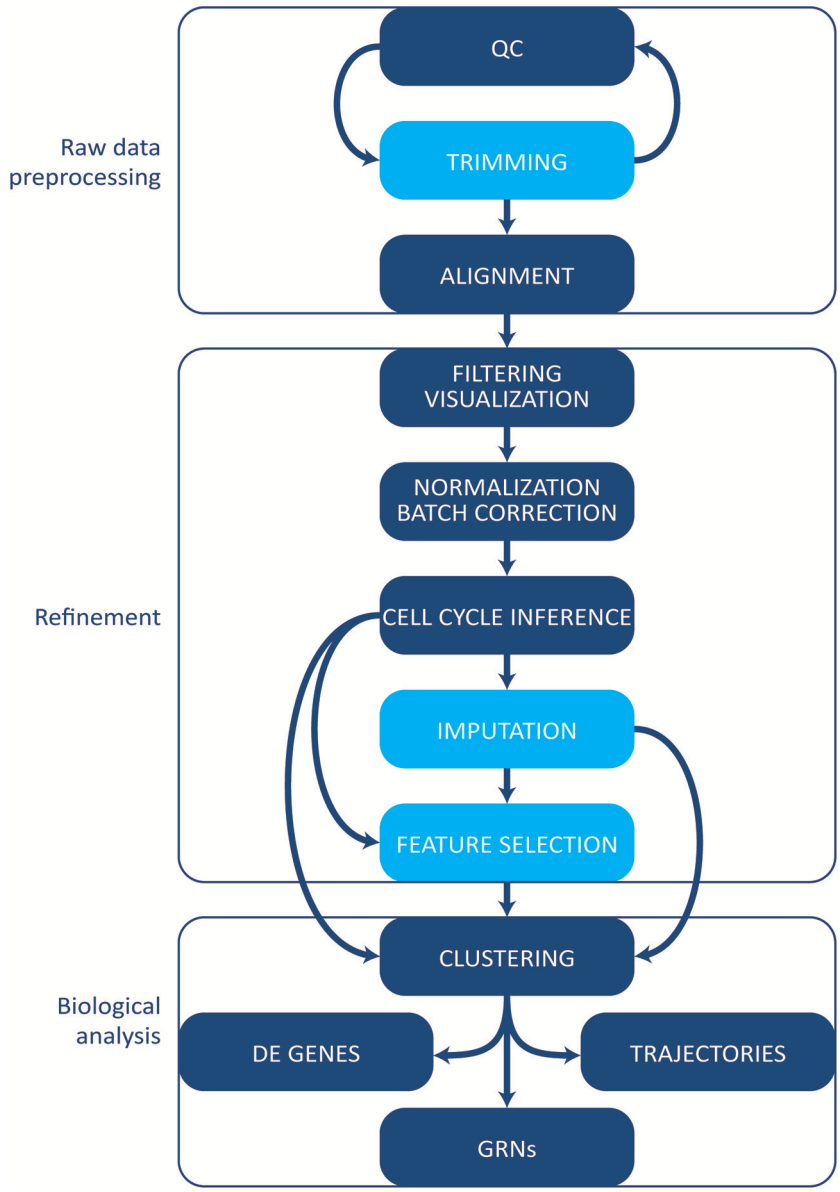

FIGURE 3 | Computational analysis pipeline. The analysis can be broadly divided into three main stages (raw data processing, refinement and biological analysis), each including several steps. Initial QC and trimming can be performed iteratively, multiple times. Light blue boxes indicate optional steps. Details are given in the text. QC, quality control; DE, differentially expressed; GRNs, gene regulatory networks.

quality or contaminating reads and trimming adapters, primers, and low quality portions of the reads. Although trimming can be bypassed if the quality of the raw data is satisfactory, it generally allows for a faster and more accurate alignment.

Popular tools used at these stages include FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for quality control and Cutadapt (https://cutadapt.readthedocs.io/en/stable/guide.html) for trimming [alternative methods are reviewed in Conesa et al. (84)]. Some tools, like AfterQC (85), perform multiple operations (QC, filtering, trimming); also, while the steps mentioned above are performed on each sample separately, some tools are able to complete operations on multiple samples at once, such as SequenceImp (86), or to aggregate multiple outputs from a large number of samples in a unified report, such as MultiQC (http://multiqc.info).

Alignment and Detection of Transcripts

After the initial processing and QC, alignment to the genome or transcriptome is performed in order to detect and quantify the transcripts in each cell. The choice of aligning to the genome

or the transcriptome depends on the users' specific objectives. Nevertheless, in both cases, if spike-ins were used, their sequence must be added to the appropriate reference before alignment. Aligning to the transcriptome is usually faster but only feasible when dealing with well-annotated species such as *Homo sapiens* or *Mus musculus*, and even then, it cannot obviously discover new transcripts, although it grants the possibility to characterize isoforms. On the other hand, alignment to the entire genome can count both known and unknown transcripts but requires "splicing aware" tools to correctly detect splicing junctions. STAR is a widely used program for transcripts alignment to the genome that allows the discovery of non-canonical splicing patterns and chimeric transcripts (87).

UMIs can be directly used to count transcripts when alignment to the genome is performed (**Figure 3**); otherwise, expression levels need to be quantified by counting the reads aligned to each gene (HTSeq or featureCounts) (88, 89). Additional feature counting is not required if full length transcripts were sequenced allowing to use pseudo-aligners, such as Salmon (90) and Kallisto (91), that have lower requirements in terms of computational resources, resulting in a faster alignment, and are more robust to sequencing errors.

Filtering

Once sequencing data have been aligned to the reference and transcripts are counted, several filtering steps are necessary to remove outliers or low-quality data, which might include wells containing zero or multiple cells, or broken or dead cells. The scater package (92) for the R environment (https://www.R-project.org/) is a useful tool to handle single cell data, perform filtering steps and preliminary exploration on the data. Some per-sample metrics are used as measures of sample quality, most notably the number of reads mapped (also referred to as library size or sequencing depth), the number of features (genes or transcripts) detected, and the percentage of reads mapping to mitochondrial genes or to spike-ins (when these are included in the design). Mapped reads and detected features should produce near-normal distributions, but the absolute values depend on the protocol used. Increased mitochondrial RNA content is usually associated with dead cells, however, cells with increased mitochondrial content and activity, as cardiac muscle cells, might physiologically produce a higher percentage of reads mapping to mitochondrial genes. The distribution of these metrics alone allows to spot outliers; however, more sophisticated approaches that considers both technical and biological features (such as genes upregulated or downregulated in broken cells) are available (93). Importantly, filtering data originating from doublets or aggregates of more cells can be coupled with demultiplexing, as done by demuxlet (94), or use methods based on the read counts, such as cellity, DoubletDecon (95), DoubletFinder (96), and DoubletDetection (https://github.com/JonathanShor/DoubletDetection).

Normalization and Removal of Confounding Effects

Normalization is required to remove systematic technical variation and ensure accurate inferences of expression levels. Theoretically, external spike-ins, which are added in the same amount to each cell, could be used to detect artificial variation introduced by the procedure (97). Although this approach is reliable in some instances (98), inconsistencies among replicates have prompted the need for alternative methods, also needed when no spike-ins are used.

Normalization designed for bulk RNA-seq relies on endogenous transcripts, global scaling, and scaling factors such as reads per million (RPM) or reads per kilobase per million (RPKM) (99) performs inconsistently for single cell data analysis, particularly when sequencing depth is low. Moreover, global scaling does not accommodate for transcripts specific biases deriving from systematic variation in the relationship between read counts and sequencing depth in scRNA-seq. To overcome this limitation and avoid over- or under-correction of some transcripts, SCnorm (100) splits transcripts in classes based on the dependence between counts and sequencing depth, then estimates a scaling factor for each class and normalizes the classes separately.

Besides library size, batch effects arising from sample processing should be minimized with an appropriate experimental design, for instance introducing replicates or with a balanced design including different conditions/individuals on the same plate or sequencing lane. Notably, to integrate data sets that are produced in different laboratories and at different times, reduction of batch effects is critical to avoid compromising the interpretation of data. Dimensionality reduction methods such as principal component analysis (PCA) can help detecting batch effects. Scater can plot PCAs and estimate the correlation between PCs and technical variables such as batch, plate, or replicate, as well as detected features or library size (92).

Once confounding factors have been identified, these need to be removed, for instance using control genes (or spike-ins) or negative control samples for which the covariates of interest are constant via the RUVSeq package (101), or by leveraging a subset of the population shared between batches (102).

While it might hold biological interest *per se*, cell cycle stage is another possible confounding factor: individual cells are typically not synchronized, and fluctuations in gene expressions across the cell cycle might mask or inflate cell heterogeneity, with an effect not limited to known cell cycle genes (103). Cell cycle stage can be treated as a latent variable, and accordingly modeled and corrected for, improving the identification of cell populations and the identification of correlated patterns of expressions across cells (103). A different approach, implemented in the cyclone tool, uses a set of six predictors to explicitly assign cell cycle stage to each cell, for G1, S, and G2/M phases (104).

Post-normalization Processing

Further post-normalization processing is required to tackle the main technical features of single cell RNA sequencing: noise and sparsity (abundance of missing values). Variability in the efficiency of RNA capture, retrotranscription, and sequencing can introduce technical noise which might mask the biological signal of interest. Modeling the contribution of noise allows to remove it or use it to select genes which are less affected by it. Endogenous oscillating genes, given their predictable nature, can

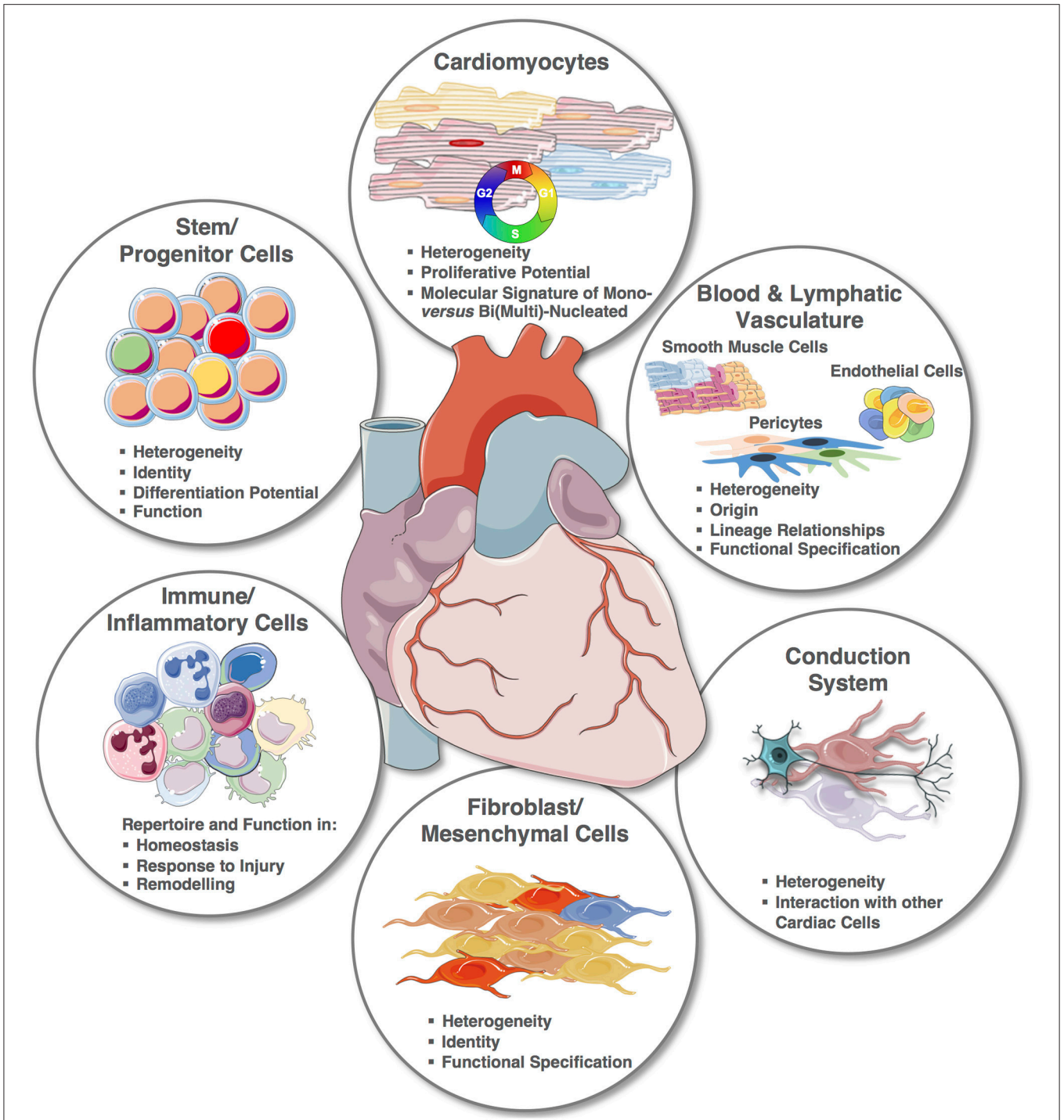

FIGURE 4 | The cellular cardiac landscape: expected deliverables and improvement using single cell and spatial transcriptomics. Single elements used to construct the figure were taken from https://smart.servier.com/ and minor modifications (e.g., color) were applied.

be used to estimate technical noise (105). A different approach to model experimental noise and the dependence between noise and read count is to use external spike-ins that are added in fixed, identical quantity to each sample and should only be subject to technical variability (97, 106, 107).

To improve signal-to-noise ratio, feature selection can be applied. Although, this is not compulsory, it reduces computational times, and is required by some tools used in further steps. Feature selection can take advantage of spike-ins: after estimating the effect of noise using spike-ins, selection of

genes with a significantly higher variability than that expected by the effect of noise alone, are used to study the heterogeneity across cells and make inferences on their biological relevance (106). Another approach to feature selection is based on the rate of dropouts, genes that are highly expressed in some cells and not detected in others, a less noisy measure than gene expression variability (108, 109).

The abundance of dropouts (or sparsity) is a relevant feature of single cell RNA-seq data, and can be alleviated by imputing missing values using the information from co-expressed genes, or from similar cells, with several tools developed to recover the "true" expression signal (110, 111). Interestingly, a recent comparison of several imputation methods (112) concludes that no imputation method outperforms all the others in every situation.

Clustering: The First Step for a Solid Biological Analysis

One of the key aims of single cell approaches is the identification of different cell populations. When the biological background is well-known, checking expression profiles of known marker genes will help retrieve known cell types. This approach requires the application of dimensionality reduction methods such as PCA or t-distributed stochastic neighbor embedding (tSNE) to the refined gene expression counts, in order to identify and visualize subgroups. However, PCA usually requires assumptions that do not hold with scRNA-Seq analyses, and tSNE is better suited for visualization purposes, but is not appropriate for dimensionality reduction in preparation of further analyses (113). An alternative dimensionality reduction approach, specifically tailored to the high proportion of dropouts in single cell RNA-sequencing, is the zero-inflated factor analysis (ZIFA) which specifically models the dropout probability (114).

The identification of unknown and possibly rare subpopulation without prior knowledge, on the other hand, requires unsupervised approaches. Generally speaking, these methods compute one or more measures of "distance" between samples (115), then group cells based on their differences or similarities, as described by these distances. Different strategies can then be employed: hierarchical clustering, for instance, organizes cells in a tree structure according to their "distance," following an agglomerative or divisive algorithm. K-means approaches, on the other hand, assign cells randomly to a predetermined number *k* of clusters, then adjust the assignment iteratively reassigning cells to the closest cluster, until no more reassignment is obtained. It is important to note that all clustering methods require some *a priori* decision from the observer, be it the height where the tree should be cut in hierarchical clustering, the *k* number of clusters or neighbors in k-means, the density parameters in density-based clustering, or the number of neighbors for each cell when constructing a graph. This depends on how fine or coarse is the cell types' definition required to address the biological question. This can switch from a smaller number of well-differentiated clusters, representing cell types or abundant populations, or a larger number of clusters reflecting small differences and highlighting rare sub-populations, and fine changes in cell states (109).

Biological Analysis Beyond Clustering and Toward Functional Inferences

The ultimate goal of identifying cell subgroups is the biological inference that can be drawn from it. This typically consists in identifying genes which are differentially expressed (DE) across cell populations, and notably pointing to marker genes specifically associated with one of the identified clusters. Some clustering tools also perform these tasks; for instance, SC3 (116) automatically determines DE genes and marker genes using non-parametric statistics. Rather than simply testing for different expression levels, methods such as SCDE (117), explicitly model, and keep into account, the probability of undetected genes.

Well-beyond this initial descriptive step a number of functional inferences can be generated on dynamic processes, including intracellular gene regulatory networks but also on intercellular mechanisms. Even when single cell experiments only take a snapshot of a process, the cells can theoretically be reordered along a "trajectory," sometimes called "pseudotime," where each cell represents a different state of the progression (118), allowing the identification of transcriptional dynamics and differentially expressed genes across cell types/states. Trajectory inference methods include a dimensionality reduction stage and, possibly, clustering followed by a trajectory modeling stage (119). Some methods, such as, TSCAN (120), infer a linear trajectory, while others, including Monocle, its new implementation Monocle2 (121), and K-Branches (122), reconstruct a branching trajectory. Not all methods aim to a unidirectional trajectory: Oscope (105), for instance, infers oscillatory trajectories, aiming to identify oscillating genes and reproduce periodic signaling pathways such as those taking place in embryonic development (123).

Dimensionality reduction is a key step of trajectory inference, and indeed a family of tools for dimensionality reduction, diffusion maps (124), inherently reconstruct a trajectory. Importantly, diffusion maps have been adapted to single cell RNA-seq by accounting for uncertainties and missing values (125), as implemented in the R package destiny (126). These are used to identify the diffusion-space direction which most probably represents true biological effects. An exhaustive list of pseudotime ordering algorithms is found at https://github.com/agitter/single-cell-pseudotime.

Systematic computational analysis of the molecular interrelationships between cells in single cell populations studied can be performed by predicting and visualizing cell-cell communication based on the creation of a repository of curated ligands and receptors, which draws on existing resources and the literature (26, 127) using the biomaRt R package (128).

The acquisition of transcriptomic data at the single cell level has enormous potential to infer gene regulatory networks (GRNs). These can be viewed a set of genes active in a specific moment or condition, and the set of transcription factors activating them (52). A first class of approaches, such as BTR

(129) and LEAP (130), requires cells to be ordered across different time points or in pseudotime trajectories, and reconstructs a network as a sequence of activations of transcription factors, aiming to predict the effect of perturbations of such sequence. A second class of methods, such as SCENIC (131), uses cells from steady states, leveraging co-expression patterns and representing GRNs as global networks, leading to the identification of key regulators for different cell states even on snapshot-type datasets and independently of any temporal or pseudotemporal ordering.

Recently, the remarkable development of technologies like spatial transcriptomics, a technology used to spatially resolve RNA-seq data (132), or FISH-based techniques (133), requires the integration of spatial information with mRNA profiling of single cells. SpatialDE allows to fully integrate spatial information and the detection of differential expression (134), enabling the identification of linear or periodic expression patterns, clustering genes based on their spatial expression patterns, and describing tissues on the basis of marker genes' expression patterns (132).

In summary, the computational approaches available allow for unprecedented biological functional inferences and highlight the need for the development of teams including wet-lab biologists, computational biologists and clinicians to exploit the technologies and data available in understanding cardiac disease.

Before moving to the review of scRNA-seq applications to cardiac studies, a short overview of single cell qRT-PCR as a rapid platform to study the expression of tens of genes in hundreds of single cells is discussed. Methods for cell selection are similar to the ones reported above for scRNA-seq. The major advantage of single cell qRT-PCR is the ability to go from cell isolation to analyzed results in less than a working day; on the other hand, scRNA-seq methods tend to be considerably more expensive and laborious, also considering the much higher requirements for data storage and computational power for data analysis. (25, 135). In deciding which approach to use, one shold consider that single cell qRT-PCR is useful to analyse up to a few hundreds cells, while scRNA-seq allows to survey up to tens of thousands of cells. Moreover, single cell qRT-PCR is tipically used to test the expression of tens of pre-selected genes, while scRNA-seq detects, in theory, the whole transcriptome, with the general limitation of sequencing depth. Albeit, full-length sequencing methods such as Smart-seq2 are potentially able to discriminate between different isoforms, most of the methods currently used, such as 10X Genomics, only produce a count of transcripts sequencing UMIs, with no isoform discrimination.

A series of qPCR assays were used to compare gene expression profile of different cells from cardiac lineages (cardiac progenitors, smooth muscle cells, CMs, endothelial cells, fibroblasts) with embryonic stem cells (mESCs), using the detected profiles to later classify unselected E10.5 cells and finely compare *in vitro* mESC- and *in vivo* embryo-derived cardiac cells. These studies highlighted a propensity of Nkx2-5+ progenitors from *in vitro* mESC to become smooth muscle cells or CMs, while the ones from embryos had preferential differentiation toward CMs or endothelial cells (24).

Data have also been obtained from freshly isolated cardiac cells of the adult heart. Single cell gene expression analysis for a selected number of genes was proven to critically augment the definition of adult cardiac progenitors, candidate cell therapeutic products and putative *in situ* cell targets for reparative and/or regenerative purposes in myocardial ischemia (136). Indeed, the combination of FACS, clonogenicity assays and single cell qRT-PCR for about 40 genes on a few hundred of cells permitted the refinement of adult cardiac Sca1+ cells into four discrete populations. Sca1+ CD31+ PDGFRa- cells were found to be enriched for endothelial lineage markers, while Sca1+ CD31- PDGFRa+ cells precisely track a cardiogenic molecular signature (25). Importantly, co-expression of Sca1 and PDGFRa with the side population phenotype, that *per se* enriches for cardiogenic and cardioprotective features, allowed to identify a cardiac subpopulation that is over 30% clonogenic (25).

Interestingly, single cell gene expression was also used to study the phenotype of human induced pluripotent stem cell (iPSC) derived CMs, an alternative cell therapeutic product for myocardial infarction, in a mouse model (137). Combining molecular imagining techniques, microfluidic single cell profiling and laser capture microdissection, iPSC-CMs were shown to contribute to heart repair via an early protective paracrine effect on the ischemic microenvironment.

As quantitative RT-PCR still represents the gold standard for gene expression analysis, its application at the single cell level is a rapid and precise tool to be used alone or in conjunction with single cell RNA-sequencing, as a preliminary exploration for selection purposes or as a validation tool (24, 138).

SINGLE CELL STATE-OF-THE-HEART

Single Cell Technologies Provide a Novel Perspective and Depth of Resolution to Study the Development of the Cardiac System

As for many other organs, the first single cell 'omics studies on the heart were used to characterize the cardiac cellular landscape during development. Within this space, the advantages of single cell transcriptomics analysis include an increased bandwidth to capture rare cell types like stem and progenitor cells, and the capacity to study cellular states at the moment when fate decisions are executed, overcoming limitations of single cell transplantation experiments or lineage tracing that enable to pick events only once they have occurred.

Characterization of cardiac progenitors in a very narrow window of time during early development (embryos of early allantoic bud, late allantoic bud, early head fold) was possible using a combination of micromanipulation for cell selection and single cell qRT-PCR for validation of cells identity, followed by scRNA-seq (43). The integration of scRNA-seq data and subsequent validation steps, including lineage tracing experiments, revealed a rapid dynamic shift of the expression profile of *Tbx5*+ cardiac progenitors with diverging patterns between first and second heart fields CMs' precursors.

A more complex and comprehensive approach was applied to study the mesodermal lineage diversification from early gastrulation (E6.5) to the generation of primitive red blood cells at E7.75 (51).The combination of FACS for single cell capture and

Smart-seq2 enabled the profiling of cellular populations that was previously challenged by the limited cellular material available. By sequencing ∼1,200 cells, including epiblast cells sorted based on viability, Flk1+ cardiac mesoderm cells and CD41+ cells that subsequently appear during blood development (139), it was possible to define for the first time the transcriptome of single cells from the developing primitive streak, and a limited number of visceral endoderm and extra- embryonic ectoderm cells, primitive streak, neural plate and head fold (51). Unsupervised clustering isolated ten clusters and correlated marker genes allowing functional inferences and identification of endothelium, blood progenitors, primitive erythrocytes or their anatomical identity (visceral endoderm, extra-embryonic ectoderm, epiblast, early mesodermal progenitors, posterior, allantoic, and pharyngeal mesoderm). Here, diffusion maps were used to make inferences on the transcriptional program underlying primitive erythropoiesis. Further, given the fundamental role of the transcription factor Tal1 in the development of all blood cells, single mesodermal Flk1+ cells from *Tal1* mutant embryos were profiled (51). The combination of loss of function studies with single cell transcriptomic analysis demonstrated, in contrast to previous retrospective knock-down and epigenetic studies (140, 141), that Tal1 promotes hemogenic differentiation of endothelial cells but does not immediately drive a cardiac fate. The difference in time of sampling (141) could justify the differences seen between a precise snap-shot of single cells transcriptomic profiling (51) vs. potentially confounding retrospective studies in bulk (140, 141). It is foreseeable that performing transcriptomic and epigenomic assays (142) in parallel to profile a high number of single cells in a fine time course will further clarify the mechanisms of early mammalian development and more specifically the fate of Flk1+ cells.

From the next stages of cardiac development (E8.5 to 10.5), profiling of 2,233 single cells using a microfluidics approach (Fluidigm, C1 single cell Auto Prep System) and Smart-seq2, combined with unsupervised and supervised clustering allowed the reconstruction of the precise spatial origins of cardiac cells solely from their transcriptional profiles (138). Interestingly, a distilled set of 65 genes was validated and found sufficient to predict location of cardiomyocytes using a multiplex qRT-PCR-based approach (24, 138).

The dynamic transcriptional programs unfolding from midgestion (E9.5) to adulthood (P21) were studied using spatiotemporal RNA-seq analyses of single cells isolated from mouse hearts and captured using microfluidics, to delineate lineage specific transcriptional regulation, including CM maturation, during heart development (143). Classification of different cell types at the single cell level with spatiotemporal resolution allowed the definition of time and chamber lineage-specific gene programs underlying normal cardiac development. Further post-natal maturation occurred by P21 with downregulation of calmodulin-interacting proteins, Bex1 and Bex4, previously shown to promote muscle regeneration (144). Albeit, one should consider the potential bias introduced by size limits inherent to the microfluidics system used to select large mature CMs, these datasets are useful to define precise developmental stages of human and mouse embryonic stem cells-derived CMs, prompting the utility for characterization of induced pluripotent stem cells-derived CMs that are becoming the gold standard in cardiac drug discovery (27, 145). Notably, analysis of single cells from Nkx2.5$^{+/-}$ murine hearts were used as a model for congenital heart disease and allowed to define lineage-specific maturation defects, including expected changes in the CM lineage but also in the endothelial lineage compartment. This supports an instructive role of CMs on endocardium development and predicting underlying mechanisms associated with human heart malformations.

The experiments herein described prove the advantage of using single cell transcriptomics in enabling the study of cellular states at the moment when fate decisions are executed, contributing to the precise deconvolution of lineage decisions previously masked by bulk analysis and, especially in early stages, difficult to study given the paucity of cells available.

Adult Heart and Cardiovascular Disease Models From a Single Cell Perspective

Compared to other areas of research—until very recently—the cardiac field has infrequently taken advantage of single cell technologies to study adult heart cells' gene expression and, even less, for epigenetic studies. Although, cells of the adult heart are not readily accessible, a number of protocols have been historically described to dissociate single CMs and single stromal cells. If some skepticism can be raised by the fact that isolation protocols are lengthy and potential side effects on gene expression are a deterrent, the use of whole tissue as comparison and validation experiments obviates any doubt. The only technical impediment remains the size of adult mature CMs (∼150 μm in length) that limits the use of microfluidic apparatuses with upper size limits usually around 50 μM (except for ICELL8).

Albeit the regenerative capacity of the adult heart is meager, there is some evidence that induction of CM proliferation could be a targetable mechanism to induce myocardial self-renewal and repair after injury (146, 147). The proliferative potential, especially the capacity to undergo cytokinesis of CMs in physiological conditions as well as in response to stress, remains unclear. Confounding factors could be the methods of analysis but, most importantly, also the absence or presence of external stimuli that can enhance CMs proliferation and self-renewal (146, 148–150). Recently, to understand the proliferative capacity of CMs, single nuclear RNA-seq was performed in single CMs from healthy and diseased hearts, including pressure overload failing murine hearts and human dilated cardiomyopathy failing hearts (151). Heterogeneity of the myocardial environment in response to stress was uncovered (151). Through weighted gene co-expression network analysis, several subgroups of CMs were characterized, including ones with a gene signature indicative of dedifferentiation and cell cycle entry. Further, two long intergenic non-coding RNAs, *Gas5* and *Sghrt,* were found in highly interconnected nodal hubs of gene regulatory networks and, subsequently, confirmed as essential regulator of CMs' cell cycle entry and de-differentiation programmes (151). These data are a first and encouraging

step toward answering additional fundamental questions: the functional implications of specific gene expression patterns in the various CMs subtypes; which subtypes are elicited or depressed in diverse pathological conditions, including early stages of dilated cardiomyopathies, hypertrophic cardiomyopathies and ischemic disease; the physical localization of CMs subpopulations and how the cellular demographics change in response to stress; finally the concasual relationships with neighboring non-CMs.

A complementary study focused on the cardiac stromal compartment and used a 10X Genomics platform to capture and sequence just over 10,000 non-myocyte cells (26). With a mean of 1,900 genes detected/cell, an expected overall complexity in the composition of the cardiac non-myocyte cellular compartment was revealed and a few additional markers and subpopulations were identified in the fibroblasts compartment and in the lymphocytes and myeloid lineages (26). Interestingly, clustering patterns for female and male cells were largely overlapping but within individual cell types, small levels of sexual dimorphism was detected for selected genes' expression, implying a diverse predisposition of the cardiac stroma response to stress (26).

A further level of complexity was obtained by comparing scRNA-seq results from healthy vs. post-ischemia mouse hearts using the SORT-seq protocol, which is essentially an integration of FACS for single cell capturing and high-throughput multiplexed linear amplification by CEL-seq2 (29, 48). In this study, cell capturing has two limitations: (1) large mature CMs have dimensions above the size of the sorting nozzle, (2) the utilized flow cytometry gates were biased toward large live cells (29). Thus, the representation of the cell types was biased, with 71% of CMs vs. the 30% expected, plus a putative selection of myocytes subtypes due the upper size limits of the sorter outlet (29). Nevertheless, all main cardiac cell types were recognized as seen using differential gene expression analysis and clustering methods. In addition, identification of new putative markers including ones for specific subpopulations were pinpointed. Amongst these, *Ckap4* was defined as a novel marker for activated fibroblasts post-ischemia in mouse, and subsequently confirmed as a marker in human ischemic hearts. CKAP4 function remains to be clarified, although knock-down experiments suggested it controls the expression of activated-fibroblasts genes. Interestingly, in single CMs' sequencing, up to 84% of reads were coming from mitochondrial genes (29). This is surely in part dependent on the high enrichment of mitochondria in CMs; however, while enrichment of mitochondrial related gene categories are amongst the features characteristic for low quality cells in a number of independent cellular models, this is not the case for myocytes (93). Thus, it could be necessary to establish a specific set of features to determine the quality of single heart myocytes based on their gene expression.

Single cell transcriptomics analysis was used to characterize the changes occurring at the single cell level in a mouse model of cardiac fibrosis (28). Co-expression analysis inherent in single cell transcriptomics studies allowed to highlight that both IL-11 and its receptor IL11RA are expressed in activated fibroblasts, the latter defined by a transcriptional profile typical of TGFβ stimulated cells and by induction of extracellular matrix encoding genes (28).

Tissue regeneration using exogenous products, including iPS-CMs, is a highly sought-after therapeutic approach but much remains to be understood regarding the level of differentiation, purity, genomic, and phenotypic stability of induced CMs especially because, until recently, gene expression studies were almost exclusively performed on bulk populations. Reprogramming fibroblasts into induced cardiomyocytes (iCMs) using combinations of cardiogenic transcription factors (152–154) offers an interesting alternative to the use of iPS-CMs to generate newly formed CMs *in vitro* and then used as cell therapy, but also for *in vivo* direct reprograming. To study and characterize the non-conventional differentiation mechanisms driving cell conversion to iCMs, single cell approaches are a suitable option allowing to unpick the heterogeneity of conversion events and the level of differentiation in single cells. Single cell transcriptomics analysis of the early stages during the reprogramming of mouse fibroblasts into iCMs uncovered a heterogeneous group of cells differentiating at unsynchronized pace (155). The definition of different cell clusters based on dimensionality reduction and unsupervised clustering algorithms was the critical step to make functional inferences. Some of these were anticipated, like the reconstruction of differentiation trajectories, the correlation between the expression of each reprogramming factor and the progress of individual cells through the reprogramming process, and the discovery of new surface markers for iCMs (155). Among other discoveries, the downregulation of factors involved in mRNA processing and splicing was found to be critical in reprogramming; interestingly, a specific splicing factor, Ptbp1, was identified and subsequently functionally validated to be a critical barrier for the acquisition of CM-specific splicing patterns in fibroblasts (155). Thus, in this settings scRNA-seq enabled the identification of targets to enhance reprogramming efficiency and new markers for the prospective isolation of iCMs but also demonstrated the immense utility of these methods in studying further models of programming and reprogramming.

An *in vitro* system was used to study the dedifferentiation potential of adult CMs to cardiac progenitor-like cells, using a genetic cell fate mapping system plus single cells transcriptome and further methylome analysis (156, 157). The authors reported a correlation between hyper-methylation of promotor regions and suppression of cardiac specific genes related with maturation, while cell cycle and stemness genes were upregulated suggesting that CMs' de-differentiation depends on epigenomic regulation of the cells transformed into progenitors. Nevertheless, this study relies on very few single cells and confirmation of the specific gene expression regulation via epigenetic modulation would benefit from analysis of the transcriptome and methylome from the same single cell.

Within the cardiac regenerative medicine space, c-kit+ adult cardiac progenitors have been proposed as a cell product albeit controversial data were reported (158). Recently, single freshly isolated adult c-kit+ CPCs were compared with short-term expanded cultures by 10X Genomics and SmartSeq2, in parallel with the meta-analysis of multiple scRNA-Seq datasets, finding substantial transcriptome alterations in the *in vitro* expanded cells (159). This increase in the transcriptome diversity included

the induction of thousands of genes related to cell cycle and metabolism, and loss of expression of identity genes, suggesting a marked change in functional characteristics of *in vitro* expanded cells, with important implications to consider when developing cell therapies than involve previous *in vitro* expansion.

Altogether, these initial explorative studies both in healthy and disease hearts demonstrate how single cell transcriptomics alone has already contributed to uncover novel cell types, further our understating of candidate cell products for cardiac cell therapies and most importantly discover novel functional pathways regulating heart function.

Predicting Achievable Outcomes and Influencing Future Clinical Applications

Thanks to the immense technological advancements of high-throughput single cell gene expression analysis and the high-resolution achieved with scRNA-seq, we can now obtain information on prevalence, heterogeneity and co-expression at the individual cell level for virtually the entire transcriptome. This enables us to get a new perspective on cellular demographics of the heart and improve intracellular and intercellular pathways definition, otherwise unthinkable with bulk analysis and in circumstances where cellular material available is limited. The almost immensurable amount of data being produced requires tight integration and interaction of biological and clinical experts with computational biologists starting from the initial experimental design. In fact, a dynamic and continued assessment of data generation and results is necessary to reach solid conclusions of any single cell 'omic study.

The complete exploitation of the value of single cell transcriptomic results comes from the integration and validation of these data by orthogonal methods. Validation of the expression of subsets of genes can be performed by standard techniques with emphasis on single cell resolution approaches ranging from gene expression validation with or without spatial resolution (e.g., single molecule *in situ* hybridization, single cell qRT-PCR, respectively), to testing the expression of the proteins encoded by the genes of interest, by flow cytometry that allows a higher bandwidth for multiplexing or immunostaining to acquire localization information. Of note, spatial transcriptomics was developed in order to retain spatial information related to the transcriptome of isolated single cells and was elected Method of the Year 2017 by Nature Methods (160). The protocol requires tissue sections that are then divided in a mosaic of tiles of $\sim$100 μm of diameter, and uses spatial barcodes permitting the localization of specific cells and their transcripts by overlaying haematoxylin & eosin stained sections onto microarrays spotted with the barcoded topographies (132, 161). The first reported application of single cell RNA-seq and spatial transcriptomics was to identify *in vivo* genes relevant to endocardial epithelial-to-mesenchymal transformation (162). More recently, spatial resolution proved to be essential in the identification of SOX9 as a key regulator of cardiac fibrosis in a mouse model of ischemic injury and injured human heart (163). Critically, rather than just allowing to localize the cell types identified by scRNA-seq, spatial transcriptomics will expedite the reconstruction of the dynamic architecture of the heart (134). Indeed, changes of gene expression and especially of transcriptomic profiles can depend on the reciprocal interactions with adjacent cells, the migration to a specific tissue site of cells' subsets and by location-specific variation of cell "states." Thus, starting from a single cells and functional micro-niches of few cells, one can envision to reconstruct the changes occurring in the cellular landscape of whole tissues and organs in 3D during specific processes including development, aging, and disease (134).

Despite the recent explorative studies, the cellular cardiac landscape, especially human, remains largely unknown (**Figure 4**). Most significantly, the integration of single cell transcriptomic and spatial transcriptomic provides the platform to reconstruct the cellular demographics of the heart with spatial resolution. For smaller animals, this will require a shorter period of time, and although the reconstruction of the entire human cardiac cellular landscape in 3D will require many iterations, every step will provide new strategic information. For example, cardiac muscle scRNA-seq will alone contribute to the clarification of CMs' heterogeneities within microniches, on the epicardial vs. endocardial fronts; as well as chamber specificities that remain largely unknown. Notably, one of the therapeutic targets for cardiac regeneration is CMs' proliferation, but for clinical translation we still need to: (1) clarify the true endogenous and spontaneous capacity of human CMs to re-enter cell cycle and undergo mitosis since results in mouse remain controversial and (2) define the stimuli that can trigger or improve their proliferation. Importantly, transcriptomic analysis of single mononucleated vs. binucleated CMs and comparison with single nuclei transcriptomics will provide inferences on their proliferative and differentiation status (146, 148–151, 164).

The identity and role of cardiac progenitors in the adult heart remains elusive with a dozen different identifiers already described, and their relationship and function still unclear (136). It is expected that an unbiased 'omic approach will allow to define their molecular signatures and likely deconvolute their ontogeny and function in the healthy and diseased heart.

The heterogeneity of vascular cells across organs and within the same organ or system has been hypothesized for a long time but the advancements in uncovering of subpopulations with distinct functions has been slow because of bulk analysis and largely limited to hypothesis driven studies that can focus on one gene/pathway at the time. The availability of single cell transcriptomics and spatial transcriptomics is providing the right tools to study endothelial cells, smooth muscle cells and pericytes' subtypes and functional changes across vessels of different diameter and function. A recent study proved feasibility and produced outstanding results by sequencing the vascular cells of the mouse brain (165) uncovering the transcriptional basis of the gradual phenotypic change along the arteriovenous axis and revealing unpredicted differences in the cells' molecular signatures with a seamless continuum for endothelial cells vs. a punctuated pattern for mural ones (165). Importantly, brain specific features were highlighted for pericytes in comparison to the lung. These important data provide the evidence for a higher organizational structure of the vasculature, providing a healthy

reference for subsequent disease studies, but also provides an invaluable hypothesis-generating data source.

Although initial scRNA-seq studies in the adult heart have already contributed to highlighting the role of specific genes in the fibrotic response after TGFβ1 stimuli (28) and ischemic injury (29), much remains to be learnt about the identity of fibroblasts, their function, heterogeneity, anatomical specificity and role in cardiac homeostasis and disease.

In recent years, a number of studies have investigated the adaptive and innate immune response following cardiac injury beyond the response to microbial causes, how these affect repair and regeneration and their potential manipulation as therapeutic targets [as reviewed in Prabhu and Frangogiannis (166), Sattler et al. (167), Toldo and Abbate (168), and Wysoczynski et al. (169)]. Single cell transcriptomics focusing on immune cells will provide an invaluable support to decode higher organizational networks triggered in cardiac disease and in physiological conditions. Finally, single cell transcriptomics will also contribute to our understanding on the conductive system of the heart, both in unbiased studies and targeted analysis.

Studying the developing heart at the single cell level has an inherent huge power, not only to further our understanding of the perfect orchestration needed for cells to go from a primordial linear tube to a four-chamber heart and uncover maps and trajectories of cellular differentiation but also, importantly, to discover how lineage-specific gene programs are altered in congenital heart disease (24, 27, 43, 51, 138). This implies a need to study and build a developing atlas of the healthy developing heart in animal models but most importantly in human, and use this as reference to study and understand the genesis of cardiac malformations within single cells, and between them.

Two studies have generated independent atlases for mouse models, providing an initial compendium of single cell transcriptome profiling representing a new resource for cell biology (170, 171). Providing gene expression data from 20 anatomical locations, the atlases allow the comparison across tissues of cell types present in every tissue such as immune cells and endothelial cells enabling hypothesis generation. Strikingly the Human Cell Atlas (HCA) initiative has triggered widespread excitement with its ambitious goals aiming to describe and define the complete cellular landscape of the human body (https://www.humancellatlas.org/) (172, 173). This will be achieved by mapping cells of the human body using a combination of single cell and spatial transcriptomics, resulting in datasets that go beyond descriptive features and will guide functional studies toward the identification of previously unpredictable subtleties of disease. Effectively, the HCA plans to integrate leading-edge technologies to: (1) defining the histological and anatomical position of newly identified cell types/states; (2) outlining lineage decisions with topographic and functional insights; (3) recapitulating precise and specific cell-to-cell interactions including autocrine and paracrine pathways.

Urgent clinical questions, that would immediately benefit from an integration of single cell transcriptome studies and spatially resolved gene expression profiling, include the definition of the changes occurring within the cellular landscape of the border zone following myocardial ischemia; the deconvolution of atherosclerotic plaque's single cell transcriptomic profiles. Inferences in ethiopathogenetic mechanisms that are invisible using standard low resolution and bulk techniques include proliferation, differentiation, survival as well as ligand/receptor pairs to infer functional networks. Additionally, transcriptomic profiling of single cells will undoubtedly enable the definition of novel biomarkers, including parameters such as frequency and pattern of gene expression within tissues and/or specific cell types.

Altogether, these new technologies will not only contribute to address hypothesis-driven questions but, most importantly, will broaden our perspective, providing hypothesis generating platforms to further our understanding on heart function in health and disease. The auspicable future of cardiac disease monitoring is based on low-risk, minimally invasive and disruptive approaches, where multi-'omics single cell approaches will play a leading role.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Extracellular Vesicles Secreted by Hypoxic AC10 Cardiomyocytes Modulate Fibroblast Cell Motility

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Extracellular vesicles (EVs) are small membrane vesicles secreted by most cell types with important roles in cell-to-cell communication. To assess their relevance in the context of heart ischemia, EVs isolated from the AC10 ventricular cardiomyocyte cell line (CM-EVs), exposed to normoxia (Nx) or hypoxia (Hx), were incubated with fibroblasts (Fb) and endothelial cells (EC). CM-EVs were studied using electron microscopy, nanoparticle tracking analysis (NTA), western blotting and proteomic analysis. Results showed that EVs had a strong preference to be internalized by EC over fibroblasts, suggesting an active exosome-based communication mechanism between CM and EC in the heart. In Matrigel tube-formation assays, Hx CM-EVs were inferior to Nx CM-EVs in angiogenesis. By contrast, in a wound-healing assay, wound closure was faster in fibroblasts treated with Hx CM-EVs than with Nx CM-EVs, supporting a pro-fibrotic effect of Hx CM-EVs. Overall, these observations were consistent with the different protein cargoes detected by proteomic analysis under Nx and Hx conditions and the biological pathways identified. The paracrine crosstalk between CM-EVs, Fb, and EC in different physiological conditions could account for the contribution of CM-EVs to cardiac remodeling after an ischemic insult.

Keywords: cardiomyocytes, fibroblasts, endothelial cells, extracellular vesicles, hypoxia, cellular communication

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INTRODUCTION

Cardiac muscle is a highly adaptable tissue that responds to physiological challenges, in part, by releasing paracrine factors including extracellular vesicles (EVs) (1). Exosomes are small nanomembranous extracellular vesicles (30–150 nm in diameter) that contain a variety of molecules including cytokines, membrane trafficking molecules, chemokines, heat shock proteins, and also several types of RNA molecules. Due to their diverse cargo, exosomes play a central role in cell-to-cell communication and we and others have previously examined the role of cardiomyocyte-derived exosomes (CM-Exo) in different stress conditions, such as glucose starvation (2), inflammation (3, 4) or ethanol treatment (5). In response to ischemia, different cardiac cell types, including cardiomyocytes, release high amounts of exosomes and other types of EVs with different cargoes (6, 7). To characterize the changes in CM-EV cargo during ischemia, we performed a comparative proteomic analysis of EVs isolated from cardiomyocytes cultured in normoxia (Nx)

or hypoxia (Hx), and mapped the biological processes activated in both conditions. We found that Hx increased the secretion of CM-EVs containing proteins related to wound healing. Consistent with this, we observed that Hx CM-EVs stimulated fibroblast motility. Our results shed more light on the contribution of CM-EVs to cardiac remodeling.

MATERIALS AND METHODS

All procedures were approved by national and local ethical committees (reference number 2016/0192).

Cell Culture

The human ventricular cardiomyocyte cell line AC10 (8) was cultured in Dulbecco's Modified Eagle's Medium-F-12 (DMEM-F12, Gibco-Invitrogen®) supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen®) and 1% penicillin/streptomycin (P/S, Millipore). Primary cultures of human umbilical cord vein endothelial cells (HUVEC) were obtained from Lonza and were grown in Endothelial Cell Growth Medium-2 (EGM-2) BulletKit™ (Lonza). Human coronary microvasculature (HCAEC; ATCC) endothelial cells were grown in Vascular Cell Basal Medium supplemented with the Endothelial Cell Growth Kit-VEGF (ATCC). Fibroblasts were obtained from skin biopsies after informed consent and were cultured in high-glucose DMEM (Thermo Fisher Scientific) supplemented with 10% FBS and 1% P/S. Cell were maintained under control conditions (Nx) in a humidified atmosphere at 37°C containing 5% CO_2. AC10 cells cultured in Hx were incubated at 2% O_2 for 48 h with glucose deprivation during the first 4 h and DMEM/F12 with depleted FBS during the next 44 h at 2% O_2. Depleted FBS was generated by ultracentrifugation of an FBS and DMEM/F12 (1:1) mix at 100,000 *g* for 16 h. The induction of Hx in cell cultures was monitored by stabilization of hypoxia inducible factor-1 alpha (HIF-1α) expression and cell viability reduction (**Figure S1**).

Extracellular Vesicle Purification

We used ultracentrifugation without sucrose-gradient centrifugation step to isolate EVs from cardiomyocytes. Accordingly, we refer to the isolated pool of vesicles as CM-EVs and not CM-exosomes. Approximately 150 mL of culture media was collected and EVs were isolated by several ultracentrifugation steps as described (9). Briefly, supernatants were centrifuged first at 2,000 *g* for 20 min (Eppendorf 5804 benchtop centrifuge, A-4-62 rotor), 10,000 *g* for 70 min (Hitachi CP100NX centrifuge, Beckman Coulter 50.2 Ti rotor) and subsequently filtered manually through a 0.22 μm filter to eliminate cell debris using a syringe. Then, EVs were pelleted by ultracentrifugation at 110,000 *g* for 120 min (Hitachi CP100NX centrifuge, Beckman Coulter 50.2 Ti rotor), filtered through a 0.22 μm filter to maintain sterility and ultracentrifuged again at 110,000 *g* for 120 min (Hitachi CP100NX centrifuge, Beckman Coulter 50.2 Ti rotor). The manipulation of EV extracts was performed in a laminar flow hood to preserve sterility. EV protein concentration was determined with the Pierce BCA Protein Assay Kit (ThermoFisher Scientific) to ensure equal amounts of protein samples. EVs were suspended in RIPA buffer [1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate in Tris-buffered saline (TBS), Sigma-Aldrich] for western blotting or phosphate buffered saline (PBS) for nanoparticle tracking, electron microscopy, flow cytometry, proteomic and functional analysis.

Extracellualar Vesicle Incorporation Experiments

Capture of labeled EVs by fibroblasts and EC was performed using procedures modified from previous reports (10). EVs were fluorescently stained with carboxyfluorescein diacetate succinimidyl diester (CFSE; 5 μM) (ThermoFisher Scientific) for 15 min at 37°C, and unincorporated dye was removed *via* ultracentrifugation. As a negative control to normalize, the same amount of PBS was added to CFSE to monitor unincorporated dye carried over after the staining steps (PBS control). Cells were seeded at 50% confluency in 24-well plates and incubated with CFSE-labeled EVs at 2 μg/mL (5.00 E + 08 particles/mL). After 3 h of incubation, cells were washed twice in cold PBS, trypsinized and analyzed by flow cytometry using a FACS Canto II at the Cytomics Unit of the Instituto de Investigación Sanitaria, La Fe.

Western Blot Analysis

EVs or cells were lysed in 100 μL of RIPA buffer containing protease (Complete, Sigma-Aldrich) and phosphatase (PhosSTOP, Sigma-Aldrich) inhibitors. Equal amounts of protein samples were suspended in non-reducing Laemmli sample buffer (BioRad) and denatured at 100°C for 5 min. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked with TBS containing 5% (w/v) nonfat dry milk powder with 0.1% Tween-20. Antibodies used were anti-CD9 (Ab 92726, Abcam), anti-TGS 101 (Santa Cruz. Sc-7964), anti-Alix (Santa Cruz. Sc-53538) and HIF-1α (610958, BD biosciences). Detection was carried out using peroxidase-conjugated secondary antibodies with the ECL Plus Reagent (Amersham, GE Healthcare, Munich, Germany). Proteins were visualized using an Amershan Imager 600 (GE Healthcare) and quantified with ImageJ software (NIH).

Nanoparticle Tracking Analysis

EV size distribution and quantification of vesicles was analyzed by Nanoparticle tracking analysis (NTA) using a NanoSight NS3000 System (Malvern Instruments, UK) Samples were suspended in 0.22 μm pre-filtered PBS and dilutions between 1:1,000 and 1:10,000 were used to achieve a particle count between 2×10^8 and 2×10^9 per mL. Measurement of the diameter was performed on 3 independent experiments and showed as mode ± standard deviation.

Electron Microscopy

Electron microscopy was performed as described previously (11). Briefly, isolated EVs were diluted in PBS, loaded onto Formwar carbon-coated grids, contrasted with 2% uranyl acetate and finally examined with a FEI Tecnai G2 Spirit transmission electron microscope. Images were acquired using a Morada CCD Camera (Olympus Soft Image Solutions GmbH). The size of the

EVs was quantified using ImageJ. The diameter of a minimum of 25 EVs in different fields was measured in 3 independent experiments and the median ± standard deviation showed.

Cell Proliferation

To test whether purified EVs from AC10 cells grown under Nx and Hx conditions altered cell viability, AC10 cells, fibroblasts and HUVEC were cultured at a density of 5×10^3 per well of a 96-well plate. Cells were incubated for 48 h with 30 μg/mL (7.00 E + 09 particles/mL) of purified EVs and the Cell Counting Kit-8 (CCK-8) assay was used to measure proliferation following the manufacturer's instructions. The optical density of the cultures was measured at 450 nm in each well 4 h after incubation with the CCK-8 assay solution.

Tube Formation Assay

Capillary-like tube formation, as a readout of angiogenesis, was measured using the tube formation assay as previously described (12). In total, 1.2×10^4 HUVEC were seeded per well into 96-well plates precoated with 50 μl of growth factor-reduced Matrigel (BD Biosciences). Cells were incubated for 6 h with 30 μg/mL (7.00 E + 09 particles/mL) of purified EVs from Nx and Hx conditions to evaluate formation of tube-like structures. The day after, images from three different viewing fields per sample were taken using an inverted microscope (Leica DM6000) with a 10× magnification. Images were analyzed with WimTube online software (WimTube: Tube Formation Assay Image Analysis Solution. Release 4.0. https://www.wimasis.com/en/WimTube).

In vitro Scratch Assay

The scratch assay is a good and valid method to measure cell migration *in vitro* (13). Human fibroblasts were seeded in 12-well culture plates at a density of 1.2×10^5 cells/well. The day after, a straight line was created on the monolayer with a pipette tip. Cells were washed once with PBS to remove the debris and treatments were added in 1 mL of corresponding medium. In total, 30 μg/mL (7.00 E + 09 particles/mL) of EVs obtained from AC10 cells cultured in Nx and Hx conditions were added to the cells. The capacity of the cells to migrate and invade the denuded area was tracked using time-lapse microscopy during 24 h. Image analysis was performed using ImageJ software.

Proteomic Analysis

Proteomic analysis was carried out as described previously (11). Briefly, CM-EVs were harvested from 75 mL of culture medium, suspended in RIPA buffer, and protein concentration was determined using the Qubit® Protein Assay Kit (Invitrogen). A total of 30 μg of protein extract in Laemmli buffer was subjected to 12.5% acrylamide 1D SDS-PAGE electrophoresis and proteins were digested with trypsin. Digestion was stopped with 1% trifluoroacetic acid and 5 μL of each sample was loaded for liquid chromatography and tandem mass spectrometry (LC-MS/MS). Data were analyzed following the ProteinPilot default parameters (ProteinPilot v4.5. search engine, ABSciex). Proteins were arranged by the Unused Protein Score, which is a measure of the protein confidence calculated from the peptide confidence for peptides from spectra.

Functional enrichment analysis (14, 15) was performed to detect associations of Gene Ontology (GO) terms (16) for each experimental group. This functional characterization method allowed us to determine whether a set of functions was over-represented in a subset of proteins. Significant biological processes were summarized and represented by TreeMaps using the REVIGO webtool (17), which enabled the comparison of results between groups and is especially useful in revealing functional patterns.

Statistical Analysis

Data are represented as mean ± standard error (SE) unless otherwise is specified. Comparisons between experimental conditions were performed with Student's unpaired *t*-test. Analyses were conducted with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Differences were considered statistically significant at $p < 0.05$ with a 95% confidence interval.

RESULTS

Analysis of CM-EVs by western blotting for the assessment of the common exosomal markers CD9, TSG101, and ALIX (**Figure 1B**), showed no difference between EVs isolated from Nx or Hx conditions. In addition morphological analysis (**Figure 1A**) and size measurement by electron microscopy showed no significant neither difference (Nx: 102.9 ± 19.81 μm vs. Hx: 88.49 ± 24.35 μm). Further characterization using the NanoSight instrument revealed that the majority of EVs under both conditions were ∼150 nm in size (Nx: 147.9 ± 14 μm vs. Hx: 136.75 ± 13.78 μm; **Figure 1C**). Total protein analysis of the EV extracts showed that Hx significantly increased the secretion of CM-EVs (**Figure 1D**), as previously described (7). To functionally characterize the protein content of CM-EVs after Nx or Hx, we used LC-MS/MS. Protein contaminants from the cell culture medium (serum albumin and keratin) were removed from the analysis. Although an equivalent amount of total protein was used (30 μg), a broader range of proteins was observed in EVs recovered from Hx cultures (**Tables S1**, **S2**): 55 proteins were identified in Nx and 99 in Hx conditions. Among the identified proteins, we found abundant extracellular matrix (ECM) proteins including collagens and laminins, and also integrins, tetraspanins, ribosomal proteins and cardiac-specific proteins. Of note, multiple relevant proteins were found only under Hx conditions, including cardiogenic and cardioprotective proteins (Dickkopf-related protein 1, neuropilin 1 and netrin 4) and proteins related to cellular stress (ATP-citrate synthase, fatty acid synthase, X-ray repair cross-complementary protein 5 and aminopeptidase N). As described previously, Hx can induce the release of cardiac EVs that contain proteins with opposing activities (6). We further mapped the biological processes and metabolic pathways represented by CM-EVs proteins in Nx and Hx, finding 101 biological processes enriched in Nx and 124 in Hx ($P < 0.05$; **Tables S3**, **S4**). The most significant biological processes identified in both conditions were visualized in a Treemap diagram using REVIGO (**Figure 1E**). CM-EVs secreted in Nx conditions contained protein cargo involved in

ECM organization, integrin-signaling, collagen catabolism, and cell adhesion and cell migration, whereas additional biological processes were over-represented in Hx CM-EVs, such as protein folding and peptide cross-linking, including pathways involved in chaperone activity and protein synthesis (**Figure 1E**).

To evaluate the functional activity of CM-EVs, we used CFSE to stain equal amounts of CM-EVs from both conditions and added them to fibroblast or HUVEC cultures to explore their ability to capture vesicles. Fluorescent cells resulting from the internalization of EVs were then quantified by flow cytometry. Results showed that CM-EVs had a stronger preference to be internalized in HUVEC than in fibroblasts, which was independent of culture conditions (**Figures 2A,B**), suggesting an active exosome-based communication mechanism between these cell types in the heart. To further explore this phenomenon, we added CM-EVs from Nx and Hx conditions to EC and evaluated their functional impact in a tube formation assay. We found that tube length and total number of loops in EC was lower when CM-EVs from Hx conditions were used than when Nx CM-EVs were used (**Figures 2C,D**), although no significant differences were found in the other parameters analyzed, such as total number of tubes and number of branching points in vascular nets (**Figures 2C,D**). Similar results were obtained when CM-EVs were added to HCAEC, in which tube length, number of total tubes and also branching points were significantly lower when Hx CM-Evs were used (**Figure S2**). To analyze the intercellular cross-talk between cardiomyocytes and fibroblasts mediated by CM-EVs, we evaluated the ability of these vesicles to modify the motility of fibroblasts in a wound healing assay. We observed accelerated wound closure when CM-EVs isolated under Hx conditions were added to fibroblasts as compared with Nx CM-EVs counterparts (**Figures 2E,F**). This occurred in the absence of any pro-mitotic effects of the CM-EVs (**Figure S3**). These responses of fibroblasts and EC to Hx CM-EVs might account for the cellular events observed during post-ischemic remodeling of the heart.

DISCUSSION

Cardiac cells, including cardiomyocytes, fibroblasts and EC, act in a coordinated fashion to support cardiac function; hence, an efficient intra-cardiac communication system is needed to control heart integrity in health and disease (6). Similar to many other eukaryotic cells, cardiac cells can release EVs, and changes in the amount and composition of these vesicles have been related to changes in physiological or pathological conditions (6, 18). The interplay between different cell populations mediated by EVs, including cardiac resident progenitor cells or cardiac progenitors, in homeostasis and pathological conditions is currently the object of intense investigation (19–21). A recent study exploring the functional activity of EVs isolated from iPSC-derived cardiovascular progenitors showed that these vesicles improved the survival of cardiac myoblasts and promoted tube formation in HUVEC *in vitro*, and also enhanced cardiac function *in vivo* in a murine model of chronic heart failure (22). In the present study, we sought to assess these biological processes by functionally analyzing EVs secreted by an adult cardiomyocyte-derived cell line (AC10) cultured in Nx and Hx conditions. We found that both Nx- and Hx-derived CM-EVs were loaded with ECM components including different types of collagens and laminins, which are typically secreted by interstitial cells to modulate normal cardiac growth and remodeling. Hx-derived CM-EVs also contained proteins related to chaperone activity and aminoacylation of proteins including T-complex protein 1 subunits α and γ and the aminoacyl-tRNA synthetase multienzyme complex. Another protein detected in Hx-derived CM-EVs was the tissue factor pathway inhibitor TFPI1, which has been shown to correlate with circulating fibrinogen levels and coronary artery disease (23), suggesting that CM-EVs might contribute to blood coagulation under similar *in vivo* conditions. GO analysis revealed the enrichment of terms associated with ECM organization, integrin-mediated signaling pathways and cell adhesion common to both Nx and Hx culture. Also, we found a major association of Hx-derived CM-EVs with two biological processes, peptide cross-linking and chaperone-mediated protein folding, which have been described in other cell types in response to pro-apoptotic stress (24). Nevertheless, our proteomic assay failed to find significant amounts of apoptotic proteins or biological processes related to apoptotic cascades, suggesting residual presence of apoptosis related vesicles in our preparations. However, since the presence of the mentioned vesicles has not been discarded, it is possible that observed effects on fibroblast and endothelial cells are partially trigger by apoptosis related vesicles. Further characterization of the secreted EVs will clarify the signaling processes involve in the effect of these EVs on the cardiac cells.

Low levels of oxygen following myocardial infarction account for the massive destruction of cardiomyocytes and EC in the myocardium. We show here a preferential EV-mediated communication between cardiomyocytes and EC, both in Nx and Hx conditions. Using a tube-forming assay, we observed lower tube formation in EC treated with Hx CM-EVs when compared with Nx CM-EVs. This result suggests that CM-EVs play a role in angiogenesis. Proteomic analysis revealed that both Nx- and Hx-derived EVs carry pro-angiogenic proteins, such as TGF-β and VEGF-C, but GO analysis indicated that Hx-derived EVs are less enriched in these factors than Nx EVs. This correlates with the results of the tube formation assay and could be related to the stress conditions of the parental cells. In this regard, the addition of CM-EVs from diabetic rats to EC cultures inhibited proliferation, migration and tube-like formation (25). Nevertheless, it has recently been described that rat CM-EVs obtained under Hx promote angiogenesis (26). The differences in regenerative potential between human and murine cells could account for these discrepancies, and further experiments are needed to elucidate these processes in detail.

To analyze the functional activity of CM-EVs on fibroblasts, we used a scratch assay to measure cell migration, finding that CM-EVs isolated from Hx cultures promoted fibroblast motility. This accelerated wound closure is consistent with the pro-fibrotic reaction in the heart after an ischemic

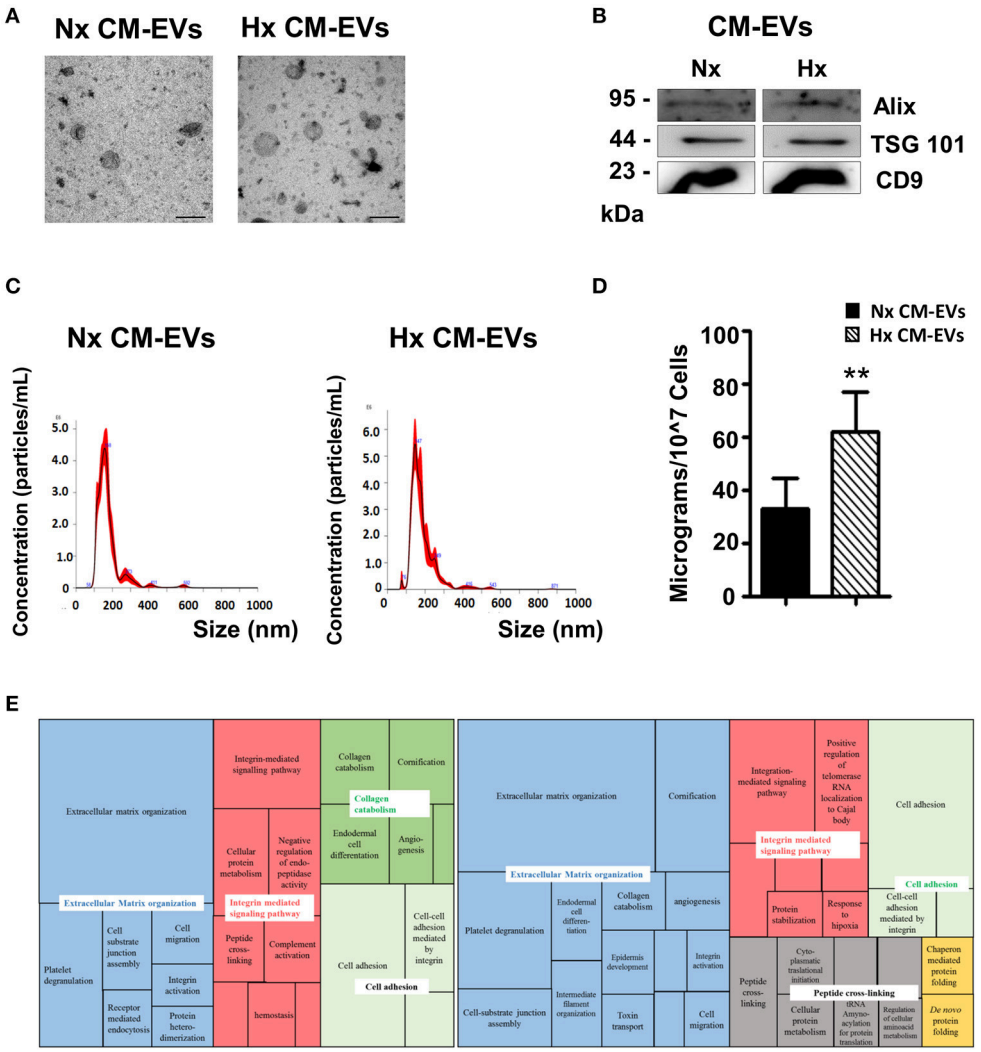

FIGURE 1 | Characterization of cardiomyocyte-derived extracellular vesicles. **(A)** Representative electron microscopy images of isolated extracellular vesicles (EVs) collected from cardiomyocyte (CM) cultures in normoxia (Nx) or hypoxia (Hx) ($n = 3$). Scale bars = 200 nm. **(B)** Representative western blot of common proteins found in EVs. EVs were lysed in RIPA buffer with complete protease inhibitors. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. **(C)** Representative images of EVs analyzed on the NanoSight NS300 instrument: particles/mL on vertical axis and size in nanometers (nm) on horizontal axis ($n = 5$). **(D)** Protein quantification of EVs harvested from equal amounts of conditioned medium ($n = 3$, $^{**}p < 0.01$). **(E)** Treemap diagram of biological processes in Nx and Hx CM-EVs using REVIGO after proteomic analysis. Extracellular matrix organization (blue), integrin-mediated signaling pathway (red), collagen catabolism (dark green), cell adhesion (light green), peptide cross-linking (gray), and protein folding (yellow).

insult. Similar deleterious effects exerted by CM through paracrine mechanisms have been described in other pathological conditions. For example, hypertrophic remodeling and fibrosis occurs in mice subjected to aortic constriction, which could be attenuated by cardiac-specific over-expression of the β3-adrenergic receptor (β3AR). The analysis of the secretome of β3AR-stimulated cardiac myocytes identified connective tissue growth factor as the main pro-fibrotic paracrine factor, which was reduced under β3AR stimulation (27). In another study, it was reported that diabetic (db/db) mice subjected to acute exercise increased their number of EVs in the heart containing miRNAs that reduce the content of MMP9, a metalloproteinase involved in cardiac fibrosis, with the consequent mitigation of this process (28). Similarly, in our proteomic analysis, we found disintegrin, metalloproteinase domain-containing protein 9 (ADAM9), laminin subunit gamma-1, TGFβI, fibronectin, laminin subunit alpha-5 and several subtypes of collagen-alpha protein in CM-EVs from Hx conditions; all of which are related to cell adhesion, cell-matrix interactions and cell motility, and that could account for the pro-fibrotic effects of CM-EVs. Overall, the results presented here contribute to the emerging picture of how CM-EVs function in cardiac remodeling after an ischemic insult. The differences between human and animal model systems should, however, be taken in consideration for the functional analysis of these vesicles.

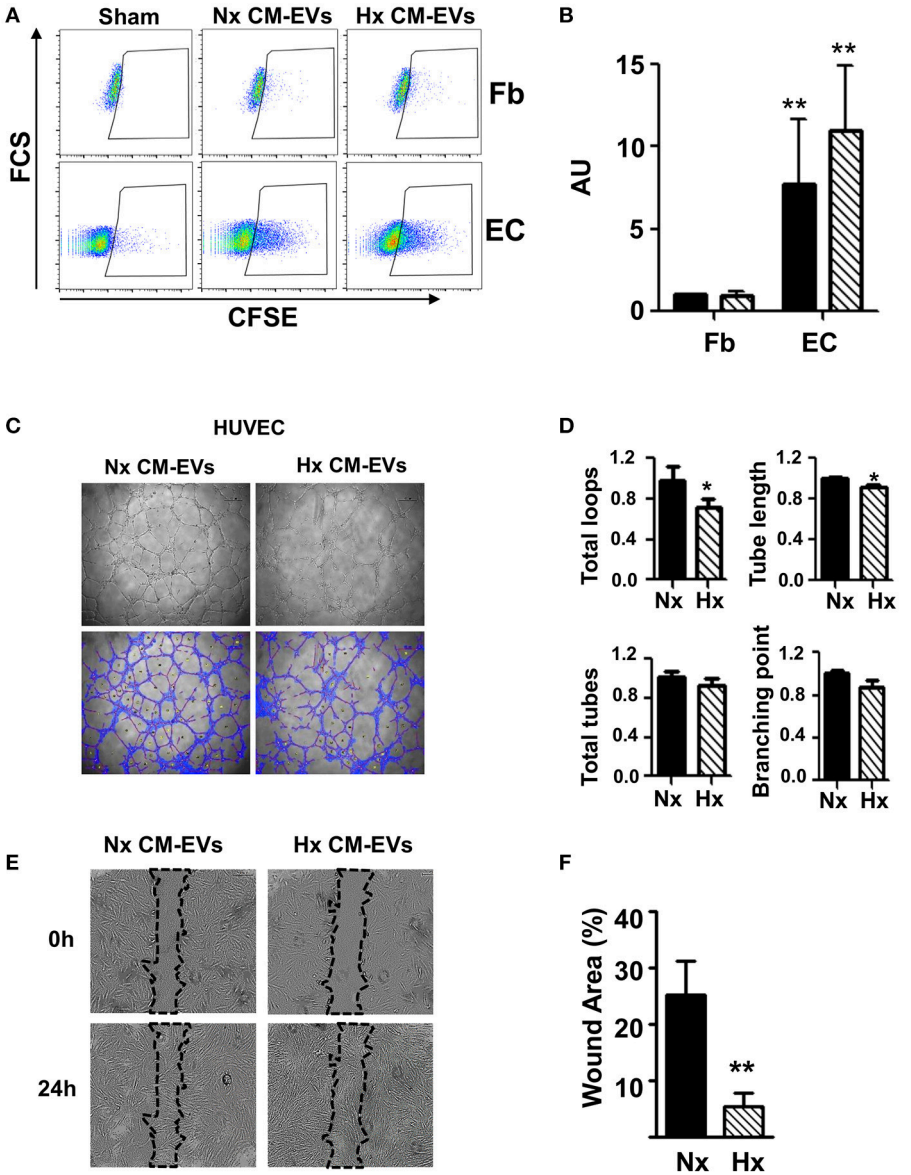

FIGURE 2 | Functional analysis of cardiomyocyte-derived extracellular vesicles. **(A)** Representative images of flow cytometry analysis of non-conditioned media derived EVs stained (Sham) with CFSE, normoxia (Nx) and hypoxia (Hx) cardiomyocyte-derived extracellular vesicle (CM-EV) captured by fibroblasts (Fb) and endothelial cells (EC). **(B)** Flow cytometry quantification of fluorescence resulting from the incorporation of CFSE-labeled CM-EVs in Nx (black bars) and Hx (dashed bars) ($n = 4$, $^{**}P < 0.01$). Data normalized to control condition. **(C)** Representative images of tube formation after 6 h of culture in the presence of Nx and Hx CM-EVs. **(D)** Quantification of total loops, loop length, total tubes and number of branching points from images taken after 6 h culture. Results are expressed in arbitrary units ($n = 3$, $^{*}P < 0.05$). **(E)** Representative images of scratch assay after 24 h of culture in the presence of Nx and Hx CM-EVs. **(F)** Quantification of wound closure from images taken after 24 h culture normalized to initial wound area ($n = 6$, $^{**}P < 0.01$).

LIMITATIONS OF THE STUDY

The major limitation of this study is the use of the AC10 cell line rather than primary cardiomyocyte cultures. However, the enormous number of cells needed for the isolation of EVs to perform the experiments would not have been feasible using primary cardiomyocytes and thus we decided to use AC10 cells. Another limitation of the study is that we did not verify the presence of proteins detected in the proteomic analysis by immunogold electron microscopy or alternative techniques, and so we cannot be sure that the proteins detected by LC-MS/MS are internalized in EVs and not non-specifically attached to isolated EVs. Finally, the low but unavoidable contamination of EV extracts with EVs originating from FBS used in cell culture should be considered. However, since we compared EVs derived from cell cultures in Nx and Hx conditions using the same batch of FBS, the differences observed in this study cannot be due to contamination with EVs from serum.

AUTHOR CONTRIBUTIONS

IO-O and AD: conception and design of the study, acquisition of data, and analysis and interpretation of data; RS, MC, MG-F, MB, EG, ST, HG-K, NG, and FG-G: acquisition and analysis of data; IO-O, AD, NG, and EP-M: critical revision of the manuscript; PS: manuscript drafting, critical revision, and final approval of the version to be submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2018.00152/full#supplementary-material

Figure S1 | (A) Representative western blot images of proteins extracted from AC10 cell cultures in normoxia or hypoxia (2% O_2). Lanes were loaded with equal amounts of protein and incubated with a hypoxia inducible factor-1 antibody. Signals were quantified by densitometry ($n = 3$). **(B)** Quantification of viable AC10 by CCK8 after different culture conditions used to isolate EVs.

Figure S2 | Tube formation assay of human coronary artery endothelial cells (HCAEC). **(A)** Representative images of tube formation after 6 h of culture in the presence of normoxia (Nx) or hypoxia (Hx) cardiomyocyte-derived extracellular vesicles. **(B)** Quantification of total loops, tube length, total tubes and number of branching points from images taken after 6 h culture. Results are expressed in arbitrary units ($n = 3$, $^*P < 0.05$).

Figure S3 | Cell proliferation assay of AC10, fibroblasts and human umbilical cord vein cells (HUVEC). Representative example of cell proliferation measured by CCK-8 assay after 48 h of culture in presence of Nx and Hx CM-EVs. Results are presented in arbitrary units ($n = 3$).

Table S1 | Protein identification in cardiomyocyte-derived extracellular vesicles in normoxia.

Table S2 | Protein identification in cardiomyocyte-derived extracellular vesicles in hypoxia.

Table S3 | Gene ontology biological processes for proteins identified in extracellular vesicles derived in normoxia.

Table S4 | Gene ontology biological processes for proteins identified in extracellular vesicles derived in hypoxia.

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REVIEW
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It Takes Two: Endothelial-Perivascular Cell Cross-Talk in Vascular Development and Disease

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The formation of new blood vessels is a crucial step in the development of any new tissue both during embryogenesis and *in vitro* models as without sufficient perfusion the tissue will be unable to grow beyond the size where nutrition and oxygenation can be managed by diffusion alone. Endothelial cells are the primary building block of blood vessels and are capable of forming tube like structures independently however they are unable to independently form functional vasculature which is capable of conducting blood flow. This requires support from other structures including supporting perivascular cells and the extracellular matrix. The crosstalk between endothelial cells and perivascular cells is vital in regulating vasculogenesis and angiogenesis and the consequences when this is disrupted can be seen in a variety of congenital and acquired disease states. This review details the mechanisms of vasculogenesis *in vivo* during embryogenesis and compares this to currently employed *in vitro* techniques. It also highlights clinical consequences of defects in the endothelial cell—pericyte cross-talk and highlights therapies which are being developed to target this pathway. Improving the understanding of the intricacies of endothelial—pericyte signaling will inform pathophysiology of multiple vascular diseases and allow the development of effective *in vitro* models to guide drug development and assist with approaches in tissue engineering to develop functional vasculature for regenerative medicine applications.

Keywords: cell-cell interaction, vascular development, endothelial, pericytes, perivascular, vascular dysfunction

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INTRODUCTION

The processes of vasculogenesis and angiogenesis play vital roles in embryonic development and vascular homeostasis during adulthood. The endothelial cell is the most basic building block of blood vessels and the growth factor mediated proliferation and migration of these cells is responsible for forming the complex vascular networks within the body. Endothelial cells do not perform these tasks in isolation and their interaction with supporting perivascular cells is one of the key processes during the formation of new blood vessels to ensure that durable vessels are formed which can support blood flow. This review will discuss the embryological origins and interactions of endothelial cells and perivascular cells and compare this with current *in vitro* approaches used to model this interaction. In addition, it will discuss how interruption of this interaction causes

a variety of genetic and acquired diseases and how novel approaches to co-culture may help to develop our understanding of this area and provide potential therapeutic options in the future.

MULTICELLULAR INTERACTIONS IN EMBRYOGENESIS

Building Blocks for New Vessels

The process of creating vascular networks involves two sequential steps: vasculogenesis, the *de novo* formation of blood vessels from progenitor cells, and angiogenesis the migration, branching, and pruning of existing blood vessels to form complex vascular networks and capillary beds (1). The endothelial cell is the most basic building block of new blood vessels and the processes of angiogenesis and vasculogenesis both require the proliferation and migration of these cells to under perfused tissues. This must be followed by the formation of strong connections between adjacent cells and the extracellular matrix (ECM) to create a durable conduit which can support blood flow. In the developing embryo there are multiple interactions between the cell and its environment responsible for controlling this process (2). This includes interactions between neighboring endothelial cells, between endothelial cells and surrounding support cells as well as the paracrine effects of growth factors released into the ECM. In addition, these newly developing vessels respond to changes in the extracellular environment including the composition of the ECM and relative levels of hypoxia or nutritional deficiencies of surrounding cells (3).

Endothelial Cells

During embryogenesis the first recognizable blood vessels occur in the yolk sac as groups of cells expressing endothelial markers including vascular endothelial growth factor receptor (VEGFR), VE-cadherin and CD31 (1, 4). These primitive endothelial cells are derived from the mesodermal layer of the embryo. They migrate to form aggregates of cells known as blood islands which are capable differentiating toward either haematopoietic or angioblastic lineages (5). As these cells begin to differentiate they align with angioblastic cells on the outside of the blood islands and haematopoietic cells in the central core. Angioblasts in the outer lining flatten and form intercellular connections to create a circumferential layer of primitive endothelial cells which is the first stage in vessel formation (1).

The formation of these blood islands in the mesoderm is controlled by growth factors released from the endodermal layer. Hedgehog signaling via the bone morphogenic protein-4 (BMP-4) pathway is one of the earliest steps that initiates endothelial differentiation from multipotent mesodermal cells and is vital in early vascular development (6–8). Fibroblast growth factors (FGF) stimulation of these cells induces the expression of early endothelial markers. The FGF driven expression of VEGFR (9–11) is an essential step in sensitizing the cells to the potent angiogenic growth factor vascular endothelial growth factor (VEGF) which is one of the key growth factors in promoting angiogenesis (12, 13).

As the blood vessel matures the endothelial layer forms a confluent monocellular layer in contact with the blood. This functions as barrier to prevent the widespread extravasation of blood and fluid however also needs to be sufficiently permeable to enable the passage of required gases, nutrient and leukocytes into the perivascular space when required. VE-cadherin one of the earliest markers expressed on the surface of developing endothelial cells. It forms part of the adherens junctions between endothelial cells to begin the formation of the monolayer. Further control of the permeability is mediated by the formation of tight junctions which are formed from claudins, occludins and junctional adhesion molecules which are upregulated as the endothelial cell matures (14).

Perivascular Cells

Perivascular or mural cells were first described histologically as cells closely associated to the endothelial layer of blood vessel and are found in all organs throughout the body. They are a phenotypically diverse family of cells with a variety of roles depending upon the anatomical location and function of the vessel (15). They can be divided into two main categories: vascular smooth muscle cells and pericytes although much heterogeneity exists within these groups.

Vascular smooth muscle cells are associated with larger conduit vessels such as arteries and veins and are separated from the endothelial layer by the basement membrane and the inner elastic lamina. They have greater expression of contractile proteins such as α-smooth muscle actin (α-SMA), adopt a stellate shape and line up in a circumferential pattern around the vessel. Pericytes, in contrast, are associated with small caliber capillaries where they are embedded in the same basement membrane as the endothelial layer. They have an elongated and flattened shape and are orientated along the long axis of the vessel with multiple finger-like projection extending through the basement membrane to make direct contact with the endothelial layer (15). These connections have a peg and socket arrangement that enables signals to be passed directly between the pericyte and endothelial layers. A single pericyte can interact with multiple endothelial cell and the relative density of pericytes surrounding a blood vessel depends upon anatomical location and function of the vessel. In skeletal muscle there is approximately a 1:100 relationship between pericytes and endothelial cells (16) whereas in the brain and the retina there is a 1:1 relationship where it is thought to play a role in maintaining the blood brain barrier (17–19). Their position in contact with multiple endothelial cells can be advantageous in co-ordinating the response of a population of endothelial cells along the vessel as intercellular signaling can be propagated to multiple cells in the endothelial layer through a single pericyte (15, 17).

Debate exists regarding the characteristics which define a perivascular cell and thus a precise definition remains elusive. Consequently, there is no single specific marker to define this population. Instead, a combination of cell surface markers are typically used and these may have variable expression depending upon the anatomical location and angiogenic state of the cell (20, 21). Common markers which are expressed in perivascular cells include neuron-glial antigen-2 (NG2), CD146, α-smooth

muscle actin (α-SMA), and platelet derived growth factor receptor-β (PDGFR-β). Endothelial and haematopoietic specific markers such as CD31, VE-cadherin, von Willebrand factor and CD45 should be absent (15, 22–25).

The precise origin of perivascular cells is more controversial than that of endothelial cells and there appears to be more than a single origin for these cells (**Figure 1**). Perivascular cells in the bowel, lung and liver have been found to have an endodermal lineage which initially develops toward an epithelial fate and subsequently undergo epithelial to mesenchymal transition into pericytes (16). In the brain, they are derived from neural crest cells (26) and vessel such as the aorta have pericytes of multiple different origins within a single vessel (16).

Perivascular cells were once thought to only provide mechanical support to the vessel however it is now clear that it has a variety of functions within the vasculature. The contractile properties of perivascular cells enable regulation of blood supply by altering the vessel diameter in response to vasoactive substances (21). In the central nervous system, where they are significantly more abundant, they are thought to play a role in the control of cerebral perfusion and restricting the permeability of the blood brain barrier (15, 21, 27). They may also play a role in tissue repair as they have the ability to transdifferentiate into fibroblast in response to injury to the surrounding tissue (16, 28) Finally, they also have an important role in intercellular signaling both to endothelial cells and to the tissues surrounding the blood vessels.

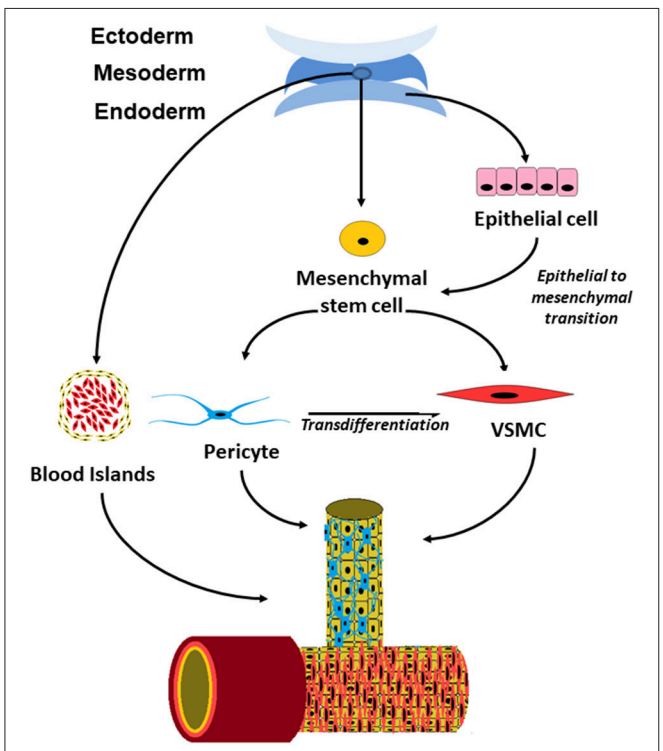

FIGURE 1 | Schematic drawing showing the origin of perivascular cells in embryonic development and postnatal vessels.

Crosstalk Between Perivascular and Endothelial Cells Is Key During Early Vasculogenesis

Signaling between the endothelial and perivascular cell layers is mediated by the release of growth factors, direct cell-cell contact at peg and socket connections and modulation of the ECM (**Figure 2**). Perivascular cell are recruited to the developing vessel as the vessel matures. Perivascular cells coating the vessel have an anti-angiogenic effect which stabilizes the vessel and limits further proliferation and migration of the endothelial cells. This is mediated by a number of signaling molecules released by the perivascular cells which act on the endothelial layer including Angiopoietins, sphingosine-1-phosphate as well as contact inhibition largely mediated by the Notch family.

VEGF

The expression of VEGF receptor on primitive endothelial cells is one of the most important steps in vasculogenesis as this enables cells to respond to the potent mitogenic and

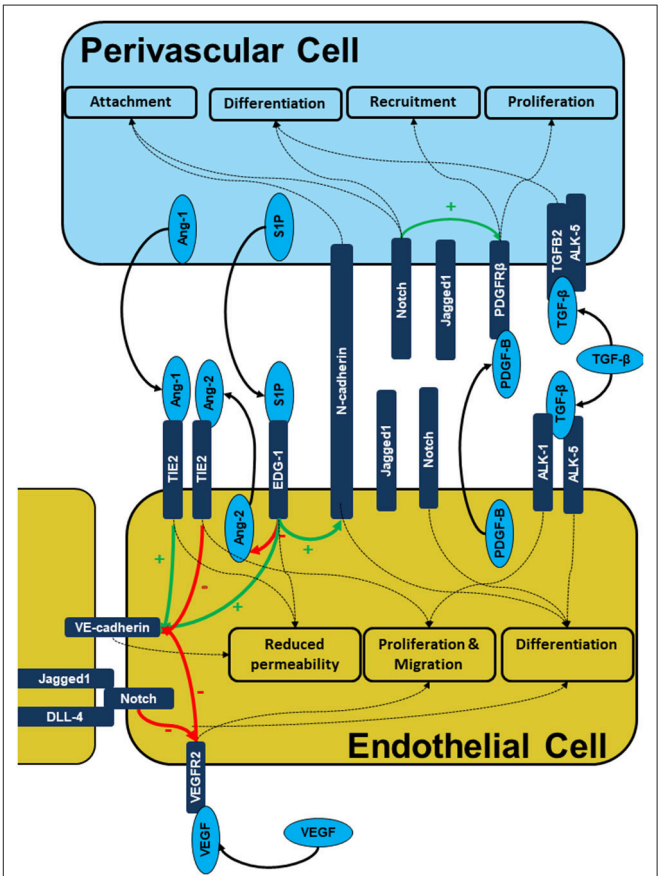

FIGURE 2 | Signaling during endothelial-pericyte cross-talk. The figure illustrates the intercellular signaling responsible for cell recruitment, differentiation, and maturation as well as vessel stability is built on multiple receptor complexes. PDGF-B/PDGFRB2, S1P/EDG-1, ANG1/2/TIE2, Cadherin, and Notch mediated signals are prerequisites of endothelial -pericyte cross-talk, cell recruitment and subsequent vessel stabilization.

chemotactic effects of VEGF (10, 29). The importance VEGF signaling pathway in early vasculogenesis is highlighted by VEGF knockout mice which die early in development without any organized vasculature present (30, 31). VEGF-A is the prototypical member of the VEGF family and is coded for by the VEGFA gene on chromosome 6. It is released by multiple cells throughout the body typically in response to hypoxia or hypoperfusion of a tissue (32).

VEGF receptors are transmembrane tyrosine kinase receptors denoted VEGFR1-R3. The most important signaling receptor in vasculogenesis and angiogenesis is the VEGFR2 which is a relatively low affinity receptor with high intracellular tyrosine kinase activity when activated. Knockout of this receptor has the same effects as VEGF-A knockouts with early embryonic lethality due to lack of organized vasculature. In contrast, VEGFR1 has a much higher affinity for VEGF-A however minimal intracellular tyrosine kinase activity upon activation (32). Knockout of the VEGFR1 receptor causes unchecked endothelial hyperplasia (33) however selective knockout of the tyrosine kinase domain of the VEGFR1 has minimal effect on vascular development. This demonstrates that this receptor acts largely as ligand trap to sequester VEGF and reduce VEGFR2 signaling and therefore angiogenesis (33).

Intracellularly activation of VEGFR2 is propagated by a number of downstream signaling pathways. This is classically via ERK signaling, however, a variety of non-canonical pathways are also known to play a role (34). Stimulation of this pathway induces endothelial proliferation and differentiation including the expression of cell adhesion molecules such as VE-cadherin which form junctional complexes between the endothelial cells and creates the tubular structure from which further blood vessel development can occur (35, 36). Control of VEGF receptor expression is maintained by exogenous signaling including concentration of FGF and transforming growth factor-β (TGF-β) as well as by negative feedback loops within the cell to internalize and degrade receptors to prevent overstimulation of the VEGFR2 pathway and excessive neovascularisation with immature and permeable vessels as is seen in VEGFR1 knockout mice.

As perivascular cells differentiate they begin to express VEGF which is expressed in response to hypoxia and TGF-β signaling (28, 37). The isoform produced by perivascular cells is most commonly VEGF-A^{165} which binds to heparin sulfate proteoglycans on the cell surface, therefore, remaining associated with the cells (37). This local release of VEGF is important in promoting endothelial cell survival and stabilizing newly formed vessels, however, does not set up a concentration gradient to encourage endothelial cell migration as occurs when more soluble isoforms are released (37, 38). Pericytes also have VEGFR1 on the cell surface which binds VEGF and sequesters it from the VEGFR2 on the endothelial cells, therefore, prevents the initiation of angiogenesis in mature, quiescent vessels (39).

TGF-β

The transforming growth factor-β family of signaling molecules consists of more than thirty molecules which have a diverse range of targets. TGF-β is the most well characterized member and is expressed by multiple cells throughout the body including both endothelial and perivascular cells. When secreted by these cells it is associated with specific TGF-β binding proteins which maintain it in an inactivated form bound to the extracellular matrix (ECM) (40). Various mechanisms can release and activate TGF-β1 including the action of proteases such as matrix metalloproteinases (MMPs) which are produced by endothelial and perivascular cells when they come into contact. Once activated TGF-β has a variety of receptors on the surface of endothelial cells and pericytes (23, 41, 42).

The effect of TGF-β is highly context dependent and varies with the local concentration of TGF-β as well as the relative expression of receptors on the cell surface of the target cells (43, 44). The two most important TGF- β receptors in angiogenesis are Activin like kinase-1 (ALK-5) which is expressed on both endothelial cells and perivascular cells and Activin like kinase-1 (ALK-1) which is restricted mainly to endothelial cells. These two receptors have opposing effects on the response of the cell to TGF-β. ALK-5 stimulation causes down regulation of proangiogenic VEGFR-2 and upregulation of the antiangiogenic VEGFR-1 leading to reduced proliferation and increased differentiation of endothelial cells (45, 46). Similarly, ALK-5 activation causes differentiation of the perivascular cells and promotes release of ECM proteins such as fibronectin from both cell types (40). Overall this encourages formation of quiescent mature vessels with significant perivascular cell coating and a well-developed stable ECM. In contrast, ALK-1 signaling which is preferentially activated at lower concentrations of TGF-β increases proliferation of endothelial cells and encourages angiogenesis to proceed (45, 47, 48).

Platelet Derived Growth Factor

Platelet derived growth factor (PDGF) is released mainly by endothelial cells and binds to the PDGFR-β on perivascular cells. Activation of this receptor stimulates proliferation of perivascular cells and acts as a potent chemoattractant causing cells to migrate toward expanding endothelial cell populations (49, 50). Absence of PDGF or PDGFR- β in mice is lethal in late gestation due to large, permeable, dilated vessels with microaneurysm formation, microvascular leakage and oedema throughout the embryo (49). Histologically these mice have widespread deficiency of perivascular cells coating the blood vessels and there is endothelial cell hyperplasia at the site of microaneurysms formation. This demonstrates the role that recruitment of perivascular cells to the perivascular niche plays in stabilizing developing vessels and reducing proliferation and angiogenesis in fully formed mature vessels (24).

VEGF signaling on perivascular cells via the VEGFR2 supresses the response of the cell to stimulation by PDGF-B by phosphorylation of the PDGFR-β receptor. This reduces the maturation and migration of perivascular cells to the site blood vessels undergoing of active angiogenesis and enables endothelial cell proliferation and vasculogenesis to continue without the inhibitory effect of pericytes (51).

Angiopoietins

Angiopoietins are a group of glycoproteins which act as ligands for the tyrosine kinase receptors TIE (Tyrosine kinase with immunoglobulin-like and EGF-like domains). The TIE-2 receptor is exclusively expressed on the surface of endothelial

cells (52). Angiopoietin-1 (ANG-1) is a ligand for the TIE receptors which is expressed by perivascular cells in response to PDGF-B stimulation (53). Activation of TIE-2 receptors causes stabilization of the vessel through the inhibition of apoptosis in endothelial cells, enhancing inter-endothelial connections and reducing endothelial layer permeability (54–56). ANG-2 is released mainly by endothelial cells and acts as an antagonist at the TIE-2 receptors preventing the anti-angiogenic activity of ANG-1 and thereby promoting angiogenesis (57).

Sphingosine-1-Phosphate

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite released by perivascular cells (58) which acts on the endothelial differentiation gene-1 (EDG-1) receptor on the endothelial cell surface. S1P specifically promotes membrane expression and trafficking of the cell-cell adhesion protein N-cadherins to the cell membrane. N-cadherins localize to the peg and socket contacts between the endothelial and pericyte where it enhances these contacts (54, 59). Similar to PDGFR-β knockouts, loss of S1P results in absence of pericyte association with the vessels and subsequent vessel dilation and hemorrhage (54). Binding of the S1P also upregulates the expression of the inter-endothelial cell adhesion molecule VE-cadherin and down-regulates the expression of the pro-angiogenic ANG-2 thereby strengthening tight junctions between cells and increasing the stability of the developing vessel (36, 58–61).

Notch Receptors and Ligands

Notch receptors are transmembrane proteins which binds to Notch ligands which are also typically membrane bound structures, therefore, having an important role in mediating signaling between adjacent cells in direct contact. Binding of a Notch ligand causes the intracellular effector domain of the Notch receptor to be cleaved which travels to the nucleus where it acts as a transcription factor (62). Endothelial cells express the Notch ligands delta-like-ligand 4 (DLL-4), Jagged-1 (JAG-1), and Jagged-2 (JAG-2) and Notch receptors 1–4 while perivascular cells express Jagged-1 and Notch receptors 1–3 (63).

Notch receptors on the endothelial cells binds JAG-1 on the perivascular cells mediating contact inhibition by downregulating expression of VEGFR2 and upregulating VEGFR1 therefore desensitizing the cell to VEGF activation, (64, 65) reducing endothelial proliferation (66–68) and encouraging the formation of a mature endothelial cell phenotype (69). The expression integrin αvβ3 is increased and which causes adhesion of endothelial cells to von Willebrand factor in the basement membrane and strengthens the structure of the developing vessel (69).

Activation of Notch receptors in the perivascular cells upregulate the expression of PDGFR-β which promotes maturation of perivascular cells with increased expression of α-SMA (68, 70). It also enhances recruitment and attachment of perivascular cells to the endothelial layer forming more stable and mature vessels (71). Notch knockout mice have early embryonic lethality due to widespread vascular abnormalities (72).

Extracellular Matrix for Vessel Formation

In embryogenesis, the ECM and particularly the vascular basement membrane plays a vital role in controlling the development of the primitive vasculature by recruiting cells to the surrounding area and modifying their transcription profile depending upon the composition of the surrounding ECM (73). The vascular basement membrane is a structure of highly crosslinked insoluble materials including collagens, laminins, and fibronectin in which endothelial cells are embedded (74). This is formed by synthesis and deposition of these substances from both endothelial cells and perivascular cells which is upregulated following contact between these two cell types (2).

The basement membrane is not a static structure; it is continually being remodeled depending upon the conditions of the vessel and surrounding tissue. The changes in composition affect the endothelial cell behavior through interaction with integrins on the cell surface. MMPs and tissue inhibitors of metalloproteinases (TIMPs) catalyze this remodeling and are in constantly shifting equilibrium (75). Close interaction of endothelial cells with perivascular cells cause TIMP expression to predominate leading to inhibition of MMP and a more stable, type IV collagen rich and highly cross-linked basement membrane (76). In this state growth factors and signaling molecules including VEGF and TGF-β remain embedded in the basement membrane and are not available for the cell to access causing it to remain in a state of quiescence. In contrast, when the basement membrane is being assembled or disassembled by MMPs these growth factors are released and activate endothelial cells by exposing them to provisional matrix components, such as vitronectin, fibronectin, type I collagen, and thrombin. Additionally, growth factors including TGF-β and VEGF which are released from their latent bound states within the ECM and encourages endothelial proliferation and migration leading to sprouting and growth of the vessel (75, 77).

Cadherins

The cadherins, N-cadherin and VE-cadherin mediate the interaction between endothelial cells and surrounding cells and structures by linking the extracellular environment to the cytoskeletal framework of the cell (78). VE-cadherin is concentrated at adherens junctions, its extracellular component mediates cell-cell interaction with cadherins on other endothelial cells (79). The inhibition of this interaction with monoclonal antibodies results in increased permeability of the endothelial layer and apoptosis of endothelial cells (80). Congenital absence of VE-cadherin causes early embryonic lethality due to a lack of organized vasculature (81).

The intracellular components of the VE-cadherin complex; β-catenin, p120-catenin and plakoglobin act on various pathways to control gene expression, proliferation, and the cytoskeleton. Binding of VE-cadherin causes VEGFR2 to be internalized and degraded therefore mediating the contact inhibition seen in confluent endothelial cell culture (82). Interaction with catenin molecules causes alteration in the cytoskeleton on binding of VE-cadherin molecules which causes the endothelial cells to change shape in response to contact with neighboring cells. The expression of VE-cadherin is under the control of multiple growth factors including VEGF causing the phosphorylation of

VE-cadherin and destabilization of the adherens junction (83, 84). This results in increased permeability of the endothelial cell layer and increased mobility of endothelial cells during angiogenesis.

N-cadherin molecules are positioned on the abluminal side of the endothelial cells mediating its contact with pericytes and disruption of this interaction with N-cadherin blocking antibodies impairs the pericyte-endothelial interaction. Although pericytes are recruited to the perivascular niche, they are weakly associated with the endothelial layer. Large extravascular cavities form and there is rupture of the endothelial layer, loss of polarity and extensive hemorrhage (85, 86).

DYSFUNCTIONAL MULTICELLULAR INTERACTIONS IN DISEASE

Congenital Disease

The failure of cell-cell interactions between endothelial cells and perivascular cells have been implicated in a variety of congenital and acquired diseases (**Table 1**). Approximately 80 per cent of rare diseases have a genetic basis, so identifying the specific mutations in underlying cell-autonomous pathways may provide insight into more common developmental and disease processes.

Hereditary Haemorrhagic Telangiectasia

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominantly inherited condition caused by mutations in the ALK-1 receptors or Endoglin receptor (ENG) which is a co-receptor in the TGF-β signaling pathway (94, 95). It is characterized by large arteriovenous malformations (AVMs) in major organs including the lungs, liver, brain and mucosa. The large fragile vessels formed in AVMs are prone to bleeding resulting in presentations with epistaxis or intracranial hemorrhage and causing shunting between the arterial and venous circulation which can present as paradoxical emboli or high output heart failure (95). The loss of TGF-β signaling in endothelial cells reduced expression of N-cadherin which impaired the formation of heterotopic cell contacts with perivascular cells. This lack of cell-cell contacts results in further reduction in TGF-β activation from the surrounding ECM and therefore reduced TGF-β signals for the perivascular cell to encourage differentiation and attachment to the endothelial layer. This impaired recruitment of perivascular cells to the developing vessel and overactive proliferation of endothelial cells results in endothelial hyperplasia. These large dilated vessels with little perivascular cell coating develop into the arteriovenous malformations which characterize the disease (95–97). Similar phenotypes are seen in spontaneous cerebral AVMs with histological analysis demonstrating dilated vessels with segmental loss of smooth muscle cells and the internal elastic lamina. This phenotype is reproducible in mice by knockout of the ALK-1 and TGF-β receptor (98).

Currently emerging treatment for HHT is targeted at increasing the number of pericytes associated with the endothelial layer. Thalidomide is one such treatment which reduces the frequency and severity of epistaxis in patients with HHT (99). In mice, thalidomide has been noted to increase the number of pericytes surrounding vessels and improves their attachment to the endothelial cell layer thereby stabilizing the vessel which is likely to play a role in the reduced bleeding from AVMs. Thalidomide treated mouse retinas have increased

TABLE 1 | Congenital syndromes of dysfunctional multicellularity.

Disease	Genetic background	Cell types involved	Cardiovascular phenotype	References
CADASIL	Notch3	Smooth muscle cells	Central nervous system arteriovenous malformations	(68)
Hajdu-Cheney syndrome	Notch2	Endothelial cells	Ductus arteriosus, atrial and ventricular septal defects Valve abnormalities	(87)
Adams-Oliver syndrome	Notch1 DLL4	Pericytes, Smooth muscle cells Endothelial cells	Hypoplastic aortic arch, middle cerebral artery and pulmonary arteries.	(88)
Singleton-Merten syndrome	Helicase C Domain 1 Dexd/H-Box Helicase 58	Endothelial cells Smooth muscle cells	Aorta calcification, subaortic stenosis	(89)
Hereditary haemorrhagic telangiectasia	Endoglin ALK1 SMAD3	Smooth muscle cells Pericytes Endothelial cells	Arteriovenous malformations and telangiectasia	(90)
Alagille syndrome 2	Notch2 JAG1	Smooth muscle cells Pericytes Endothelial cells	Atrial septal defect Pulmonary stenosis Tetralogy of Fallot Hypertension	(91)
Von Hippel-Lindau syndrome	Hypoxia-inducible factor-2 alpha, *VHL* tumor suppressor gene	Endothelial cells Smooth muscle cells Pericytes	Stage-specific changes in vessel branching and an advanced progression toward an arterial phenotype	(92)
Idiopathic basal ganglia calcification	PDGF-B PDGFR-β Type III sodium dependent phosphate transporter 2	Pericyte Endothelial cells	Perivascular calcium deposits Cerebral aneurysm Arteriovenous malformations	(93)

expression of PDGF-B by endothelial cells (94) providing a potent chemotactic and maturation signal to neighboring pericytes.

Idiopathic Basal Ganglia Calcification

Idiopathic basal ganglia calcification is a rare disorder, most commonly inherited in an autosomal dominant fashion. It presents with neurological and psychiatric abnormalities at a young age with associated areas of calcification within the basal ganglia of the brain. A loss of function mutations in PDGF-B is associated with this condition in humans (100). This is thought to cause reduced pericyte recruitment to cerebral vasculature which increases the permeability of the blood-brain barrier. Mouse models of the disease involving knockdown of PDGF-B function result in similar reduction in perivascular coverage of the cerebral vasculature and progressive brain calcification (100).

Notch Mutations

Mutations in Notch receptor genes or ligands lead to a variety of congenital disorders in humans. Adams-Oliver syndrome is a rare inherited disorder, with varying degrees of defective vasculature defects including hypoplastic aortic arch, middle cerebral artery, and pulmonary arteries. The spectrum of vascular defects has been thought to be due to a disorder of vasculogenesis, perivascular dysfunction and abnormal vascular coverage during development (88, 101, 102). To support this idea, transient inhibition of Notch signaling in perivascular cells inhibited their differentiation and led to localized hemorrhages in newly forming vasculature during embryonic development (103). Alagille syndrome is a multi-systems inherited disease caused by loss of function mutations in JAG1 or NOTCH2 (91). Pulmonary artery involvement including hypoplastic pulmonary arteries and pulmonary stenosis are the common vascular manifestation of the disease. There is also a high rate of intracranial hemorrhage up to 16% suggesting the presence of fragile intracranial vessels (91).

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is a degenerative disorder characterized by early-onset strokes and dementia; it is caused by loss of Jag1 in the endothelium or defective Notch3 in vascular smooth muscle cells. This results in alterations of vascular smooth muscle cells; NOTCH1 and NOTCH3 mutant mice develop arteriovenous malformations and show hallmarks of CADASIL (68).

TIE Mutations

Hereditary cutaneomucosal venous malformation is a rare condition which is inherited in an autosomal dominant fashion due to abnormalities in the TIE-2 receptor gene (*TEK*). This causes small and usually asymptomatic venous malformations mostly in the mucous membranes and face and much less common involving the internal organs (104). The mutation is a hyperphosphorylating mutation within the TIE-2 receptor which results in a patchy coating of perivascular cells along the vessel wall associated with hyperplasia of the endothelial layer producing large and dilated vessels (105–107).

Acquire Disease

Retinopathy

Diabetic retinopathy is a common condition affecting over one third of patients with diabetes worldwide (108). Chronic hyperglycaemia causes endothelial cells to release reactive oxygen species and inflammatory cytokines and reduces the expression of growth factors including PDGF-B (109). These changes coupled with direct effects of hyperglycaemia on the basement membrane and pericytes cause the vessel to become denuded of pericytes (110–112). This is one of the earliest histological findings in diabetic retinopathy which is followed by microaneurysm formation and oedema which can be detected clinically as the fragile neovasculature ruptures or leaks (113).

As described previously physical contact between pericytes and endothelial cells is required for activation of TGF-β and deficiency of TGF-β signaling impairs vessel maturity and increases permeability (114, 115). Additionally, ANG-1 released by pericytes has an important role in maintaining quiescence of the endothelial layer and reducing the permeability of the vessel. Blockage of the ANG-1 pathway using the competitive antagonist ANG-2 in normoglycaemic mice induces pericyte loss similar to diabetic retinopathy and subsequent endothelial hyperplasia. Models of diabetic retinopathy can also be induced in mice by reducing PDGF-B and TGF-β signaling in the absence of hyperglycaemia (114–117).

Pathological neovascularization also occurs in the setting of age-related macular degeneration (AMD). AMD is a condition where there is neovascularization of the macula of the retina which causes progressive blindness. Wet AMD is associated with macular oedema as the fragile and permeable vessels allow the leakage of fluid into the subretinal space (118).

The mainstay of treatment to date has been VEGF inhibition with monoclonal antibodies against VEGF which reduces the permeability of these vessel and induces regression of the vasculature. This treatment has significantly reduced the rates of progression and blindness associated with this condition (119). However, a proportion of patients become resistant to anti-VEGF treatment after an initial period of success. Pericytes have been implicated in providing survival signal to the developing blood vessels overcoming the inhibition of VEGF leading to ongoing angiogenesis within the macula. Combination of monoclonal antibodies against VEGF and PDGF have shown promise in pre-clinical trials to reduce the extent of neovascularization (120) and currently a trial of combination therapy is ongoing (121, 122). Similarly, treatments targeting other combinations of signaling molecules involved in this pathway are currently being trialed in diabetic retinopathy. This includes antibodies targeting both VEGF and Angiopoietin-2 (Clinicaltrials.gov-BOULEVARD, Clinicaltrials.gov-RUBY) and treatments targeted at VEGF, PDGF-B and FGF (clinicaltrials.gov-squalamine).

Cancer

One of the hallmarks of cancer is the ability to induce angiogenesis to augment the blood supply to the rapidly dividing cancerous cells (123). Therefore, targeting this pathway with inhibitors of angiogenesis has become an important target in oncological therapy. Monoclonal antibodies blocking VEGF

and inhibitors of tyrosine kinase receptors have become a mainstay in cancer regimes for multiple indications (124). However, after a period of treatment, a proportion of cancers become resistant to the anti-VEGF treatment and escape this inhibition. Thus, further efforts have been invested in developing therapies to target the pericyte-endothelial cell interaction to prevent this resistance developing. Less selective tyrosine kinase inhibitors with action against multiple tyrosine kinases has shown promise in this area. Sunitinib targets the VEGF and PDGF receptors and is a potent inhibitor of angiogenesis. It reduces the coverage of pericytes along the developing vessel causing reduction in angiogenesis overall and therefore improved survival in renal cell cancers (125, 126). Preventing migration of perivascular cells to the perivascular niche of developing cancer vasculature reduces the survival signal to endothelial cells leading to apoptosis and preventing further angiogenesis (127).

Manipulation of TIE2/Angiopoietin pathway has also been targeted in preclinical models of renal cell cancer by using antibodies which selectively block the effect ANG-2, therefore, potentiating the effects of ANG-1 which will stabilize the vasculature and prevent further angiogenesis and therefore limit further growth of the tumor (128). This is currently being trialed in phase 1 human trials (129).

MULTICELLULAR INTERACTIONS IN CULTURE

Modeling rare vascular genetic disease *in vitro* is possible using pluripotent stem cell technology which provides insights into the mechanisms involved in these diseases. The use of multicellular constructs provides a more analogous environment to the *in vivo* environment than can be replicated by the exogenous application of growth factors as it is able to model and regulate a greater variety of interaction between cells in a more physiological manner.

Endothelial Cells

The earliest stage of *in vitro* blood vessel formation involves expansion of endothelial cells in culture to form a network of tubular structures replicating the early steps of vasculogenesis in the embryo. Multiple cell types and sources have been used to provide endothelial cells for this purpose. Fully differentiated primary endothelial cells have been used, most commonly these are sourced from the human umbilical vein (HUVEC). These cells already have a fully differentiated endothelial phenotype and therefore will immediately begin forming tubular structures in culture (35). However, use of these cells is limited by replicative senescence which develops after limited passages in culture making them sub-optimal for large-scale replication. Less well differentiated cells including late outgrowth endothelial cells (OEC) or multipotent cells such as mesenchymal stem cells (130) show greater replicative potential and are also partially differentiated toward vascular lineage, therefore, require less manipulation of growth factors to produce fully differentiated endothelial cells.

Pluripotent cells including embryonic stem cells or inducible pluripotent stem cells have attracted most interest recently (131, 132). The ability to produce more than one cell type from the a single cell source enables the development of complex vascular structures incorporating endothelial cells and supporting cells from the same cell source. This raises the possibility of individualized regenerative medicine approaches being possible in the future and additionally, makes it possible to create models of inherited genetic disease states *in vitro* by inducing pluripotency in cells of patients with specific genetic diseases and creating models of vasculogenesis using these cells.

There are three main methods used to differentiate pluripotent cells toward endothelial phenotype (131). Each of these methods attempt to replicate parts of the embryonic development of endothelial cells and vascular networks. The first method requires embryoid body formation which are three dimensional aggregates of pluripotent stem cells which form in suspended culture. These replicate the blastocyst formation seen *in vivo* (133, 134) and begin to differentiate with all three primitive germ layers represented; ectoderm, mesoderm and endoderm. Aggregates of cells expressing early endothelial markers developed within the embryoid body resembling blood islands (135) and these can be expanded and selected for by plating on suitable matrix with the addition of exogenous growth factors (136, 137).

The second commonly used method is co-culture of pluripotent stem cell with a feeder layer of mesenchymal cells such as bone marrow stromal cells. The feeder later provides growth factors to the stem cells encouraging differentiation toward mesoderm and expression of key endothelial markers (8, 138, 139). This also recapitulates the embryonic development of endothelial cells by providing Indian hedgehog (IHH) signaling which acts through BMP4 to upregulate endothelial markers such as VE-cadherin, CD31 and VEGFR2. These effects are abolished by neutralizing either IHH or BMP4 signaling and IHH signaling is rescued by exogenous BMP4 (8).

The third method is by 2D culture of inducible pluripotent stem cells on culture plates coated with protein substrate and culture media enriched sequentially with specific growth factors to direct differentiation toward mesodermal lineage and then endothelial lineage. The two most common protocols used to differentiate pluripotent cells toward endothelial lineage have numerous similarities and both attempt to recreate the embryonic development in the blastocyst. Mesodermal differentiation in the embryo relies on BMP-4, nodal and Wnt signaling pathways (132). *In vitro* supplementation of the media with BMP-4 and inhibitors of GSK3β, which upregulates the Wnt/B-catenin pathway, or Activin A which acts on nodal receptors are used to achieve this mesodermal transition (29, 140–142).

Following mesodermal induction, the addition of VEGF to the media is needed to start endothelial differentiation. Activators of the protein kinase A pathway such as forskolin or 8-bromo-cAMP appears to enhance the effects of VEGF and produce a greater purity of endothelial cells (29, 143, 144). The endothelial cells formed from these differentiation procedures have multiple markers of endothelial lineage including VE-cadherin, CD31 and

von Willebrand factor, however, there remain a proportion of cells which differentiate toward other lineages and therefore to obtain a pure culture of endothelial cells requires cell sorting and re-plating of endothelial cells.

Perivascular Cells

The source of perivascular cells used in multicellular culture also varies, however, these are typically mesenchymal in origin. This includes aortic smooth muscle cells, adipose derived stem cells, osteoblasts (145) and mesenchymal stem cells (146). Pericytes have also been developed using embryoid body method from pluripotent stem cells. Similarly, to the endothelial cell protocols following formation of the embryoid bodies in suspended culture they can be dissociated and sorted to using cell surface markers to identify a population of pericytes which can then be plated and expanded (147).

It is also possible to produce perivascular cells from iPSC by manipulation of growth factors in the growth media. As with endothelial cells, the first step requires induction of mesoderm which is followed by supplementation with PDGF-B along with TGF- β or Activin A to direct differentiation toward a perivascular cell fate. This is highly efficient at creating a culture of perivascular cells which can be used in co-culture experiments with endothelial cells. (140, 148) Orlova et al used a single source of hiPSC and exposed them to a short period of supplementation with VEGF to begin vascular specification which yielded a mixed population of CD31+ and CD31- cells. Following cell sorting the endothelial and perivascular cells were able to be produced from the CD31+ and CD31- fractions, respectively (149). In co-culture, the perivascular cells were able to control sprouting and proliferation of the endothelial cells in a TGF-β dependent manner similar to that seen in the developing embryo.

Co-culture

Combining endothelial cells and pericytes to create multicellular models of vasculogenesis can recreate a greater degree of the complexity of intracellular signaling seen *in vivo* than is provided by supplementing the media with supra-physiological levels of growth factors. Intrinsic intercellular regulatory mechanisms which are as yet incompletely understood and therefore difficult to replicate may provide a more physiological environment in which to model vasculogenesis.

Culture of mesenchymal stem cells (MSC) alongside the developing vascular network of endothelial cells increases the survival of vessels and helps to maintain these vessels in long-term culture (150). This coculture has been shown to upregulate VE-cadherin on the cell surface of endothelial cells and reduce the rate of proliferation and apoptosis of endothelial cells (151, 152). This results more stable, less permeable cells which form a more durable vascular network. Chen et al used co-culture of HUVEC and mature perivascular cells obtained from skeletal muscle to create vascular networks in Matrigel plugs (28). The inclusion of perivascular cells supported the formation of complex capillary structures within the plugs which was not seen with culture of endothelial cells alone (28).

Additionally, Koike and colleagues produced a model of vasculogenesis by combining HUVEC with mesenchymal stem cells in a 3D type 1 collagen and fibronectin construct and implanting these into mice. The implantation of HUVEC alone formed tubules initially but it was unable to support perfusion and subsequently, these tubules regressed. In contrast during co-culture experiments, the mesenchymal precursor cells migrated to a perivascular position started to express perivascular cell markers including α-SMA and produced stable long-lasting blood vessels which were able to support flow for over 60 days (150).

Melero-Martin and colleagues have produced similarly robust vascular networks in Matrigel plugs by using blood and cord blood derived OEC and MSC derived from the bone marrow. OEC and MSC have a greater replicative capacity than primary cell types and can be harvested from the same donor from blood and bone marrow. This enables sufficient quantities of genetically identical cells to be produced which enables more accurate modeling of inherited diseases and has potentially be used in regenerative medicine to vascularize tissue engineered grafts (153).

The development of inducible pluripotent stem cells (iPSC) by Takahashi and colleagues (154) provided further tools to build multicellular constructs which could now be derived from a single cell without many of the ethical difficulties which surrounded embryonic stem cells. Samuel et al developed endothelial and mesenchymal precursor cells in parallel from human iPSCs and by combining these cells in culture were able to form robust long-lasting functional blood vessels when implanted in mice (155). This parallel development of endothelial cells and the supporting perivascular cells was a step forward, however, still requires cells to be dissociated and sorted using flow cytometry before being plated and expanded.

Kusuma et al have started to overcome this difficulty by creating a bipotent population of cells from hiPSC which are able to differentiate toward endothelial and perivascular cell lineage (156). The difficulty with this approach *in vitro* is that proliferation is inhibited when perivascular cells and endothelial cells come into contact. Therefore, they initially focused on developing VE-cadherin positive endothelial cells. They used high concentrations of VEGF and inhibited TGF-β signaling which causes perivascular cell differentiation and mediates contact inhibition of endothelial cells (15, 157, 158). This created two populations of early vascular cells both of which expressed $CD146^{+}$ and $CD105^{+}$ and were negative for the haematopoietic marker CD45. However, the endothelial precursors expressed VE -cadherin while the perivascular precursors expressed PDGFRβ. When plated in hydrogel these cells were able to self-assemble into tubular-like structures of endothelial cells with perivascular cells surrounding these vessel forming a multicellular vascular network (156). Although these networks were not durable and typically regressed within approximately 2 weeks it provided a significant step forward in developing engineered multicellular vascular networks (159).

Use of patient-specific hiPSC-derived endothelial cells is now being used for disease modeling some of the inherited vascular disorders. The model of CADASIL is one of the first attempts to gain a better understanding of the developmental biology and cross-talk of endothelial cells and perivascular cells with

NOTCH3 mutations. Similarly, NOTCH1 mutations have also been studied with pluripotent stem cell platforms (160). This *in vitro* approach may also facilitate the development of therapeutic gene editing interventions for vascular malformations and arteriopathies in the future.

The use of three cells together in culture has recently introduced and this extra complexity appears to have further benefits to promote stable vasculature. Caspi and colleagues used coculture of HUVEC, embryonic fibroblasts and embryonic stem cell derived cardiomyocytes on a biodegradable scaffold. The embryonic fibroblasts differentiate into perivascular cells and produced increased complexity of vascular networks. Additionally, there was upregulation of several growth factors in this model including VEGF, PDGF-B and ANG-1 compared to single cell culture and the endothelial cell survival was higher (161). Similarly, bone grafts have been produced by combining HUVEC and CD146+ perivascular cells with human inducible pluripotent mesenchymal stem cells in a calcium phosphate cement. This triculture demonstrated more complex vascular networks compared to culture with mesenchymal stem cell and endothelial cells alone. There was more rapid induction of VEGF signaling and there was improved bone mineral density all of which suggested benefits from combining multiple cell types (162).

CONCLUSIONS

Development of functional blood vessels requires precise interaction between the tubule forming endothelial cells and the surrounding environment including growth factors, ECM and perivascular cells. The crosstalk between perivascular and endothelial cells is complex and incompletely understood. This cross talk plays a vital role in normal blood vessel development and homeostasis while abnormalities in this relationship have been implicated in multiple congenital and acquired diseases.

Knowledge of the molecular process involved in vasculogenesis and angiogenesis provides insight into the pathophysiological mechanism multiple vascular diseases including diabetic retinopathy and cancer. Therapies targeting the interaction between endothelial cells and neighboring perivascular cells are already in use or in late stages of development (94, 121). However, an in-depth understanding of the crosstalk between these cells will allow the development of more accurate vascular models to enable the identification of novel drug targets and further guide drug development.

Improved understanding of vascular development will also provide useful in the field of regenerative medicine where ensuring perfusion of stem cell derived tissue is one of the key challenges in producing viable implantable grafts. The development of vascular networks using multiple cell types is being used increasingly commonly and appears to provide greater complexity, stability and durability of the vasculature. Approaches using pluripotent cells are particularly intriguing given the possibility to develop these structures from a single cell source (156). This has the potential to develop more accurate models of vascular disease as well as personalized regenerative medicine applications.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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REVIEW
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Changing Metabolism in Differentiating Cardiac Progenitor Cells—Can Stem Cells Become Metabolically Flexible Cardiomyocytes?

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The heart is a metabolic omnivore and the adult heart selects the substrate best suited for each circumstance, with fatty acid oxidation preferred in order to fulfill the high energy demand of the contracting myocardium. The fetal heart exists in an hypoxic environment and obtains the bulk of its energy via glycolysis. After birth, the "fetal switch" to oxidative metabolism of glucose and fatty acids has been linked to the loss of the regenerative phenotype. Various stem cell types have been used in differentiation studies, but most are cultured in high glucose media. This does not change in the majority of cardiac differentiation protocols. Despite the fact that metabolic state affects marker expression and cellular function and activity, the substrate composition is currently being overlooked. In this review we discuss changes in cardiac metabolism during development, the various protocols used to differentiate progenitor cells to cardiomyocytes, what is known about stem cell metabolism and how consideration of metabolism can contribute toward maturation of stem cell-derived cardiomyocytes.

Keywords: heart, progenitor cells, substrate metabolism, mitochondria, differentiation, cardiomyocytes

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STEM CELL THERAPY FOR THE HEART

Myocardial infarction (MI) is the primary cause of disease-related death in the world with no reliable therapy (1). Acute coronary syndromes, like MI, account for half of all cardiovascular deaths in the industrialized world, with around 20% of patients developing heart failure (HF) and having a 1-year mortality rate (2, 3). Current therapeutic strategies focus on reperfusion, thrombolysis and reducing the workload of the heart using pharmacological agents or surgical procedures (4–6). Recent advances in treatment have improved time to reperfusion, but progress in identifying efficient therapies to offer more than symptom alleviation and support the surviving myocardium is yet to result in substantial clinical benefit (7, 8). The only current long-term solution is heart transplantation but with the limited numbers of donors, and the need for chronic immunosuppressants (9), the search to find an alternative solution to the problem of end stage HF is becoming increasingly urgent. MI can lead to a loss of up to 1 billion cardiomyocytes, which cannot be replaced due to the insufficient degree of regeneration in the adult heart (10). Although the heart is no longer considered a post-mitotic organ, the turnover of cardiomyocytes in the adult heart is around 1% per year (11) which is insufficient to counter the loss caused by MI. Stem cell therapy (SCT) has the potential to regenerate the damaged tissue and restore its

contractility, harnessing the self-renewal and differentiation potential of stem cells (SCs) (12). *In vivo*, the transplanted cells can act *via* a combination of the following mechanisms; (a) replicate themselves and/or differentiate to mature cardiomyocytes; (b) stimulate the endogenous cardiac cells to regenerate; (c) exert a beneficial effect via paracrine mechanisms of action (13) (**Figure 1**).

TYPES OF STEM CELLS FOR THERAPY

A wide range of cells have been tested both in animal models or early-stage human clinical trials in order to find the appropriate source for SCT (14, 15). These include bone-marrow derived cells (16–18), cardiac stem or progenitor cells (19–25), human embryonic stem cell-derived cardiomyocytes (26–29) and human inducible-pluripotent stem cell-derived cardiomyocytes (30, 31).

Bone marrow-derived stem cells were claimed to differentiate into cardiomyocytes that spontaneously beat after 2 weeks in culture (17) or into myotubules that, when injected into infarcted hearts, stimulated angiogenesis and generated cardiac-like cells (16). In addition, it was reported that when bone marrow-derived stem cell growth factor receptor-positive/linage negative (c-kit+/lin-) cells were injected into infarcted tissue, they generated new cardiac cells and blood vessels and re-muscularised the damaged region (18). However, later studies showed that bone marrow-derived cells do not trans-differentiate into cardiomyocytes and that retained transplanted cells adopted a mature haematopoetic fate (32, 33). Bone-marrow derived mesenchymal cells have also been shown to improve cardiac function following MI, although repair is now thought to result from the delivery of a cocktail of beneficial cytokines which induce angiogenesis, limit scar fibrosis and may activate endogenous cardiac progenitors (34–36). Other key types of mesenchymal stem cells (MSCs) such as umbilical cord MSCs (37, 38), adipose-derived MSCs (39–41) and amniotic fluid MSCs (42), chosen for their ease of isolation and differentiation, have also been tested for therapeutic potential after infarction. As with bone marrow cells, any beneficial effect was deemed to be paracrine.

In 2003, a population of cardiac progenitor cells called stem cell growth factor receptor-positive (c-kit+) cells were identified (19). *In vitro*, these proliferative cells can self-renew and differentiate into myocytes. When injected into infarcted hearts, c-kit+ cells were shown to differentiate into cardiomyocytes resulting in myocardium regeneration and improved heart function (19). Subsequent studies supported the beneficial effect after cardiac injury, but suggested that the c-kit+ cells were of bone marrow origin (43) or were mast cells (44), endothelial cells (45) or maybe a mixed population of both (46). Studies looking at the issue from a developmental perspective suggested that c-kit+ cells contribute to new cardiomyocytes after injury in the neonate, but they were unable to do so in the adult heart (47), in line with previous observations regarding the role of c-kit+ cells in the neonatal heart (48). In 2011, the Anversa group used c-kit+ cardiac progenitor cells (CPCs) in a phase-I Stem Cell Infusion in Patients with Ischemic cardiomyopathy (SCIPIO) clinical trial showing encouraging results for post-MI treatment (49). However, in 2014 the results were questioned for their integrity (50). In 2013, Ellison et al. showed that c-kit+ CPCs were necessary and sufficient for cardiac recovery in rodent models of diffuse myocardial damage causing acute heart failure (51). This was subsequently challenged by studies using c-kit+ lineage tracing mouse models, and reporter lines, where it was shown that cardiac c-kit+ cells contribute to cardiomyocytes only minimally, but mainly and substantially generate cardiac endothelial cells (52–54). The debate continues, with recent publications showing that selected c-kit+ cardiac cells contain a low level (about 1%) of clonal cells that can be expanded and differentiated into spontaneously beating cardiomyocytes (55) and that c-kit expression can be identified on both CPCs and a subpopulation of cardiomyocytes and is upregulated in response to pathological stress (56).

Also in 2003, another population of cardiac progenitor cells was identified, the stem cell antigen 1 (sca-1+) cells, in the mouse heart, having stem-like self-renewal characteristics and the ability to home to the injured myocardium (20). Later it was shown that this cell population led to increased ejection fraction and neoangiogenesis, after injection into the acutely infarcted mouse heart (57). It was also shown that sca-1+ CPCs contribute to the generation of cardiomyocytes during normal aging and after injury sca-1+ cells were induced to differentiate to three cardiac cell types (44). In line with these observations, a genetic deletion of sca-1 caused primary cardiac defects in heart contractility, an impaired damage response and reduced CPC proliferation (58). Although the human sca-1 isoform does not exist, a sca-1-like cell population has been isolated from the human heart using the murine antibody and has been extensively studied by the Goumans group (59). In a similar manner to the isolation of a clonal c-kit+ population, clonal cells have been identified within selected sca-1+, PDGRFα+ mouse cells and have been shown to improve cardiac function after MI, but again by a largely paracrine mechanism (60).

In addition, a population of progenitor cells were derived, via the formation of cardiospheres, from cells migrating from adult human and murine heart explants, called cardiosphere-derived cells (CDCs) (21). It was reported that human CDCs have the ability to self-renew in culture and express the endothelial kinase insert domain receptor (KDR) and other known stem cell markers (CD-31, CD-34, c-kit, and sca-1) (21). This heterogeneous cell population has also been shown to have beneficial effects, both in animal models (22, 61) and in a clinical trial (62), but again via the release of paracrine factors.

CPCs have been also identified by markers of embryonic origin, like Insulin gene enhancer protein 1 (Isl1) and NK2 homeobox 5 (Nkx2.5). Isl1 is a cardiac transcription factor expressed in second heart field progenitors and cardiac neural crest cells, involved in cardiovascular development, and leading to severely deformed hearts in rodents after genetic deletion (24, 63). Nkx2.5, a homeobox-containing transcription factor, has been identified via its involvement in cardiac looping (64, 65). Isl1+ and Nkx2.5+ CPCs have been shown to differentiate into

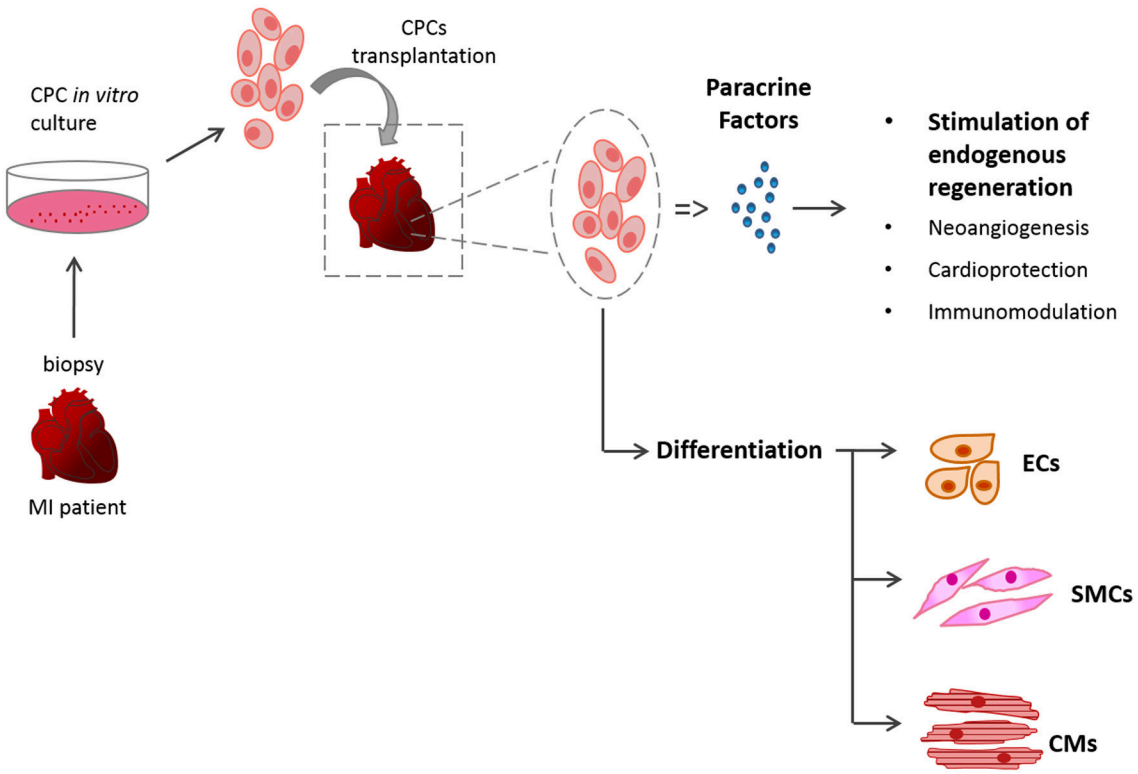

FIGURE 1 | Schematic of SCT. The mechanisms of action of the transplanted cardiac stem cells (CSCs) can be by differentiation of the donor cells or via paracrine mechanisms.

major cardiac lineages, mainly contributing to proepicardium during development (25).

Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of blastocysts from donated fertilized eggs (66). Human ESCs have the ability to continuously proliferate in an undifferentiated state and, when given the appropriate signals, will differentiate into any cell type. An interesting tool for cell therapy that originated from this field, are the *in vitro*–generated, stem cell–derived cardiomyocytes (SC-CMs). These have been shown to integrate structurally and functionally with healthy host cardiac tissue *in vivo* in various studies (26, 67, 68). These cells show great promise, but there are ethical concerns using hESCs in the clinic and the risk of teratoma formation (69). In 2007, Yamanaka's group were the first to report the reprogramming of human somatic cells into induced pluripotent stem cells (iPSCs), by overexpression of the transcription factors: Oct4, Sox2, KLF4, and c-myc (70). The reprogrammed hiPSCs resembled hESCs and had the ability to self-renew while maintaining pluripotency (70). Human iPSCs can be produced from patient-specific somatic cells, therefore overcoming the problem of immune rejection and the ethical concerns of using hESCs (69). hiPSCs have been shown to improve cardiac function, albeit with limited donor cell retention (30, 31) and used extensively as *in vitro* human-cell-based models to study basic biology and development (71), to model diseases (72) and to screen for drugs (73, 74). This is particularly important for the heart, since adult cardiomyocytes do not survive *in vitro*, as morphological and functional changes occur in long-term culture and so there has been no easy way to determine whether the effect of genetic mutations or of drug compounds that were observed in animal models would also be seen in a human cardiomyocyte. However, despite the promising *in vivo* results, the initiation of beating in SC-derived cardiomyocytes does not mean that these cells have the maturity or metabolic characteristics of mature cardiomyocytes found in the healthy heart (75). Studies have shown that SC-derived cardiomyocytes have immature calcium handling (76) and a response to drugs more akin to cardiomyocytes from the failing heart (77).

The effect of the transplantation environment on enhancing the maturation of human pluripotent SC-derived cardiomyocytes has been studied in rats. Despite their capacity to survive and form grafts, they failed to improve adverse remodeling or overall cardiac function after chronic MI (28). Approaches to enhance their efficacy, via preconditioning the cells and host environment, are currently being investigated [reviewed here (78)].

CARDIAC METABOLISM

The heart is a fascinating organ that beats 100,000 times a day and pumps 7,200 L of blood through the body, in the same period using 35 L of O_2 for energy production. It requires

about 6 kg of adenosine triphosphate (ATP), which it utilizes at a rate of 30 mg per second to sustain myocardial contraction and maintain ion homeostasis (79, 80). Since the heart has a low capacity for energy storage (81), an array of metabolic networks guides ATP production rates, based on demand. The heart has been characterized as a metabolic omnivore, being able to use a variety of substrates for energy production [see reviews (82, 83)]. Glucose, pyruvate, triglycerides, glycogen, lactate, ketone bodies, fatty acids (FAs) of different chain-lengths and certain amino acids are among the energy-providing substrates of the heart. It is responsible for almost 10% of the whole body fuel consumption; with FAs accounting for 70% of ATP production and carbohydrates for the remaining 30%.

Energy, in the form of ATP, can be produced in the cytosol via glycolysis (**Figure 2**); catabolism of glucose derived from carbohydrates. The end-product of glycolysis is pyruvate, which can be further reduced to produce lactate. In case of carbohydrate shortage, gluconeogenesis of pyruvate, re-oxygenation of lactate or glycerol metabolism, can be used as sources of glucose synthesis (82, 84). Alternatively, pyruvate can enter the mitochondria in the form of acetyl-coenzyme A (Acetyl-CoA) and be oxidized in the TCA cycle (also known as the Krebs cycle), in a process called oxidative phosphorylation (85). The reducing equivalents of this chained reaction act as hydrogen carriers (Nicotinamide Adenine Dinucleotide Hydrogen; NADH and Flavin Adenine Dinucleotide Hydrogen; $FADH_2$) and enter the electron transport chain (ETC). There the coupled-transfer of electrons and H^+ creates an electrochemical proton gradient that leads to the production of ATP.

ATP can also be generated by the degradation of lipids (including triglycerides) into FAs, which are metabolized in the mitochondria via beta-oxidation (**Figure 2**), which converts Fatty Acyl-CoA to Acetyl-CoA for flux into the TCA cycle and ATP synthesis as above (86). Under aerobic conditions, more than 95% of ATP production comes from oxidative phosphorylation (86) and in the healthy heart 50–80% of the energy is generated via beta-oxidation (87). Oxidative phosphorylation yields 36 ATP/glucose molecule, being more efficient than glycolysis (2 ATP/glucose). Lipids, due to their reduced state are more oxygen-demanding than glucose (producing 2.8 ATP/O_2, vs. 3.7 ATP/O_2), but they are more energy-dense with a much higher yield of ATP/carbon (depending on the chain length of the parent FA) (79, 88).

The heart has a remarkable ability to adapt to changes in its physiological state by selecting the most efficient substrate, depending on the conditions of its environment (82). For example, as FAs require more oxygen than glucose, to generate the same amount of ATP (82, 89), upregulation of the hypoxia-inducible factor (HIF) under hypoxia has been shown to increase

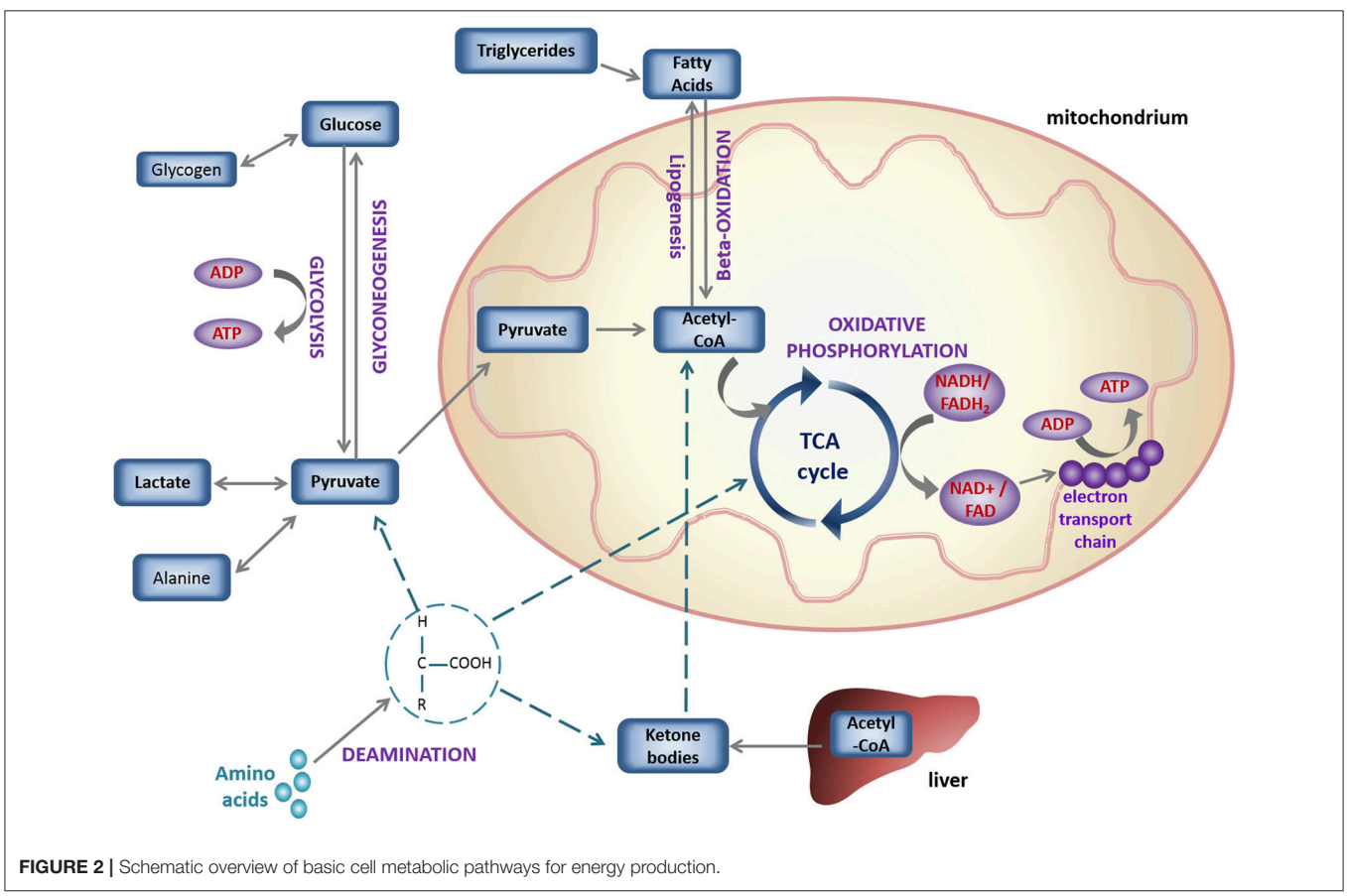

FIGURE 2 | Schematic overview of basic cell metabolic pathways for energy production.

glycolysis and suppress mitochondrial oxidative metabolism (83, 90), shifting toward the more oxygen-efficient fuel: glucose. A network of interrelated signaling pathways control the flux of glucose and fatty acid metabolism to enable the heart to switch substrates rapidly. This was first described as the glucose-fatty acid cycle by Randle in 1963 (91), but the complexity of this network is yet to be fully explored (92). Under conditions of starvation, acetyl-CoA in the liver can form ketone bodies, that are water-soluble forms of this metabolite, and can be efficiently used by the brain and other oxidative organs (82) (**Figure 2**).

Cardiac Metabolism During Development—Metabolic Switch (1)

The fetal heart is adapted to an environment of low oxygen and low fatty acid content, so fetal cardiomyocytes are highly dependent on glycolysis for ATP production (93). In addition, lactate availability allows for energy production via lactate oxidation. During development, the heart undergoes a major metabolic alteration; the main physiological changes during the transition to the post-natal stage are the increased workload, and the demand for growth, that cannot be met by glucose and lactate consumption alone (94, 95). Interestingly, immediately after birth some studies suggest that the main energy biogenesis mechanism is still glycolysis (96). The post-natal increase in both circulating levels of free FAs (due to dietary alteration and lipid content in maternal milk) and in O_2 levels mediates a switch from glycolysis-dependence, to predominantly relying on oxidative metabolism as mature cardiomyocytes (93, 97) (**Figure 3**). After birth, the "fetal switch" to oxidative metabolism of glucose and fatty acids has been linked to the loss of the regenerative phenotype (98). Neonatal mouse hearts can regenerate in the first postnatal week but this is lost after day 7 (99). Puente et al. investigated exposure of neonatal mice to hyperoxia (100% oxygen) or mild hypoxia (15% oxygen) and found that hyperoxia induced cardiomyocyte cell cycle arrest after birth whereas hypoxia prolonged the regenerative window (98). They hypothesized that this effect resulted from increased oxidative stress that accompanied the induction of oxidative metabolism and showed that treatment with an antioxidant resulted in a significant increase in cardiomyocyte mitosis in the first weeks after birth. During the early postnatal period, as cardiac energy demands increase, the number of mitochondria in cardiomyocytes increases dramatically (100, 101). A recent study has shown that the HIF1 signaling localisation pattern controls the embryonic switch toward oxidative metabolism, disruption of which affects cardiac maturation. The cardiac compartment where HIF was absent, the trabeculae, has increased oxidative metabolism, as well as higher mitochondrial content (102). Several studies demonstrated an increase in PPAR coactivator 1α (PGC-1), as well as Peroxisome Proliferator-Activated Receptor α (PPARα), mRNA levels in mice or rats during development (103, 104). The regulatory mechanism involves genes encoding several key mitochondrial ETC proteins, specifically the transcription factors nuclear respiratory factors-1 and−2 (NRF-1 and−2) (103, 105). Disruption of the ETC function, during cardiac development, leads to disrupted mitochondrial organization in the cardiomyocytes, resulting in perturbed sarcomere formation and contraction (106). Various

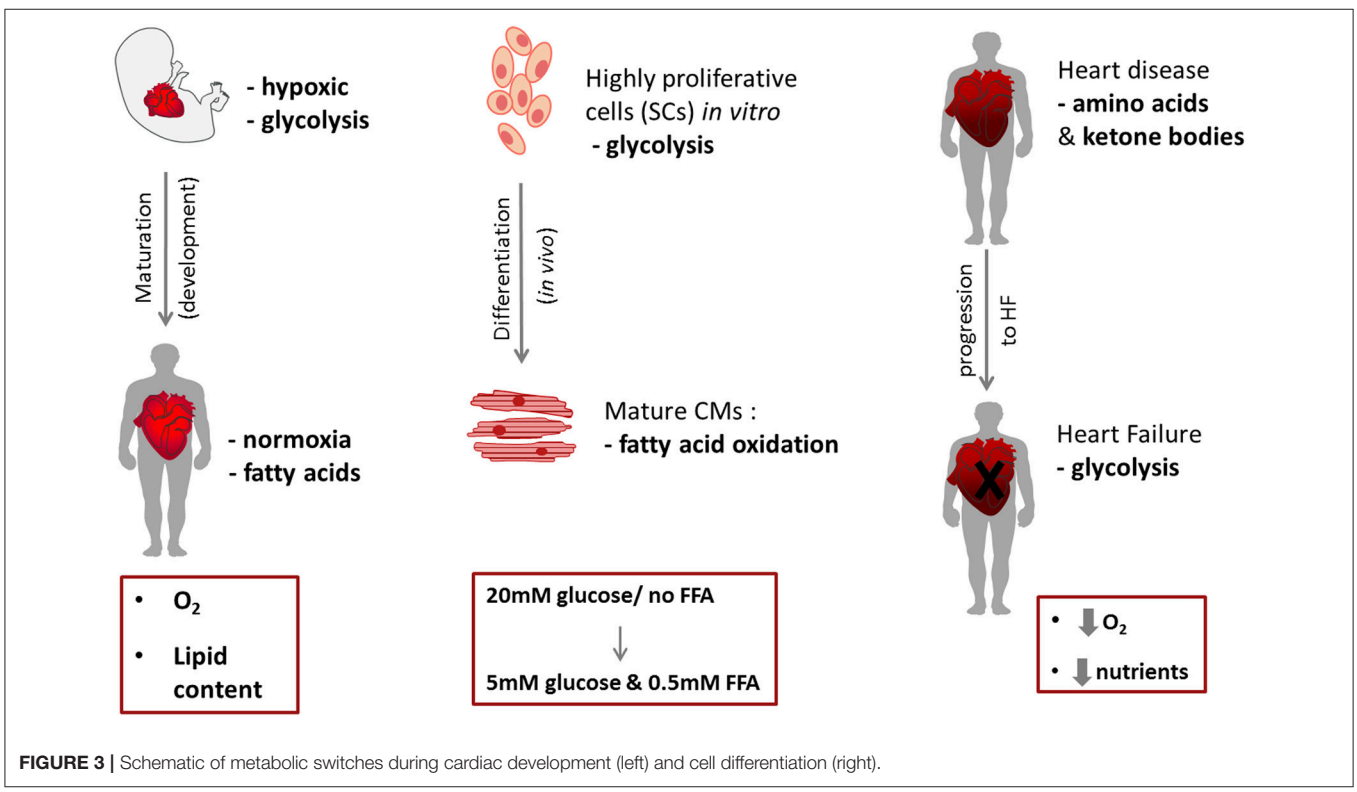

FIGURE 3 | Schematic of metabolic switches during cardiac development (left) and cell differentiation (right).

mouse models have revealed that disruption in mtDNA processes leads to embryonic lethality (107). In addition, signaling pathways involving the Nuclear factor of activated T-cell (NFAT) family of transcription factors play a role in mitochondrial biogenesis and cardiac function. Mice with nfatc3 and nfatc4 deletion had abnormal mitochondrial structure and reduced oxidative capacity, eventually dying after E10.5 (108). The ERRα/ERRγ axis plays a crucial role in mitochondrial energy production, as well, and their deletion leads to premature lethality in mice (109). Disruption of the ERRγ gene leads to impaired mitochondrial function, eventually blocking the switch from glycolysis to oxidative phosphorylation and leading to early prenatal death in mice (110).

Cardiac Metabolism in MI and HF—Metabolic Switch (2)

Following MI, tissue ischaemia leads to hypoxia, which in turn activates long-term HIF signaling in the myocardium (111). During MI, the ischemic region relies solely on glycogen, as an anaerobic fuel for energy production (112). The absence of oxygen for OXPHOS means that NADH and FADH2 are accumulated, affecting fatty acid enzyme reactions and leading to a build-up of fatty acid intermediates and resulting in disruption of mitochondrial cristae and function (87). Reduction in fatty acid oxidation upregulates anaerobic glycolysis and lactate production leading to intracellular acidosis (87). HIF1α activation upregulates the transcription of Bcl-2 adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), which triggers selective mitochondrial autophagy (113, 114). Under normal conditions, BNIP3 expression is suppressed by the nuclear factor kappa beta (NF-κB) pathway (114). In addition, HIF1α upregulates the transcription of pyruvate dehydrogenase kinase 1 (PDK1), which inactivates pyruvate dehydrogenase, thereby blocking the entry of pyruvate into the mitochondria for oxidative phosphorylation (113). These HIF-1 mediated changes play key roles in reducing mitochondrial oxidative metabolism and thereby in reducing the generation of reactive oxygen species (ROS) (115). Following reperfusion, oxidative metabolism is restored but the resulting induction of ROS can lead to mitochondrial damage (116). As the heart progresses to failure, flux from pyruvate into the TCA cycle decreases (117). Mitochondria can be found with membrane and ETC defects (118, 119), as well as reduced respiratory capacity (119, 120) and reduced oxidative phosphorylation (121–123). These findings are consistent with the concept that, during HF, the metabolic state of the heart resembles that of the fetal stage, switching to glycolysis rather than mitochondrial oxidative metabolism (124); with increased glucose uptake (125) and glycolysis (86) and either no change or a decrease in glucose oxidation (125, 126).

The aforementioned perturbations in cardiac metabolism lead to a dangerous environment, due to the excess of lactate and oxidative metabolic intermediates. Progressive heart failure induces, at a cellular level, increased conversion to lactate, increasing cell acidosis (127). However, amino acids such as aspartate play a role in reducing damage that occurs as a result of lactate accumulation via upregulating the conversion of pyruvate to alanine. Glutamate and aspartate improve the production of ATP as they produce intermediates that feed the TCA cycle for further oxidation (128). Studies have reported the reduced availability of amino acids in heart failure patients, leading to the depletion of some valuable amino acid derivatives that are important for normal cardiac function (l-carnitine and creatine) (129). However, there is also evidence of their accumulation during HF, as seen in a study on failing mouse hearts where genes associated with amino acid catabolism were downregulated during compensated hypertrophy and overt failure (130). Transcriptomic analyses reflected downregulation of genes involved in amino acid degradation pathways [proline, alanine, tryptophan, and mainly branched-chain amino acids (BCAAs)] (131–133). Using a genetic mouse model, Sun et al. demonstrated that a deficiency in BCAA catabolism induced heart failure under mechanical overload, resulting from increased oxidative stress (132). Similarly, Li et al. found that whilst chronic accumulation of BCAAs did not affect cardiac energetics and function in the healthy mouse heart, glucose oxidation was decreased which increased ischaemic injury after myocardial infarction (134).

The importance of mitochondrial integrity for maintenance of cardiac function has been also highlighted through several conditions which are characterized by mitochondrial mutations or abnormalities, causing among others: cardiomyopathy, neuromuscular dysfunction, diabetes mellitus and even sudden death (122, 135). In addition, the efficiency of the ETC is found to decline with age, which in turn decreases ATP generation (135, 136).

Cardiac Metabolism From Stem Cells to Adult Cells and Vice Versa—Metabolic Switch (3)

Metabolic changes during embryonic development have been reviewed in detail by Johnson et al. (137). Mammalian oocytes contain low levels of glycogen and fat but have substantial stores of amino acids and protein which have been estimated to be sufficient for the energetic needs of the first few days of development (137). The preimplantation embryo relies on the mitochondria inherited from the oocyte and on pyruvate oxidation for ATP production (138, 139). There is a gradual switch in metabolism during the preimplantation stage (morula, blastocyte) from aerobic oxidation to anaerobic glycolysis in preparation of the low-oxygen environment in the uterine wall (137). As the preimplantation embryo undergoes cell division there is a reduction in mitochondrial DNA copy number (mtDNA) and mitochondrial density (138) and an upregulation of glucose transporter 3 and hexokinase gene expression until anaerobic glycolysis increases to maximal rates as the blastocyst implants into the uterus (137). ESCs originate from the inner cell mass of preimplantation blastocyst, therefore, these cells show high rates of glycolysis and low oxidative phosphorylation to support the rapid cell proliferation (138, 140). Similarly, ESCs derived from *in vitro* cultured ICMs (141) or ESCs cultured *in vitro* (142) show high rates of glycolysis and low oxidative phosphorylation. A comparison of the metabolomics and energetics of iPSCs with those of ESCs showed many

similarities and confirmed that, during reprogramming, somatic cells convert from an oxidative state to a glycolytic state in pluripotency (143).

The reprogramming factors, c-Myc and Lin28, have been shown to enhance glycolysis and nuclear reprogramming (140) and reprogrammed pluripotent stem cells show upregulation of glycolytic enzymes such as the glucose transporter GLUT1, hexokinase (HK), phosphofructokinase (PFK) and LDH (144). In addition, hiPSCs have increased levels of intracellular glucose-6-phosphate, which feeds into the pentose phosphate pathway (PPP) to generate the reducing factor NADPH, which is used for biosynthesis of FAs and nucleotides necessary for rapid proliferation (144). Stimulation of glycolysis with D-fructose-6-phosphate or by PDK1 activation has been shown to enhance the efficiency of iPSC reprogramming (145). Mitochondrial morphology changes from being elongated and tubular-shaped with well-developed electron-dense cristae in somatic cells, to being spherical with small-undeveloped cristae in hiPSCs, with a transition from an extensive cytoplasmic networks in somatic cells, to a predominantly peri-nuclear location in hiPSCs (146). Prieto et al. found that this fragmentation was not associated with mitophagy but was induced by activation of dynamin-related protein 1 (Drp1), which fragments mitochondria in a GTPase-dependent manner (147). They showed that Erk activation in early reprogramming induced Drp1 phosphorylation which was critical to the reprogramming pathway. Studies in human and mouse ESCs have also found a small number of rounded and immature mitochondria with under-developed cristae (106, 148). Moreover, multipotent stem/progenitor cells show the same characteristics; for example, mitochondria in HSCs are relatively inactive and ATP content increases in lineage-committed progenitors compared with HSCs (149).

Due to the ongoing controversy as to whether the heart contains resident progenitor cells (150), little is known about the metabolism of cardiac stem cells in the adult heart. However most adult endogenous progenitor/stem cells reside in hypoxic niches (151), in a quiescent state, and therefore the metabolic rate is presumed to be low. CPCs isolated from the heart, by marker selection, tissue digestion or from explants, and expanded *in vitro*, are proliferative and grown in culture medium containing high levels of glucose and little or no FAs. Basal cellular homeostasis involves processes like protein turnover, DNA repair, and vesicle trafficking and therefore proliferating cells, in addition to homeostasis maintenance, need energy for anabolic processes such as cell division and growth. Proliferative cells, such as cancer cells, have been suggested to predominantly rely on glycolysis for ATP production, irrespective of oxygen presence; this metabolic paradox, first observed by Warburg in 1956 (152) has been termed "aerobic glycolysis." Apart from Warburg's observations on cancer cells, mouse fibroblasts (153) and human (154) and mouse (155) lymphocytes have been shown to utilize "aerobic glycolysis," when stimulated to proliferate. Various studies have supported the conclusion that the major function of aerobic glycolysis is to supply glycolytic intermediates for anabolic reactions in cells, thus being the metabolic pathway of choice during cell proliferation [for Review see (156)]. Although cells in high glucose medium predominantly metabolise glucose, they can use other substrates if provided. Human bone marrow MSCs have an active oxidative metabolism with a range of substrates and can produce more ATP from substrate oxidation than glycolysis with certain substrates (157). For example, the ketone body, acetoacetate, can be oxidized at up to 35 times the rate of glucose. Many proliferating mammalian cells, such as human MSCs, also consume glutamine to provide material for biosynthesis (158). Glutamine, as a carbon source, can supply the TCA cycle with intermediates that can be used for the production of new macromolecules in cells. A recent study by Hosios et al., in 2016, argued that glutamine contributes most to protein synthesis, suggesting that anaplerosis of glutamine in the TCA cycle is serving mainly amino acid biosynthesis (159).

When stem/progenitor cells differentiate to cardiomyocytes they need to increase the number of their mitochondria and upregulate FA metabolism (**Figure 3**). A study comparing hiPSC-derived cardiomyocytes (hiPSC-CM) to hiPSCs, showed an increase in mitochondrial relative abundance and activity (mitochondria membrane potential) as a result of cardiac differentiation (160) and that additional mitochondrial maturation in hiPSC-CMs could be achieved by long-term culture (3 months). Comparison of substrate metabolism in ESCs with that in ESC-derived cardiomyocytes has shown increased oxidative metabolism after differentiation (161) and that the respiratory capacity of cardiomyocytes was higher than in ESCs, resulting in an increased ADP:ATP ratio in the cardiomyocytes (106). The mitochondria become elongated with abundant and organized cristae in cardiomyocytes and formed into networks which filled the cytoplasm (106).

WHY STUDY STEM CELL METABOLISM

The metabolism of any cell type *in vitro* depends on energy requirements and substrate availability. Cardiac progenitor cells, when expanded *in vitro*, reside in a high glucose environment and rely on aerobic glycolysis for energy generation. CPCs will experience a shift in substrate availability following transplantation *in vivo* (**Figure 3**), being transferred from the culture medium, which contains about 5–25 mM glucose (depending on the culture protocol) and no FFA, to substrates in plasma that vary substantially. Glucose levels in mice have been measured at between ∼3.4 and 9.6 mM (2.8–7.5 mM in rats) (162, 163) and ∼0.18–0.6 mM FFA (164). In humans, healthy plasma glucose levels are around 5 mM and FFA 0.5 mM (165). This alteration is bound to cause changes in the metabolic machinery of the cells which might be one of the stimuli that induce differentiation following transplantation, but may also result in increased release of ROS and cellular damage.

The various protocols used for CPC differentiation focus on pharmacological reagents and cytokines, and do not refer to or take into account the substrate composition. This is a striking fact especially knowing how metabolic changes affect the function of the cells (166) and how transition from glycolysis to FA oxidation affects cell maturation (93), and *vice-versa* (144). iPSC-CM have been shown to integrate structurally and functionally with healthy host cardiac tissue *in vivo* in

various studies (26, 67, 68). Despite the promising *in vivo* results, the initiation of beating in iPSC-CM does not mean that these cells have the metabolic characteristics of mature cardiomyocytes found in the heart. Studies have shown that SC-CMs have immature calcium handling (76, 167) and a response to drugs more akin to cardiomyocytes from the failing heart (77). Despite the observed mitochondrial remodeling and upregulation of oxidative metabolism previously discussed, newly differentiated iPSC-CM in culture have been shown to retain a predominantly glycolytic metabolism (168). One of the major roles of iPSC-CM is that of a drug-testing platform and this requires the differentiated cells to acquire a fully mature phenotype (74). For example, a recent iPSC-CM study showed that the electrophysiological responsiveness of iPSC-CM was dependent on their maturation state (169).

ASSESSING CELL METABOLISM

The main approaches for the investigation of substrate metabolism include the measurement of metabolic fluxes using radio-labeled substrates and of oxygen consumption.

Radio-Labeled Substrates Assays

Glucose oxidation rates are commonly measured using the method of the Collins et al. (163) with cell culture media containing D-U-^{14}C-glucose. Glucose oxidation results in the production of $^{14}CO_2$ which is trapped for analysis using a scintillation counter. For ^{3}H-FA oxidation **t**he cells are incubated in media supplemented with the radioactive FA tracer of interest. FA oxidation rates are determined by the production of ^3H_2O from the mitochondria (170, 171). Media aliquots contain both ^3H_2O and ^{3}H-FA, so the ^3H_2O is separated via a Folch extraction (170). Glycolytic rates are determined through the conversion of ^{3}H-glucose to ^3H_2O via enolase which converts 2-phosphoglycerate to phosphoenolpyruvate and releases ^3H_2O as a by-product that is collected using a Dowex mesh (Sigma, UK) anion exchange column, allowing for the ^{3}H-glucose to bind to the column and ^3H_2O to be eluted (171).

Oxygen Consumption

The rate of mitochondrial oxygen consumption (OCR) can be measured using the XF Extracellular Flux Analyzer (Seahorse Bioscience), the Oroboros O2K or the Clark-type oxygen electrode (172, 173). Seahorse XF Analyzers measure the concentration of dissolved oxygen and pH to quantify the oxygen consumption and extracellular acidification rate in the media in multi-well plates. Four injection ports in each well allow for addition of reagents. However, the Seahorse requires cells to be adhered to the wells, multiple additions of substrates are not possible and the individual wells are not self-contained and thus cells can be affected by gases from adjacent wells (172). The Clark-type oxygen electrode is embedded in individual reaction chambers and cells are added in suspension in the respiration media (173). Unlimited reagents and substrates can be added by manual addition. This system is more automated in the Oroboros O2K which also includes optical sensors to allow for detection of fluorescent dyes so that parameters such as ATP production or mitochondrial membrane potential may be measured (172).

The OCR can be measured under baseline un-stimulated conditions in media containing the substrate of interest (such as pyruvate; palmitate; malate; or oleic acid). Addition of the ATP synthase inhibitor oligomycin provides a measure of contaminating ATP synthase activity from damaged mitochondria and of proton leak, whilst the metabolic uncoupler, carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) permits measurement of fully uncoupled or maximal respiration (173). Finally, mitochondrial inhibitors such as rotenone (complex I) and antimycin (complex III) will completely inhibit mitochondrial respiration. More exquisite interrogation of the function of mitochondrial metabolism can be performed using substrates such as glutamate which assesses the second span of the Krebs cycle, succinate which enters the electron transport chain at complex II, or β-hydroxybutyrate, a fatty acid-derived substrate which bypasses β-oxidation and enters the Krebs cycle as acetyl CoA (116, 174).

Extracellular Measurement of Glucose Consumption and Lactate Production

Lactate production can be measured using the Seahorse Flux Analyzer from the extracellular acidification rate (ECAR) of the media. Glucose and lactate levels can also be determined using enzymatic assays or the ABX Pentra 400 Chemistry Analyser (Horiba Ltd. USA). Glycolysis yields two molecules of pyruvate, which can be converted to either lactate or acetyl CoA. Therefore, the ratio of glucose consumption to lactate production can be used as an indicator of the level of utilization of pyruvate in oxidative metabolism (175).

CARDIAC DIFFERENTIATION *IN VITRO*

Various methods and strategies have been applied to develop the optimal protocol for directing *in vitro* cardiac differentiation of stem cells. *In vitro* differentiation of adult endogenous CPCs is very challenging, due to their limited plasticity. Despite a variety of differentiation studies, the ability of adult progenitors for differentiation, is still under debate (176, 177). Different approaches, utilizing various differentiation factors have been used on both pluripotent SCs and CPCs, with the main ones being; DMSO, 5-Azacytidine, Ascorbic Acid, members of the TGF-β superfamily, oxytocin, dexamethasone and retinoic acid (see **Table 1**).

More specifically, 5-azacytidine (5-Aza) is a demethylating agent that allows for the exposure of genes that are normally silenced, due to hypermethylation, by inhibiting DNA methyltransferase (191, 192). Several *in vitro* studies suggested that 5-Aza can induce cardiac differentiation, on different MSC types, such as human umbilical cord-derived MSCs (186) and adult human bone marrow-derived MSCs (17, 187). Other studies have demonstrated the inefficiency of 5-Aza as a cardiac differentiation agent, showing transdifferentiation to skeletal muscle cells, rather than cardiac cells (193) as well as unsuccessful differentiation of adipose-derived stem cells (ASCs) (194) and adult mouse Sca-1+ CPCs (182). Ascorbic Acid (A.A.), is an antioxidant compound which has been shown to increase the expression of cardiac genes and their proteins

TABLE 1 | Factors and media used to differentiate adult stem cells to cardiomyocytes.

Differentiation approaches	References	Differentiated cell type	Cell culture medium and differentiation factors	Glucose concentration
TGF-β1 family	(178)	Human atria Sca-1$^+$; clonally isolated & magnetic-sorting	5 μM 5-Aza, TGF-β1 1 ng/ml, 10^{-4} M AA, IMDM/Ham's F12 GlutaMAX, 2% serum, 1% MEM amino acids, 1% insulin–transferrin–selenium	
	(179)	Human fetal and adult atrial biopsies, Sca-1$^+$ magnetic-sorting		12.5 mM
	(180)	Whole mouse heart Sca-1$^+$/ CD45$^-$ magnetic-sorting CDCs (Isl1$^+$)		
Oxytocin	(181)	Mouse or rat whole hearts, c-kit$^+$/CD45$^-$/Tryp$^-$ magnetic-sorting	DMEM 100 nM oxytocin acetate, 50 μg/ml AA, 2% serum, 1 μM dexamethasone, beta-glycerol phosphate 10 mM, TGF-β1, 5 ng/ml, BMP2 10 ng/ml, BMP4 10 ng/ml, Dkk1 150 ng/ml	5.5. mM
	(182)	Mouse whole heart Sca-1+ magnetic-sorting	IMDM, 10% serum, 100 nm oxytocin	22.5 mM
	(183)	Rat, mouse ventricles side population cells		
5-Azacytidine	(184)	Mouse bone marrow stroma MSCs	IMDM, 20% serum, 3 μmol/L 5-Aza	20 mM
	(185)	Human adipose MSCs	RPMI + 15% FCS, 1–9 μmol/L 5-Aza	9.4 mM
	(20)	Mouse whole heart Sca-1$^+$ magnetic-sorting	Medium 199, 2% FBS, 3 μM 5-Aza	5.5. mM
	(186)	Human umbilical cord MSCs	LG-DMEM, 10% FBS, 10 μM 5-Aza	5.0. mM
	(187)	Human bone marrow MSCs	LG-DMEM, 20% serum, 3 μM 5-Aza	4.4 mM
	(188)	Rat bone marrowMSCs	DMEM, 10% FBS, 10 μM 5-Aza	5.0. mM
Dexamethasone	(189)	Human atrial or ventricular c-kit$^+$ -sorted	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(19)	Rats c-kit$^+$/ Lin$^-$ magnetic-sorting	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(190)	Dog left ventricle c-kit$^+$/ Lin$^-$ and Sca-1$^+$/ Lin$^-$ magnetic-sorting	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(53)	mouse whole heart c-kit$^+$	DMEM, 10% serum, 10 nM dexamethasone	9 mM

5-Aza, 5-azacytidine; AA, Ascorbic acid; BMP, Bone morphogenic protein; Dkk1, Dickkopf-related protein 1; DMEM, Dubecco Modified Eagle Medium; MDMD, Iscove's Modified Dulbecco's Medium; LG-DMEM: low glucose DMEM; MEM, Minimum Essential Medium; RPMI, Roswell Park Memorial Institute medium; TGFβ, transforming growth factor β.

and to lead to beating cardiomyocytes in mouse ES cells (195). Cao et al. in 2012 demonstrated that A.A. was able to induce cardiac differentiation and maturation in several human and mouse iPSC lines (196). In contrast, treatment of BM-derived MSCs with A.A. triggered their proliferation and differentiation into osteoblasts and adipocytes (197). Another key player in cardiac differentiation is transforming growth factor-beta 1 (TGF-β1) which is thought to drive cardiac differentiation by inducing the cardiac transcription factor Nkx2.5 (198). Goumans et al. used TGF-β1 to induce differentiation of adult atrial Sca-1$^+$ CPCs, in combination with 5-Aza and A.A (178, 179). The mechanism of action involves phosphorylation of Smad2, that leads to the expression of cardiac-specific proteins (179). Dexamethasone is a glucocorticoid compound with immunomodulatory properties. Initial studies showed that it stimulates differentiation and maturation of osteogenic progenitor cells (199). The osteogenic effect of dexamethasone has since been demonstrated on MSCs (200–202). Interestingly, various groups have used it as the main agent of differentiation of adult selected c-kit+ CPCs to cardiomyocytes (19, 181, 189, 190). One of the most successful *in vitro* differentiation protocols for adult cardiac progenitors uses staged treatment with oxytocin, BMP2/4, TGF-β, and DKK1 which has been shown to induce cloned c-kit+ progenitor cells to form beating cardiomyocytes (181)

Cardiac directed differentiation protocols used for pluripotent SCs can be divided into two main groups, the Embryoid Body (EB)-based method (203, 204) and the monolayer-based method (30, 205–208). These protocols are discussed briefly here but a more comprehensive review has been given by Mummery et al. (209). Although cardiac differentiation protocols vary, most monolayer-based methods involve stage-specific activation and inhibition of signaling pathways that control heart development, replicating the early cardiac developmental stages in the early embryo (mesoderm induction, mesoderm cardiac specification, and generation of cardiomyocytes). The signaling pathways that

are involved in most directed cardiac differentiation protocols are Activin/Nodal and bone morphogenetic protein (BMP) signaling (203), which are members of the (TGFβ) signaling pathway and the fibroblast growth factor (FGF) (30, 203), and the Wnt signaling pathways (205–208). Activin A, BMP4, and Wnt3 induce the mesoderm, and upregulate the expression of Brachyury T, whereas inhibition of Wnt signaling at later stages of differentiation has been shown to induce mesoderm cardiac specification (209). Following these steps, cells are generally cultured with media in the absence of growth factors, to allow cardiomyocyte maturation to give spontaneous beating. The distinct effects of Wnt signaling during various stages of cardiac development has been thoroughly investigated, both *in vitro* and *in vivo* (210). More specifically, it was shown with *in vivo* gene function studies in the mouse that Wnt initially enhances mesoderm commitment, while it later hinders the induction of cardiogenesis, and that it stimulates the proliferation of Isl1+ cardiac progenitors (211, 212). In addition, a study in zebrafish embryos demonstrated the switch from the inductive to the inhibitory role, of Wnt on cardiac formation, during a short 1-hour window prior to gastrulation (213). In chick embryos, treatment with Wnt antagonists *in vivo* enhanced expression of cardiac muscle differentiation markers and increased expression of Isl1 and Nkx2.5 in splanchnic mesoderm (214). *In vitro,* Murry's lab in 2010 showed that supplementation with exogenous Wnt at the point of initiation of cardiogenic differentiation of hESC enhanced the cardiac marker expression, while the same effect was induced by antagonism of endogenous Wnts at a later stage (208).

Differentiation protocols were first developed using hESCs and then were translated to iPSCs. **Table 2** summarizes and compares some of the published differentiation protocols and shows a shift from the EB-based model using hESCs to the monolayer-based model using hiPSCs, which is the commonly model used currently. **Table 2** also takes note of the fact that all these protocols are differentiated in variable concentrations of glucose (4.5–25mM) and insignificantly low amounts to no fatty acids (at most 2 μM). In general, spontaneously beating EBs are generated from cells in suspension using a serum-based media to induce spontaneous cardiac differentiation (27, 204, 216). The beating areas are then hand-picked from the rest of the EB or flow-sorted. Laflamme et al., played a key role in the enhancement of cardiac differentiation protocols by shifting from the serum-induced EB-based differentiation protocol that generated 10–15% spontaneously beating EBs (218) to the serum-free monolayer-based differentiation generating >30% cardiomyocytes using Activin A and BMP4 (27). In 2011, Burridge et al. systematically compared 45 variables added to EBs formed by forced aggregation which they tested on four hESC and seven hiPSC lines (222). Their optimized method included addition of BMP4 and FGF2, with polyvinyl alcohol to aid EB formation, serum and insulin to induce oxidative metabolism and with staged exposure to physiological (5%) oxygen. The growth factor-directed differentiation enabled cells to be differentiated as monolayers, thereby introducing more straightforward, and hopefully more reproducible, methods of differentiation (209). Lian et al. also optimized the Wnt-based protocol by removing insulin during early stages of differentiation (205). They had found that when iPS cells were differentiated using a Gsk3β inhibitor, Activin A, and BMP4, the presence of insulin in the early stages had a strong inhibitory effect but that this was not seen when cells were differentiated by manipulation of Wnt signaling. They identified an interplay between insulin signaling and Wnt signaling which coordinates to influence differentiation to cardiomyocytes and therefore introduced staged removal and re-addition of insulin to their differentiation protocol (205).

MEDIA COMPOSITION DURING/AFTER DIFFERENTIATION

As shown in **Tables 1**, **2**, the majority of differentiation protocols for CPCs to cardiomyocytes had a no lipids in the media composition whilst the glucose concentration in culture media ranged from 4.5 mM to 25 mM (Here it should be mentioned that the addition of serum at percentages ranging from 2 to 20% does not allow for an absolutely clear image of the media composition). The variability of glucose concentration is striking, especially bearing in mind that a glucose level of 25 mM *in vivo* is considered hyperglycemic and leads to loss of mitochondrial networks (224, 225).

The Role of Hypoxia and of Reactive Oxygen Species in Differentiation

Stem cells *in vivo* occupy an hypoxic niche and their energy yielding metabolism is likely to be hypoxic with a high reliance on glycolysis for ATP generation (226, 227). There are conflicting reports on the relationship between hypoxia and differentiation of stem cells. Hypoxia alone can revert hESC- or iPSC-derived differentiated cells back to a stem cell-like state, by re-activation of an Oct4-promoter reporter (228). In contrast, exogenous expression of HIF has been shown to promote cardiac differentiation of ESC (229). Transient hypoxia during *in vitro* cardiac differentiation upregulated the Wnt signaling pathway with increased expression of the endogenous Wnt proteins (wnt3, wnt3a, wnt9a, and wnt11), which was lost when the cells were transferred back to normoxia. This resulted in increased expression of cardiac markers such as Isl-1 and Troponin C but decreased expression of βMHC and a failure to develop the contractile phenotype (230). To further complicate the picture, Gaber et al. found a dose-dependent increase in expression of the DNA damage marker γH2AX and of senescence in ESCs differentiated under increasing exposure to hypoxia (231). These differential reports may result from the different cell types (ESC or iPSC) and species studied. Fynes et al. found that hypoxic culture of mESCs primed the cells for differentiation and resulted in increased differentiation along the mesoderm and endodermal lineages whereas hiPSCs were pushed toward a more naïve pluripotent state by hypoxia and were then primed for ectoderm differentiation (232).

As previously discussed, the loss of regenerative potential of the heart in the first weeks of life has been attributed

TABLE 2 | Factors and media used to differentiate pluripotent stem cells to cardiomyocytes.

Different-iation method cell type	Growth factor or small molecules	Culture media	Glucose concent-ration	% of Cardiac markers	References
EB-Method hESCs iPSc	Cells in suspension -optionally differentiated with serum-based media, DMSO, all-trans retinoic acid or 5-Aza	80% KO-DMEM, 1 mMol L-glutamine, 1.1-1.2mMol B-ME,1% NEAA, 20% FBS	4.5mM	8.1% spontaneously beating EBs. In beating EBs, 29.4% cTnI+ cells. 25% spontaneously beating EBs by day 8 and 70% by day 16 of differentiation 5-Aza enhanced levels of cardiac α-MHC 10 to 15% positive for sarcomeric MHC	(204) (215) (216) (217) (218)
Monolayer hESCs hiPSC	Guided differentiation: h-Activin A, h-BMP4 +/- staged addition of h-Wnt3a & h-DKK1	RPMI+B27	11.1mM Note: 2uM of oleic acid	> 30% CMs Differentiated cells underwent Percoll gradient centrifuge for CM enrichment (69 ± 10% CMs) Increase in sarcomeric MHC+ cells from 4 to 27%	(27) (70) (208)
EB-Method hESCs hiPSC	END-2 method (Insulin depletion, PGI2, p38 inhibition)	80% KO-DMEM, 2 mMol L-glutamine,10ng/ml bFGF, 1.2 mMol B-ME, 7.5% FCS	4.5mM	50% beating CM	(219) (220)
EB-Method hESCs	Guided differentiation: h-Activin A, h-BMP4, h-bFGF, h-VEGF, h-DKK1	StemPro-34 (Base) + [L-glutamine, AA, optional MTG, P/S]	>25mM	KDR+ selected cells 35 ± 6% cTNT+, enriched to 57±4% by monolayer culture. DKK1 on day 4 gave 2-fold enrichment of CTNT+ cells KDR+/PDGFRA+ cells, 50–70% cTNT+ cells in beating EBs. 80% cTNT+ cells by monolayer culture	(221) (203)
EB-method iPSCs	Forced aggregation EBs with guided differentiation:BMP4, FGF2, Staged O_2 levels	RPMI (L-glutamine) 20% FBS on day 3 Insulin on days 0-2 and day 4	9–11mM Note: 2uM FA	Contracting EBs contained 64–89% of cardiac troponin I^+cells	(222)
Monolayer hiPSCs	Guided differentiation: h-Activin A, h-BMP4, h-bFGF	StemPro-34 + [L-glutamine,MTG, AA, P/S]	>25mM	Spontaneously beating sheets of CMs 40 ± 15% CMs	(30)
Monolayer hESCs hiPSCs	Guided differentiation method: h-Activin A, h-BMP4, h-bFGF, + Matrigel or CHIR99021, IWP4/IWP2	RPMI+B27(-insulin) [d0-6 of differentiation]; RPMI+B27 [from d7 of differentiation]	11.1mM Note: 2uM oleic acid	Matrigel addition on day−2 and day 0 generated 80% cTnT+ CMs Spontaneously beating sheets of CMs, 87% cTNT+ CMs	(223) (207) (206) (205)

B-ME, beta-mercaptoethanol; CM, cardiomyocytes; MTG, monothioglycerol; P/S, penicillin/streptomycin; AA, ascorbic acid; NEAA, non-essential amino acids.

to DNA damage resulting from the increase in ROS that accompanies upregulation of mitochondrial metabolism (98). However, ROS have also been shown to signal differentiation to several cell types, including cardiomyocytes. Transient expression of an NADPH oxidase-like enzyme with induction of ROS during embryoid body development of ESCs enhanced cardiomyogenesis, which was shown to occur *via* PI-3-kinase regulation and to be inhibited by the addition of radical scavengers (233). Similarly, NADPH oxidase (NOX)-derived ROS induced cardiac differentiation via a p38 mitogen-activated protein kinase (MAPK)-dependent pathway (234). Inhibition of mitochondrial biogenesis using shRNA targeting of PGC-1α, in hESC differentiation to cardiomyoytes, repressed mitochondrial respiration, and beating frequency (161). Levels of ROS increased during differentiation but were repressed by knockdown of PGC-1α. However, decreasing ROS levels by differentiating cells under hypoxia decreased the rate of mitochondrial biogenesis, which was stimulated by induction of ROS (161). Interestingly, in addition to reducing the beating frequency, decreasing ROS levels increased the action potential and calcium transient amplitude, but made the cells vulnerable to metabolic stress. ES cells cultured in physiological levels of glucose (5 mmol) maintained their stemness and showed reduced levels of ROS, but failed to differentiate to fully-formed cardiomyocytes (235). This was associated with reduced levels of NOX4 and MAPK which were rescued by addition of the pro-oxidant ascorbic acid.

STRATEGIES TO MATURE CELLS

A range of strategies have been used to mature iPS- and ESC-CMs, including time in culture (236), mechanical and electrical stimulation (237, 238), addition of small molecules (239), substrate stiffness (240), genetic approaches (241–243) and growth in 3D tissues (244–246). These are reviewed in detail elsewhere (74, 247) but very few consider the medium in which the cells are cultured.

CHANGING MEDIA COMPOSITION TO BE MORE PHYSIOLOGICALLY RELEVANT

There is a change in energy substrate availability and metabolism during heart development from the embryo into the adult stage. However, this change in metabolism is not mimicked in most published differentiation protocols. Maturation of ESC-CMs was induced by treatment with the thyroid hormone, tri-iodo-L-thyronine, which induced an enhancement in contractile kinetics, in rates of calcium release and reuptake and in sarcoendoplasmic reticulum ATPase expression, and a significant increase in maximal mitochondrial respiratory capacity and respiratory reserve capability (239). However, in that study the newly formed cardiomyocytes were cultured in serum-free medium with no fatty acids. Similarly, where maturation of ESC-CMs was induced using members of the Let-7 family of microRNAs, increasing cell size, sarcomere length, force of contraction, and respiratory capacity, no fatty acids were added to the serum-free culture medium (243).

A few recent studies have begun to change media composition to induce a particular disease phenotype. Kim et al. used a lipogenic medium comprising insulin, dexamethasone and 3-isobutyl-1-methylxanthine to induce fatty acid metabolism in iPSC-CM derived from patients with arrhythmogenic right ventricular dysplasia. They saw a mild increase in lipogenesis with minimal apoptosis after 4–5 weeks of treatment and increased expression of PPARα. Cell maturation revealed that metabolic derangement was implicated in the onset of arrhythmia (168). Drawnell et al. induced a diabetic phenotype by treating differentiated iPSC-CM with a maturation medium containing insulin and fatty acids, but no glucose for three days, followed by treatment with a diabetic milieu of glucose (10 mM), endothelin-1 (10 nM), and cortisol (1 μM) (248). This induced an increase in sarcomere length, and in velocity and duration of action potential, in addition to increases in the myosin light chain genes, MYL2, MYL3 and MYL4; of genes involved in regulation of sarcoplasmic reticulum calcium content and an associated repression of fetally enriched genes. Correia et al. reported that shifting hPSC-CMs from glucose-containing to galactose- and fatty acid-containing medium induced fast maturation into adult-like cardiomyocytes with higher oxidative metabolism, enhanced contractility and more physiological action potential kinetics (249). Perhaps the most comprehensive investigation into the effects of changing media is that of Rana et al., who modified a basal medium with combinations of high or low glucose with galactose or fatty acids (250). They found that exclusion of glucose from the medium was needed to induce iPSC-CM to switch from glycolysis to OXPHOS for their ATP production.

TISSUE ENGINEERING APPROACHES

It is hypothesized that culturing cells in 3D would better mimic conditions *in vivo*, such that differentiated cells would adopt a more mature phenotype. There are four main types of tissue engineering strategies that have been developed to construct contractile heart muscle equivalents: stacking monolayer cell sheets to form multi-layered heart muscle (251), cells seeded onto decellularized native tissue, cells seeded onto synthetic or biologic scaffolds (252–257) and entrapment of cells in naturally occurring biogels or hydrogels (258–260). Bian et al. cultured neonatal rat cardiomyocytes in a 3D environment and after 3 weeks saw aligned, electromechanically coupled cardiomyocytes with capillary-like structures, improved calcium handling properties. The cells had action potentials which showed enhanced conduction velocities and directional dependence on the local cardiomyocytes orientation (261). Cardiac muscle strips which were fabricated from hESC-CMs and stromal cells in collagen-based biomaterials, showed higher passive and active twitch force, aligned sarcomeres, regularly dispersed connexin-43 and N-cadherin and increased expression of maturation markers (262).

Scaffolds can be formed from synthetic polymers or from natural materials such as the cardiac extracellular matrix (ECM). Besides its structural role of giving support to surrounding cells, the ECM also has important signaling roles in cardiac development and remodeling. In recent years, it has been shown to help regulate cell survival, proliferation, migration and differentiation, for example by modulating the activity, bioavailabilty or presentation of growth factors to cell surface receptors (263, 264). ECM macromolecular proteins such as collagen (259, 265, 266), elastin (267), fibrin (268, 269) and glycoaminoglycans (270, 271) can be extracted from tissue and integrated in 3D scaffolds (267, 271) or hydrogels (268) to explore further the retention and maturation of SCs seeded. Material properties are more controllable in synthetic polymers which can be modified to incorporate adhesion peptides or release biological molecules (272).

In the hydrogel approach, cells are encapsulated in the scaffold during synthesis of the gel which allows homogenous seeding. Hydrogels have also been shown to induce cell maturation and differentiation which makes them an attractive system for basic studies of cardiac development and potential for the delivery of therapeutics to the heart (273, 274). Suspension of CDCs in a hydrogel formed from serum and the glycosoaminoglycan hyaluronic acid, increased the oxygen consumption rate from that of cells in suspension and increased cell retention after transplantation *in vivo* (275). Porous scaffolds can be generated by freeze-drying suspensions poured in molds (267, 276). This type of manufacturing gives flexibility in shape and composition, but limits cell seeding efficacy as most seeded cells remain attached to the scaffold surface (267). Fibrous

scaffolds can be manufactured from a large variety of materials by electrospinning which gives control over the nano-scale structure and mechanical properties of the scaffold, but again limits the seeding efficiency (277). Zhang et al. found that, compared to 2D hESC monolayers, hESC-CM in a 3D fibrin-based cardiac patch exhibited significantly higher conduction velocities, longer sarcomeres and enhanced expression of genes involved in cardiac contractile function (244). Potentially the "ultimate scaffold material" is that of decellularized heart tissue since it has the potential to give rise to appropriately structured scaffolds for organ replacement (278).

One noteworthy 3D construct is the engineered heart tissue (EHT) which is a three-dimensional, hydrogel-based muscle construct that is restrained between posts, thereby allowing the cells to contract against a force. EHTs can be generated from isolated heart cells from adult hearts, such as chicken (259) and rat (258) as well as from hESC (279) and hiPSC (280). iPSC-CM EHTs have showed to increase mitochondrial mass, DNA content, and protein abundance (proteome) compared to their 2D counterparts. Moreover they were found to generate more energy via oxidation of glucose, lactate, and fatty acid with a decreased reliance on anaerobic glycolysis, generating 2.3-fold more ATP by oxidation than 2D hiPSC-CMs (245). Mills et al. have developed a technique for growing 3D cardiac organoids in 96 well plates (281). The cells form dense muscle bundles in serum-free conditions developed to promote metabolic and proliferative maturation. They found that addition of palmitate increased the force of contraction and expression of ventricular myosin light chain 2. Interestingly, addition of insulin promoted cell cycling and so was not included in the maturation medium comprising 1 mM glucose and 0.1 mM palmitate. By comparing contractile properties of organoids grown in maturation medium with those in control medium, they found that maturation medium reduced activation time and relaxation time, thereby recapitulating changes seen during human cardiomyocytes development.

Thus, three-dimensional culture conditions have induced binucleation, rod-like cell shape, increased sarcomere alignment, more mature electrophysiology, and calcium handling properties (247). Complex, multi-cellular cell sheets are now being developed from mixtures of iPSC-CMs with fibroblasts and endothelial cells (282) or with mesenchymal cells (283) which exhibit more mature physiology or drug responses but these are still grown in non-physiologic culture media (282, 283). What is needed now, is routine modulation of the culture medium, as discussed above, to cells in 3D constructs to fully induce metabolic maturation.

CONCLUSIONS

The field of CPC biology has come a long way over the last 20 years. Beating sheets of cardiomyocytes can now be generated routinely from ESCs and iPSCs in large numbers. However, the maturity of the cardiomyocytes remains a cause for concern if they are to be used to validate new drug compounds, detect cardiotoxicity or recapitulate the cardiac physiology of patients with genetic disorders. Long term culture can aid maturation, as can culture in 3D constructs, but a fully mature cardiomyocytes should express the appropriate level of substrate transporters and mitochondrial proteins. Further work is required to determine appropriate cell culture conditions to enable pluripotent stem cell-derived cardiomyocytes to achieve the metabolic flexibility of the adult heart.

AUTHOR CONTRIBUTIONS

All authors contributed conception and design for the manuscript. SM-M and CL wrote the first draft of the manuscript. All authors wrote sections of the manuscript and contributed to manuscript revision, read and approved the submitted version. SM-M and CL are joint first authors.

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Regenerating the Cardiovascular System Through Cell Reprogramming; Current Approaches and a Look Into the Future

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Cardiovascular disease (CVD), despite the advances of the medical field, remains one of the leading causes of mortality worldwide. Discovering novel treatments based on cell therapy or drugs is critical, and induced pluripotent stem cells (iPS Cells) technology has made it possible to design extensive disease-specific *in vitro* models. Elucidating the differentiation process challenged our previous knowledge of cell plasticity and capabilities and allows the concept of cell reprogramming technology to be established, which has inspired the creation of both *in vitro* and *in vivo* techniques. Patient-specific cell lines provide the opportunity of studying their pathophysiology *in vitro*, which can lead to novel drug development. At the same time, *in vivo* models have been designed where *in situ* transdifferentiation of cell populations into cardiomyocytes or endothelial cells (ECs) give hope toward effective cell therapies. Unfortunately, the efficiency as well as the concerns about the safety of all these methods make it exceedingly difficult to pass to the clinical trial phase. It is our opinion that creating an *ex vivo* model out of patient-specific cells will be one of the most important goals in the future to help surpass all these hindrances. Thus, in this review we aim to present the current state of research in reprogramming toward the cardiovascular system's regeneration, and showcase how the development and study of a multicellular 3D *ex vivo* model will improve our fighting chances.

Keywords: reprogramming, vascular cells, regeneration, cardiovascular system, induced pluripotent stem cells (iPS Cells)

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INTRODUCTION

The cardiovascular or circulatory system (CVS) consists of the heart, the blood vessels and almost 5l of blood that continuously gets pumped throughout the body transferring everything that is needed to maintain homeostasis of nutrients, wastes and gases. A severely damaged CVS is incompatible with life making its successful treatment, especially at an early stage, crucial as each year passes. When one or more of its components are malfunctioning the end-result is complicated diseases—some of them extremely difficult to diagnose.

Stem cell technology represents a big hope for treating unmet clinical needs, including in the context of cardiovascular disease. The ability to self-renew indefinitely and to differentiate

in all the three germ layers makes them an attractive candidate both for drug development and personalized cell therapies. Using a variety of source cells, we can now generate endothelial cells (ECs), cardiomyocytes (CMs), vascular smooth muscle cells (VSMCs), and pericytes (PCs) or even progenitor cells to be used for transplantation and to create engineered organs. At the same time, we are able to further study developmental vasculogenesis and angiogenesis *in vitro* and identify possible mechanisms of pathogenesis by comparing models created by patient cells.

Not to be carried away, we note the limitations and challenges currently present in the use of the ESC—and iPS—derived cell lines both *in vitro* and *in vivo*. Issues with tumorigenesis are present with the vast majority of the cell lines due to the genetic stability of the clones. All iPS cell lines are genetically screened and subsequently characterized *in vivo* with tumorigenesis assays with the successful establishment giving a positive result; in contrast, the iPS-derived cell lines ought to present a negative result. Still, the high levels of proliferation of the cells in their early passages cause concerns when it comes to their clinical application; it is worth mentioning that Mandai et al—who just last year were the first to succeed in transplanting a sheet of retinal pigment epithelial (RPE) cells differentiated from iPS Cells in a patient with neovascular age-related macular degeneration—excluded their second patient due to detecting copy-number alterations in the iPS Cells they derived from them (1). Similarly, the high variability between different lines in respect to both maturity and subtype needs to be addressed. It is well-established that iPS Cells carry the identical genetic anomalies related to the source donor—a fact which makes them ideal for disease modeling. Several types of CVDs have already been modeled including: Hypertrophic cardiomyopathy (HCM), Dilated cardiomyopathy (DCM), Barth syndrome (BTHS), Long-QT (LQT), Catecholaminergic polymorphic ventricular tachycardia (CPVT) and Arrhythmogenic right ventricular cardiomyopathy (ARVC) but, as it will be discussed further on, the models are incomplete (2–4). To address these problematics in the last few years, teams from all over the world come up with new ideas every day: genetic manipulation using the CRISPR/Cas9 technology, direct reprogramming of somatic cells bypassing the pluripotent state, creation of small molecule cocktails for *in situ* direct reprogramming of local cell populations to name a few.

In this review, we discuss what the current state of the stem cell field is and how close or far away we are from designing a potential strategy for clinical cardiovascular therapies that combines successfully a multicellular model.

PLURIPOTENCY REPROGRAMING

In 1981, Evans, Kaufman and Martin reported the establishment of the first mouse embryonic stem cells (ESCs) in culture (5, 6), even though it took 17 years until Thompson et al. developed the first human ESCs lines in 1998 (7). Being able to study the differentiation of cells *in vitro* creates, for the first time, the opportunity to extensively look at the underlying mechanisms, as well as the opportunity to develop new and advanced treatments.

During those decades it was universally acknowledged that specialized cells reach a point when they cannot differentiate or de-differentiate any more making the process terminal. In 1987, Davis et al. transfected fibroblasts with the cDNA of MyoD and it gave rise to a population of myocytes (8). That was the first challenge of the irreversibility of differentiation and 19 years later the field of stem cells was revolutionized by Yamanaka, Takahashi et al. with the establishment of the first mouse (9) and human (10) induced pluripotent stem cells (iPS Cells) in 2006 and 2007, respectively. Subsequently, the iPS Cells were incorporated into high quality research with teams differentiating them into neurons, cardiomyocytes, hepatocytes endothelial cells etc. Strategies for furthering the field of personalized medicine started developing as the clinical significance of patient specific iPS cell lines is undeniable.

The original protocol developed by Yamanaka utilizing a retroviral vector transduction of the four reprogramming factors *Oct4, Sox2, Klf4,* and *C-myc* (OSKM) has been modified since aiming to increases in efficiency of reprogrammed cells and/or the generation of footprint-free iPS cell lines that lack integration of any viral vector sequences into their genomes (**Figure 1**). *C-Myc* as a known oncogene was substituted with *Wnt3* improving the efficiency of the generation of mouse iPS Cells (miPS Cells) colonies (11). Another group reported the addition of *Lin28* and *Nanog* with the OSKLN derived iPS Cells appearing similar to both Embryonic Stem Cells (ESCs) and OSKM-derived iPS Cells (12). Other delivery methods were also applied: Non integrating viruses like adenovirus (13) and Sendai virus (14) were developed for the reprogramming of human fibroblasts or blood cells into iPS Cells but the efficiencies of the reprogramming were 0.0002 and ∼1% respectively. Traditional molecular manipulation methods have also been used successfully among them Cre/LoxP (15) and piggyback (16, 17). Others have established mRNA (18), miRNA (19–21), proteins (22), episomal plasmid transfections (12), or minicircle vectors with a varied combination of genetic modulation (23). Since the CRISPR/Cas9 technology was established in eukaryotic cells (24, 25) steps were taken into combining the two revolutionary technologies and creating a new more versatile approach to the genetic editing of human iPS Cells (26, 27). The ability to simultaneously differentiate cells and genetically modify them—as first described in Howden et al. (28)—raises hope for the disease remodeling field.

Soon, it became apparent that even when following the same protocol, both the efficiency of the reprogramming and the stability varied between iPS cell lines, presenting quite a challenge, especially with patient specific lines. The cause of that variability is most probably due to the parental line or even disease-specific mutations, therefore, making it clear that the genetic background is crucial to the differentiation potential of a donor-line; curiously surpassing even that of the source-specific variation (29, 30). Large scale screenings and extensive study of the pathways involved in pluripotency led to the discovery of both different transcription factors (31–33) that can be combined

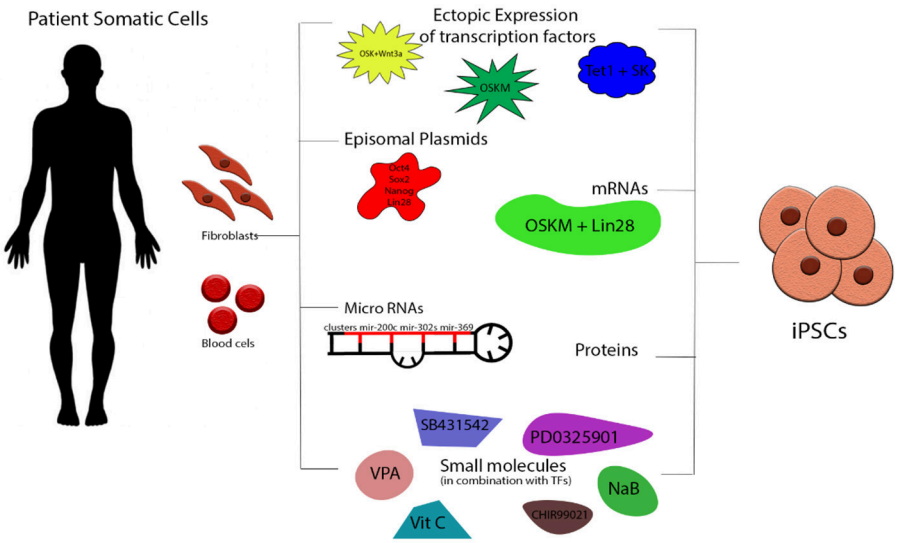

FIGURE 1 | Schematic presentation of iPS Cells generation. Somatic cells (fibroblasts, peripheral blood cells etc) can be isolated from healthy donors and patients and be directly reprogrammed into iPS Cells by the ectopic expression of transcription factors via retro-/lenti-/adeno/Sendai viral transduction. Most commonly used are the combinations of OSKM, OSKNL, OSKL, and TSK. Other strategies involved episomal plasmids (OSLN), mRNAs (OSKML), miRNAs (variety from cluster 200s, 300s, 367/9s), proteins and small molecules including histone modifiers, metabolic modulators, and signaling pathway inhibitors. O, Oct4; S, Sox2; K, Klf4; C, c-Myc; N, NANOG; L, Lin28; T, TET1.

and also small molecules that enhance the reprogramming efficiency (34). Epigenetic modifiers like inhibition of histone demethylation (35), inhibition of transforming growth factor-β (TGF-β), MEK and ROCK signaling pathways as well as metabolic modulation and induction of glycolysis (36) were shown to improve the iPS Cells induction (**Table 1**).

No one can deny that the establishment of patient specific iPS Cells technology gives breath to novel ideas for drug discovery by making *in vitro* screening of side effects as well as new drug development. The question is; is this enough? In respect to the cardiovascular field, as it will be further discussed, the answer is edging toward no.

CARDIOMYOCYTE REPROGRAMMING

Adult CMs have a very low regenerative ability, mainly coming from the differentiation of cardiac progenitor cells instead of the replacement of the damaged ones via cell division as it was showcased in genetic-fate mapping projects in 2007 (41, 42). Extensive damage leads to scar formation from the activated fibroblasts causing cardiac remodeling and heart failure (HF). Heart transplantation which has been the standard treatment for patients with end-stage HF is still plagued by several issues such as donor shortage, major post-surgery complications such as stroke, bleeding and infection due to chronic immunosuppression (43). The question, as a result, remains. How would we be able to overcome these and regenerate the heart?

The last decade or so, several potential strategies based on stem cells and cell reprogramming have been proposed as an answer to that question. Cell transplantation of ESCs—or iPS Cells-derived CMs (iPS Cells-CMs) is a very promising tool for "remusculising" a failing heart, as showcased in several studies both in small rodents and in non-human primates (44, 45). Alternately, generation of CMs from endogenous sources *in situ* through differentiation of resident cardiac progenitors or the transdifferentiation of local populations like cardiac fibroblasts or pericytes (46, 47) is another promising approach. Last but not least, cardiac tissue engineering has been evolving rapidly in parallel trying to create fully functional 3-dimensional (3D) biometric constructs from cells derived from iPS to replace the damaged myocardium (48, 49).

Cardiac cells were some of the first cells that were derived from mouse ESCs back in 1985 (50) and subsequently, with the road to pluripotency open, multiple teams in the last decade have been able to differentiate iPS Cells into cardiac progenitors and CMs. The first murine iPS Cells-CMs were derived in 2008 by three groups using the embryonic bodies (EBs) method. Specifically, Mauritz et al. compared the differentiation of an established ESC line and that of an iPSC line toward CMs and they reported a successful conversion, albeit with a much lower efficiency (51). Schenke-Layland et al. exposed EBs to collagen type IV (CollV) and selected Fetal Liver Kinase$^+$ (Flk$^+$) cells through magnetic separation, which, in turn, were differentiated into functional CMs, SMCs, ECs, and hematopoietic cells (52). Narazaki et al. also, modified their protocol and cultured their Flk$^+$ cells on OP9 stroma cells inducing self-beating CMs (53). For human cells, the first iPS Cells-CMs were reported by Zhang et al. in 2009 when they used OSK and Lin28 to generate iPS Cells and then differentiated them using the EB method (54). Many techniques have been described since, and the cells generated have all the

TABLE 1 | Small molecules that enhance iPS Cells generation.

Small molecule	Process affected	Combination with other methods	References
Valproic acid (VPA)	Histone deacetylase inhibition	OS	(35)
SB431542 + PD0325901	TGFβ- and MEK inhibition	OSKM	(37)
SB431542 + PD0325901 + thiazovivin	TGFβ- and MEK and ROCK inhibition	OSKM	(37)
A-83-01 + PD	TGFβ-inhibition	O	(38)
NaB + PS48	TGFβ-inhibition Histone deacetylase inhibition	OSKM	(39)
PS48	PI3K/Akt activation	OSKM	(39)
Vitamin C	Enhances epigenetic modifiers, promotes survival by antioxidant effects	OSKM	(40)

O, Oct4; S, Sox2; K, Klf4; C, c-Myc.

advantages that come by that type of differentiation: they can be patient-specific and compatible, making them ideal candidates both for disease study, remodeling and cell therapy (55). Apart from the EB method, differentiation in a monolayer has also been described with high efficiencies by multiple groups. The last 5 years iPS Cells-CMs in varied stages of maturity are produced in larger scales with the help of bioreactors. The murine myocardial infraction model (56) has been used widely to confirm the derived cells' capability of heart tissue regeneration and reduction of scarring. As the technology evolved, rodent models were gradually replaced by non-human primates (44) and pigs (57), and in 2015 Menasché et al. reported the first human ESC-derived cardiac progenitors transplant to patients with advanced ischemic heart failure (58, 59). Even though complications like ventricular arrhythmias may occur post-transplant, their success is considerable. So if we take into consideration the similarities between ESCs and iPS Cells, the baseline of a patient-specific robust cell therapy strategy is set.

In parallel, the concept of transdifferentiating cardiac fibroblasts or other non-myocytes that localize in the heart tissue into CMs also attracts a lot of attention. The first attempts to reprogram cells *in vivo* started in 2009, when Takeuchi and Bruneau demonstrated that overexpression of *Gata4*, *Tbx5*, and the interacting chromatin remodeling protein, Baf60c, converts non-cardiogenic mesoderm into beating CMs in the embryo by a mechanism involving the induction of Nkx2-5 by Gata4 and Baf60c (60). The exogenous production of CMs was revolutionized a year later when Ieda et al. reported the discovery of a 3-factor cocktail, Gata4, Mef2c, and Tbx5 (GMT), successfully reprogramming murine cardiac fibroblasts (mCFs) into induced CM-like cells *in vitro* (61). Shortly thereafter, three independent studies proved that the non-myocyte pool in the adult mouse heart consisting mainly of CFs can be transdifferentiated *in vivo* via injecting directly the GMT cocktail into the mouse heart with (GMHT) or without Hand2, and reprogram *in vivo* CFs into adult induced-CMs (62–64). This resulted in the regeneration of the myocardium and the improvement of cardiac function.

Unfortunately, the low efficiency of most of the *in vitro* reprogramming protocols—especially when using human cells—as well as concerns for the integration of viral DNA into the host, paved the way of constant modifications, additions and alternations in the GMHT protocol (65). Transcription factors were added (66–68), replaced (69–71), supplemented with small molecules (67, 72) and miRNAs (73) and finally omitted in favor of miRNAs (74), which do not incorporate into the host chromosome, presenting a much safer future clinical application. More recently, Huang et al. proposed a chemically-induced reprogramming *in vivo* with the combination of CHIR99021; RepSox; Forskolin; VPA; Parnate; TTNPB and Rolipram, successfully inducing CMs from CFs in adult mice and resulting into a reduction of the fibrotic tissue after myocardial infraction (75) (**Figure 2**). The mechanisms surrounding reprogramming are still left to be elucidated but all these changes contribute little by little to increasing our understanding and, as will be discussed later, to the design of a strategy to combat CVD.

ENDOTHELIAL CELL REPROGRAMMING

The vascular endothelium controls vascular function and structure, mainly via nitric oxide (NO) production and play a pivotal role in the CVS. EC dysfunction is attributed to be the cause of severe complications, many times proven to be fatal. As the years pass, a growing list of pathological conditions and diseases including hypertension, hypercholesterolemia, diabetes mellitus, congestive heart failure, hyperhomocysteinemia, and even the aging process itself, are associated with EC dysfunction (76).

Differentiation of ECs is governed by several factors, including the immediate microenvironment, interactions with surrounding cells, and the local release of cytokines and growth factors. In the early stages of embryonic development, angiogenesis occurs through the expansion of the vascular plexus with vessel sprouting. The primitive vascular plexus remodels into a highly organized vascular network in which larger vessels ramify into smaller ones and become surrounded by mural cells, which stabilize the newly formed vessels and provide strength to control blood flow and blood pressure (77). In adults with pathological conditions such as cancer or ischemia, or even wound healing, the ECs have the ability to reactivate the angiogenic process making them invaluable for survival.

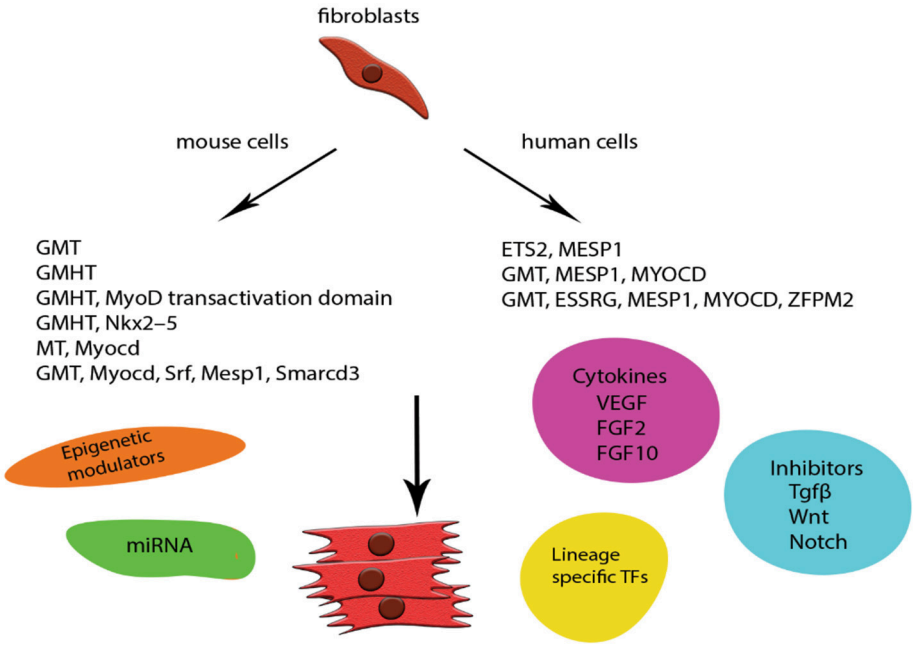

FIGURE 2 | Schematic representation of examples of direct *in vitro* reprogramming to cardiomyocytes. CMs were one of the first cell lines to be transdifferentiated from mouse and human fibroblasts. The low efficiency of the original GMT protocol especially in human cells was addressed by modifications and addition of more lineage specific factors. Safety concerns were addressed by incorporating new techniques like delivery of miRNA and episomal plasmids coding specific TFs. Small molecules are also widely used with the addition of cytokines and TGFβ inhibition enhancing the final populations and giving rise to robust protocols. G, Gata4; M, Mef2c; T, Tbx5; H, Hand2.

ESC-derived ECs were one of the first to be developed after the establishment of the first colonies with the traditional methods: formation of EBs from the mesoderm germ layer, from which both hematopoietic cells and ECs emerge, and their subsequent exposure to a variety of growth factors, which enhances their differentiation inside the EBs (78, 79). Two-dimensional (2D) cultures under feeder or feeder-free conditions are also very popular and, even though the efficiencies leave something to be desired, advances in the phenotypic stability are being made every day. ESCs gave their place to iPS Cells while linage specific additions to the OSKM factors gave rise to a variety of protocols generating EC progenitors or mature ECs. Especially in the case of endothelial progenitor cells (EPCs), despite the invaluable therapeutic potential they present, the many subtypes that exist create a tricky unit to work with (80–82). A similar challenge is presented with the more mature ECs with different subtypes presenting differences in proliferation and functionality (83, 84). Cell sorting for endothelial progenitor markers like CD34, CD105, Neurophilin1 (NRP1), Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), and PECAM1 (CD31) as well as more mature ones such as VE-Cadherin (CD144) are used to enhance the populations and ensure high purities of the target population (**Table 2**). Severe limitations like the length of time it takes in generating iPS Cells from the source cells and, in turn, differentiating them into new cell types, challenged the scientific groups. The idea of bypassing the pluripotency state and going down the road of direct reprogramming through epigenetic and linage-specific modulations was reported in 2012 by Margariti et al. when the OSKM factors were transferred to human fibroblasts for 4 days and generated a population of partial-iPS which did not form tumors *in vivo* and then differentiated them into functional ECs able to revascularize tissue engineered vessels (89). Li et al. used only two of the Yamanaka factors—*Oct4* and *Klf4*—to transdifferentiate human fibroblasts to endothelial-like cells capable of expressing CD31, von Willebrand Factor (vWF) and CD144 that were functional *in vivo* as well (90). Wong et al. lays emphasis on the importance of epigenetics and describes the potential of using miRNAs (91). Different miRNAs are described to enhance endothelial differentiation including miR-99b,−181a, and−181b (92),−199b (93),−21 (94) of which the overexpression is reported to increase endothelial marker expression and functionality. Apart from fibroblasts, other cell sources have been identified during the last 10 years like mature amniotic cells (95), blood (96), SMCs (97). In addition, genes that have been dubbed as "master key regulators" like Quaking (98–100) and ETV2 (101–104) due to their invaluable role in endothelial function hold a potential to be useful in developing new direct reprogramming strategies.

Direct reprogramming via small molecules and chemical compounds alone has been reported in many cell lines including neuronal cells, glial cells, neural stem cells, brown adipocytes, hepatocytes, CMs, somatic progenitor cells by the regulation of cell signaling pathways and/or histone modification (105). ECs have not been successfully derived yet but further elucidation of the pathways involved both in cell signaling and, in their metabolism, can lead the way. Already scientific groups

TABLE 2 | Examples of efficient derivation of iPS Cell-ECs or EC progenitors.

Method	Ectopic TF overexpression	Small molecules	Cell sorting	Efficiency	References
EB	–	–	VE-cadherin	18 ± 4%	(85)
Small molecules	–	BMP4 Activin CHIR VEGFA	PECAM-1	20–30%	(86)
Small molecules	–	FGF2 BMP4 $VEGF_{165}$	NRP-1 PECAM-1	≥60%	(82)
Small molecules	–	GSK3 inhibitor BMP4 VEGFA	VE-Cadherin	80%	(87)
Small molecules	–	GSK3 inhibitor BMP4 FGF2 VEGFA	–	99% of CD31+ and 96.8% VE-cadherin+	(88)

are turning to single-cell RNA-seq to delve deeper into the heterogeneity of the iPS-derived cell populations. They aim to assess the protocols in use as well as to further study the differentiation process; with Paik et al. publishing a large scale screening of iPS-ECs earlier this year (106).

SMOOTH MUSCLE CELLS

Smooth muscle cells (SMCs) are highly specialized cells whose major function when matured is the contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. Since the late 70s, SMCs have been widely accepted as the main contributors in the pathogenesis of atherosclerosis (107, 108). More specifically, the theory suggested that, in response to vascular injury, SMCs migrate from the media into the intima, where they turn into foam cells and produce extracellular matrix. Almost 30 years later that view was challenged when many scientific teams presented evidence that SMC progenitor cells and hematopoietic stem cells differentiate into SMCs in the intima (109–112). That, in combination with the widely discussed heterogeneity of origin [as different developmental stage SMCs appear with different phenotypes and different source populations (113, 114)], made them the center of attention. Stem cell technology provided the ideal way of studying the different mechanisms of their derivation as well as an opportunity of further understanding the way both mature SMCs and progenitors contribute to the pathophysiology of CVDs.

Again, the EB formation method was used for ES-derived SMCs with Haller et al. reporting that the exposure of the EBs in retinoic acid and dibutyrylcydic adenosine mono-phosphate (db-cAMP) induced differentiation of spontaneously contracting cell clusters in 67% compared with 10% of untreated controls (115). Huang et al. also reported their success in differentiating ESCs into SMCs by adding trans retinoic acid in a monolayer culture with 93% of them expressing SM α-actin and SM-MHC (116). Further studies into cell signaling during the process of differentiation of ESCs and MSCs to SMCs showed that both TGF-β and the Notch pathway, as well as the Bone Morphogenic Proteins (BMPs) (117), are important for the expression of the vascular SMC markers (118, 119). Histone deacetylation plays a main role in vascular homeostasis as well as neuronal, controlling the migration proliferation and differentiation toward SMCs (120–122).

With the introduction of iPS Cells technology, different protocols have been applied into directing the iPS Cells toward SMCs depending on the desired linage (**Figure 3**). More recently, Steinbach et al. in 2016 described the stepwise administration of key members of the TGF-β superfamily to generate lateral plate-derived vascular SMCs (VSMCs) from human iPS Cells (127). Yang et al. used a combination of Fibroblast Growth Factor (FGF), VEGF and TGFβ to generate VSMCs reporting as well a diversity in their endpoint culture (128).

Direct differentiation protocols have also been applied: most notably, Karamariti et al. used PiPS Cells and 4 days later, the PiPS-SMCs were expressing a full panel of VSMC markers including calponin, SMA-α and SM22α as well as elastin and collagen, characteristically seen in the VSMCs of large arteries (129).

The existence of different VSMCs lineages, occurring as a result of germ layer formation during embryological development, further adds to the complexity of iPS Cells differentiation techniques, as mentioned before. The current studies have provided only a glimpse to a very complicated system but the potential is there. Even though most of the CVD models or tissue engraftments are based on induced-CMs or induced-ECs, we believe VSMCs are an important part of the cardiovascular physiology that should not be bypassed.

PERICYTES AND THEIR POTENTIAL

Perivascular pericytes, or mural cells, envelop the vascular tube surface and are integral in the formation of blood vessels. They are multipotent cells that are heterogeneous in

their origin, function, morphology and surface markers. Thus, many controversies have been sparked in respect of their characterization. They are integral for the regulation of blood flow, the stability and permeability of vascular structure and angiogenesis (130–132). Blood vessels lacking pericytes become hyperdilated and haemorrhagic, leading to pathological changes ranging from diabetic retinopathy to embryonic death. They are known to have high level of plasticity and differentiate into other cell types. They also have tissue- specific properties which have been extensively reviewed (132–134).

Dar et al. used EB formation and demonstrated the isolation of $CD105^{+}CD90^{+}CD73^{+}CD31^{-}$ multipotent clonogenic mesodermal precursors. After expansion, the cells expressed markers, like CD146, Neural/glial antigen 2 (NG2), and Beta-Type Platelet-Derived Growth Factor Receptor (PDGFRβ) (135). Orlova et al. described in 2014 the generation of iPS Cells-derived ECs that were expressing a plethora of markers and were functional *in vivo;* at the same time, using TGFβ3 and BMP4, they differentiated the CD31- fraction of their selection toward pericytes (86).

Another tool in their application into regeneration of the vasculature is cell therapy using pericytes isolated from patients; as they are abundant in various sites on the human body. In 2013, Katare used the mouse myocardial infraction model and demonstrated that transplantation of pericytes, expanded from redundant human leg veins, relocated around the vessels of the peri-infarct zone and released a variety of transcription factors. These enhanced ECs and CMs survival and proliferation

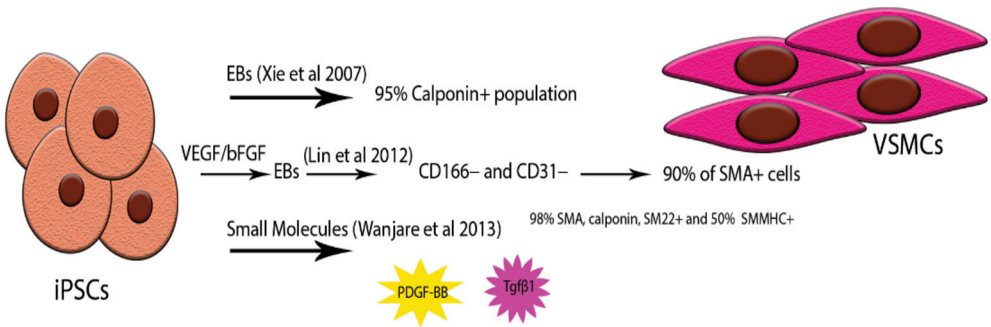

FIGURE 3 | Examples of highly pure iPSC-VSMCs. Due to the heterogeneity of origin as well as the many subtypes of VSMCs acquiring highly pure populations has proven to be challenging. Three types of protocols have been used successfully; simple embryonic body formation and selection of the Calponin positive population (123, 124), combination of EBs and monolayer culture by pre-treating the hiPS Cells with VEGF (125), forming the EBs and then expanding the CD166- /CD31- population in a monolayer again, small molecule treatment of iPS Cells monolayers with PDGF-BB and Tgfβ1 (126).

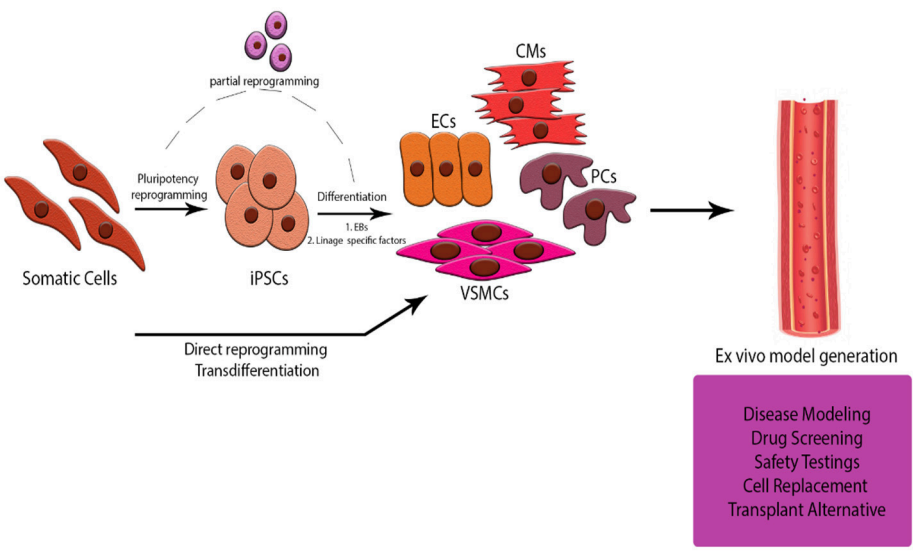

FIGURE 4 | Schematic Representation of the abstract. Generation of an *ex-vivo* tissue model for studying the cell to cell interaction in a 3D CVS model is offering multiple advantages over current animal models and co-culture *in vitro* systems. Using cells derived from iPS Cells or somatic cells of donors will help us look deeper into the pathogenesis of diseases, customize and safely test novel drug treatments and cell therapies for patients, even develop strategies for alternative sources of tissue for transplants.

(VEGF, angiopoietin) and others inhibited cardiac hypertrophy and fibrosis while promoting angiogenesis (MiR-132 inhibits Ras-GAP, angiotensin 1 receptor (AT1R) and MeCP2 (136).

ESCs and iPS Cells have been used as a source of pericytes (135) but two main concerns have been raised that have yet to be addressed in their entirety. The first is that the efficiencies of the protocols described are usually really low and the second is the lack of distinct pericyte specific markers due to the large heterogeneity of the populations. Most commonly used and widely accepted are PDGFR-β, CD44, CD90, NG2, and α-SMA but they are all strongly present in other cell types so functional assays and extensive expression profiling is needed to complete a characterization. The fact that there is no standardized way of getting a homogenous population of cells is a hindrance to the design of novel therapies however possible epigenetic and secretome screening may help us study the mechanisms of pathogenesis they are involved in.

DISCUSSION

It is commonly acknowledged that, thus far, disease models are incomplete. *In vitro* co-culture systems are difficult to maintain and although they are very informative, they cannot accurately model the complex and structured *in vivo* environment. At the same time, animal models are a very useful tool in research for cell therapies and drug development but the different species to species physiology creates major barriers in the clinic. The ideal for many is a 3D *in vitro* model that will allow for safe testing of therapies and will be modeled with human cells. Naturally, the most promising cell types to be used are human iPS Cells or reprogrammed cells.

With respect to the cardiovascular field, analyzing the three points of structure is crucial to planning future strategies. First and foremost, EC dysfunction is widely acknowledged as one of the leading causes of complications for patients suffering from diabetes and other vascular diseases. Secondly, CM damage combined with the heart's low regeneration ability has proved to be one of the most difficult points to address during the study of the pathophysiology of a disease both *in vitro* and *in/ex vivo* as well as in the development of therapies (137). Thirdly, SMCs are deeply integrated into the pathogenesis of the atherosclerotic plaque but the knowledge of the mechanism behind their differentiation during different stages of the disease is lacking (114). Last but not least, pericytes may have been relatively overlooked—possibly due to their heterogeneity—but are integral to the preservation of vascular rheology and homeostasis (134). Keeping these in mind, it is obvious to see that an attempt of excluding a component may lead into not being able to complete the puzzle. Tissue engineering technology is advancing rapidly and experimentation of new biomaterials and re-vascularization strategies is a fact. Engineering 3D cardiac tissue with a physiologically relevant microenviroment is quite challenging. Most promising are the re-vascularization strategies of the bioengineered graft and the maturity of the cells that will be used, since the reprogrammed cells—especially the CMs—are usually more immature types, and not what we would see in a functional human heart. As presented extensively by Costa- Almeida et al. constant vascularization is critical based on cellular strategies combining EC transplantation with support cells, which will produce growth factors, cytokines, hormones and other bioactive molecules essential to the stability of the scaffold (138).

If we take a step back we will see that currently we are getting closer but we still have a long way to go. *In vitro* culture (and co-culture) models are very useful for studying different cell type interactions but we are still missing the complexity of the cell signaling interplay in tissue. Future research should be focusing not only in getting novel insights into the process of angiogenesis but in combining our knowledge of the interaction of heterotypic cells to develop *ex vivo* models of the CVS. Considering the difficulty of acquiring mature cells from patients, iPS Cell-derived or reprogrammed cells are ideal candidates for modeling these. Concerns about the phenotypical stability of the differentiated cells should also be addressed by further studying the epigenetic process in which we erase the cell memory and direct them in a different path. The advantages are significant for personalized and regenerative medicine as well as drug development and testing, revealing a potential role of these models for their manipulation into patient-specific scaffolds for heart and vessel damage (**Figure 4**).

AUTHOR CONTRIBUTIONS

MT conception and design, manuscript writing. SK design, final approval of manuscript. RC final approval of manuscript. CY final approval of manuscript. AM conception and design, financial support, final approval of manuscript.

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Cardiomyocyte—Endothelial Cell Interactions in Cardiac Remodeling and Regeneration

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The heart is a complex organ consisting of various cell types, each of which plays an important role in both physiological and pathophysiological conditions. The cells communicate with each other through direct cell-cell interactions and paracrine signaling, and both homotypic and heterotypic cell interactions contribute to the organized structure and proper function of the heart. Cardiomyocytes (CMs) and endothelial cells (ECs) are two of the most abundant cardiac cell types and they also play central roles in both cardiac remodeling and regeneration. The postnatal cell cycle withdrawal of CMs, which takes place within days or weeks after birth, represents the major barrier for regeneration in adult mammalian hearts, as adult CMs exhibit a very low proliferative capacity. Recent evidence highlights the importance of ECs not only as the most abundant cell type in the heart but also as key players in post-infarction remodeling and regeneration. In this MiniReview, we focus on blood vascular ECs and CMs and their roles and interactions in cardiac physiology and pathologies, with a special emphasis on cardiac regeneration. We summarize the known mediators of the bidirectional CM-EC interactions and discuss the related recent advances in the development of therapies aiming to promote heart repair and regeneration targeting these two cell types.

Keywords: cardiomyocyte, endothelial cell, cell-cell interaction, cardiac regeneration, cardiac remodeling, cardiac cell therapy, cardiovascular gene therapy

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THE MULTICELLULAR COMPOSITION OF THE HEART

The heart is a complex multicellular organ with specialized structures and cells to take care of their own "subprojects." Its main function, to maintain the blood circulation, depends on its pump function, making cardiomyocytes (CMs) the central cardiac cell type in both normal and pathological conditions. Cardiomyocytes are generally divided into pacemaker cells and force-producing ventricular and atrial CMs. In order for the heart to function properly, additional cell types, such as blood and lymphatic endothelial cells (ECs), vascular smooth muscle cells (SMCs), fibroblasts, and pericytes are needed. Besides their structural role in the interior surfaces of blood vessels, vascular ECs are metabolically active, control vasomotor tone, and regulate angiogenesis (1, 2). Fibroblasts are connective tissue cells that produce constituents of extracellular matrix, while vascular SMCs and pericytes regulate blood flow in the cardiac vasculature. The proportions of cardiac cell types have been debated for decades, as technical limitations and the lack of cell-type specific markers have impeded accurate analysis. Traditionally, fibroblasts have been considered

the most abundant cell population (3–5). Fairly recently, ECs were however reported to represent the major non-myocyte population (6), suggesting that their physiological and thereby therapeutic importance may be greater than previously appreciated. Of the ECs, about 95% are blood vascular and 5% lymphatic (6). There is recent evidence that lymphatic vessels also play an important role in the heart, especially after myocardial infarction (7, 8). However, no studies are available at the moment on the possible interplay between lymphatic ECs and CMs.

In the myocardium, CMs are physically connected and communicate with each other through gap junctions, adherens junctions, and desmosomes (9). However, they also communicate with other cell types in the heart through both direct physical contacts and paracrine signaling (**Figure 1**). Pathological conditions, such as hypertension or myocardial infarction (MI), elicit maladaptive responses in both CMs and non-myocytes, contributing to the deterioration of cardiac function. Cardiac fibrosis is one example of fibroblast-mediated phenomena playing a central role in the pathogenesis of heart failure (10). Heterotypic interactions between different cell types are known to cause or aggravate cardiac pathologies. Cell-cell interactions between fibroblasts and CMs that contribute to e.g., arrhythmogenesis have been extensively investigated and are reviewed in depth in (11–13). The interactions between the two most abundant cell types, ECs, and CMs, have however not been equally well characterized. In this MiniReview we focus on the importance and mechanisms of communication between blood vascular ECs and CMs as well as their therapeutic implications in cardiac repair and regeneration.

REMODELLING OF CARDIAC VASCULATURE AND CARDIOMYOCYTE HYPERTROPHY

Cardiac remodeling, i.e., remodeling of the left ventricular wall, can take place in physiological or pathological conditions (14). While physiological remodeling mainly includes CM hypertrophy, is reversible and improves cardiac function, pathological remodeling is accompanied with fibrosis and CM hypertrophy, atrophy, and apoptosis, and leads to deterioration of cardiac output. In physiological hypertrophy, the heart preserves its oxygen supply and balances the proportional increases in CM size and the extent of coronary microvasculature through angiogenesis. In heart failure, however, the pathological progression is associated with an imbalance between oxygen supply and demand, as CM hypertrophy is not matched by a corresponding increase in the vasculature (15). Identification of the mechanisms behind this mismatch between CM and EC growth could lead to discovery of new approaches to treat heart failure.

CARDIAC REGENERATION

Since the adult mammalian heart exhibits a very limited regenerative capacity (16, 17), a cardiac injury such as MI leads to fibrotic scarring and subsequent remodeling of the surrounding myocardium, eventually progressing to heart failure [see (10, 18)]. Some lower vertebrates, such as the zebrafish, as well as neonatal rodents can however regenerate their hearts (19, 20). Furthermore, it seems that the human heart also possesses this intrinsic regenerative capacity at the time of birth (21). The postnatal irreversible cell cycle arrest in mammalian CMs is connected to oxygen-rich postnatal environment and increased oxidative energy metabolism in cardiomyocytes, which promote oxidative stress and DNA damage (22). More detailed understanding of the mechanisms is however needed to unlock regenerative therapeutic possibilities [see (23–25)].

During cardiac regeneration in the zebrafish and neonatal mouse, new CMs are produced through proliferation of remaining CMs (20, 26, 27). Additionally, regeneration requires revascularization of the injured area, which occurs through proliferation and migration of vascular ECs from existing vessels in the surrounding myocardium (28). Remarkably, in zebrafish the ECs invade the injured area already in less than 1 day and rapid revascularization is indispensable for the induction of CM proliferation, suggesting that revascularization not only provides nutrients to the regenerating tissue, but also regulates the regenerative response. In liver and lung, ECs have been shown to be crucial for regeneration acting via paracrine secretion of angiocrines (29, 30). In line with this, both ECs and CMs in adult mouse hearts are unable to activate the injury-responsive neonatal gene regulatory networks, while MI-induced gene expression changes in fibroblasts and leukocytes are similar in neonatal and adult mouse hearts (31). This further highlights CMs and ECs as the main targets for regeneration-inducing therapies and calls upon detailed investigations clarifying their mutual interactions in the process of cardiac regeneration.

PARACRINE SIGNALLING BETWEEN CARDIOMYOCYTES AND ENDOTHELIAL CELLS

Cardiokines Affecting Endothelial Cells

Cardiomyocytes produce and secrete proteins and peptides for paracrine signaling with other cardiac cells and for endocrine signaling with peripheral tissues. It has been estimated that the heart secretes 30–60 different proteins or peptides, which are called cardiokines or cardiomyokines (32); however, the number may increase markedly as analysis techniques improve. Cardiokines include growth factors, endocrine hormones, cytokines, extracellular matrix proteins, and peptides, which are important for maintenance of normal heart growth and function and as signal mediators in response to various stresses. The well-known stress-induced cardiokines include natriuretic peptides A (ANP) and B (BNP), which act as cardioprotective factors mainly for CMs, but also exert effects on ECs (33).

The main cardiokine regulating EC activation and proliferation is vascular endothelial growth factor (VEGF) that binds to and activates the VEGF receptor 2 (VEGFR2, also known as KDR and FLK1) in ECs (34). VEGFR2 signaling induces angiogenesis, growth of new blood vessels from the pre-existing ones, improving circulation, and thereby oxygen

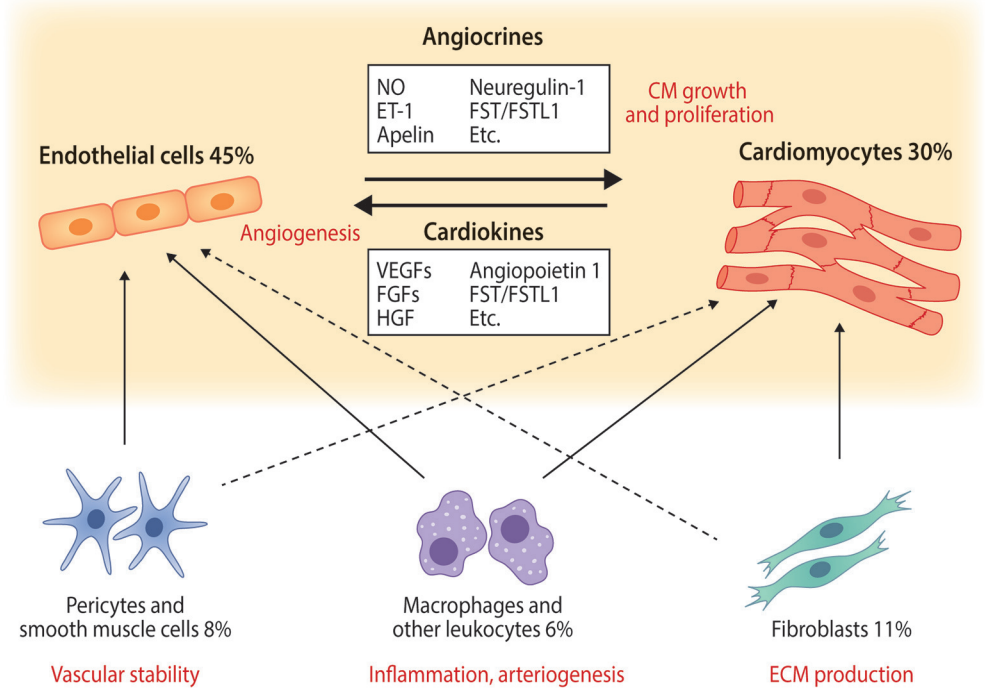

FIGURE 1 | The main cardiac cell types and their interactions with endothelial cells and cardiomyocytes. Selected known paracrine factors mediating endothelial cell—cardiomyocyte cross-talk (angiocrines and cardiokines) are illustrated. The relative abundances of each cell type in normal adult mouse ventricular tissue are from (6) and represent those quantified in adult mouse heart. Solid arrows represent well known cell-cell interactions and dashed lines illustrate less well characterized but potentially important interactions. ET-1, endothelin-1; FGFs, fibroblast growth factors; FST, follistatin; FSTL1, follistatin-like 1; HGF, hepatocyte growth factor; NO, nitric oxide; VEGFs, vascular endothelial growth factors.

and nutrient availability in deprived areas to match the need of e.g., growing or infarcted heart. Additional extensively studied cardiokines inducing cardiac angiogenesis include the other VEGF family members VEGF-B (35), VEGF-C (36), and placental growth factor (PlGF) (37), as well as fibroblast growth factors (FGFs) (38), hepatocyte growth factor (HGF) (39), and angiopoietin-1 (40). CMs also produce and secrete several members of the TGF-β superfamily, which elicit both cardioprotective and detrimental effects on the heart. Of these, follistatin like-1 is one of the best characterized, and it has been shown to affect both CMs and ECs [see (41)].

Angiogenesis-Induced Cardiac Hypertrophy

The first evidence indicating that angiogenesis induces cardiac hypertrophy came from a study by Tirziu et al. (42), where the authors showed that vascular growth without any other stimuli increases cardiac mass. Both VEGF-B and PlGF have been shown to induce cardiac angiogenesis accompanied by modest cardiac hypertrophy with preserved or improved function and, importantly, no progression into heart failure even in old transgenic animals (43–45). Currently, the molecular mechanisms mediating angiogenesis-induced CM hypertrophy are not known. Nitric oxide (NO) has been suggested to be important in this regulation, however, its blockade could not fully reverse the phenotype (44).

Cardiac Angiocrines Mediating EC-CM Crosstalk

It is estimated that humans have about 100,000 km of blood vessels, which most likely makes vasculature the largest "endocrine organ." In response to physiological or pathological stimuli, ECs secrete a set of proteins and other factors, angiocrines, which act on the neighboring cell types. Recent data from other tissues provide strong evidence that ECs establish an instructive vascular niche, which stimulates organ growth and regeneration via paracrine signaling (29, 30, 46, 47). However, very little is known about the cardiac EC secretome (cardiac angiocrines) in response to physiological or pathological stimuli. While the concept of EC to CM signaling and its role in cardiac function has been extensively reviewed by Brutsaert already 15 years ago (48), we are still in the early phase of understanding the mechanisms of this crosstalk.

A growing number of EC-derived factors acting on CMs have been identified. One of the most studied over the years is NO, which is produced by many cell types in the heart and affects both vasculature and CMs. NO induces relaxation of vascular SMCs resulting in vasodilation (49), and can regulate contractile responses of CMs (50). Of the secreted proteins, neuregulin-1 (NRG-1), which belongs to the epidermal growth factor family and is expressed and released by cardiac microvascular ECs and perivascular cells, has received a lot of interest over the last 10 years. NRG-1 binds and activates ErbB receptors in CMs

promoting cardioprotection and regeneration, and both NRG-1 and ErbB2 have been shown to promote CM proliferation and growth (51, 52). Apelin is another secreted peptide, which is highly EC-specific and expressed in sprouting vessels in the heart (53). It regulates cardiomyocyte hypertrophy and myocardial response to infarction through its receptor APJ (54, 55). Endothelin-1 (ET-1) was first identified as a potent vasoactive substance produced by porcine aortic ECs (56). It is produced by ECs, CMs, and fibroblasts, and its receptors are expressed in CMs, where it regulates CM contractility and maladaptive cardiac remodeling (57). There are also reports on other potential cardiac angiocrines such as prostacyclin, periostin, thrombospondins, follistatin/follistatin-like 1, angiotensin II, and connective tissue growth factor (58, 59), but their role and function in EC-CM crosstalk needs to be further validated with e.g., EC-specific gene deletion mouse models. In addition, pathological insults will lead to production of e.g., inflammatory and extracellular matrix proteins by several cell types in the heart, including ECs, but these are beyond the scope of this MiniReview.

Physical Cell-Cell Contacts and Extracellular Vesicles

In addition to paracrine mediators, also direct physical cell-cell contacts may play a role in EC-CM communication. Connexins are important in cell-cell communication and Cx43, Cx40, and Cx37 are expressed in both ECs and CMs [reviewed in (60)]. They form gap junctions between adjacent CMs enabling efficient electrical conduction. In ECs, connexins regulate e.g. leukocyte trafficking. Based on *in vitro* co-culture studies, connexins have also been suggested to mediate communication between ECs and CMs; however, more evidence is needed to prove the existence and relevance of direct EC-CM contacts *in vivo*. Furthermore, exosomes and other extracellular vesicles can carry non-coding RNAs such as microRNAs, long non-coding RNAs and circular RNAs, which have been shown to regulate cardiac remodeling and regeneration (61). Their roles and mechanisms in EC-CM communication have however not been characterized in detail.

THERAPEUTIC APPROACHES

With the pressing and unmet need for effective treatments to reduce cardiovascular mortality, various approaches are being investigated as potential reparative and regeneration-inducing treatments. Therapeutic strategies aiming to induce vascular and cardiac regeneration include gene therapy and drug development, while cardiac repair is being pursued with cell therapy and tissue engineering approaches (**Figure 2**).

Cardiovascular Gene Therapies

The growing understanding of the molecular mechanisms regulating vascular and cardiomyocyte growth and homeostasis enables the development of targeted therapies for cardiovascular diseases. Gene therapy experiments in both small and large animals have provided promising results for enhancing cardiac vasculature and cardiomyocyte function. The most commonly used vectors for therapeutic gene delivery, adenoviral and adeno-associated viral vectors (AAVs) as well as plasmids, exhibit a good safety profile also in clinical trials. However, the clinical efficacy of cardiovascular gene therapies has been somewhat disappointing [reviewed in (62)]. Plasmids exhibit a rather low transfection efficiency, whereas adenoviral vectors have marked immunogenicity and induce high transient transgene expression. For ischemic heart disease, long-term transgene expression with low immunogenicity is needed, making recombinant AAVs currently the best choice. Although the AAV serotype AAV9 has the best tropism for cardiomyocytes (63), the lack of truly tissue-specific AAV vectors is a topical problem. Importantly for cardiovascular diseases, there are no rAAVs at the moment, which would transduce ECs efficiently.

Some randomized clinical trials with VEGF and FGF gene therapies have improved myocardial vascularity; however, convincing evidence on functional benefits is still missing [see (62, 64)]. Clinical trials targeting heart failure to enhance cardiomyocyte function have mainly used gene delivery of either SERCA2a or SDF-1, but the results have not fulfilled the expectations, possibly due to insufficient gene delivery (62, 65). This will likely improve in the near future, as re-engineered vectors will increase transduction efficiency and specificity, and more validated targets advance to clinical stage.

RNA Therapeutics

RNA-based drugs include short interfering RNAs, antisense oligonucleotides, messenger RNAs and CRISPR gene-editing technology (66). The first members of RNA therapeutics have gained FDA approval or entered advanced phases of clinical trials; most of them are for various cancers or viral infections (66). Thus far only one RNA drug targeting cardiovascular diseases is in clinical trials: AZD-8601 is a chemically modified RNA encoding VEGF for the induction of therapeutic revascularization in the heart (67). Its phase 1 trial (NCT02935712) has been completed and phase 2 trial (NCT03370887) has started recruiting patients (www.clinicaltrials.gov). It will be administered by epicardial injections during coronary artery bypass grafting surgery. In preclinical studies, several miRNAs have been shown to regulate EC and CM survival and proliferation and are thus expected to become potential therapeutic targets as the delivery and targeting techniques improve (68, 69).

Heart Repair by Cell Therapies and Tissue Engineering

Stem cell therapy for cardiac repair has been under intensive investigation and produces modest, although variable, improvements in cardiac function in clinical trials (70–73). The transplanted cells do not, however, differentiate into cardiac cells and engraft into the myocardium, but instead their effects are attributable to paracrine pro-survival factors. Transplantation of cardiomyocytes alone or together with other cell types has been studied in various preclinical models (74–77) including non-human primates (78, 79), and the results show engraftment of transplanted CMs into the myocardium and in most cases also improved cardiac function. The observed ventricular arrhythmias (78) call for caution before proceeding to clinical trials. In the majority of cell therapy experiments,

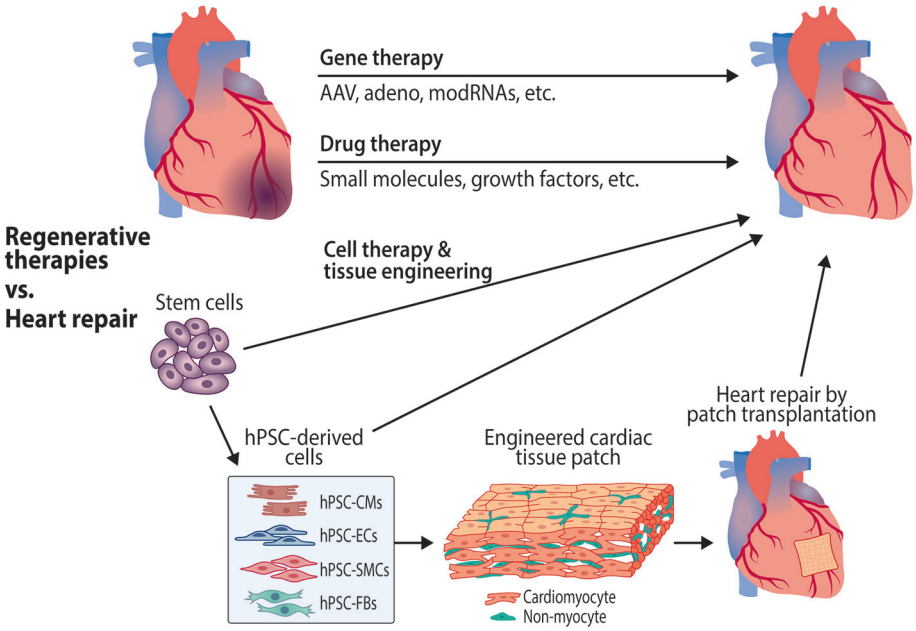

FIGURE 2 | Therapeutic approaches targeting cardiomyocytes and endothelial cells to promote cardiac regeneration and repair. Gene therapies to induce revascularization and cardiac regeneration are being investigated using various approaches, including adeno-associated (AAV) and adenoviral vectors, as well as modified RNAs (modRNAs). Potential regenerative drug therapies that are in preclinical stages of drug discovery and development include small molecule compounds and biologics such as growth factors. Cell therapies include the use of stem cells, cardiovascular progenitor cells and stem cell-derived cardiovascular cells to repair the damaged myocardium. Tissue engineering approaches use stem cell-derived cardiomyocytes and other cell types to create organized tissue patches for transplantation. hPSC, human pluripotent stem cell; hPSC-CMs, hPSC-derived cardiomyocytes; hPSC-ECs, hPSC-derived endothelial cells; hPSC-SMCs, hPSC-derived smooth muscle cells; hPSC-FBs, hPSC-derived fibroblasts.

the cells have been administered as injections and exhibit no organization at the time of transplantation, which may predispose to arrhythmias. A more refined approach, tissue-engineering, which produces cell sheets or patches with a more organized structure, i.e., cardiomyocyte alignment and established cell-cell connections, is proceeding to clinical trials in the near future [reviewed in (80)].

The requirement for fast neovascularization of the infarcted area (28) and experimental evidence showing that ECs improve the survival and spatial organization of CMs *in vitro* (81) suggest that concomitant transplantation of ECs together with CMs could improve the survival of transplanted cells and revascularization of the graft. However, intramyocardial injections of CMs in combination with ECs and SMCs did not improve cardiac function in a porcine model of MI despite enhancing vasculogenesis in the peri-infarct region (77). There is some evidence of improved outcome when ECs have been included in tissue-engineered patches (82), and patches containing CMs, ECs, and SMCs were recently shown to reduce infarct size and improve cardiac function in swine (83). However, ECs might be dispensable, as transplantation of patches consisting of CMs and fibroblasts also produces highly organized and progressively vascularized grafts in rat hearts (84).

Furthermore, the beneficial paracrine effects of cell therapy have prompted research on extracellular vesicles that carry pro-survival signals such as non-coding RNAs, which could be used to achieve similar effects as cell therapy without the delivery of cells. Prolonged cell-free delivery of hPSC-CM-derived extracellular vesicles into infarcted rat hearts promotes cardiac recovery (85). Furthermore, exosomes collected from cocultures of hPSC-derived CMs, ECs, and SMCs were reported to be more efficient in protecting cultured cardiomyocytes than exosomes from hPSC-CMs cultured alone (83) highlighting the importance of non-myocyte-derived signals in CM survival.

Drug Discovery

The efforts to discover regeneration-inducing pharmacological agents are still in their infancy. Considering the lack of regenerative gene expression response in both CMs and ECs (31), a potential regenerative treatment strategy would induce production of new CMs and promote revascularization at the same time. It is therefore very likely that a combination of compounds will be required. These may include compounds promoting cardiomyocyte proliferation, such as GSK3 inhibitors and p38 MAPK inhibitors (86) as well as proangiogenic factors or small molecule compounds, such as VEGFs or relaxin receptor agonists (87). Furthermore, compounds targeting the cardiac transcription factor GATA4, which promotes cardiac regeneration (88), might exhibit regenerative potential (89, 90).

Due to potential adverse effects in off-target organs, regeneration-inducing therapeutics should be specifically targeted to the heart. To avoid invasive administration, cardiac targeting with heart-homing nanoformulations has provoked interest. For example, porous silicon nanoparticles

coated with ANP and loaded with a novel GATA4-targeted cardioprotective compound were shown to enrich in the ischaemic endocardium and inhibit prohypertrophic signaling after systemic administration (91). Although the current targeting efficiency requires improvement, nanoparticle-based drug delivery would not only enable tissue or cell type-specific targeting, but also prolonged or sequential release of several drugs, as well as enable the delivery of poorly soluble or instable therapeutics (92–94).

Cardiac heterocellularity also needs to be implemented in *in vitro* models used in drug discovery and development. More relevant *in vitro* systems are needed not only for screening and lead optimization of cardiovascular drugs, but also for screening cardiovascular and other drug candidates for cardiotoxicity and proarrhythmic effects. The advances in the production of human pluripotent stem cell-derived cardiovascular cells and microfabrication provide an unprecedented possibility for large-scale drug screening in humanized multicellular cardiac cell culture models and heart-on-a-chip systems (95, 96).

SUMMARY AND CONCLUDING REMARKS

Cardiac regeneration requires an orchestrated multicellular response, the mechanisms of which are not yet fully understood (97). The current focus in the field of cardiac repair and regeneration has shifted from stem cells toward promoting regenerative processes in the endogenous cardiac cells, of which CMs and ECs are the most important considering regeneration. Detailed understanding of the mechanisms and crosstalk between these two cell types will be key in developing regenerative therapies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Cardiomyocytes and Macrophages Discourse on the Method to Govern Cardiac Repair

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In response to pathophysiological stress, the cardiac tissue undergoes profound remodeling process that incorporates the elimination of dying resident cells, compensatory hypertrophy of functional cardiomyocytes, growth and remodeling of the vascular compartment and formation of a fibrotic scar. Accumulating evidences indicate that cardiac remodeling is, at least in part, controlled by a complex crosstalk between cardiomyocytes and macrophages. The strategic location of abundant macrophages to the proximity of cardiomyocytes suggest that they could regulate the fate of cardiomyocytes in the injured heart. As such, macrophages appear as critical support cells for cardiomyocytes and play central roles in cardiac hypertrophy, fibrosis and remodeling. Notably, the cardiac tissue expands heterogeneous population of cardiac macrophages through local proliferation of resident macrophage as well as recruitment and differentiation of blood-derived monocytes. It has also been suggested that cardiac-resident macrophages display distinct functional properties from that of monocyte-derived macrophages in cardiac tissue. Furthermore, macrophages are an overflowing source of biological entities with non-canonical roles on cardiac conduction or cardiomyocyte proliferation by regulating action potential diffusion or cardiac cell cycle reentry. Alternatively, stressed cardiomyocytes can trigger the release of a broad repertoire of instructive signals that can regulate macrophage number, skew their phenotype and therefore direct their beneficial or deleterious actions. In this review, we highlight recent discoveries describing how the intricate dialogue between cardiomyocytes and macrophages can shape the deleterious or healing signaling mechanisms in the injured cardiac tissue.

Keywords: heart, inflammation, macrophages, heart failure, myocardial infarction

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Pathological conditions (hypertension, heart valve disease, myocardial infarction, cardiomyopathy) result in cardiomyocyte hypertrophy and cardiac remodeling progression associated with systolic and diastolic dysfunction. This adverse ventricular remodeling precipitates the occurrence of heart failure, arrhythmia, or sudden death. Hypoxia, neuro-humoral activation, and mechanical stress initiate multiple signaling pathways leading to cardiomyocyte death and hypertrophic growth of surviving cardiomyocytes. Nevertheless, the heart also entails different types of non-myocyte cells, including vascular cells, fibroblasts and inflammatory cells. Notably, emerging evidences indicate that cardiac remodeling is regulated by direct and indirect communications between cardiomyocytes and inflammatory cells. In response to

pathophysiological stress, the abundance of inflammatory cells and particularly, that of macrophages, and their proximity to cardiomyocytes, position them to critically regulate cardiomyocyte homeostasis in the injured heart. As such, macrophages appear as critical support cells for cardiomyocytes and play central roles in cardiac hypertrophy, fibrosis and remodeling. In this review, we highlight recent advances, mainly related to experimental observations, in our understanding of the decisive interaction between macrophages and cardiomyocytes.

ORIGIN OF CARDIAC MACROPHAGES

During early gestation, around embryonic day 7, primitive hematopoiesis occurs and macrophages expand in the extra-embryonic yolk sac. Subsequently, erythromyeloid precursors emerge from the yolk sac, and generate fetal macrophages. The onset of vascularization is associated with erythromyeloid precursors migration to the fetal liver. Concomitantly, hematopoietic stem cells (HSCs) arise from the aorta-gonad-mesonephros and migrate to the fetal liver. In the perinatal period, bone marrow serves as a major reservoir of HSCs and synthesizes the complete repertoire of immune cells (1).

The development of genetic fate-mapping techniques allows to classify and track distinct macrophage subsets, and when associated with parabiotic and adoptive-transplant studies, permits to discern the origin of tissue macrophages. Using this combination of methods, it has become obvious that most of cardiac macrophages derives from precursor of embryonic origin, but not from circulating monocytes after birth (2). Murine cardiac macrophages ($CD45^+CD11b^+F4/80^+CD64^+$ or $CD68^+$) are further categorized by expression of CCR2 (C-C chemokine receptor type 2), MHC-II (major histocompatibility complex II), and the lymphocyte antigen 6 complex (Ly6C). High and low expression of Ly6C can be used to discriminate inflammatory and reparative macrophages but the use of both CCR2 and MHC-II is useful to distinguish three separate and discrete cardiac macrophages pools from different origins. In steady state, the adult heart contained two resident macrophages subsets, MHC-$II^{low}CCR2^-$ and MHC–$II^{high}CCR2^-$ cells. These macrophages are separate from the blood monocyte pool and originate mainly from yolk sac- and fetal liver- embryonic progenitors (2, 3). The third macrophage pool expresses CCR2 (MHC-$II^{high}CCR2^+$) but is smaller numerically and is derived entirely from blood monocytes (2, 3). In the steady state, tissue-resident macrophages constitute the main subset of cardiac macrophages and are preserved through tissue proliferation (2). Nevertheless, it has been suggested that self-renewal of cardiac-resident macrophages declines with aging and that a substantial pool of cardiac resident macrophages is replenished by monocyte-derived macrophages, even in the lack of pathological triggers and inflammation (4). Indeed, using alternative gating strategy based on MHC-II and CX3CR1 expression, it has been shown that the proliferation capacity of embryo-derived cardiac macrophages is progressively reduced from the peri-natal period (4).

Of note, comprehensive transcriptional analysis of resident macrophages suggests that resident macrophages isolated from various organs are transcriptionally different from each other (5, 6). It is therefore likely that the tissue niche provides instructive signals coordinating macrophage phenotype and that the cardiac environment shapes the gene repertoire and controls macrophage-related functions. Furthermore, alteration of this cardiac niche is expected to affect the transcriptional network of cardiac macrophages resulting in a continuum of polarization states in the pathological heart.

Physiological and pathological settings adjust the number of each cardiac macrophage pools. Macrophage number could depend on local expansion of resident macrophage or recruitment and differentiation of blood-derived monocytes. In the absence of circulating monocytes, such as in CCR2 deficient mice, resident cells can repopulate the pool of cardiac macrophages. After transient depletion of macrophages with clodronate liposomes, circulating monocytes infiltrate the myocardium and are able to differentiate into long lasting populations of cardiac macrophages. Furthermore, CCR2– macrophages disappeared after a cardiac insult and blood monocytes fully replenish heart macrophages (2, 7). Hence, after a cardiac insult, it is likely that bone marrow-derived monocyte populations represent the major cardiac macrophage substitute. Notably, in the bone marrow, a $CCR2^+CD150^+CD48^-$ $Lineage^-Sca\text{-}1^+c\text{-}Kit^+$ hematopoietic subset has been identified as the main precursor of myeloid cells after ischemic insult (8). The cardiac tissue produces mobilizing factors such as granulocyte/macrophage colony-stimulating factor or CCL (CC motif chemokine ligand)2 and CCL7 that act distally to foster monocyte mobilization from the bone marrow to the blood (9–11). Bone marrow-derived HSCs lead to the emergence of two principle monocyte subsets depicted by the expression Ly6C. Classical $Ly6C^{high}$ monocytes appear to originate from $Ly6C^+$ monocyte progenitors, and non-classical $Ly6c^{low}$ monocytes, which likely differentiate from $Ly6C^{high}$ monocytes, through a Nr4a1-dependent transcriptional program (12–14). Bone marrow derived Ly6C monocytes are recruited into the injured cardiac tissue and differentiate to macrophages. In addition to the bone marrow, the spleen also acts as a reservoir for $Ly6C^{high}$ monocytes and participates to cardiac macrophage repopulation [(15); **Figure 1**]. One can also speculate that additional compartment could participate to the inflammatory cell influx within the cardiac tissue. In this line of reasoning, the pericardial adipose tissue has been shown to contain a high density of lymphoid clusters (16). The white adipose tissue has also been identified as a reservoir of mast cell progenitors, that do not originate from the bone marrow, and are able to home to cardiac tissue through a stem cell factor dependent signaling (17). The putative contribution of adipose tissue to the monocyte and/or macrophage pool remains to be defined. Altogether, these results indicate that, following the disruption of homeostasis; multiple complementary pathways could participate to the dynamic regulation of the heterogeneous population of cardiac resident macrophages.

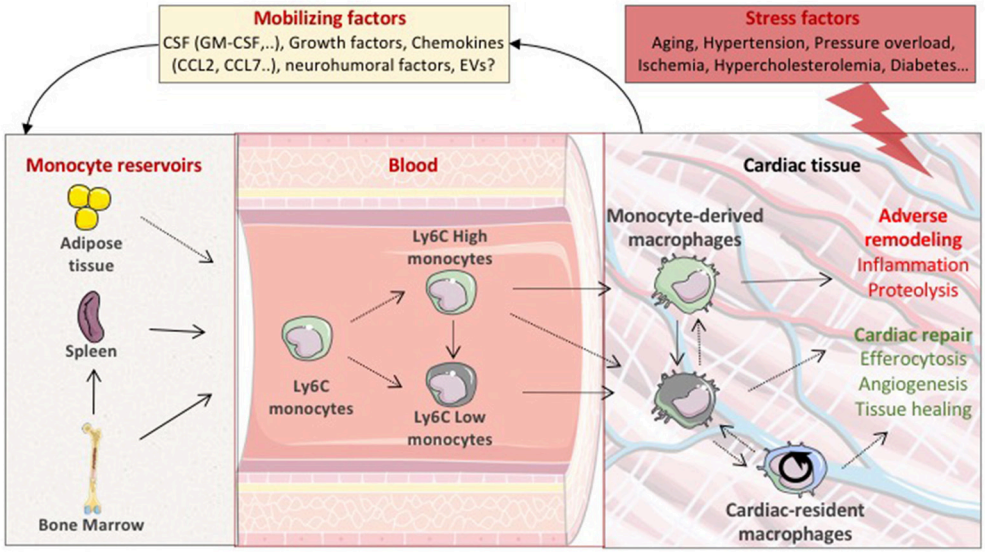

FIGURE 1 | Life cycle of macrophages during cardiac stress. In pathological settings, the stressed cardiac tissue sends mobilizing factors to monocyte reservoirs including bone marrow and spleen, leading to the emergence of two principle monocyte subsets depicted by the expression of Ly6C (Ly6C High or Low). Circulating Ly6C positive monocytes are then recruited into the injured cardiac tissue and differentiate to macrophages. Local proliferation of cardiac resident macrophages also participates to the expansion of cardiac macrophage population. Cardiac-resident macrophages and monocyte-derived macrophages are expected to display distinct functional properties precipitating or preventing adverse ventricular remodeling. CSF, colony-stimulating factors; GM-CSF, granulocyte macrophage colony stimulating factor; EVs, extracellular vesicles.

In macrophage-depleted mice, infarct healing is impaired, and myocardial rupture occurs suggesting a net beneficial role of the heterogeneous population of cardiac macrophages in cardiac repair (18–20). Resident macrophages are expected to display functional properties distinct from that of monocyte-derived macrophages. Transcriptional analysis revealed that monocyte-derived macrophages synchronize cardiac inflammation, but are less efficient in antigen processing and clearance of dying cells (2). In particular, $CCR2^{+}$ macrophages contain distinct components of the NLRP3 inflammasome and are instrumental in IL-1β release (2). On the same note, chemoattractants CXCL (C-X-C motif chemokine ligand)2 and CXCL5 produced by $CCR2^{+}$ resident macrophages have been shown to specifically contribute to the initial neutrophil extravasation into the ischemic area (21). Inhibition of monocyte and monocyte-derived macrophage recruitment improves outcome after acute MI and myocardial infarction-induced heart failure. Conversely, cardiac-resident macrophages ($MHC\text{-}II^{low}CCR2^{-}$ and $MHC\text{-}II^{high}CCR2^{-}$) seem to display robust proangiogenic and mitogenic properties, suggesting a reparative potential for these macrophage subsets [(22); **Figure 1**].

Nevertheless, it must be acknowledged that there is no consensus to harmonize the phenotypic classification of macrophages, which induces uncertainty in the characterization as well as function of the distinct sub-populations of cardiac macrophages. An ambiguity amplified by the use of enzymatic digestion methods designed to isolate cardiac inflammatory cells that alter the levels of cell-surface markers and by the shared expression of some markers such as CD11b, Ly6C or F4/80 on dendritic cells or myeloid derived suppressor cells (23).

Although macrophages are clearly present in human heart (24), information regarding their function, origin and heterogeneity in steady state and pathological conditions are lacking. Nevertheless, two distinct populations of macrophages have been identified in left ventricular myocardial specimens from patients with dilated and ischemic cardiomyopathies (25). CCR2+ and HLA-DR High (the human homolog of MHC II) macrophages are monocyte-derived cells with specific pro-inflammatory potential whereas CCR2– and HLA-DR High macrophages are resident cells with reparative properties [(25); **Table 1**].

MACROPHAGES AND OUTSIDE-IN SIGNALING WITHIN CARDIOMYOCYTES

Canonical Role of Macrophages

The interaction between macrophages and cardiomyocytes hypertrophy and survival has been studied in experimental model of acute injury (mainly acute myocardial infarction) or heart failure following pressure overload, hypertensive treatment, or myocardial infarction. Whatever the incentive stimulus, the failing heart contains increased number of macrophages that expand by both local proliferation of resident macrophage and further recruitment and differentiation of circulating monocyte (2, 7, 26, 27). Interactions of cardiac macrophages with other non-myocyte cells including inflammatory cells, vascular cells and fibroblasts shape the dysfunctional heart. These cardiac macrophages also secrete factors that directly or indirectly alter the cardiac extracellular matrix network. Hence, the net impact of cardiac macrophages on cardiomyocyte likely relies

TABLE 1 | Immunophenotypic properties of different subsets of embryo- and monocyte-derived cardiac macrophages.

		Origin of cardiac Mφ	Inflammatory cell signature markers	Mφ signature markers	Specific signature markers
Mice	Gating strategy 1	Embryo-derived Mφ	CD45+, CD11b+, Auto+, Ly6C–	CD64+, Mertk+, F4/80+	MHCII High/Low and CCR2–
		Monocyte-derived Mφ	CD45+, CD11b+, Auto+, Ly6C–	CD64+, Mertk+, F4/80+	MHCII High and CCR2+
	Gating strategy 2	Embryo-derived Mφ	CD11b+, Ly6C Low, CD11c Low-Int	CD14+, CD64+, F4/80+, Mertk+	CX3CR1+ and MHCII–
		Monocyte-derived Mφ	CD11b+, Ly6C Low, CD11c Low-Int	CD14+, CD64+, F4/80+, Mertk+	CX3CR1± and MHCII+
Human	Gating strategy 3	Resident Mφ	CD45+	CD14+, CD64+, Mertk+, CD68+	CCR2– and HLA-DR High
		Monocyte-derived Mφ	CD45+	CD14+, CD64+, Mertk+, CD68+	CCR2+ and HLA-DR High

Gating strategy 1, 2, and 3 from Epelman et al. (2), Molawi et al. (4), and Bajpai et al. (25), respectively. Mφ indicates macrophages; Auto, auto-fluorescence; int, intermediate.

on pleiotropic effects involving regulation of distinct and complementary processes. Furthermore, the molecular cascades involved in macrophage specific effects on cardiomyocytes are poorly understood. Nevertheless, the cardiac influx of macrophages on pressure overload, for example, precedes signs of hypertrophy and heart failure, suggesting a direct contribution to cardiomyocyte hypertrophy and survival [(28); **Figure 2**].

Considering their abundance, diversity, and phenotypic plasticity, macrophages represent a major reservoir of cytokines. Inflammatory signals induce interleukin(IL)6 release by macrophages and neutralizing IL-6 reduces cardiomyocyte hypertrophy (29, 30). Acutely, IL-6 also prevents cardiomyocytes against oxidative stress-induced cellular damages. IL-6 receptor cooperates with at least one subunit of the signal-transducing protein gp130. Of note, complete loss of gp130 results in massive induction of myocyte apoptosis and dilated cardiomyopathy in response to pressure-overload (31). Alternatively, in chronically exposed myocytes, IL-6 family signaling reduces the basal contractility and the beta-adrenergic responsiveness of the myocytes (32).

Specific subtype of pro-inflammatory CCR2 positive macrophages is a major purveyor of IL-1β (2). Interestingly, in a phase 3 trial, the use of canakinumab, an IL-1 β neutralizing antibody, has been shown to decrease the incidence of repetitive atherothrombotic event in patients with myocardial infarction already on state of the art treatment (33). Inflammatory macrophages also release IL-18, another member of the IL-1 family (34, 35). IL-18 enhances cardiomyocyte hypertrophy and exacerbates cardiac dysfunction as well as fibrosis in infarcted and non-infarcted heart (36, 37). IL-18 could also precipitate adverse ventricular remodeling by reducing capillary density in the ischemic tissue (38). Toll-like receptor activation induces the production of tumor necrosis factor (TNFα) from macrophages. In a genetic heart failure model, TNF-α has been shown to confer cardioprotection by maintaining normal intercalated disc structure and mitochondrial integrity as well as cardiomyocyte function (39). Transforming growth factor (TGF)β produced by cardiac macrophages triggers extracellular matrix deposition (40) but is also a direct mediator of the hypertrophic growth response of the heart (41). Myeloid-derived growth factor is secreted by macrophages and stimulates cardiomyocyte survival (42). In addition, macrophages synthesize IL-10 and leukemia inhibitory factor, which subsequently abrogate apoptosis of hypoxic cardiomyocytes (43).

Beyond cytokine production, macrophages are an overflowing source of biological entities with pro-hypertrophic and survival potential. For example, Galectin-3, a macrophage-derived mediator, contributes to cardiac hypertrophy (44). Removal of micro(mi)RNA-155 in macrophages reduces cardiac hypertrophy and cardiac dysfunction following pressure overload (28). Mineralocorticoid receptor deletion in macrophages abrogates cardiac hypertrophy after aortic constriction (45) or in the setting of hypertension (46, 47). Mice with macrophage harboring prolyl hydroxylase domain protein 2 deficiency display attenuated cardiac hypertrophy and contractile dysfunction after hypertensive treatment (48).

Non-canonical Role of Macrophages

Cardiac Conduction

Recent work also reveals that resident cardiac macrophages establish direct connections to cardiomyocytes via connexin 43 gap junctions in the atrioventricular node. *In vitro*, neonatal cardiomyocytes cause rhythmic depolarizations in macrophages, which in turn, alters the electrophysiological properties of cardiomyocytes. Since macrophages are poorly polarized cells, their coupling with cardiomyocytes depolarizes them. While in the "working" cardiomyocytes an increase of the resting potential inactivates the sodium channels and slows the conduction, the depolarization of the atrioventricular node cardiomyocyte depends on the calcium channels, whose voltage-dependent inactivation is shifted toward higher positive voltages. Activation of a rhodopsin 2 channel specifically expressed in macrophages causes sodium entry and depolarization, improving nodal conduction. Conversely, inactivation of connexin 43 specifically in macrophages, or the congenital deficiency of macrophages, affect the propagation of cardiac electrical impulses, attesting to the existence of a functional connection between resident macrophages and specialized cardiomyocytes within the nodal tissue (24). Macrophages could also regulate conduction abnormalities beyond the atrioventricular node, including the course of atrial fibrillation

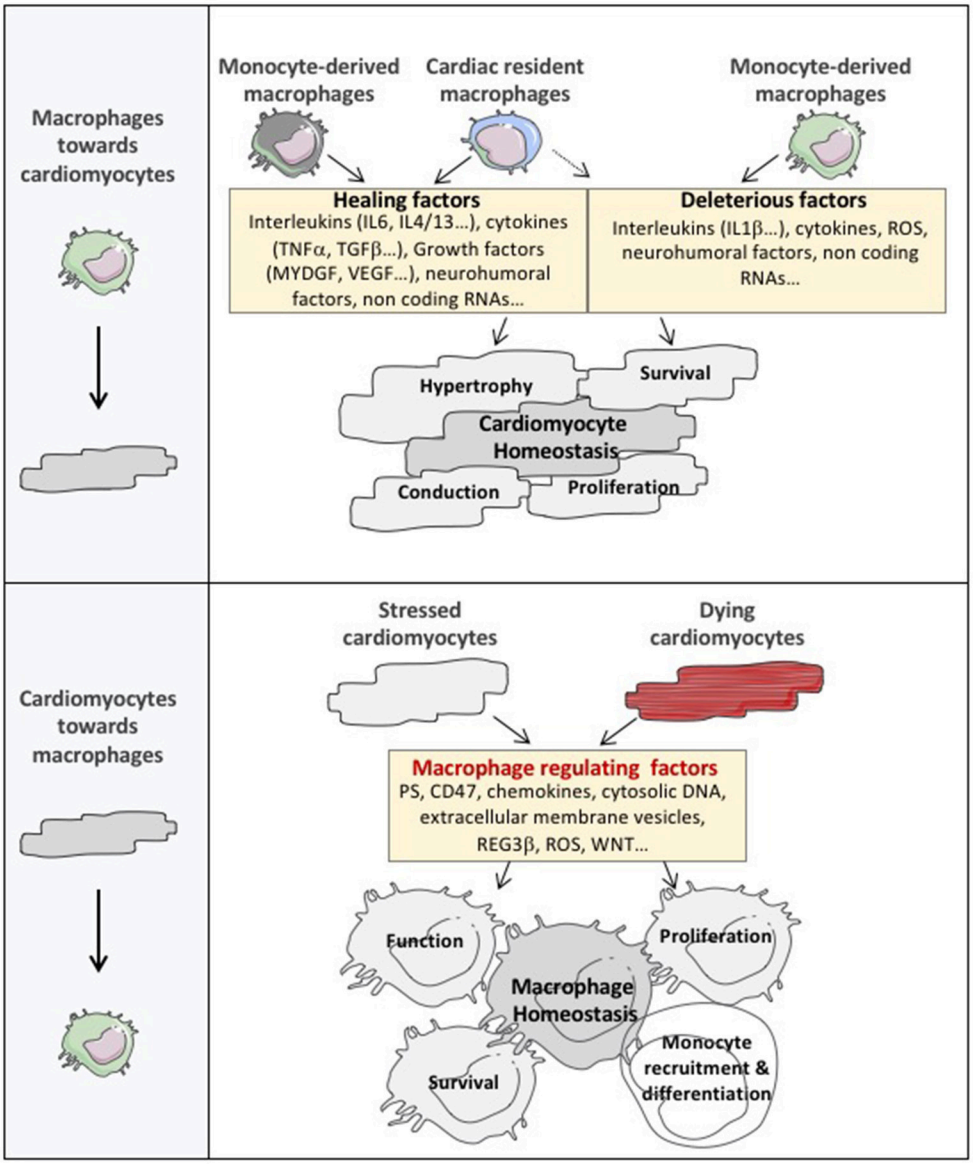

FIGURE 2 | The cardiomyocyte and macrophage cross-talk in cardiac tissue. The expanded cardiac macrophage population plays an important role in the regulation of cardiac dysfunction by secreting factors that directly or indirectly alter cardiomyocyte homeostasis. Notably, considering their abundance, diversity, and phenotypic plasticity, macrophages represent a major reservoir of factors controlling cardiomyocyte hypertrophy, survival and contractility. Furthermore, cardiac macrophages exert non-canonical roles on cardiac conduction or cardiomyocyte proliferation by regulating action potential diffusion or cardiac cell cycle reentry. Nevertheless, the communication between cardiomyocytes and macrophages operates in both directions and cardiomyocytes dialogue toward cardiac macrophages to drive their number, function and phenotype through the release of a broad catalog of macrophage regulating factors. MYDG, myeloid derived growth factor; PS, phosphatidyl serine; ROS, reactive oxygen species; DNA, deoxyribonucleic acid; REG3β, regenerating islet-derived protein 3-beta.

or ventricular arrhythmias induced by ischemia. Clinically, atrioventricular block is a common indication for implantation of cardiac pacemakers, but many cases of atrioventricular block occur for unknown reasons. Macrophage phenotype and number are modified after acute myocardial infarction and heart failure, conditions associated with sudden cardiac death and ventricular arrhythmias. In this regard, recent work has shown that diabetic inflammation causes secretion by resident macrophages of IL-1β, which destabilizes the electrical activity of cardiomyocytes potentially promoting the occurrence of ventricular arrhythmias (49). Other cardiac inflammatory diseases, including Chagas disease, Lyme disease, and myocarditis, initiate conduction defects. In these pathological conditions, cardiac macrophages could also participate to the development of atrioventricular block in addition to cardiomyocytes and specialized conductive

tissue deregulations. Conduction system development or genetic abnormalities could also be dependent of cardiac macrophage-related actions.

Cardiomyocyte Regeneration

Change in monocyte/macrophage gene expression is associated with decrease in cardiomyocyte proliferation with aging (50). In the neonatal heart, clodronate liposome-induced phagocytic cell depletion abrogates heart regeneration. Human fetal and adult mononuclear phagocytes show distinct pro-inflammatory features after LPS stimulation (2). Furthermore, it has been shown that in response to injury, resident CCR2– reparative cardiac macrophages dominate in the neonatal cardiac tissue and directly foster cardiomyocyte proliferation (22). Comparative analyses have been performed in many species revealing that the regenerative capacity of the cardiac tissue sparsely exists across the animal kingdom, and seems to correlate with immature immune system. As an example, zebrafish exhibits unchallenged ability to regenerate cardiac tissue. Interestingly, another teleost, the medaka, does not share this capacity. Comparative transcriptomic analyses following cardiac cryoinjury in Zebrafish and medaka point to major differences in immune cell dynamics between these two models. Delayed macrophage recruitment in zebrafish hampers cardiomyocyte proliferation and scar resolution. Delayed and reduced macrophage recruitment is observed in medaka, along with inefficient cardiac regeneration. Stimulating Toll-like receptor signaling in medaka activates immune cell dynamics and galvanizes cardiomyocyte proliferation and scar resolution (51). During the regenerative process, cardiomyocytes undergo a transcriptional reversal of cell differentiation program characterized, at least in part, by recrudescence of embryonic gene expression. Interestingly, IL-13 has been identified as a potential upstream regulator of the core network, which induced cardiomyocyte cell cycle entry, in part by the STAT3 pathway (52). Altogether, these studies provide further insight into the complex role of the immune response during cardiac repair, and suggest that macrophage could serve as a platform to release cytokines and growth factors, promoting cardiomyocyte proliferation, at least in neonatal mammalian heart.

CARDIOMYOCYTES AND OUTSIDE-IN SIGNALING WITHIN CARDIAC MACROPHAGES

The communication between cardiomyocytes and macrophages operates in both directions and cardiomyocytes dialogue toward cardiac macrophages using different languages (**Figure 2**).

First, dying cardiomyocytes send some "eat me" signals to stimulate clearance of dying cells and prevent collateral myocyte loss and infarct expansion. Efficient phagocytic clearance of cardiac apoptotic cells by macrophages involved the coordinated role of 2 major mediators of efferocytosis, the myeloid-epithelial-reproductive protein tyrosine kinase (MerTK) and the milk fat globule epidermal growth factor 8 (MFG-E8). Macrophage defective for efferocytosis leads to cardiac dysfunction (53, 54). Interestingly in ischemic heart, dying cardiomyocytes send unappropriated signals, such as upregulation of integrin-associated protein CD47 or shedding of macrophage MerTK, that impair phagocytic removal by cardiac macrophages (55, 56). Notably, resident $MHCII^{low}CCR2^{-}$ cells are characterized by elevated expression of MerTK and, release pro-reparative cytokines, such as TGF-β, in response to apoptotic cell engulfment (57). Of interest, non-professional phagocytes including cardiac myofibroblasts, have also been shown to efficiently engulf dead cells in the infarcted heart. These cardiac myofibroblasts secrete MFG-E8 promoting efferocytosis and acquisition of anti-inflammatory properties (58). Communication between professional and non-professional phagocytes likely coordinates phagocytosis of dying cells in the vicinity of the damage (59). As a prototypic example, in epithelial tissue, following clearance of apoptotic cells, macrophages release soluble growth factors, such as insulin-like growth factor 1, which redirect the phagocytosis of neighbor epithelial cells toward uptake of smaller vesicular components (60). Nevertheless, the existence of this crosstalk between macrophages and non-professional phagocytes remains to be detailed in the pathological cardiac tissue.

Second, in pathological conditions, activated cardiomyocytes can release a broad panel of instructive signals that could control macrophage number and skew their phenotype. For example, neutrophils and macrophages produce the cytokine oncostatin M, which galvanizes the cardiomyocytes to produce the regenerating islet-derived protein 3-β. Subsequently, regenerating islet-derived protein 3-β improves monocyte-derived macrophage number in the cardiac tissue (61). Other molecular entities delivered by hypoxic cardiomyocytes such as inhibitors of the WNT pathway, radical oxygen species or the release of cytosolic nucleic acid also modulate the number and phenotype of cardiac macrophages (62–64). In hypertensive heart failure model, microarray and immunohistochemistry analysis revealed a cardiomyocyte specific upregulation of 12/15-lipoxygenase, a major enzyme metabolizing arachidonic acid into hydroxy-eicosatetraenoic acids. Cardiomyocyte overexpression of 12/15-lipoxygenase increases cardiac CCL2 contents leading to accumulation of inflammatory macrophages and systolic dysfunction (65). Extensive amount of evidences highlights that cell-to-cell communication involves extracellular vesicles (EVs). EVs regulate major biological functions, including inflammation. Presence of EVs in cardiomyocyte cytoplasm has been revealed in human hearts. Culture cardiomyocytes are also able to release EVs *in vitro* (66). Acute myocardial infarction in mice transiently enhances EV levels in the left ventricle. Both large (252 ± 18 nm) and small (118 ± 4 nm) EVs are produced and mainly emerge from cardiomyocytes. Of note, large EVs, but not small EVs, are able to control IL-6, CCL2, and CCL7 release from cardiac monocytes (67). Hypoxic cardiomyocytes have also been shown to release high rate of small EVs (mainly exosomes) containing excessive expression of TNF-α (68). Mixt EV population or small EVs (mainly exosomes) have emerged as critical agents of cardiac repair triggered by different type of cell therapy (69–71). EV-related effects could be mediated by cell-surface

receptor activation or delivery of multiple components within the targeted cells. Notably, small EV isolated from cardiosphere-derived cells are enriched in several miRNAs, such as miR-181b, and have been shown to specifically target CD68-positive cardiac macrophages switching their phenotype toward a reparative state (72). Cardiac EVs have also been identified in fragments of the interventricular septum obtained from patients undergoing extracorporeal circulation for aortic valve replacement suggesting that cardiomyocyte-derived EVs could constitute a major component of the cardiomyocyte/macrophage crosstalk (67). One can also speculate that these cardiac EVs could reach the bone marrow and/or the spleen, where they could stimulate a distinct myeloid-biased progenitor subset or monocyte mobilization. Nevertheless, the specific role of large or small cardiac EVs on different subset of inflammatory compounds as well as characterization of their core signaling mediator need to be further explored. Finally, the mechanical deformation of cardiomyocytes by pathways dependent on Mitogen Activated Protein Kinases is sufficient to increase the population of macrophages in the healthy myocardium (26).

FUTURE DIRECTIONS

Hence, cardiomyocytes and macrophages form an interspersed network and dialogue with each other to profile the multiple processes shaping the cardiac tissue. In pathological settings, the exact roles of resident embryonically derived- and monocyte-derived macrophage subsets on cardiomyocyte homeostasis remain to be fully defined. Of note, the use of strategies designed to inhibit CCR2 signaling or endothelial cell-dependent adhesion is of interest to abrogate monocyte-derived macrophages upregulation but likely induces non-specific effects on other inflammatory cell subtypes and, more importantly, hampers the direct impact of monocytes on cardiac remodeling. Such side effects may lead to confounding outcomes and misinterpretation of cardiac macrophages related-actions. For example, monocyte-derived macrophages likely display both positive and negative potential on cardiomyocyte and cardiac tissue. As such, $CD206^{+}F4/80^{+}CD11b^{+}$ macrophages exhibit strengthened reparative abilities after myocardial infarction. This macrophage subpopulation emerges from bone marrow HSCs through a kinase TRIB1 dependent pathway (73). In addition, IL-4 administration increases the number of reparative macrophages and enhances the post-myocardial infarction prognosis in mice (73). In the stressed heart, one can also speculate that the inflammatory landscape could skew the status of cardiac-resident macrophages from a reparative to a deleterious phenotype. Therefore, studies using specific resident macrophage depletion or distinct resident macrophage gene deletion need to be developed. Nevertheless, cardiac tissue provides a revealing example where macrophage number and phenotype, duration of macrophage-related signaling, from acute to chronic, could balance the protective and pathogenic transition of cardiac tissue.

Inflammation-dependent remodeling process has been predominantly described in acute injury, such as acute myocardial infarction. However, the impact of these inflammatory signalings remain mostly undefined in chronic pathological settings (74). Notably, further studies need to explore the mechanisms underlying the direct roles of macrophages on cardiomyocyte survival and hypertrophy and if specific macrophage sup-population could regulate the progression from compensated hypertrophy to heart failure. Finally, the precise identification of the molecular and cellular signals send from cardiomyocytes toward macrophages would likely improve our understanding of cardiac macrophage physiology and both their beneficial and deleterious actions.

AUTHOR CONTRIBUTIONS

IG, VD, and J-SS perform literature search and writing. All authors approved the manuscript.

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