Engineering Human Cardiac Muscle Patch Constructs for Prevention of Post-infarction LV Remodeling

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Tissue engineering combines principles of engineering and biology to generate living tissue equivalents for drug testing, disease modeling, and regenerative medicine. As techniques for reprogramming human somatic cells into induced pluripotent stem cells (iPSCs) and subsequently differentiating them into cardiomyocytes and other cardiac cells have become increasingly efficient, progress toward the development of engineered human cardiac muscle patch (hCMP) and heart tissue analogs has accelerated. A few pilot clinical studies in patients with post-infarction LV remodeling have been already approved. Conventional methods for hCMP fabrication include suspending cells within scaffolds, consisting of biocompatible materials, or growing two-dimensional sheets that can be stacked to form multilayered constructs. More recently, advanced technologies, such as micropatterning and three-dimensional bioprinting, have enabled fabrication of hCMP architectures at unprecedented spatiotemporal resolution. However, the studies working on various hCMP-based strategies for *in vivo* tissue repair face several major obstacles, including the inadequate scalability for clinical applications, poor integration and engraftment rate, and the lack of functional vasculature. Here, we review many of the recent advancements and key concerns in cardiac tissue engineering, focusing primarily on the production of hCMPs at clinical/industrial scales that are suitable for administration to patients with myocardial disease. The wide variety of cardiac cell types and sources that are applicable to hCMP biomanufacturing are elaborated. Finally, some of the key challenges remaining in the field and potential future directions to address these obstacles are discussed.

Keywords: tissue engineering, cardiac patch, myocardium, heart failure, myocardial infarction, regenerative medicine, cardiac regeneration and remodeling

INTRODUCTION

Despite advancements in preventive medicine, cardiovascular disease (CVD) remains a leading cause of morbidity and mortality worldwide (1, 2) with estimated 17.9 million people died of cardiovascular disease in 2016, accounting for 31% of all deaths globally (3). The molecular and cellular basis for progressive heart failure is the result of the inability of damaged and apoptotic myocytes to be replaced. The regenerative capacity of mammalian hearts declines rapidly after birth, and <1% of cardiomyocytes (CMs) in the hearts of adult humans are replaced each year (4);
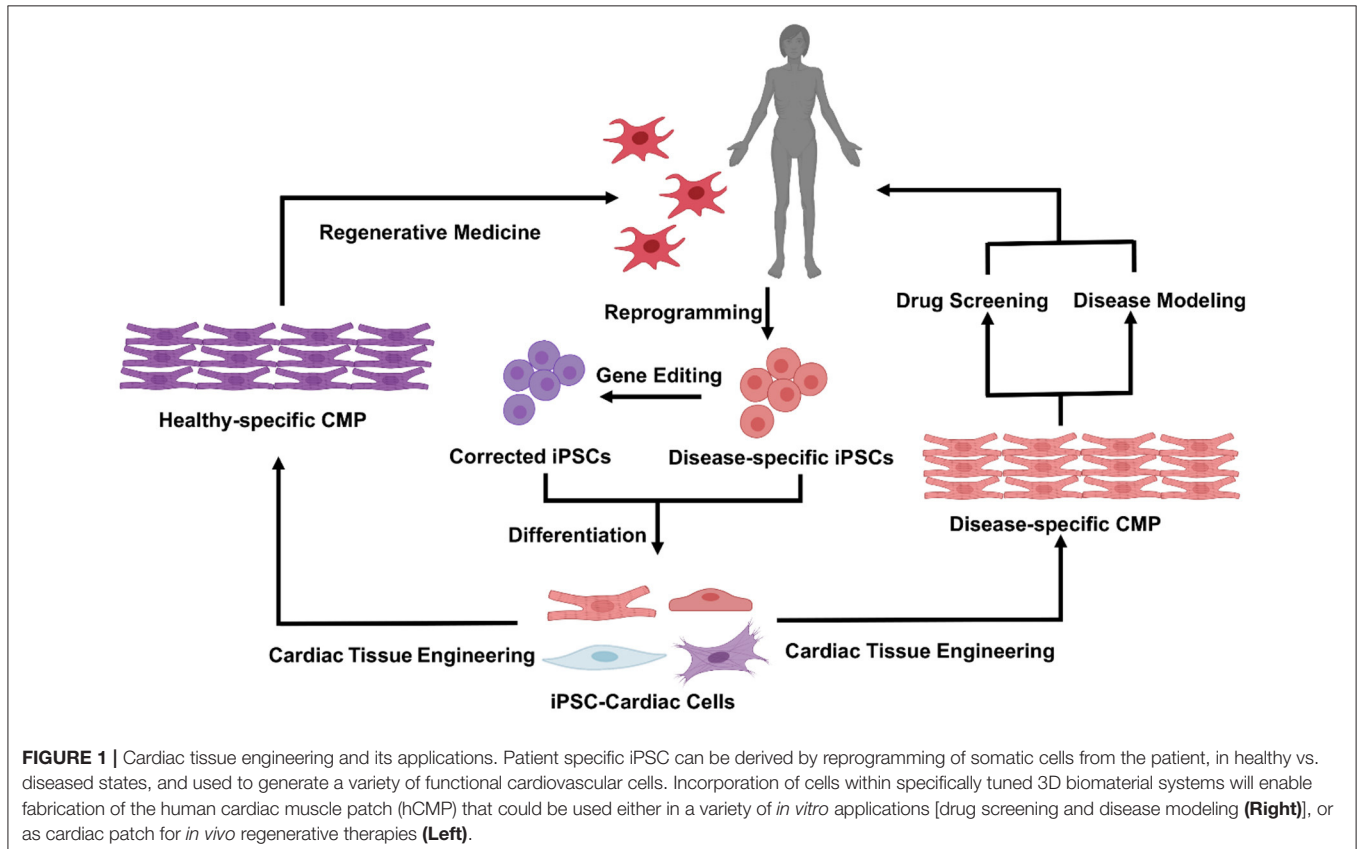
thus, myocardial injury leads to adverse cardiac remodeling and fibrosis as the injured myocardium is replaced by fibrotic scar tissue (5, 6). The only available procedure for end-stage patients is whole-heart transplantation which is restricted by an inadequate supply of donors. Therefore, alternative strategies for limiting post-injury cardiac remodeling and remuscularizing the damaged myocardium are urgently needed (7, 8). Administration of exogenous stem cells was initially considered a promising approach (9). The cells were expected to differentiate into CMs after transplantation (10, 11); however, the results from subsequent preclinical and clinical studies indicated that the benefits were only marginal and likely attributable to the cells' paracrine activity, because the proportion of cells that were retained and survived at the site of injury (i.e., the engraftment rate) was drastically low (12, 13).
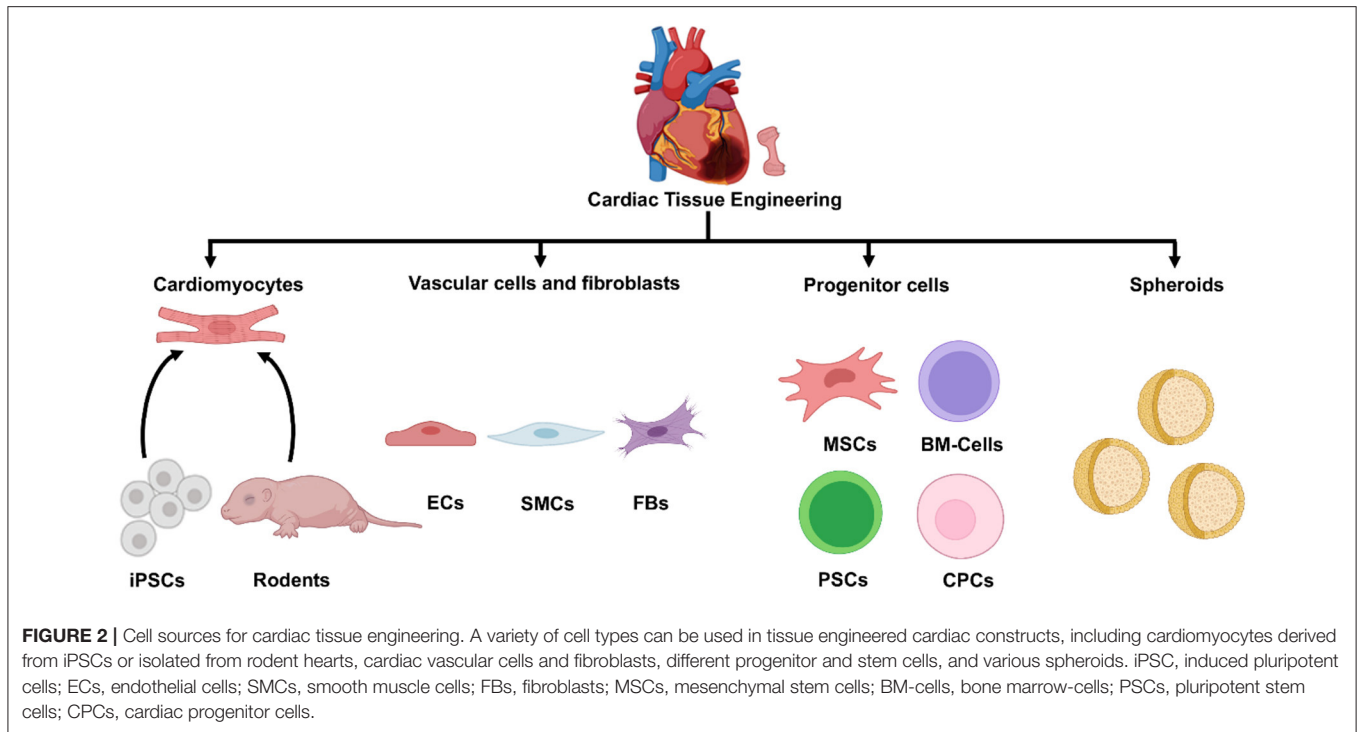
Tissue engineering combines the principles of engineering and life sciences to better understand the structure-function relationships in normal and pathological tissues and to generate living tissue equivalents for drug testing (14, 15), disease modeling (16–18), and regenerative medicine [(6, 19, 20); **Figure 1**]. In the cardiovascular field, the first proof-of-concept study was conducted in 1997, when embryonic chick CMs were suspended in collagen solution, and the mixture subsequently solidified and contracted coherently between two glass tubes (21). With the emergence of techniques for reprogramming human somatic cells into induced pluripotent stem cells (iPSCs)

and differentiating them into CMs (22) and other cardiac cells, the field has progressed to the development of engineered human cardiac muscle patch (hCMP) constructs (6). These engineered patch constructs are often associated with higher rates of engraftment and appear to support the injured myocardium more effectively than transplanted cells (2, 23). Nevertheless, vascularization of the hCMP (either during manufacture or via the infiltration of native vessels after transplantation) is not extensive and efficient enough to support the high metabolic demand of the heart (13, 24). Consequently, the thickness/dimensions of most hCMPs are limited to just a few hundreds of micrometers (13, 25). In this review, we discuss many of the most recent advancements in cardiac tissue engineering, with a primary focus on techniques for generating thicker and more integrative hCMP systems.

Cell Types and Sources for hCMP Fabrication

CMs are the fundamental contractile units of the myocardium and occupy 70–85% of myocardial volume in adult mammals (26); thus, many investigations of cell therapy have been conducted with CMs alone, either as dissociated cells or contiguous sheets (27, 28). However, hCMPs are designed to comprehensively recapitulate the physical structure and signaling pathways present in native heart tissue (13, 29) and, therefore, are typically composed of multiple cardiac cell types,

FIGURE 1 | Cardiac tissue engineering and its applications. Patient specific iPSC can be derived by reprogramming of somatic cells from the patient, in healthy vs. diseased states, and used to generate a variety of functional cardiovascular cells. Incorporation of cells within specifically tuned 3D biomaterial systems will enable fabrication of the human cardiac muscle patch (hCMP) that could be used either in a variety of *in vitro* applications [drug screening and disease modeling **(Right)**], or as cardiac patch for *in vivo* regenerative therapies **(Left)**.

FIGURE 2 | Cell sources for cardiac tissue engineering. A variety of cell types can be used in tissue engineered cardiac constructs, including cardiomyocytes derived from iPSCs or isolated from rodent hearts, cardiac vascular cells and fibroblasts, different progenitor and stem cells, and various spheroids. iPSC, induced pluripotent cells; ECs, endothelial cells; SMCs, smooth muscle cells; FBs, fibroblasts; MSCs, mesenchymal stem cells; BM-cells, bone marrow-cells; PSCs, pluripotent stem cells; CPCs, cardiac progenitor cells.

including CMs, endothelial cells (ECs), smooth muscle cells (SMCs), and cardiac fibroblasts (6, 26, 30). Other cell types or clusters of multiple cell types (e.g., progenitor cells and spheroids) have also been incorporated into cardiac patches and evaluated in preclinical models of myocardial injury (**Figure 2**).

Cardiomyocytes (CMs)

Healthy adult human CMs were largely unavailable for early hCMP studies due to scarcity of healthy heart donors and their non-proliferative phenotype. Thus, most of what we have explored and learned about the structural and functional properties of engineered cardiac tissues was initially investigated in experiments using primary rodent CMs (31). Pioneering work with fetal CMs from 15-day-old mouse embryos demonstrated that the cells were engrafted and survived after administration into the mouse hearts (32). Rhythmically contracting hydrogels were generated by plating neonatal rat heart cells on collagen, which enabled researchers to study how factors, such as cell density and collagen concentration, influenced contractile activity (33) and to demonstrate that these cellular constructs generated electrocardiography (ECG)-like potentials (34). Subsequent studies confirmed that patches composed of fetal rat ventricular cells and gelatin could survive and continue to contract when implanted subcutaneously in the adult rat legs. The engineered graft formed junctions with native heart cells when delivered to the scarred region of cryoinjured hearts, but whether the treatment could improve cardiac function remained uncertain (35). Patches consisting of fetal rat cardiac cells suspended in an alginate scaffold were among the first to preserve measures of cardiac function

and impede adverse cardiac remodeling when administered to infarcted rat hearts (36).

Embryonic stem cells (ESCs) and iPSCs are the most readily available sources of human-lineage CMs, because they can proliferate indefinitely and be differentiated into cells of different lineages (37). The first human vascularized, contracting hCMP was generated by combining CMs and ECs derived from human ESCs with mouse embryonic fibroblasts in porous sponges composed of 50% poly-l-lactic acid (PLLA) and 50% polylactic-glycolic acid (PLGA) (38). Experiments in both rodent (19, 39, 40) and swine (23) models of myocardial injury suggest that iPSC-based hCMPs are associated with higher levels of cell survival and engraftment than those associated with iPSC-based cell injection, and that the cells' paracrine activity can be modestly beneficial. Both ESC- and iPSC-derived CMs are, however, structurally and functionally more similar to fetal or neonatal cells than to the adult mature CMs. This could hinder downstream applications of hCMPs composed of immature PSC-CMs. The electrical immaturity of PSC-CMs may induce ventricular arrhythmias as demonstrated in non-human primate models, raising a safety concern for clinical translation (41, 42). Therefore, various methods for improving stem cell-derived CM maturity before transplantation, including electrical stimulation, and treatment with neurohormonal factors, have been frequent topics of research (43–46). hCMP maturation can be influenced by the purity of the initial CM population, as evidenced by increases in conduction velocity and contractile force (44). Further, mechanical conditioning of cardiac patches has been shown effective in maturation and functional improvement of encapsulated CMs. For instance, hCMPs consisting of a collagen matrix and CMs differentiated from mouse ESCs, more closely

resembled native myocardial tissue after undergoing 7 days of *in vitro* cyclic stretching (47).

Cardiac Vascular Cells and Fibroblasts

Cardiac ECs and SMCs not only promote the vascularization and survival of transplanted hCMPs but are also key mediators of the signaling mechanisms that regulate CM activity (23, 48, 49). Cardiac fibroblasts can further improve CM survival by remodeling the extracellular matrix (ECM) and releasing cytoprotective paracrine factors (6, 9, 50–52). Except for providing a reliable source of CMs, hiPSCs demonstrate great potential in offering vascular cells and fibroblasts. Robust protocols for differentiating hiPSCs to ECs (53), SMCs (50), and cardiac fibroblasts (54, 55) have been established. The inclusion of ECs and stromal cells, combined with uniaxial mechanical stimulation during manufacturing, has been shown to improve the hCMP maturity (56). However, early protocols for hiPSC-EC differentiation are not efficient and the phenotype of generated hiPSC-ECs remain rather unstable. Refined EC differentiation protocols, utilizing spatiotemporal 3D environments, have prolonged the maintenance of EC phenotype to up to 4 weeks (57). With deeper insights into the signaling pathways affecting cardiac cell differentiation, novel protocols for EC and SMC differentiation have yielded efficiencies exceeding 80% within 6-day time periods (58). Great efforts have also been devoted to deriving arterial EC from hiPSC, which demonstrated arterial-specific functional characteristics unlike generic ECs, thus offering reliable cell sources for hCMP (59). Cardiac fibroblasts have been successfully differentiated from hPSCs, which resembled native cardiac fibroblasts in morphology, gene expression, and proliferation. Notably, hPSC-derived cardiac fibroblasts could generate three-dimensional (3D) ECM scaffolds and their co-culture with hPSC-CMs could increase action potential propagation rate compared to co-culture with dermal fibroblasts (60). Despite development of refined protocols for generation of vascular cells and fibroblasts of arterial lineage, the optimal combination and proportion of cell types for recapitulating the complex 3D environment of native heart tissue continues to be an active area of research (52, 61–64).
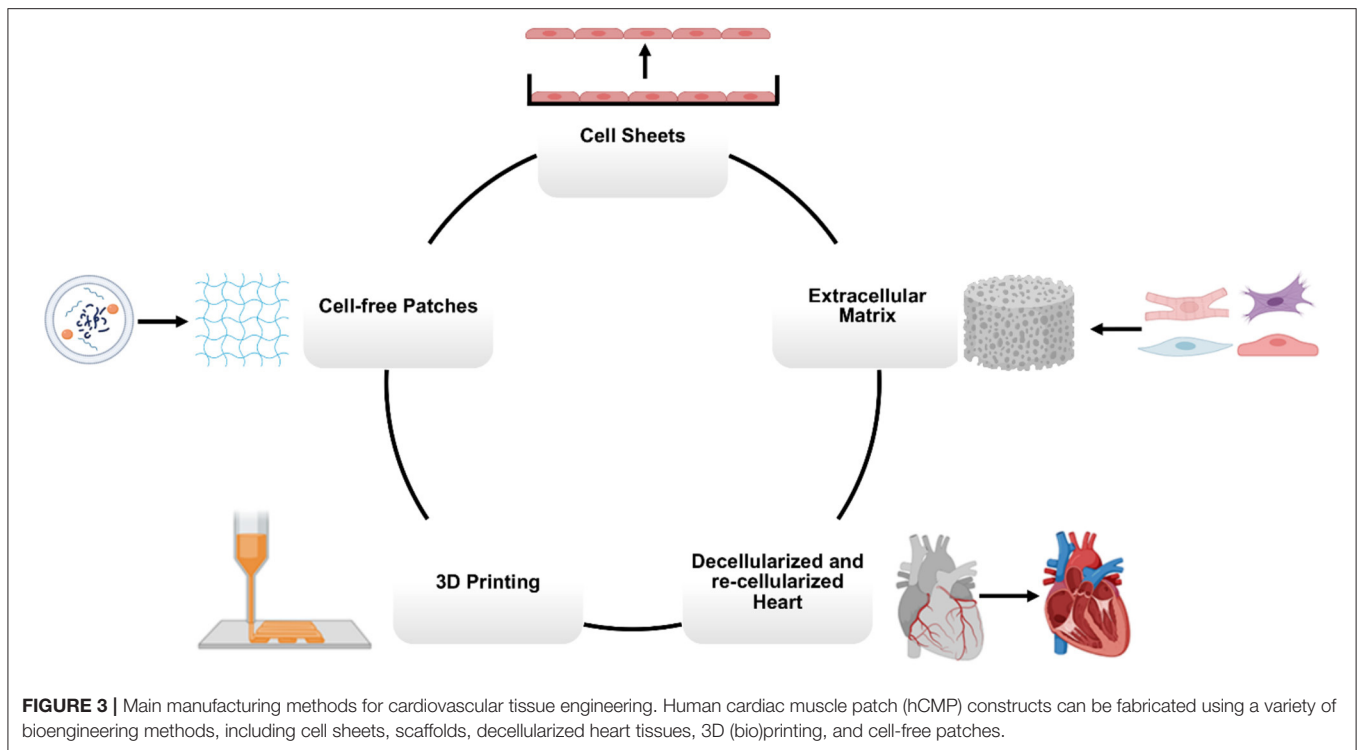
Pluripotent Stem Cells

Pluripotent Stem Cells (PSCs), including ESCs and iPSCs, have revolutionized the field of cardiac tissue engineering by providing a platform of unlimited numbers and types of cells due to their ability to self-renew indefinitely and the plasticity to differentiate into any type of cells (6, 45). ESCs were initially derived from inner cell mass of developing blastocyst (65), thus raising ethical issues due to destruction of fertilized human embryos. ESCs could also be collected via parthenogenesis which alleviates the ethical issues (66). In contrast, iPSCs were initially reprogrammed from somatic cells via overexpression of four transcription factors, Oct4 (octamer-binding transcription factor 4), Sox2 (sex determining region Y-box 2), Klf4 (KLF family of transcription factor 4), and Myc (67). Different combinations of transcription factors, such as Oct4, Sox2, Nanog, and Lin28 (68) or only Oct4 and Sox2 (69) can be also used to generate iPSCs. Despite the controversy on immunogenicity of autologous iPSCs

(70–73), devoid of ethical issues qualifies them as one of the most promising cell sources for cardiac tissue engineering.

PSCs have revolutionized biomedicine by providing robust platforms for regenerative medicine, drug screening, and disease modeling, all requiring reproducible and efficient protocols for differentiating PSCs to CMs. Initially, contractile CMs were spontaneously differentiated from hESCs in 3D embryoid bodies (EBs) with low efficiencies (74–76). These early protocols suffered from line-to-line variability, inclusion of undefined components, and heterogeneous EB sizes (77, 78). To advance the yield and purity of CM differentiation protocols, in-depth knowledge of the pathways involved in embryonic heart developments was utilized to optimize the process. CM differentiation stages typically involve early mesendoderm priming, cardiac progenitor specification, and differentiation into CM subtypes such as ventricular and atrial like phenotypes (79–81). Modulation of TGFβ signaling superfamily via serial application of activin A and bone morphogenetic protein 4 (BMP4) yielded >30% CMs (82); manipulation of Activin/Nodal and BMP4 signaling pathways could efficiently induce cardiac mesoderm, resulting >60% CMs in mouse PSCs and >50% in human PSCs (83). Robust CM differentiation protocols have been developed by mimicking biphasic pattern of WNT pathway with chemical compounds. Temporal modulating Wnt pathway via GSK inhibitor CHIR99021 and Wnt inhibitor IWP2 generated 80–98% CMs (84). Suspension culture in stirred tank bioreactors was utilized to upscale hPSC expansion and lineage differentiation (85, 86). Efforts have been made to develop suspension culture based hPSC-CM production with good manufacturing practice standards (79, 87). Pioneering works of hCMP with PSC-CMs have laid foundation for optimization of manufacturing methods and demonstrated beneficial efficacy after transplantation into animal models with higher engraftment and survival rate than those associated with iPSC-based cell injection (38, 47, 56). Notably, hCMP fabrication typically incorporates PSC-derived cardiac cells with refined protocols to differentiate PSC into ECs, SMCs, and fibroblasts which benefit hCMPs via pre-vascularization and secretion of ECM proteins (50, 52).

Progenitor Cells

The results from early clinical trials suggested that the modest benefits associated with transplanted bone marrow-derived cells and cardiac progenitor cells (CPCs) occurred via the cells' paracrine activity, rather than by directly repairing the damaged heart tissue (30). Nevertheless, bone marrow mesenchymal stem cells (MSCs) have been suspended in 3D hydrogels and tested in a rat model of myocardial infarction (MI) (88). The development of a program for generating human ESC-derived CPCs under Good Manufacturing Practice (GMP) conditions (89) led to the first clinical trial of hESC-CPCs, in which the cells were administered by suspending them in fibrin and suturing the patch to the surface of the infarcted heart. Heart function improved symptomatically and no safety concerns were observed 3 months after transplantation (90). Further research was conducted to investigate the efficacy of ESC-derived cardiovascular progenitors administered in a fibrin patch in six patients with severe ischemic left ventricular dysfunction. Results demonstrated uneventful

FIGURE 3 | Main manufacturing methods for cardiovascular tissue engineering. Human cardiac muscle patch (hCMP) constructs can be fabricated using a variety of bioengineering methods, including cell sheets, scaffolds, decellularized heart tissues, 3D (bio)printing, and cell-free patches.

recoveries with no complications such as tumor formation, arrhythmias or alloimmunization (91). These trials demonstrated feasibility and safety of clinical translation of hESC-derived cardiovascular progenitors, paving the way for further efforts of efficacy studies. Recently, CPCs have shown promise as a highly reproductive cell source in additive cardiac tissue manufacturing, as an alternative to the non-proliferative, mature CMs, to create highly cellularized function hCMPs (92–94).

Spheroids

During the iPSC differentiation process, the cells tend to aggregate into spheroids containing multiple cell types, which can be used for high-throughput screening of heterocellular interactions and drug testing (6, 9). Spheroids could also enhance differentiation and promote maturation, with CMs comprising 80–100% of the cells in microtissues, while refined CM differentiation protocols in monolayer cultures could yield 80–98% CMs (84, 95). Furthermore, spheroids containing proportions of iPSC-derived CMs, ECs, and fibroblasts somewhat recapitulate the morphological and physiological properties of human myocardium, as well as the response to treatment with pharmacological agents, which suggests that spheroids could provide a valuable platform for *in vitro* disease modeling (96, 97). Measurements of engraftment and survival also appear to be greater when cells are administered in the form of spheroids, rather than disaggregated cellular suspensions (98, 99). In another study, engrafted spheroids composed of CPCs expressing the ISL1-LIM-homeodomain transcription factor differentiated into CMs and ECs and contributed to the formation of new blood vessels in infarcted mouse hearts (100). The maturation

of iPSC-CM spheroids can be increased via mechanical and electrical stimulation (101, 102), but measurements of isometric force and electrical conduction are hindered by the lack of a functional architecture (9). The use of cardiac spheroids in advanced biomanufacturing procedures, such as bioprinting, has recently attracted increasing attention, as they can provide improved printed cell viability, fusion, and function to fabricate large-scale cardiovascular constructs (103, 104).

Advanced Cardiac Tissue Manufacturing Strategies

Conventional methods for manufacturing hCMPs include generating contiguous sheets of cardiac cells (mainly CMs) or suspending cells of a variety of types in scaffolds of biocompatible material (94, 105–108). More recently, the emergence of 3D printing technologies, combined with cell-containing "bioinks" and computer-aided design (CAD), has enabled researchers to define the architecture of hCMPs with previously unattainable precision (**Figure 3**).

Cell Sheet Approaches to Fabricate hCMPs

Cell sheets are typically produced by culturing cells on dishes coated with a temperature-sensitive polymer, such as poly(N-isopropyl acrylamide) (PIPAAm), which releases the attached cells when the temperature is reduced from 37 to 32°C, thereby maintaining the ECM and intercellular connections produced during the culture period (109). Since cell sheets lack the exogenous/synthetic scaffold, concerns regarding the potential immunogenicity of the scaffold material are abolished. The sheets can be stacked to generate 3D hCMPs that contract

spontaneously (110). Furthermore, the cells of adjacent layers form connections that facilitate communication between layers, including gap junctions, which are required for electrical coupling (111). Although, constructs composed of more than four cellular layers are typically resistant to vascularization, the vascularity of fabricated cell sheets can be improved by the inclusion of stromal cells or omentum during manufacture (112, 113). Further, CM survival and contractile function were significantly improved when iPSC-CM sheets were covered with an omentum flap after transplantation into infarcted pig hearts (114–116). However, transplanted cell sheets tend to remain electromechanically isolated from the native myocardium, which suggests that the observed benefits likely occur primarily through the activation of paracrine mechanisms (117). Clinical trials utilizing cell sheets of skeletal myoblast demonstrated safety and feasibility with no complications, paving the way for further therapeutic efficacy studies (118, 119). Notably, the Japanese health ministry recently approved the use of iPSC-derived CM sheets in a small study of patients with heart disease, which represents only the second clinical application of iPSC-derived cells (115, 120).

Extracellular Matrix

The biomaterials used in scaffold-based hCMPs (121) are designed to mimic the native cardiac ECM which consists of a highly specialized, 3D network of structural (e.g., collagen) and non-structural (e.g., glycoproteins, proteoglycans, and glycosaminoglycans) proteins that support cardiac function (122, 123). The patch biomaterial is aimed to provide the architecture for cell attachment and cell-cell or cell-matrix interactions (122, 124) and facilitate the coordinated transmission of electrical and mechanical signals (9). Therefore, several design criteria for scaffold biomaterials have been established. *Biocompatibility* of materials is critical to minimize immunogenicity and cytotoxicity, while promoting cell attachment, differentiation and proliferation (30, 125). *Biodegradability* is another important aspect of scaffolding biomaterials, allowing the material to degrade at an appropriate rate while new ECM proteins are being synthesized (106, 126). *Biomechanical and biophysical properties* of materials such as stiffness, elasticity, and porosity enable effective electromechanical coupling of cardiac scaffolds and facilitate mass transport within the constructs (9, 94, 127, 128). Toward that goal, strategies utilizing natural hydrogels (e.g., collagen, fibrin, and Matrigel), synthetic polymers (e.g., polyglycolic acid, polycaprolactone, polylactic acid, and poly-D, L-lactic-co-glycolic acid), or nanofibrous materials as scaffolds have been attempted (9, 129–135). Natural materials have the advantages of promoting cell attachment and viability without cytotoxicity. Nevertheless, physical properties of natural materials could vary from batch to batch and source to source. Immunogenicity is also a concern for xeno-transplantation. Synthetic materials could be designed to precisely control mechanical properties and degradation rate, thus inducing minimal immunogenicity. However, the novel composition of synthetic materials often struggles to fully support cell adhesion and survival. Various scaffold architectures and geometries that have been tested include cylindrical constructs, which typically contain only small numbers of cells, and therefore, are usually scaled to much larger hCMPs at clinically relevant dimensions (2 cm × 4 cm × 1.25 mm) (23). Other structures used are various rodent-sized ventricular organoids (9).

Decellularized Heart Tissues

The role of the ECM in cardiac differentiation, organization, and vascularization is well-established by many studies with decellularized heart tissues (136). The ECM from decellularized human ventricles is shown to induce CM gene expression in human CPCs and MSCs. Further, human umbilical vein ECs formed endocardial and vascular linings in these constructs, and fully differentiated human CMs aggregated into muscular bundles with mature calcium dynamics and electrical coupling (137). Whole rat hearts have been also decellularized, seeded with cardiac and endothelial cells, and perfused in bioreactors, demonstrating macroscopic contractions after only 4 days of culture. By day 8 in culture, the recellularized hearts displayed pumping function in response to electrical stimulation, equivalent to about 2% of adult rat hearts (138). Even in the absence of seeded cells, application of a decellularized porcine myocardial tissue resulted in significant improvements in cardiac functional parameters when evaluated in a rat model of MI, and the patch was vascularized by cells that had migrated from the native tissues (139).

3D Bioprinting—Additive Biomanufacturing

3D bioprinting technologies utilize CAD modeling to guide the assembly of living cells and other biological materials into large-scale tissue and organ analogs with precisely controlled, native-like architectures (92, 140–143). In an early work, tissue printing technology was utilized to generate a construct composed of human cardiac-derived CPCs and alginate. The printed construct remained committed to cardiac lineage with high viability after culturing for 7 days (144). In subsequent studies, human CM progenitor cells were bioprinted into a matrix of hyaluronic acid and gelatin to form a patch with six perpendicularly printed layers and a total surface area of 4 cm^2. When evaluated in a murine MI model, the patch improved measures of cardiac function and prevented remodeling. The expression of cardiac and vascular differentiation markers increased during the 4-week follow-up period (145). Scaffold-free bioprinted hCMPs have been also generated by loading spheroids, one-by-one, onto an array of needles, allowing them to fuse, and then removing the hCMP and culturing it until the needle holes were filled in with surrounding tissue. The construct remained engrafted and displayed evidence of vascularization 1 week after implantation into the infarcted rat hearts (104). A more recent, customized device has been developed that can load an entire layer of spheroids onto the needle array simultaneously, which will substantially reduce the time required to print larger engineered constructs (146).

The resolution of traditional bioprinting techniques cannot accommodate the structural details that facilitate interactions with individual cells. To address this limitation, a more advanced technique, multiphoton-excited (MPE) 3D bioprinting, has emerged, allowing the control of the architecture of

photoactive polymers to resolutions of <1 μm and, consequently, reproducing the structural features of the ECM with high fidelity (147). MPE 3D-printed hCMPs composed of iPSC-derived CMs, ECs, and SMCs in a photoactive gelatin scaffold began generating calcium transients and beating synchronously within 1 day post-manufacturing. The printed patches were associated with significant improvements in cardiac function (left ventricular ejection fraction and fractional shortening), infarct size, apoptosis, vascularity, and cell proliferation when tested in mice with surgically induced MI (62). Photoactivated 3D bioprinting has also been conducted with a bioink containing both ECM proteins and hiPSCs to generate two-chambered structures with both inlet and outlet vessels. The hiPSCs proliferated and differentiated into mature cardiac cells *in situ* to form a living pump with contiguous walls of human cardiac muscle that mimicked the chambers and large vessels of a native heart (148).

Cell-Free Cardiac Patches

Cell- and tissue-based treatments will likely require specialized methods of storage and transportation to maintain cell viability (149). Since most of the benefits associated with transplanted cells and engineered tissues appear to evolve from the paracrine factors produced by the cells, rather than the cells themselves (150), strategies that exploit the regenerative activity of these paracrine factors may be more easily translated to the clinics. The three primary categories of secreted, biologically active cellular products are growth factors (typically proteins that function as signaling molecules), non-coding RNA (short, single-stranded oligonucleotides that regulate gene expression), and extracellular vesicles of endosomal origin (151). Artificial cardiac patches have been manufactured by encapsulating the factors secreted by cardiac stromal cells in PLGA and then embedding the capsules in decellularized porcine myocardium. Applying these factor-laden patches to the infarcted hearts of both immunocompetent rats and pigs resulted in reduced scarring and improved cardiac function, without inducing an immune response (152). In other works, the controlled release of miRNAs from injected hydrogels has shown improvement in cardiac function in infarcted mouse hearts (153). Further, hydrogel patches containing the extracellular vesicles produced by human iPSC-CMs reduced infarct size and cardiac hypertrophy in a rat MI model (154). Collectively, these results suggest that the controlled release of paracrine factors from cell-free patches or injected biomaterials may be a feasible alternative approach to transplanted hCMPs for the treatment of myocardial disease.

Increasing hCMP Thickness/Dimensions

Despite substantial improvements in the components and protocols used for hCMP manufacturing, few studies have been conducted with constructs of clinically relevant size. Even patches with comparatively large surface areas (e.g., 8 cm^2) are relatively thin (1.25 mm) (23), with inability of direct perfusion limiting thickness of hCMP to 1–2 mm (155, 156). Thus, production methods continue to be optimized and enhanced for the development of larger and thicker hCMP constructs. The inability to engineer and maintain thick and
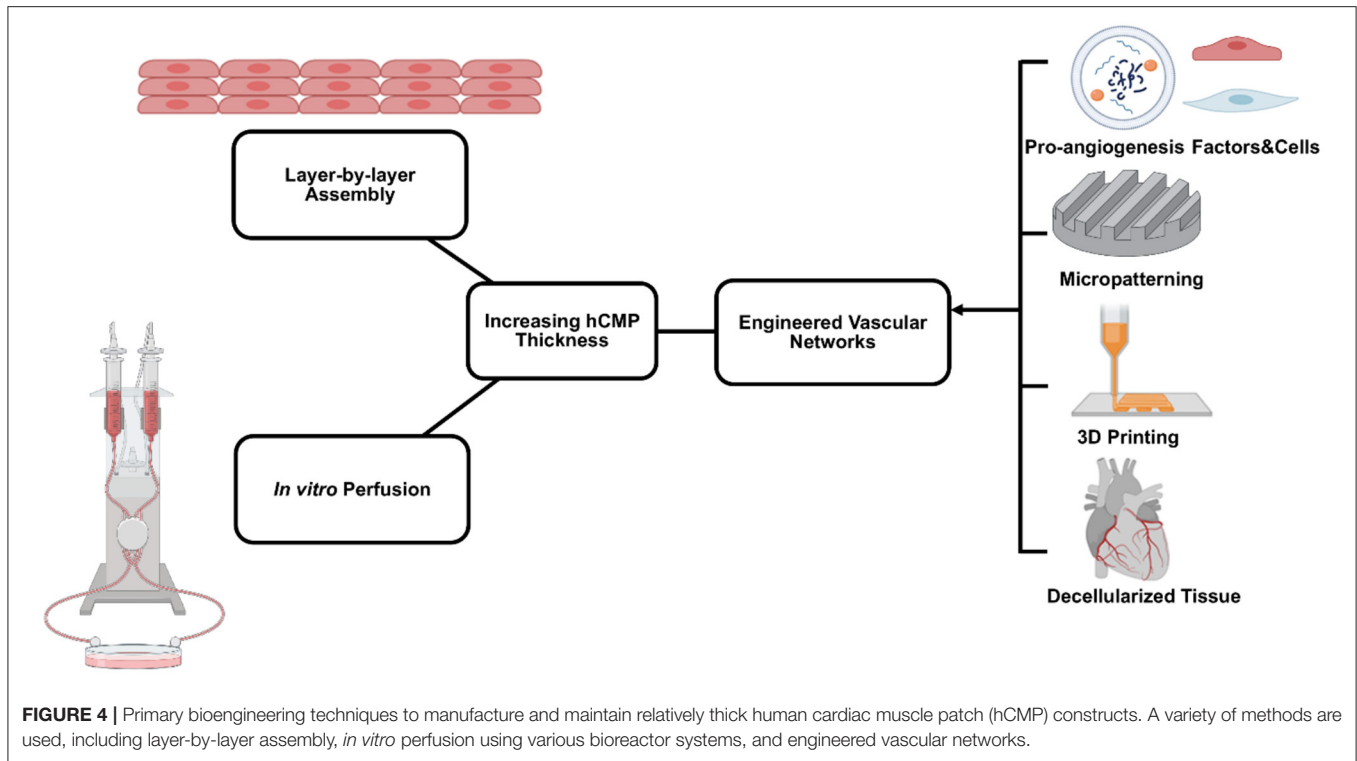
viable hCMP jeopardizes the clinical applications due to their inability to mimic characteristics of native myocardium, such as generating adult-like forces and action potentials (157, 158). In practice, hCMP thickness is often limited by the diffusion of oxygen and nutrients from the vasculature after transplantation, which requires CMs to be within 100–200 μm distance from the capillaries (159). Diffusion can be facilitated by including molecular crystals (e.g., sucrose) in the matrix solution and leaching them out after the matrix solidifies to increase the porosity of the scaffold (160). However, hCMPs of clinically relevant thicknesses require formation of a dense internal vascular network that couples with the native circulation after transplantation (9, 157). Vascularization can be increased by including combinations of vascular and other cell types (ECs, SMCs, and/or fibroblasts) (23, 62, 161) during manufacturing and/or via the nanoparticle-mediated (151) extended release of pro-angiogenic factors [e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and the Wnt activator CHIR99021; (162, 163)], which can promote infiltration of the native circulatory system. Further, the spatial orientation of the vascular network can be controlled with more technologically advanced fabrication methods (e.g., micropatterning and 3D bioprinting) to enhance the mass transport and perfusion [(164); **Figure 4**].

Layer-By-Layer Assembly

In theory, the layer-by-layer assembly technique enables hCMPs of any desired thickness to be generated simply by stacking the required number of individual CM sheets. However, since the individual layers of multilayered hCMPs are grown in isolation before assembly, intercellular connectivity is likely to be greater between cells within the same layer than between the cells of adjacent layers. Techniques for enhancing the formation of physical and electromechanical connections between layers include the use of graphene oxide (GO)-based thin films, which improved not only adhesion but also electrical coupling, maturation and cell organization (165), as well as the Tissue-Velcro platform, in which cardiac cells are cultured on 2D cell meshes that incorporate a microfabricated hook-and-loop system. After layering, the hooks and loops of adjacent layers interlocked and the hCMP contracted in response to electrical stimulation (166). The vascularization of multilayered hCMPs can be promoted by sandwiching vascular cells between the CM layers (167, 168). Coherent vascular networks have been generated in stacked hCMPs by using resected tissue as a vascular bed. When the resected vasculature was overlaid with sheets of cardiac cells (including ECs), connected to a bioreactor, and perfused with culture medium, ECs in the cardiac-cell layers connected to capillaries in the vascular bed and formed tubular structures. The vascularized hCMPs could survive after transplantation into the necks of rats via blood vessel anastomosis (169).

In vitro Perfusion

Techniques for maintaining adequate oxygen and nutrient availability during the manufacturing process include the

FIGURE 4 | Primary bioengineering techniques to manufacture and maintain relatively thick human cardiac muscle patch (hCMP) constructs. A variety of methods are used, including layer-by-layer assembly, *in vitro* perfusion using various bioreactor systems, and engineered vascular networks.

development of cartridges that reproduce the convective-diffusive properties of oxygen transportation present in native myocardial tissue (170). Another approach is culturing cells on a simulated capillary network with medium containing an oxygen carrier (perfluorocarbon) that mimics hemoglobin (171, 172). The hemoglobin mimic significantly increased the expression of cardiac markers and improved the contractile performance of constructs composed of primary neonatal CMs and fibroblasts. Small intestinal submucosa could be utilized to enhance perfusion with rat primary endothelial cells forming a network in pre-existing vessel structures (173). AngioChip technology (174) incorporates a perfusable, 3D microchannel network that recapitulates the native vascular interface and, because it is compatible with current practices in both laboratories and industry, can be adapted to produce a variety of tissue types. Perfusion can also be improved by culturing cardiac tissue constructs under dynamic conditions [e.g., on a rocking platform; (158)]. This approach was used during the manufacture of large (2 cm × 4 cm) patches for subsequent testing in a swine MI model and was associated with improvements in not only cell viability, but also in measures of hCMP maturation (23). Notably, some evidence suggests that the contractile properties of hCMPs can be enhanced by perfusing them in rhythmic pulses, rather than a continuous static flow (175).

Engineered Vascular Networks

Rather than relying solely on infiltration from the native circulatory system, thicker hCMPs will likely require at least some amount of engineered vascularity before transplantation.

Vascularization can be induced during the manufacturing process by encapsulating a sacrificial gelatin mesh in scaffold material and then melting the gelatin mesh away, leaving behind a network of interconnected microfluidic channels. When seeded with human microvascular ECs, the sacrificial scaffolds produced a rudimentary endothelial network (176). An alternative strategy mimics the endogenous angiogenic process by using a sustained-release preparation of the angiogenic factor thymosin β to promote and guide the outgrowth of vessels from explanted arteries and veins to form a capillary bed within a hydrogel scaffold (177). Vessel growth can also be directed with micropatterned polyglycerol sebacate scaffolds. After transplantation, host blood cells infiltrated into the microvessels as the scaffold degraded (178). Micropatterning has also been used to organize ECs into "cords," which guide the formation of capillaries that integrate with the host tissue after transplantation (179).

One of the novel reports of 3D printed vessels used a thermal inkjet printer to print mixtures of human microvascular ECs and fibrin, forming micro-sized fibrin channels lined with confluent cells (180). Vascular networks have also been bioprinted with an advanced extrusion system that produced a sheath of photoactive, cell-laden bioink around an alginate core. After UV crosslinking, the alginate was dissolved with a Ca^{2+}-chelating agent, allowing the cells to proliferate, spread, and form a perfusable biomimetic vascular network (140). However, the penetration of UV radiation is limited. Polymerization can be induced at greater depths via enzymatic reactions, such as the thrombin-induced cleavage of fibrinogen

into fibrin. This strategy has been combined with the co-printing of vascular and cellular inks in cast ECM material to generate markedly thick (>1 cm) engineered osteogenic tissues (181).

Transplantation of hCMP Constructs

hCMP Delivery Methods

Delivery methods for cells and tissues could be categorized into invasive and non-invasive approaches, both often featuring low engraftment rates (12). Transplantation of hCMPs mainly falls into invasive delivery, requiring open-chest surgery, and suturing/attaching the patch onto the epicardium (23, 62). This method typically offers an enhanced engraftment and survival rate compared with cell injection into the injured myocardium. The widespread application of intra-myocardial cell injections has demonstrated a poor cell retention rate with induced damages to both exogenous and host cells due to a dramatic increase in flow velocity and shear stress (182). Studies have shown that repeated cell injections increase the therapeutic effects (183). However, invasive delivery methods are not optimal for repeated applications due to requirements of dedicated facilities and highly trained staff to perform open-chest surgery. As a result, invasive delivery of cells and hCMPs has challenged clinical translation of these therapies (184).

Alternatively, non-invasive delivery methods, such as intravascular (or intravenous) delivery and injectable hydrogel approaches, have been tested. Intravascular delivery has shown recirculation and redistribution of injected cells to other organs besides the target site (6). These methods have demonstrated improved cardiac function potentially due to the paracrine signaling mechanism as previously discussed (185). This method has the advantages of being non-invasive and possibility of repeated administrations, qualifying them a good choice from a clinical viewpoint (184). Recently, a novel minimally invasive method demonstrated epicardial delivery of hydrogels through the pericardial space (186). A pericardial device (catheter) delivers the hydrogel components through separate (coaxial) lumens and combines them after exiting the device, forming a stable hydrogel construct between the pericardium and epicardium layers. This method could help minimize the risks of extensive myocardial injury, thrombotic occlusion, and arrhythmia. Injectable hydrogels have been also increasingly tested as a minimally (or non) invasive delivery approach for cardiac patch systems (187, 188). In a clinical study, acellular alginate-based hydrogels were injected in MI patients, demonstrating the preservation of the LV indices and ejection fraction (189). However, a more recent large clinical trial, investigating the effect of injectable alginate hydrogels in patients with advanced HF, reported about 9% death within 30 days post-injection, while the control group had no fatalities (190). Therefore, while significant progress has been made in developing injectable cardiac patch systems, more efforts are required for their further improvement for efficient clinical use. These include further enhancement in biomechanical and biochemical properties of the hydrogel constructs to mimic the native tissue, improving cell viability

and biomolecules activity, controlled degradation and immune response, and enhancement in the *in vivo* tracking of the patch (187, 191, 192).

Animal Models to Test hCMPs

Preclinical studies have utilized different animal models, including mice, rats, guinea pigs, swine, and non-human primates (**Table 1**). Choice of a suitable animal model with high predictive validity for safety profile and therapeutic outcomes is crucial for the clinical translational purposes. Initial studies of therapeutic efficacy of transplanted human cells or cardiac tissues were performed in immunocompromised rodents, including athymic rats and severe combined immunodeficiency mice, due to genetic manipulations and easy handling. However, the disparity of anatomy and physiology between rodents and human dampens the reliability of therapeutic outcomes (197). Subsequent preclinical studies utilize large animal models, including non-human primates and swine, demonstrating remuscularization and therapeutic efficacy with safety concerns of ventricular arrhythmias (42, 196, 198). Large animal models are more reliable and relevant for preclinical studies compared with rodent models. However, the high cost associated with large animal studies would limit their applications (41).

Mechanisms of Action of hCMPs

The ultimate goal of cardiac patch transplantation is to replace the injured myocardium with exogenously functional cardiac muscle. Preclinical studies from mice (199, 200), rats (201), guinea pigs (52, 193), porcine (50), and non-human primates (41) have demonstrated some degree of myocardial remuscularization of the fibrotic scar tissue with transplanted cardiac tissues. The clinical translation of this approach has met some challenges, first of which is the requirement of large quantities of exogenous CMs (and/or other cardiac cells) used to replenish lost tissue. Robust protocols for CM differentiation and scalability of CM production, together with cryopreservation and retrieval procedures following current good manufacturing practice (cGMP), have enabled generation of well-characterized PSC-CMs as an off-the-shelf therapy (202). Furthermore, it remains a critical issue to ensure long-term graft retention for maximal therapeutic efficacy based on the hypothesis that contractile force is correlated to the electromechanically integrated CMs (202). Gene manipulation has been adopted to increase cell retention. Overexpression of CCND2 (cyclin D2), a cell cycle activator, increased cell cycle activity and proliferation rate in hiPSC-CMs, thus improving engraftment rate from average 10 to 25% with a significant remuscularization of injured myocardium in mice (203). CM retention could also be enhanced via co-administration of pro-survival factors, such as Matrigel, cyclosporine A, pinacidil, ZVAD-fmk, insulin-like growth factor-1 (IGF-1), CHIR99021, and fibroblast growth factor 1(FGF1) (50, 82, 163).

The lack of electromechanical coupling between hCMPs and host tissue is another challenge on the way of clinical translation of patch-based therapies. Super-aligned carbon nanotubes were utilized during the fabrication of cardiac tissues to enhance electrophysiological homogeneity due to the

TABLE 1 | Summary of various hCMP studies conducted in animal models of heart injuries.

Animal model	Cell type and number	Delivery method/Material	Vascularization	Observations	Follow up
NOD/SCID gamma mice with MI (101)	Five Spheroids composed of hiPSC-CMs (2×10^5/spheroid)	Engineered fibrin cardiac patch	N/A	Improved LVEF and FS; enhanced engraftment	4 weeks
NOD/SCID gamma mice with MI (163)	1×10^6 hiPSC-CM	Engineered fibrin cardiac patch containing nanoparticles releasing CHIR99021 and FGF1	N/A	Enhanced cardiac function; reduced infarct size; increased engraftment with enhanced cell cycle activity of hiPSC-CMs	4 weeks
Guinea pigs with cryoinjury (193)	1×10^8 hESC-CMs	IM	N/A	Improved mechanical function; reduced incidence of arrhythmias	4 weeks
Guinea pigs with MI (52)	5×10^6 hiPSC-CM and 2×10^6 hiPSC-ECs	Engineered fibrin cardiac patch	Inclusion of iPSC-ECs	Improve left ventricular function by 31%; remuscularization; vascularization; electrical coupling with host myocardium	28 days
Rats with MI (35)	4×10^7 Fetal rat ventricular muscle	Engineered gelatin cardiac patch	N/A	No significant change in cardiac function; surviving grafts enhanced angiogenesis	5 weeks
Rats with MI (36)	3×10^5 Fatal rat CMs	Engineered alginate cardiac patch	N/A	No significant change; Engraftment with intensive neovascularization; integration; attenuate LV dilatation	9 weeks
Normal athymic rats Sprague Dawley (56)	ESC-CMs and iPSC-CMs; HUVEC; MSCs; MEFs	Engineered collagen cardiac patch	Inclusion of stromal supporting cells	Form grafts containing microvessels; Enhanced formation of vessel-like structures	1 week
Rats with MI (88)	1×10^6 Bone marrow mesenchymal stem cells	Engineered silk fibroin/hyaluronic acid cardiac patch	N/A	Therapeutic efficacy; improved LV wall thickness; high viability; neovascularization	8 weeks
Nude athymic rats with MI (39, 194)	2.5×10^6 hESC-CMs	Engineered collagen cardiac patch	N/A	No significant changes of LVEF; Preserved heart function revealed by tagged magnetic resonance imaging; high engraftment	4 weeks
Athymic rats with MI (40)	2.2×10^6 iPSC-CMs; 3.4×10^5 human pericytes	Engineered fibrin cardiac patch	Inclusion of human pericytes	Improved cardiac function; reduced infarct size; engraft onto host heart	4 weeks
Rats with acute MI (139)	Acellular	Decellularized porcine myocardial slice	Utilization of decellularized porcine myocardium mimicking native ECM	Improved cardiac function; attach to host myocardium; prevent LV wall thinning; vascularization	4 weeks
Rats and porcine with MI (152)	Acellular; synthetic cardiac stromal cells	Cardiac patch composed of a decellularized porcine myocardium		Enhanced cardiac recovery with reduced scar and promoted angiogenesis in rat model; therapeutic efficacy in porcine model	3 weeks
Porcine with MI (113)	1.5×10^7 Skeletal myoblasts from mini-pigs	Cell sheets covered with omentum flap	Utilization of omentum flap for revasularization	Improved cardiac function; Reduced infarct size; increased angiogenesis	8 weeks
Porcine minipigs with MI (114)	iPSC-CMs	Cell sheets	N/A	Improved cardiac performance and attenuated left ventricular remodeling; poor engraftment	8 weeks
Normal porcine mini-pigs (115)	iPSC-CMs	Cell sheets with an omentum flap	Inclusion of an omentum flap	Enhanced survival and engraftment; rich vasculature	8 weeks
Immunosuppressed Yorkshire pigs with MI (51)	4×10^6 hiPSC-VC (ECs and SMCs)	Engineered fibrin cardiac patch	Inclusion of iPSC-VCs (ECs and SMCs)	Increased BZ contractile function and ATP turnover rate with attenuated regional wall stress, neovascularization and improved BZ perfusion	4 weeks

(Continued)

TABLE 1 | Continued

Animal model	Cell type and number	Delivery method/Material	Vascularization	Observations	Follow up
Porcine with acute MI (50)	6×10^6 total iPSC-cardiovascular cells (CMs, ECs, and SMCs; each 2×10^6)	IM + fibrin patch containing IGF	Inclusion of iPSC-ECs, SMCs	Improved left ventricular function; reduced infarct size; Integration with host myocardium; vascularization;	4 weeks
Porcine with ICM (116)	5×10^6 iPSC-CMs	Cell sheets with omental flap	Utilization of omentum flap	Enhanced therapeutic effects and survival;	3 months
Porcine with MI (23)	iPSC-Cardiac cells (4×10^6 CMs, 2×10^6 ECs, and 2×10^6 SMCs)	Engineered fibrin cardiac patch	Inclusion of iPSC-ECs, -SMCs	Improved left ventricular function; reduced infarct size; reduced LV wall stress; no significant changes in arrhythmogenicity	4 weeks
Porcine with MI (195)	Acellular; exosomes derived from iPSC-Cardiac cells (iPSC-CMs, -ECs, -SMCs)	IM	N/A	Improve myocardial recovery without increasing the frequency of arrhythmogenic	4 weeks
Non-human primate *Macaca nemestrina* with MI (41)	1×10^9 (1 billion) hESC-CMs	IM	N/A	Remuscularization; vascularization; electromechanical coupling; arrhythmias	3 months
Non-human primate *Macaca fascicularis* with MI (196)	1×10^9 iPSC-CMs	IM	N/A	Improved cardiac function; Survive for 12 weeks; electrical coupling; ventricular tachycardia (transient)	12 weeks
Non-human primate macaque monkeys with MI (42)	~750 million hESC-CMs	IM	N/A	Improved cardiac function; formation of electromechanical junctions; ventricular arrhythmias	3 months
One patient with severe heart failure (90)	4×10^6 hESC-derived Isl-1$^+$ SSEA-1$^+$ cells	Engineered fibrin cardiac patch	N/A	Symptomatically improved LVEF; new-onset contractility; no complications	3 months
Seven patients with ischemic cardiomyopathy (UMIN000008013) (118)	3×10^8 autologous skeletal muscle	Cell sheet	N/A	Improved LVEF; improved patient status	26 weeks
Six patients with severe ischemic left ventricular dysfunction (NCT02057900) (91)	8.2×10^6 hESC-derived cardiovascular progenitors	Engineered fibrin cardiac patch	N/A	Uneventful recoveries; no safety concerns, such as tumor, arrhythmias, alloimmunization	1 year
Four patients with dilated cardiomyopathy (UMIN000000660) (119)	$4.5–7.5 \times 10^8$ autologous skeletal muscle	Cell sheet	N/A	Improved LVEF in two patients; reduced cardiac hypertrophy	3 months

FGF1, fibroblast growth factor 1; LVEF, left ventricular ejection fraction; FS, fractional shortening; IM, intra-myocardial delivery; HUVECs, human umbilical vein endothelial cells; MSCs, human marrow stromal cells; MEFs, mouse embryonic fibroblasts; IGF, insulin growth factor; ICM, ischemic cardiomyopathy.

anisotropic conductivity of their aligned structure (204). In another study, electrospun nanofibrous scaffolds with enhanced conductivity were demonstrated to promote electrical coupling of the patch, showing potential for fabrication of clinically relevant hCMP products (205). One major aspect that must be carefully studied, when working on electromechanical integration of exogenous CMs with host myocardium is the likely potential to trigger arrhythmias, as discussed in the previous sections (41).

Low CM survival rates, as little as 10%, have been usually associated with functional benefits from hCMP transplantations (39, 51, 194). Patch constructs composed of non-CMs have also shown improved cardiac function (51). These data collectively suggest an alternative mechanism of action, based on paracrine signaling, where cell secreted signals, including extracellular vesicles, mediate cardiac repair via increasing CM survival and neovascularization and decreasing apoptosis and inflammation (206). Paracrine signaling was further evidenced by a study where exosomes secreted from combination of iPSC-derived CMs, ECs, and SMCs yielded cardioprotective effects similar to that obtained from direct cells injection in a porcine model of MI (195). Further, acellular hCMPs composed of non-viable/irradiated cells (39) or decellularized porcine myocardial tissues (139) have shown therapeutic benefits after transplantation, through the proposed mechanism of mechanical stabilization. Thus, the therapeutic benefits associated with hCMP transplantation could be a combination of any or all of mechanisms including remuscularization, paracrine signaling, and mechanical stabilization (202, 207).

Safety Concerns

Occurrence of arrhythmias is a critical safety concern for clinical applicability of cardiac patch therapies. Sine large animal models more closely approximate the human heart's physiology, function, and anatomy, they have been increasingly used to assess arrhythmias in cardiac patch studies (41). Preclinical studies of non-human primates demonstrated transient non-fatal arrhythmias during 2 weeks after transplantation, followed by a subsequent decrease in those irregularities (41, 196). Ventricular arrhythmias occurred in porcine models have been more frequent and lethal than those in non-human primate models (2 out of every 7 pigs) (198). It is currently viewed that disorder of impulse generation at the interface between engrafted CMs and host myocardium could induce ventricular arrhythmias (42, 198). Further investigation of arrhythmic complications will be necessary before translation into patients.

Autologous cell transplantation offers an advantageous option based on the hypothesis that autologous cell transplantation could induce no immunological response due to the acceptable human leukocyte antigens (HLA) match. However, accumulating evidence prove that the clinical translation of autologous cells could become complicated by limitations, such as variability from batch to batch, requiring a detailed characterization and quality control of each batch. The initial hypothesis that

transplantation of autologous iPSC derivatives would induce no immunogenicity has been challenged by some recent studies (70, 71).

For a long time in clinical practice, inhibition of the immunological responses has been mainly through the use of immunosuppressive drugs, such as cyclosporine, dexamethasone, and FK-506. With side effects induced by such immunosuppressants, there is a trade-off between efficacy and toxicity. High-dose immunosuppression would result in toxicity and associated complications, while low-dose immunosuppression would lead to allograft rejection. The choice of transient or chronic exposure of immunosuppressants would be based on the presumed mechanism of action, as discussed previously (184, 208). In addition, immunological tolerance could be induced via host conditioning by activation/adoptive transfer of regulatory T cells (209). Short-term administration of nanobiologics targeting macrophages could benefit long-term allograft survival (210). Other strategies have been adopted to reduce immunogenicity. Modern genome-editing tools [e.g., zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-9 system; (37, 211, 212)] have enabled the development of minimally immunogenic iPSC lines by knocking out key components of major histocompatibility complexes (MHCs) I and II [Beta2 microglobulin (B2M) and MHC II transactivator (CIITA)] (213). This is done by combining MHC gene inactivation with the overexpression of CD47, a ubiquitous membrane protein that directly regulates T-cell immunity (214), and by disrupting the genes for HLA (215). These genetically modified hiPSCs could be used to generate a stockpile of "off-the-shelf" cardiac cells and hCMPs for administration to patients in emergency situations without the need for concomitant immunosuppressive therapies.

Summary—Current Challenges and Future Perspectives

The potential benefits of hCMPs for the treatment of myocardial injury and disease are readily observable in preclinical studies, and at least one small study in patients is currently underway (120). Conventional methods for manufacturing hCMPs include suspending cells in scaffolds of biocompatible material or growing 2D sheets in culture and stacking them to form multilayered constructs. More advanced technologies, such as micropatterning and CAD-guided 3D printing with bioinks have given researchers the tools to control the architecture of hCMPs at resolutions that match the scale of interactions between individual cells or between cells and the ECM. Most studies, however, have been conducted with hCMPs at relatively small scales that are not suitable for administration to patients. The size and (especially) thickness of hCMPs are often restricted by the diffusion limitations of oxygen, nutrients, and other biologically active molecules, which requires CMs to be within 100–200 μm distance from the capillaries. Thus, the clinical translation of hCMP technology will require the development of protocols for manufacturing larger and thicker constructs that are adequately vascularized and fully couple

with the circulatory and electromechanical systems of the native myocardium.

All authors contributed to the article and approved the submitted version.

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