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Proteomic-based approaches to cardiac development and disease

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Congenital malformations, or structural birth defects, are now the leading cause of infant mortality in the United States and Europe (Dolk *et al*., 2010; Heron *et al*., 2009). Of the congenital malformations, congenital heart disease (CHD) is the most common (Dolk *et al*., 2010; Heron *et al*., 2009). Thus, a molecular understanding of heart development is an essential goal for improving clinical approaches to CHD. However, CHDs are commonly a result of genetic defects that manifest themselves in a spatial and temporal manner during the early stages of embryogenesis, leaving them mostly intractable to mass spectrometry-based analysis. Here, we describe the technologies and advancements in the field of mass spectrometry over the past few years that have begun to provide insights into the molecular and cellular basis of CHD and prospects for these types of approaches in the future.

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Introduction

Congenital heart disease (CHD) is the leading cause of infant mortality in the United States and Europe [1,2]. Since the initiating event in the majority of CHDs takes place during early development of the human heart, studies to understand CHD etiology have relied on vertebrate model systems, most notably the mouse [3–10]. Much effort has, therefore, gone into identifying the genes and networks required for these stages of normal mouse development at the RNA level [11–19]. However, only 10% of RNAs that show twofold or more changes in levels of expression are associated with alterations in protein abundance. Conversely, changes in protein levels are often not associated with changes in RNA levels [20,21,22••,23]. It is, therefore, evident that research into the molecular roots of CHD should involve direct assessments of protein expression levels and interaction networks.

Tandem mass spectrometry (MS/MS)-based analysis has proven invaluable in studying the temporal and spatial distribution of proteins during development in a range of animal model systems [19,24•,25–29]. This type of proteomic-based approach not only allows for the generation of a compendium of proteins of a given cell type at a given stage of development, but also provides information for the characterization of post-translational modifications (PTMs). Hence, such a type of approaches may also identify the growth factor signaling pathways that control a protein's function. By using multiple or parallel reaction monitoring, it is further possible to determine the precise amount of a protein in a given cell or tissue type [30,31]. Despite these powerful advantages, proteomic-based approaches still face limitations, most notably the amount of material that is required for an in-depth analysis from small tissues that express modest levels of a given protein relative for example, to tissue culture cells. Thus, the field of cardiac biology has been largely limited to analysis of serum and plasma biomarkers (e.g. Refs. [32–34]).

To circumvent this issue, technologies, procedures, and workflows have been developed to increase the efficiency of protein recovery from murine tissue and cell types, as well as the application of proteomics to surrogate systems which may in principle mimic human cardiac differentiation. These systems include cardiomyocyte differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), as well as direct reprogramming of differentiated cells (e.g. cardiac fibroblasts) into cardiomyocytes.

Proteomic-based approaches in embryonic heart tissue

The identification and characterization of endogenous proteins and protein complexes *in vivo* under physiological conditions are essential to gain a basic understanding of normal cardiac development and the pathology of CHD [19]. However, use of these approaches has been limited due to lack of optimized mass spectrometry-based protocols and workflows for the analysis of such small samples in early stage tissues and embryos. To identify endogenous interactomes utilizing targeted MS, approaches have focused on optimization of protein extraction buffers and cell/tissue lysis conditions, as well as increasing the efficiency of immuno-isolation. One approach has been to tag the endogenous protein through

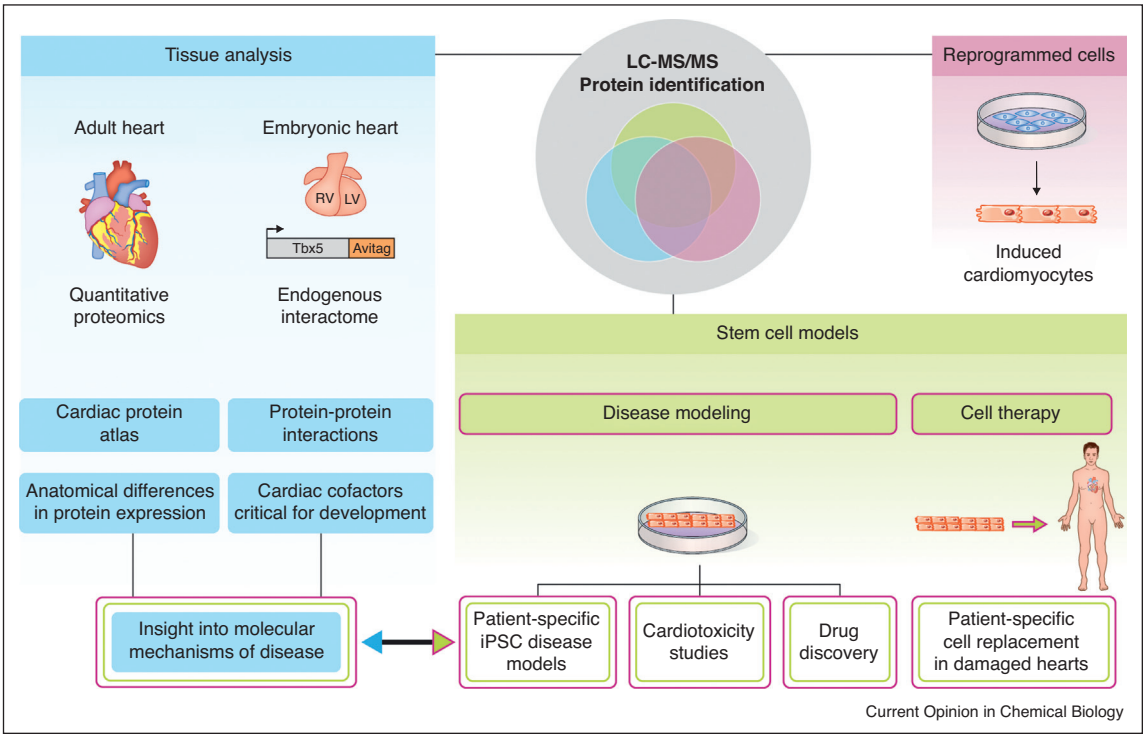
homologous recombination with the Avi-tag [35,36,37••]. The Avi-tag is an artificial epitope tag that combines the minimal invasiveness of a small peptide tag with the specificity and strength of the biotin–streptavidin attachment, the strongest non-covalent peptide–ligand interaction in nature, exceeding any antibody–antigen interaction [38–41]. Therefore, the approach offers high-affinity and high-specificity isolation of the targeted protein (Figure 1). This type of approach has proven highly effective in the isolation of the transcription factor TBX5, mutations in which cause Holt–Oram syndrome [42–45]. This study demonstrated that TBX5 interacts biochemically and genetically with the nucleosome remodeling and deacetylase (NuRD) transcriptional repressor complex, thus defining a TBX5–NuRD interaction essential to cardiac development [37••].

Differentiation of embryonic stem cells into cardiomyocytes

In vivo developmental biology systems have identified some of the molecular mechanisms that underlie CHD. However, the utility of these systems for proteomic-based analysis is limited by the amount of tissue one can obtain from a given species at a given developmental stage. Furthermore, because of the species-specific differences in various aspects of cardiac biology and development, findings from many systems cannot be applied directly to human biology. This is notably relevant to those incidences of cardiac-related deaths attributed to the limited ability of the damaged heart tissue to regenerate [46–48]. The production of *de novo* cardiomyocytes, either by human ESCs or human iPSCs, or direct reprogramming, holds the potential for cardiac repair, novel cardiac drug discovery, identification of drug response predictors, and elucidation of the molecular mechanisms during development that underlie cardiac diseases [49–51].

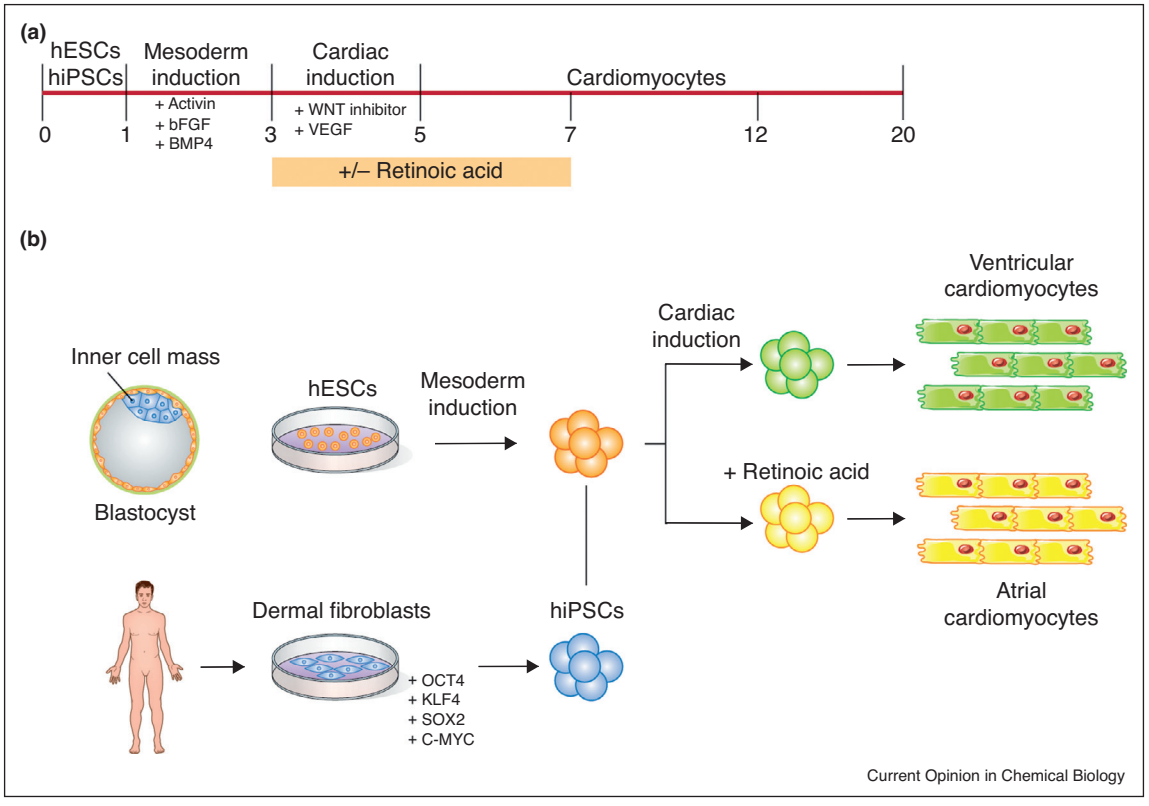
The first of these technologies pursued was the identification and propagation of ESCs, first in the mouse and later in human. ESCs, derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency [52–54]. Reports on the *in vitro* differentiation of mouse ESCs into cardiac progenitors demonstrated that these cells can give rise to multiple cardiac cell lineages [55–58]. Subsequent studies showed effective differentiation of human ESCs into the cardiac lineages (Figure 2) [59–62]. By combining these types of approaches with the Avi-tag/BirA system, it was

Figure 1



Schematic of proteomic-based approaches in cardiac tissue and cells.
Mass spectrometry-based approaches in cardiac tissue have led to the generation of a global protein atlas of the healthy human heart. The isolation of endogenous interactomes utilizing gene recombination in the mouse has identified cardiac cofactors that are critical for embryonic development. Cell-based systems that produce *de novo* cardiomyocytes have multiple applications to treating human disease including engraftment into the heart, drug discovery, and cardiotoxicity studies. All of these approaches can provide insight into the molecular mechanism of disease.

Figure 2



Pluripotent stem cells differentiated into cardiomyocytes.
(a) Cardiomyocyte production timeline. **(b)** Embryonic stem cells harvested from the inner cell mass of a blastocyst are induced into the mesodermal and cardiac lineages with the treatment of various growth factors. Fibroblasts harvested from the dermis of a human patient are reprogrammed into pluripotent stem cells with the addition of 4 defined factors: OCT4, KLF4, SOX2, and C-MYC. These cells are then induced into the mesodermal and cardiac lineages. Production of atrial-specific cardiomyocytes requires treatment with Retionic Acid at defined stages of differentiation while cultures without Retionic Acid differentiate into ventricular cardiomyocytes.

possible to use targeted MS to isolate the temporal changes in the TBX20 interactome [63,64••]. Loss of function mutations in TBX20 can cause dilated cardiomyopathy, atrial septal defects, or mitral valve disease, while gain of function mutations of TBX20 have been reported in patients with Tetralogy of Fallot (i.e. pulmonary outflow tract obstruction, ventricular septal defects, overriding aortic root, and right ventricular hypertrophy) [65–69]. Analysis of TBX20 during ESC cardiomyocyte differentiation showed a temporal regulation of the TBX20 interactome and led to the identification of CASZ1 [70,71] as a TBX20 interacting protein, a cardiac interaction that when disrupted leads to dilated cardiomyopathy [63,64••].

In addition to providing an *in vitro* system for analyzing temporal protein regulation, ESC differentiation led to the expectation that human ESCs may provide an understanding of cardiac disease mechanisms and, therefore, lead to effective therapeutic treatments for patients. A recent study utilizing mouse and human ESCs combined with a shot gun liquid chromatography LC–MS/MS approach identified 246 cell surface markers during key stages of mesoderm specification and early cardiac development. This led to the identification of FZD4 as a marker of lateral plate mesoderm, further enhancing cardiomyocyte enrichment [72]. However, the use of human embryos faces many ethical controversies that hinder the application of human ESCs. In addition, it is difficult to generate patient or disease-specific ESCs, which would greatly aid in their effective application. Therefore, new technologies were pursued that could meet all of these goals and greatly minimize the ethical implications.

Differentiation of induced pluripotent stem cells into cardiomyocytes

iPSC technology reprograms a fully differentiated somatic cell (usually taken from dermal fibroblasts) into a pluripotent stem cell that retains all of the genetic

characteristics of its host (e.g. a human patient). iPSC generation requires transduction with four defined transcription factors: Oct3/4, Sox2, Klf4, and c-Myc [73,74]. iPSCs can then be differentiated into functional cardiomyocytes (cardiac troponin T-positive cells) utilizing embryonic growth factor signals that induce mesoderm and subsequent cardiac specification [75]. Until recently, most of the human iPSC-to-cardiomyocyte studies have produced mixed cardiovascular populations that contain ventricle-like cells together with pacemaker and atrial-like cells [76–78]. Atrial and ventricular cardiomyocytes derive from different mesoderm populations and consequently exhibit distinct molecular and functional profiles essential for their diverse physiological roles in the heart (Figure 2) [79••,80].

To effectively model and treat diseases that affect specific regions of the heart (such as atrial fibrillation), it is essential to develop differentiation strategies that promote the generation of each of the cardiomyocyte subtypes [80]. Importantly, Lee *et al.* [80] showed in cardiac-differentiated human iPSCs that retinoic acid signaling at the mesoderm stage of development is required for atrial specification. This work was expanded upon by Cyganek *et al.* [79••] to quantitatively analyze the proteomes of atrial versus ventricular human iPSC-derived cardiomyocytes (iPSC-aCMs and iPSC-vCMs) using stable-isotope labeling by amino acids in cell culture (SILAC) and LC–MS/MS. Analysis of equal portions of SILAC-labeled iPSC-aCMs and unlabeled iPSC-vCMs, and *vice versa*, allowed calculation of abundance ratios based on their mass differences and subsequently displayed the protein expression differences between the two cardiomyocyte subtypes. The authors identified 3568 proteins present in their samples, 94 of which showed significantly higher expression and 178 of which showed significantly lower expression in iPSC-aCMs compared with iPSC-vCMs. To validate their findings, the authors compared their data to a previous study of the proteomes of human fetal [81••] and adult [82••] atrial and ventricular tissues and found an enrichment of atrial proteins in the iPSC-aCMs whereas iPSC-vCMs showed enrichment in ventricle-related proteins [79••,81••,82••]. Significantly, they discovered a subset of differentially expressed proteins that were observed in the iPSC-aCMs and vCMS, as well as in the fetal tissues that were not differentially expressed in the adult tissues, which suggests that these genes are important during cardiac development [79••].

Direct reprogramming of fibroblasts into induced cardiomyocytes (iCM)

Direct reprogramming is the process of converting fibroblasts into cardiomyocyte-like cells (iCMs) without an embryonic/pluripotent intermediate, but rather through a direct transformation of cell types [83,84]. In mouse fibroblasts, this can be achieved by retroviral overexpression of three cardiac lineage-specific transcription factors, *Mef2C* (M), *Gata4* (G), and *Tbx5* (T) (MGT) (Figure 3) [83–85,86••,87–95]. Proteomic analysis and quantitation of proteins using isobaric labeling with tandem mass tags (TMT) demonstrated systematic and temporally distinct alterations in the levels of specific functional classes of proteins during the initial 72 hours of reprogramming. Surprisingly, few, if any of these proteins, are cardiac-related but rather are extracellular matrix proteins, translation factors, and chromatin-binding proteins [96••]. New questions stemming from these findings include how expression of these classes of proteins bypasses the embryonic steps and when in the process a fibroblast cell first expresses markers of or becomes a true cardiomyocyte [97•].

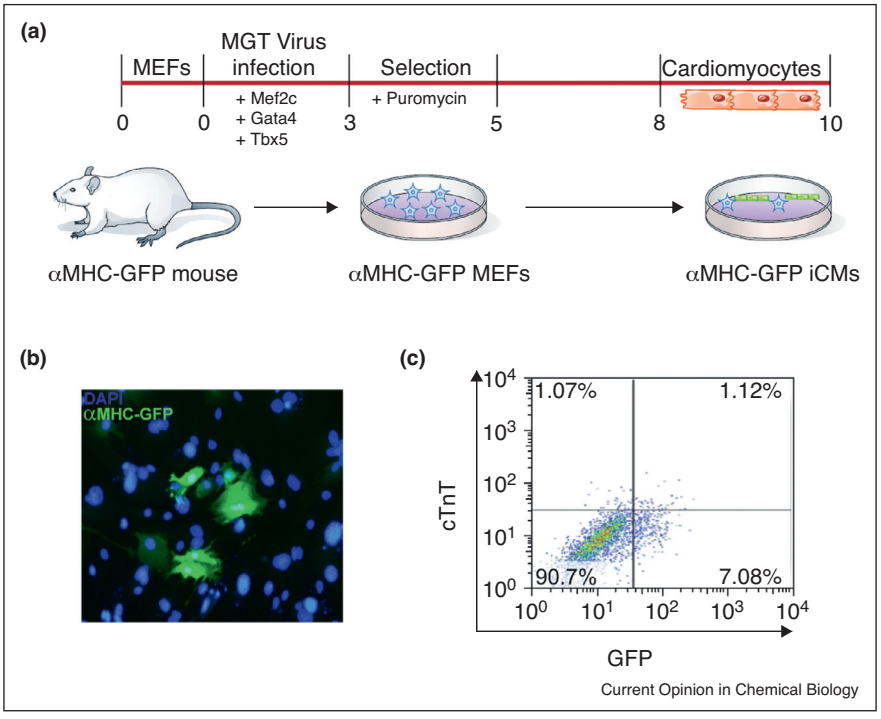
Perspectives

Though great progress has been made in applying proteomic mass spectrometry-based approaches to cardiac development and disease, the field is still in its infancy and is plagued by the absence of large in-depth data sets from specific stages of heart development or from tissue derived from models of human disease states. Recently, two studies utilizing quantitative mass spectrometry in human fetal and adult hearts generated a global protein atlas of the healthy human heart [81••,82••]. Lu *et al.* identified cardiac proteins expressed in human atria and ventricles during fetal development [81••]. This work was further enhanced by Doll *et al.* [82••], which generated a proteomic map from 16 different anatomical cardiac regions as well as three different cardiac cell types. These two studies have created valuable datasets that can be used for comparison to disease states (Figure 1).

Data from these types of approaches during embryonic development are emerging, but the utility of the results can be limited by not knowing which proteins and protein pathways are conserved and which have diverged between human and vertebrate model systems such as the mouse and, in industry, the pig (Sus scrofa). Furthermore, though surrogate systems have proven invaluable in assessing cardiomyocyte maturation, it is still not known what type of cardiomyocyte is produced through ESC or iPSC differentiation or through direct reprogramming. These resources and issues will need to be addressed in the coming years.

Finally, the majority of proteomic approaches to date have focused on whole tissue or cardiomyocytes, while it is now apparent that cardiac fibroblasts also play an essential role in heart development and disease [84,98,99]. Moreover, there appears to be many more cell types in the heart than initially believed [84,100]. Thus, it will be necessary to expand current technologies [101] as well as develop new approaches that allow isolation and characterization of pure populations of these cell types.

Figure 3



Direct cardiomyocyte reprogramming system.
(a) *In vitro* reprogramming timeline and experimental schematic. Mouse embryonic fibroblasts are harvested from αMHC-GFP transgenic mice which only express green fluorescent protein in mature cardiomyocytes. Fibroblasts are exposed to retroviral overexpression of the cardiac-specific transcription factors, Mef2C, Gata4, and Tbx5 (MGT). Induced cardiomyocytes can be observed after eight days in culture following selection for cells that successfully incorporated the MGT virus. **(b)** Immunostaining analysis of induced cardiomyocytes at day 10 of culture shows GFP positive cells which marks mature cardiomyocytes and DAPI to mark nuclei. **(c)** FACS analysis of αMHC-GFP positive cells and cardiac troponin T (cTnT) positive cells, an additional mature cardiomyocyte marker, illustrates the reprogramming efficiency.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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